

In Vitro Evaluation of Gilaburu (*Viburnum Opulus L.*) Juice on Different Cell Lines

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Abstract

The purpose of this study is to investigate the effects of gilaburu juice on cell viability (3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide, MTT) and angiogenesis (tube formation assay) using different cell lines (human cancer cell lines A549, Caco-2, HeLa and normal cell lines MDCK and HUVEC) in vitro. In addition, the genotoxic effects of gilaburu juice is evaluated using COMET assay on HUVEC cells. Our results demonstrate that gilaburu juice could inhibit the growth of Caco-2 and HeLa cancer cell lines, but could not significantly inhibit normal cell lines and A549 cancer cell lines. It disrupted tube formation of HUVEC cells. Gilaburu juice appears to have no genotoxic potential to the DNA of HUVEC cells. The results obtained in this study confirm the potential application of commercial gilaburu juice as a functional food in prevention of cancer.

Keywords: Gilaburu juice, European cranberry bush, *Viburnum opulus*, angiogenesis, cancer

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Introduction

The bark and fruit of *Viburnum opulus* (European cranberrybush), a member of the Caprifoliaceae family, are widely-used in pharmacology. It is also known with some other names such as European cranberrybush, American cranberrybush, crampbark, guelder rose, gueldres-rose, cherry-wood, rose elder, snowball and whitten tree (Sagdic, Ozturk, Yapar, & Yetim, 2014). The fruit juice of the European cranberrybush (*Viburnum opulus* L.) is a traditional drink in the middle Anatolia region of Turkey, especially in the city of Kayseri (Soylak, Elci, Saracoglu, & Divrikli, 2002). Gilaburu is the local name of *Viburnum opulus*. Gilaburu juice is manufactured as a commercial product by Kayseri Pazarı Bio. Bitkisel Ürünler San. ve Tic. A.Ş. in Kayseri (Ulger et al., 2013). Gilaburu fruit is used as a remedy for health problems, due to the presence of total phenolics, ascorbic acid, flavonoids and anthocyanins in its contents. The fruit has been used to treat kidney, heart, lung, stomach, digestive tract, liver and biliary diseases and disorders, as well as neurosis, coughs and colds, and high blood plessure (Zayachkivska et al., 2006; Van et al., 2009; Česonienė, Daubaras, Vencloviene, & Viškelis, 2010; Kraujalytė, Leitner, & Venskutonis, 2012; Moldovan, David, Chişbora, & Cimpoiu, 2012). The juice obtained from the body and the shell of the plant can be used both internally and externally. Systemic administration is used in mild asthma, epileptic seizures, hypertension, certain heart conditions, cramps, menstrual pain, mumps, postpartum pain, sleep disorders, and certain conditions of the nervous system, while external use is reserved for skin conditions such as eczema. Another field where it is applied is that of limb or abdominal cramps or other parts in women, especially during pregnancy, and menstrual pain (Felter & Loyd 2004; Velioglu, Ekici, & Poyrazoglu, 2006; Sagdic, Aksoy, & Ozkan, 2006; Altun & Yılmaz, 2007). *In vitro* studies confirm that the *Viburnum* species or their extracts have antioxidative and antimicrobial effects (Ersoy et al, 2017; Kraujalytė, Venskutonis, Česonienė, & Daubaras, 2013; Eryılmaz et al., 2013; Česonienė, Daubaras,

Kraujalytė, Venskutonis, & Šarkinas, 2014). Gilaburu has activity on alleviation of testicle and sperm damages has been studied in vivo (Sarıözkan et al., 2017). In the literature, there are only a few studies concerning gilaburu. These studies mostly concentrate on the anthocyanin and polyphenolic content of gilaburu. Gilaburu is a good source of polyphenolics (Česonienė, Daubaras, & Viskelis, 2008; Rop et al., 2010), phenolic acids (Velioglu, Ekici, & Poyrazoglu, 2006), Flavonoids and anthocyanins (Česonienė, Daubaras, Viškelis, & Sarkinas, 2012; Velioglu, Ekici, & Poyrazoglu, 2006), ascorbic acids (Rop et al., 2010) and L-malic acids. The presence of a high concentration of phenolics correlates with a strong radical scavenging capacity of gilaburu extracts (Sagdic, Aksoy, & Ozkan, 2006; Altun, Sever-Yılmaz, & Saltan-Çitoğlu, 2007; Česonienė, Daubaras, & Viskelis, 2008). Karaçelik et al. (2015) studied antioxidant components of *V. opulus* juice and extracts by using on-line HPLC-UV-ABTS radical scavenging and LC-UV-ESI-MS methods. They analyzed antioxidant components profile of *V. opulus* juice and identified main antioxidants of the juice. They demonstrated that the major components of *V. opulus* juice were catechin, epicatechin, procyanidin B2, procyanidin trimer, quercetin-deoxyhexose, chlorogenic acid, and proanthocyanidin dimer monoglycoside (Karaçelik et al., 2015).

Cancer is a devastating disease, which affects millions of lives every year. Cancer development includes a multi-step process, such as induction of genetic instability, abnormal expression of genes, abnormal signal transduction, angiogenesis, metastasis, and immune evasion (Rop et al., 2010; Khazir et al., 2014). Angiogenesis, the formation of new blood vessels by endothelial cells, involves the degradation of basement membrane, cell proliferation and migration and tube formation (Folkman 2003; Chan et al., 2009; Chen et al., 2011). Numerous studies have demonstrated that polyphenols from edible plants have antioxidative, anti-inflammatory, antiproliferative, and anti-angiogenic activities. The literature has shown that polyphenols may affect different steps of angiogenesis. Many *in*

in vitro and preclinical studies have shown that polyphenols inhibit angiogenesis via modulation of endothelial cell and vascular smooth muscle cell proliferation, migration, and tube formation (Chen et al., 2011). Many antioxidants have also been identified as natural mutagens and carcinogens, apart from their role as natural anti-mutagens and anti-carcinogens (Ames 1983; Stavric 1994; Middleton, Kandaswami, & Theoharides, 2000; Franke et al., 2004). A number of recent reports have observed that gilaburu has high level antioxidant activity and antimicrobial potential (Sagdic, Aksoy, & Ozkan, 2006; Altun & Yilmaz, 2007; Ulger et al., 2013). For this reason, in the present study, we evaluate the genotoxic effect of gilaburu juice on HUVEC cells.

The objectives of the current study are: (1) investigation of gilaburu juice *in vitro* antiproliferative properties against three human cancer cell lines and two healthy cell lines; (2) investigation of the genotoxic potential of gilaburu juice on HUVEC cells; and (3) an assessment of anti-angiogenic activity of gilaburu juice on HUVEC cells.

Material and Methodology

Material

A549 (human type II lung epithelium, ECACC Cat no. 86012804), Caco-2 (human colon adenocarcinoma, ECACC Cat no. 09042001), HeLa (human cervix adenocarcinoma, ECACC Cat no. 93021013), MDCK (Madin Darby Canine Kidney, ECACC Cat no. 84121903) cell lines were obtained from ECACC (European Collection of Cell Cultures, Salisbury, UK). HUVEC cells were purchased from the ATCC (American Type Cell Collection, USA, Cat no. CRL-1730TM). Fetal calf serum were purchased from Hyclone (Hyclone, Logan, UT, USA). Minimum Essential Medium Eagle (MEM), Nutrient Mixture F12 HAM medium, Minimum Essential Medium Eagle (MEM), Nutrient Mixture F12 HAM

medium, Penicilin-streptomycin, 0,25 % Tripsin-EDTA, MEM Non-essential amino acid solution, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide], matrigel were purchased from Sigma-Aldrich (Missouri, USA). Endothelial cell basal medium-2 (EBM-2; Cambrex Bio Sciences, Walkerville, US).

Plant Material Preparations

Gilaburu juice (65% gilaburu pulp, 45% water and pH: 3.09) (Ulger et al., 2013) was obtained from a commercial supplier Kayseri Pazarı Bio Bitkisel Ürünler San. ve Tic. A.Ş., Turkey. Gilaburu juice was kept in amber colored bottles and stored at -20 °C until use. Gilaburu juice was diluted in medium to the working concentrations (10, 20, 40, 80 and 100 µl/ml). The working concentrations were adjusted to pH 7,4 with 5N NaOH.

Cell Culture Treatment

Five different cell lines were used for the experiments: A549 (human type II lung epithelium) cells, Caco-2 (human colon adenocarcinoma) cells, HeLa (human cervix adenocarcinoma) cells, MDCK (Madin Darby Canine Kidney) cells and Human umbilical vein endothelial cells (HUVEC). The first four cell lines were obtained from ECACC (European Collection of Cell Cultures, Salisbury, UK). Caco-2, HeLa, MDCK cells were cultured in Minimum Essential Medium Eagle (MEM) supplemented with 10% (v/v) fetal calf serum, penicillin (100 U/ml)-streptomycin (100 µg/ml) and sodium hydrogen carbonate. A549 cells were cultured in sterile Nutrient Mixture F12 HAM medium containing 10% (v/v) fetal calf serum, penicillin (100 U/ml)-streptomycin (100 µg/ml) and sodium hydrogen carbonate. HUVEC cells were purchased from the ATCC (American Type Cell Collection), which were incubated and grown in Nutrient Mixture F12 HAM medium supplemented with 20% heat-inactivated fetal calf serum and endothelial cell growth supplement (ECGS, 0.05

mg/ml). The cells were maintained at 37°C in a saturated humidified atmosphere containing 95% air/ 5% (v/v) CO₂.

Assay of Cell Viability

The cytotoxic effects of *Gilaburu* juice was determined by using *in vitro* colorimetric MTT[3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay (Mosmann 1983). Cells were seeded into 96-well microtiter tissue culture plates having a final volume of 100 µl. After 24 h of attachment, the cells were treated with different concentrations (10-100 µl/ml) of *gilaburu* juices in culture medium. Eight replicate wells per concentration were used and repeated in triplicate at different intervals. Untreated medium controls (blank) and solvent controls (ultra pure water) were assayed also in parallel. The cells were incubated for 24, 48 and 72 hours with *Gilaburu* juice one by one and MTT dye was added 2 h prior to completion of incubation periods. The medium from each well was discarded and resulting formazan crystals were solubilised by adding 100 µl of dimethylsulphoxide (DMSO) and quantified by measuring absorbance at 570 nm in an ELx808 Absorbance Microplate Reader (Bio-Tek, USA).

Tube Formation Assay For Angiogenesis

The anti-angiogenic effect of *Gilaburu* juice on HUVEC differentiation was assessed by examining *in vitro* tube formation on Matrigel as previously described (Quchi et al., 2004). Similar protocol was used as described in earlier for HUVEC cells (Koparal, Ulus, Zeytinoglu, Tay, & Turk, 2010). The surface of the 96-well plates were coated with 0.1 ml Matrigel. Human umbilical vein endothelial cells (HUVEC) were harvested endothelial cell basal medium-2 (EBM-2; Cambrex Bio Sciences, Walkerville, US) with 2% FCS for 4h. 4 x 10⁴ HUVEC cells in EBM-2 medium containing different concentrations of *gilaburu* juice

were seeded on 1:1 matrigel. The cells were incubated to attach and differentiate at 37 °C for 12 h. The endothelial cell-deriving tube-like structures were visually examined with inverted phase-contrast microscope (Olympus IX70) using 10X objective and pictures were recorded digitally with Olympus DP 71 camera.

Assay of Genotoxicity

Alkaline single-cell microgel electrophoresis (SCGE comet) assay was applied to detect DNA stand breaks in single cells (Lim et al., 2011). Approximately 1.5×10^5 cells were seeded in a 6-well cell culture plate. After 24 h. the cells were exposed for 24 h. different concentrations of gilaburu juice (20, 40, 80 $\mu\text{g/ml}$). Hydrogen peroxide (25 μM) was used positive control for comet assay. Slides were prepared by method as described (Singh, Mccoy, Tice, & Schneider, 1988) and modified by Bajpayee et al., (2005). Two slides were prepared from each well. Single cells were embedded in low-temperature-gelling agarose and pipetted onto an agarose-coated microscope slide. The slide was then immersed in lysis solution (200 mM NaOH, 1mM EDTA) for one hour at 4°C in dark. Slides were placed in alkaline electrophoresis solution (300 mM NaOH, 1mM EDTA; PH ≥ 13). Voltage was applied (25 V, 300 mA, 20 min). Each slide was stained with 20 $\mu\text{g/ml}$ ethidium bromide solution. The slides were analyzed using fluorescence microscope at a magnification of 400X (Olympus BX 50). For each sample 50 comets randomly selected and analysed BAB Bs200ProP / BsComet DNA Comet Assay image system (Ankara, Turkey). The results represent mean and \pm standart error (SE) of three independent experiments.

Statistical Analysis

All data expressed as means \pm Standard deviation (SD) using SPSS software. The values were presented as a viability compared with control wells (the mean optical density of untreated

cells was set 100% viability), calculated One Way Anova. All independent cytotoxicity experiments were repeated at least three times. In all cases, $p < 0.05$ was considered significant.

Results

Assay of Cell Viability

All the cells in the experiment were treated with increasing concentrations of gilaburu juice (10, 20, 40, 80, 100 $\mu\text{l/ml}$) for 24, 48 and 72 h, or as a control, with ultra pure water. After 24, 48 and 72 hours, the cell viability was determined by MTT assay. The results obtained in the present study show that there is no cytotoxic effect of gilaburu juice on A549 cells at 10-80 $\mu\text{l/ml}$ concentrations over a 72 hour period. Gilaburu juice reduced the proliferation of A549 cells by 89, 84 and 81% at a concentration of 100 $\mu\text{l/ml}$, compared with a vehicle treated control, over 72 hours (Fig. 1). Among the three cancer cells tested, the viability of Caco-2 and HeLa cells were the most affected by the gilaburu juice. As shown in Fig. 2A and 2B, gilaburu juice showed a dose-dependent decrease on cell viability in Caco-2 and HeLa cells at 40, 80 and 100 $\mu\text{l/ml}$ concentrations. Gilaburu juice significantly decreased the viability of HeLa and Caco-2 cells at concentrations of 40, 80 and 100 $\mu\text{l/ml}$, but in MDCK and HUVEC cells, only weak reductions (15% and 19%) were seen upon treatment with 80 and 100 $\mu\text{l/ml}$ (Fig. 3A and 3B).

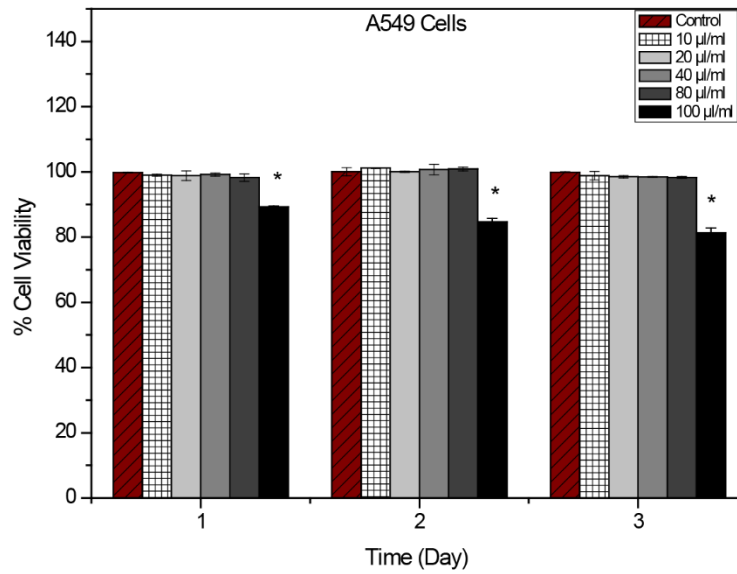


Figure 1. Dose dependent cell viability results of gilaburu juice on A549 cells by MTT assay. Gilaburu juice treatment for 3 days, Values are means \pm SD of triplicates, Asterisks (*) represent means significant difference from the control group by the Tukey test ($p < 0.05$)

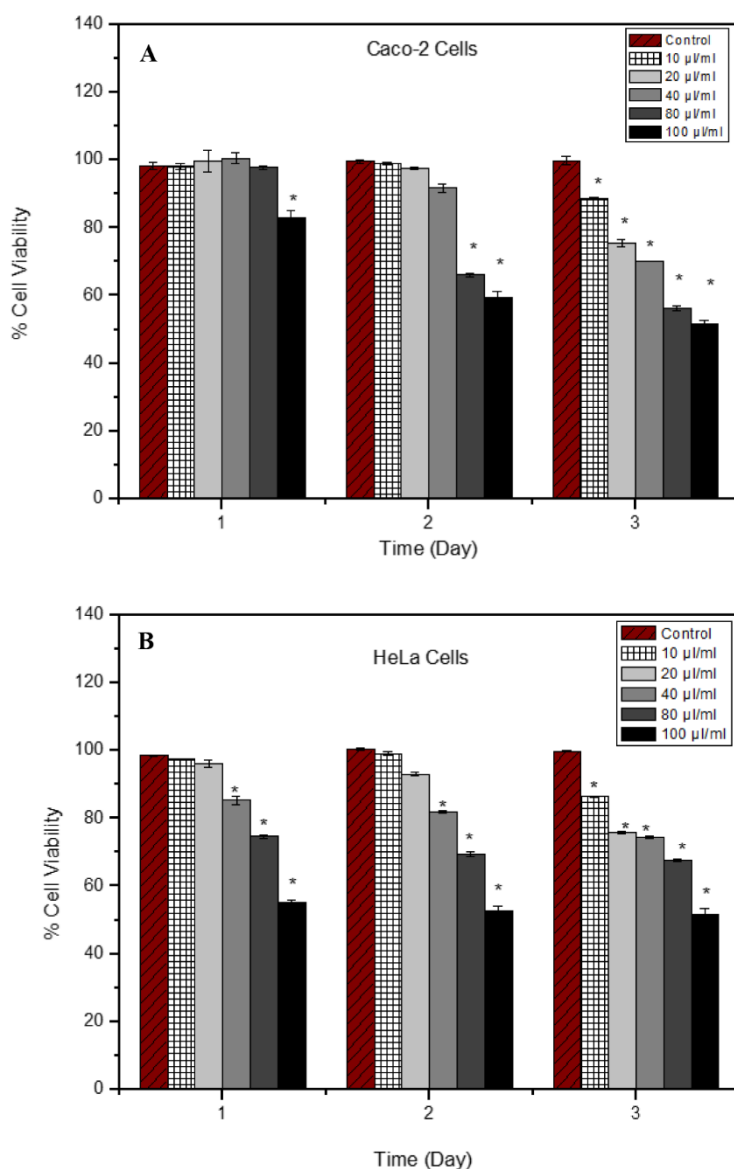


Figure 2. Dose dependent cell viability results of gilaburu juice on Caco-2 cells (A) and HeLa cells (B) by MTT assay. Gilaburu juice treatment for 3 days, Values are means \pm SD of triplicates, Asterisks (*) represent means significant difference from the control group by the Tukey test ($p < 0.05$)

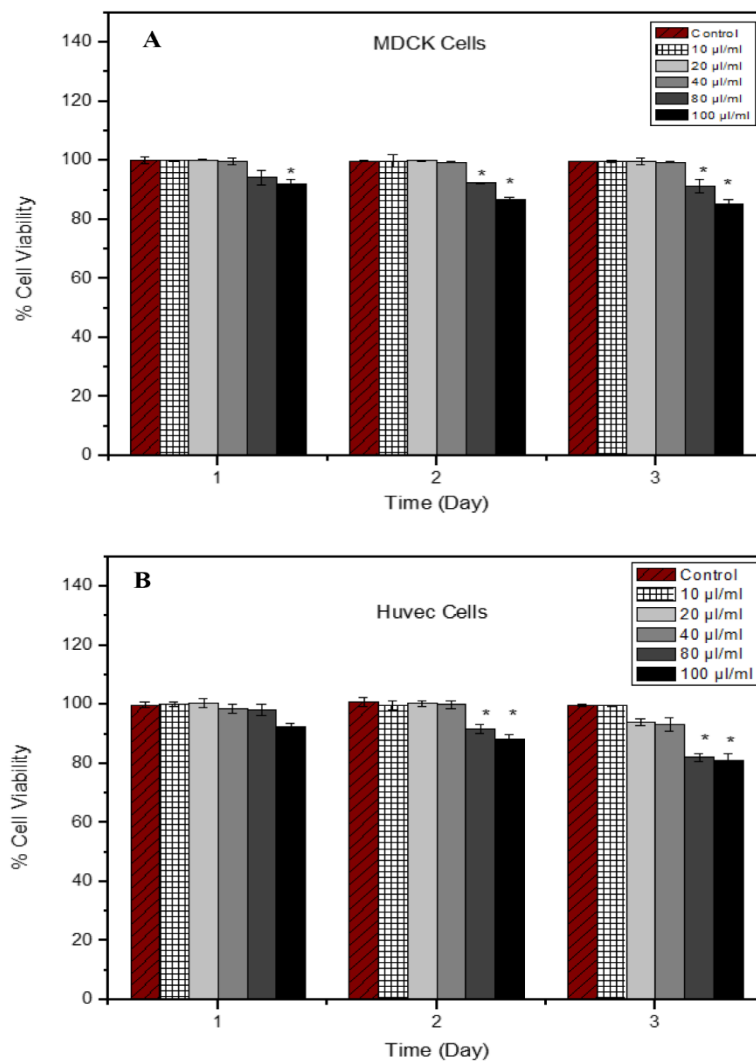


Figure 3. Dose dependent cell viability results of gilaburu juice on MDCK (A) and HUVEC (B) cells by MTT assay. Gilaburu juice treatment for 3 days, Values are means \pm SD of triplicates, Asterisks (*) represent means significant difference from the control group by the Tukey test ($p < 0.05$).

***In Vitro* Tube Formation**

Gilaburu is mainly constituted of polyphenolic compounds. Many *in vitro* and clinical studies have demonstrated that polyphenols, like anthocyanins, modulate angiogenesis (Chen

et al., 2011). To test whether or not gilaburu juice has any anti-angiogenic effect, an *in vitro* tube formation test was used on HUVEC cells. At the end of the incubation period, the HUVEC cells attached to each other and formed tube structures in the control group (ultra pure water). As seen in Figure 4 (a-e), these tubes are strong and robust. The gilaburu juice did not inhibit tube formation within the range of 10-80 $\mu\text{l/ml}$ (Fig. 4a-e). In contrast, a 100 $\mu\text{l/ml}$ concentration of gilaburu juice inhibited tube formation of the HUVEC cells (Fig. 4f). These tubes were broken, shortened, and much thinner at many sites. As shown in Fig. 4f, anti-angiogenic effect of gilaburu juice was shown at a 100 $\mu\text{l/ml}$ concentration.

Figure 4.

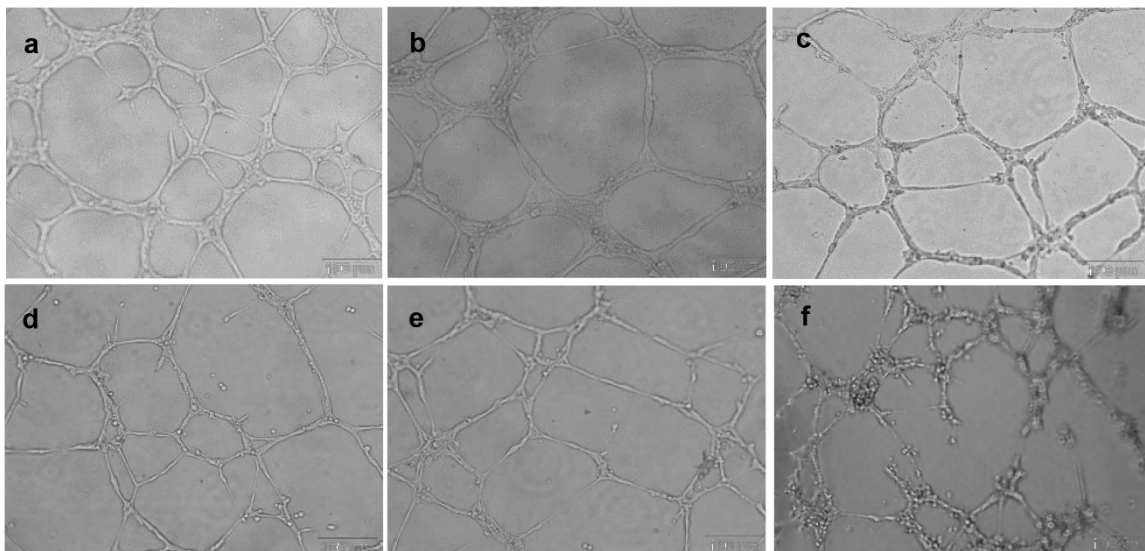


Figure 4. The effect of *Viburnum opulus* on HUVECs tube formation. (a) Control cells and (b) Solvent (ultra pure water) control cells. (c-e) HUVECs were treated *V. opulus* (gilaburu) juice at c) 20 $\mu\text{l/ml}$, d) 40 $\mu\text{l/ml}$, e) 80 $\mu\text{l/ml}$, f) 100 $\mu\text{l/ml}$ for 12 hours. Images shown are of independent triplicate assays.

Genotoxicity

Undamaged DNA retains a highly-organized association with matrix proteins in the nucleus, but when DNA is damaged, this organization is disrupted. Our results are designed to

evaluate the potential genotoxic effect of gilaburu juice on a normal human endothelial cell line (HUVEC). Cell viability was higher than 99 % at all concentrations investigated. H₂O₂ and ultra pure water were utilized as positive and negative controls, respectively. DNA comets were evaluated by measuring the tail lengths of 50 comets. Damage to DNA, obtained through alkaline comet assay of the HUVEC cells treated with gilaburu, is shown in Fig. 5. DNA damage in the HUVEC cells by the comet parameters is shown in Fig. 6. When the Huvec cells were exposed to 20-40-80 µl/ml concentrations for a short time period (24 hours), there was no significant increase in DNA damage from gilaburu juice (tail lengths) compared to the negative control (ultra pure water). The results obtained in this study show that gilaburu juice has no genotoxic potential to the DNA of HUVEC cells.

Figure 5.

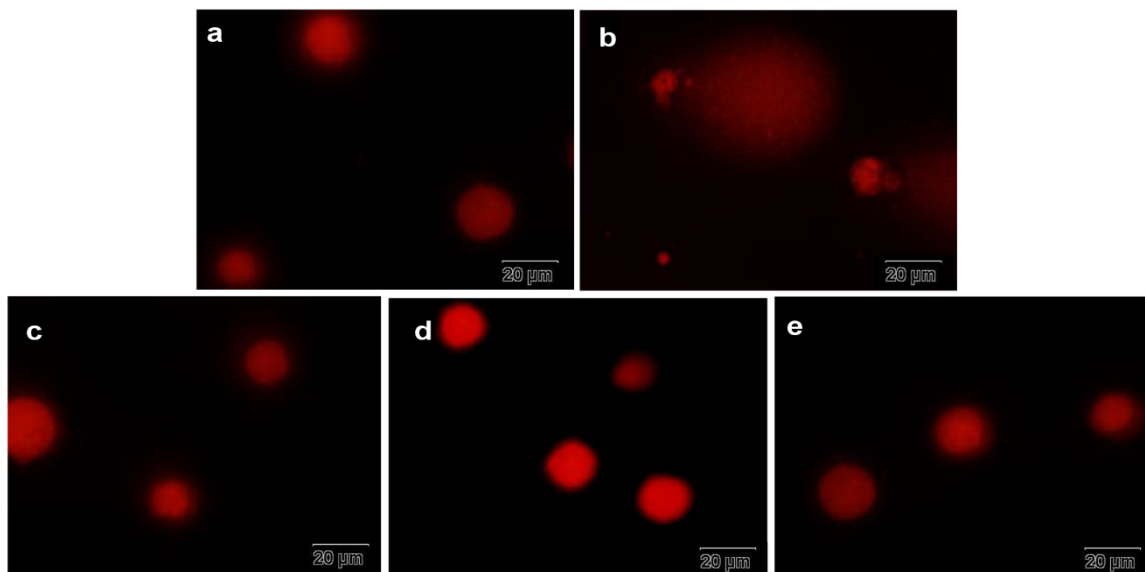


Figure 5. The morphology of HUVEC cells. a) negative control (ultra pure water), b) positive control (H₂O₂) and after exposed to gilaburu juice at c) 20 µg/ml, d) 40 µg/ml, e) 80 µg/ml for 24 h.

Figure 6.

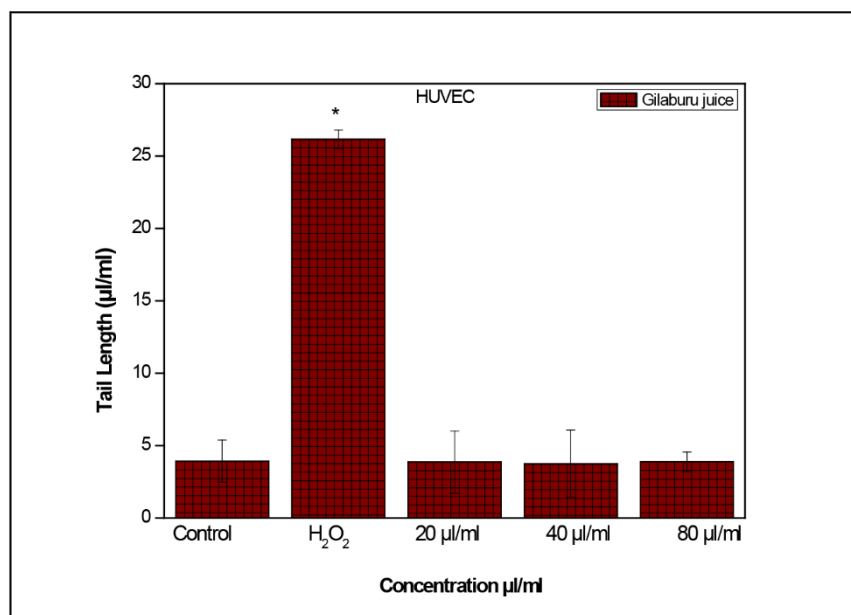


Figure 6. The effect of gilaburu juice on HUVEC DNA detected by the comet assay. *P<0,05 significant difference with regard to the corresponding control.

Discussion

Many natural products contain active compounds with possible therapeutic benefits in the treatment of diseases. The viburnum species are known to have edible fruits containing polyphenolic substances (Calis, Yuruker, Ruegger, Wright, & Sticher, 1995; Sever-Yılmaz, Altun, Orhan-Erdoğan, & Saltan-Çitoğlu, 2013). Gilaburu juice is a rich source of certain components such as L-malic acid, amentoflavone and anthocyanins (Sever-Yılmaz, Altun, Orhan-Erdoğan, & Saltan-Çitoğlu, 2013). Natural products may act as a chemoprotective agent against cancer cells. Therefore, the screening of plants and their biological activity is important. Thus, the toxic effects of gilaburu juice on cells have been demonstrated by the MTT assay. Gilaburu juice showed a dose and time dependent inhibitory effect on cell viability in Caco-2 and HeLa cells ranging from 20 µl/ml up to 100 µl/ml for periods of 24, 48 and 72 hours. Gilaburu juice caused the lowest cell viability with values ranging from 56,2% to 83,8 % at concentrations of 80 and 100 µl/ml after 72 hours of treatment on Caco-2

cells. Our Caco-2 MTT data is in accordance with that reported by Ulger et al., (2013) who concluded that gilaburu juice might be useful for the prevention of colon cancer at the initiation stage. The data that we obtained *in vitro*, clearly shows that gilaburu juice inhibits the growth of not only Caco-2 cells, but also of HeLa cells. A possible reason is that the antiproliferative activity of gilaburu juice might depend on the anthocyanin contents of *V. opulus*. Zhao et al., (2004) report that anthocyanins have inhibitory effects on the growth of colon cancer cell lines HT-29.

Similar results have also been obtained from different *Viburnum* species (Ulger et al., 2013). Fukuyama, Minoshima, & Kishimoto, (2005) demonstrated that *Viburnum luzonicum* display inhibition effects on HeLa S3 cancer cells. Another study concerning the cytotoxicity of the *Viburnum* species, found that compounds of *Viburnum odoratissimum* caused cytotoxic effects on human nasopharyngeal carcinoma tumor cells and human gastric cancer cells (Shen et al., 2004). On the other hand, in this study, gilaburu juice did not cause any significant decrease on the cell viability of MDCK and HUVEC normal cell lines (Fig. 3A and 3B). In our investigation, in which five different mammalian cell lines were used (A549, Caco-2, HeLa, MDCK and HUVEC) it was found that cells of different tissue origin behave in different ways and that certain cells are more robust than others concerning tolerance to gilaburu juice. The different sensitivity to growth inhibition on cancer cells may be explained by the different origins of the cell lines or/and different disruptions in the signaling pathways or changes of expression of enzymes related to apoptosis associated with tumor resistance (Waheed et al., 2013).

Angiogenesis is vital for tumor growth and is necessary for cancer progression and metastasis (Folkman 2003). Gilaburu is mainly constituted by polyphenolic compounds. Many *in vitro* and preclinical studies have demonstrated that polyphenols, like anthocyanins, modulate angiogenesis (Chen et al., 2011). Nizamutdinova et al., (2009) reported that

anthocyanins inhibit ROS accumulation and VEGF production in TNF- α stimulated endothelial cells. Furthermore, a number of studies demonstrate that anthocyanins can inhibit angiogenesis and cancer invasion (Favot, Martin, Keravis, Andriantsitohaina, & Lugnier, 2003; Ding et al., 2006). Thus, gilaburu juice may also have an anti-angiogenic effect. To address the theory of the anti-angiogenic and beneficial effects of gilaburu juice on HUVEC cells, a tube formation assay was used as a model to mimic the functional aspects of angiogenesis. In our tube formation assay, Gilaburu juice exhibited inhibitory effects on HUVEC tube formation at concentrations of more than 80 μ l/ml.

V. opulus (gilaburu) fruit contain high amounts of total phenolics and ascorbic acid (Rop et al., 2010) flavonoids, L-malic acid and anthocyanins (Velioglu, Ekici, & Poyrazoglu, 2006; Deineka, Sorokopudov, Deineka, Shaposhnik, & Kol'tsov, 2005). Chlorogenic acid is the most important polyphenol in the European cranberry bush. *V.opulus* (gilaburu) juice has been shown to have anti-inflammatory, antioxidant, anti-bacterial, anti-viral (Wang et al., 2009) anti-carcinogenic activities (Altun, Sever-Yılmaz. & Saltan-Çitoğlu, 2007; Shi et al., 2013). Chlorogenic acid can prevent the formation of mutagenic N-nitroso compounds and inhibit damage to DNA *in vitro* (Olthof, Hollman, & Katan, 2001). Polyphenolic compounds, such as anthocyanins, are of great interest for their radical scavenging activity and their strong antioxidant properties. An intake of dietary antioxidants that act as radical scavengers is believed to be effective in preventing many diseases (Jang, Park, Park, Park, & Lee, 2008). For this reason, we evaluated the potential genotoxic effect of gilaburu juice on normal human endothelial cell lines (HUVEC). The DNA comets were evaluated by measuring the tail lengths of 50 comets.

The results obtained in this study show that gilaburu juice has no genotoxic potential to DNA of HUVEC cells. The effect may be related to the chlorogenic acid and anthocyanin content of gilaburu. As the results shown in Fig. 6. İndicate, gilaburu juice has no genotoxic

effect on normal human cells (HUVEC) at concentration ranges of 20-80 µl/ml compared to the control. To our knowledge, there has been no published data on an assessment of the genotoxicity of gilaburu (*V. opulus*).

Conclusions

The present study was carried out to investigate the cytotoxicity, genotoxicity and anti-angiogenic effects of Gilaburu (*V. opulus*) juice on cancer and normal cells. This study describes the cytotoxic, genotoxic and anti-angiogenic effects of *Viburnum opulus* juice on cell lines, for the first time. Taken together, the findings of the current study indicate that gilaburu juice shows antiproliferative activities against human cervical cancer cells and human colon adenocarcinoma cells, except for A549 and normal healthy cell lines (MDCK and HUVEC). A possible reason for this is that the antiproliferative activity of gilaburu juice on Caco-2 and HeLa cells might depend on certain main bioactive components and their interaction or their synergistic effects. Further investigation needs to be conducted in regard to this concern. Furthermore, gilaburu juice did not show effects on HUVEC cells. The results obtained from this study confirm the potential application of commercial *Viburnum opulus* (gilaburu) juice to be functional in regard to cancer prevention.

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