

RESEARCH ARTICLE

Induction of Apoptosis and Cell Cycle Arrest by *Dorema Glabrum* Root Extracts in a Gastric Adenocarcinoma (AGS) Cell Line

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Abstract

Objective: *Dorema glabrum* Fisch. & C.A. Mey is a perennial plant that has several curative properties. Anti-proliferative activity of seeds of this plant has been demonstrated in a mouse fibrosarcoma cell line. The aim of the present study was to evaluate cytotoxicity of *D. glabrum* root extracts in a human gastric adenocarcinoma (AGS) cell line and explore mechanisms of apoptosis induction, cell cycle arrest and altered gene expression in cancer cells. **Materials and Methods:** The MTT assay was used to evaluate IC₅₀ values, EB/AO staining to analyze the mode of cell death, and flow cytometry to assess the cell cycle. Quantitative real-time polymerase chain reaction (qRT-PCR) amplification was performed with apoptosis and cell cycle-related gene primers, for cyclin D1, c-myc, survivin, VEGF, Bcl-2, Bax, and caspase-3 to determine alteration of gene expression. **Results:** Our results showed that n-hexane and chloroform extracts had greatest toxic effects on gastric cancer cells with IC₅₀ values of 6.4 µg/ml and 4.6 µg/ml, respectively, after 72 h. Cell cycle analysis revealed that the population of treated cells in the G1 phase was increased in comparison to controls. Cellular morphological changes indicated induction of apoptosis. In addition, mRNA expression levels of Bax and caspase-3 were increased, and of bcl-2, survivin, VEGF, c-myc and cyclin D1 were decreased. **Conclusion:** Our study results suggest that *D. glabrum* has cytotoxic effects on AGS cells, characterized by enhanced apoptosis, reduced cell viability and arrest of cell cycling.

Keywords: *Dorema glabrum*- cytotoxic- gastric cancer- apoptosis- cell cycle

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Introduction

Dorema glabrum Fisch. & C.A. Mey is a perennial plant that grows in loamy or rocky slopes of southern Caucasus especially, Azerbaijan, Armenia and Iran. Even though, already it has been reported that the distribution of *D. glabrum* is restricted to Transcaucasia region (Nakhichevan and Armenia zone), recent studies have shown that this plant is growing in some locations in North-West of Iran (Ajani et al., 2008; Asnaashari et al., 2011). This plant belongs to Apiacea and the gum-resin of this species is used for treating diarrhoea and as a diuretic (Delnavazi et al.). Herbs of this group have also antispasmodic, expectorant, carminative, diaphoretic, emmenagogue, stimulant, vasodilator (Mood, 2008; Yousefzadi et al., 2011), antioxidant (Delnavazi et al., 2015), antimicrobial and antifungal (Kumar et al., 2006), and hepatoprotector (Govind, 2011) activities. The plants of this group are widely used as a green vegetable or as a folk medicine for treatment of many disorders (Ibadullayeva et al., 2011).

Based on the folk beliefs of Azeri and Armenian people, *Dorema* species can remedy many abnormalities

especially catarrh, bronchitis and also for treating diarrhoea and as a diuretic (Mir-Babayev et al., 1993). It appears that widespread use of the plant for medicinal and local purposes is the main reason of extreme reduction of the natural resources of *D. glabrum* (Gabrielian, 1981; Ibadullayeva et al., 2011). It has been shown that methanol extract of *D. glabrum* seed has anti-proliferative effect on WEHI-164 mouse fibrosarcoma cell line and could induce apoptosis in this cell line (Amirkhiz et al., 2013; Bannazadeh Amirkhiz et al., 2013). Moreover, cytotoxic activity of *Dorema ammoniacum* another member of this group has been reported (Yousefzadi et al., 2011).

Gastric cancer is the fourth most common cancer and second leading cause of cancer death worldwide (Crew and Neugut, 2006). The gastric adenocarcinoma is the most prevalent type of gastric cancer (Alberts et al., 2003). Gastric adenocarcinoma (AGS) cell line is one of the widely studied cell line that is proper for apoptosis and cell cycle experiments (Bohlooli et al., 2012; Jafari et al., 2012).

The current study was conducted to evaluate cytotoxic effects of *Dorema glabrum* Fisch. & C.A. Mey root extracts (n-hexane, ethyl acetate, chloroform, and methanol) on

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AGS (human gastric adenocarcinoma) cell line.

Materials and Methods

The human gastric adenocarcinoma (AGS) cell line was provided from Pasteur Institute of Iran. All reagents, chemicals and media were used and prepared freshly.

Plant preparation

The roots of *D. glabrum* were collected from "Ghaflankuh" mountains located in East-Azerbaijan (northwest of Iran) during its flowering stage in June 2012. The plant was authenticated by a botanist Dr. Yousef Ajani and its voucher specimen (No. 2120 MPIH) was deposited at the herbarium of Institute of Medicinal Plants, ACECR, Karaj, Iran.

Extraction

The air-dried and comminuted roots (2.4 kg) were undergone extraction by using maceration method, sequentially, with n-hexane, chloroform, ethyl acetate and methanol (3×5 L each) at the room temperature. The attained extracts were concentrated using a rotary evaporator under reduced pressure at 45 °C and then dried in a vacuum oven at 40 °C for 24 h.

Cell Culture and Treatment

Cancer cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), penicillin 100 unit/ml and streptomycin 100 µg/ml. Cells were cultured at 37 °C in a moistened atmosphere of 5% CO₂ and 95% air. Then, cells trypsinized and plated in 96-well plates at a density of 1×10⁴ cells per well in 150 µl medium and incubated overnight; next, cells were treated with a FBS-free medium containing 1 mg/ml of each compound by 1/4 serial dilutions. Then, plates were incubated for 24, 48, and 72 h. The cytotoxicity of *D. glabrum* extracts was determined by MTT assay.

MTT Assay

Culture media were removed 4 h before completion of the incubation time, then 200 µl of 0.25 mg/ml MTT (Merck, Germany) was added to each well. Plates were incubated again for an additional 4 h in order to complete the incubation time. The supernatants were removed and 200 µl DMSO was added to wells and plates were shaken for 10 min. The absorbance was measured at 540 nm by a plate reader (Synergy HT, BioTek).

Apoptotic Cell detection by EB/AO Staining

Cells were cultured in 96-well plates at a confluence of 1×10³ cells/well and incubated overnight and then treated with extracts in their IC₅₀ doses. Then plates were centrifuged for 5 min (129 g, 1,000 rpm) at 4 °C. The EB/AO dye mix (100 µg/ml ethidium bromide and 100 µg/ml acridine orange) was dissolved in PBS and 20 µl of the dye mix was added to wells. Cells were analyzed and counted by an inverted fluorescence microscope (IX 71, OLYMPUS). Live cells were determined by normal green color which were resulted by up-taking of acridine orange (green fluorescence) and repelling of ethidium bromide

(red fluorescence).

Apoptotic cells display apoptotic bodies, and perinuclear condensation of chromatin stained by ethidium bromide. While live cells were identified as normal nuclear chromatin stained by acridine orange. Necrotic cells were detected by uniform ethidium bromide staining of the cells (Ribble et al., 2005). Images were captured with a digital camera (DP 71, OLYMPUS) equipped microscope. Experiments were done in triplicate, quantifying a minimum of 100 cells each time.

Flow Cytometric analysis of the Cell Cycle

Cells were cultured in 6-well plates at a confluence of 1×10⁶ cells/well. Cells were treated with extracts for 24 h with respective IC₅₀ values. Then, cell cycle phases and DNA content were analyzed by flow cytometry. Briefly, cells were collected and fixed with ice ethanol 70% for 2 h. Fixed cells were centrifuged (300 g, 4 °C, 5 min) and washed with cold PBS, and then stained with diamidino-2-phenylindole (DAPI, 10 µg/mL, Triton X-100 0.1% v/v in PBS). Then cells were filtered by a nylon mesh with a pore size of 30 µm. Cell cycle analysis was done using a flow cytometer (Partec CyFlow space, Germany). The distribution of cells in different cell cycle phases was assessed by means of Partec FloMax software.

mRNA expression analysis by qPCR

Cells were cultured in a 6-well plates at a confluence of 2×10⁵ cells/well and kept at 37 °C in a moistened air of 5% CO₂ for an overnight. Cells were then treated for 48h with chloroform, ethyl acetate, n-hexane and methanol extracts at 8.2, 48.7, 9.4 and 256.1 µg/ml concentrations respectively.

For RNA extraction from cells Trizol reagent (Cat. No: 15596-026, Invitrogen, CA, USA) was used according to manufacturer's protocol. First-strand cDNA was generated from the cells' extracted RNA by RevertAid First Strand cDNA Synthesis Kit, Fermentas (Cat No: #K1621, Maryland, USA) based on the kit's procedure.

Primers (Bax, cyclin D1, VEGFA, Bcl-2, Caspase 3, c-myc, survivin and the homo sapiens ribosomal protein L38 (RPL38) as a housekeeping gene were designed using Primer Express 3.0 (PE Applied Biosystems, Foster City, CA, USA). See Supplementary Table 1 for the details of primers used in quantitative real-time PCR. For accuracy and specificity all primers were blasted in NCBI website: <http://www.ncbi.nlm.nih.gov/tools/primer-blast/>. Primers were synthesized by the custom oligonucleotide synthesis service, Metabion (Martinsried, Germany).

Quantitative analysis was done by StepOne Real-Time PCR System (Applied Biosystems 7500, Foster City, CA, USA) by using the PowerSYBR Green PCR Master Mix (Cat. No: 4309155, Applied Biosystems, Foster City, CA, USA).

Individual reaction mix contained an overall volume of 25 µl (master mix 12.5 µl, cDNA 3 µl, primer 3 µl, and H₂O 6.5 µl). The quantitative RT-PCR settings were: 50 °C for 2 minutes, 95 °C for 10 minutes, then 60 cycles of 95 °C for 30 seconds, and 60 °C 30 seconds and 72 °C for 30 seconds. Relative quantities of target mRNA in test sample was measured and standardized to the

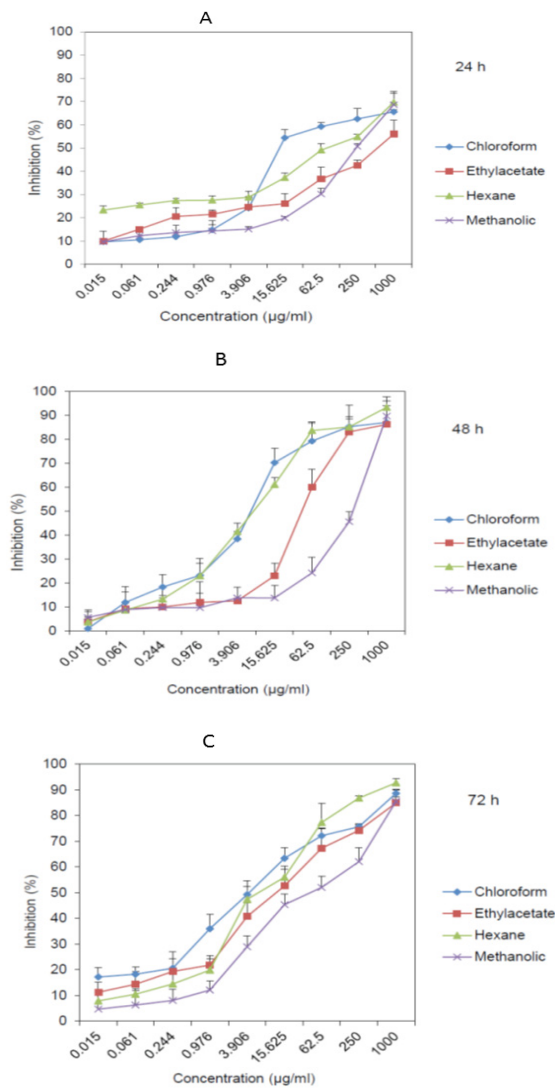


Figure 1. Cytotoxic Effect of *D. Glabrum* Root Extracts (N-Hexane, Ethyl Acetate, Chloroform, and Methanol) on AGS Cells Analyzed by MTT Assay after 24 h (A), 48 h (B), and 72 h (C)

housekeeping gene, RPL38 mRNA transcript level. The comparative Ct method was used to assess expression as previously described by Livak and Schmittgen (Livak and Schmittgen, 2001).

Statistical Analysis

IC50 values were calculated using Sigma Plot 12 software. Data values for the growth inhibition study are presented as mean±SD, except in figures where error bars

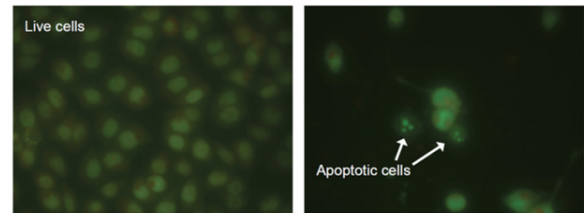


Figure 2. Representative Micrograph of Cells after Treatment with Ethylacetate Extract of the *D. Glabrum* Root in IC50 Concentration. Apoptotic Cells are Condensed and Fragmented in Comparison to the Live Untreated Cells

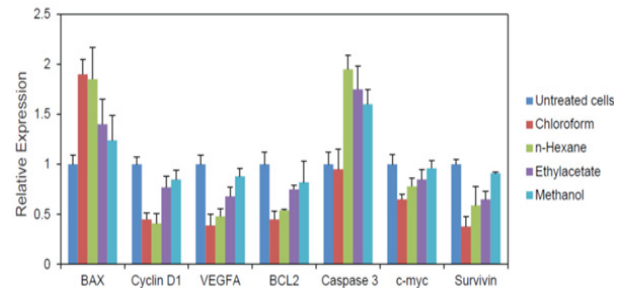


Figure 3. Relative mRNA Expression Levels of Genes Involved in Apoptosis and Cell Cycle which Performed Using qPCR. The Data are Expressed as the Means ± SEM of a Representative Experiment Performed in Triplicate (n=3)

represent standard error of mean (SEM).

Results

Cytotoxic effect of the *D. glabrum* has been analyzed with MTT assay. n-hexane and chloroform extracts had most toxic effect on gastric cancer cells with IC50 values of 6.4 µg/ml and 4.6 µg/ml after 72 h respectively (Figure 1 and Table 2). Chloroform extract was the most potent with least IC50 of 14.3 µg/ml after 24 h (Table 2).

In order to determine the apoptosis induction of the *D. glabrum*, EB/AO staining was conducted. Quantification of the cells showed that after incubation of the cells with *D. glabrum* extracts, they displayed a series of morphological changes including condensation and fragmentation of chromatin and nucleus, and formation of apoptotic bodies which were designated as typical evidence of apoptotic bodies. In contrast, control cells displayed a normal appearance (Figure 2 and Table 3).

In this study, the DNA content of *D. glabrum* treated

Table 1. Primers Used in Quantitative PCR and the Amplicon Sizes (bp: Base Pair)

Target	Forward primer	Reverse primer	Amplicon Size (bp)
Bax	5'-GCCCTTTTGCTTCAGGGTTTC	5'-CATCCTCTGCAGCTCCATGT	168
Cyclin D1	5'-GGCGGAGGAGAACAACAGA	5'-TGTGAGGCGGTAGTAGGACA	181
VEGFA	5'- TGTCTAATGCCCTGGAGCCT	5'- GCTTGTACATCTGCAAGTACG	175
Bcl-2	5'- CAGGATAACGGAGGCTGGGATG	5'- AGAAATCAAACAGAGGCCGCA	70
Caspase 3	5'- GCGGTTGTAGAAGAGTTTCGTG	5'- CTCACGGCCTGGGATTTCAA	101
c-myc	5'- CCCTCCACTCGGAAGGACTA	5'- GCTGGTGCATTTTCGGTTGT	96
Survivin	5'-TTCAAGGAGCTGGAAGGCTG	5'-AGCAATGAGGGTGGAAAGCA	151
RPL38	5'-TCACTGACAAAGAGAAGGCAGAG	5'- TCAGTGTGTCTGGTTCATTTTCAGTT	88

Table 2. IC50 Values of Dorema Glabrum Fisch. and C.A. Mey Root Extracts Analyzed by MTT Assay after 24 h, 48 h, and 72 h. Values are in µg/ml.

Extract	24 h	48 h	72 h
Chloroform	14.3 ± 3.2	8.2 ± 1.2	4.6 ± 1.6
Ethyl acetate	124.6 ± 15.6	48.7 ± 3.5	14.7 ± 2.8
n-Hexane	65.3 ± 7.4	9.4 ± 1.4	6.4 ± 1.5
Methanol	261.6 ± 21.7	256.1 ± 18.2	59.8 ± 8.4

Table 3. Apoptosis Induction of Dorema Glabrum Fisch. and C.A. Mey Root Extracts on AGS Cell Line. Cells Were Treated with IC50 Values of Extracts. Data are Presented as Percentage of Cells

Column1	Normal live cells	Apoptotic cells	Necrotic cells
Untreated cells	96.4 ± 1.4	2.4 ± 0.9	0.8 ± 0.2
Chloroform	45.6 ± 2.8	53.4 ± 1.5	2.1 ± 0.8
Ethylacetate	48.4 ± 2.7	50.1 ± 2.8	1.8 ± 0.6
Hexane	46.8 ± 5.3	53.6 ± 4.2	1.2 ± 0.7
Methanolic	52.6 ± 3.1	47.6 ± 1.8	1.2 ± 0.4

cells during cell cycle phases were measured in order to obtain information about the cell cycle progression. The percentage of cells in G1, S, and G2/M phases were calculated using Partec FloMax software and is shown in Table 4. After 24 h of D. glabrum treatment, population of cells in the G1 phase increased in comparison to controls. The increase of cell population at the G1 phase was accompanied by a decrease of cell population in the G2/M phase of cell cycle (Table 3).

Q-PCR results showed that all extracts reduced mRNA expression of intracellular Bcl-2, survivin, VEGF, cyclin D1 and c-myc genes and increased Bax, and caspase-3 genes expression more distinctly than the untreated control cells. Chloroform extract had the most potent effect on the down-regulation of the Bcl-2, survivin, VEGF, cyclin D1 and c-myc genes, and up-regulation of the Bax, and caspase-3 genes.

Discussion

Gastric cancer is one of the most common cancers worldwide. Because of high incidence rate in some areas, finding an effective treatment is the challenge of researchers. Introducing natural plants with curative features would be proper replacement for chemical anti-cancer drugs. Hence, natural plants are less toxic to healthy body; finding new plants with cytotoxic effects could be promising. We found that D. glabrum could inhibit cell proliferation, induce apoptosis and arrest cell cycle in G1 phase among via four extracts of D. glabrum. n-hexane and chloroform extracts showed most cytotoxic effects with low IC50 values 6.4 µg/ml and 4.6 µg/ml after 72 h (Figure 1) respectively. After verifying cytotoxicity, we aimed to determine the mode of cell death. EB/AO staining was conducted to quantify number of live, necrotic and apoptotic cells. By analysis of morphological changes including condensation and fragmentation of chromatin and nucleus, and also formation of apoptotic

Table 4. Population of the Cells was Analyzed by Flow Cytometry. Cells were Treated with Extracts of Dorema Glabrum in Respective IC50 Values. Cell Cycle Analysis of the Cells Demonstrates that All Extracts Induce Cell Cycle arrest in G1 Phase

Extract (µg/ml)	G1 (%)	S (%)	G2/M (%)
Untreated cells	42.1± 5.7	18.7±6.4	38.4±4.2
Chloroform (14.3)	62.3±3.8	15.3±5.1	22±1.7
Ethyl acetate (124.6)	58.6±3.4	17.5±2.7	24.6±5.3
n-Hexane (65.3)	60.7±3.8	16.3±2.5	23.2±2.4
Methanol (261.6)	53.6±4.6	17.8±1.8	29.4±3.6

bodies it revealed that cell underwent apoptosis pathway. These results prompted us to evaluate the mRNA expression of relevant genes in apoptosis process.

Bcl-2 (B-cell lymphoma 2) is considered as an important anti-apoptotic protein and is therefore classified as an oncogene. Targeting Bcl-2 by therapeutic agents, block its inhibitory effects on apoptosis and therefore malignant cells could undergo apoptosis. Our results showed that all extracts could down-regulate Bcl-2 expression, nevertheless, chloroform and n-hexane were more potent in reducing its expression.

Survivin is a member of the inhibitor of apoptosis (IAP) family. Survivin by inhibiting caspase activation could suppress apoptosis. It has been found by inhibition of survivin activation pathways, apoptosis increases and tumor growth reduces. We showed that chloroform extract decreased survivin expression much more than the other extracts. However, methanol extract was not more effective.

Second pathway responsible for caspase activation is the release of cytochrome c from mitochondria (Wong, 2011). The release of cytochrome c to cytoplasm activates caspase-3 via the formation of apoptosome complex. This complex includes cytochrome c, APAF-1 and caspase-9 (Jackson and Combs Jr, 2008). Pro-apoptotic agent Bax is also affect this pathway. Bax promotes apoptosis by binding to and antagonizing the Bcl-2 protein (Kumar et al., 2013). Our experiments showed that D. glabrum extracts could up-regulate caspase-3 and Bax expression and thereby promote apoptosis induction (Figure 3).

In order to validate our data, we decided to analyze the effect of extracts on cell cycle distribution. The flow cytometric results revealed that 24 h incubation of cells with extracts, increase the population of cell in G1 phase. These findings encouraged us to evaluate expression levels of cyclin D which is one of important regulators in cell cycle G1/S transition.

Cyclin D1 regulates CDK4 or CDK6, which are required for cell cycle G1/S transition. Overexpression of this gene modifies cell cycle progression and triggers tumorigenesis (Nakamura et al., 2013). All extracts down-regulated the expression of cyclin D1, especially, n-hexane extract effect was higher than the other extracts.

Myc (c-Myc) functions as a regulator in cell cycle progression, apoptosis and cellular transformation (Petrich et al., 2014). Myc enhances unregulated expression of many genes that are involved in cell division, therefore it is considered as a promising target for anti-cancer

drugs. *D. glabrum* extracts reduced mRNA levels of Myc, nevertheless, methanol extract did not have significant effect on its expression.

Vascular endothelial growth factor (VEGF) stimulates vasculogenesis and angiogenesis and therefore, provides oxygen to tissues when blood circulation is insufficient. Tumors that can express VEGF could grow and metastasize. Our experiments demonstrated that *D. glabrum* extracts could down-regulate mRNA expression of VEGFA in AGS cells. Chloroform and n-hexane extracts had the most effect and methanol had the least effect in decreasing VEGFA expression levels.

In conclusion, these findings may suggest that *D. glabrum* has some cytotoxic effects on gastric cancer cells and could induce apoptosis and cell cycle arrest in G1 phase. It may be used as adjuvant chemotherapy agent in gastric originated cancers. Nevertheless, the concentrations used in the current study are achieved by an in vitro study and the possibility to extrapolate these concentrations to clinical practice needs more investigations. *D. glabrum* needs to be investigated further, especially with animal tumor models to confirm its anticancer and chemotherapeutic activity in vivo.

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Conflict of interest

We declare that there is no conflict of interest.

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