

Cytotoxic Effects of *Rhododendron ponticum* L. Extract on Prostate Carcinoma and Adenocarcinoma Cell Line (DU145, PC3)

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Abstract

Rhododendron species, having been used in traditional medicine for the treatment of inflammation, pain, cold, asthma, skin and gastro-intestinal disease, are distributed widely around the world. Mad honey obtained from the nectar of common rhododendron (*Rhododendron ponticum* L.), which is distributed throughout Black Sea region of Turkey, contains grayanotoxins, which are toxic diterpenes. The grayanotoxins, mostly grayanotoxins I and III, which are present in leaves, flowers and nectar responsible for toxicity of mad honey. The aim of this study was to investigate *in vitro* cytotoxic effects of the extract on prostate carcinoma cell lines. During the flowering period of common rhododendrons gathered from the Altınordu District of Ordu and dried under suitable conditions, extracted with distilled water and lyophilized. The cytotoxic activity of the extract of common rhododendron against human prostate carcinoma (DU145) and human prostate adenocarcinoma (PC3) cell lines by using the MTT and Neutral Red assay was evaluated. It was determined that the extract of common rhododendron had a dose-dependent cytotoxic effect. IC₅₀ of the extract was found to be 283.3 and 169.9 µg/mL in MTT assay, and 307.6 and 346.0 µg/mL in Neutral Red assay for DU145 and PC3 respectively. The fact that common rhododendron has cytotoxic effects on prostate carcinoma cells suggests that it may be a potential therapeutic agent for anticarcinogenic activity.

Keywords: Cancer cell line, Cytotoxic effect, DU145, PC3, *Rhododendron ponticum* L.

Rhododendron ponticum L. Extractinin Prostat Karsinomu ve Adenokarsinoma Hücre Hatları (DU145, PC3) Üzerindeki Sitotoksik Etkileri

Öz

Yangı, ağrı, soğuk algınlığı, astım, deri ve gastrointestinal hastalıkların tedavisinde geleneksel olarak kullanılan Ormangülü türleri (*Rhododendron*) dünya çapında yaygın olarak dağılım göstermektedir. Türkiye'nin Karadeniz bölgesinde dağılmış Ormangülünün (*Rhododendron ponticum* L.) nektarından elde edilen delibal, toksik diterpen olan grayanotoksinleri içerir. Delibalin toksisitesinden sorumlu olan grayanotoksinler, çoğunlukla grayanotoksinler I ve III, yapraklarda, çiçeklerde ve nektarlarda bulunur. Bu çalışmanın amacı, orman gülü ekstraktının prostat karsinom hücre hatları üzerine *in vitro* sitotoksik etkilerini araştırmaktır. Çiçeklenme döneminde Ordu Altınordu ilçesinden toplanan orman gülü, uygun koşullar altında kurutuldu, distile su ile ekstrakte ve sonrasında liyofilize edildi. İnsan prostat kanseri (DU145) ve insan prostat adenokarsinoması (PC3) hücre hatlarına karşı orman gülü ekstraktının sitotoksik etkinliği MTT ve Nötral Kırmızı testleri ile değerlendirildi. Orman gülü ekstraktının doza bağımlı bir sitotoksik etki yaptığı tespit edildi. Ekstraktın IC₅₀'si, DU145 ve PC3 için MTT analizinde, sırasıyla 283.3 ve 169.9 µg/mL ve Nötral kırmızı analizinde, sırasıyla 307.6 ve 346.0 µg/mL olarak bulundu. Orman gülü, prostat karsinom hücreleri üzerinde sitotoksik etkilere sahip olması, antikanserojenik aktivite için potansiyel bir terapötik madde olabileceğini düşündürmektedir.

Anahtar sözcükler: DU145, Kanser hücre hattı, PC3, *Rhododendron ponticum* L., Sitotoksik etki



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INTRODUCTION

Rhododendron species occur in virtually every inhabited part of the world [1], especially, southwestern China, islands between Asia and Australia, northern Turkey, southern Europe, southern India, eastern, northern and western North America [2]. Two dominant species of *Rhododendron* are yellow-flowered rhododendron (*R. flavum* Don.) and purple-flowered rhododendron (*Rhododendron ponticum* L.) in the western and eastern Black Sea Region, respectively [3]. *Rhododendron ponticum* is extensively naturalized in the British Isles. It is a native of the area to south of the Black Sea [1]. Purple-flowered rhododendron, also called common rhododendron, is native to the Black Sea Region forests of Turkey [3]. Besides *R. ponticum*, there are four other *Rhododendron* species growing naturally in Turkey, including *R. ungueri*, *R. luteum*, *R. caucasicum*, and *R. smirnovii* [4,5]. *Rhododendron ponticum* and *R. luteum* are used to produce "Mad honey" by honeybees from the nectar of the plants. Mad honey consumed by humans and young leaves or flowers of these species consumed by livestock causes intoxications in Turkey [6-10].

Rhododendron species, growing naturally in Turkey, contain non-terpenic hydrocarbons, alcohols, esters and ketones as the major components. H₂O extracts of these plants are very poor in volatiles and has only traces of some organics [9]. *Rhododendron* family contains more than 750 plant species, most of which contain grayanine type tetracyclic diterpenes (grayanotoxins = andromedotoxins) [11]. The medical use of *Rhododendron* genus is limited due to its grayanotoxin (GTX) content all over the world. Some species of the genus are used alternative medicine to treat muscle, skin, lung, bowel, gastric and metabolic diseases in Turkey and China, although they contain GTXs [5,12-15]. Mad honey obtained from these plants, is used as a sexual stimulant in Anatolia [6]. Flavonoids in the plants are mainly responsible for analgesic, anti-inflammatory, anti-diabetic activities, while GTXs, a class of toxic diterpenoids, create toxic, insecticidal, and cytotoxic effects [6,12,16]. Main cytotoxic mechanism of the GTXs is carried out interfering with the transmission of the action potential by blocking sodium channels in excitable cell membranes. Thus, excitable nerve and muscle cells are maintained in a state of depolarization [6,7,10]. GTX I is the most toxic and has been reported to possess *in vitro* cytotoxicity and/or selectivity towards cancerous hepatoma and leukemia cells [17]. However, very little is known about the mechanism of action associated with the cytotoxic effect [12]. *Rhododendron brachycarpum* extract showed anticancer activity on human cancer cell lines (A549, AGS, Hep3B, MCF7) in the MTT assay [18]. Recently, it has been demonstrated that *R. luteum* has anti-proliferative effects on human hepatocellular carcinoma (HepG2) and colon adenocarcinoma (WiDr) [5].

The purpose of this study was to determine the cyto-

toxic effects of *R. ponticum* L. extracts collected from Ordu province of Turkey on prostate carcinoma and adenocarcinoma cell lines (DU145, PC3).

MATERIAL and METHODS

Sample Collection

During the flowering period of common rhododendrons, the samples were gathered from the Altinordu District (Saraycik) of Ordu province in Black Sea Region of Turkey (Fig. 1). Voucher No: 60522 (Herbarium of Ankara University, Faculty of Science, Department of Biology, Ankara, Turkey) (Fig. 2).

Plant Material and Preparation of Distilled Water (dH₂O) Extract

The plant was identified immediately after collection. It was dried under suitable conditions and then separated into leaf and flower (Fig. 2). The flowers were powdered by milling and extracted using the maceration method with distilled water (5 g/100 mL). The extract was first filtered through a 0.22 µm syringe filters (Sartorius Minisart® RC15 Syringe Filter 17761). The aqueous extract was lyophilized (Alpha1-2 LD Christ) to yield a crude aqueous extract [15]. The lyophilized extracts were stored and packed in freezer bags at -20°C until tested.

Cell Line and Cell Culture

DU145 (ATCC® HTB-81 TM) and PC3 cells (ATCC® CRL-1435TM) were maintained, cells were placed into 75 cm² tissue culture flasks (BD Falcon, Rockville, MD, USA), and grown at 37°C under a humidified 5% CO₂ atmosphere in RPMI 1640 medium (Gibco®) with 2-mM L-glutamine, 10% fetal bovine serum, and 1% penicillin-streptomycin (10.000 U/mL penicillin and 10 mg/mL streptomycin) (Thermo Fisher Scientific, Waltham, MA, USA).

Cell Viability Assay

Cell viability was measured using thiazolyl blue (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide; MTT) and Neutral Red (NR) assays. Cells were seeded in 96-well plates (3x10⁵ cell/mL) plates and cultured overnight. Cells were treated with various concentrations of rhododendron's lyophilized extract (25, 50, 100, 250, 500, 1000, 1250 and 2500 µg/mL in RPMI) and the medium only and 0.1% Triton X-100 served as negative and positive controls for 24 h. Each concentration was tested in triplicate. Stock solutions for MTT and Neutral Red were prepared at a concentration of 5 mg/mL in PBS and 40 µg/mL in DMEM. MTT and NR test working solutions (15 µL for MTT, 100 µL for NR) were added to each well for 2 h at 37°C. After treatment, the solution was removed and Neutral red assay plates were washed with PBS. Then, they were incubated with 100 µL of a solubilizing solution (DMSO for MTT, Destain for NR) at 37°C overnight. Cell viability was

measured at 540 nm wavelength using the SpectraMax i3/i3x Multi-Mode Detection Platform (Molecular Devices, Sunnyvale, California, USA.).

The untreated cell control was set to 100% viability (MaxV). The dead cell control applied Triton-X was set to viability (MinV). The degree of cytotoxicity of the extract treated cells was expressed as percentage of the untreated cell control. A plot of cytotoxicity versus sample concentrations was used to calculate the concentration, which showed 50% cytotoxicity (IC_{50})^[19].

Formulation used in calculation has been indicated as below:

$$\text{Cytotoxicity (\%)} = [1 - (\text{test} - \text{MinV}) / (\text{MaxV} - \text{MinV})] \times 100$$

Statistics

All study data were obtained from three independent experiments. Results were expressed as means \pm SD of

replicates. IC_{50} values were calculated using the GraphPad Prism software, version 7.03, from linear regression analysis.

RESULTS

Evaluation of Cytotoxicity Using the MTT and NR Assays

In our previous study, we found that the extract used in this study contained 55.75 $\mu\text{g}/\text{kg}$ GTX I and 7.4 $\mu\text{g}/\text{kg}$ GTX III^[15]. In this study, it was determined that the extracts containing GTX I and GTX III had a dose-dependent cytotoxic effect on prostate carcinoma and adenocarcinoma cell lines.

To determine the cytotoxic activities of *R. ponticum* L. extract, an *in vitro* assay was performed using human prostate carcinoma (DU145) and human prostate adenocarcinoma (PC3). The 50% inhibitory concentration (IC_{50}) of the extract was found to be 283.3 $\mu\text{g}/\text{mL}$ in MTT assay and



Fig 1. The display of Black Sea provinces belonging to *R. ponticum* L. habitat and the Altınordu District of Ordu province in current study on the map of Turkey



Fig 2. *R. ponticum* L. gathered from the Altınordu District

307.6 $\mu\text{g}/\text{mL}$ in Neutral Red assay for DU145 cell line and 169 $\mu\text{g}/\text{mL}$ in MTT assay and 346 $\mu\text{g}/\text{mL}$ in Neutral Red assay for PC3 cell line. The IC_{50} values demonstrated that the *R. ponticum* L. extract exhibited cytotoxic effect on the Prostate cells (Fig. 3, 4, 5).

PC3 cells incubated 24 h with 25, 50, 100, 250, 500, 1000, 1250, 2500 $\mu\text{g}/\text{mL}$ *R. ponticum* L. extract the cell viability percentages were found 86.91%, 82.59%, 66.47%, 50.18%, 43.94%, 38.32%, 29.54%, and 20.45% in NR assay, 68.07%, 62.10%, 53.98%, 42.94%, 40%, 33.86%, 29.05% and 24.45% in MTT assay, respectively. DU145 cells incubated 24 h at dosages of 25, 50, 100, 250, 500, 1000, 1250, 2500 $\mu\text{g}/\text{mL}$ *R. ponticum* L. extract the cell viability percentages were found 93.41%, 84.68%, 72.40%, 65%, 41.09%, 28.56%, 20% and 1.39% in NR assay, 96.88%, 82.04%, 68.91%,

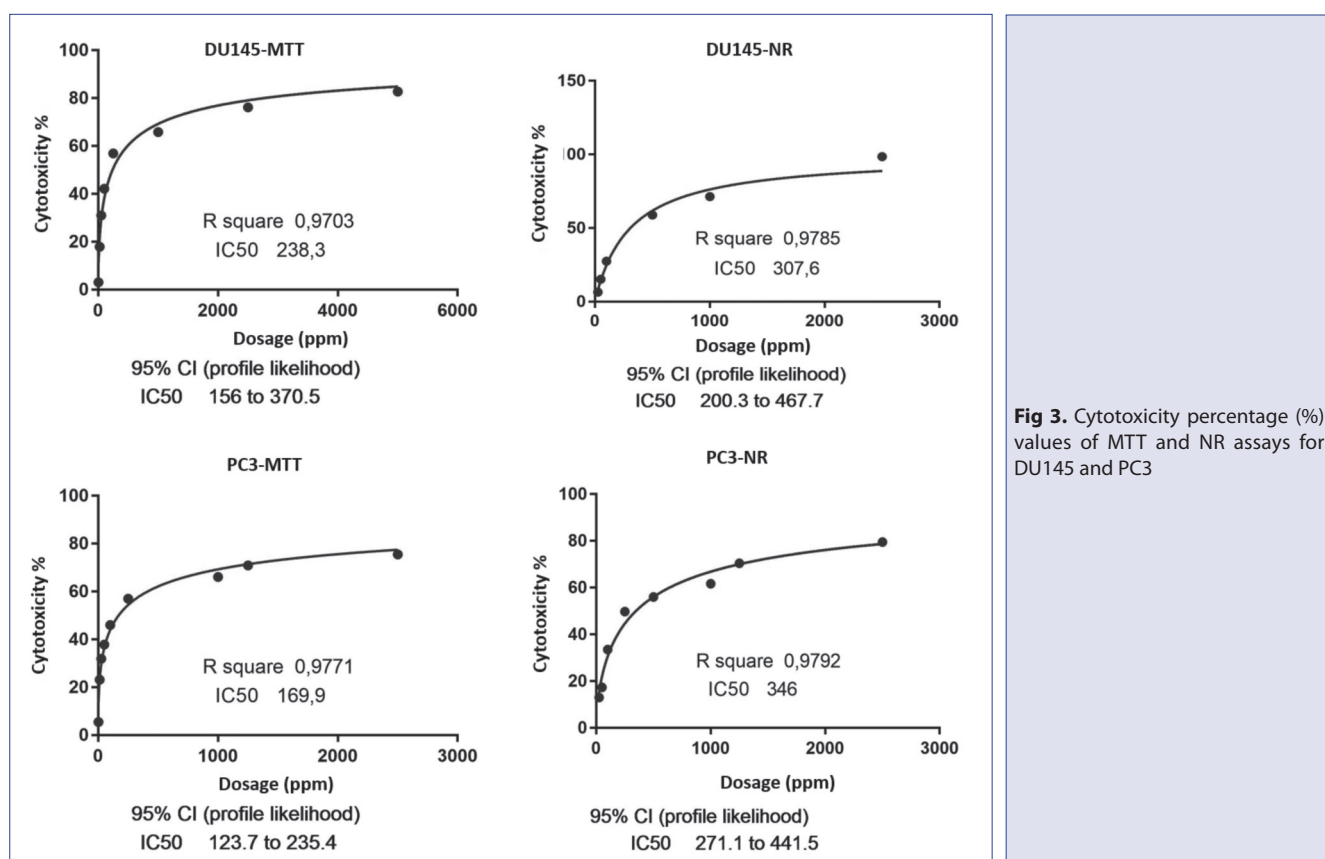


Fig 3. Cytotoxicity percentage (%) values of MTT and NR assays for DU145 and PC3

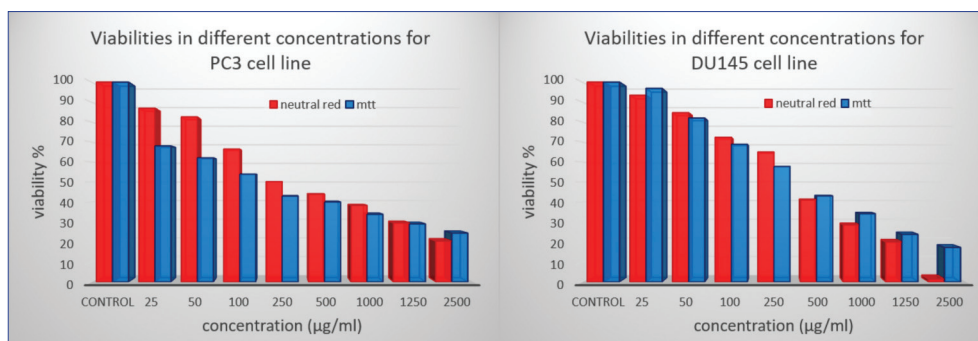


Fig 4. MTT and NR assays are measure of mitochondrial and lysosomal activities, respectively. According to MTT and NR assays, the common rhododendrons were more effective on the mitochondrial pathways at lower doses and lysosomal pathways at higher doses in PC3 and DU145 cell lines. In the NR and MTT assays, cell viability was reduced as a concentration of 2500 µg/mL was approached in PC3 and DU145 cell lines, but the reduction was more pronounced in NR in DU145 cell line

57.80%, 43.05%, 34.15%, 23.82%, and 17.24% in MTT assay, respectively (Fig. 4, Table 1).

DISCUSSION

Plants has been shown to be an excellent source of new drugs, throughout medical history. They have always been useful sources of antitumor compounds [20,21]. Currently, most of the used anticancer drugs are derived from natural sources, including plants [21,22]. There is an increasing interest in the anti-proliferative properties of natural products because they are believed to be

low toxic effects on mammals and are widely used as traditional medicines in the world [13,23]. They are used against inflammation, pain, skin diseases, common fever, and gastrointestinal system disorders in the traditional medicines of Asian, North American, and European countries [12,16]. The extracts of some *Rhododendron* species and some isolated bioactive compounds reportedly possess some anti-proliferative activities [5,18,24-26].

In studies on anticancer effects of *Rhododendron* species, *R. luteum* exhibited anti-proliferative effects on human hepatocellular carcinoma (HepG2) and colon adeno-

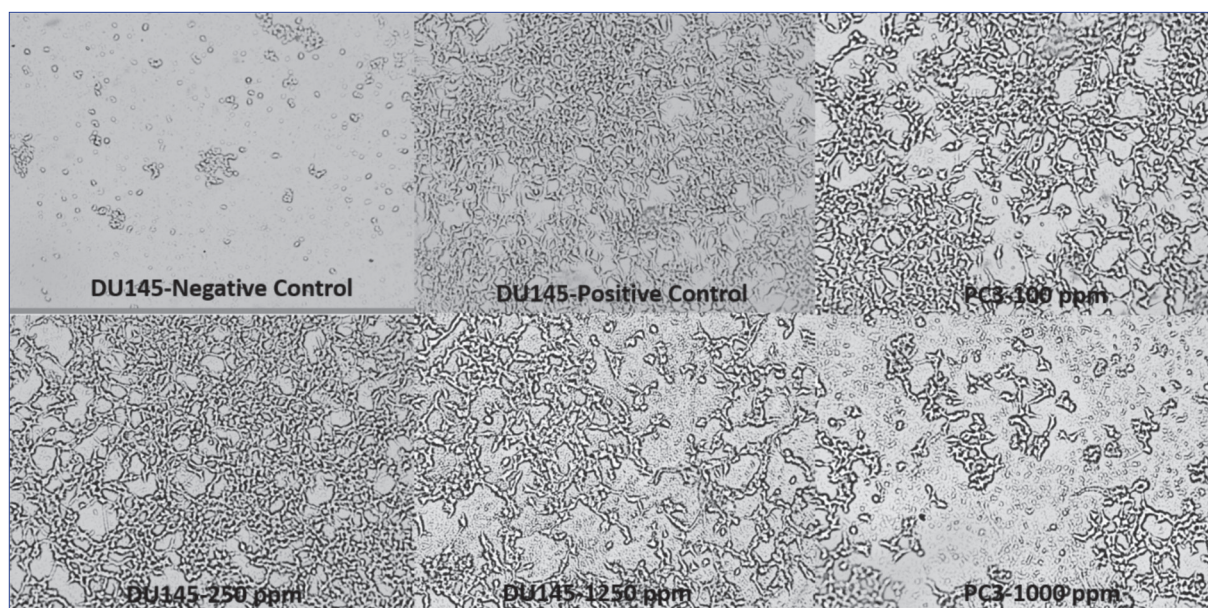


Fig 5. Images of cells at different doses of the common rhododendron extracts in PC3 and DU145 cell lines

Table 1. Percent of cell viability (PC3 and DU145 cell lines)

Dose ($\mu\text{g/mL}$)	PC3		DU145	
	MTT	NR	MTT	NR
25	68.07 \pm 14.29	86.91 \pm 5.93	96.88 \pm 8.19	93.41 \pm 13.28
50	62.10 \pm 10.09	82.59 \pm 3.24	82.04 \pm 0.72	84.68 \pm 2.05
100	53.98 \pm 6.52	66.47 \pm 4.11	68.91 \pm 2.46	72.40 \pm 4.13
250	42.94 \pm 9.79	50.18 \pm 7.35	57.80 \pm 2.78	65.00 \pm 16.09
500	40.00 \pm 6.91	43.94 \pm 5.72	43.05 \pm 5.62	41.09 \pm 12.31
1000	33.86 \pm 8.70	38.32 \pm 12.01	34.15 \pm 10.21	28.56 \pm 16.62
1250	29.05 \pm 5.29	29.54 \pm 4.66	23.82 \pm 15.63	20.00 \pm 2.50
2500	24.45 \pm 7.62	20.45 \pm 2.88	17.24 \pm 17.39	1.39 \pm 14.02
Mean \pm SD				

carcinoma (WiDr) cancer lines [5]. *Rhododendron ponticum* L. exhibited antiproliferative effects on rat glioma cell line (F98) [15]. *Rhododendron formosanum* Hemsl. exhibited antineoplastic potential against non-small cell carcinoma (NSCLC) [24]. Another *Rhododendron* species, *R. brachycarpum* exerted anticancer activity on human cancer cell line such as A549, AGS, Hep3B, and MCF-7 [18]. Grayanotoxins (GTXs), which are the most actively tetracyclic diterpenoid compounds of *Rhododendron* species, are believed to be responsible for anticancer activity. The effect is more related to the ability in induction of apoptosis or of cell cycle arrest in cancer cells [12,24,25,27]. Grayanotoxins also lead to the inhibition of all catalytically active mammalian carbonic anhydrase (CA, EC 4.2.1.1) isoforms, especially on cytosolic isoforms CA I and II [28]. CAs is known to be almost expressed in all living organisms and sixteen their isoforms have been identified in mammals, many of which are involved in a wide range of physiological processes. These isoforms are important

drug target in some human disease due to their potential effects on the processes [29].

In our previous study, GTX I and GTX III levels were determined in the extract of *R. ponticum* L. It has been found that the extract contains 55.75 $\mu\text{g/kg}$ GTX I and 7.4 $\mu\text{g/kg}$ GTX III [15]. The extract was able to inhibit the proliferation of DU145 cells with IC_{50} value of 283 $\mu\text{g/ml}$ and PC3 cells with value of 169 $\mu\text{g/mL}$ after exposure time of 24 h. However, according to the criteria of the American National Cancer Institute (NCI), the limit of activity for crude extracts at IC_{50} of proliferation is less than 30 $\mu\text{g/mL}$ after exposure time of 72 h [30]. In this study, the values were found to be higher than that specified by NCI. In a study, *R. ponticum* was extracted with different solvents. Acetone extract showed the highest percentage of cell death (91.18%). The percentage of cell death in water extract was found to be lower (7.76%) [26]. This may be due to exposure time being 24 h and extraction being by water in this study.

The extract showed cytotoxic effects on DU145 and PC3 prostate cells, possibly by reduced activity in mitochondria. The degeneration was mostly of a hydropic one and/or involved acute cell swelling. Hydropic degeneration and acute cell swelling are known to be more related with impaired cellular oxygenation^[31]. When cellular oxygen is decreased, mitochondrial oxidative phosphorylation stops, and the cells switch to anaerobic metabolism or die. The early events in acute cell swelling are potentially reversible, but if the cell fails to restore mitochondrial function, acute cell swelling becomes irreversible, leading to cell necrosis^[32]. Also, CA IX and CA XII have important roles in tumorigenesis such as regulation of pH inside and outside the tumor cells, inhibition of phosphorylation of various proteins in cancer cells^[28]. That CAs are important for survival in hypoxic tumors and that inhibition of CAs by the GTXs may have negatively affected survival in tumor cells in this study.

Our study for the first time demonstrated the cytotoxic effect of the *R. ponticum* L. extract on the cancer cell lines. In the NR test, GTX I and III found in *R. ponticum* extract reduced activity in mitochondria, which is the respiratory center of the cell, so it was obvious that acute cell swelling in DU145 and PC3 prostate cells occurred due to disruption of cellular oxygenation in the cells. According to the current results, *R. ponticum* may be a new and promising natural therapeutic agent for treatment of several cancers. Prostate cancer, the second leading cause of malignancy-related death, is one of the most common health problems in men^[33]. Particularly, the antiproliferative effect on prostate cells might be a new remedy for cure. However, further *in vivo* experimental models and investigations into mechanism of molecular action are needed to confirm these anticarcinogenic activities.

CONFLICT OF INTERESTS

The study has not been supported by any organization. The authors declare no conflict of interests.

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