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ORIGINAL ARTICLE / ÖZGÜN MAKALE

ANTIOXIDANT ACTIVITY AND ANTI-CANCER EFFECTS OF BILBERRY (*VACCINIUM MYRTILLUS* L.) FRUIT EXTRACT ON GASTRIC CANCER, AGS CELL LINE

YABANMERSİNİ (VACCİNİUM MYRTİLLUS L.) MEYVE ÖZÜ'NÜN MİDE KANSERİ, AGS HÜCRE HATTI ÜZERİNDE ANTİOKSİDAN AKTİVİTESİ VE ANTİ-KANSER ETKİLERİ

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ABSTRACT

Objective: *Vaccinium myrtillus L. fruits are consumed as food. This research was aimed to evaluate V. myrtillus methanol extract antioxidant and cytotoxic activities and determine its anti-cancer potential to further*

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study against gastric cancer.

Material and Method: *V. myrtillus fruit (Bilberry) methanol extract was examined for its antioxidant activities by ABTS•+ and DPPH• assays. The phytochemical analysis of the extract was studied by HPLC method. The cytotoxic effect of V. myrtillus fruit methanol extract on gastric cancer cell line AGS was measured by Cell Titer-Glo assay. Additionally, as healthy control, fibroblast like human mesenchymal stem cell line was used for testing anti-cancer efficacy.*

Result and Discussion: *V. myrtillus fruit methanol extract showed 0.1413 and 0.0439 mg/mL IC50 values as antioxidant activity by ABTS•+ and DPPH• assays, respectively. Malvidin-3-O-Glucoside was detected as an anthocyanin compound by HPLC method. Cytotoxicity analysis showed that among different concentrations (0.5-10 mg/ml), the most significantly, 2 mg/ml of Bilberry extract treatment decreased the viability of AGS gastric cancer cells while sparing healthy MSC cells. This data suggests the further analysis of Bilberry extract on several cancer cell lines as well as the determination of a potential active substance in the extract.*

Keywords: *Anti-cancer, antioxidant activity, bilberry, gastric cancer, Vaccinium myrtillus*

ÖZ

Amaç: *Vaccinium myrtillus L. meyvesi Türkiye'de gıda olarak tüketilmektedir. Bu araştırma V. myrtillus metanol ekstresinin antioksidan ve sitotoksisite aktivitelerini ve mide kanser hücresindeki potansiyel etkilerini belirlemeyi amaçlamıştır.*

Gereç ve Yöntem: *V. myrtillus meyvesi metanol ekstresinin antioksidan aktiviteleri spektrofotometrik olarak ABTS+ ve DPPH• yöntemleriyle gerçekleştirilmiştir. Fitokimyasal analiz HPLC yöntemi ile araştırılmıştır. V. myrtillus metanol ekstresinin mide kanseri hücre hattı AGS üzerindeki sitotoksik etkisi, Cell Titer-Glo testi ile ölçülmüştür. Ek olarak, anti-kanser etkinlik analizi için fibroblast benzeri insan mezenkimal kök hücre hattı sağlıklı kontrol hücreleri olarak kullanılmıştır. Morfolojik değişiklikler, faz kontrast mikroskobu kullanılarak incelenmiştir.*

Sonuç ve Tartışma: *V. myrtillus meyve metanol ekstresinde ABTS+ ve DPPH• testleri ile sırasıyla 0.1413 ve 0.0439 mg/mL IC50 değerleri hesaplanmıştır. Bir antosiyanin bileşiği olarak malvidin-3-O-glukozit HPLC yardımıyla tespit edilmiştir. Sitotoksite analizi, farklı konsantrasyonlar (0.5-10 mg/ml) arasında 2 mg/ml V. myrtillus meyve metanol ekstresinin, sağlıklı MSC hücrelerini korurken AGS mide kanseri hücrelerinin canlılığını azalttığını göstermiştir. Bu veriler, Yaban mersini özünün çeşitli kanser hücre dizileri üzerinde daha fazla analiz edilmesini ve ekstresindeki potansiyel aktif maddelerin belirlenmesini önermektedir.*

Anahtar Kelimeler: *Anti-kanser, antioksidan aktivite, mide kanseri, Vaccinium myrtillus, yaban mersini*

INTRODUCTION

Vaccinium myrtillus L. belongs to the Ericaceae family and is known as bilberry, blueberry, whortleberry, and huckleberry; it is called "yaban mersini" and "ayı üzümü" in Turkey [1, 2]. *V. myrtillus* is a type of shrub that grows naturally in the Black Sea region in Turkey; native to mountain and forest areas in Northern and Central Europe [3]. This species is a small plant with a height of up to 30 cm, shed its leaves in the winter. The fruit berry type is dark red - purple color [1].

Prepared from *V. myrtillus* leaves infusion (5%) is used for constipation, antiseptic, strengthening, and diabetes in Turkey [1]. In general herbal tea of leaves and stems of *V. myrtillus* are used for antioxidant activity, and also alcoholic extracts of these leaves and stems have been shown antibacterial activity [2, 4].

Fruits of *V. myrtillus* are consumed as food worldwide. The fruits are consumed for their health protection properties, as anti-inflammatory, anti-hypertensive, anti-microbial, and anti-cancer agents [1, 5]. The fruits include considerable quantities of antioxidant and micronutrients compounds, such as

polyphenols. These polyphenols, anthocyanins (delphinidin, cyanidin, petunidin, pelargonidin, peonidin and malvidin with the sugar part consists of glucose, arabinose, and galactose) the main ones, which belong to the flavonoid group and are responsible for the pigmentation of these fruits. Phenolic acids (caffeic, p-coumaric, ferulic, chlorogenic acids), flavonols (quercetin, myricetin), and flavonol (catechin) have been determined in fruits [5–7]. Because of these polyphenols, the fruits have antioxidant, astringent, antibacterial, and antiseptic properties. Also included are the ability to reduce the permeability and fragility of capillaries, inhibition of platelet aggregation, inhibition of urinary infection, and strengthening of collagen matrices through cross-links [8–11]. In recent studies have shown that extracts of fruit can be used in the prevention and treatment of chronic pathologies such as diabetes, cardiovascular disease, and obesity and confirm that high antioxidant and antiradical activity of bilberry fruits [2, 5, 12, 13]. Moreover, anthocyanin containing fruit extracts have the capacity to inhibit tumor formation and reduce cancer cell proliferation. It was observed that fruits inhibited MCF-7 cells by 50% at a concentration of 0.3-0.4 mg/ml. [14–21].

In this study, the total phenolic content (TPC) of methanol extract of *V. myrtillus* fruit has been determined. The extracts were investigated *in vitro* antioxidant and cytotoxic activities. To analyze cytotoxicity, cell viability was measured on the gastric cancer cell line, AGS and healthy MSC cell line treated with different doses (0.5-1-2-5-10 mg/ml) of methanol extract of *V. myrtillus* fruit. According to cell viability assay, at 2 mg/ml concentration significant cytotoxic effects of methanol extract of *V. myrtillus* fruit were determined in gastric cancer cell line while no toxicity was recorded in healthy MSC cells.

MATERIAL AND METHOD

Materials

Ascorbic acid, Trolox, 3-O-Glucoside, $ABTS^{\bullet+}$ *ve* $DPPH^{\bullet}$ radicals were purchased from Sigma, Germany. Methanol was supplied by Merck, Germany. Dulbecco's Modified Eagle's Medium (DMEM), 10% Fetal bovine serum, 1% Penicillin-Streptomycin were purchased from Gibco. To examine the effects of All other reagents used were of analytical grade.

Preparation of Samples

Fruits of *V. myrtillus* were purchased from a local market in Ankara, Turkey, and authenticated by Dr. Derya Çiçek Polat from Ankara University. Samples were pureed and they were extracted with methanol using a stirrer (250 g sample, 400 mLx3) (Heidolph MR3001). After being filtered, the extracts were concentrated in a vacuum at 40°C (Heidolph WB2000). The methanol extract yield of *V. myrtillus* was calculated as 9.32%.

Antioxidant Activity

The reaction mixture contains 100 μM DPPH• in methanol and different concentrations of *V. myrtillus* fruit extract (1-0.5 and 0.25 mg/mL). Free radical capture by measuring the absorbance value at 517 nm after 30 min at room temperature. The procedure was developed based on Blois's method [22]. Ascorbic acid was used as a positive control and the trials were carried out in triplicate.

The reaction mixture consists of 2.45 mM potassium persulfate and 7mM $ABTS^{\bullet+}$ aqueous solution. This mixture is allowed to stand in the dark overnight at room temperature. The $ABTS^{\bullet+}$ solution was diluted with ethanol. Samples were diluted 1/100 with $ABTS^{\bullet+}$ solution. 6 min later at 734 nm, the inhibition rates were determined by measuring the absorbance value [23]. Trolox was applied as a positive control and the trials were carried out in triplicate.

Determination of Phenolic Content

Folin Ciocalteu technique was used to detect total phenolics (gallic acid equivalent) of *V. mytrtillus* fruit methanol extract. The reaction mixture with extract was allowed incubate at 45°C and the absorbance was determined at 765 nm at room temperature. A linear calibration curve ($R^2 = 0.9913$) was used to determine the phenolic content [24].

HPLC Analysis

HPLC study was completed with an Agilent C18 column (250 × 4.6 mm, i.d. 5 μm). The mobile phases are A: Water:Formic acid (95:5); B: Acetonitrile and the gradient elution set up in the time frame 0–40 min, B%95–80 and the flow rate 1-0.5 mL/min. The detection wavelength was 520 nm and the injection volume was 20 μL. Malvidin-3-O-Glucoside was used for reference substance and injected at 0.2 mg/mL concentration [25].

Cell Viability Assay

In the study, AGS (ATCC®CRL-1739 ™; gastric adenocarcinoma) and MSC (UE7T-13 cells, # RBRC-RCB2161; RIKEN, Japan) lines were used. Growing and expansion media of AGS and MSC; DMEM with 2 mM L-Glutamine, 10% Fetal bovine serum, 1% Penicillin-Streptomycin. To examine the effects of *V. myrtillus* fruit methanol extract, AGS and MSC cells were seeded in 96 black well plates at a density of $5x10^3$-$1x10^4$, respectively. After 24 h the media of the cells were discarded. AGS and MSC cell lines were treated with *V. myrtillus* fruit methanol extract at various concentrations (0.5-1-2-5-10 mg/ml) and incubated for 48 h under standard culture conditions (37°C and 5% CO_2). The Cell Titer-Glo viability assay was performed according to (cat no: G7570, Promega) manufacturer's instructions. SpectraMax i3x Multi-Mode Detection Platform was used to determine the percentage of viable cells. The MSC cell line was used as a healthy control cell. Control groups of both cells were treated with the extract solvent (DMEM media). Each concentration was prepared in triplicate.

Statistical Analysis

Significance was determined according to *unpaired student t-test* using GraphPad (GraphPad Prism 7.0 program) for two group comparisons (*p* < 0.05, and p** < 0.001*).

RESULT AND DISCUSSION

Antioxidant Activity and Total Phenolic Content

Bilberry is a fruit known for its antioxidant properties and is widely consumed as a food. In addition, in many previous studies, the high antioxidant capacity of the fruit was revealed many times. In the results obtained from this study, it is seen that the antioxidant capacity of the bilberry extract used is high. The IC_{50} findings were presented in Table 1. When the obtained activity results are compared with the results obtained in previous studies, it can be considered that the results are consistent. In a previous study, the fruit extract was able to inhibit lipid peroxidation (IC_{50} = 50.28 μg/mL) and to scavenge superoxide anion (IC_{50} < 25 μg/mL). [25–28]. Studies explaining that the high antioxidant capacity of bilberry extracts is related to the phenolic compounds they contain [9, 29–31]. In another previous study, the changes in antioxidant capacity of bilberry extracts according to the seasons were examined and it was revealed that fruits collected in July had the highest antioxidant capacity, although there was not much difference [2].

Table 1. $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ scavenging activities *V. myrtillus* fruit methanol extract.

	V. myrtillus	**References**
	IC_{50} ±SD (mg/mL)	
$ABTS^{\bullet+}$	0.1413 ± 0.0075	0.0112 ± 0.001 (Trolox)
$DPPH^{\bullet}$	0.0439 ± 0.0043	0.0135 ± 0.001 (Ascorbic acid)

The phenolic content of *V. myrtillus* extract was measured as 2908 mg gallic acid/100g and found to exhibit moderate antioxidant capacity on the $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ analyzes.

HPLC Analysis

The phytochemistry of the *V. myrtillus* extracts was analyzed using HPLC which headed to the discovery of several compounds. The main anthocyanin component of *V. myrtillus* fruit extract was found to be the malvidin-3-O-glucoside as seen in Figure 1 and Figure 2. Malvidin is thought to be the major component in the extract.

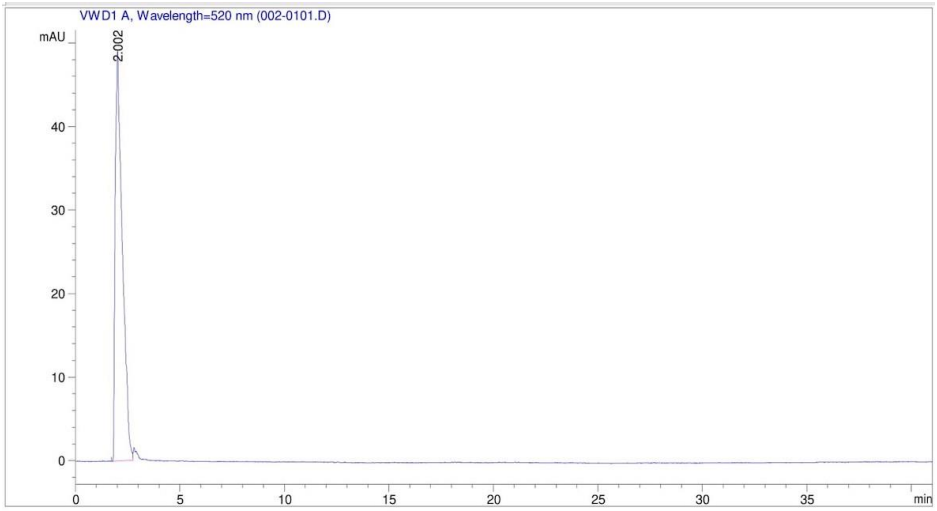

Figure 1. HPLC Standard Chromatogram

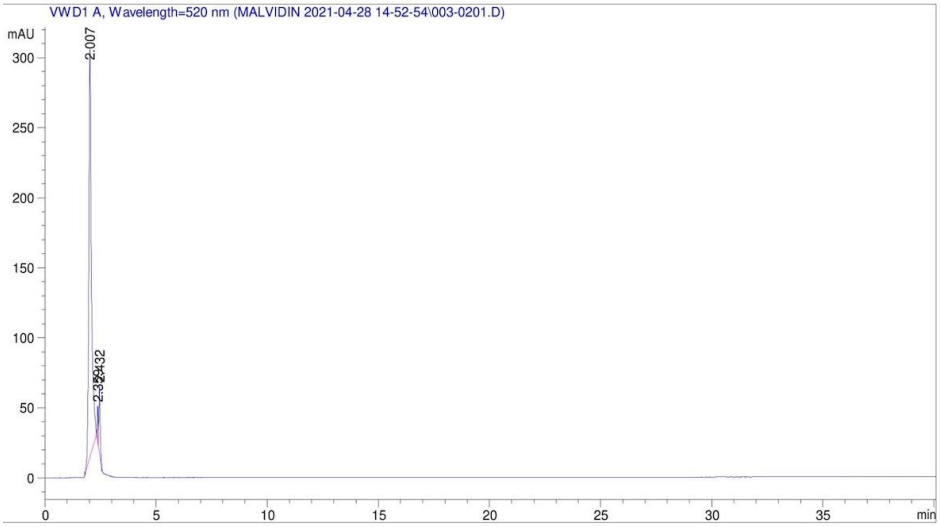

Figure 2. The HPLC chromatogram of *V. myrtillus* Methanol extract

Anti-cancer Activity

Morphological analysis displayed that 48h exposure of AGS and MSC cells with 5 or 10 mg/ml *V. myrtillus* fruit methanol extract, the cell growth was significantly inhibited, and cells became shrunken; cell blebbing and cytoplasmic degradation also observed. While there was no change in the morphology of the MSC cells treated with the 1, 2 mg/ml extract concentrations, changes in the MSC cell morphology were observed when treated 5 or 10mg/ml. The untreated (0 mg/ml) control cells preserved their healthy morphology (Figure 3A). The effects of *V. myrtillus* fruit methanol extract on the viability of the AGS cell line and MSC cells were studied after 48 h of treatment. The Cell Titer-Glo viability assay was performed. According to the result, the cell viability of the cells decreased gradually in a dose dependent manner. In comparison to the control cells, inhibition of viability of

bilberry extract treated AGS cells was ≅8% with 0.5 mg/mL, ≅33% with 1 mg/mL, ≅51% with 2 mg/mL, ≅69% with 5 mg/mL, and ≅97% with 10 mg/ml (Figure 3B). In short, cytotoxicity analysis showed that among different concentrations (0.5-10 mg/ml), 2mg/ml of Bilberry extract treatment decreased the viability of AGS gastric cancer cells the most significantly while sparing healthy MSC cells.

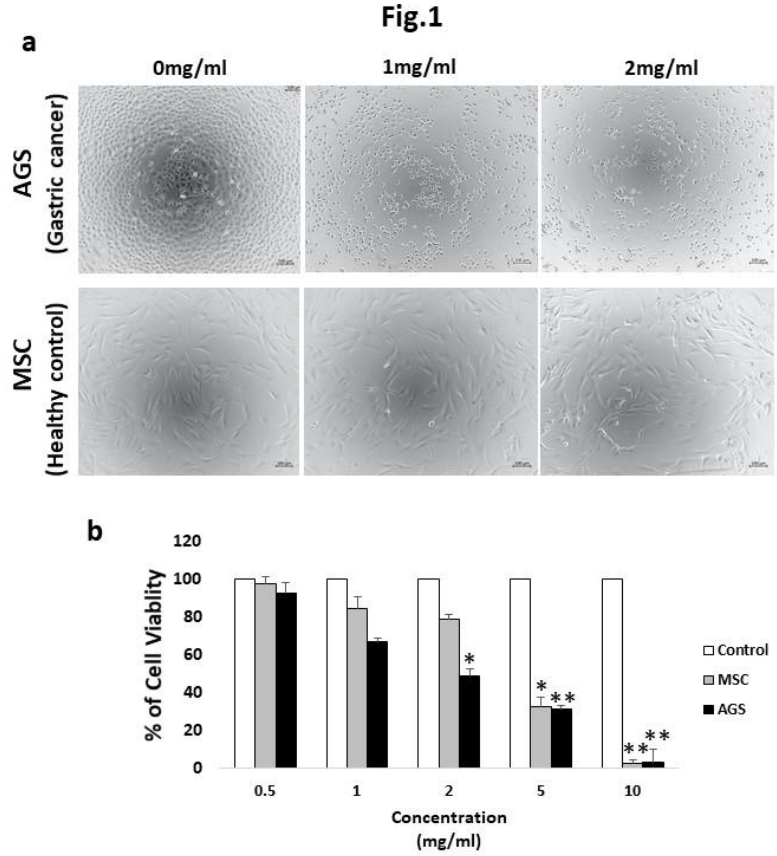

Figure 3. Cell viability results of AGS (gastric cancer) and MSC (mesenchymal stem cell; healthy control) cell lines treated with the bilberry extract for 48 h. a) Bright field image (10X Magnification). b) % Cell viability ratio. The results were expressed as the mean ± SD from triple replicates ($p^* < 0.05$, $p^{**} < 0.001$).

Bilberry (*Vaccinium myrtillus* L.) is known as one of the richest natural sources of anthocyanins especially delphinidins and cyanidins and contains other important phenolic components such as flavanols, tannins, ellagitannins, and phenolic acids [32]. In this study, the total phenolic content of the fruit extract was investigated, and an average value was determined. It is known that a significant part of the biological activities of extracts rich in phenolic substances are caused by these compounds.

Anthocyanins are the bio-flavonoid phytochemicals that give rich coloring to vegetables, flowers, and fruits, such as berries, pomegranates, and grapes. The most common anthocyanins in plants are pelargonidin, delphinidin, peonidin, petunidin, malvidin and cyanidin and are usually seen as 3-glycosides of these compounds [33–35]. Numerous studies have suggested that anthocyanins possess a powerful antioxidant, anti-inflammatory, anti-cancer activity [36, 37]. Anthocyanins have been proven to upregulate tumor suppressor genes, induce apoptosis in cancer cells, repair and protect genomic DNA integrity [33]. Anthocyanins stimulates redox-sensitive caspase 3-related apoptosis and Bad/Bcl-2 pathway dysregulation in chronic lymphocytic leukemia cells with no effect in healthy cells [38].

Several types of researches concluded that anthocyanin containing extracts significantly inhibited the growth of various cancer cells such as breast cancer, MCF-7, human colon cancer, HT-29, human cervical carcinoma, HeLa at different concentrations [16–18]. Although gastric cancer is the third cancer type worldwide [19, 20] there are no studies about the effect of the Bilberry extract on gastric cancer cells.

In recent times, a bilberry extract was proven to inhibit cell growth in MCF7-GFP-tubulin cells [16]. It seems to a similar effect with our results, but low doses inhibited cell proliferation in MCF7-GFP-tubulin breast cancer cells while higher doses inhibited cell proliferation in AGS gastric cancer cells. It can be related to gastric cancer being a more aggressive type than MCF7-GFP-tubulin breast cancer cells.

Another important point is that other studies did not look at the effects of Bilberry extract on normal cells. In this study, it was determined that blueberries are rich in phenolic compounds, and it was found that the major anthocyanin was malvidin-3-O-glucoside, which is known anti-cancer effect, by HPLC method in bilbery extract. It was demonstrated in previous studies that malvidin and its derivatives prevent oxidation in the cell, and it was thought that this may be related to cell life and its protective effect against cancer [39]. In addition, the anticancer activity of malvidin and its derivatives on some cell lines was investigated and its mechanisms were elucidated in detail [40–42]. The findings in previous studies also support the data obtained in this study. The studied bilberry extract is also rich in malvidin-3-o-glucoside, as can be understood from the HPLC results, this can explain its antioxidant and cytotoxic effects on the cancer cells.

In vitro studies have shown the significant cell killing ability of Bilberry extract at a certain concentration (2mg/ml) in AGS gastric cancer cells while no significant effect was measured for healthy human cells. Higher doses of extract increased the death cell population without cancer specificity, indicating cytotoxicity. Therefore, it was determined the optimum dose (2mg/ml) among tested, decreases cancer cell viability and did not damage normal cells. The extract can be further analyzed for

a possible active ingredient. More cancer cells can be included as a panel and response to treatments can be determined. Taken together, this study reveals a potential anti-cancer extract for a detailed analysis.

AUTHOR CONTRIBUTIONS

Concept: *N.K., M.E.O., A.E.K., D.Ç.P.;* Design: *N.K., M.E.O., A.E.K., D.Ç.P.;* Control: *M.E.O.* Sources: *N.K., M.E.O., T.S.;* Materials: *A.E.K., D.U.;* Data Collection and/or processing: *A.E.K., D.U., T.S.;* Analysis and/or interpretation: *N.K., M.E.O., A.E.K.;* Literature review: *M.E.O., A.E.K., D.Ç.P.;* Manuscript writing: *M.E.O., A.E.K., D.Ç.P.;* Critical review: *N.K., M.E.O.;* Other: -

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ETHICS COMMITTEE APPROVAL

The authors declare that the ethics committee approval is not required for this study.

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