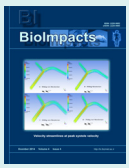
Eskandani *et al.*, ***BioImpacts,*** 2014, 4(4), 191-198
doi: 10.15171/bi.2014.019
http://bi.tbzmed.ac.ir/

Geno/cytotoxicty and Apoptotic Properties of Phenolic Compounds from the Seeds of *Dorema Glabrum* Fisch. C.A

Morteza Eskandani[1,2], Elmira Dadizadeh[3], Hamed Hamishehkar[4], Hossein Nazemiyeh[1,3*], Jaleh Barar[1,3*]

[1] Research Center for Pharmaceutical Nanotechnology, Tabriz University of Medical Sciences, Tabriz, Iran
[2] Student Research Committee, Tabriz University of Medical Sciences, Tabriz, Iran
[3] Faculty of Pharmacy, Tabriz University of Medical Sciences, Tabriz, Iran
[4] Drugs Applied Research Center, Tabriz University of Medical Sciences, Tabriz, Iran

Article Info

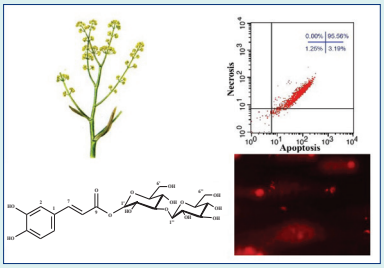


Article Type:
Research Article

Article History:
Received: 10 Dec. 2014
Revised: 15 Dec. 2014
Accepted: 27 Dec. 2014
ePublished: 30 Dec. 2014

Keywords:
Dorema glabrum
Phenolic compounds
Cytotoxicity
Genotoxicity

Abstract



Introduction: *Dorema glabrum* (Apiaceae) is a rare and monocarpic species distributed in Transcaucasia and North West of Iran. We aimed to explore anti-cancer potency of bioactive compounds from the seeds of *Dorma glabrum*.

Methods: Methanol extract was subjected to phytochemical investigation using normal phase Sep-pak and reversed-phase HPLC, and cytotoxic effect of isolated compounds on CAOV-4 cell line was evaluated. Furthermore, Annexin V/PI staining and comet assay were used to study genotoxicity of compounds.

Results: Diglucosyl caffeoyl ester **(1)**, Glucopyranosylcaffeic acid **(2)** and skimmin **(3)**, were identified. MTT cytotoxicity assay showed growth inhibition of CAOV-4 cells due to treatment with compunds **(1)**, **(2)** and **(3)** with an IC_{50} of 99.7, 87.3 and 70.03 µg/ml at 48 h, respectively. Annexin V-FITC/PI staining showed occurrence of early/late apoptosis in the **(1)**-treated cells, while **(2)**-and **(3)**-treated cells necrosis/late apoptosis was dominant event. Single/double strands DNA breakages were observed by comet assay in all treatments.

Conclusion: This work provides sufficient information about anti-cancer properties of the diglucosyl caffeoyl ester from the seeds of *D. glabrum*.

Introduction

In spite of different treatment strategies, cancer remains among the main causes of mortality worldwide.[1] Despite various therapeutic modalities for the management of cancer (i.e. chemotherapy, surgery, radiotherapy and adjuvant hormonal therapy), chemotherapy is considered to be one of the most important cancer therapeutic strategy. Acquired resistance to available chemotherapy agents represents one of the critical hurdles in combating the disease, hence circumventing such resistance, and introducing novel anti cancer drugs is a major requirement for improving the efficiency of the cancer therapy regimen. Natural remedies have been a main source of numerous current chemotherapeutic agents, and are promising resource for the forthcoming potent bioactive and anti-cancer compounds such as phenolics, glycosides, steroids, polysaccharides, flavonoids, terpenoids and alkaloids.

The genus Dorema belong to the Apiaceae (*alt.* Umbelliferae), is represented in the flora of Iran by seven species, among them *Dorema glabrum* Fisch. C.A. Mey, *D. aucheri* Boiss and *D. ammonicum* D. Don are endemic,[2] mainly distributed in northwestern, southwestern and central Asia.[3,4] Most of the species in this genus are similar to each other, morphologically. They have thick roots, are monocarp, and possess simple and large umbel. Their corymb is the main distinction between Feula genuses.[5] The plant has widespread uses in traditional medicine in origin regions, and/or as food additive, as well. There exist common believe and scientific observation regarding to the pharmaceutical properties of the genus Dorema, and their gum-resins, specially. The "Ammoniacum" from *D. ammoniacum* is the naturally-exuding gum resin latex and well-known therapeutic agent with antispasmodic and expectorant properties which is used for the treatment of skin inflammatory diseases,[6] and showed antimicrobial activity.[7] Also, the other species "*D. aucheri*" exhibited beneficial role in thyroid function,[8] increase the blood level of antioxidant enzymes (SOD and GPX) and vitamins (E

***Corresponding authors:** Hossein Nazemiyeh, Email: nazemiyehh@tbzmed.ac.ir, Jaleh Barar, Email: jbarar@yahoo.com

and C) concentration dose dependently,[9] and also showed antibacterial properties against *Staphylococcus areus*, *Staphylococcus epidermis* and Streptococcus *pneumonia.*[10] The other members of this genus possess carminative, diaphoretic, mild diuretic, emmenagogue, stimulant, vasodilator,[11,12] antimicrobial and antifungal[12-14] and hepatoprotector[15] properties.

Dorema glabrum Fisch. C.A. Mey is a perennial herb that grows up to 2.5 m height in loamy or rocky slopes of Nakhichevan, Republic of Azerbaijan, Armenia and Iran. Though, according to Rechinger, distribution of *D. glabrum* is restricted to Transcaucasia region (Nakhichevan and Armenia zone).[3] Recent works show that this plant can be found in some locations in Northwestern of Iran[5,16] including Aras region. In Nakhichevan, this herb is used as a green vegetable or as a folk medicine as a diuretic and anti-diarrheal agent, as well as for the treatment of bronchitis and catarrh.[17] Some recent biological surveys have shown antioxidant[18] and anti-lipidemic effects[19] of the aerial parts of *D. glabrum*. Also, we showed the cytotoxic and apoptotic properties of the alcoholic total extracts of seeds of *D. glabrum* in previous comprehensive studies.[20,21]

Limited studies have been reported phytochemical constituents from the different part of Dorema species. Bukreeva and Nurmukhamedova reported some phloroacetophenone glycosides from the roots of *D. aitchisonii*[22] and *D. hyrcanum,*[23] respectively. Iranshahi et al, also cnfirmed some sesquiterpene derivatives from the aerial parts and the roots of *D. kopetdaghense.*[24] However, only one investigation recently has been reported phloroacetophenone glycosides from the roots of *D. glabrum.*[19] Likewise, previous reports on essential oil composition of Dorema species are limited to the elucidation of α-eudesmol and δ-cadinene from the aerial parts of *D. aucheri,*[25] and (Z)-ocimenone, (E)-ocimenone and cyclocitral from the fruit of *D. ammoniacum.*[12] We also reported d-cadinene and b-bisabolene as the main compounds of the essential oil composition of the roots of *D. glabrum*, previously.[26]

In this investigation, we identified potent anti-cancer compounds of the seeds of *D. glabrum* based on a bioassay-guided isolation, and investigated their anti-cancer properties using MTT survival, and Annexin V apoptosis assays on CAOV-4 human ovarian adenocarcinoma cells. Also, genotoxicity properties of isolated compounds were analyzed by alkaline comet assay. In this context, we elucidated three phenolic compounds including diglucosyl caffeoyl ester, glucopyranosylcaffeic acid and skimmin.

Material and Methods

Plant materials

The fruit samples (seeds) of *D. glabrum* were collected from Aras river bank (Jolfa- East Azarbayjan, Iran; 2008) and Voucher specimen (Tbz-FPh 541) representing this collection was deposited in the Herbarium of the Faculty of Pharmacy, Tabriz University of Medical Sciences.

Materials and reagents

Hex, DCM and methanol were purchased from Caledon (Canada). Chloroform, ethyl acetate and TLC plates (Pre-coated silica gel, $F_{254,}$ 0.25 mm; Merck) were obtained from Merck (Germany). Sep-Pak cartridges (C18, 35 cc; Vac cartridge, 10 g) were purchased form Waters Corporation, USA. HPLC instrument that used in this investigation were from Shimadzu (Japan), as well.

General experimental procedures

^{1}H, ^{13}C-NMR spectra were recorded in Methanol-d4 on a Brucker 200MHz spectrometer operating at 200.13 MHz for ^{1}H-NMR and 50.32 MHz for ^{13}C-NMR. Tetramethylsilane (TMS) was used as internal standard. HPLC analysis was performed using a Shimadzu LC8-A (Japan) system coupled to a photodiode array detector (SPD-10A). Detection was done at λ_{max} 235, 254, 280, and 325 nm. UV data for individual compounds were extracted from the online UV spectra provided by the instrument software.

Extraction and fractionation

Two hundred grams of air dried and powdered seeds were extracted by Hex (8 h), DCM (10 h) and methanol (8 h) using a Soxhlet apparatus. The extracts were concentrated by a rotary evaporator under reduced pressure at 45 °C to obtain a dry extract. The cytotoxicity of the extracts was initially assessed by MTT assay. Methanol extract showed the most potent anti-proliferative properties, hence was selected for fractionation. Methanol extract fractionation was performed by solid phase extraction (SPE) method.[27] Briefly, methanol extract (2 g) was loaded on a Sep-pak cartridge and fractions were eluted with a step gradient of 200 ml MeOH/H_2O (20: 80, 40: 60, 60: 40, 80: 20, and 100: 0,) mixtures. The Sep-pak procedure was repeated for at least 3 times to get enough amount of each fraction. The solvents of each fraction were removed *in vacuo* and 40 °C. Once again, the yielded fractions were subjected to MTT assay, and the fractions with dominant anti-proliferate activity (eluted by 20:80 and 40:60 MeOH: H_2O; 1.495 and 1.38 g, respectively) were further evaluated and fractioned using high pressure liquid chromatography with different procedure and program.

Preparative HPLC

The resulted anti-proliferate potent fractions by Sep-pak were analyzed by preparative HPLC eluted with a linear gradient of MeOH/water and monitored using a photo-diode-array detector at the range of 190 to 400 nm. Only for 2 fractions (20, 40, all MeOH/H_2O), HPLC was carried out by different methods and time gradient. For 20% fraction (system A: mobile phase: 0-50 min, MeOH from 15 to 30% in H_2O; 50-62 min, 30% MeOH in H_2O; 62-64 min, MeOH from 30 to 15% in H_2O; 64-75 min MeOH 15% in H_2O, flow rate 20 ml/min), for 40% MeOH/H_2O fraction (system B: mobile phase: 0-60 min, MeOH from 30 to 45% in H_2O; 60-70 min, 45% MeOH in H_2O; 70-72 min, MeOH from 45 to 30% in H_2O; 72-82 min MeOH

30% in H_2O, flow rate 20 ml/min) were used. Then the solvents of eluted fractions were removed by rotary evaporator at the ambient temp and *in vacuo.* All collected sub-fractions were monitored on TLC plates and the comparable compounds were integrated. Cytotoxicity of all mixed fractions was evaluated and the most potent anti-proliferate fraction were gathered for chemical structure determination and extensive biological investigations.

Determining the chemical structures

The structure of purified compounds were elucidate by UV-visible, ^{1}H-NMR and ^{13}C-NMR spectroscopy techniques. For ^{1}H-NMR and ^{13}C-NMR spectroscopy the sufficient amount of yielded compounds were dissolved in methanol-d_4.

Cell culture

CAOV-4 human ovarian adenocarcinoma cells were seeded at the density of 2 $\times 10^4$ cell/cm^2 on 96-well plates and were incubated in a humidified incubator (95% air and 5% CO_2) at 37 °C. Cell culture media consisted of RPMI 1640 complemented with 10% FBS. Cells were fed every other day and sub-cultured once a week.

Cell viability assessment

To assess the influence of purified compounds studied here on the cellular viability, the CAOV-4 were seeded and cultured up to 40-50% confluency in the 96-well plates prior to treatment. The cells were exposed with a range of compounds concentrations and each group was incubated for 24, 48 and 72 h at 37 °C, and doxorubicin (32 µM) was used as positive control. Then, the cells of each group were washed once with phosphate buffered saline (PBS) and culture medium in each well was replaced with 150 µl fresh media plus 50 µl MTT reagent (2 mg/ml in PBS). After 4 h incubation at 37 °C, the media was removed and the cells were exposed to 200 µl DMSO and 25 µl of Sorenson buffer (0.1 M glycine, 0.1 M NaCl, pH 10.5). The plates were incubated at 37 °C to dissolve formazan crystals and then UV absorbance was measured at 570 nm using a spectrophotometric plate reader, ELx 800 (Biotek, CA, USA).[28]

Single cell-thin layer gel- electrophoresis (alkaline comet assay)

Alkaline comet assay was used to assess the compounds direct interactions with whole chromatin and its DNA breakage properties. Doxorubicin (32 µM) was used as positive control. Alkaline comet experiment was carried out based on previous works.[29-31] Briefly, CAOV-4 were isolated from flask and the supernatant was removed by centrifuging at 800 rpm for 8 min. Normal melting point agarose (1%) pre-coated slides were then used as surface for the low melting point agarose (%0.5)-embedded cells (1×10^4) and subjected to a lysis step [4 h incubation at 4 °C in 2.5 M NaCl, 100 mM Na_2EDTA, 1% triton X-100 (pH>12)] without third agarose layer[32] and then washed 3 times in an ice-cold alkaline electrophoresis solution (300 mM NaOH, 1 mM Na_2 EDTA of pH>13) for 30 min to allow DNA unwinding and removal of histones. This was followed by electrophoresis in same alkaline buffer conducted for 20 min (0.6 V/cm, current 300 mA). After that the slides were washed with neutralization buffer (40 mM Tris-HCl, pH 7.5) and stained with a drop of ethidium bromide. Prior to coverage of slides with a 20 × 20 cover slip, the slides were washed two or three times to remove excess background color and finally immediate microscopic analyses (Olympus IX81 fluorescence microscope equipped with XM10 monochrome camera; wavelength 546 nm; barrier 580 nm) were completed.[29,30] The images were analyzed by CASP software. DNA strand breaks were expressed as the percentage of total fluorescence DNA migrated in the tail for each nucleus [% (DNA in tail/DNA in head)] (Eq. 1).

$$DNA\ strand\ cleavage\ \% = \frac{DNA\ in\ tail}{DNA\ in\ head} \times 100 \quad (1)$$

Annexin V-FITC/PI apoptosis assay

For determination of phosphatidyl serine externalization and detection of the extent of apoptotic death, Annexin V Apoptosis Detection Kit APC (FITC) was used (eBioscience, San Diego, USA). Briefly, treated cells with isolated compounds and doxorubicin (32 µM) were detached by tripsinization and washed three times with 500 µl 1X binding. Later, the supernatant was removed and the cells were resuspended in 100 µl of Annexin V binding buffer and stained with 8 µl of Annexin V-FITC. Following 15-min incubation at room temperature in the dark, the cells were washed 3 times with 1X binding buffer (1000 RPM, 5 min) and resuspended in 200 µl 1X binding buffer and then 8 µl propidium iodide (PI) staining solution were added. The incubation lasted for 5 min at room temperature in the dark. The cells were analyzed using Becton Dickinson FACS Calibur System (San Jose, USA) with emission filters of 515–545 nm for FITC (green) and 600 nm for PI (red). A total of 10,000 cells per sample were acquired, and the data were analyzed with CELLQuest software (Becton Dickinson).

Statistical analysis

All expressed data in Figures and text represent the mean of at least three repeated experiments (error bars represent mean ± standard deviation). An independent Student's *t*-test was used to compare mean differences between two independent groups and a one-way ANOVA was used to multiple comparisons. When the differences between the means were significant, post-hoc pairwise comparisons were carried out using Tukey multiple comparison tests (SPSS; version 13.0). The statistical significance was defined as $p<0.05$.

Results

Determining the chemical structures

Phytochemical analyses of the compounds with anti-prolifirative properties led to the isolation of two caffeic

acid derivative compounds and a coumarin glycoside (Fig. 1). The isolated compounds were identified as diglucosyl caffeoyl ester (**1**), 4-O-β-D-glucopyranosylcaffeic acid (**2**), and umbelliferone 7-O-β-D-glucoside (skimmin) (**3**). The structures of these compounds were characterized using NMR spectroscopic data (^{1}H-NMR, ^{13}C-NMR), and also by comparing with those reported in the literature.

Chromatographic and spectroscopic data

*Diglucosyl caffeoyl ester (**1**)*

Yellow gummy residue; Rt: 7.30 min (System A of HPLC); ^{1}H-NMR (MeOH-d_4, δ/ppm, *J*/Hz): 7.60 (1H, d, *J*=15.8, H_7), 7.08 (1H, d, *J*=2.1, H_2), 6.96 (1H, dd, *J*=8.1, 2.2 H_6), 6.81 (1H, d, *J*=8.1, H_5), 6.32 (1H, d, *J*=15.8, H_8), 5.58 (1H,d, *J*=7.3, $H_{1'}$), 3.52–4.1 (6H, m, $H_{2'-6'}$), 4.56 (1H, d, *J*=7.5, $H_{1''}$), 3.52–4.17 (6H, m, $H_{2'-6'}$); ^{13}C-NMR (MeOH-d_4, δ/ppm): 176.01 (C9), 143.57 (C4), 141.23 (C3), 140.09 (C7), 125.65(C1), 118.33 (C6), 116.33 (C8), 115.39 (C5), 112.21 (C2), 103.60 (C1″), 93.01(C1′), overlapped (C5″), 76.65 (C3″), 83.31 (C3′), overlapped (C5′), 70.34 (C2′), 73.70 (C2″), 69.98 (C4′), 70.34 (C4″), 60.82 (C6′), 61.36 (C6″).

*4-O-β-D-glucopyranosylcaffeic acid (**2**)*

Pale brown powder; Rt: 50.6 min (System B of HPLC); ^{1}H-NMR (MeOH-d_4, δ/ppm, *J*/Hz): 7.58 (1H, d, *J*=15.83, H_7), 7.16 (1H, d, *J*=7.8, H_5), 6.96 (1H, d, *J*=2.1, H_2), 6.86 (1H, dd, *J*=7.62, 2.1 H_6), 6.28 (1H, d, *J*=15.8, H_8), 4.79 (1H, d, *J*=7.4, $H_{1'}$), 3.52–4.23 (6H, m, $H_{2'-6'}$); ^{13}C-NMR (MeOH-d_4, δ/ppm): 170.36 (C 9),150.21 (C4), 149.89 (C3), 143.77 (C7), 130.82 (C1), 122.03 (C6), 118.13 (C5), 117.84 (C8), 116.11 (C2), 103.24 (C1′), 78.29 (C5′), 75.89 (C3′), 73.13 (C2′), 70.34 (C4′), 62.93 (C6′).

*Umbelliferone 7-O-β-D-glucoside (skimmin) (**3**)*

Yellow solid ; Rt: 50.3 min (System B of HPLC); ^{1}H-NMR (MeOH-d_4, δ/ppm, *J*/Hz): 7.93 (1H, d, *J*=9.4, H_4), 7.61 (1H, d, *J*=8.4, H_5), 7.1 (1H, dd, *J*=8.4, 2.1 H_6), 6.88 (1H, d, *J*=1.89, H_8), 6.33 (1H, d, *J*=9.4, H_3), 4.62 (1H,d, *J*=7.4, $H_{1'}$), 3.52–4.19 (6H, *m*, $H_{2'-6'}$); ^{13}C-NMR (MeOH-d_4, δ/ppm):162.33 (C2), 159.67 (C7), 152.5 (C9), 140.8 (C4), 129.40 (C5), 126.90 (C6), 113.9 (C10), 113.42 (C3), 104.0 (C1′), 102.98 (C8), 76.86(C3′), 76.40(C5′), 73.53 (C2′), 70.21 (C4′), 61.93 (C6′).

MTT cytotoxicity assay

The MTT assay results CAOV-4 cells treated with compound (**1**), (**2**) and (**3**) is shown in Fig. 2. The results showed that all compounds were able to inhibit CAOV-4 cells growth rate in a dose-dependently with an IC_{50} of approximately 99.7 (197 µM), 87.3 (254 µM) and 70.03 (216 µM) µg/ml compound (**1**), (**2**) and (**3**) at 48 h, respectively. In addition, light microscopic observations illustrated that the treated and untreated cells have distinct morphologic differences in normal and dead cells number and appearance. Chromatin condensation, cell shrinkage and membrane blobbing are the most important macroscopic morphological changes due to the compounds treatment after 48 h (Fig. 3).

Alkaline comet assay

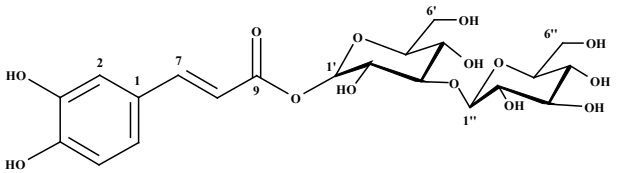
Here, the results of the genotoxicity assay revealed some degree of DNA breakage with different treatments. Figure 3 shows the typical results obtained by means of the comet assay for CAOV-4 cells treated with compounds (**1**), (**2**) and (**3**) as well as the hydrogen peroxide as a positive control. Significant double strand breakage of DNA was observed in all treatment groups by comet assay. No double or single strands DNA breakage was observed within untreated CAOV-4 cells while significant breakage was seen within those treated H_2O_2. Statistical analysis of tail DNA/head DNA confirmed significant differences (p<0.05) between untreated and all compounds treated CAOV-4 (Fig. 4). However, insignificant differences (p>0.05) between all compounds treated CAOV-4 were seen.

Flow cytometric analysis of apoptosis

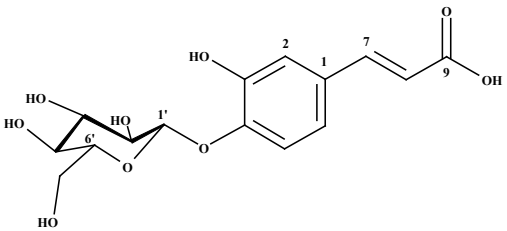
Incidence of early/late stages of apoptosis within CAOV-4 cells treated with compound (**1**) was observed. Treated cells with compounds (**2**) and (**3**) showed significant increases (p<0.05) in the proportion of cells entering necrosis and late apoptotic stages.in which compound (**1**) apoptotic impacts were significantly greater than intact others (Fig. 5).

Discussion

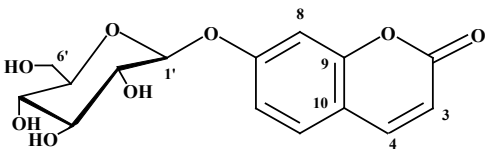
This study was planned to evaluate the biological effects of compounds isolated from *D. glabrum* seeds. To pursue



Diglucosyl caffeoyl ester (**1**)



4-O-β-D-glucopyranosylcaffeic acid (**2**)



Umbelliferone 7-O-β-D-glucoside (skimmin) (**3**)

Fig. 1. Structures of the isolated compounds from the methanol extract of *D. glabrum* seeds

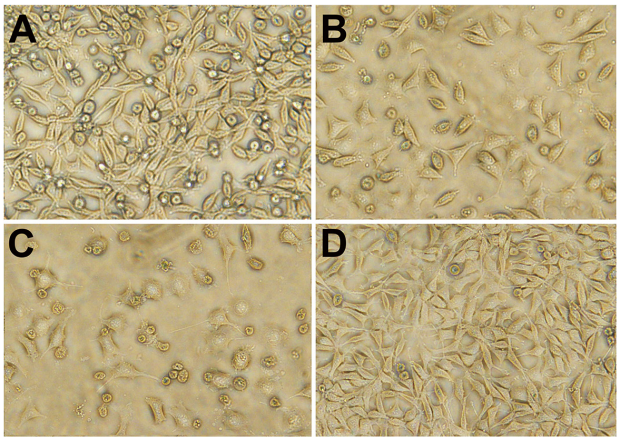


Fig. 2. CAOV-4 cell morphology after 48 hours treatments with 100µg/ml of (A) diglucosyl caffeoyl ester (**1**), (B) glucopyranosylcaffeic acid (**2**), (C) skimmin (**3**). Panel (D) shows morphology of untreated cells.

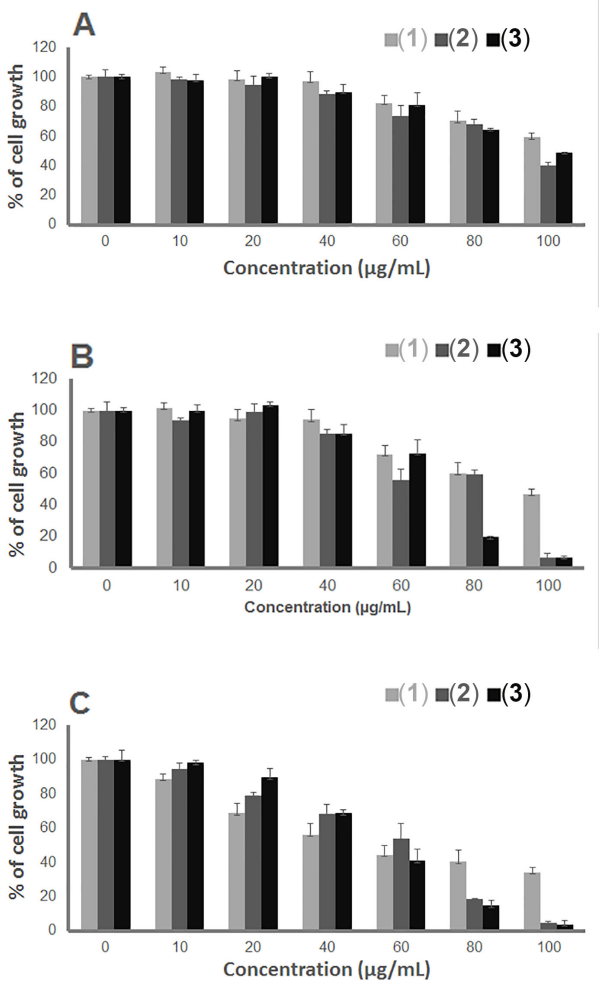


Fig. 3. Inhibition properties of compounds (**1**), (**2**) and (**3**) on CAOV-4 ovarian carcinoma. A, B and C shows MTT result of treatment of compounds after 24, 48 and 72 h, respectively. Error bars represent SD (n=3)

this aim, we extracted three compounds from the seeds of plant based on the bioassay-guided isolation platform and the purified compounds were elucidated by UV/ vis, ^{1}H and ^{13}C-NMR. Diglucosyl caffeoyl ester (**1**) is reported for the first time from the seeds of *Dorema glabrum* in this investigation, which has previously been isolated from the cranberries.[33] The appearance of two anomeric proton resonances at δ 5.58 (1H, d, *J*=7.3 Hz) and δ 4.56 (1H, d, *J*=7.5 Hz) revealed presence of two sugar moieties in the structure. Existence of signals at 83.31(C3′) and 70.34 (C2′), showed a β (1→3) linkage of two sugar moieties. Also, appearance of a clear carbon resonance at δ 176.01 approved a carboxyl group in its structure. The compound 1 was hydrolyzed and its sugar studied in the presence of reference sugar samples according to the published procedure.[34] Based on the TLC result, sugar moiety was glucose. Beside, two signals at 7.60 ppm and 6.32 ppm (1H, d, J=15.8,) that implied to a Trans unsaturated system, considering the rest of signals in aromatic region [7.08 (1H, d, *J*=2.1, H_2), 6.96 (1H, dd, *J*=8.1, 2.2 H_6), 6.81 (1H, d, *J*=8.1, H_5)], could led us to the caffoeyl structure. To the best of our knowledge, no pharmacological activity has been reported related to the compound 1. 4-O-β-D-glucopyranosylcaffeic acid (**2**) is also a rare phenolic compound. During our comprehensive literature review we only found an investigation that reported 2 from rhizoma of *Davallia mariesii* Moore in 1990.[35] Umbelliferone 7-O-β-D-glucoside (skimmin) (**3**) is a known simple coumarin derivative which was extracted previously from different species.[36-41] However, this is the first report regarding the presence of skimmin in the seeds of *D. glabrum*. Some limited pharmacological properties have been reported for the skimmin. Recently, Zhang et al showed that skimmin could down-regulate the TGF-beta1 and TGF-beta Receptor I expression and so could suppress diabetic nephropathy, and may slow down the renal fibrosis by regulating TGF-beta1 signal pathway.[42,43] There has very limited studies been reported anti-tumor activity of non-glucoside umbelliferone.[44] However, to the best of our knowledge, this study is first report on the anti-cancer activity of skimmin.

CAOV-4 cells were cultured in the presence of the compounds and were monitored under light microscope. Light microscopic observations illustrated that the treated and untreated cells have distinct morphologic differences. Chromatin condensation and cell shrinkage are the most important macroscopic morphological changes due to the compounds treatment after 48 h. The cytotoxicity effects of compounds were also studied in the CAOV-4 human ovarian adenocarcinoma cells. All examined compounds were able to inhibit CAOV-4 cells growth rate as well as doxorubicin in a dose-dependently. However, compound (**1**) exhibited significant influence on the proliferation of the cells in terms of the concentration which has been used (197 µM).

The interaction of plant-derived compounds with intercellular organelles (e.g., DNA and microtubules) and production of reactive intermediates in numerous cell lines have been described previously.[45] Comet assay as a

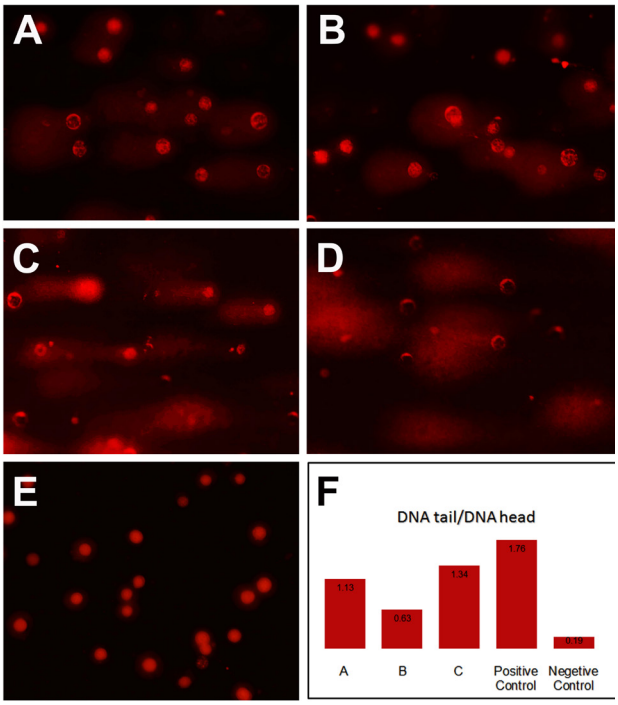


Fig. 4. Photographic illustrations of comet assay after 48 h incubation. A) compound (**1**), B) compound (**2**), C) compound (**3**), D) treated CAOV-4 cells by 200 μM H_2O_2 (positive control), E) untreated CAOV-4 cells, and F) shows bar graph of (DNA tail/DNA head); the DNA cleavage level in examined compound treated cells is not as high as treated cells with 200 mM H_2O_2 but is significantly (p<0.001) higher than untreated cells.

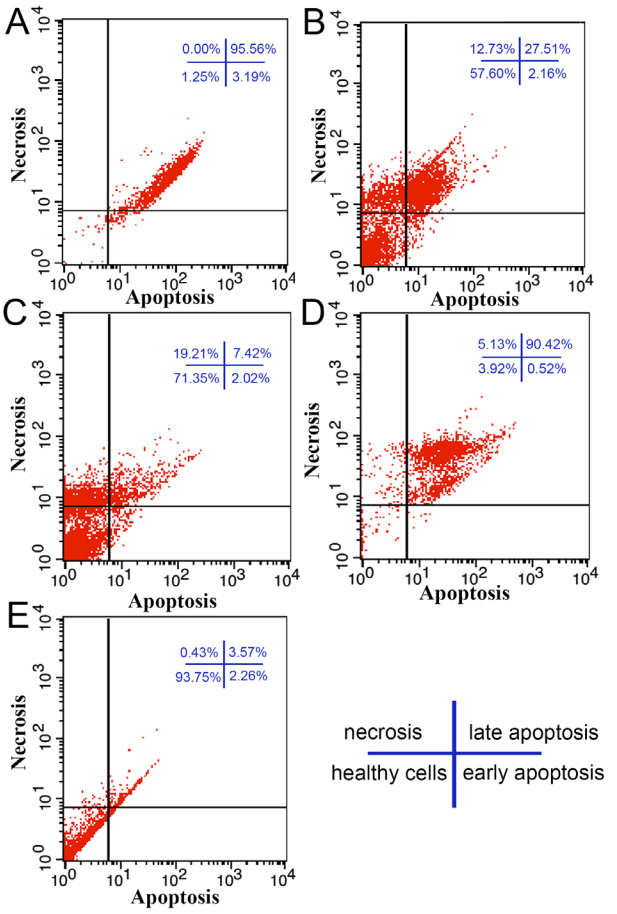


Fig. 5. FITC-labeled annexin V flow cytometric detection of apoptosis in CAOV-4 cells. A) Compound (**1**), B) compound (**2**), C) compound (**3**), D) treated CAOV-4 cells by 32μM of doxorubicin as positive control, E) untreated CAOV-4 cells. Treated cells with compounds (**2**) and (**3**) showed significant rises (p<0.05) in the proportion of necrotic and late apoptotic cells, in which compound (**1**) apoptotic impacts were significantly greater than intact others.

robust and powerful tools could detect the interaction of compound with heritage materials.[46] In this investigation the direct interaction of compounds was also examined using alkaline comet assay. Significant double strand breakage of DNA was observed in all groups by comet assay. According to the obtained results, significant double strand breakage of DNA was observed in all treatment groups by comet assay. However, insignificant differences (p>0.05) between CAOV-4 treated with all compounds were seen.

Finally, to validate the results obtained by genotoxicity assay (comet assay), we also exploited annexin V-FITC flow cytometry for detection of early and late apoptosis as reported previously by Bratton et al.[47] To clarify apoptosis occurrence, we followed translocation of phosphatidylserine (PS) from the inner layer of cell membrane to the external using FITC-labeled annexin V flow cytometry.[48] Results showed that treated cells with compounds (**2**) and (**3**) showed significant increases (p<0.05) in the proportion of cells entering necrosis and late apoptotic stages, whereas compound (**1**)'s apoptotic impacts were significantly greater than others. The results obtained from FACS revealed that compound (**1**) led to cell death mainly by activation of apoptosis pathways, whilst in the case of compounds (**2**) and (**3**) main mechanism of the cell death was through the necrosis pathway. Therefore, we speculate that compound (**1**) may have apoptotic properties at the treated dosage, and cell death prompted by the compound may be associated with activation of apoptosis pathways.

Conclusion

This research reported three potent anti-cancer phenolic compounds from the seeds of *D. glabrum* including diglucosyl caffeoyl ester, glucopyranosylcaffeic acid and skimmin for the first time. Also their cytotoxicity and apoptotic properties were evaluated on CAOV-4 cells using MTT assay and Annexin V staining, respectively. In addition, alkaline comet assay have been used to investigate the genotoxicity and DNA destructive properties of the compounds. Our results showed that diglucosyl caffeoyl ester (compound 1) apoptotic impacts were significantly greater than others, while the others (compounds 2 and 3) cause the cells entering necrosis.

Acknowledgments

This work was supported by Research Center for Pharmaceutical Nanotechnology (RCPN), Tabriz University of Medical Sciences (grant No: 90011, which is a part of PhD thesis No: 90/011/101/1).

Ethical issues

None to be declared.

Competing interests

The authors declare no competing interests.

References

1. WHO. The top 10 causes of death. Switzerland: WHO; **2014** [cited 2014]; Available from: http://www.who.int/mediacentre/factsheets/fs310/en/index2.html
2. Mozaffarian V. *Dictionary of Iranian plant names*. Tehran: Farhang Moaser; **2003**.
3. Rechinger K. Dorema. In: Hedge IC LJ, Rechinger KH, editors. *Flora Iranica*. Graz: Akademische Druck-und Verlagsanstalt; **1987**.
4. Pimenov M. Monografitcheskaya reviziya roda Dorema D. Don (Umbelliferae). *Biull Mosk Ova Ispyt Prir (Biol)* **1988**; 93: 76–90.
5. Mozaffarian V. *Flora of Iran*. Tehran: Publication of Research Institute of Forests and Rangelands; **2007.**
6. Amanzadeh Y. *Ammoniacum gum*. Editorial C, editor. tehran: Ministry of Health and Medical Education Publications; **2002**.
7. Rajani M, Saxena N, Ravishankara MN, Desai N, Padh H. Evaluation of the Antimicrobial Activity of Ammoniacum Gum from Dorema ammoniacum. *Pharm Biol* **2002**; 40: 534-41. doi: 10.1076/phbi.40.7.534.14686
8. Azarneushan F, Karami M, Golizadeh L, Davary K. The effect of Dorema aucheri-Hydroalcoholic extracts on thyroids hormones in adult male rats. *Journal of Shahrekord University of Medical Sciences* **2010**; 12: 84-8.
9. Khoshvaghti A, Valizadeh MR, Vasei M, Nazifi S, Akbarpour B. The Effects of Dorema aucheri Hydroalcoholic Extract on Blood Levels of Antioxidant Enzymes (SOD and GPX) and Vitamins (E and C) in vivo. *Journal of Faculty of Veternary Medicine Istanbul University* **2013**; 39: 230-7.
10. Sharifi A, Naghmachi M, Bahrami S. Antimicrobial Activities of *Dorema Auchri*. *Armaghan e Danesh* **2010**; 15: 378-87. [in Persian]
11. Ghollassi Mood S. A contribution to some ethnobotanical aspects of Birjand Flora (Iran). *Pak J Bot* **2008**; 40: 1783-91.
12. Yousefzadi M, Heidari M, Akbarpour M, Mirjalili MH, Zeinali A, Parsa M. In vitro Cytotoxic Activity of the Essential Oil of *Dorema ammoniacum* D. Don. *Middle-East J Sci Res* **2011**; 7: 511-4.
13. Shahidi G, Moein M, Foroumadi A, Rokhbakhsh Zamin F. Cytotoxic activity of medicinal plants used in Iranian traditional medicine on two strains of Saccharomyces cerevisiae. *DARU* **2002**; 10: 162-4.
14. Kumar VP, Chauhan NS, Padh H, Rajani M. Search for antibacterial and antifungal agents from selected Indian medicinal plants. *J of Ethnopharm* **2006**; 107: 182-8. doi: 10.1016/j.jep.2006.03.013
15. Govind P. Medicinal plants against liver diseases. *Int Res J Pharm* **2011**; 2: 115-21.
16. Ajani Y, Ajani A, Cordes JM, Watson MF, Downie SR. Phylogenetic analysis of nrDNA ITS sequences reveals relationships within five groups of Iranian Apiaceae subfamily Apioideae. *Taxon* **2008**; 57: 383-401.
17. Mir-Babayev NF, Houghton PJ. Plants of the Republic of Azerbaijan with Potential Medicinal Applications. Part III. *Pharmaceutical Biology* **2002**; 40: 16-22. doi: 10.1076/phbi.40.1.16.5863
18. Dehghan G, Fatholahi G, Sheikhzadeh N, Ahmadiasl N. Hypocholesteremic and antioxidant effects of Dorema glabrumextract in rats fed high cholesterol diet. *Journal of Iranian Chemical Society* **2009**; 6: 114-43.
19. Delnavazi MR, Hadjiakhoondi A, Delazar A, Ajani Y, Yassa N. Azerosides A and B: Two new phloroacetophenone glycosides from the roots of Dorema glabrum Fisch. & C.A. Mey. *Med Chem Res* **2014**; 1-10. doi: 10.1007/s00044-014-1138-2
20. Bannazadeh Amirkhiz M, Rashtchizadeh N, Nazemieh H, Abdolalizadeh J, Mohammadnejad L, Baradaran B. Cytotoxic effects of alcoholic extract of dorema glabrum seed on cancerous cells viability. *Adv Pharm Bull* **2013**; 3: 403-8. doi: 10.5681/apb.2013.064
21. Bannazadeh Amirkhiz M, Rashtchizadeh N, Nazemiyeh H, Abdolalizadeh J, Mohammadnejad L, Baradaran B. Investigating Apoptotic Effects of Methanolic Extract of Dorema glabrumSeed on WEHI-164 Cells. *ISRN Pharmacology* **2013**; 1-9. doi: 10.1155/2013/949871
22. Bukreeva TV, Pimenov MG. 2,6-Dihydroxy-4-methoxyacetophenone 2-O-β-D-gentiobioside from the roots of Dorema aitchisonii. *Chemistry of Natural Compounds* **1991**; 27: 638-9. doi: 10.1007/BF00630378
23. Nurmukhamedova MR, Nikonov GK. Glycosides of Dorema hyrcanum. *Chem Nat Comp* **1976**; 12: 92-3. doi: 10.1007/BF00570207
24. Iranshahi M, Shaki F, Mashlab A, Porzel A, Wessjohann LA. Kopetdaghins AE. Sesquiterpene Derivatives from the Aerial Parts and the Roots of Dorema kopetdaghense. *J Nat Prod* **2007**; 70: 1240-3. doi: 10.1021/np070043u
25. Masoudi S, Esmaeili A, Ali khalilzadeh M, Rustaiyan A, Moazami N, Akhgar MR, *et al.* Volatile constituents of Dorema aucheri Boiss., Seseli libanotis (L.) W. D. Koch var. armeniacum Bordz. and Conium maculatum L. three Umbelliferae herbs growing wild in Iran. *Flavour and Fragrance Journal* **2006**; 21: 801-4. doi: 10.1002/ffj.1722
26. Asnaashari S, Dadizadeh E, Talebpour AH, Eskandani M, Nazemiyeh H. Free Radical Scavenging Potential and Essential Oil Composition of the Dorema glabrum Fisch. C.A. Mey Roots from Iran. *Bioimpacts* **2011**; 1: 241-4. doi: 10.5681/bi.2011.035
27. Nazemiyeh H, Bahadori F, Delazar A, Ay M, Topcu G, Nahar L, *et al.* Antioxidant phenolic compounds from the leaves of Erica Arborea (Ericaceae). *Nat Prod Res* **2008**; 22: 1385-92. doi: 10.1080/14786410701824007
28. Eskandani M, Nazemiyeh H. Self-reporter shikonin-Act-loaded solid lipid nanoparticle: formulation, physicochemical characterization and geno/cytotoxicity evaluation. *Eur J Pharm Sci* **2014**; 59: 49-57. doi: 10.1016/j.ejps.2014.04.009
29. Eskandani M, Golchai J, Pirooznia N, Hasannia S. Oxidative stress level and tyrosinase activity in vitiligo patients. *Indian J Dermatol* **2010**; 55: 15-9. doi: 10.4103/0019-5154.60344
30. Singh NP, McCoy MT, Tice RR, Schneider EL. A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp Cell Res* **1988**; 175: 184-91.
31. Hamishehkar H, Khani S, Kashanian S, Ezzati Nazhad Dolatabadi J, Eskandani M. Geno- and cytotoxicity of propyl gallate food additive. *Drug Chem Toxicol* **2014**; 37: 241-6. doi: 10.3109/01480545.2013.838776
32. Vandghanooni S, Eskandani M. Comet Assay: A Method to Evaluate Genotoxicity of Nano-Drug Delivery System. *BioImpacts* **2011**; 1: 87-97.
33. Marwan AG, Nagel CW. Identification of the

Hydroxycinnamtic Acid Derivatives in Cranberries. *J Food Sci* **1982**; 47: 774-8. doi: 10.1111/j.1365-2621.1982.tb12712.x
34. Tom J. Mabry, K. R. Markham, Thomas MB. *The Systematic Identification of Flavonoids*. Verlag: Springer; **1970**.
35. Cui CB, Tezuka Y, Kikuchi T, Nakano H, Tamaoki T, Park JH. Constituents of a fern, Davallia mariesii Moore. I. Isolation and structures of davallialactone and a new flavanone glucuronide. *Chem Pharm Bull (Tokyo)* **1990**; 38: 3218-25.
36. Ding XF, Feng X, Dong YF, Zhao XZ, Chen Y, Wang M. Studies on chemical constituents of the roots of *Angelica pubescens*. *Journal of Chinese Medicinal Materials* **2008**; 31: 516-8.
37. Dawa ZM, Zhou Y, Bai Y, Gesang SL, Xie P, Ding LS. Studies on chemical constituents of *Saussurea laniceps*. *China Journal of Chinese Materia Medica* **2008**; 33: 1032-5.
38. Feng BM, Gong XJ, Shi LY, Jiang G, Pei YH, Wang YQ. Studies on phenolic compounds from *Stellera chamaejasme*. *China Journal of Chinese Materia Medica* **2008**; 33: 403-5.
39. Brown D, Asplund RO, McMahon VA. Phenolic constituents of Artemesia tridentata spp. Vaseyana. *Phytochemistry* **1975**; 14: 1083-4. doi: 10.1016/0031-9422(75)85191-0
40. Petruľová-Poracká V, Repčák M, Vilková M, Imrich J. Coumarins of Matricaria chamomilla L.: Aglycones and glycosides. *Food Chem* **2013**; 141: 54-9. doi: 10.1016/j.foodchem.2013.03.004
41. Reisch J, Achenbach SH. A Furanocoumarin glucoside from stembark of Skimmia japonica. *Phytochemistry* **1992**; 31: 4376-7. doi: 10.1016/0031-9422(92)80484-V
42. Zhang S, Xin H, Li Y, Zhang D, Shi J, Yang J*, et al.* Skimmin, a Coumarin from Hydrangea paniculata, Slows down the Progression of Membranous Glomerulonephritis by Anti-Inflammatory Effects and Inhibiting Immune Complex Deposition. *Evid Based Complement Alternat Med* **2013**; 2013: 819296. doi: 10.1155/2013/819296
43. Zhang S, Yang J, Li H, Li Y, Liu Y, Zhang D*, et al.* Skimmin, a coumarin, suppresses the streptozotocin-induced diabetic nephropathy in wistar rats. *Eur J Pharmacol* **2012**; 692: 78-83. doi: 10.1016/j.ejphar.2012.05.017
44. Yang XW, Xu B, Ran FX, Wang RQ, Wu J, Cui JR. [Inhibitory effects of 11 coumarin compounds against growth of human bladder carcinoma cell line E-J in vitro]. *Zhong Xi Yi Jie He Xue Bao* **2007**; 5: 56-60.
45. Kuptsova N. *Pharmacogenetics of the DNA Repair and Oxidative Stress Pathways in Outcomes of Cancer Patients*. ProQuest; **2008**.
46. Eskandani M, Hamishehkar H, Ezzati Nazhad Dolatabadi J. Cyto/Genotoxicity study of polyoxyethylene (20) sorbitan monolaurate (tween 20). *DNA Cell Biol* **2013**; 32: 498-503. doi: 10.1089/dna.2013.2059
47. Bratton SB, Salvesen GS. Regulation of the Apaf-1–caspase-9 apoptosome. *Journal of Cell Science* **2010**; 123: 3209-14.
48. Eskandani M, Abdolalizadeh J, Hamishehkar H, Nazemiyeh H, Barar J. Galbanic acid inhibits HIF-1α expression via EGFR/HIF-1α pathway in cancer cells. *Fitoterapia* **2015;** 101: 1-11. doi: 10.1016/j.fitote.2014.12.003