Research Article

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Cytotoxic, genotoxic and apoptotic effects of *Viburnum opulus* on colon cancer cells: an *in vitro* study

[*Viburnum opulus*'un kolon kanseri hücreleri üzerindeki sitotoksik, genotoksik ve apoptotik etkileri: Bir *in vitro* çalışma]

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Abstract

Objective: Intake of various fruits is quite significant for maintaining the human body, due to their supply of useful constituents. *V. opulus* has been found to have outstanding antioxidant activity while showing a pro-oxidant effect at high doses. Due to this feature, *V. opulus* would be anticipated to have a healing impact on cancer treatment. In this study, it has been proposed to examine the cytotoxic, genotoxic, and apoptotic effects of *V. opulus* on human colorectal cancer cell.

Method: Different concentrations of *V. opulus* methanolic extract (5–2000 µg/mL) were incubated for 24 h with colorectal cancer cell line (Lovo). The cell viability, intracellular reactive oxygen species (iROS), DNA damage, and apoptosis were measured after incubation.

Results: The obtained results of this research demonstrate decreased cell viability and increased DNA damage, iROS, and apoptosis levels of *V. opulus* in Lovo cells in a concentration-dependent manner in the range of 14.88–52.06%. There were strong positive relationships between apoptosis, genotoxicity, and cytotoxicity in *V. opulus* methanolic extract treated cancer cell line.

Discussion: This *in vitro* research clearly demonstrated that *V. opulus* methanolic extract induces DNA damage, apoptosis, and cytotoxicity in a dose-dependent manner in cancer cells due to its pro-oxidant activity.

Conclusion: Although *in vitro* results are favorable, *in vivo* and further studies are needed.

Keywords: apoptosis; colorectal cancer; cytotoxicity; genotoxicity; *Viburnum opulus*.

ÖZ

Amaç: Çeşitli meyvelerin tüketimi, büyük miktardaki fonksiyonel bileşenler aracılığıyla insan sağlığının korunması için oldukça önemlidir. *V. opulus*'un yüksek antioksidan aktiviteye sahip olduğunu göstermesine rağmen, bu bitki yüksek dozlarda pro-oksidan etki de göstermektedir. Bu özelliğinden dolayı *V. opulus* kanser tedavisi üzerinde iyileştirici bir etkisi olmaktadır. Bu çalışmanın amacı *V. opulus*'un kolon kanseri üzerindeki sitotoksik, genotoksik ve apoptotik etkilerinin incelenmesi önerilmiştir.

Gereç ve yöntem: Farklı konsantrasyonlarda olan *V. opulus* metanolik ekstraktı (5–2000 µg/mL) kolorektal kanser hücre hattında (Lovo) 24 saat inkübe edilmiştir. İnkübasyondan sonra hücre canlılığı, hücre içi reaktif oksijen türleri (iROS), DNA hasarı ve apoptoz ölçülmüştür.

Bulgular: Bu çalışmanın elde edilen sonuçları, *V. opulus* ekstraktının kolorektal kanser hücre hattındaki (Lovo)

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hücre canlılığını azalttığını, DNA hasarını ve apoptoz düzeylerini doza bağımlı bir şekilde %14.88 ile %52.06 aralığında arttırdığını göstermiştir. *V. opulus* metanolik ekstrakt ile tedavi edilen kanser hücrelerinde DNA hasarı, apoptoz ile sitotoksisite arasında güçlü pozitif ilişkiler görülmektedir.

Tartışma: Bu *in vitro* çalışma, *V. opulus* metanolik ekstraktının kanser hücrelerinde doza bağımlı bir şekilde prooksidan aktivitesi yoluyla apoptoz, DNA hasarı ve sitotoksisiteye neden olduğunu açık bir şekilde kanıtlamaktadır.

Sonuç: Her ne kadar *in vitro* sonuçlar iyi olsa da *in vivo* ve daha ileri çalışmalar gereklidir.

Anahtar Kelimeler: apoptoz; genotoksisite; Kolorektal kanser; sitotoksisite; *Viburnum opulus*.

Introduction

Colorectal cancer is a major global health issue. According to 2018 data published by the World Health Organization, colorectal cancer is the third most commonly diagnosed cancer following lung and breast cancers, while it takes second place in cancer deaths worldwide [1]. Diet and increased average life expectancy along with environmental factors increase the risk of colorectal cancer in humans in America and Europe, where it is industrially more developed. Age is the most critical risk factor in the development of sporadic colorectal cancer. The incidence begins to increase after age 45, and almost 95% of the cases occur after 50 years of age [2].

Consuming a variety of fruits is quite important for an individual, as they contain significant amounts of functional constituents, suchlike total phenolic, and total antioxidant content, which are present in *Viburnum opulus* fruit [3]. *V. opulus* from the Caprifoliaceae family can be commonly observed grown naturally in Europe and sometimes also in Asia and North Africa [4]. The *V. opulus* fruit has a red color, an astringent bitter-sour taste, and is regarded as somewhat toxic due to the presence of saponin glycosides and viburnin [5]. The fruit of *V. opulus* is very acidic and contains a large number of polyphenols, ascorbic acid, and L-malic acid. The *V. opulus* fruits contain phenolic acids, such as hydroxybenzoic acids, tannins, coumarins, catechols, iridoid glycosides, anthocyanins, ascorbic acid, chlorogenic acid, salicin, (+)-catechin, (−)-epicatechin, cyanidin-3-glucoside, cyanidin-3-rutinoside, and quercetin [6].

The purpose of this study is to investigate the effects of *V. opulus* methanolic extract on viability, DNA damage, intracellular reactive oxygen species (ROS), and apoptosis on human colon cancer cell. These potential properties are believed to be due to the phytochemical content of *V. opulus*, in particularly, the content of flavonoids, which appear to have high pro-oxidant activity.

Material and methods

Chemicals

ABTS (2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt), methanol, DMSO (Dimethylsulphoxide) MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), Phosphate buffered saline, Folin–Ciocalteu, Ethidium bromide, Acridine orange, Ascorbic acid, Potassium acetate, Sodium acetate, Acetic acid, Gallic acid, Quercetin, H_2DCF-DA, and Aluminum nitrate were purchased from Sigma-Aldrich, Merck, and Gibco.

Plant extraction

V. opulus fruits were collected from Bunyan, Kayseri, Turkey. 50 g pulp samples were extracted with 50 mL methanol at room temperature for 24 h. Then the extract was centrifuged. After centrifugation, separated supernatants were evaporated at 45 °C [7].

After evaporation, the extract was lyophilized. The extract was stored in an amber bottle at −80° C until studies were conducted.

Antioxidant assays

Total antioxidant capacity (TAC): For the determination of the total antioxidant capacity of *V. opulus*, two reagents were used. The reagent 1 includes 0.4 M sodium acetate buffer (pH 5.8) and 0.4 M acetic acid while reagent 2 includes 30 mM acetate buffer (pH 3.6), 10 mM ABTS, H_2O_2, and 10% ethylene glycol. 1 mM ascorbic acid is used as the standard, diluted with Tris buffer. 10 µL of the extract was mixed with reagent 1 and measured photometrically at 660 nm. After measuring, 20 µL of reagent 2 was added and incubated for 5 min at room temperature with a shaker. After this incubation process, the absorbances were measured spectrophotometrically at 660 nm [8].

Total polyphenol capacity: For the determination of the total polyphenol capacity of *V. opulus* extract, gallic acid was used as the standard. The extract samples were diluted with 1:1 PBS. 200 µL of Folin–Ciocalteu reagent was added to 40 µL of the extract, and the mixture was incubated at room temperature for 8 min. After the incubation, 60 µL 7.5% Na_2CO_3 was added to the mixture. The solutions were incubated at 25 °C for 2 h. The results of the sample were compared to gallic acid [9]. The absorbance of *V. opulus* extract was measured by spectrophotometry (Varioskan Flash Multimode Reader, Thermo, Waltham, USA) at 746 nm.

Total flavonoid content: The content of total flavonoid was determined by spectrophotometry using quercetin as standard. *V. opulus* extract was mixed with methanol, 10% aluminum nitrate, 1 M aqueous potassium acetate. The mixture was incubated at RT for 40 min, then measured at 415 nm [10].

CUPRAC: Cupric ion (Cu^{2+}) reducing capacities of *V. opulus* extract was applied by modifying the cupric ion reducing antioxidant capacity (CUPRAC) method by Apak [11]. Prepared extracts were dissolved in 10 mL ethanol. 20 µL of these solutions were completed to 67 µL with ethanol. The solutions were vortexed after adding 61 µL of 10 mM $CuCl_2$ solution, 61 µL of 7.5 mM neocuproine reagent, and 61 µL NH_4Ac buffer (1 M, pH:7), and the solutions were incubated for 30 min at room temperature. After the incubation, the absorbance of the solutions was measured at 450 nm with the reference solution 1 mM ascorbic acid.

Cell line and treatment

Colon cancer cell line Lovo (ATCC® CCL-229®) colorectal adenocarcinoma from human were grown in F12K medium, and 10% Fetal bovine serum, and 1% Penicillin-Streptomycin antibiotic. Adherent monolayer Lovo cells were incubated with saturated moisture in 5% CO_2 at 37 °C.

Cytotoxicity

The cytotoxic effects of *V. opulus* extract was determined by using *in vitro* MTT (3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay [12]. Lovo cell line was cultured in a 96-well plate (7×10^3 cells/mL). After 24 h incubation, the medium was treated for 24 h with different concentrations of *V. opulus* (5–2000 µg/mL; l). After overnight incubation with the extract, the wells were treated with 15 µL of 5 mg/mL MTT. This mixture was incubated for 4 h at 37 °C. After the incubation, 100 µL of DMSO was added to each well and incubated at room temperature for 20 min. After the incubation, the formed purple solution was quantified colorimetrically at 540 nm (Varioskan Flash Multimode Reader, Thermo, Waltham, USA).

To calculate the half-maximal inhibitory concentration (IC_{50}), the following formula has been used:

$$y = ax + b$$
$$IC_{50} = \frac{(0.5 - b)}{a}$$

Intracellular reactive oxygen generation

The level of intracellular reactive oxygen species was measured in fluorometry using H_2DCF-DA fluorescence dye. After different concentrations of *V. opulus* were incubated for 24 h, the cells were washed three times with dPBS. The 5 µM H_2DCF-DA was incubated at 37 °C for 30 min in the dark. After the incubation, the cells were washed and resuspended in dPBS. Intracellular ROS contents were measured using a fluorimeter (Varioskan Flash Multimode Reader, Thermo Scientific, USA).

DNA damage

The single-cell gel electrophoresis assay (Comet Assay) to evaluate the genotoxic effects of *V. opulus* extract on Lovo cells were performed with a minor modification to Singh et al. [13]. Lovo cells were seeded into 6-well plates to be incubated with different doses of *V. opulus* under IC_{50} for 24 h. After incubation, the cells were removed with trypsin-EDTA and centrifuged at 400×*g*. Following the supernatant was aspirated, it was washed with dPBS at 400×*g* for 5 min.

Fifteen microliter of cell suspension was mixed with 85 µL of 0.6% low melting agarose and added to the slide pre-coated with 1% normal melting agarose. After freezing the gel at +4 °C, it was incubated with lysis buffer at +4 °C, and it was incubated for 40 min in the dark in an alkaline solution to unwind DNA in the presence of cooling. Slides were electrophoresed at +4 °C for 25 min (condition: 26 V, 300 mA). Afterward, slides dehydrated with ethanol were stained with 2 µg/mL ethidium bromide, and images were recorded. All DNA figures were scored using the Comet assay IV software with a fluorescence microscope (Leica DM 1000, Solms, Germany).

Apoptosis

The nuclear morphological changes associated with apoptosis were analyzed by double staining AO/EB, as explained by McGahon et al. [14]. Lovo cells were seeded in the 6-well plate for 24 h. The incubated cells were treated for 24 h at 37 °C with *V. opulus* concentrations below $IC_{50,}$ which was determined as a result of cytotoxicity assay. As a negative control, 1% DMSO was used. The cells were harvested and washed twice with PBS. The acridine orange/ethidium bromide solution was added to the cell suspension, and the nuclear morphology was evaluated by fluorescence microscopy (Leica DM 1000, Solms, Germany). Images were captured at randomly selected areas, and a minimum of 100 cells were counted. The inhibition rates of the concentrations were calculated with the formula as following:

$$Inhibition\ rate = \left(\frac{\text{Number of deadce cells}}{\text{Number of total cells}}\right) \times 100$$

Statistical analysis

All experiments in the study were performed a minimum of four times, and the results were given as mean ± standard deviation (mean ± SD). Statistical analyzes were made with one-way ANOVA. $p<0.05$ was considered statistically significant. The IC_{50} value of *V. opulus* was calculated by non-linear regression analysis. All statistical analysis of the study was done with the Statistical Package for Social Sciences (version: 23.0) program.

Results

Phenolic content

The quantification of the phenolic compounds content of the fruit from *V. opulus* methanol extract is shown in Figure 1. The concentrations of the extract that were determined are 1, 2, 4, and 8 mg/mL. The extracts were stated using the standard curve equation $y=0.0849x + 0.2424$, $R^2=0.9711$ in term of gallic acid equivalent. The amount of phenolic compounds ranged from 0.098 to 7.83 mg gallic acid/mL *V. opulus* extract.

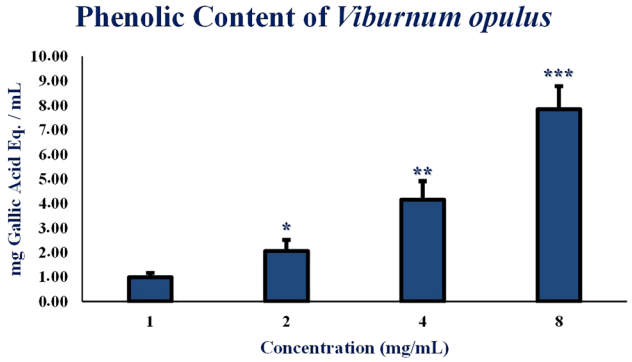

Figure 1: Phenolic content in *Viburnum opulus* concentrations is 1–8 mg/mL. Statistically significant differences of compared to control; * for p<0.05, ** for p<0.01, *** for p<0.001.

Flavonoid content

Flavonoids are a large group of phenolic compounds among several important constituents of *V. opulus* fruit. The concentrations of the *V. opulus* methanol extract that were determined are 1, 2, 4, and 8 mg/mL. The extract was stated using standard curve equation y=0.314x−0.2084, r^2=0.9773 in term of quercetin equivalent. It can be seen in Figure 2 that the total flavonoid content of the extract is the dose-dependent manner in the range of 0.918–1.537 mg quercetin/mL *V. opulus* methanol extract.

Total antioxidant capacity

With the ABTS method, the total antioxidant capacity of *V. opulus* methanol extract was detected. The concentrations of the extract that were determined are in the range of 1–8 mg/mL. The extract was stated in term of ascorbic acid equivalent using a standard curve equation y=−0.6425x + 0.6309, R^2=0.9939.

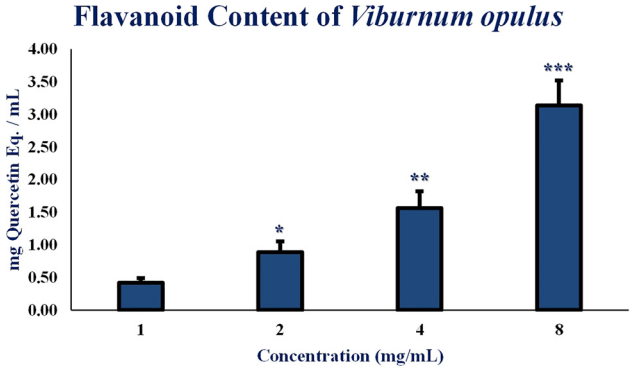

Figure 2: Flavonoid content of *V. opulus* concentrations is 1–8 mg/mL. Statistically significant differences of compared to control; * for p<0.05, ** for p<0.01, *** for p<0.001.

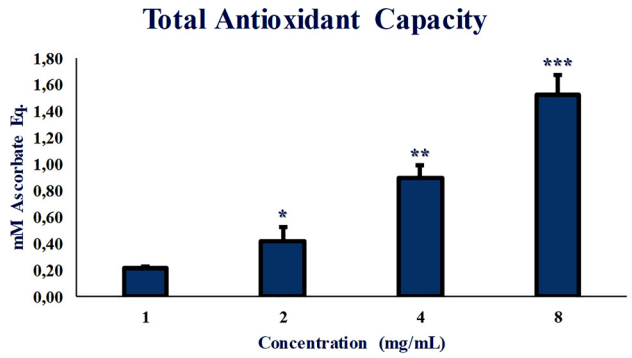

Figure 3: Total antioxidant capacity of *V. opulus* concentrations is 1–8 mg/mL. Statistically significant differences of compared to control; * for p<0.05, ** for p<0.01, *** for p<0.001.

The total antioxidant content of the extract ranged from 0.11 to 0.90 mmol/L ascorbic acid eq. (Figure 3).

Cell viability

To detect the cell viability, Lovo cells in the experiment were treated with increasing concentrations of *V. opulus* methanol extract (5; 10; 50; 125; 250; 500; 750; 1000; 2000 µg/mL) for 24 h. The control was without the extract treatment. After 24 h, the cell viability was determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay.

Viability assay showed that the *V. opulus* methanol extract induced the cell viability in a concentration-dependent manner in the range of 5–2000 µg/mL. The results of the cytotoxicity assay are presented in Figure 4. The extract was able to decrease the

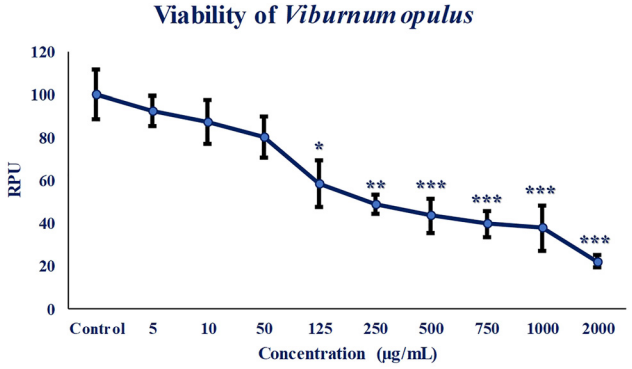

Figure 4: Effect of *V. opulus* on cell viability. Lovo cells were treated with concentrations (5–2000 µg/mL) of *V. opulus* for 24 h. The calculated percent of cell cytotoxicity was normalized according to the control. All results are given as mean ± standard deviation (mean ± SD). Statistically significant differences of relative values compared to control; * for p<0.05, ** for p<0.01, *** for p<0.001.

proliferation of Lovo cells. The IC_{50} of the extract is calculated with the standard curve equation y=−8.7575x + 109.04. The IC_{50} doses of *V. opulus* extract for Lovo cells were found 225.26 µg/mL.

Intracellular ROS generation

The intracellular ROS generation was measured using the H_2DCF-DA probe by the fluorometric method. All doses (5–2000 µg/mL) significantly reduced intracellular ROS production in Lovo cells (Figure 5).

DNA damage

The effect of different concentrations of *V. opulus* (below IC_{50} doses) in colon cancer cell on DNA damage after 24 h incubation was measured by the Comet Assay method. If there is no damage in DNA, round occurs, and if DNA is damaged, comet occurs (Figures 6, 7).

Apoptosis

The apoptotic effect of *V. opulus* methanol extract concentrations 10; 50; 100, and 200 µg/mL was detected by staining the cancer cell with acridine orange/ethidium bromide double dyes.

Figure 5 represents the results of acridine orange/ethidium bromide staining as the percentage of apoptotic cells to total cells. The values are indicated as percentage mean of apoptotic cells to total cells in each dose point. The inhibition rate of the concentrations 10; 50; 100; 200 µg/mL

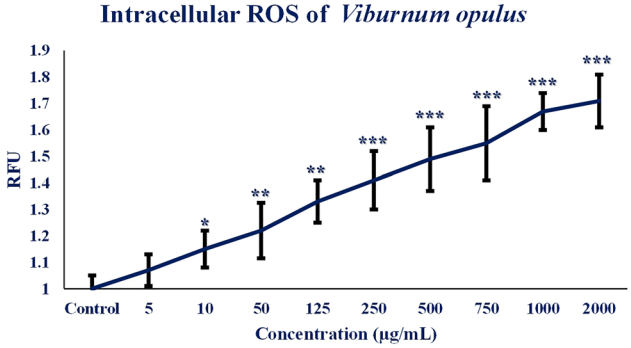

Figure 5: Effect of *V. opulus* on intracellular reactive oxygen species generation. Different concentrations of *V. opulus* were incubated in Lovo cells for 24 h. The pro-oxidant activity of *V. opulus* was determined by using reactive oxygen generating assay. Statistically significant differences of relative values compared to control; * for p<0.05, ** for p<0.01, *** for p<0.001.

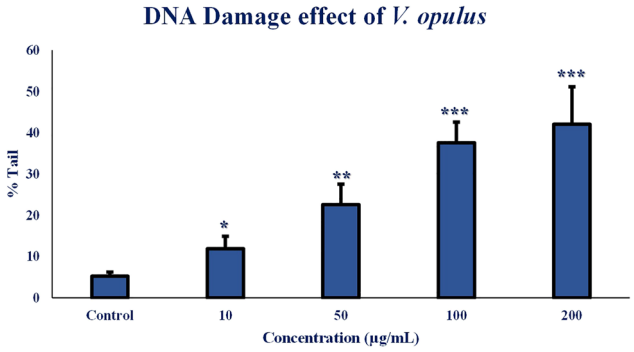

Figure 6: Lovo cells were treated with different concentrations of *V. opulus* (10 to 200 µg/mL) for 24 h. Statistically significant differences of relative values compared to control; * for p<0.05, ** for p<0.01, *** for p<0.001.

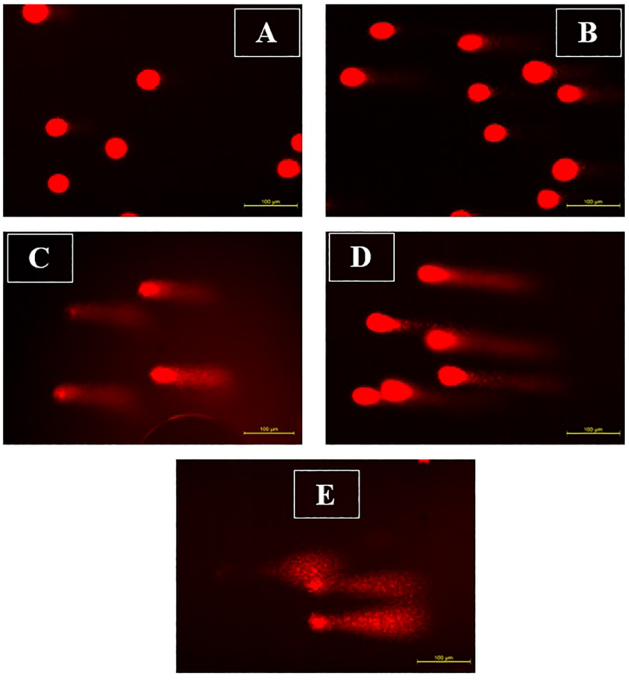

Figure 7: Increased *V. opulus* concentrations damaged the DNA of Lovo cells. Thus% tail density increased statistically significantly. (A) Control without *V. opulus* treatment. (B) Treatment with 10 µg/mL *V. opulus* methanol extract. (C) Treatment with 50 µg/mL *V. opulus* methanol extract. (D) Treatment with 100 µg/mL *V. opulus* methanol extract. (E) Treatment with 200 µg/mL *V. opulus* methanol extract under fluorescence microscope by 200X (Leica). Green color shows the normal morphology of living cells, while the yellow and orange color shows apoptotic cells.

are in the range of 14.88–52.06%. The apoptosis-inducing potential of the extract was tested in cells using the acridine orange/ethidium bromide double staining assay. The morphology changes in apoptotic cells, including morphology change and nuclear fragmentation are shown

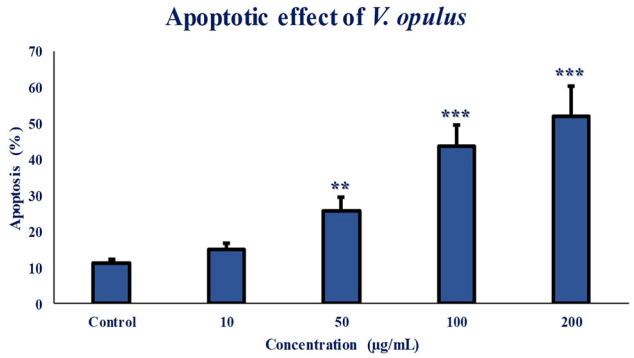

Figure 8: Effects of *V. opulus* on cell apoptosis in a colorectal cancer cell line (Lovo) treated with different concentrations (10; 100; 500; 1000 µg/mL) of *V. opulus* extract for 24 h.

in Figure 6. After the 24 h incubation, as the concentration of *V. opulus* increased, living cells decreased while apoptotic cells increased. These morphological changes of yellow-orange cells suggest that apoptosis increased in cells treated with *V. opulus* methanol extract in a dose-dependent manner in the range of 14.88–52.06% in the Lovo cell line (Figures 8, 9).

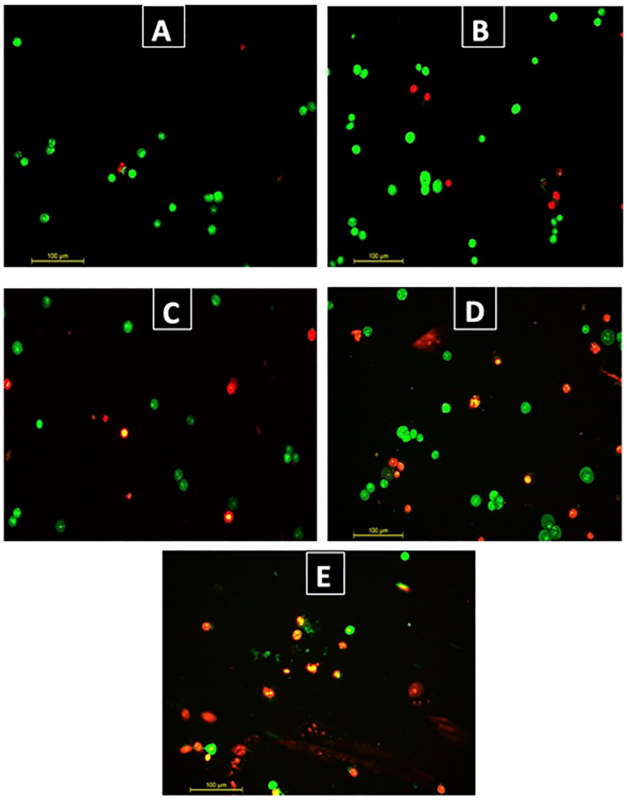

Figure 9: Acridine orange/ethidium bromide staining has shown a decreased apoptosis induced by stress in Lovo cell line treated with *V. opulus* methanol extract. (A) Control without *V. opulus* treatment. (B) Treatment with 10 µg/mL *V. opulus* methanol extract. (C) Treatment with 50 µg/mL *V. opulus* methanol extract. (D) Treatment with 100 µg/mL *V. opulus* methanol extract. (E) Treatment with 200 µg/mL *V. opulus* methanol extract under fluorescence microscope by 200X. Green color shows the normal morphology of living cells. It shows apoptosis in yellow and orange cells.

Discussion

Colorectal cancer is a severe health problem in the world. In 2019 WHO data, death from colorectal cancer is the second in all cancer cases in the world [1]. The search for alternative treatments continues due to the inadequacy of the current drugs and combinations in the therapy of cancer. Antioxidants are found in natural diet, which include vitamins, carotenes, and minerals. Several antioxidants inhibit reactive oxygen compounds in metabolism. The positive effect of an antioxidant is to neutralize reactive oxygen species (ROS) and release it from the free radicals that may damage DNA and support tumorigenesis. The antioxidants are the first line of choice to take care of the stress [15, 16]. The purpose of this research was to determine the amount of phenolic and flavonoid contents along with the antioxidant activity of *V. opulus* fruit methanol extract and to the purpose of the cytotoxic effects of the extract on human colon cancer cell line (Lovo) in culture. The antioxidative potential of *V. opulus* methanol extract was determined using photometric methods as well as phenolic and flavonoid content, total antioxidant capacity, and CUPRAC. The content of phenolic compounds was detected with gallic acid as the standard of *V. opulus* methanol extract in the range of 0.098–7.83 mg gallic acid/mL. The total flavonoid content of the *V. opulus* methanol extract is in the range of 0.918–1.537 mg quercetin/mL. Judging by these findings, *V. opulus* can be classified rich in phenolic and flavonoid content. The total antioxidant content of the extract is in the range of 0.11–0.90 mmol/L ascorbic acid eq. The results showed that the concentration of the extract increased CUPRAC levels in a dose-dependent manner in the range of 1.39–16.30 mmol/L. The IC_{50} value of the extract was found 225.26 µg/mL. This assay also confirmed the reduction on cell viability, and the decrease in cell number was due to the cytotoxic effects of *V. opulus* methanol extract against the colorectal cancer cell line. The cytotoxic effect of *V. opulus* may be derived from alkaloids, phenolic compounds, flavonoids, or their synergistic combinations that are found in *V. opulus* methanol extract. It is reported to play an important role of oxygen-derived radicals in the etiology of cancer development [5].

Previous studies in the literature have shown that *V. opulus* has no genotoxic activity [17]. But in our study,

V. opulus has increasingly inflicted DNA damage as the dose increased. The most important difference between the literature and our study is that high doses of proxy effects cause DNA damage.

The apoptosis in this study was determined by the detection of acridine orange/ethidium bromide double-stained condensed nuclei by fluorescent microscopy. Apoptotic, necrotic, and live cells can be distinguished using acridine orange/ethidium bromide dye. While living-healthy cells are green, the morphology of apoptotic cells has changed, and it is yellow-orange. Necrotic cells are colored red. Our results show that apoptosis and necrosis increase, and viability decreases between 5 and 2000 µg/mL doses colorectal cancer cell in a concentration-dependent manner in the range of 14.88–52.06%. Recent studies have shown that *V. opulus* has higher level of antioxidant activity and antimicrobial potency [18, 19]. From the other studies, the phenolic compound of the antioxidant content of *V. opulus* varied in the range of 5.4–10.6 mg GAE/g [20]. Another study found total phenolic content as 351.26 ± 27.73 mg GAE/100 mL [15]. In our research, we found the phenolic content also in these ranges. *V. opulus* have antioxidant properties by low doses and pro-oxidant properties by high doses. Hence, *V. opulus* may have a positive effect on cancer treatment. *V. opulus* fruit extract modulates cell viability, cell proliferation, cell death, adhesion, inflammation, oxidative stress, and signal transduction pathways. *V. opulus* extract prepared with an organic solvent has a flavonoid-rich fraction and a total polyphenolic fraction, which inhibits the viability of colon cancer cell lines [21, 22]. In general, antioxidants inhibit oxidative damage due to their capability of inhibiting intracellular ROS. It should be noted, high doses of antioxidants show pro-oxidant activity with the presence of transition metals (iron-II and iron-III) [23]. To our knowledge, this study is the first *in vitro* study to show the pro-oxidant effects of *V. opulus*.

Conclusion

Intracellular ROS increased due to the high-dose pro-oxidant property of *V. opulus* extract. So, cytotoxicity, DNA damage, and apoptosis are increased. Experimental animals and clinical studies are needed for *V. opulus* to be an option in cancer treatment.

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Informed consent: Informed consent was obtained from all individuals included in this study.

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