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The Effect of *Rosa pimpinellifolia* Extract on the Proliferation of Human Tumor Cells

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ABSTRACT

The aim of this study was to evaluate the antioxidant properties of *R. pimpinellifolia* and its cytotoxic effect against human colon (WiDr), liver (HepG2) and lung (A549) cancer cells compared with a normal fibroblast (BJ) cell line. The antioxidant properties of extract was determined using spectrophotometric methods, while the cytotoxic effect of extract was evaluated using MTT assay. The total phenolic content, total flavonoid content and reducing power values of extract were calculated as 16.4±0.4 mg gallic acid equivalent, 5.2±0.2 mg quercetin equivalent and 34.3±2.4 mg trolox equivalent per g sample, respectively. Although the extract exhibits selective cytotoxic effect on three cancer cell lines in a dose-dependent manner compared to BJ cells, the highest selectivity index value (2.7-fold) was obtained for the WiDr cell line. In conclusion, this is the first study to demonstrate the cytotoxic effect of *R. pimpinellifolia* on cancer cells. To demonstrate the molecular mechanism of this cytotoxic effect and to carry out *in vivo* studies may be a guide for future clinical studies.

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Rosa pimpinellifolia Ekstraktının İnsan Tümör Hücrelerinin Çoğalmasına Etkisi

ÖZET

Bu çalışmanın amacı, *R. pimpinellifolia*'nın antioksidan özelliklerini ve insan kolon (WiDr), karaciğer (HepG2) ve akciğer (A549) kanser hücreleri üzerindeki sitotoksik etkisini normal bir fibroblast (BJ) hücre hattına kıyasla değerlendirmektir. Ekstraktın antioksidan özellikleri spektrofotometrik yöntemler kullanılarak belirlenirken, sitotoksik etkisi ise MTT testi kullanılarak değerlendirildi. Ekstraktın toplam fenolik içeriği, toplam flavonoid içeriği ve indirgeme gücü değerleri g örnek başına sırasıyla 16.4±0.4 mg gallik asit eşdeğeri, 5.2±0.2 mg kuersetin eşdeğeri ve 34.3±2.4 mg troloks eşdeğeri olarak hesaplandı. Ekstrakt BJ hücrelerine kıyasla üç kanser hücresi hattı üzerinde doza bağımlı bir şekilde seçici sitotoksik etki sergilemesine rağmen, en yüksek seçicilik indeks değeri (2.7 kat) WiDr hücre hattı için elde edildi. Sonuç olarak, bu çalışma *R. pimpinellifolia*'nın kanser hücreleri üzerindeki sitotoksik etkisini gösteren ilk çalışmadır. Bu sitotoksik etkinin moleküler mekanizmasını göstermek ve *in vivo* çalışmaların yapılması gelecekteki klinik çalışmalar için yol gösterici olabilir.

Araştırma Makalesi

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Anahtar Kelimeler
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INTRODUCTION

Cancer is a major public health problem worldwide, and approximately 10 million new cases are detected annually (Faezizadeh et al., 2016). There are various traditional treatment options, such as surgery, radiotherapy, and chemotherapy, to treat cancer. As one of the most commonly used treatments, chemotherapy has been in clinic for about 70 years for the purpose of suppressing cancer cell proliferation (Demir et al., 2019). However, toxicity of chemotherapeutics on normal cells and drug resistance

on cancer cells affect the success percentage of chemotherapy (Faezizadeh et al., 2016). The worldwide high incidence of cancer and disadvantages of chemotherapy necessitates the development of new clinical strategies (Abotaleb et al., 2020). Increasing evidence supports the potential use of some phytochemicals that can kill cancer cells by modulating the expression of metabolic enzymes (Zhang, 2015). In recent years, the plant extracts with low toxicity on normal cells and with greater effect on cancer cells have led the scientific community to investigate the anticancer properties of herbal products (Faezizadeh et al., 2016; Zarei and Yaghoobi, 2019). Because natural products are an important source of phytochemicals and other bioactive compounds, they are an important resource for therapeutic products that can prevent or cure a large number of diseases (Mármol et al., 2020). Today, many epidemiological and clinical studies have revealed the positive relationship between herbal medicines consumption and human health (Lall et al., 2015; Mármol et al., 2020). For this reason, natural products rich in bioactive substances have become an area of interest for the pharmaceutical, food and cosmetic industries in recent years (Aliyazicioglu et al., 2017; Demir et al., 2018a). Recently, the emergence of side effects of synthetic antioxidants in chronic use has further increased this interest (Cosmulescu et al., 2017.

The *Rosa* genus with more than 100 species is one of the most populated members of the Rosaceae family and is widely distributed in Europe, Asia, the Middle East and North America (Fattahi et al., 2012; Kilinc et al., 2020). This genus is one of the most used plants in folk medicine for the treatment of different diseases, such as kidney and bladder stones, diarrhea, gum bleeding, arthritis, rheumatism, gout, colds, throat, asthma, hemorrhoids and pains (Guimarães et al., 2014; Koczka et al., 2018; Kilinc et al., 2020). *Rosa* species contain various biologically active compounds, such as sugars, organic acids, pectins, phenolics, tannins, carotenoids, tocopherols, fatty acids, vitamins, minerals and amino acids (Fattahi et al., 2012; Roman et al., 2013; Bhave et al., 2017). These species have many biological acitivities, such as antioxidant, antimutagenic, antidiabetic, antiproliferative, anti-inflammatory, anti-arthritic, analgesic, cardioprotective, antimicrobial, gastroprotective, neuroprotective, hepatoprotective, immunosuppressive, renoprotective, anti-aging, anti-ulcerogenic, anticarcinogenic and immunomodulatory effects and these activities are attributed to the above mentioned compounds (Khazaei et al., 2020; Mármol et al., 2020). In recent years, the investigation of the antiproliferative effects of Rosaceae species on cancer cells has become a very popular research area. Although there are many studies investigating the cytotoxic effect of different *Rosa* species in various cancer cells, such as breast (4T1 and MCF-7) (Olsson et al., 2004; Radovanovic et al., 2013; Berkoz et al., 2019), colon (WiDr and HT-29) (Olsson et al., 2004; Demir et al., 2017), prostate (LNCaP) (Lee et al., 2008) and melanoma (B16) (Fujii et al., 2011), there is no study determining the cytotoxic effect of *Rosa pimpinellifolia* on cancer cells in the literature. The aim of this study was to investigate for the first time the selective cytotoxic effect of *R. pimpinellifolia* extract on human lung (A549), liver (HepG2) and colon (WiDr) cancer cells, which represent the most common types of cancer in the world, compared with a normal fibroblast (BJ) cell line.

MATERIALS and METHODS

Chemical and Reagents

All the chemicals and solvents used in extract preparation, antioxidant activity analysis and cytotoxicity experiments were purchased from Sigma-Aldrich (St. Louis, MO, USA). All the chemicals and solutions used in cell culture studies were purchased from Lonza (Verviers, Belgium) and Biological Industries (Kibbutz Beit Haemek, Israel).

Preparation of Extract

The fruits of *Rosa pimpinellifolia* were collected from Gumushane province of Turkey. The fruit samples were left to dry at room temperature for 20 days. The fruit parts of the dried plant were carefully powdered using a mechanical grinder (Retsch ZM 200, Haan, Germany). For preparing stock extract, 1 g of the powder material was weighed and mixed with 20 mL of dimethyl sulfoxide (DMSO) and then mixture was continuously stirred at 45ºC for 24 h. At the end of the period, the mixture was filtered through filter paper (Whatman No.1) and 0.2 µM filter and stored at -20ºC by aliquoting (Turan et al., 2018; Kilinc et al., 2020).

Determination of Total Phenolic Content

Total phenolic content (TPC) of extract was determined using Folin-Ciocalteu method (Slinkard and Singleton, 1977). Briefly, the sample (12.5 µL) was mixed with 125 µL of sodium carbonate and 62.5 µL of Folin reagent. After 30 min of reaction at room temperature, the absorbance was measured at 760 nm in a plate reader spectrophotometer (Molecular Devices Versamax, California, USA). Gallic acid was used as standard. Results are presented as mg gallic acid equivalent per g sample (GAE g^{-1} sample) (Demir et al., 2019).

Determination of Total Flavonoid Content

Total flavonoid content (TFC) of extract was determined by aluminium chloride method (Moreno et al., 2000). The sample (20 µL) was mixed with 172 µL

of 80% ethanol, 4 µL of 10% aluminum chloride and 4 µL of 1 M potassium acetate solution. After 40 min of reaction at room temperature, the absorbance was measured at 415 nm in a plate reader spectrophotometer (Molecular Devices Versamax, California, USA). Quercetin was used as standard. Results are presented as mg quercetin equivalent per g sample (QE g^{-1} sample) (Turan et al., 2017).

Reducing Power Analysis

The ferric reducing power of extract was determined using the method of developing by Oyaizu (1986). The sample (40 µL) was mixed with 100 µL of 0.2 M phosphate buffer, and 100 µL of potassium ferricyanate solutions. After 20 min of reaction 50°C, 100 µL of 10% trichloroacetic acid was added to the mixture and the mixture was centrifuged at 1800×g for 10 min. The supernatant (100 µL) was transferred to a flat-bottomed 96-well microplate and mixed with 100 µL of distilled water and 20 µL of iron (III) chloride. After 5 min of reaction at room temperature, the absorbance was measured at 700 nm in a plate reader spectrophotometer (Molecular Devices Versamax, California, USA). Trolox was used as a standard. Results are presented as mg trolox equivalent per g sample (TE g^{-1} sample) (Misir et al., 2018).

Cell Culture

A549, WiDr, HepG2 and BJ cell lines were obtained from American Type Culture Collection (Manassas, VA, USA). All cell lines were grown in a humidified incubator at 37°C with a 5% CO_2 atmosphere. Eagle's minimum essential medium (EMEM) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin was used for the culture of these cell lines.

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyl-Tetrazolium Bromide (MTT) Assay

The cytotoxic effects of the *Rosa pimpinellifolia* extract and cisplatin (was used as a positive control) on three human cancer and one normal cell lines were assayed using the MTT assay (Mosmann, 1983) with slight modifications (Misir et al., 2020). Cancer cells were seeded in 96-well plates at $5x10^3$ cells per well, while BJ cells were seeded at $2.5x10^3$ cells per well and overnight incubated at 37ºC. After over night incubation, the cells were exposed with different concentrations of extract (5-500 µg mL^{-1}) and cisplatin (0.1-12 µg mL^{-1}) for 72 h. DMSO concentration to which cells were exposed was kept below 0.1% (v/v), and only cells treated with 0.1% (v/v) DMSO formed the negative control group. After incubation, MTT solution (0.25 mg mL^{-1}) was added to each well and after 2 h the formazan precipitate was dissolved in 200 µL of DMSO. Optical density (OD) of each well was measured at 570 nm using a microplate spectrophotometer (Versamax Molecular Devices, CA, USA). The survival curve of the cells were calculated after comparison with control, and percent viability was determined as follows:

Cell viability (%) = ($OD_{treatment\ group}$ / $OD_{control\ group}$) x 100

The dose-response curves were drawn using logarithmic concentrations against %cell viability and the IC_{50} value of extract and cisplatin were calculated for each cell line. The IC_{50} values calculated for extract and cisplatin in all cell lines were used to determine the selectivity index (SI) value with the following formula (Demir et al., 2018b):

SI = BJ cells IC_{50} / Cancer cells IC_{50}

Statistical Analysis

All experiments were performed at four times. The distribution of the data was examined with the Kolmogorov-Smirnov test. Data showing normal distribution were expressed as arithmetic mean±standard deviation. Statistical analyzes between the groups were revealed by ANOVA and post-hoc Tukey tests. $p<0.01$ was regarded as significant.

RESULTS and DISCUSSION

Oxidative stress is the condition that results from the deterioration of the oxidant-antioxidant balance in favor of the oxidant (Tusat et al., 2017). Increased oxidative stress damages the biomolecules, such as carbohyrate, protein, lipid and nucleic acids (Jiménez et al., 2016). The role of oxidative stress in the pathogenesis of various human diseases, including cancer, aging, and atherosclerosis, is increasingly accepted (Mavi et al., 2004; Jiménez et al., 2016). Thus, compounds with antioxidant properties that can modulate cellular redox balance are among the most promising targets for functional food science. Since plants can contain large amounts of these compounds, it has come to the fore in recent years, and the determining the antioxidant activity of plants has become one of the most popular research areas (Tumbas et al., 2012). Therefore, the antioxidant properties of the *R. pimpinellifolia* extract were determined by TPC, TFC and reducing power methods and the results are shown in Table 1.

Table 1. Antioxidant properties of *R. pimpinellifolia* extract (n=4)

Çizelge 1. *R. pimpinellifolia ekstraktının antioksidan özellikleri (n=4)*

Antioxidant Parameters	
Total Polyphenolic Content (mg GAE g^{-1} sample)	16.4±0.4
Total Flavonoid Content (mg QE g^{-1} sample)	5.2±0.2
Reducing Power (mg TE g^{-1} sample)	34.3±2.4

In the studies examining the antioxidant properties of *R. pimpinellifolia* extract, Mavi *et al.* (2004)

demonstrated that the TPC value of *R. pimpinellifolia* extract, collected from Turkey, is 58.8 mg GAE g^{-1}, while Fattahi *et al.* (2012) reported that the TPC and TFC values of *R. pimpinellifolia* extract, collected from West Azerbaijan, are 1.77 mg GAE g^{-1} and 0.41 mg QE g^{-1}, respectively.

In other studies examining the antioxidant properties of different *Rosa* species extracts, collected from Hungary, Poland, Serbia, Transylvania, Tunisia, Turkey and West Azerbaijan, the TPC and TFC values are reported to vary between 2.26-76.6 mg GAE g^{-1}, and 0.44-26.47 mg QE g^{-1}, respectively (Jablonska-Rys et al., 2009; Ghazghazi et al., 2010; Fattahi et al., 2012; Roman et al., 2013; Koczka et al., 2018; Paunović et al., 2019; Kilinc et al., 2020). The antioxidant results of this study are in agreement with the previous literature data. It is stated that the phenolic and flavonoid content of natural products may vary depending on the genotype, environment, soil conditions, harvest time and the extraction method used (Ozkan et al., 2017). Small differences between antioxidant activity results and available literature data are thought to be due to these reasons.

Colon cancer accounts for approximately 10% of all cancers and cancer-related deaths diagnosed worldwide each year, and it is the second most common cancer in women and the third most frequently diagnosed cancer in men (Dekker et al., 2019). Lung cancer is the most common cause of cancer deaths in the world and each year approximately 1.8 million new cases of lung cancer are diagnosed worldwide (Wadowska et al., 2020). Liver cancer is the fifth most common cause of malignancies and the third cause of cancer deaths, with less than 9% 5-year survival rate in patients (Alnajjar and Elsiesy, 2015). Therefore, this study aimed to determine the cytotoxic effect of *R. pimpinellifolia* extract on various cell lines, including A549, HepG2 and WiDr, representing common cancer types in the world, compared with a normal fibroblast (BJ) cell line. The cytotoxic effect of extract on these cell lines was determined using MTT assay and the growth curves of the cells are shown in Figure 1.

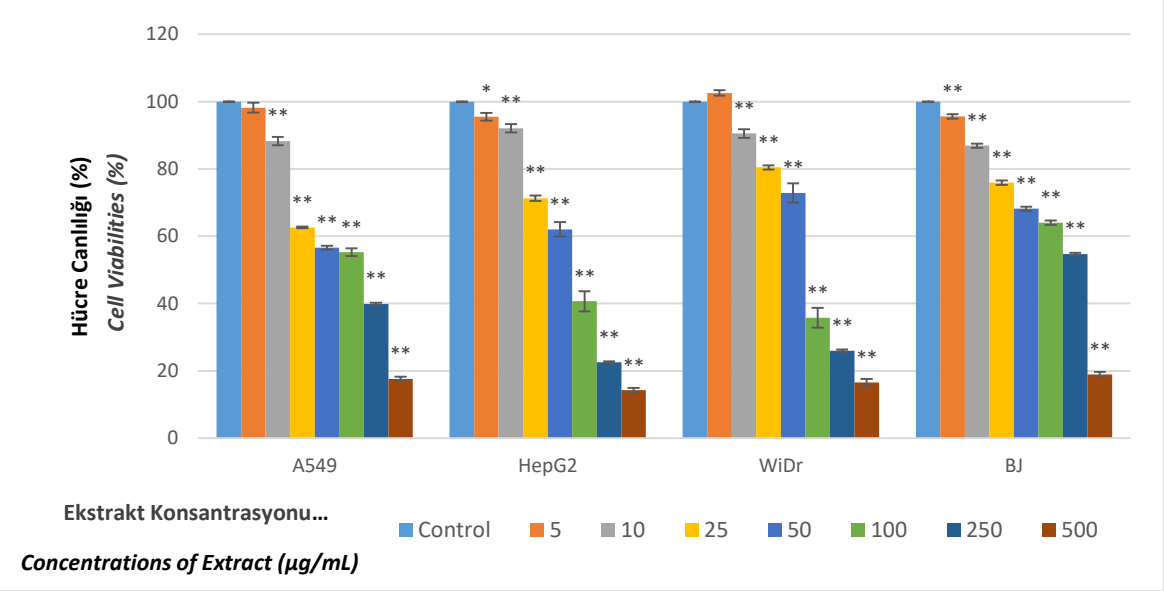

Figure 1. The cytotoxic of *R. pimpinellifolia* extract on cancer and BJ cell lines (n=4). *Statistically significant compared to its own control group (p=0.006), **Statistically significant compared to its own control group (p=0.0001).

Şekil 1. *R. pimpinellifolia ekstraktının kanser ve BJ hücre hatları üzerindeki sitotoksik etkisi (n=4). *Kendi kontrol grubu ile kıyaslandığında istatistiksel olarak anlamlı (p=0.006), **Kendi kontrol grubu ile kıyaslandığında istatistiksel olarak anlamlı (p=0.0001).*

When all cells were evaluated together, statistically significant cytotoxic effect of extract was emerged starting at a concentration of 10 µg mL^{-1} and extract exhibited cytotoxic effect in all studied cancer cells in a dose-dependent manner. In order to make the results more understandable, the IC_{50} values (µg mL^{-1}) of extract and cisplatin (used a positive control in cytotoxicity experiments) in all studied cell lines were calculated and presented in Table 2.

When Table 2 is examined, it is seen that the IC_{50} values for extract in cancer cells ranged from 72.1 to 153.4 µg mL^{-1}. Many studies have been shown that the cytotoxic effect of different *Rosa* (*Rosa agretis, Rosa*

Table 2. IC_{50} values (µg mL^{-1}) calculated for *R. pimpinellifolia* extract and cisplatin (n=4)

Çizelge 2. *R. pimpinellifolia ekstraktı ve cisplatin için hesaplanan IC_{50} (µg mL^{-1}) değerleri (n=4)*

	Extract	Cisplatin
A549	153.4±3.3	0.75±0.06
HepG2	74.3±5.2	3.57±0.06
WiDr	72.1±4.9	0.88±0.05
BJ	195.1±3.8	3.61±0.10

canina, Rosa damascena, Rosa micrantha, Rosa roxburghii Tratt) extracts on various human cancer cell lines, such as cervix (HeLa), breast (MCF-7), colon (HT-29 and HCT-115), esophageal (CaEs-17), gastric (SGC-7901), lung (NCI-H460 and A549) and liver (HepG2) cancer cells. In these studies, it is seen that the IC_{50} values of *Rosa* extracts in cancer cells vary between 150-498 µg mL^{-1} (Zamiri-Akhlaghi et al., 2011; Liu et al., 2012; Tumbas et al., 2012; Guimarães et al., 2014; Erguven et al., 2015; Nadpal et al., 2016). Thus, it is remarkable that the IC_{50} values indicating the cytotoxic activity of *R. pimpinellifolia* extract on HepG2 and WiDr cell lines are quite low compared to the literature.

Selectivity is one of the most important criteria for a compound to be evaluated as a chemotherapeutic (Demir et al., 2018b). For this reason, one normal cell line (BJ) was used along with three cancer cells in the study. The SI of extract and cisplatin for all studied cancer cells were calculated using the formula described in the "Materials and Method Section" of the IC_{50} values obtained for each cell and results were presented in Table 3.

Table 3. Selectivity index of *R. pimpinellifolia* extract and cisplatin

Çizelge 3. *R. pimpinellifolia ekstraktı ve cisplatin için hesaplanan seçicilik indeksleri*

	Extract	Cisplatin
A549	1.3	4.8
HepG2	2.6	1.1
WiDr	2.7	4.1

The results showed that the extract exhibited a highly selective cytotoxic effect, especially in the WiDr and HepG2 cell lines. It can be said that a compound or extract whose effect is investigated has a selective cytotoxic effect only if the SI value is greater than 2. (de Oliveira et al., 2016). From this point of view, the SI value of the extract in these two cell lines were satisfied this condition. Many studies have been shown that the selective cytotoxic effect of different *Rosa* (*Rosa beggeriana, Rosa canina* and *Rosa damascena*) extracts on various human cancer cell lines, such as breast (MCF-7), cervix (HeLa), colon (WiDr), liver (LCL) lung (A549) and prostate (PC-3), compared with normal fibroblast cells. In these studies, it is seen that the SI values of *Rosa* extracts in cancer cells vary between 1.3-3.8 (Artun et al., 2016; Turan et al., 2018; Zarei and Yaghoobi, 2019; Kilinc et al., 2020). This finding of the study were also in agreement with the previous reports. Preliminary experiments were performed to determine the optimum cell number per well for the MTT test. In these experiments, increasing amounts of each cell line were added to each well and the MTT protocol was applied. As a result of these experiments, seeding of 5000 cells per well giving absorbance values between 0.5 and 1 for WiDr, HepG2 and A549 cell lines was accepted as optimum. Since the BJ cell line is a cell of connective tissue fibroblast origin and has a more bulky and elongated appearance compared to other cells, the optimum cell number was determined to be 2500 per well in order to avoid contact inhibition. These cell numbers used are similar to cell numbers in similar comprehensive studies in the literature (Balsevich et al., 2012; Turan et al., 2017).

Rosa species are reported to be rich in phenolics, such as gallic acid, vanilic acid, chlorogenic acid, caffeic acid, syringic acid, coumaric acid, ferulic acid, sinapic acid, salicylic acid, elagic acid, trans-cinnamic acid, protocatechuic acid, quercetin, rutin, myricetin, kaempferol, and catechin derivatives (Tumbas et al., 2012; Cosmulascu et al., 2017). Various studies reported that phenolics exhibit antiproliferative effect on various types of cancer cells, such as lung, colon, breast, prostate, liver, gastric and cervix (Lall et al., 2015; Zhou et al., 2016). The antiproliferative activities of these compounds are explained by several cellular mechanisms, such as their ability of modulating apoptosis, necroptosis, ROS formation, angiogenesis, metastasis, autophagy, ferroxitosis, cell cycle arrest, mitochondrial function and endoplasmic reticulum stress (Zhou et al., 2016; Abotaleb et al., 2020). Therefore, we think that the selective cytotoxic effect of *R. pimpinellifolia* extract may be due to the synergistic effect of its phenolic content.

CONCLUSIONS

The results confirm that the phenolic and flavonoid contents of *Rosa pimpinellifolia* are rich and its antioxidant power is high. It can be concluded that *R. pimpinellifolia* might be a good candidate for inhibiting proliferation of especially WiDr and HepG2 cells. This is the first study to demonstrate the effect of *R. pimpinellifolia* on the proliferation of cancer cells. In future studies, it is necessary to determine the cytotoxic active components of *R. pimpinellifolia*, and to conduct characterization studies and to conduct anticancer efficacy studies in different cancer cell lines and experimental animal models.

Researchers Contribution Rate Declaration Summary

The authors declare that they have contributed equally to the article.

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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