

Cytotoxic Effect of *Rhododendron luteum* Leaf Extract on Human Cancer Cell Lines

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ABSTRACT

Rhododendron luteum belongs to the genus *Rhododendron*, members of which are frequently used in folk medicine. Various studies have investigated the cytotoxic effect of different *Rhododendron* species, but there have been limited studies of the cytotoxic effect of *R. luteum*. The aim of this study was to investigate the antioxidant properties and cytotoxic effect of *R. luteum* leaf extract on the human cancer cells. The total phenolic content (TPC), total flavonoid content (TFC), and reducing power of the extract were evaluated using spectrophotometric procedures. The cytotoxic effect of the extract on five cancers (human breast, colon, lung, prostate, and liver carcinoma) and human fibroblast cells was determined using the MTT assay. TPC and reducing power values of extract were found 173.2±3.97 mg gallic acid equivalents, and 629.5±3.49 mg trolox equivalents per g sample, respectively. *R. luteum* leaf extract exhibited selective cytotoxicity especially against colon (1.9 fold) and liver (2.2 fold) cancer cells compared to normal fibroblast cells. This is the first study to reveal the cytotoxic effect of *R. luteum* leaf extract. Further studies are now needed to identify the cytotoxic molecules in the extract and their mechanisms.

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Rhododendron luteum Yaprak Ekstraktının İnsan Kanser Hücre Serilerindeki Sitotoksik Etkisi

ÖZET

Rhododendron luteum, *Rhododendron* cinsine dahil bir bitki olup, bu cinse ait bitkiler geleneksel tedavide sıklıkla kullanılmaktadır. *Rhododendron* türlerinin sitotoksik etkilerini araştıran çok sayıda çalışma olmasına rağmen, *R. luteum* türünün sitotoksik etkilerini inceleyen çalışmalar sınırlıdır. Bu çalışmanın amacı *R. luteum* yaprak ekstraktının antioksidan özelliklerini ve insan kanser hücreleri üzerindeki sitotoksik etkisini araştırmaktır. Ekstraktın toplam fenolik içeriği (TPC), toplam flavonoid içeriği (TFC) ve indirgeyici gücü spektrofotometrik prosedürler kullanılarak değerlendirildi. Ekstraktın beş kanser (insan meme, kolon, akciğer, prostat ve karaciğer karsinomu) ve insan fibroblast hücreleri üzerindeki sitotoksik etkisi MTT analizi kullanılarak belirlendi. Ekstraktın TPC ve indirgeyici güç değerleri g örnek başına sırasıyla 173.2±3.97 mg gallik asit eşdeğeri ve 629.5±3.49 mg troloks eşdeğeri olarak bulundu. *R. luteum* yaprak ekstraktı normal fibroblast hücrelerine kıyasla özellikle kolon (1.9 kat) ve karaciğer (2.2 kat) kanser hücrelerine karşı seçici sitotoksik etki sergiledi. Bu çalışma *R. luteum* yaprak ekstraktının sitotoksik etkisini ortaya koyan ilk çalışmadır. Ekstraktta bulunan sitotoksik moleküllerin tanımlanması ve etki mekanizmalarının belirlenmesi için daha kapsamlı çalışmalara ihtiyaç vardır.

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Araştırma Makalesi

INTRODUCTION

Cancer is one of the leading causes of morbidity and mortality worldwide (Yuan et al., 2017). According to a recent report by the World Health Organization (WHO), the average number of newly diagnosed cancer cases in the world exceeds 14 million per year, resulting in more than 60% deaths (8.8 million in 2015) (Russo et al., 2017). In the United States alone, approximately 1,688,780 new cancer cases and 600,920 deaths were reported in 2017 (Yuan et al., 2017). Early diagnosis and prevention of cancer has therefore become one of the priorities for governments, healthcare institutions and international organizations in all countries (Russo et al., 2017). Although billions of dollars are spent on cancer research every year, unfortunately the exact cause of cancer is not known yet. Normal cells have the ability to repair most of the DNA damage which can cause mutation. However, irreparable DNA damage causes cells to grow uncontrollably over time. It is suggested that the formation of cancer cells is caused by the deterioration of the cell turnover balance in the body. The removal of this imbalance is the main purpose of all cancer therapies (Prakash et al., 2013). In addition to surgery and radiation therapy, chemotherapy is a widely used method for the clinical treatment of cancer. Chemotherapeutic drugs can kill cancer cells that multiply rapidly, but these drugs can also damage normal cells and cause high rates of complications. Despite the significant advances recorded in chemotherapy, survival rates are not satisfactory due to drug resistance that can develop in cancer cells over time (Yuan et al., 2017).

Plants have been used as natural medicines by humans from ancient times. It is estimated that the vast majority of the world's population is resorting to traditional medicines to treat primary health problems. Plant extracts and plant derived active ingredients have an important place in the traditional treatment applied (Prakash et al., 2013). It is emphasized that beneficial activities resulting from antioxidant properties of medical plants can be used to reduce toxic side effects of radiotherapy and chemotherapy used in cancer treatment. In addition, the use of secondary herbal metabolites is now questioned in the treatment of cancer (Nema et al., 2013). *Rhododendron* is one of the largest vascular plants and covers most of the Northern Hemisphere (Popescu and Kopp, 2013). The genus *Rhododendron* pertains to the *Ericaceae* family of plants and includes more than 1000 species (Demir et al., 2016a). *Rhododendron* species are used as an alternative medicine in the treatment of various diseases, such as stomach ailments, gonorrhoea, spasm, eczema, diarrhea, dysentery, arthritis, bronchitis and hypertension (Lin et al., 2014; Demir et al., 2016a). *Rhododendron* species are reported to be rich in terpenoids, saponins, alkaloids, tannins, and phenolic compounds (Popescu and Kopp, 2013). Antioxidant,

antimicrobial, anti-inflammatory, analgesic, immunomodulator, antidiabetic, hepatoprotective, and cytotoxic effects of *Rhododendron* species have been shown in previous studies (Yaylaci et al., 2007; Qiang et al., 2011; Popescu and Kopp, 2013; Demir et al., 2016a). Several studies have investigated the cytotoxic effect of different species of the genus *Rhododendron*. Park and Kim (2008) demonstrated that the essential oil fraction obtained from *Rhododendron mucronatum* exhibits a cytotoxic effect in human immortal keratinocyte (HaCaT) cancer cells. Manikumar et al. (2011) reported that the acetone extract obtained from *Rhododendron ponticum* leaves exhibits a cytotoxic effect on the human prostate cancer (PC-3) cell line. Recently, Demir et al. (2016a) demonstrated that *Rhododendron luteum* flower extract has a selective cytotoxic effect against human colon and liver cancer cells. The purpose of this study was to determine the cytotoxic effect of *Rhododendron luteum* leaf extract in human prostate, breast, colon, lung, liver cancer cell lines, and human normal foreskin fibroblast cells.

MATERIAL and METHODS

Chemicals

All the chemicals used in the analysis of antioxidant activity were in analytical purity and were provided from Sigma-Aldrich (St. Louis, MO, USA). All chemicals used in cell culture studies were provided from Lonza (Verviers, Belgium) and Biological Industries (Kibbutz Beit Haemek, Israel).

Plant Extraction

Rhododendron luteum plant samples used in the study were collected from Caykara-Trabzon, Turkey in the Spring of 2016. The plant samples were dried in room temperature for 20 days. The leaf parts were then carefully separated and converted into a fine powder using a blender and milling procedures. One g of the powdered samples was mixed with 20 mL of dimethyl sulfoxide (DMSO). After thorough vortexing, the mixture was incubated for 24 h with continuous shaking at 150 rpm at 45°C. After incubation, the mixture was centrifuged at 2000×g for 10 min. The supernatant was filtered with Whatman No. 1 filter paper and then passed through 0.2 µm filters (Demir et al., 2016a). The resulting DMSO extract of *R. luteum* leaf was aliquoted for use in experiments and stored in the dark at -20°C.

Determination of Total Phenolic Content (TPC)

The total phenolic content of the extract was determined spectrophotometrically according to the Folin-Ciocalteu method (Slinkard and Singleton, 1977). Gallic acid was used as a standard and the TPC value was calculated as mg gallic acid equivalent (GAE)/g sample.

Determination of Total Flavonoid Content (TFC)

The total flavonoid content of the extract was determined using aluminum chloride method (Moreno et al., 2000). Quercetin was used as a standard and the TFC value was calculated as mg quercetin equivalent (QE)/g sample.

Determination of Reducing Power

The reducing power of extract was determined using previously described method by Oyaizu (1986). Trolox was used as a standard and the reducing power value was calculated as mg trolox equivalent (TE)/g sample.

Cell Culture

Prostate adenocarcinoma (PC-3), hepatocellular carcinoma (HepG2), colon adenocarcinoma (WiDr), breast adenocarcinoma (MCF-7), lung carcinoma (A549) human cancer and normal foreskin fibroblast were supplied by the American Type Culture Collection (Manassas, VA, USA). All cells were cultured in Eagle's Minimum Essential Medium (EMEM) supplemented with 10% heat inactivated fetal bovine serum and 1% gentamicin solution with a 5% CO₂ supply at 37°C.

Drug Preparation and Treatment

Cisplatin was used as a reference anti-cancer compound in cytotoxicity experiments (Demir et al., 2017). Quercetin was used as a single flavonoid for cytotoxicity experiments because it is one of the major flavonoids in *Rhododendrons* (Popescu and Kopp, 2013). Both were dissolved in DMSO to obtain 1000 µg/mL stock solution. External working concentrations were prepared by further dilution with DMSO. Final solvent concentrations of compounds were no higher than 0.5% in culture media in any experiment. That concentration was not sufficient to affect cell morphology or viability.

Cytotoxicity Experiments

MTT assay (Mosmann, 1983) was employed to measure the cytotoxic effects of *R. luteum* leaf extract, quercetin, and cisplatin on five cancer and a normal cell lines. Briefly, cells were seeded into a flat-bottomed 96-well cell culture plates. The cells were then treated with varying concentrations of *R. luteum* leaf extract (0–100 µg/mL), quercetin (0–50 µg/mL), and cisplatin (0–10 µg/mL) for 72 h. Subsequently, 10 µL of MTT dye (0.25 mg/mL) was placed inside each well. The crystals that emerged were then dissolved in DMSO. Finally, absorbance was measured at 570 nm with a microplate reader (Molecular Devices Versamax, California, USA). Optical densities were employed to calculate percentage viabilities in treated cells compared to untreated control cells. Log-concentrations versus % cell viabilities were plotted with a logarithmic graph, which was then used to determine the IC₅₀ values.

The IC₅₀ values of extract, quercetin, and cisplatin in the cancer and normal cell lines were used to elicit a selectivity index with the following formula (Demir et al., 2016b):

$$\text{Selectivity Index} = \frac{\text{Fibroblast cells IC}_{50}}{\text{Cancer cells IC}_{50}}$$

Statistical Analysis

All experiments were performed at least three times, the results were expressed as mean ± standard deviation. Normal distribution was determined using the Kolmogorov-Smirnov test. One-Way ANOVA was performed to analyze intergroup differences. P<0.05 was regarded as significance level.

RESULTS and DISCUSSION

The main disadvantage of synthetic medicines used in medical treatment is the risk of creating side effects (Prakash et al., 2013). Natural products are in high demanded in primary medical services in developing countries because they are traditionally used and believed to have fewer side effects (Nema et al., 2013). Phenolics are secondary metabolites that play a key role in growth, metabolism and protection in plants. More than 8000 phenolic compounds isolated from various plant species have been identified. Phenolic compounds are known to have high antioxidant effects. Phenolics are believed to originate from the antioxidant properties of many useful biological functions (Niedzwiecki et al., 2016; Ozkan et al., 2017). The determination of the antioxidant activity of the tested natural product is therefore considered as a starting point for more extensive biological activity studies. Many *in vitro* tests are used to determine the antioxidant capacity of plant extracts and it is recommended that this activity be identified by at least two different methods (Ozkan et al., 2017; Aliyazicioglu et al., 2017). The antioxidant properties of the *R. luteum* leaf extract were therefore determined using three different methods and the results are presented in Table 1.

Table 1. Antioxidant properties of *R. luteum* extract (n=3)

Antioxidant Parameters	
TPC (mg GAE/g sample)	173.2±3.97
TFC (mg QE/g sample)	51.5±0.16
Reducing Power (mg TE/g sample)	629.5±3.49

The TPC value of ethanolic *Rhododendron ponticum* bark extract was found 34.8 g catechin equivalent/100 g extract (Yaylaci et al., 2007), while the TPC and TFC values of %80 methanolic bark extract of *Rhododendron yedoense* var. *poukhanense* were 160 mg GAE and 2.2 mg QE per g dry extract, respectively (Lee et al., 2011).

Lin *et al.* (2014) found that the TPC and reducing power values of the methanolic extracts prepared from the leaves of 10 different *Rhododendron* species naturally grown in Taiwan were 165-319 mg GAE and 225-387 mg catechin equivalents per gram, respectively. Jing *et al.* (2015) reported that the TPC and TFC values of the 70% ethanolic extract of *Rhododendron anthopogonoides* were 165 mg GAE and 231 mg rutin equivalents per gram sample, respectively. Demir *et al.* (2016a) demonstrated that TPC, TFC and reducing power values of *R. luteum* flower extract were as 54.2 mg GAE, 18.5 mg QE, and 164.2 mg TE per g sample, respectively. The antioxidant activity results of this study are largely consistent with previous studies. The difference between our antioxidant activity results and those of other studies may be due to the plant species, type of used standart compound, type of extraction methods, geographic region, harvest season, and post-harvesting conditions.

Cancer is a major public health problem in both developed and developing countries. Cancer cells have abnormal growth rates and are able to invade and harm normal cells. Every year, millions of people are diagnosed with cancer and many lose their lives. According to the American Cancer Society, cancer-related deaths account for 2-3% of annual worldwide deaths (Prakash *et al.*, 2013). The five most common cancer types in males are lung, prostate, colon, stomach and liver cancers, while in females breast, colon, lung, scervix, and stomach cancers (Rayan *et al.*, 2017). The accepted treatment modalities for cancer treatment are surgery, radiation or chemotherapeutic drug applications. These treatments can be administered alone or in combination. Chemotherapeutic agents are frequently used in the treatment of cancer (Taraphdar *et al.*, 2001). Since standard chemotherapeutic drugs develop resistance to cancer cells over time and severe side effects may be seen, new treatment options need to be investigated (Li *et al.*, 2012). Synthesis and modification of novel anti-cancer drugs that can be used in clinical oncology is one of the most popular research areas. However, only a small fraction of synthetic drug candidates can attend clinical use. New prototypes with potential biological anti-cancer effects are therefore needed. Natural products are emerging as potential prototypes for the next generation of anti-cancer drugs (Taraphdar *et al.*, 2001). Today, more than 60% of the anticancer drugs used clinically are obtained from natural products (Rayan *et al.*, 2017). Recent work on plant-derived anti-tumor compounds has revealed an impressive array of structure-function relationships (Taraphdar *et al.*, 2001). When the literature is examined, it is seen that the studies on the cytotoxic effect of the extracts obtained from *Rhododendron* species are limited (Park and Kim, 2008; Manikumar *et al.*, 2011; Demir *et al.*, 2016a). Especially, it has been revealed that some natural product extracts can

increase apoptosis in cancer cells without harming normal cells in recent years. Screening for cytotoxic effects of plant-derived extracts or compounds isolated from them in cancer cells is therefore important (Taraphdar *et al.*, 2001). Selectivity (no toxic effects on healthy cells) and effectiveness (high efficacy against multiple cancers) are the desired two main proterties from an effective and acceptable anticancer agent (Demir *et al.*, 2016a). The cytotoxic effect of the *R. luteum* leaf extract was therefore examined on five common cancer cell lines and a normal cell line. The concentration-dependent effect of the extract on cell viability is shown in Figure 1. The IC₅₀ values obtained from the growth curves are presented in Table 2.

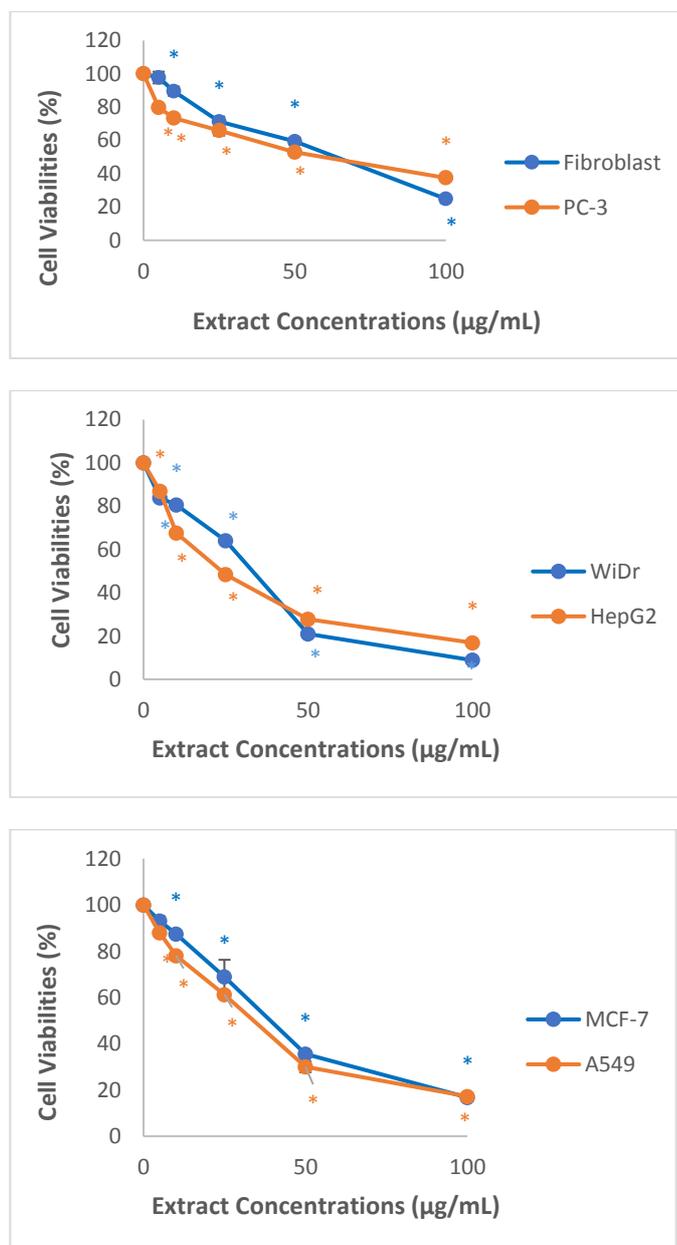


Figure 1. The anti-growth effect after the treatment with the extract for 72 h against human cancer and normal fibroblast cells by the MTT assay (n=3)

Table 2. Cytotoxic activity (IC₅₀, µg/mL) of *R. luteum* extract and other test compounds (n=3)

	Test Compounds		
	<i>R. luteum</i> extract	Quercetin	Cisplatin
A549	34.1±0.8	3.6±0.2	0.7±0.1
MCF-7	38.8±1.5	8.3±0.3	1.6±0.1
HepG2	24.5±1.1	5.1±0.1	2.5±0.1
PC-3	61.2±3.7	4.2±0.1	0.7±0.1
WiDr	27.6±0.6	8.3±0.1	1.1±0.1
Fibroblast	53.4±2.3	15.5±0.2	4.8±0.2

Table 3. Selectivity index of *R. luteum* extract and other test compounds

	Test Compounds		
	<i>R. luteum</i> extract	Quercetin	Cisplatin
A549	1.6	4.3	6.9
MCF-7	1.4	1.9	3.0
HepG2	2.2	3.0	1.9
PC-3	0.9	3.7	6.9
WiDr	1.9	1.9	4.4

The IC₅₀ values of the extract in the cancer cell lines range from 24.5 to 61.2 µg/mL. According to the US National Cancer Institute, extracts with a small IC₅₀ value of 30 µg/mL against tumor cell lines *in vitro* conditions are considered promising for anticancer drug development (de Oliveira et al., 2016). The present results showed that *Rhododendron luteum* leaf extract was more cytotoxic on colon and liver cancer cells with their lower IC₅₀ values (<30 µg/mL) than the other cancer cells tested.

The selectivity index of the extract and other test compounds are shown in Table 3. The most selective cytotoxic effect of the extract was seen on HepG2 and WiDr cells. The term selectivity index indicates how selectively the extract or drug molecule can eliminate cancer cells compared to normal cells (Demir et al., 2016b).

Previous studies with *Rhododendron* species have shown that the 70% ethanolic extract of *Rhododendron brachycarpum* leaves has a cytotoxic effect on human lung (A549), stomach (AGS), breast (MCF-7) and liver (Hep3B) cancer cell lines (Byun et al., 2005). Way et al. (2014) reported that different fractions of the methanolic extract of *Rhododendron formosanum* leaves exhibit cytotoxic effect in human lung cancer cells by inducing apoptosis. In addition, Bilir et al. (2018a) demonstrated that the aqueous extract of *Rhododendron ponticum* flowers exhibits cytotoxic effects on human prostate cancer (PC3 and DU145) cell lines. Studies have also investigated the antiproliferative activity of various compounds isolated from *Rhododendron* species. Jang et al. (2005) reported that grayanotoxin I isolated compound from *Rhododendron brachycarpum* has a strong antiproliferative effect in human lung, ovarian,

melanoma, colon and glioblastoma cancer cells. Seephonkai et al. (2011) demonstrated that ferruginenes C isolated from *Rhododendron ferrugineum* exhibits a cytotoxic effect on human acute promyelocytic leukemia, cervix, and breast cancer cells. Li et al. (2012) reported that hyperin isolated from Manchurian *Rhododendron* leaf exhibits antiproliferative effect in human endometrial cancer cells through mitochondrial apoptosis pathway. It is reported that 15-oxoursolic acid isolated from *Rhododendron arboreum* exhibits cytotoxic effect on human renal, liver, lung, and ovarian cancer cells (Ali et al., 2017). Recently, Bilir et al. (2018b) reported that the aqueous extract of *Rhododendron ponticum* flowers exhibits cytotoxic effect on the glioma cells.

Rhododendron species were shown to be rich in phenolic compounds, such as 5-hydroxy-6,7-dimethoxyflavone, kaempferol, quercetin, isorhamnetin, myricetin, quercitrin, rutin, taxifolin, catechin derivatives, phloretin, cyanidin, delphinidin, coumaric acid, and caffeic acid (Qiang et al., 2011; Popescu and Kopp, 2013). It is emphasized that phenolic compounds can exhibit anti-cancer activity through arresting the cell cycle, stimulating apoptosis, suppressing glucose uptake, eliminating drug resistance, inhibiting angiogenesis, and producing various epigenetic changes (Zhou et al., 2016). Quercetin was used as a single phenolic compound in our study since it is one of more abundant polyphenols in *Rhododendrons* (Qiang et al., 2011; Popescu and Kopp, 2013). Our findings show that the IC₅₀ values of extract were higher than those of quercetin. For this reason, the cytotoxic effect of extract on five cancer cell lines may not derive only from quercetin, and this result may explain the synergistic effect of all extract constituents.

CONCLUSIONS

This study is the first report about the cytotoxic effect of *R. luteum* leaf extract on cancer cells. Particularly, the selective cytotoxic effect of extract on liver and colon cancer cells may be a new remedy for cure. However, further experimental models and investigations into mechanism of molecular action are needed to confirm these cytotoxic activities.

Conflicts of interest statement

None of the authors had any financial or personal relationships with other individuals or organizations that might inappropriately influence their work during the submission process.

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