



Antiproliferative and apoptotic effects of *Onobrychis albiflora* extract on HCT-116 cells

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ABSTRACT

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In this study, the cytotoxic effects of the extracts of methanol: water (80:20) prepared from the above-ground parts of the varieties of *Onobrychis albiflora* Hub.-Mor., *Onobrychis argyrea* Boiss. subsp. *argyrea*, *Onobrychis galegifolia* Boiss. and *Onobrychis tournefortii* (Willd.) Desv. species to HCT-116 cells were investigated. With cytotoxicity analysis that with these species inhibitor concentrations (IC₅₀) which resulted in a 50% reduction in the proliferation of HCT-116 cells were identified. In continuation of the study; the antiproliferative and apoptotic effects of *Onobrychis albiflora* extract on HCT-116 cells were evaluated by Caspase 3, Annexin V / PI Apoptosis / Necrosis analysis, Apoptin Green and 7-AAD Apoptosis / Necrosis analysis. The antiproliferative, apoptotic and necrotic effects of *Onobrychis albiflora* extract on HCT-116 cells also were determined.

Keywords:

Apoptosis
Flow cytometry
HCT-116
HEK-293
Necrosis

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1. Introduction

The use of natural resources by humans for medical purposes was always the case since the existence of humanity. Human beings have benefited from natural resources, especially plants, to protect their health and to cure their diseases. Medicinal plants are of utmost importance as they are used as medicines themselves, as

well as being the source of new medicines. Nearly 50% of the approved drugs used in the field of cancer in the last 30 years are produced directly or indirectly from natural products (Veeresham, 2012).

Paclitaxel, docetaxel, vinblastine and vincristine are just some of them (Cragg et al., 2005; Lee and Xiao, 2005; Cragg et al., 2012; Lee and Xiao, 2012). The fact

that plants contain compounds with different biological effects, and that some plant components have multiple effects and that plants are an unlimited source for new drug molecules are the most important reasons for their widespread use in the development of new drugs that can be used in the treatment.

Onobrychis Miller is a genus of perennial plants of the Fabaceae family. *Onobrychis* species, which have been cultivated since ancient times, are widespread in the Near East Flora, including our country. It is a plant which is more resistant to cold and drought and it is more fertile in arid, calcareous soils which are not suitable to other plants (Acikgoz, 2001).

Both in this respect and in terms of having high nutritional value, it is common for our country to be used as fodder plant especially in Eastern Anatolia Region. The genus *Onobrychis* is distributed in Europe, America, West Asia and North Africa with more than 160 species. In our country, 55 species belonging to the genus *Onobrychis* are grown naturally and approximately 50% are endemic (Avcı et al., 2016). In these plants species; *O. argaea*, *O. elata*, *O. argyrea* and *O. tournefortii* have entered to the List of Medicinal and Aromatic Plants of Turkey (Acikgoz, 1998). Although there are some studies on antioxidant and anticancer effects of *Onobrychis* species, there is no study on the anticancer effect of *O. argyrea* and *O. albiflora* which we used in our study.

According to World Health Organization 2014 data, 8.8 million people died due to cancer worldwide. Colon cancer is the fourth highest incidence in the world, with the third highest mortality rate (WHO, 2014).

Many previous studies have been identified that, *Onobrychis* contains large number of flavonoids, tannins, benzofuran derivatives and other chemicals (Marais et al., 2000; Karamian and Asadbegy, 2016). According to Phytochemical studies on the genus *Onobrychis* it was found that the species contained compounds with an anticarcinogenic effect like that flavonoids; afzelin (Zhu et al., 2015), quercetin (Hashemzaei et al., 2017), vitexin (Scarpa et al., 2017), myricetin (Zheng et al., 2017), benzofuran derivatives; ebenfuran-III (Halabalaki et al., 2000) and 2 arylbenzofuran (Katsanou et al., 2007); phenolic acids; cinnamic acid (Zhu et al., 2016), gallic acid (Pang et al., 2017), vanilic acid (Anbalagan et al., 2017), caffeic acid (Tyszka et al., 2017), coumaric acid (Roy et al., 2016), ferulic acid (Eitsuka et al., 2016).

The cytotoxic effects of ferulic acid and coumaric acid on human colorectal cancer cell line HCT-15 were tested and both compounds were shown to be effective in killing colorectal cancer cells (Roy et al., 2016). In a previous study on CaCo-2 colon cancer cells, the pro-apoptotic effects of vitexin have been evaluated and it has been shown that vitexin inhibits the proliferation of CaCo-2 cells as a result of the activation of Caspase 9, 8 and 3 (Scarpa et al., 2017). In another study, HT-29 colon cancer cells were treated with cinnamic acid and MTT analysis

revealed that the IC₅₀ value was ~1 mM. In addition, Bcl-2 expression was decreased and apoptosis was induced in cells (Zhu et al., 2016). In this study, the cytotoxic effects of *O. argyrea*, *O. galegifolia*, *O. tournefortii* and *O. albiflora* extracts on HCT-116 colon cancer cells were investigated.

2. Materials and methods

Plant material

Onobrychis species were collected from the localities given in Table 1 from Sivas. Identification of species made by Dr. Mehmet Tekin who is Assoc. Prof. in Trakya University, Faculty of Pharmacy, Department of Pharmaceutical Botany and it is stored in the Herbarium of Cumhuriyet University. Dried and powdered aerial parts of the plant (30 g) were extracted with 300 ml of methanol: water (80:20) at room temperature during 8 hours for 3 days by continuous stirring. Each extract was filtered and concentrated to dryness under reduced pressure and low temperature (40–50 °C) on a rotary evaporator to yield crude extracts. Concentrated extracts weighed 10 mg, homogenization of the blender in 1 ml distilled water. The obtained homogenates were centrifuged at 1500 rpm for 15 minutes at 4 °C. The pellet and supernatant portions were separated; the supernatants used as stock solution were sterilized by filtration. Serial dilutions were prepared in medium.

Table 1. *Onobrychis* species were collected from the localities from Sivas.

Species	Localities	Collector and Herbarium No
<i>O. albiflora</i>	Sivas:Sincan -Kangal, 3. Ian roadside 1220 m,	M. Tekin, 1291
	39028' 01,9" N; 370 50' 34,S" E	
<i>O. arevrea</i> subsp. arwea	Sivas Zara - Divrigi. 55 Ian from Divrigi1691 m,	M. Tekin, 1293
	39036' 58)" N ; 37044' 28,4" E	
<i>O. galegifolia</i>	Sivas: Divrigi-SIncan.vz-S Ian from Sincan. 1312	M. Tekin, 1294
	m, 390 30' 21,7" N; 370 49' 45,4" E	
<i>O. toumefortii</i>	Sivas: Sivas-Hafik. around the village of Emre,	M. Tekin, 1290
	1317 m, 390 49' 37,S" N; 37017' 05)" E	

Cell culture

Studied with Human colon cancer (HCT-116) and human embryonic kidney (HEK-293) cell lines. Cultivation for HCT-116 cells were used McCoy's 5A medium (Multicell) containing, 1% antibiotic / antimycotic solution (Multicell), 10% Fetal Bovine Serum (South America Origin, Biosera), 5% L-Glutamine. And cultivation for HEK-293 cells were used high glucose DMEM medium (Multicell), containing 1% antibiotic / antimycotic solution (Multicell), 10% Fetal Bovine Serum (South America Origin, Biosera).

Cytotoxicity measurement

The cultivation of the cells was carried out to 96-well plates at 0.5×10^5 cells per well and allowed to stand for 24 hours to be confluent. Then, the media was aspirated on the wells. Different concentrations of the extracts dissolved in distilled water (5 mg/ml, 4 mg/ml, 3 mg/ml, 2 mg/ml, 1 mg/ml and 0.5 mg/ml) with equal amounts of media on HEK-293 and HCT-116 cells added. Triplicate was performed for 24 and 48 hours for all concentrations. At the end of incubation periods, the medium on the cells was aspirated. The MTT (Roche) agent was added at a rate of 1:20 (MTT agent: medium) and incubated at 37°C and 5% CO₂ for 4 hours. Following incubation, MTT was aspirated. 1:1 (medium: DMSO) solvent solution was added and incubated for 1 hour in the dark with the orbital shaker. Cells exposed to Triton™ X-100 (Sigma-Aldrich) were used as positive controls. The absorbance of the color change was measured by spectrophotometer at 570 nm. The inhibitory concentration (IC₅₀), statistics and graphics of the extracts which resulted in a 50% reduction in the 24 and 48 hours proliferation of both cell lines were recorded using the GraphPad Prism 6 program.

Annexin V-FITC early apoptosis and necrosis analysis

In this study, Annexin V-FITC Early Apoptosis Detection Kit (Cell signaling technology, Cell Proliferation Kit I) was used. 1×10^6 HCT-116 cells cultured in a T25 flask were incubated for 24 hours with an IC₅₀ dose of the extract dissolved in distilled water of *O. albiflora*. At the end of incubation, the cells were removed by trypsin (Multicell) and centrifuged at 500 g for 5 min and the supernatant was removed after centrifugation. The cell pellet was separated into 96 μ l aliquots by mixing with 1X Annexin V binding buffer contained in the kit. 1 μ l of Annexin V-FITC conjugate and 12.5 μ l Propidium Iodide (PI) were added to each 96 μ l aliquot. It was incubated on ice for 10 minutes in the dark. Final volume was added to 250 μ l by adding 1X Annexin V binding buffer. For PI 560 nm and for FITC 488 nm flow cytometry (BD Influx Cell Sorter) was analyzed with appropriate laser channels.

Apoptin green and 7-AAD apoptosis / necrosis analysis

Apoptin green, 7-AAD and CytoCalcein Violet Apoptosis / Necrosis Kit (Abcam, ab176749) were used in our study. 1×10^6 HCT-116 cells cultured in a T25 flask were incubated for 24 hours with an IC₅₀ dose of the extract dissolved in distilled water of *O. albiflora*. At the end of incubation, the cells were removed by trypsin (Multicell) and centrifuged at 500 g for 5 min and the supernatant was removed after centrifugation. The cell pellet was mixed with 200 μ l assay buffer contained in the kit. 2 μ l of Apoptin Green indicator, 1 μ l 7-AAD (7-aminoaktinomycin D) and 1 μ l CytoCalcein Violet 450 agents were added to the mixture. Incubations of 1 hour at room temperature were achieved. Before flow cytometry analysis, it was further diluted with 300 μ l assay buffer. Apoptin Green Indicator using

the FL1 channel (Ex/Em = 490/525 nm), 7-AAD using the FL3 channel (Ex/Em=550/650 nm), and CytoCalcein Violet USING450 Ex/Em=405/450 nm was analyzed by flow cytometric. HCT-116 cells that were not exposed to *O. albiflora* extract were used as the control group.

Analysis of fluorometric caspase 3

Caspase 3 (Abcam39383) kit was used in our study. HCT-116 cells were cultured in 96 well plates to each well containing 0.5×10^5 cells. After the cells were confluent, they were incubated with *O. albiflora* for 24 hours with an IC₅₀ dose of 24 hours. The contents of the wells were aspirated after incubation. 50 μ l of lysis buffer was added to each well. After incubation for 10 minutes on ice, 50 μ l of 2x (containing 10 Mm DTT) assay buffer and 5 μ l of 1Mm DEVD-AFC substrate were added. It was incubated again at 37°C for 2 hours. Caspase 3 fluorescence values of the cells were recorded with the Ex / Em: 400 nm / 505 nm spectrophotometer (SpectraMax i3x, Molecular Devices). HCT-116 cells and cells exposed to Triton™ X-100 (Sigma-Aldrich) were used as negative and positive control groups.

3. Results

Cytotoxicity measurement

On the HCT-116 cells of the herbs, the graphs of the viability curves (Figs. 1 and 2), which were formed as a result of the 24 and 48 hours incubation, were shown and the table for the IC₅₀ values was created (Table 2). Accordingly, as a result of 24 hours incubations, the IC₅₀ value for *O. albiflora* extract was 0.001396 g/ml (Fig.1A), 0.003287 g/ml for *O. argyrea* (Fig.1B), for *O. galegifolia* 0.002707 g/ml (Fig.1C) and 0.002048 g/ml for *O. tournefortii* (Fig.1D).

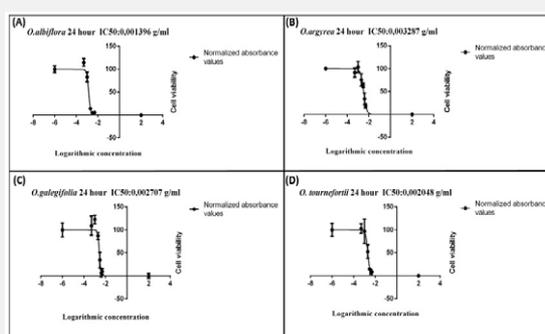


Fig. 1. The graphs of the viability curves which were formed as a result of the 24 and 48 hours incubation.

The analysis was repeated to determine the viability levels of HCT-116 cells incubated with extracts for 48 hours. As a result, the IC₅₀ value was determined for *O. albiflora* extract 0.001322 g/ml (Fig. 2A), 0.001217 g/ml for *O. argyrea* (Fig. 2B), 0.002755 g for *O. galegifolia* g/ml (Fig. 2C) and *O. tournefortii* for 0.002236 g/ml (Fig. 2D).

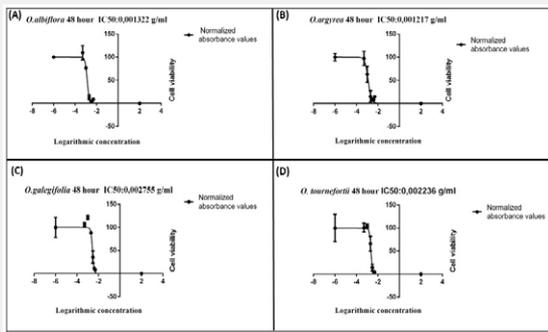


Fig. 2. The graphs of the viability levels of HCT-116 cells incubated with extracts for 48 hours.

Table 2. IC50 values of the HCT-116 cells of the herbs after 24 and 48 hours incubation.

	HCT-116	
	24 hours IC50 (mg/ml)	48 hours IC50 (mg/ml)
Onobrvchis albiflora	1.396	1.322
Onobrvchis ealeeifolia	2707	2.755
Onobrvchis tournefortii	2.048	2.236
Onobrvchis arzvrea	3.287	1.217

HEK-293 cell line was used to evaluate the effects of herbs on healthy cells. For 24 hours of incubation of the same doses of herbal extracts on HEK-293 cells, the IC50 value was determined for *O. albiflora* extract was 0.001649 g/ml (Fig. 3A) and 0.003749 g/ml for *O. argyrea* (Fig. 3B), 0.002563 g/ml (Fig. 3C) for *O. galegifolia* and 0.0009797 g/ml for *O. tournefortii* (Fig. 3D).

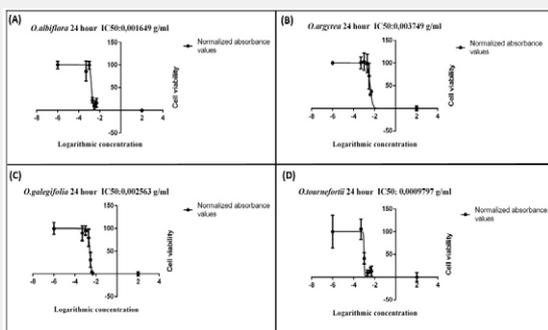


Fig. 3. The graphs of the viability levels of HEK-293 cells incubated with extracts for 24 hours.

When exposed to the extracts for 48 hours, the IC50 values was detected 0.001601 g/ml (Fig. 4A) for *O. albiflora* extract, 0.003611 g/ml (Fig. 4B) for *O. argyrea*, 0.003989 g/ml (Fig. 4C) for *O. galegifolia*, 0.001269 g/ml (Fig. 4D) for *O. tournefortii*. HEK-293 cells in 24 and 48 hours incubation of the herbs were determined as a result of the table for the values of IC50 (Table 3). As a result of MTT analysis, the most effective herb on HCT-116 cells was *O. albiflora*.

Table 3. IC50 values of the HEK-293 cells of the herbs after 24 and 48 hours incubation.

	HEK-293	
	24 hours IC50 (mg/ml)	48 hours IC50 (mg/ml)
Onobrvchis albiflora	1.649	1.601
Onobrvchis ealeeifolia	2.563	3.989
Onobrvchis tournefortii	0.9797	1.269
Onobrvchis argyrea	3.749	3.611

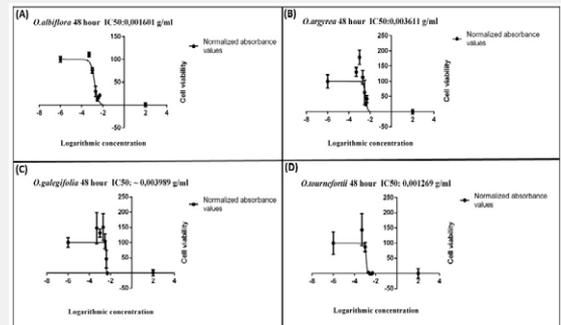


Fig. 4. The graphs of the viability levels of HEK-293 cells incubated with extracts for 48 hours.

Annexin V-FITC early apoptosis and necrosis analysis

The apoptotic effect of *O. albiflora* on HCT-116 cells was analyzed by flow cytometry using Annexin V-FITC and phosphatidylserine (PS). The distribution of apoptotic and necrotic cells belonging to the experimental group was shown (Fig. 5).

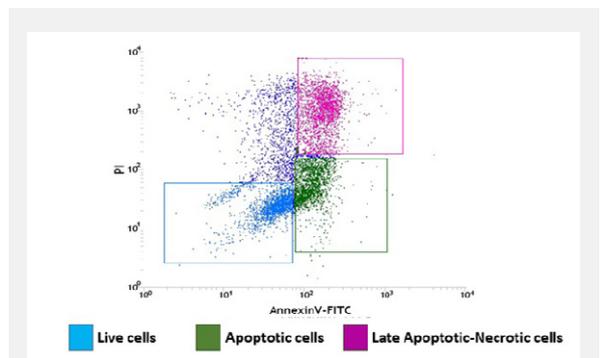


Fig. 5. The graphs of live, apoptotic, late apoptotic cells in the experimental group.

Accordingly, the ratio of apoptotic cells was 25.95 %, the ratio of late apoptotic-necrotic cells was 31.21 %, and the percentage of living cells was 24.18 % (Table 4). In addition, flow cytometry analysis with Annexin V-FITC and PS was performed in the control group HCT-116 cells, which were not exposed to any dose of the herbs (Fig. 6). While the percentage of living cells in the control group was 80.6 %, the rate of apoptotic cells was 8.56 % and the rate of late apoptotic-necrotic cells was 6.35 % (Table 5).

Table 4. The Ratio of the late apoptotic-necrotic, anapoptotic and live cells in the control group HCT-116 cells.

	Cell population	% Value
All cells	7.167	100 %
Late Apoptotic-Necrotic	2.237	31.21 %
Apoptotic	1.860	25.95 %
Live cells	1.733	24.18 %

Table 5. The Ratio of the late apoptotic-necrotic, anapoptotic and live cells in the control group.

	Cell population	% Value
All cells	18.857	100 %
Late Apoptotic-Necrotic	1.198	6.35 %
Apoptotic	1.615	8.56 %
Live cells	15.199	80.60 %

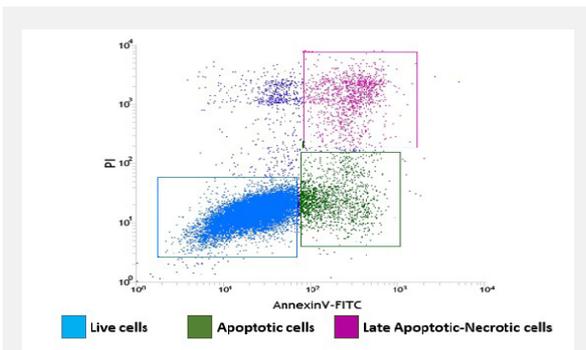


Fig. 6. The graphs of live, apoptotic, late apoptotic cells in the control group HCT-116 cells.

Apoptin green and 7-AAD apoptosis / necrosis analysis

Apoptin green, 7-AAD and CytoCalcein violet 450 were agents that include staining of early stage apoptosis cells, late apoptotic-necrotic cells and live cells. Created the peak ratio diagrams for the CytoCalcein violet 450 (respectively, Fig. 7A and 7B), Apoptin green indicator (respectively, Fig. 8A and 8B) ve 7-AAD (respectively, Fig. 9A and 9B).

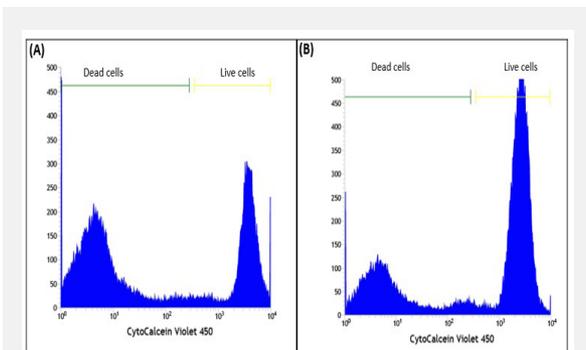


Fig. 7. The graphs of dead and live cells for the CytoCalcein violet 450.

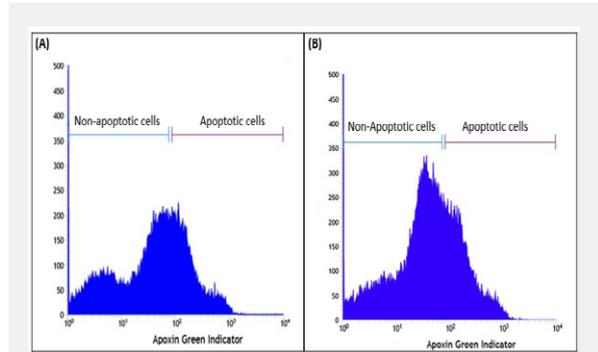


Fig. 8. The graphs of dead and live cells for the Apoptin green indicator.

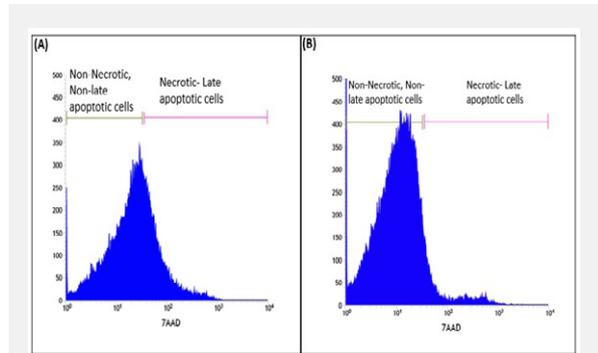


Fig. 9. The graphs of dead and live cells for the 7-AAD.

According to this, 39.82 % of the experimental group cells were alive (Table 6), while 66.29 % of the control cells were alive (Table 7). When we evaluated the ratio of apoptotic cell numbers, while this rate was recorded as 35.33 % (Table 8) in experimental group cells, 32.8% (Table 9) of the control group cells were determined as apoptotic. Necrotic-late apoptotic cell ratios were 33.86 % in the experimental group (Table 10) and 9.47 % in the control group (Table 11). Also combined peak rate diagrams of all results were generated (Fig. 10, 11, 12).

Table 6. Live and dead cells in the experimental group.

	Cell population	% Value
Live cells	14.496	39.82 %
Dead cells	21.660	59.61 %

Table 7. Live and dead cells in the control group.

	Cell population	% Value
Live cells	27.128	66.29 %
Dead cells	13.555	33.12 %

Table 8. Apoptotic and non-apoptotic cells in the experimental group.

	Cell population	% Value
Non-apoptotic cells	22.727	62.55 %
Apoptotic cells	12.839	35.33 %

Table 9. Apoptotic and non-apoptotic cells in the control group.

	Cell population	% Value
Non-apoptotic cells	27.002	65.98 %
Apoptotic cells	13.169	32.18 %

Table 10. Necrotic-late apoptotic cell ratios in the experimental group.

	Cell population	% Value
Non-necrotic, non-late apoptotic cells	23.471	64.59 %
Necrotic-late apoptotic cells	12.305	33.86 %

Table 11. Necrotic-late apoptotic cell ratios in the control group.

	Cell population	% Value
Non-necrotic, non-late apoptotic cells	36.759	89.93 %
Necrotic-late apoptotic cells	3.874	9.47 %

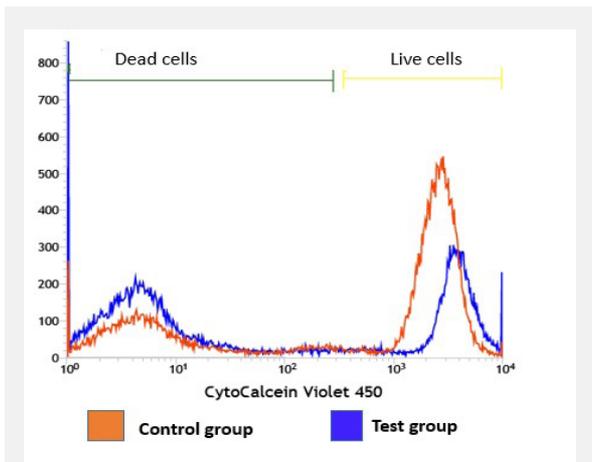


Fig. 10. The graphs of dead and live cells for the CytoCalcein violet 450 in the test and control groups.

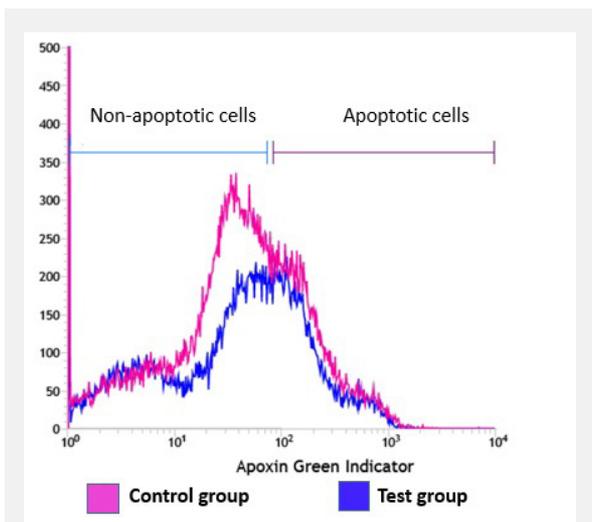


Fig. 11. The graphs of dead and live cells for the Apoxin green indicator in the test and control groups.

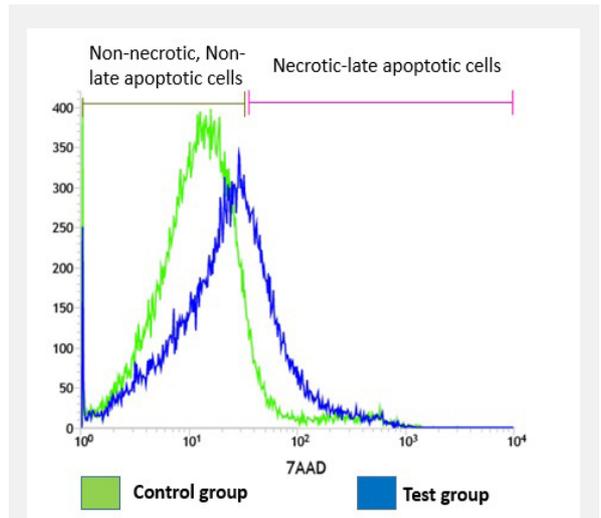


Fig. 12. The graphs of dead and live cells for the 7-AAD in the test and control groups.

Analysis of fluorometric caspase 3

Fluorometric caspase 3 analyses were performed in order to determine the apoptosis level of *O. albiflora* extract in the mechanism of death on HCT-116 cells. In fluorometric caspase 3 analyses, the value of caspase 3 expression of the negative control group was determined as 7.5×10^6 and the fluorescence value of the experimental group was determined as 15.3×10^6 . In addition, the fluorescent value of the positive control group was recorded as 19.9×10^6 (Fig. 13).

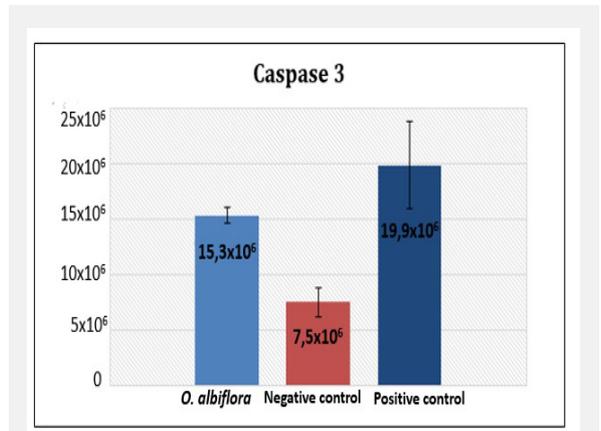


Fig. 13. The graphs of the value of the fluorometric caspase 3 in the *O. albiflora* and negative control, positive control.

4. Discussion

Anticancer drugs are expected to be selective against tumor cells and should not show any toxic effects to healthy cells but, it is observed that they can also kill healthy cells (Desai et al., 2008). Therefore, our study was continued with *O. albiflora* extract which has the least toxic effect against HEK-239 cells while, showed the most toxic

effect against HCT-116 cells, according to MTT analysis results. After 24 hours of incubation *O. albiflora* extract on HCT-116 cells IC₅₀ value was determined as 1.4 mg/ml and 48 hours incubation value was 1.3 mg/ml. The results revealed that the cell viability and concentration of plant extract were inversely proportional during both of incubation periods. In terms of IC₅₀ values, the 24 hours incubation period was not significantly different compared to the 48 hours incubation period. Considering the 24 hours incubation period would be advantageous in both in vivo and in vitro studies in terms of time, it was deduced to be appropriate to continue with 24 hours incubation period. In the flow cytometry analysis performed with Annexin V-FITC and PS, 80, 60 % of the cells in control group were alive and 8.56 % were apoptotic, while on research group which was subjugated to *O. albiflora* extract; 24.18 % of cells were alive and 25.95 % was apoptotic. These results showed *O. albiflora* extract induces HCT-116 cells to apoptosis significantly. To confirm that, *O. albiflora* induces apoptosis in HCT-116 cells, flow cytometry analysis with Apoptin green and 7-AAD was performed again. While the ratio of apoptotic cells was determined as 32.8 % in the control group, this rate increased to 35.33 % in the experimental group cells. In addition, the rate of necrotic/late apoptotic cells in the control group cells was 9.47 % and this ratio increased to 33.86 % in the experimental group cells. These results confirmed that cells exposed to a dose of 1.4 mg/ml of *O. albiflora* extract entered the early stage of apoptosis and were shown to be parallel to Annexin V-FITC early apoptosis/necrosis analysis.

Apoptosis, which is defined as programmed cell death, is composed of two basic pathways as extrinsic and intrinsic. Both pathways converge in a final pathway that involves activation of a number of proteases called caspases which break down regulatory and structural molecules and result in cell death. Therefore, caspase-3 expression in HCT-116 cells was measured. In the fluorometric caspase 3 analyses, the value of caspase 3 expression of the negative control group was determined as 7.5×10^6 , while the fluorescence value of the experimental group was almost doubled to 15.3×10^6 . In addition, the fluorescence value of the positive control group showed a significant parallelism with 19.9×10^6 (Fig. 13).

It has been scientifically determined that the risk of developing many chronic diseases such as cancer, diabetes, cardiovascular diseases, chronic inflammation, cataract, atherosclerosis, Alzheimer's disease is reduced by having fed with food rich of phenolic compounds known to have antioxidant effects. Phenolic compounds are one of the largest classes of secondary metabolites and can be grouped under many classes such as flavonoids, phenolic acids, coumarins, tannins, lignans, stilbenes (Choi et al., 2004; Goncalves et al., 2005; Conforti et al., 2008; Guha et al., 2011). Phenolic compounds, due

to their potent antioxidant effects, provide the integrity of the biological system by protecting structures such as carbohydrates, proteins, lipids and DNA from the harmful effects of oxidation. In addition, many studies show that phenolic compounds prevent cancer through different mechanisms. Therefore, their effects are not only dependent on their antioxidant properties but also on their interactions with cellular mechanisms. Preclinical studies show that phenolic compounds exhibit effects such as inhibition, deceleration of initiation and progression of different types of cancer such as prostate, liver, colon and also inhibiting cell proliferation, inhibiting tumor growth and development (Basli et al., 2017). It is supported by studies that phenolic compounds, especially flavonoids reduce the incidence of cancer, and have an important role in cancer treatments especially in complementary therapy (Nicodemus et al., 2001). In many similar studies, when cell viability analyzes was taken as reference, results are significantly parallel to our conducted experiments in our study.

The prepared extract was prepared with a mixture of methanol: Water (80:20). When applying, these extracts were used by dissolving in water. This method is also supported by the reference of another study in which the effects of Nettle extract on colon cancer cells have been investigated (Korkmaz, 2010).

Interest and use of phytotherapy has been increasing recently. Traditionally, there are different methods of use in the form of extracts prepared by maceration or infusion or decoction of various parts of the plants. This way of use is widespread, but not sufficiently reliable. Because use of the dosages in traditional methods are not determined by scientific approaches. This may cause unexpected acute or chronic problems. For example, D-Pinitol, the main source of the Fabaceae family, has been shown to have an insulin-like lowering effect as well as anticancer properties (Bates et al., 2000). Without dose information, it can be thought that plants with D-Pinitol which are used by conventional methods can cause serious problems by damaging sugar metabolism in case of overdose.

Although the number of drugs and investigations made with the active ingredients isolated from the extracts of various plants is increasing day by day, it is not possible for people to isolate and use the chemicals used in traditional plants. In this aspect, the effect is expected from the whole plant. In many current studies, the individual medical effects of the compounds in plant contents are investigated. However, this method does not anticipate the consequences of interactions of individual chemical compounds with each other in the case of usage of all chemicals in the content. Our study is original and important in this aspect. Considering the data obtained in our study, and in case of continuation of analysis, it will light the way for examination of many other endemic species for their potential use for medical purposes.

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