

Identification of phenolic compounds, antioxidant activity and anti-cancer effects of the extract obtained from the shoots of *Ornithogalum narbonense* L.

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Abstract: This study aimed to examine the anti-cancer and antioxidant properties and identify the phenolic content of methanolic extract obtained from the shoots of the *Ornithogalum narbonense* L. (OR) species, which is used for folk-medicine and food in the Sanliurfa region of Turkey. The antioxidant activity of the extract was investigated using total phenolics, flavonoids, ABTS and CUPRAC methods. Phenolic component analysis of the plant extract was performed by LC-MS/MS. The anti-cancer property of OR extract was investigated on human colon (DLD-1), endometrium (ECC-1) cancer cells and embryonic kidney (HEK-293) cells. Cytotoxic effects were defined with MTT, apoptotic activity with DNA fragmentation ELISA and AO/EB fluorescent staining, the genotoxic effect with the comet assay and the intracellular oxidative status with TAS and TOS methods. As a result of the study, it was determined that OR extract showed an antioxidant effect, and as a result of the content analysis made with LC-MS/MS, phenolic components were determined, the most abundant being cosmosiin, followed by cinnamic acid, p-coumaric acid and quinic acid. OR extract showed cytotoxic activity on DLD-1 and ECC-1 cancer cells, while the cytotoxic effect on HEK-293 cells was determined to be low. It was determined that through OR extract, by increasing the intracellular amount of free radicals on cancer cells, led to DNA damage, which consequently led to apoptosis of the cancer cells.

Key words: *Ornithogalum narbonense* L.; Genotoxicity; Cytotoxicity; LC-MS/MS; Apoptosis.

Introduction

Despite significant advances in the development of cancer-preventative drugs in the last decade, cancer remains one of the primary causes of death throughout the world (1). Colorectal cancer is a significant disease affecting millions of people each year throughout the world and is thought to be the third most commonly diagnosed cancer in males and the second in females (2). In the western world, with 655,000 deaths per year, colorectal cancer is ranked third in the causes of cancer-related deaths (3). Endometrial carcinoma is the most frequently seen cancer of the female genital system (4, 5). In the USA, it has been stated to be the most frequently encountered gynaecological malignancy, with 60,050 new cases determined in 2016 alone (6).

Multiple strategies are used to treat human cancers, including surgery, chemotherapy, radiotherapy and a combination of these treatments. As surgery cannot be applied to advanced cancer patients, chemotherapy and/or radiotherapy are widely used as alternative or additional treatments and have a significant role in anti-cancer treatment. Nevertheless, failures of these two anti-cancer treatments are often seen in clinical cases (7).

One of the most important reasons that greater success has not been achieved in the struggle against cancer is that the anti-cancer drugs have a cytotoxic effect on cancer cells, they also show a toxic effect on healthy cells, thereby leading to devastation of organs inclu-

ding affected cells are located. This is an important factor directing people to phytotherapy. Herbal medicines have been used for hundreds of years in the provision of healthcare services, playing a significant role both in the treatment and management of various diseases and disorders moreover they have retained their popularity in the world in general (8).

Plants contain bioactive compounds with several biological activities, primarily antioxidant and anti-cancer properties. Of these compounds, the phenolic compounds have several benefits on health and may be useful in the prevention of various diseases (9, 10). Phenolic compounds are the primary group of phytochemicals found in plants. A hydroxyl group and an aromatic ring form the basic structure of phenols. Depending on the number of phenolic units and the place and number of the hydroxyl group, the phenolic family includes more than 8000 compounds (11, 12).

Interest is increasing in the subject of the biological effects of phenols as innumerable studies have found an association between the phenolic content of fruit, vegetables, flowers, leaves and seeds and antioxidant and anti-cancer activities (13). Antioxidants are chemical compounds which inhibit free radical intermediary products created during oxidative reactions. The majority of natural antioxidants are formed of phenolic compounds (14).

Therefore, the aim of this study was to examine the anti-cancer and antioxidant properties and pheno-

lic content analysis of the *Ornithogalum narbonense* L (OR) plant for the first time. *Ornithogalum* belongs to the Liliaceae family, and may be seen especially in fields and on waste ground. It is a perennial, bulb, herbaceous plant which can grow to 80 cm and has white flowers. It is commonly known as the Star of Bethlehem, or “ahbandır” and “akbaldır” in Turkish (15). This plant is gathered by people in spring in Şanlıurfa (Siverek) in south-east Turkey and the shoots are used as food. The mucilaginous structure is also said by local people to have a therapeutic effect on digestive system disorders. The purpose of this study was to investigate the phenolic composition analysis, antioxidant, cytotoxic, genotoxic and apoptotic effects of methanolic extract obtained from the aerial shoots of the OR plant for the first time in the literature in addition, to investigate whether this could be a potential new herbal medicine in cancer treatment.

Materials and Methods

Materials

TAS and TOS assay kits were purchased from Rel assay (Turkey). Cell death detection ELISA^{PLUS} kit was purchased from Roche (Germany). All the chemicals used in this study were purchased from Sigma (Europe).

Obtaining the plant samples and preparation of the extract

In this study, the shoots of the *Ornithogalum narbonense* L. (OR) plant were gathered on 05 March 2016, from a property located in Siverek-Karacadag, Sanliurfa, Turkey (N:37°46.664', E:039° 44.737', A: 1230 m). The plant species were determined by Prof. Dr. Ö. Faruk Kaya from the Biology Department. A herbarium record was taken. The OR plant samples were dried and rendered into powder form, then 200 grams of the plant sample was mixed in a solvent of methanol and water in the ratio of 80:20 respectively. The extract was kept overnight in a waterbath at 40°C. Then the mixture was filtered and the methanol was evaporated in a rotary evaporator and the sample was dried with a lyophilizer freeze dryer (Telstar). The extracts were then stored in a deep freeze at -80°C until assay.

Determination of the amount of total phenolic compound

The amount of total phenolic compounds in the extracts was determined according to the Slinkard and Singleton method with Folin-Ciocalteu reactive. After adding 4.5 mL distilled water to the tubes, 0.1 mL Folin-Ciocalteu reactive was added. After a waiting period of 3 mins, 0.3 mL of 2% Na₂CO₃ solvent was added. The tubes were mixed in a vortex and then kept for 2 hours in the dark at room temperature. Absorbance was read at 760 nm with a microplate reader. The results were stated as GAE (gallic acid equivalent).

Determination of the amount of total flavonoid

The amount of total flavonoid in the OR extracts was determined with the Zhishen assay (16). After the addition of 75 µL 5% NaNO₂ and 1.25 mL distilled water to 0.25 mL plant extract, the mixture was left for 6 minutes at room temperature. Then, 150 µL 10% AlCl₃ solvent

was added and we waited a further 5 minutes. With the addition of 0.5 mL 1 M NaOH solvent and 275 µL distilled water, the tubes were well mixed and absorbance values were read at 510 nm with spectrophotometry. The total flavonoid content of the OR extract was expressed as quercetin equivalents (QE) mg/g.

ABTS radical scavenging activity

The ABTS Radical Scavenging Activity method is based on the determination of the scavenging activity of ABTS radical, which is a strong radical cation of antioxidants. In 1 mL distilled water, 7.4 mM ABTS (2,2'-Azino-bis (3-ethylbenzenothiazoline-6-sulphonic acid) was dissolved and 1 mL 2.6 mM potassium persulphate was added. This mixture was left in the dark at room temperature for 12-16 hours. Then, 1 mL was taken from the mixture and 60 mL methanol was added, 2850 µL was taken and 150 µL plant extract was placed over it. This was left in the dark for 2 hours. The absorbance value was read at 734 nm with spectrophotometry. The concentration obtained after calculation of the percentages of the plant extract and standards, was stated as the results in the form of percentage ABTS radical scavenging activity.

CUPRAC (Cupric Reducing Antioxidant Capacity) Method

The cuprac method is an antioxidant determination method based on the reduction of copper-II neocuproin to copper-I neocuproin by antioxidants (17). In this order, 1 mL 1.0 x 10⁻² M Cu (II) chloride solvent, 1 mL 7.5 x 10⁻³ M neocuproin solvent and 1 mL 1 M ammonium acetate buffer (pH=7.0) solvent were added to the test tube. Then after the addition of x mL standard antioxidant solvent, 2- x mL distilled water was added to complete a final volume of 5 mL. The tubes were closed and left at room temperature for 30 mins. Absorbance values were measured at 450 nm corresponding to the reference mixture. A concentration and absorbance graph was obtained from the absorbance values of the plant extract and standards.

Quantitative analysis of phenolic and flavonoid compounds by LC-MS/MS

The LC-MS/MS system formed by combining the Shimadzu Nexera model UHPLC device with the Shimadzu LC-MS/MS 8040 model triple quadrupole mass spectrometer device was used in the qualitative and quantitative analysis of 37 phytochemicals. The liquid chromatography system used was formed of the LC-30 AD model gradient pump, the DGU-20A3R model degasser, the CTO-10ASvp model column oven and the SIL-30AC model autosampler. Chromatographic differentiation was applied in the Inertsil ODS-4 model C18 (100 mm x 2.1 mm, 2 µm) column. During analysis, the column oven was set at 35°C. In the elution gradient, ultrapure water was used for the movement phase A and acetonitril for movement phase B. For better chromatographic differentiation and to facilitate ionisation, 10 mM ammonium formate and 0.1% formic acid was added. Following several trials to achieve optimum differentiation of the analytes, the most appropriate UHPLC gradient profile was found to be 5-20% B (0-10 mins), 20% B (10-22 mins), 20-50% B (22-36 mins), 95% B

(36-40 mins), 5% B (40-50 mins). The mobile phase flow rate was defined as 0.25 mL/min and injection volume 4 μ L. The triple quadrupole mass spectrometer is fitted with an electrospray ionisation (ESI) source which works in both negative and positive modes. The LC-ESI-MS/MS data recorded on the device was gathered and processed using LabSolutions software (Shimadzu, Kyoto, Japan). The device was operated in multiple reaction monitoring (MRM) for numerical definition of the amounts of the analytes and molecular (parent) ions were combined with one or two fragmented ions (product ions) (the first was used for quantitative purposes and the other for qualitative purposes). The other parameters optimised in mass spectrometry were the interface temperature; 350 °C, DL temperature; 250 °C, heat block temperature; 400 °C, nebuliser gas flow (N₂); 3 L/min and dry gas (N₂); 15 L/min

Cell culture

DLD-1 (Human colon adenocarcinoma), ECC-1 (Human endometrium carcinoma) and HEK-293 (Human embryonic kidney cell) cell lines were incubated in DMEM and RPMI-1640, supplemented with 10% fetal bovine serum, 10 IU/ml penicillin/streptomycin and 2 mM L-glutamine at 37 °C with 5% CO₂ in a 25-ml flask. The cells were used for the assays performed in this study when 80-90% confluence was reached.

Viability assay (MTT assay)

The cytotoxicity and IC₅₀ values of OR were determined by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay which is based on the cleavage of tetrazolium salts by mitochondrial succinate-tetrazolium reductase in viable cells to form formazan dye. Cancer and normal cells were plated in 96-well plates at a density of 1 x 10⁴ cells/well and allowed to attach. After incubation at 37°C, 5% CO₂ for 24 hours with exposure to the OR extract and 5-FU at different concentrations (2-200 μ g/ml), the medium was then removed and the cells were lightly washed with PBS. After the addition of MTT (5 mg/mL) and incubation at 37°C for 4 hrs, the resulting formazan crystals were dissolved in DMSO. Cell viability was measured in terms of absorbance at 570 nm using a microplate reader (M5, Spectra Max) and the decrease in cell viability was expressed as the percentage compared with the control group designated as 100%. Three individual wells were measured for each treatment point. Cytotoxicity data (where appropriate) were fitted to a sigmoidal curve and a four-parameter logistic model was used to calculate the IC₅₀, which is the concentration of material causing 50% inhibition in comparison to the untreated controls.

Single cell gel electrophoresis assay (Comet assay)

The comet assay is a sensitive assay to examine DNA fragmentation at the level of the individual cells. The alkaline comet assay was used to study the potential OR extract induced DNA damage in cancer cell genotoxicity. In the study, 100 and 200 μ g/ml OR plant extract was planted over 1 x 10⁵ DLD-1 and ECC-1 cells in 24-well plates. Nothing was planted for the negative control group and 100 μ g/ml 5-FU chemotherapy drugs were added to the positive control group. After incuba-

tion for 48 hours, 2 slides were prepared for each group. The Comet assay was performed according Kocyigit *et al.*, (2016) as follows: approximately 2 x 10⁴ cells were suspended in low melting point agarose (LMA) (75 μ l of 1.0%), and stratified onto semi-frozen slides previously covered with a slim stratum of normal melting point agarose (1.0%). Another stratum of 0.5% LMA was placed over the second layer. The cells were dissolved for 2 h at 4°C in a solution (100 mM EDTA, 2.5 M NaCl, 10% DMSO, 1% Triton X-100, 10mMTris, pH 10.0). Following dissolution, the slides were exposed to electrophoresis in buffers (0.3 M NaOH, 1mM EDTA, pH 13.1) for 30 min. Then, the slides were neutralized within a Tris buffer (0.4 M Tris-HCl, pH 7.5). The slides were carefully dried at 25°C in an incubator and marked with ethidium bromide (10 μ g/ml in distilled water, 70 μ l/ slide). The slides were screened using a fluorescent microscope (Olympus CKX-51, Japan) imaging system. In each sample, 100 random cells were scored on a scale of 0-4 based on fluorescence outside the nucleus. The scoring scale used was as follows: 0, no tail; 1, comet tail, half the width of the nucleus; 2, comet tail equal to the width of the nucleus; 3, comet tail longer than the nucleus; and 4, comet tail twice the width of the nucleus. Scoring the cells in this way has been shown to be as accurate as using computerized image analysis (18).

Apoptotic analysis with ELISA

The ELISA method analyzes the amount of apoptosis occurring in the cells by quantitatively analyzing the oligo-nucleosome fragments which form during apoptosis. In this study, apoptosis was determined using the Cell Death Detection ELISA^{PLUS} colorimetric kit (Roche Applied Science, Germany). In each well of 96-well plates, 5x10³ cells were planted 3 times, and after waiting 24 hours for the cells to attach, 100 and 200 μ g/ml of the extract and 100 μ g/ml chemotherapy substances were added. After incubation for 48 hours, the amount of apoptosis in the cells was calculated according to the manufacturer's instructions. By accepting 1.00 for the sample to which no substance was added (negative control cells), mono and oligo-nucleosome enrichment, which expresses DNA fragmentation, was calculated as proportional to the enrichment factors of the other samples.

Dual AO/EB fluorescent staining for apoptosis

Dual acridine orange/ethidium bromide (AO/EB) fluorescent staining assay was performed according to Liu *et al* (19). In this assay, fluorescent staining visualized under a fluorescent microscope, can be used to identify apoptosis-associated changes of cell membranes during the process of apoptosis and this method can also accurately distinguish cells in different stages of apoptosis (19). Cancer cells were plated in 12-well plates at a density of 5 x 10⁵ cells/well and allowed to attach. After incubation for 24 hrs at 37°C, 5% CO₂, with exposure to the OR extract at different concentrations (2-200 μ M), the medium was then removed and the cells were lightly washed with PBS and trypsinised. When the cells had sloughed off, the suspensions (25 μ l) were transferred to glass slides. Dual fluorescent staining solution (1 μ l) containing 100 μ g/ml AO and 100 μ g/ml EB (AO/EB, Sigma, St. Louis, MO) was added

to each suspension, and the slide was covered with a coverslip. The morphology of apoptotic cells was examined and 500 cells were counted within 20 min using a fluorescent microscope (OLYMPUS, CKX-51 Japan). The dual acridine orange/ethidium bromide (AO/EB) staining method was repeated at least 3 times.

Determination of intracellular total oxidant status (TOS), total antioxidant status (TAS) and oxidative stress index (OSI)

Total oxidant status (TOS) and total antioxidant status (TAS) were detected in cell homogenates using commercially available kits (Rel Assay, Turkey) with an autoanalyzer (Cobas integra 800, Roche). TOS and TAS results were presented in $\mu\text{mol H}_2\text{O}_2$ equivalent/L (20) and mmol Trolox equivalent/L, respectively (21). The ratio of the TOS to the TAS revealed the oxidative stress index (OSI) which is used as an indicator for total oxidative stress (18). The OSI value was calculated according to the following formula: $\text{OSI} = [(\text{TOS}, \mu\text{mol HP equiv / g}) / (\text{TAS}, \mu\text{mol Trolox equiv / L})] \times 10$.

Statistical analyses

The results were expressed as mean \pm standard deviation. Descriptive statistics were used to analyze the mean, standard deviation, variation, and level of statistical significance between groups. Differences between the groups were tested using One-way ANOVA. A value of $p < 0.05$ was considered statistically significant.

Results and Discussion

Determination of total phenolic and flavonoid compounds

Total phenolic and flavonoid compounds in plants are basic compounds that provide antioxidant activity as secondary metabolite synthesised compounds. The total phenolic content of the OR extract in this study was determined as $173.26 \pm 13.5 \mu\text{g/mL GAE/mg}$, and total flavonoid content as $22.13 \pm 0.9 \mu\text{g/mL quercetin}$ equivalent.

Examination of the antioxidant capacity

The antioxidant activity of the OR plant extract was examined with the ABTS and CUPRAC methods. The ABTS and CUPRAC activity results of the OR plant extract and standards are shown in Figures 1. The ABTS radical scavenging activity of the OR plant extract was determined to be higher than that of the BHT standards.

Quantitative analysis of phenolic and flavonoid compounds of *O. narbonense* L. by LC-MS/MS

Plant extracts have been used for centuries in the

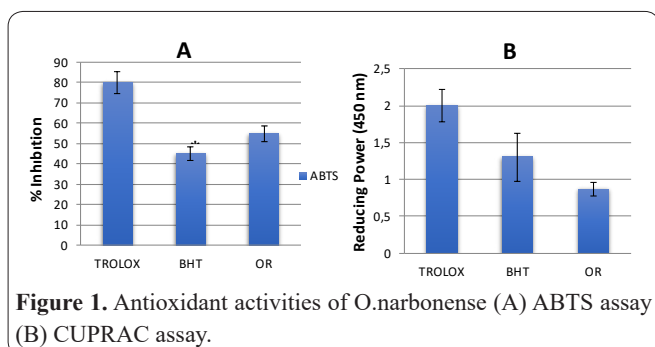


Figure 1. Antioxidant activities of *O.narbonense* (A) ABTS assay (B) CUPRAC assay.

treatment of many diseases and the beneficial effects could be due to the phenolic compound content (22, 23). As the most widely occurring groups of phytochemicals, phenolic compounds have strong antioxidant properties and are of considerable physiological and morphological importance to the plant itself (24). In many studies, an association has been shown between the anti-cancer, anti-inflammatory, antimicrobial activities and antioxidant of many herbs, plants and species and their phenolic content. Thus for potential application in the treatment of diseases, the identification and quantification of phenols from different sources have become increasingly important. Extraction with organic solvents is the generally-used sample preparation technique for phenols, and also identification and quantification are mainly performed through spectrophotometric and chromatographic techniques (22-26).

In this study, the phenolic compounds of *Ornithogalum narbonense* L. in Turkey, were identified and quantified by using a previously validated LC/MS-MS method (40). To the best of our knowledge, there has been no previous study in literature which has evaluated the phenolic compounds of *O.narbonense*. Representative chromatograms of the standards are shown in Figure 2.

It was possible to identify each peak via retention time in addition to the MS spectra of samples and authentic standards. The characteristics of each peak from the LC-MS/MS analysis of authentic standards/samples are presented in Table 1. With 6 phenolic acids, *O.narbonense* was found to be the richest in terms of the number and amount of phenolic compounds. Furthermore, the extract also contains a high level of the bioactive flavonoid, cosmosiin (apigenin-7-glucoside) ($1013,32 \mu\text{g/g}$ extract).

MTT test results

The cytotoxic effects of the OR extract and 5-FU chemotherapeutic substance used as the positive control on DLD-1, ECC-1 and HEK-293 cells were examined with the MTT method and the results are shown in Figures 3-5.

It was observed that the OR plant extract and 5-FU showed a dose-related cytotoxic effect on DLD-1 and ECC-1 cancer cells (Figure 1). The IC_{50} values of the OR extract and 5-FU on the normal and cancer cells are shown in Table 2. The OR plant extract was shown to have a higher cytotoxic effect on DLD-1 cells. Although the cytotoxic effect of the OR extract on cancer cells is lower than the cytotoxic effect of 5-FU, it was seen to have effects at low doses in cancer cells (176.26 - $278.97 \mu\text{M}$), and the cytotoxic effect on HEK-293 cells was seen at high doses ($1234.5 \mu\text{M}$) (Table 2).

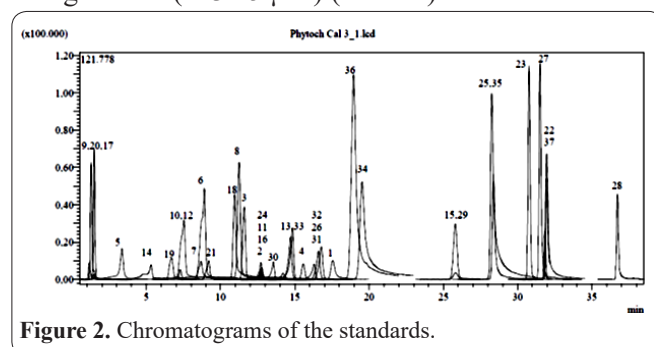


Figure 2. Chromatograms of the standards.

Table 1. Identification and Quantification of Phenolic Compounds of Methanol Extract of *O.narbonense* by UHPLC ESI-MS/MS.

	µg/kg		µg/kg		µg/kg
Coumarin	5.17	tr-Ferulic acid	7.0	Naringenin	1.41
Hesperidin	0.5	Chlorogenic acid	26.73	Rutin	1.73
p-coumaric acid	42.02	Rosmarinic acid	N.D	Quercetin	N.D
o-coumaric acid	N.D	Protocatechuic acid	1.93	Quercitrin	N.D
Gallic acid	2.78	Cinnamic acid	125	Apigenin	24.94
Caffeic acid	1.55	Sinapinic acid	N.D	Chrysin	N.D
Vanillic acid	11.36	Fumaric acid	4.53	Liquiritigenin	N.D
Salicylic acid	N.D	Vanillin	1.38	Isoquercitrin	12.39
Quinic acid	38.89	Pyrocatechol	4.1	Cosmosiin	1013.32
4-OH-Benzoic acid	N.D	Malic acid		Rhoifolin	1.19
Myricetin	N.D	Syringic acid	N.D	Nicotiflorin	N.D
Kaempferol	N.D	Hesperetin	N.D	Fisetin	N.D
Luteolin	N.D				

N.D; Not detected.

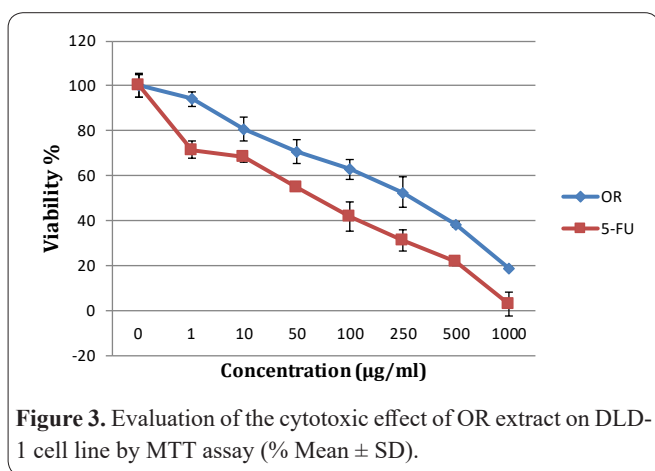


Figure 3. Evaluation of the cytotoxic effect of OR extract on DLD-1 cell line by MTT assay (% Mean ± SD).

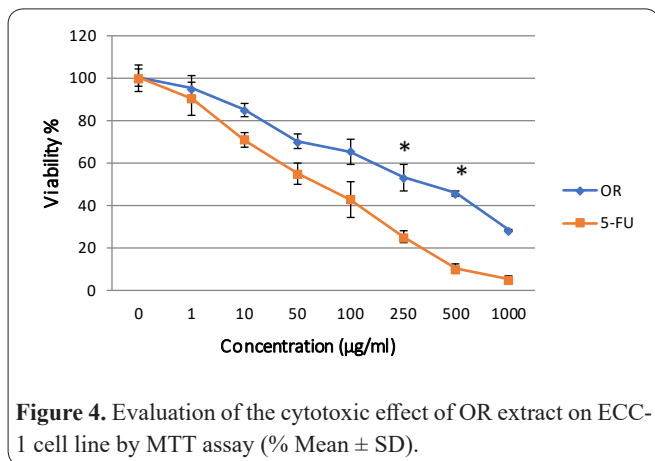


Figure 4. Evaluation of the cytotoxic effect of OR extract on ECC-1 cell line by MTT assay (% Mean ± SD).

The apoptotic effects of OR plant extract on cancer cells determined with ELISA

Whether or not an apoptotic effect was seen of plant extracts which showed a cytotoxic effect at 100 and 200 µg/ml on cancer cells were examined morphologically under a fluorescent microscope with the acridine orange/ethidium bromide (AO/EB) staining method and ELISA. To quantify the finding that exposure to OR plant extract caused apoptosis in DLD-1 and ECC-1 cancer cells and for further support, cell death was determined with ELISA. The apoptotic effects of OR extract and 5-FU on DLD-1 and ECC-1 cells are shown in Figure 6.

In comparison with the controls, exposure of the DLD-1 and ECC-1 cells to OR extract at concentrations of 100 and 200 µg/ml resulted in a 1.65-fold and 2.1-

fold increase in induction of apoptosis in ECC-1 and a 1.8-fold and 2.4-fold increase in induction of apoptosis in DLD-1 (Figure 6).

Table 2. IC₅₀ values of cell proliferation inhibition of OR extracts (µg/mL).

	DLD-1	ECC-1	HEK-293
OR	176.26±4.30	278.97±6.50	1234.5±11.22
5-FU	29.24±2.11	38.34±3.22	63.25±7.44

The values presented are mean ± standard deviation, n = 3. Results were analyzed using descriptive statistics.

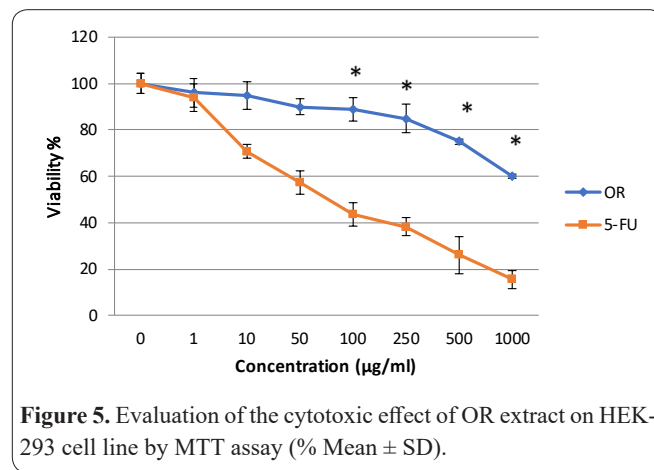


Figure 5. Evaluation of the cytotoxic effect of OR extract on HEK-293 cell line by MTT assay (% Mean ± SD).

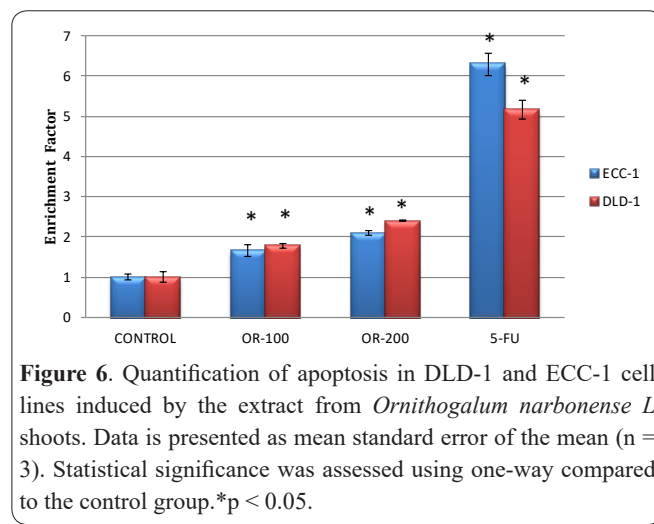


Figure 6. Quantification of apoptosis in DLD-1 and ECC-1 cell lines induced by the extract from *Ornithogalum narbonense* L shoots. Data is presented as mean standard error of the mean (n = 3). Statistical significance was assessed using one-way compared to the control group.*p < 0.05.

Morphological determination of the apoptotic effects of the OR extract with the AO/EB fluorescence staining method

The apoptotic effects of the OR extract and 5-FU (at a dose of 200µg/ml) on DLD-1 and ECC-1 cells were reexamined morphologically with the AO/EB fluorescence staining method (Figure 7).

The determination of the genotoxic effects of OR extracts on cancer cells with the Comet assay

The DNA damage occurring in DLD-1 and ECC-1 cells caused by the OR extracts were measured with the Comet assay method. It was determined that DNA damage was caused in DLD-1 and ECC-1 cells by OR extract in a dose-related manner. Compared to the controls, the OR extract at doses of 100 and 200 µg/ml was determined to lead to DNA damage at a significant level in both cells ($p < 0.05$) (Figure 8).

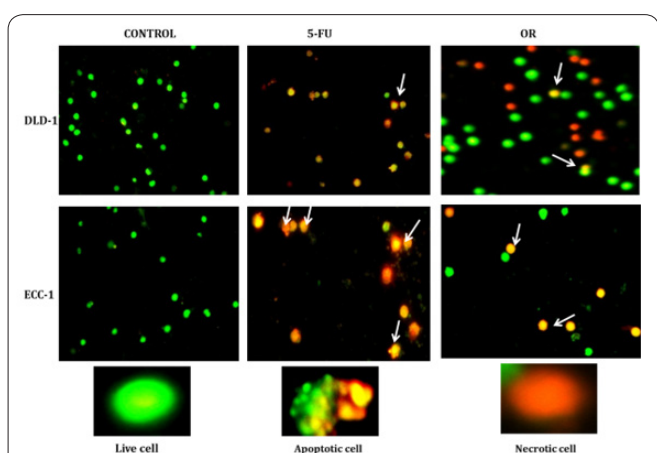


Figure 7. Fluorescence morphological image of the apoptotic effect of OR extract on DLD-1 and ECC-1 cell line. Control group cells: the circular nucleus is uniformly distributed in the center of the cell. Apoptotic cells: the nucleus showed yellow-green fluorescence with AO/EB staining and was concentrated. It was determined that OR extract showed an apoptotic effect on DLD-1 and ECC-1 cells.

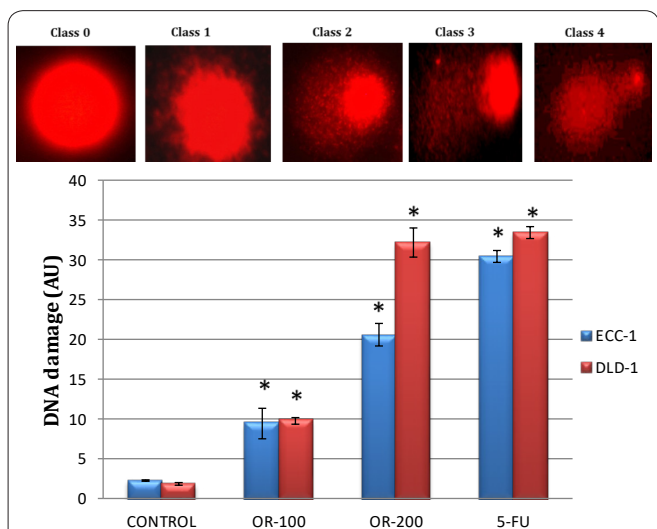


Figure 8. DNA damage rate shown with the mean of comet formation in DLD-1 and ECC-1 cells treated with OR extract. Data are presented as mean standard error of the mean (n = 3). Statistical significance was assessed using one-way compared to the control group. * $p < 0.05$.

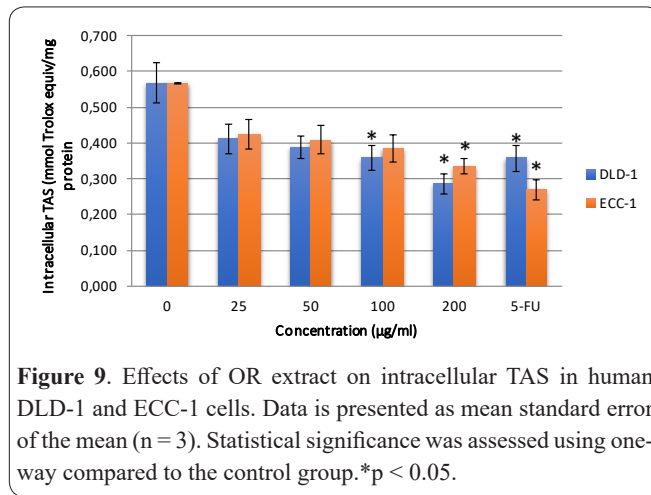


Figure 9. Effects of OR extract on intracellular TAS in human DLD-1 and ECC-1 cells. Data is presented as mean standard error of the mean (n = 3). Statistical significance was assessed using one-way compared to the control group. * $p < 0.05$.

Determination of Intracellular TAS, TOS, and OSI

In both cell lines, the TAS level showed a decrease associated with a dose increase, compared to the controls. It was determined that the OR extract caused a greater drop in the TAS level in DLD-1 cells. The greatest decrease in TAS level was observed to be at 200 µg/ml OR dose, close to the 5-FU level (Figure 9).

Compared to the control group, an evident increase in TOS levels was determined in the DLD-1 and ECC-1 cells related to dose increase of the OR extract leading to an increase in free radicals (Figure 10).

The oxidative stress index (OSI) value is calculated by dividing TOS by TAS ($\mu\text{mol H}_2\text{O}_2$ equivalent/ mmol Trolox equiv), so there is no definitive unit to express the OSI results. Therefore, these results are reported as an arbitrary unit (AU). As seen in Figure 11, and according to the calculation result, there was an ascending pattern, showing an increase in oxidative stress and reduction in antioxidants, which eventually induces the process of apoptosis.

As improved results have not been obtained with the classic medical drug treatments for cancer, which remains a fatal disease, and with the increase in success rates of treatment of cancer types as a result of the application of alternative treatments sourced from plants, the scientific world has turned to natural resources to eliminate the negative effects of living with cancer.

The majority of these natural resources are plant species containing bioactive compounds. Plant chemicals are defined as phytochemicals some of which are bioactive compounds which can be obtained from various parts of the plant species and reduce the risk of several

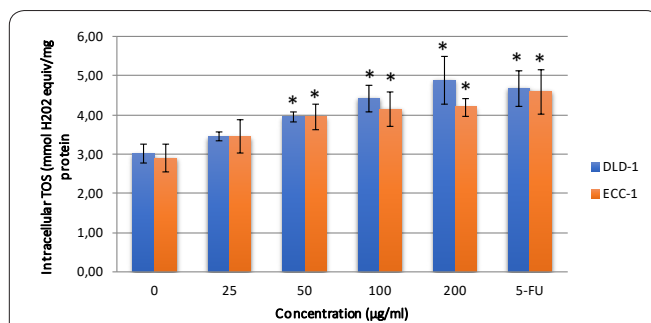


Figure 10. The effects of the OR extract on intracellular TOS in human DLD-1 and ECC-1 cells. Data are presented as mean standard error of the mean (n = 3). Statistical significance was assessed using one-way compared to the control group and standard. * $p < 0.05$.

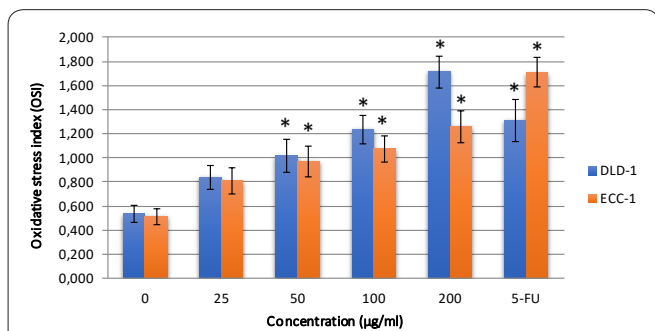


Figure 11. The effects of the OR extract on intracellular OSI in human DLD-1 and ECC-1 cells. Data are presented as mean standard error of the mean (n = 3). Statistical significance was assessed using one-way compared to the control group. *p < 0.05.

chronic diseases. Phytochemicals can show anti-cancer or anti-tumour activity effects in different ways. Promising results have been obtained against various malignancies in cancer treatment with phytochemicals (27). The discovery of secondary metabolites derived from plants as potential new drugs represents an inexhaustible chemical source. In particular, phenolic compounds that are found in plant species have anti-cancer or anti-tumour activity. Compounds isolated from plant species cannot be used directly as drugs but they could lead to the development a new potential drugs (28).

Therefore, the detection species containing high amounts of phenolic compounds that show high antioxidant activity is important for cancer treatment and inhibiting cancer development by preventing the oxidation of cells (18, 29). In recent studies, there has been a focus on the effects of plant-origin compounds in the regulation of the cell cycle and apoptotic pathways (30, 31). Therefore, in this study it was aimed to investigate for the first time in literature, the phenolic compound profile of the methanol extract obtained from aerial shoots of the *Ornithogalum narbonense* L., (OR) species and its antioxidant and anti-cancer properties. In a study of the OR plant by Zengin *et al* (32), ethyl acetate extract of samples of the bulb of this plant was determined to have antioxidant and anti-tyrosinase activity and a high rate of phenolic content. Makasci *et al* (33) reported that the leaf extract of *Ornithogalum alpigenum* Stapf was highly effective in free radical scavenging activity. In a study by Dastan *et al* (34), there was shown to be an antioxidant and antibacterial effect at a significant rate in n-hexane, ethyl acetate, methanol and water extracts of *Ornithogalum cuspidatum* Bertol. Water-soluble polysaccharide fractions of *Ornithogalum caudatum* have also been determined to show a significant level of anti-tumour activity against solid sarcoma tumours (35). The human diet is rich in plant polyphenols and they are known to have anti-cancer properties by intervening at different stages of cancer development (36). The basic effect mechanism responsible for chemopreventative and therapeutic effects against cancer is formed by the strong antioxidant effect of polyphenols (37).

In the first stage of this study, the total soluble phenolic and flavonoid substances were determined to identify the presence of a phenolic compound, which is known to have an antioxidant effect, in the extract obtained from the shoots of the OR plant. The total phenolic content of the OR extract was found to be $173,26 \pm 13,5$

µg/mL GAE/mg extract, and total flavonoid content was $22,13 \pm 0,9$ µg/mL quercetin equivalent. Furthermore, the antioxidant activities were examined by CUPRAC and ABTS methods. The ABTS radical scavenging activity was seen to be higher than the BHT standard, and lower than the Trolox standard (Figure 1). With the CUPRAC method, the antioxidant capacity value of the OR extract was determined to be lower than that of the standards (Figure 1B).

The phenolic profile of the OR was determined by a previously developed and validated LC-MS/MS method. As a result of the analysis, among the 20 phenolic compounds determined in the content, cosmosiin was found to be the most abundant. While the other 19 compounds were found at levels between 0,5-125 mg/ml, cosmosiin was determined at the high level of 1013,32 mg/ml (Table 1). As a result of the first stage of the study, it was understood that the OR extract contained phenolic and flavonoid compounds which showed antioxidant activity and therefore this extract could be a natural antioxidant source.

In recent studies there has been focused on the effects of plant-origin compounds in the regulation of the cell cycle and apoptotic pathways (30, 31), Polyphenols found in plants show anti-cancer property through anti-proliferation, halting the cell cycle, inhibiting metastasis and angiogenesis and providing induction of apoptosis (38). These effects have been found to have been shown by inhibiting key signal pathways which provide cell proliferation such as NF-κB, HIF-1, phosphonic acid 3 kinase/ Akt, Wnt signal proteins of polyphenols (39).

In this study, it was investigated whether or not the OR extract has a therapeutic property by evaluating the cytotoxic, genotoxic and apoptotic effects on human colon adenocarcinoma (DLD-1) and human endometrium adenocarcinoma (ECC-1) cancer cells. When the cytotoxic effect of OR extract was examined on the cancer (DLD-1 and ECC-1) cells and the normal cells, the cytotoxic effect value (IC_{50}) on DLD-1, ECC-1 and HEK-293 cells were determined as 176.26, 278.97 and 1234.5 µg/ml respectively, and the values of 5-FU chemotherapeutic substance used as a positive control on the same cells were 29.24, 38.34 and 63.249 µg/ml respectively (Figures 3-5). According to these results, while OR extract showed the most toxic effect on DLD-1, the cytotoxic effect on HEK-293 cells was lower than on the cancer cells, and sensitivity was determined to be very high compared to the 5-FU chemotherapeutic substance. To demonstrate whether or not the OR extract, which had shown a cytotoxic effect on cancer cells, showed an apoptotic effect, morphological examination was made with ELISA and the AO/EB staining method. The OR extract was observed to have an apoptotic effect on cancer cells. While the rate of apoptosis on the DLD-1 cells was seen to increase associated with an increase in dose of the OR extract, the apoptotic effect on ECC-1 was higher. In both the ELISA and the AE/EB morphological examinations, OR extract was determined to trigger apoptosis of cancer cells (Figures 6, 7).

Various mechanisms lead to apoptosis of cells. One of the most important of these reasons is DNA damage. If the DNA damage that occurs in the cell is not repaired, it leads to cell apoptosis. The DNA damage caused by OR extract in DLD-1 and ECC-1 cancer cells was

measured with the Comet assay method. The Comet assay is a frequently preferred method in the measurement of DNA damage as it is simple, rapid, sensitive, can be applied to different types of cells and DNA damage, and most importantly it does not require radioactive marking. The level of DNA damage on DLD-1 and ECC-1 showed an increase related to the dose of the studied extract. The OR extract caused the highest level of DNA damage on DLD-1 cells and was observed to be at a level close to 5-FU (Figure 8).

There is a certain intracellular free oxidant/antioxidant balance and when this balance is impaired with the effect of various factors, there is an increase in oxidative stress within the cell. Increasing intracellular oxidants cause apoptosis of the cell by leading to damage in lipid and protein biomolecules, primarily DNA. In this study, the oxidative stress index was calculated with colorimetric total oxidant (TOS) and total antioxidant status (TAS) methods to determine whether or not OR extract caused intracellular oxidative stress. It was determined that the OR extract increased the intracellular oxidative stress level by reducing the TAS levels and increasing the TOS levels in the DLD-1 and ECC-1 cells (Figures 9-11).

The results of the study showed that the OR extract caused cell apoptosis in cancer cells (DLD-1 and ECC-1) through an increase in the amount of intracellular free radicals leading to associated DNA damage. This effect of OR extract could be caused by cosmosiin being the most abundant in the content. Therefore, there is a need for further studies to be conducted by purifying the cosmosiin phenolic compound. These results have shown that the OR extract is an effective inhibitor on cell proliferation in DLD-1 and ECC-1 cells through concentration-related cytotoxic and apoptotic effects.

Compliance with ethical standards

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Ethical statement

This article does not include any studies on human participants or animals performed by any of the authors.

Conflict of interest statement

The authors declare that they have no conflict of interests.

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