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

Emine Kübra BİLİR 1  Hidayet TUTUN 2,3  Sedat SEVİN 1  Görkem KİSMALI 1  Ender YARSAN 1

1 Ankara University, Faculty of Veterinary Medicine, Department of Pharmacology and Toxicology, TR-06110 Ankara - TURKEY  
2 Mehmet Akif Ersoy University, Faculty of Veterinary Medicine, Department of Pharmacology and Toxicology, TR-15030 Burdur - TURKEY  
3 Ankara University, Faculty of Veterinary Medicine, Department of Biochemistry, TR-06110 Ankara - TURKEY

Article Code: KVFD-2017-19219  Received: 03.12.2017  Accepted: 13.03.2018  Published Online: 14.03.2018

How to Cite This Article


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 Altinordu 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.
**INTRODUCTION**

*Rhododendron* species occur in virtually every inhabited part of the world [10], 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 [9]. *Rhododendron ponticum* is extensively naturalized in the British Isles. It is a native of the area south of the Black Sea [11]. 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. ungerii*, *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 grayanane type tetracyclic diterpenes (grayanatoxins = andromedotoxins) [11]. The medical use of *Rhododendron* genus is limited due to its grayanane (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) [9].

The purpose of this study was to determine the cytotoxic 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 (Saraycık) 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® RL-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 (IC50) [19].

Formulation used in calculation has been indicated as below:

\[
\text{Cytotoxicity (\%) = } \left[1 - \frac{\text{test} - \text{MinV}}{\text{MaxV} - \text{MinV}}\right] \times 100
\]

Statistics

All study data were obtained from three independent experiments. Results were expressed as means±SD of replicates. IC50 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 μg/kg GTX I and 7.4 μg/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 (IC50) of the extract was found to be 283.3 μg/mL in MTT assay and 307.6 μg/mL in Neutral Red assay for DU145 cell line and 169 μg/mL in MTT assay and 346 μg/mL in Neutral Red assay for PC3 cell line. The IC50 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 μg/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 μg/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%,
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 [11,23]. 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 adenocarcinoma (SW480) cell lines. The extracts of *R. ponticum* had cytotoxic effects on DU145 and PC3 cell lines. 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. The extracts of *R. ponticum* had cytotoxic effects on DU145 and PC3 cell lines.
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].

Grayanatoxins (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]. Grayanatoxins 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 μg/kg GTX I and 7.4 μg/kg GTX III [15]. The extract was able to inhibit the proliferation of DU145 cells with IC₅₀ value of 283 µg/ml and PC3 cells with value of 169 µg/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 IC₅₀ of proliferation is less than 30 µg/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 related death, is one of the most common health problems. Prostate cancer, the second leading cause of malignancy-

natural therapeutic agent for treatment of several cancers. According to the current results, R. ponticum extract on the cancer cell lines. 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.

**REFERENCES**


