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Antioxidant Properties of *Primula vulgaris* Flower Extract and Its Cytotoxic Effect on Human Cancer Cell Lines

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ABSTRACT

Primula vulgaris is a plant belonging to *Primula* genus, and these species are often used in traditional therapy. Although there are numerous studies on the antioxidant and cytotoxic effects of different *Primula* species, there are limited studies of these properties of *P. vulgaris* extracts. The aim of this study was to determine the antioxidant properties and cytotoxic effects of the extract prepared with dimethyl sulfoxide from *P. vulgaris* flowers. The total phenolic content, total flavonoid content, and reducing antioxidant power of the extract were determined using spectrophotometric methods. The cytotoxic effect of the extract on human colon (WiDr), lung (A549), liver (HepG2), breast (MCF-7), and prostate (PC-3) cancer cells and a normal human fibroblast cell line was assessed using the MTT assay. Total phenolic content and reducing power values of extract were found 33.02±0.92 mg gallic acid equivalents, and 64.86±1.18 mg trolox equivalents per g sample, respectively. Extract exhibited selective cytotoxic effect on all studied cancer cells compared to normal fibroblast cells, and the IC_{50} values of the extract in the cancer cell lines range from 191.8 to 375.3 µg/mL. This work is one of the pioneering work that reveals the powerful antioxidant properties of *P. vulgaris* flower extract and its selective cytotoxic effect on cancer cell lines. Further studies are needed to identify the active molecules in the extract and the cytotoxic action mechanisms of these molecules.

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Primula vulgaris Çiçek Ekstraktının Antioksidan Özellikleri ve İnsan Kanser Hücre Serileri Üzerindeki Sitotoksik Etkisi

ÖZET

Primula vulgaris, *Primul*a cinsine mensup bir bitki olup, bu türler sıklıkla geleneksel tedavide kullanılmaktadırlar. Farklı *Primula* türlerinin antioksidan ve sitotoksik etkilerini konu alan çok sayıda çalışma olmasına rağmen, *P. vulgaris* ekstraktlarının bu özellikleri ile ilgili sınırlı sayıda çalışma bulunmaktadır. Bu çalışmanın amacı *P. vulgaris* çiçeklerinden dimetil sülfoksit ile hazırlanan ekstraktın antioksidan özelliklerinin ve sitotoksik etkilerinin belirlenmesidir. Ekstraktın toplam fenolik madde miktarı, toplam flavonoid madde miktarı ve indirgeyici antioksidan güç tayini spektrofotometrik yöntemler kullanılarak belirlendi. Ekstraktın sitotoksik etkisi ise insan kolon (WiDr), akciğer (A549), karaciğer (HepG2), meme (MCF-7), ve prostat (PC-3) kanser hücre serileri ile normal insan fibroblast hücre serisi üzerinde MTT yöntemi kullanılarak değerlendirildi. Ekstraktın toplam fenolik madde içeriği ve indirgeyici antioksidan güç değerleri g örnek başına sırasıyla 33.02±0.92 mg gallik asit eşdeğeri ve 64.86±1.18 mg troloks eşdeğeri olarak bulundu. Ekstraktın çalışılan tüm kanser hücre serileri üzerinde normal fibroblast hücrelerine göre seçici sitotoksik etki gösterdiği belirlendi ve IC_{50} değerleri 191.8-375.3 µg/mL arasında hesaplandı. Bu çalışma

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Anahtar Kelimeler
Antioksidan aktivite,
Kanser,
Primula vulgaris,
Sitotoksisite

Araştırma Makalesi

P. vulgaris çiçek ekstraktının kuvvetli antioksidan özelliklerini ve kanser hücre serileri üzerindeki seçici sitotoksik etkisini ortaya koyan öncü çalışmalardan birisidir. Ekstrakttaki etken molekülleri ve bu moleküllerin sitotoksik etki mekanizmalarını belirleyebilmek için daha ileri çalışmalara ihtiyaç duyulmaktadır.

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INTRODUCTION

Cancer is a global health problem and 21% and 9% of deaths in developed and developing countries are reported to be cancer-related, respectively. World Health Organization (WHO) estimates about 27 million new cases and 17.5 million cancer-related deaths annualy by 2050 (Hussain et al., 2016). Chemotherapy is one of the most used treatments against cancer along with radiotherapy and resection. However, there are many disadvantages of chemotherapy, among which, drug resistance in cancer cells and toxicity in the normal cells are more common. Current research therefore focuses on the new generation drug development studies which has fewer side effects (Unnati et al., 2013; Demir et al., 2016a).

Plants have been used against many human and animal diseases for both protective and therapeutic purposes since ancient times (Unnati et al., 2013). Today, approximately 50% of the chemotherapeutic drugs are derived from plants (Demir et al., 2016a). According to the WHO data, more than 80% of people living in developing countries apply natural products for their primary health problems. Recent surveys show that more than 60% of cancer patients use natural products for therapy (Unnati et al., 2013; Turan et al., 2017a). The herbal medicines are regarded as important source of developing of new anticancer drugs due to their multiple therapeutic effects, such as inhibiting cancer activating enzymes and hormones, stimulating DNA repair mechanism, promoting production of protective enzymes, inducing apoptosis, cell cycle arrest, and immunity (Shukla et al., 2015; Turan et al., 2017b). However, very few of the medical plants have been scientifically evaluated in terms of their anticancer properties (Turan et al., 2017a). The development of new generation anticancer drugs from natural products has been therefore met with great interest both in scientific and commercial environments in recent years (Unnati et al., 2013; Turan et al., 2017a).

Primula genus comprises more than 400 species, belonging to family of *Primulaceae*, and is spread in the northern hemisphere temperate and cold zone (Ozkan et al., 2017). *Primula* species are traditionally used against some disorders, such as bronchitis, asthma, insomnia (Turan et al., 2017a). It is reported that *Primula* species are rich in saponins, alkaloids, tannins, terpenes, and phenolic compounds (Mostafa et al., 2014; Ozkan et al., 2017). The antioxidant, antimicrobial, antigenotoxic, anti-inflammatory, hypoglycemic, and wound healing properties of *Primula* species have been extensively studied (Orhan et al., 2012; Aslam et al., 2014; Mostafa et al., 2014; Ozkan et al., 2017; Turan et al., 2017a). In studies involving the cytotoxic activity of *Primula* species on cancer cells, the cytotoxic effect of aqueous extracts of *Primula vulgaris* flowers and leaves were examined using the brine shrimp method and the LC_{50} values were found to be 311 and 40 µg/mL, respectively (Turker et al., 2008). Behzad *et al.* (2016) demonstrated that the methanolic extract of *Primula auriculata* exhibits apoptotic properties by providing caspase activation in human colon cancer (HT-29) cell line. Recently, Turan *et al.* (2017a) reported that *P. vulgaris* leaf extracts have cytotoxic effects in human liver (HepG2), prostate (PC-3), lung (A549), breast (MCF-7), and colon (WiDr) cancer cells, and the IC_{50} values of the extract in these cell lines range from 133.3 to 253.8 µg/mL. However, no previous studies have investigated the cytotoxic effect of *P. vulgaris* flower extract on cancer cells. The purpose of this study was to determine the *in vitro* antioxidant properties of *P. vulgaris* flower extract and its possible cytotoxic effect in human prostate, breast, colon, lung, liver cancer cell lines, and human normal foreskin fibroblast cells.

MATERIALS and METHOD

Chemicals

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

Plant Extraction

Primula vulgaris plant samples used in the study were harvested in the summer from Trabzon, Turkiye. The plant samples were dried at room temperature, the flower parts were then carefully separated and converted into a fine powder using a milling procedures. Next, 1 g of the powdered samples was mixed with 20 mL of dimethyl sulfoxide (DMSO). After 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 passed through 0.2 µm filters. The resulting DMSO extract of *P. vulgaris* was aliquoted for use in experiments and stored in the dark at -20°C (Turan et al., 2017a; Demir et al., 2018).

Determination of Total Phenolic Content (TPC)

The total phenolic content of the extract was determined spectrophotometrically according to the modified Folin-Ciocalteu method (Slinkard et al., 1977). 12.5 µL of sample was mixed with 62.5 µL of 1:10 diluted Folin reagent and 125 µL of 20% sodium carbonate solution and incubated at room temperature for 30 min in the dark. At the end of the incubation time the absorbance measurement was performed in a microplate reader (Molecular Devices Versamax, California, USA) at 760 nm. Gallic acid was used as standard and 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 the previously described colorimetric method (Moreno et al., 2000). 20 µL of sample; 172 µL of 80% ethanol, 4 µL of 10% aluminum chloride and 4 µL of 1 M potassium acetate solution were mixed and incubated at room temperature for 40 min in the dark. At the end of the incubation time the absorbance measurement was performed in a microplate reader (Molecular Devices Versamax, California, USA) at 415 nm. Quercetin was used as standard and TFC value was calculated as mg quercetin equivalent (QE)/g sample.

Determination of Reducing Power

The reducing power of extract was determined according to method by Oyaizu (1986). 40 µL of sample, 100 µL of 0.2 M phosphate buffer, and 100 µL of potassium ferricyanate were mixed and incubated in the dark at 50°C for 20 min and finally chilled under water. 100 µL of 10% trichloroacetic acid was added to the mixture and the mixture was centrifuged at 2000×g for 10 min. 100 µL of the supernatant was transferred to a 96-well microplate and 100 µL of distilled water and 20 µL of iron (III) chloride were added onto the supernatant. Preppared plates were incubated at room temperature for 5 min. At the end of the incubation, the absorbance measurement was performed in a microplate reader (Molecular Devices Versamax, California, USA) at 700 nm. Trolox was used as standard and the reducing power 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% CO_2 supply at 37°C.

Drug preparation and treatment

Cisplatin was dissolved in DMSO and used as a positive control and reference chemotherapeutic drug in cytotoxicity experiments (Demir et al., 2016b; Demir et al., 2017a). Final solvent concentrations of compounds were no higher than 0.5% in culture media in any trial. That concentration was not sufficient to affect cell morphology or viability.

Cytotoxicity experiments

MTT assay with a 72-h treatment time was employed to measure the cytotoxic effects of *P. vulgaris* extract, and cisplatin on five cancer and a normal cell lines (Mosmann, 1983; Demir et al., 2017b). Briefly, cells were seeded into a flat-bottomed 96-well cell culture plates. The cells were then treated with varying concentrations of *P. vulgaris* extract (0-500 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 IC_{50} values. The IC_{50} values of extract and cisplatin in the both cell lines were used to elicit a selectivity index with the following formula (Demir et al., 2016a):

$$\text{Selectivity Index} = \text{Fibroblast cells } IC_{50} / \text{Cancer cells } IC_{50}$$

Statistical analysis

All experiments were performed mininmum of three times, the results were presented as mean±standard deviation. Normal distribution was determined using the Kolmogorov-Smirnov test. One-Way ANOVA was prformed to analyze intergroup differences. $p<0.05$ was regarded as significant.

RESULTS and DISCUSSION

Oxidative stress is a pathological condition that results from oxidant-antioxidant balance impairment and is associated with many diseases, such as cancer, cardiovascular, and autoimmune disorders (Aliyazicioglu et al., 2011; Yalcin et al., 2016). Antioxidant activity is therefore important for human health in recent years, and it has been argued that many biological activities are originated from

antioxidant effects (Aliyazicioglu et al., 2017). Especially in recent years, phytochemicals found in natural products have been shown to play an important role in protecting oxidative stress related diseases (Kaur et al., 2001). It is believed that phenolics in natural products might prevent humankind against oxidative stress associated chronic diseases through their antioxidant action. The determination of the antioxidant activity of a tested natural product is therefore considered as a starting point for extensive studies (Demir et al., 2017a; Aliyazicioglu et al., 2017). Various *in vitro* methods have been described to determine the antioxidant activities of natural product extracts and at least two different methods are recommended. Total phenolic content, total flavonoid content, and reducing power analysis are antioxidant activity determination methods based on electron transfer. TPC and TFC are often used to examine the antioxidant properties of natural product extracts since they are useful, rapid, and cost-effective. Moreover, direct relationship were found between the TPC and TFC values and antioxidant capacity of many natural product extracts. The reducing power assay is also frequently preferred to estimate the antioxidant power of a compound (Aliyazicioglu et al., 2017; Ozkan et al., 2017; Turan et al., 2017a). The antioxidant properties of the DMSO extract prepared from *P. vulgaris* flowers were therefore determined using TPC, TFC and reducing power analysis and the results are shown in Table 1.

Table 1. Antioxidant activity of *P. vulgaris* extract (n=3)

Antioxidant Parameters	
Total Polyphenolic Content (mg GAE/g sample)	33.02±0.92
Total Flavonoid Content (mg QE/g sample)	11.9±0.26
Reducing Power (mg TE/g sample)	64.86±1.18

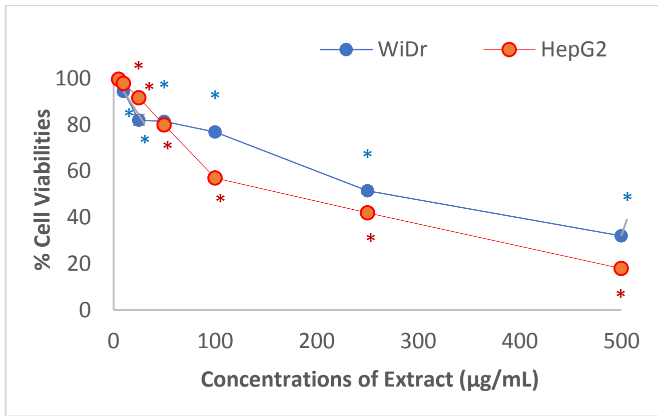
Orhan *et al.* (2012) reported that the TPC value of the aqueous extract of *P. vulgaris* leaves is 7.55 mg GAE/g sample, while Demir *et al.* (2014) demonstrated that the TPC value of the ethanolic *P. vulgaris* extract was 122.8 mg GAE/g sample. Ozkan *et al.* (2017) reported that the TPC and ferric reducing antioxidant power value of water extract of *P. vulgaris* are 15.02 mg GAE and 82.6 µM TE per g sample, respectively. Turan *et al.* (2017a) demonstrated that the TPC, TFC, and reducing power value of DMSO extract of *P. vulgaris* are 18.9 mg GAE, 4.88 mg QE, and 32.3 mg TE per g sample, respectively, while Aslam *et al.* (2015) reported that the TPC value of ethanolic extract of *P. denticulata* is 15.9 mg GAE/g sample. Antioxidant activity values are consistent with the literature. We think that the small differences may have originated from the plant species, type of extraction method, type of solvent, geographic region, harvest season, and post-harvesting conditions.

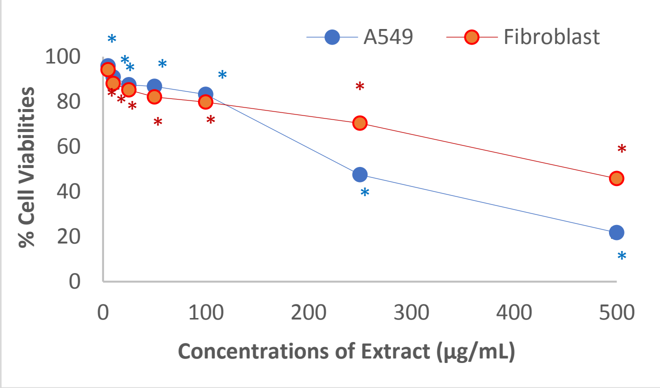
Cancer is one of the most important causes of disease-caused deaths in the world. WHO reports that between 2005 and 2015 approximately 84 million people lost their lives due to cancer (Behzad et al., 2014). While chemotherapy is one of the most used method for the treatment of cancer, it has many serious side effects, including drug resistance, toxicity, and low specificity (Turan et al., 2015a; Turan et al., 2017c). Natural products are therefore looked at as potential raw materials for new drug discovery, and phenolic compounds found in natural products come to the forefront with their structures and activities. Investigation of the anticancer effects of both natural extracts and compounds isolated from such products has become one of the popular research fields in recent years (Demir et al., 2016b; Demir et al., 2017a). Selectivity (no toxic effects on normal cells) and effectiveness (high efficacy against multiple cancers) are the desired two main proterties from an effective and acceptable anticancer agent (Demir et al., 2016b). When the literature is examined, is seen that the studies on the cytotoxic effect of the extracts obtained from *Primula* species are limited (Turker et al., 2008; Behzad et al., 2016; Turan et al., 2017a). The cytotoxic effect of the *P. vulgaris* flower 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 IC_{50} values obtained from the growth curves are presented in Table 2.

Table 2. Cytotoxic effects (IC_{50}, µg/mL) of *P. vulgaris* extract and cisplatin (n=3)

Cell Lines	Extract	Cisplatin
A549	228.6±11.2	0.71±0.06
HepG2	191.8±3.8	2.49±0.06
MCF-7	240.5±7.3	1.57±0.02
PC-3	375.3±12.9	0.53±0.01
WiDr	287.8±3.5	1.28±0.01
Fibroblast	492.8±1.3	4.86±0.23

The IC_{50} values of the extract in the cancer cell lines range from 191.8 to 375.3 µg/mL.
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 A549 cells. The term selectivity index indicates how selectively the extract or drug molecule can eliminate cancer cells compared to normal cells (Demir et al., 2016a).
In previous studies with *Primula* species have shown that the cytotoxic effect of the ethyl acetate, chloroform, and benzene fractions of ethanolic extract of *Primula macrophylla* is examined by brine shrimp method and the most effective activity is observed in the ethanolic crude extract (Najmus-Saqib et al., 2009).





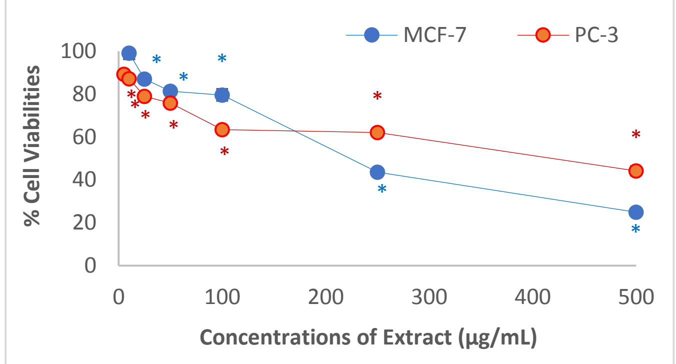


Figure 1. The anti-growth effect after the treatment with the extract for 72 h against human cancer and normal fibroblast cells by the MTT assay (n=3). *Represents significant result compared to untreated cells.

Table 3. Selectivity index of *P. vulgaris* extract and other test compounds

	Test Compounds	
	P. vulgaris extract	Cisplatin
A549	2.2	6.8
MCF-7	2.1	3.1
HepG2	2.6	2.0
PC-3	1.3	9.2
WiDr	1.7	3.8

Behzad *et al.* (2014) reported that the methanolic extract of *Primula auriculata* exhibits a selective cytotoxic effect on MCF-7, HepG2, and HT-29 cancer cell lines compared to normal bovine kidney cells. There is no cytotoxic effect in the A549 cell line up to the concentration of 100 µg/mL of the extract in the same study. Recently, Demir *et al.* (2018) demonsrated that the *Primula vulgaris* flower extract has a selective cytotoxic effect on human cervix (HeLa) cancer cells by arresting their cell cycle at the S phase, and inducing the number of apoptotic cells. There are few studies that examine the cytotoxic activity of *Primula* genus in the literature. However, the methanolic extract of *Dionysia termeana*, a member of the *Primulaceae* family, has been reported to exhibit cytotoxic effects in human myelogenous leukemia (K562), and T-lymphocytic leukemia (Jurkat) cell lines in a dose-dependent manner via inducing apoptosis (Amirghofran et al., 2007). Studies have also investigated the cytotoxic effect of various compounds isolated from *Primula* species. Tokalov *et al.* (2004) demonstrated that some compounds isolated from *Primula denticulata*, such as 5-hydroxyflavone, 2'-hydroxyflavone, 5,2'-dihydroxyflavone, and 5,8 dihydroxyflavone, have cytotoxic effect on human acute myeloid leukemia (HL-60) cells via inducing cell cycle arrest and apoptosis.

Phenolics are an important class of secondary herbal metabolites and reported to exhibit strong antioxidant properties. The antioxidant effect of these molecules is attributed to their ability to donate electrons to reactive oxygen species, chelating metal ions, and stimulating antioxidant and detoxifying enzymes (Turan et al., 2015b). Polyphenolic compounds have been reported to exhibit antioxidant, anticancer, antimutagenic, anti-atherosclerotic, antimicrobial, and anti-inflammatory effects due to these properties (Turan et al., 2017d). It has been suggested that the anticancer effect of phenolics may derive from their ability to modulate carcinogen metabolism, regulation of gene expression, induction of cell cycle arrest and apoptosis, and inhibition of signal transduction pathways (Demir et al., 2017a). It is demonstrated that *Primula* species have been shown to be rich in phenolic compounds, such as kaempferol, quercetin, rutin, 5-hydroxy pyrogallol, apigenin, catechin derivatives, gallic acid, rosmarinic acid, *p*-coumaric acid, protocatechuic acid, *p*-OH benzoic acid, vanillic acid, caffeic acid, ferulic acid, and ellagic acid (Mostafa et al., 20014; Ozkan et al., 2017). There are many reports of antiproliferative effects of these phenolics in different cancer cells (Ravishankar et al., 2013; Zhou et al., 2016). Studies examining the cytotoxic properties of extracts from various natural products show that the overall effect of the extract has first been examined, and the results have been attributed to synergistic effects. Effective compounds was isolated

from crude extract, and its biological effects was also investigated (Turan et al., 2017a). It is believed that all these phenolic compounds of the *P. vulgaris* flower extract contribute to its cytotoxic effect.

CONCLUSION

One limitation of our study is that *in vitro* studies can not be extrapolated to possible activity *in vivo*. Further studies are now necessary to obtain a more detailed understanding of the exact interaction of the signaling pathways involved.

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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