

In Vitro Cytotoxic Effect of Various Fruits on Human Cervical Carcinoma Cells

Farklı Meyvelerin İnsan Servikal Kanser Hücreleri Üzerindeki Sitotoksik Etkileri

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Abstract

Objective The aim of this study was to evaluate the antiproliferative activities of some the fruits, traditionally used in folk medicine. Therefore, the fruits of *Fructus cynosbati* (Rosehip), *Rubus fruticosus* (Blackberry), *Sorbus torminalis* (Wild service tree), *Vaccinium myrtillus* (Blueberry) and *Vitis labrusca* (Isabella grape) were used in the present study.

Materials and Methods Methanol extracts of the fruits were obtained and added to ARPE-19 (no-transformed) and HeLa (transformed) culture media at various concentrations. Taxol and DMSO were used as positive and negative controls, respectively. After 48 h of cell incubation, the cytotoxic effect of the extracts was assessed by MTT. Cell viability values were calculated for each group. The statistical analysis of the data was performed using unpaired t-test. p values less than 0.05 were considered significant.

Results *V. labrusca* showed dose dependent cytotoxic activity against both cell lines. In additions, *V. myrtillus* and *R. fruticosus* extracts exhibited moderate cytotoxic effect on both cell lines at higher concentrations. Extracts of *F. cynosbati* and *S. torminalis* were found to decrease the cell viability in a dose-dependent manner in HeLa cells and the results were statistically significant ($p < 0.05$). Additionally, *S. torminalis* extract was found to be the most effective extract against the HeLa cells.

Conclusion These results demonstrated that *S. torminalis* methanol extracts has a potent in vitro cytotoxic effect toward the human cervical cancer cell line HeLa. Further work needs to be done on isolation and purification of specific bioactive compounds in *S. torminalis* methanol extract.

Keywords HeLa, Phytotherapie, *Sorbus torminalis*, MTT.

Özet

Amaç Çalışmanın amacı geleneksel olarak halk hekimliğinde kullanılan bazı meyvelerin antiproliferatif aktivitelerini araştırmak olup, *Fructus cynosbati* (Kuşburnu), *Rubus fruticosus* (Böğürtlen), *Sorbus torminalis* (Akçaağaç yapraklı üvez), *Vaccinium myrtillus* (Yabannersini) ve *Vitis labrusca* (Kokulu üzüm)'ün meyveleri çalışmada kullanılmıştır.

Materyal ve Metod Meyvelerin metanol özütleri elde edilerek, çeşitli konsantrasyonlarda ARPE-19 (normal) ve HeLa (kanser) hücre kültür ortamlarına eklenmiştir. Pozitif ve negatif kontrol olarak sırasıyla, Taxol ve DMSO kullanılmış olup, özütlerin sitotoksik etkisi, 48 saatlik hücre inkübasyon süresinden sonra MTT testi ile değerlendirilmiştir. Verilerin istatistiksel analizi, unpaired t-testi kullanılarak gerçekleştirilmiş olup, p değeri 0.05'ten küçük olan sonuçlar anlamlı olarak kabul edilmiştir.

Bulgular *V. labrusca* her iki hücre hattına karşı doza bağlı sitotoksik aktivite göstermiştir. Ayrıca *V. myrtillus* ve *R. fruticosus*, özütlерinin artan konsantrasyonlarda her iki hücre hattında orta derecede sitotoksik etkili olduğu saptanmıştır. Buna ek olarak, *F. cynosbati* ve *S. torminalis* özütlерinin sadece HeLa hücrelerinde canlılığı doza bağlı olarak azalttığı görülmüş ve sonuçlar istatistiksel olarak anlamlı bulunmuştur ($p < 0.05$). Fakat çalışmada *S. torminalis* özütünün HeLa hücrelerine karşı en etkili özüt olduğu saptanmıştır.

Sonuç Bu sonuçlar, *S. torminalis* metanol özütünün servikal kanser hücrelerine karşı in vitro koşullarda güçlü sitotoksik etkisinin olduğunu göstermiştir. Sonuç önemli olup, *S. torminalis* metanol özütü içindeki spesifik biyoaktif bileşenlerinin izolasyonu ve purifikasyonu için yeni çalışmalara ihtiyaç vardır.

Anahtar Kelimeler HeLa, Fitoterapi, *Sorbus torminalis*, MTT.

INTRODUCTION

Cancer is a significant health problem that affects large number of people in many countries in the world. Also it is a terrifying disease characterized by uncontrolled growth and propagation of abnormal cells.¹ Cervical cancer is a major health problem worldwide and the most common malignant tumor among women.² Chemotherapy is a method commonly used in cancer treatment. However, their high toxicity rate among the both of normal and cancer cells is the lack of desired effect of current therapies.¹ Indications have shown that active principle compounds from natural products may serve as potential chemotherapeutic agents with less toxicity to normal cells.³ Especially, phenolic compounds such as phenolic acids, flavonoids-flavonols, anthocyanins, tannins, and ascorbic acid are contained in many fruits with huge amount, and may act as strong antioxidants. The popularity of natural products in treatments is rising worldwide. Over 80% of people prefer to use plant products for healthcare needs.⁴

The fruits have been used in traditional and alternative medicine for a long time to treat various diseases.⁵ Therefore, the fruits of *Fructus cynosbati* (Rosehip), *Rubus fruticosus* (Blackberry), *Sorbus torminalis* (Wild service tree), *Vaccinium myrtillus* (Blueberry) and *Vitis labrusca* (Isabella grape) were chosen to work in the present study. Those plants above are widely occurring perennial herb in eastern black sea vicinity.

Fructus cynosbati is belonging to *Rosaceae* family. Various organs of the plant have been traditionally used as a diaphoresis, a diuretic expectorant agent and as agents of tuberculous enterorrhea, chronic enterogastritis and bronchitis and a preservative.⁶ In the middle ages, *Fructus spp.* has been used in the removal of parasites such as tapeworm, kidney, bladder and bile diseases, diabetes and diarrhea.⁷ *Rubus fruticosus* is wild small tree which also belong to *Rosaceae* family. They are one of the most diverse genera of plants, comprising 12 subgenera of which four have high value as fruiting species.⁸ Berry fruits have a very desirable

flavor because of sourish taste and sweet aroma for consumers, therefore they are mostly known on food industry. However, they have precious potential contents of nutraceuticals such as polyphenols, anthocyanins, and ascorbic acid.⁹ Unnatural high load of oxidants and free radicals could cause deleterious effects. They assault to lipids, proteins, and DNA, damaging cell membranes, enzymes, and genetic material.¹⁰ Generally, natural defense mechanism called antioxidants may eradicate the free radical impression. *Rubus spp.* extracts had polyphenols or other compounds that occur synergetic effects with together, could inhibit proliferation of cancer cells in vitro.¹¹ *Sorbus torminalis* belonging to the family *Rosaceae*. *Sorbus* genus has a wide usage area in landscape architecture. Also, in food industry the fruit is mostly used to produce liqueurs and schnapps. It has astringent characteristic at ripening, can be eaten when it is over ripe. *Sorbus spp.* has rich structure in bioactive compounds such as anthocyanin. It was determined that antitumor and anti-metastasis activity was formed on lung cancer.¹² In another study, *Sorbus spp.* extract has no effect on human hepatocellular carcinoma (HepG2) and human lung adenocarcinoma (A549) cells but has been tested to show potent antiproliferative activity on human colon adenocarcinoma (HT29) cells.¹³ *Vaccinium myrtillus* known as blueberry or bearberry in conventional. It exists within the family of *Ericaceae* and has many sub-species. Well-known health benefits, nutritional value, and excellent sensory evaluation made blueberries more popular in the last decade. Many studies about them have been reported to impact against pharmacological because of, their significant source of vitamins and other bioactive substances. Clinical study consuming blueberry phytochemicals could inhibit proliferation and metastatic potential of breast and colon cancer cells.^{14,15} The synergistic effect of phenolic compounds and ascorbic acid associate with inhibition of cancer cell growth and proliferation and induce apoptosis.¹⁶⁻¹⁸ *Vitis labrusca* is a member of *Vitaceae* family. Grapes contain high levels of phytochemicals such as antioxidant which have been correlated with a decreased risk of chronic diseases. They have also great antiproliferative

activities. The antiproliferative activity was assessed by the inhibition of MCF7, NCI-H460, HCT116, and MKN45 cancer cell proliferation.¹⁹

MATERIAL and METHODS

Plant Material Collection and Extraction

F.cynosbati, *R.fruticosus*, *S.torminalis* and *V.myrtillus* were collected from Ikizdere Mountains of Rize, during July-October 2018. *V. labrusca* is bought from public bazaar. Respectively, the frozen fruits were crushed and extracted with methanol. The extracts were filtered through a Whatman paper and the filtrates were then concentrated using a rotary evaporator (LabTech.EV311) at 40°C. Stock solutions of the extracts (50-200 mg/mL) were prepared in DMSO (dimethyl sulfoxide) and all the extracts were stored at -20°C for further analysis. For all the experiments, the working solutions were prepared by diluting the stocks of the extracts in complete media to the desired concentrations immediately before use. The final DMSO (as a negative control) concentration during the assays was kept below 0.5%

Cell Lines and Cell Culture

Human cervical cancer epithelial cell line (HeLa) was kindly provided by Prof. Dr. Fikretin Şahin (Yeditepe University, İstanbul, Turkey); and the diploid ARPE-19 retinal pigment epithelial cell line was kindly provided by Dr. Muradiye Acar (Turgut Özal University, Ankara, Turkey). Dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), taxol, D-PBS, and trypan blue (0.4%) were obtained from Sigma. Cell lines were maintained in RPMI-1640 (Hyclone) or DMEM (Gibco) with 10% fetal bovine serum (FBS) (Gibco) and antibiotics (100 µg/mL streptomycin + 100 U/mL penicillin) (Gibco) in T25 flasks at 37°C in 5%CO₂. Confluent cells were detached using 0.25% trypsin-EDTA (Gibco) solution for serial passage. Once cells reached 80-90% confluence, they were harvested and seeded into new sterile flasks. Lastly, cells were allowed to attach overnight for incubation. Cells were used in cytotoxicity assays

as stated below.

Morphological Assessment of Cells

By Inverted Microscope

Stock solutions (50-200 mg/mL) of fruits were diluted in the medium in order to generate working concentrations. HeLa (1×10⁴ cells/well) and ARPE-19 cells (2×10⁴ cells/well) were seeded into 24-well culture plates in 100 µL of growth medium in triplicate. After overnight incubation, various concentrations of fruits (800, 400, 200, 100, 50, and 25 µg/mL) or solely of the corresponding DMSO (max. 0.4%), were added to the wells. Taxol (5 nM) was used as a positive control. The cultures were maintained at 37°C for 24 h.

After 24 h, the effect of extracts on cell morphology was determined using an inverted light microscope with a 10× objective (Olympus CKX41). The changes in cellular morphology were photographed using a digital microscope camera (Olympus SC30).

Cytotoxic Studies Using MTT Assay

This assay was performed according to a slight modification of the procedure as reported by Mosmann.²⁰ Briefly, 1×10⁴ cells/well were seeded into 96-well microtiter plates in 100 µL of growth medium in triplicate and allowed to adhere overnight. The next day, different concentrations of each fruits (800, 400, 200, 100, 50 and 25 µg/mL) or solely of the corresponding DMSO were added into the cells. After 24 h incubation period, 10 µL of filter sterilized MTT (Sigma) solution (5 mg/mL in water) was added to each well and the cells were incubated for additional 4 h. After the medium was removed, formazan crystals formed in viable cells during the MTT treatment and these were dissolved by adding 100 µL of DMSO/well. The plates were then further incubated at 37°C for another 20 min and the absorbance was measured at 570 nm using the ELISA microplate reader (Termo, Multiskan GO). All experiments were performed three times in triplicate.

Statistical Analysis

Each set of experiments was repeated three times (n=3) whereas each assay was performed in duplicates, with the results being averaged. Growth inhibition was calculated in terms of percentage by the formula:

$$\frac{[(\text{absorbance of control well} - \text{absorbance of sample well}) / \text{absorbance of control well}] \times 100}{\text{absorbance of control well}} \times 100$$
. The results were analyzed using unpaired t-test. The results were considered significant if the p value was lower than 0.05.

RESULTS AND DISCUSSION

Many studies reported on the increased cancer cell death via induction of apoptosis by using several plant extracts or the natural compounds.²¹ Most of the chemopreventive agents cause various side effects that kill both cancerous and normal cells. Various studies are shown that using of plants derived agents reduces this risk. In the present study, in order to determine the anticancer capability of *F. cynosbati*, *R. fruticosus*, *S. torminalis*, *V. myrtillus* and *V. labrusca* in vitro, methanol extracts of those fruits were examined for the cell growth inhibition in HeLa and ARPE-19 cells lines.

Morphological Studies

The HeLa and ARPE-19 cells used in this study were morphologically plastic adherent cells. The morphological features of the cells were examined under an inverted microscope with fruit extracts (400 µg/mL), taxol, or DMSO after 24 h incubation.

As shown in Figure 1, no morphological changes were observed in the cell lines (HeLa, ARPE-19) treated with negative controls (DMSO or media). Meanwhile, positive control taxol (5 nM) caused rounding and detachment of the HeLa cells from the surface, but there were no changes in the morphology of the ARPE-19 cells. *F. cynosbati* extract (400 µg/mL) showed very mild effect on HeLa cells without affecting the ARPE-19 cell line. Additionally, exposure of the HeLa and ARPE-19 cell lines with other

fruit extracts did not induced any morphological alterations in those cells (data not shown). Since we had very limited amount of the methanol extract from *S. torminalis*, it was not tested for the morphological changes in the cells.

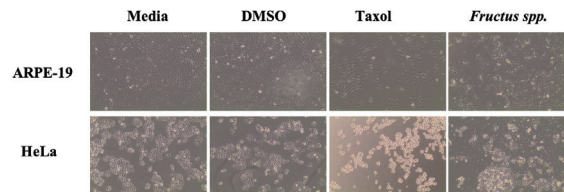


Figure 1. Morphological changes in the cell lines after treatment with the *Fructus cynosbati* extract and controls (Media, DMSO, Taxol/5nM, *F. cynosbati* extract/400 µg/mL) for 24 h.

Viability results

The cell viability of the HeLa and ARPE-19 cell lines was assessed by MTT method described by Mossman²⁰, with minor modifications.

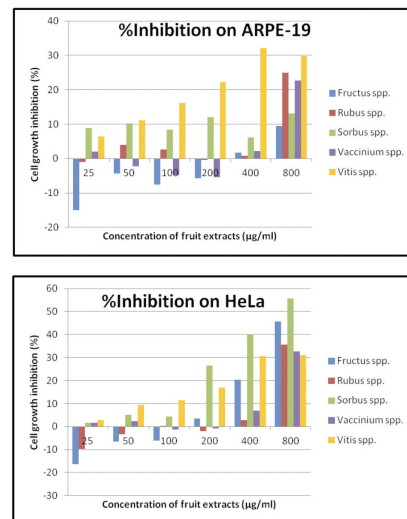


Figure 2. The anti-growth effect using MTT assay after treatment with the fruit extracts for 48 h against ARPE-19 and HeLa cell lines (concentrations between 800-25 µg/mL)

As shown in Figure 2, treatment of the cell lines with the various concentrations (between 800-25 µg/mL) of *V.*

labrusca methanol extract resulted in a concentration-dependent decline in cell viability. In addition, *V. myrtillus* and *R. fruticosus* extracts exhibited moderate cytotoxic effect on both cell lines at higher concentrations. However, the extracts of *F. cynosbati* and *S. torminalis* were found to decrease the cell viability in a dose-dependent manner only in HeLa cell line without affecting the normal ARPE-19 cells. Even though, *F. cynosbati* extract induced cytotoxic effect only on human cervical cancer epithelial cell line, as the concentration decreased below 800 µg/ml, there was no significant difference between ARPE-19 and the HeLa cells.

Meanwhile, it was determined that, *S. torminalis* extract at the concentrations of 800 µg/ml, was highly cytotoxic and led to 56 % cell death in HeLa, as compare to only 13 % in control cell ARPE-19. The IC50 value was found to be 264 µg/mL for HeLa cells but this value was not detectable for the normal ARPE-19 cell line. In terms of the cytotoxicity, the statistical analysis revealed that the difference between the ARPE-19 and HeLa cell lines was significant ($p < 0.05$). Cytotoxic effects of *S. torminalis* methanol extract against cervical cancer cell line HeLa has not yet demonstrated. This is the first report described the anticancer activity of *S. torminalis* fruit extract on human cervical cancer in vitro. Only a few studies have evaluated the growth inhibition of *S. torminalis* on human cancer cell. Among those, one study indicated that ethanol extract from *Sorbus rufopilosa* induced cell growth inhibition in colon cancer cells (HT-29) in a dose-dependent manner by inducing the apoptosis.¹³ The others found that invasive and migratory potentials of hepatocellular carcinoma Hep3B cells were inhibited with the water extract obtained from the stem and the cortex of *Sorbus commixta*.²² Finally, an in vivo study they demonstrated that *Sorbus aucuparia* extract led to a significant inhibition in the development of Lewis lung carcinoma in mice.¹²

Taken together, the findings of this study suggest for the first time, to the best of our knowledge, that *S. torminalis*

methanol extract obtained from the fruits caused dose-dependent inhibition in human cervical cancer cell line (HeLa).

CONCLUSIONS

Many fruit extract compounds have been reported for the antiproliferative activities and some of them showed their anti-proliferative effects on different cancer cell lines by inhibiting the cell growth.

The present study demonstrated a dose dependent cytotoxic effect of *Fructus spp.*, *Rubus spp.*, *Sorbus spp.*, *Vaccinium spp.*, and *Vitis spp.* methanol extracts on human cervical cancer cell (HeLa). The findings provided evidence that the methanol extract from *S. torminalis* might have some compound/compounds that may induce specific growth inhibition effect on HeLa cancer cells. It is likely that active components in *S. torminalis* extract have good potential to be developed into useful treatment for human cancer cell HeLa. Further work is needed to identify the activity of the components that may induce antiproliferative effect in HeLa cells.

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