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Evaluation of anticancer, antioxidant activity and phenolic compounds of Artemisia absinthium L. Extract

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Abstract: In the treatment of cancer, which remains a fatal disease, increasingly successful treatment rates of alternative therapies using the power of plants have directed the scientific world towards natural plant resources. This study aimed to examine the anti-cancer and antioxidant properties and identify the phenolic content of the methanolic extract obtained from *Artemisia absinthium* L. (AR) species, which is used as folk-medicine. 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 AR 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 AR 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 chlorogenic acid, followed by quinic acid, cinnamic acid, rhoifolin and malic acide. AR 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 by increasing the intracellular amount of free radicals on cancer cells, this led to DNA damage, which consequently led to apoptosis of the cancer cells.

Key words: *Artemisia absinthium* L.; Genotoxicity; Cytotoxicity; LC-MS/MS; Apoptosis.

Introduction

Various factors such as modern-day sedentary lifestyles, nutrition, stress and environmental pollution have led to an increase in free radical production in the body and thus various diseases including fatal cancer occur. The intake of natural antioxidant substances is known to be effective in the prevention of free-radical-origin diseases (1-6). The primary source of natural antioxidants is plants (fruit, vegetables and medicinal herbs) as they contain phenolic compounds, vitamins and terpenoids (7).

In recent years, plant-origin substances which show anti-inflammatory and anti-cancer properties inhibiting the oxidative process, have been used as a support to conventional cancer therapies (6, 8-11). The increase in treatment success rates of cancer types as a result of the application of these alternative therapies that take their power from plants has directed the scientific world to natural sources to eliminate the negative effects on life of cancer. The vast majority of these natural resources are plants containing biologically active compounds (12-15).

The differentiation of cancer cells is low in antineoplastic drugs currently used in chemotherapy for cancer patients. Just as antineoplastic cells destroy cancer cells which proliferate pathologically in the body, they can also rapidly destroy normal cells which are multiplying. Moreover, the cancer cells of chemotherapy patients can develop resistance to the chemotherapeutic drugs or fatal toxicity can be created. Therefore, the basic methods applied in current cancer treatment and the drugs used are thought to be insufficient (16-17).

Recent studies have investigated the anti-cancer effects of plant drugs used against cancer. There have been studies in many countries throughout the world to determine plant-origin natural compounds which can be used against cancer (18-23). It has been reported that these natural products are on average 20-fold less toxic than chemotherapy drugs (24). Furthermore, the ongoing success of plant-origin anti-cancer drugs such as taxol, etoposide and vincristine which have been discovered and developed in the last 40 years has continued this trend. Therefore, the antioxidant and anti-cancer properties of various plants have been investigated to use in the treatment of various diseases, primarily cancer (25-27).

The aim of this study was to investigate the antioxidant and anti-cancer properties of AR is a plant of the asteraceae family, which is herbaceous, perennial and grows by running water, in fields and on slopes and cliffs. In Turkey it is commonly known by different names such as Pelin, Pelin Otu, Acı Pelin and Horasani. Since ancient times it has been used as an aromatic and long-lasting herb as medication, generally as an antihelminthic, antiseptic, balsamic, antispasmodic and antidepressant, against cholera and digestive disorders and has also been used in the treatment of leukaemia and sclerosis because of its diuretic properties. In some studies, antioxidant and anti-cancer properties have also

been determined (28-32) .

As *Artemisia absinthium* L. (AR) has extremely important ethnopharmaceutic properties, a scan of literature was made and to the best of our knowledge, there has been no previous similar study, and thus AR was examined in this research. Through analysis of the phenolic composition of the methanolic extract obtained from the above-ground portion of the AR plant and by examining the antioxidant, cytotoxic, genotoxic and apoptotic effects on colon (DLD-1) and endometrium (ECC-1) cancer cells, an evaluation was made of whether or not this could be a candidate as a new herbal drug in the treatment of cancer.

Materials and Methods

Materials

TAS and TOS assay kit 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 (Europa).

Obtaining the plant samples and preparation of the extract

The above ground parts of *Artemisia absinthium L*. (AR) were collected in August 2016 from Binboga mountain, K.Maras, Turkey. They were identified by Maruf Balos (Botanist, Department of Biology, University of Harran, Turkey) by authentic herbarium techniques. A voucher specimen (M.B:4200) was deposited in Department of Biology, University of Harran, Turkey. The AR plant samples were dried and rendered into powder form, then 200gr of the plant sample was mixed in a solvent of methanol and water in the ratio of 80:20. This mixture was kept overnight in a waterbath at 40°C. Then the mixture was filtered and the alcohol was evaporated in a rotary evaporator (Heidolph, laborota 4002) at 40°C and the sample was dried with a lyofilizator 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, 0.1mL Folin-Ciocalteu reactive was added. After a waiting period of 3 mins, 0.3mL of 2% Na_2CO_3 solvent was added. The tubes were mixed in a vortex and then kept for 2 hours in the dark at room temperature (RT). 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 AR extracts was determined with the Zhishen assay (33). After the addition of 75 µL 5% $NaNO_2$ and 1.25 mL distilled water to 0.25mL plant extract, the mixture was left for 6 minutes at room temperature. Then, 150 µL 10% $AlCl_3$ solvent was added and a further 5 minutes were waited. 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, 1mL 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.

Cupric Reducing Antioxidant Capacity (CUPRAC)

The cuprac method is an antioxidant determination method based on the reduction of copper-II neocuproin to copper-I neocuproin by antioxidants (34). In this order, 1 mL 1.0 x 10^{-2} M Cu (II) chloride solvent, 1 mL 7.5 x10^{-3} M neocuprin 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 5mL. 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 were 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 LCMS 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×2.1 mm, 2µm) column. During analysis, the column oven was set at 35°C. In the elusion gradient, ultrapure water was used for movement phase A and acetonitryl 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 µL.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 ºC, DL temperature; 250 ºC, heat block temperature; 400 ºC, 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 normal embriyonic 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% CO_2 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 IC_{50} values of AR were determined by the MTT 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 10^4 cells/well and allowed to attach. After incubation at 37ºC 5% CO_2 for 24 hours with exposure to the AR extract and 5-FU at different concentrations (2-200 µg/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 IC_{50}, 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 AR-induced DNA damage in cancer cell genotoxicity. In the study, 100 and 200 µg/ml AR plant extracts were planted over 1 x10^5 DLD-1 and ECC-1 cells in 24-well plates. Nothing was planted for the negative control group and 100 µg/ml 5-FU chemotherapy drugs were added to the positive control group. After incubation for 48 hours, 2 slides were prepared for each group. The Comet assay was performed according to Singh et al. (35) with slight modifications Kocyiğit et al. (36) as follows: approximately 2 × 10^4 cells were suspended in low melting point agarose (LMA) (75 µl 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 µg/ml in distilled water, 70 µl/ 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 twice the width of the nucleus. Scoring the cells in this way has been shown to be as accurate as using computerized image analysis (37).

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, 5x10^3 cells were planted 3 times, and after waiting 24 hours for the cells to attach, 100 ve 200 µg/ml plant extracts and 100 µg/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 EB/ AO fluorescent staining for apoptosis

The dual EB/AO assay was performed according to (38). 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 (38). Cancer cells were plated in 12-well plates at a density of 5 x 10^5 cells/well and allowed to attach. After incubation for 24 hrs at 37ºC, 5% CO_2, with exposure to the AR extract at different concentrations (2-200 µM), the medium was then removed and the cells were lightly washed with PBS and trypsinised. When the cells had sloughed off, the suspensions (25 µl) were transferred to glass slides. Dual fluorescent staining solution (1 µl) containing 100 µg/ml AO and 100 µg/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.

Table 1. Identification and Quantification of Phenolic Compounds of Methanol Extract of *Artemisia absinthium L*. by LC-MS/MS.

		µg/kg			µg/kg
1.	Coumarin	14,25	20	Malic acid	81,11
2.	Hesperidin	38,86	21	Syringic acid	2,08
3.	p-coumaric acid	22,35	22	Hesperetin	14,48
4.	o-coumaric acid	N.D	23	Naringenin	6,9
5.	Gallic acid	N.D	24	Rutin	62,57
6.	Caffeic acid	65,96	25	Quercetin	N.D
7.	Vanillic acid	8,56	26	Quercitrin	2,15
8.	Salicylic acid	4,48	27	Apigenin	4,5
9.	Quinic acid	383,76	28	Chrysin	N.D
10.	4-OH-Benzoic acid	N.D	29	Liquiritigenin	N.D
11.	tr-Ferulic acid	3,57	30	Isoquercitrin	37,35
12.	Chlorogenic acid	1031,96	31	Cosmosiin	N.D
13.	Rosmarinic acid	15,13	32	Rhoifolin	93,25
14.	Protocatechuic acid	4,68	33	Nicotiflorin	N.D
15.	Cinnamic acid	100,61	34	Fisetin	N.D
16.	Sinapinic acid	1,62	35	Luteolin	N.D
17.	Fumaric acid	N.D	36	Myricetin	N.D
18.	Vanillin	7,53	37	Kaempferol	N.D
19.	Pyrocatechol	N.D		**N.D:** Not detect.	

Determination of intracellular TOS, TAS and OSI

Total oxidant status (TOS) and total antioxidant status (TAS) were detected in cell homogenates using commercially available kits (Rel Assay, Turkey). TOS and TAS results were presented in µmol H_2O_2 equivalent/mg (39) and mmol Trolox equiv/mg protein, respectively (40). The ratio of the TOS to the TAS revealed the oxidative stress index (OSI) which is used as an indicator for total oxidative stress (37). The OSI value was calculated according to the following formula: OSI = [(TOS, µmol HP equiv / mg protein) / (TAS, µ mol Trolox equiv / mg protein)] X 10**.**

Statistical analyses

The results were expressed as mean ± standard deviation. Descriptive statistics were used to analyze the mean, standard deviation 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

Analysis of phenolic compounds of *Artemisia absinthium* by LC –MS/MS

Plant extracts have been used for centuries in the treatment of many diseases and the beneficial effects could be due to the phenolic compound content (41, 42). 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 (43). 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 has become increasingly important. Extraction with organic solvents is the generally-used sample preparation technique for phenols, and identification and quantification is mainly performed through spectrophotometric and chromatographic techniques (41-45). In this study, the phenolic compounds of *Artemisia absinthium* L. in Turkey, were identified and quantified using a previously validated LC-MS/MS method.

It was possible to identify each peak via retention time in addition to the MS/MS spectra of samples and authentic standards. The characteristics of each peak from the LC-MS/MS analysis of samples were presented in Table 1. Of the 37 compounds identified as phenolic compounds, 23 were found to be in the plant extract. With 6 phenolic acids, *A.absinthium* was found to be richest in terms of the number and amount of phenolic compounds. Furthermore, the extract also contains a high level of the bioactive Chlorogenic acid (1031,96 µg/g extract).

Determination of the amount 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 and flavonoid compounds results of the AR plant extract are shown in Table 2.

Evaluation of the antioxidant capacity

The antioxidant activity of the AR plant extract was examined with the ABTS and CUPRAC methods. The ABTS and CUPRAC activity results of the AR plant extract and standards are shown in Figures 1. The ABTS radical scavenging activity of the AR plant extract was determined to be higher than that of the trolox and butyl hydroxy toluen (BHT) standards.

Table 2: Total phenolic and flavonoid compounds in *A.absinthium L.*

	T.Flavonoid (QE mg/g)	T.Phenolic (GAE / mg)
*A.absinthium L.*extract	28,86±3,4	177,29 ±9,25

The values presented are mean ± standard deviation, (n = 3).

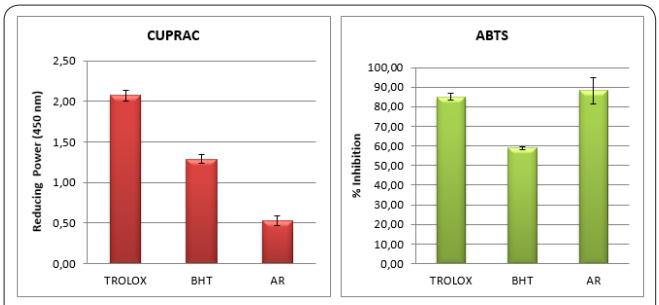

Figure 1. Antioxidant activities of *A. absinthium L.*extract by CUPRAC and ABTS assay.

MTT test results

The cytotoxic effects on DLD-1 (colon cancer), ECC-1(endometrium cancer) and HEK-293 (normal embriyonickidney cell) cells of the AR plant methanolic extract and the 5-FU chemotherapeutic agent used as positive control were examined with the MTT method and the results are shown in Figures 2-4.

It was observed that the AR plant extract and 5-FU showed a dose-related cytotoxic effect on DLD-1 and ECC-1 cancer cells (Figure 2,3). The IC_{50} values of the

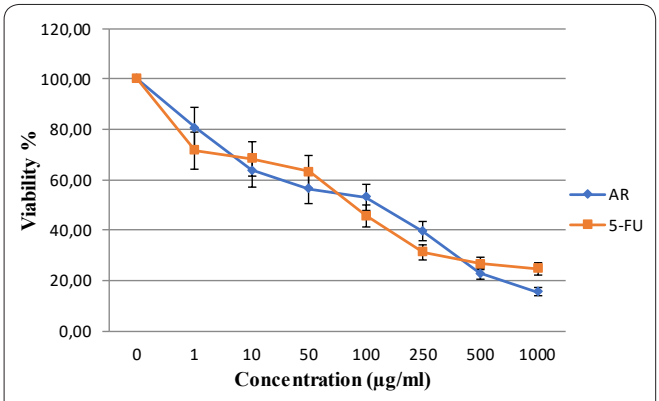

Figure 2. Evaluation of cytotoxic effect of AR extract on DLD-1 cell line by MTT assay (% Mean ± SD). Statistical significance was assessed using one-way as compared to the 5-FU group.*p < 0.05.

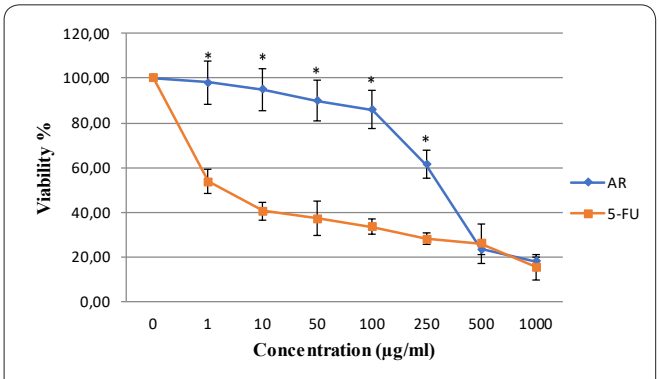

Figure 3. Evaluation of cytotoxic effect of OR extract on ECC-1 cell line by MTT assay (% Mean ± SD). Statistical significance was assessed using one-way as compared to the 5-FU group.*p < 0.05.

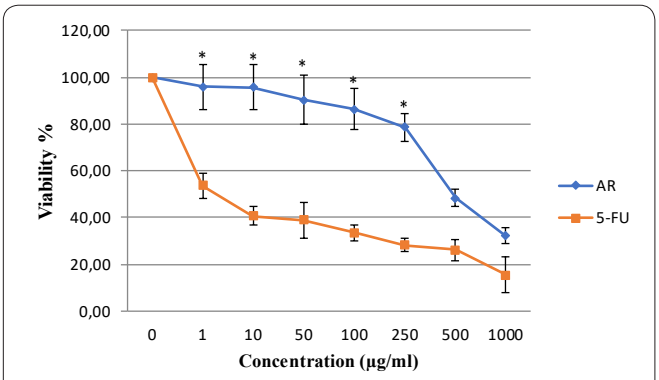

Figure 4. Evaluation of cytotoxic effect of AR extract on HEK-293 cell line by MTT assay (% Mean ± SD). Statistical significance was assessed using one-way as compared to the 5-FU group.*p < 0.05.

Table 3. IC_{50} values of cell proliferation inhibition of *A. absinthium* extracts (µg/mL).

	DLD-1	ECC-1	HEK-293
AR	3,92±0,1	480,25±18,3	652,7±13,4
5-FU	3,87±0,6	0,871±0,02	0,929±0,01

The values presented are mean ± standard deviation, (n = 3).

AR extract and 5-FU on the normal and cancer cells were shown in Table 3. The AR plant extract was shown to have a higher cytotoxic effect on DLD-1 cells. Although the cytotoxic effect of the AR extract on ECC-1 cancer and HEK-293 normal cells is lower than the cytotoxic effect of 5-FU, it was seen to have effects at low doses in DLD-1 cells (3,92 µg/ml), and the cytoxic effect on normal HEK-293 cells (Figure 4) was seen at high doses (652,7 µg/ml).

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

To quantify the finding that exposure to AR 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 AR extract and 5-FU on DLD-1 and ECC-1 cells are shown in Figure 5.

In comparison with the controls, exposure of the DLD-1 and ECC-1 cells to AR extract at concentrations of 100 and 200 µg/ml resulted in a 2,1-fold and 2,7-fold increase in induction of apoptosis in ECC-1cell and a 2,7-fold and 3,3-fold increase in induction of apoptosis

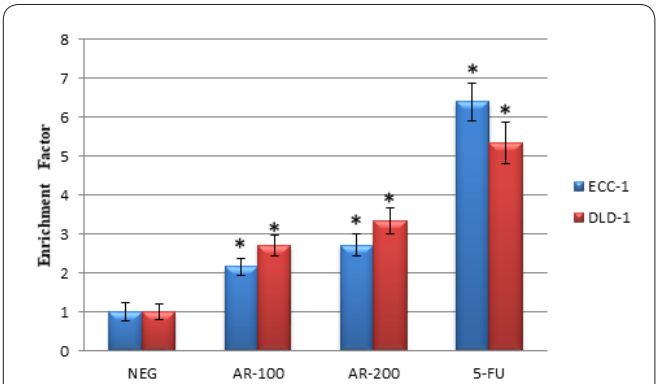

Figure 5. Quantification of apoptosis in DLD-1 and ECC-1 cell lines induced by extract from *A. absinthium*. Data is presented as mean standard error of the mean (n = 3). Statistical significance was assessed using one-way as compared to the negative group.*p < 0.05.

in DLD-1 cell (Figure 5).

Morphological determination of the apoptotic effects of AR plant extracts with the AO/EB fluorescence staining method

The apoptotic effect on DLD-1 and ECC-1 cells of AR plant extract and 5-FU at a dose of 100µg/ml was examined morphologically with the AO/EB fluorescence staining method (Figure 6). It was determined that AR plant extract showed an apoptotic effect on DLD-1 and ECC-1 cells.

The determination of the genotoxic effects of AR extracts on cancer cells with the comet assay

The DNA damage occurring in DLD-1 and ECC-1 cells caused by AR plant extracts was measured with the comet assay. It was determined that DNA damage was caused in DLD-1 and ECC-1 cells by AR extract in a dose-related manner. Compared to the controls, the AR 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 7).

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 AR extract caused a greater drop in the TAS level in DLD-1and ECC-1 cells. The greatest decrease in TAS level was observed to be at 200 µg/ml

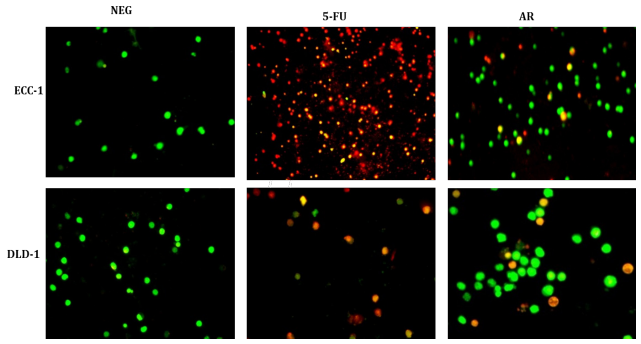

Figure 6. Fluorescence morphological image of the apoptotic effect of AR extract on DLD-1 and ECC-1 cell line. Control group cells: the circular nucleus uniformly distributed in the center of the cell. Apoptotic cells: nucleus showed yellow-green fluorescence by AO/EB staining and concentrated.

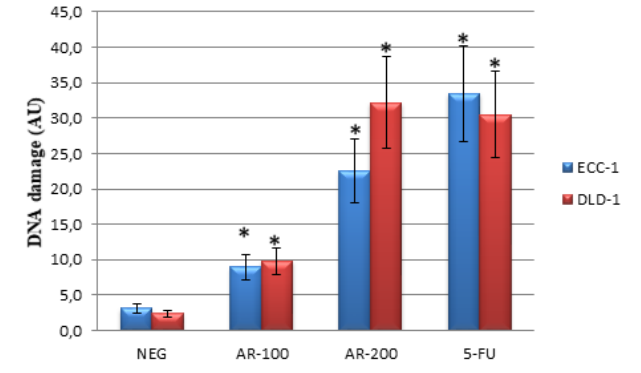

Figure 7. DNA damage rate by the mean of comet formation in DLD-1 and ECC-1 cells treated with AR extract. Data is presented as mean standard error of the mean (n = 3). Statistical significance was assessed using one-way ascompared to the negative group.*p < 0.05.

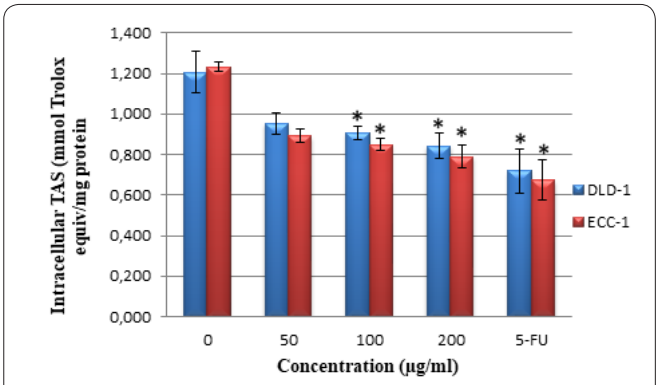

Figure 8. Effects of AR extract on intracellular TAS in human DLD-1 and ECC-1 cells. Data is presented as mean standard error of the mean (n = 3). Statistical significance was assessed using one-way as compared to the control group.*p < 0.05.

AR dose, close to the 5-FU level (Figure 8).

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 AR extract leading to an increase in free radicals (Figure 9).

The oxidative stress index (OSI) value is calculated by dividing TOS by TAS (µmol H_2O_2 equivalent/ mmol Trolox equiv), so there is no definitive unit to express the OSI results. Therefore, these results are reported as arbitrary unit (AU). As seen in Figure 10, 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 permanent recovery 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.

Discussion

No effective results have been obtained as yet in the fight against cancer. Phytotherapy is applied to people additional to chemotherapy and radiotherapy. In addition, supportive therapy routes are being researched as the application of nutritional therapy with a healthy

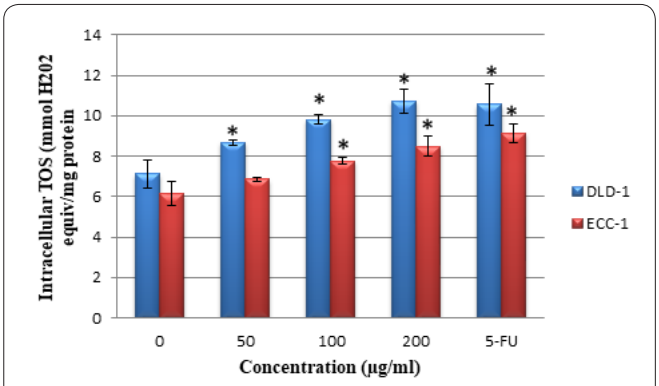

Figure 9. Effects of OR extract on intracellular TOS in human DLD-1 and ECC-1 cells. Data is presented as mean standard error of the mean (n = 3). Statistical significance was assessed using one-way as compared to the control group and standard.*p < 0.05.

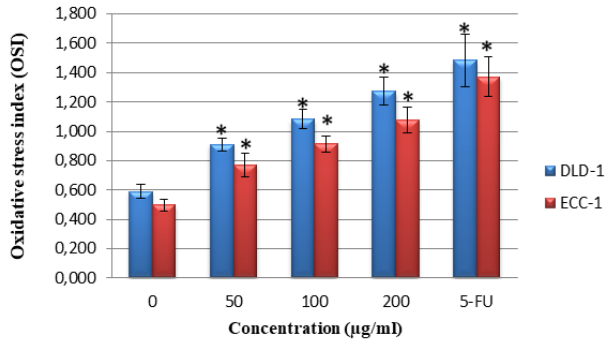

Figure 10. Effects of OR extract on intracellular OSI in human DLD-1 and ECC-1 cells. Data is presented as mean standard error of the mean (n = 3). Statistical significance was assessed using one-way as compared to the control group.*$p < 0.05$.

diet, due to the low success rates of conventional cancer treatments and that there have been no side-effects or recurrence in the majority of patients (46).

Plants contain bioactive compounds with several biological activities primarily antioxidant and anti-cancer properties. Plant chemicals are defined as biologically active plant compounds that can be obtained from parts of the plant and can reduce the risk of several chronic diseases. Antioxidant and anti-cancer activity can be shown in the effects of plant chemicals in different aspects.

The anti-cancer property was found in plants originates from polyphenols, primarily phenolic compounds, flavanols and flavonoids (47-49). Encouraging results have been obtained from these types of phytochemicals in the treatment of various malignancies (50). Researchers believe that polyphenols are helpful in killing cancer cells and halting the progression of cancer. For example, it has been suggested in some studies that the polyphenols in green tea play an important role in protection against colon cancer (51).

Of the phytochemicals found in plants, phenolic compounds in particular have anti-cancer or anti-tumour properties (49, 51-52). Phenolic compounds are the main group of phytochemicals found in plants. The basic structure of phenols is formed of an aromatic ring and a hydroxyl group. Therefore, the identificaton of plants containing phenolic compounds that show high antioxidant activity is important for the treatment of cancer and preventing the formation of cancer by preventing the oxidation of cells (36, 53-55).

In this study, the phenolic compound profile and antioxidant and anti-cancer properties were investigated of the methanol extract obtained from the above-ground portion of the *Artemisia absinthium L*., (AR) plant. The AR plant is used as a herbal folk medicine. In a study by Velioglu et al (56), there was reported to be a strong correlation between the total phenolic content and antioxidant activity. It is thought that greater antioxidant activity could be shown through synergic effects of different phenolic compounds. In previous studies, the oil from the tips of *Artemisia dracunculus* has been reported to show antioxidant activity (57, 58).

The total soluble phenolic and flavonoid substances were tested in this study to determine the presence of phenolic compound with an antioxidant effect in the methanol extract obtained from the AR plant. The amount of total phenolic compound obtained from the AR extract was found to be 177.29 ±9.25µg/mL GAE/mg extract, and the amount of total flavonoid µg/mL quarcetin equivalent was 28.86±3.4.

In addition, the LC/MS-MS with phenolic compound profile of the plant extract was determined. As a result of the analysis, 23 phenolic compounds were determined, the most abundant being Chlorogenic acid, followed by Quinic acid, cinnamic acid, rhoifolin and malic acid. While 21 of these compounds were found to in the range of 1.62-100.61 µg/kg, Chlorogenic acid was determined at the high level of 1031.96 µg/kg (Table 1).

Chlorogenic acid is an ester formed of caffeic acid and quinic acid. As a polyphenol compound isolated from the leaves and fruit of dicotyledon plants, chlorogenic acid is a basic phenolic compound found mostly in coffee. This compound, which is known to be an antioxidant has been determined to slow down the expression of glycose into the blood circulation after eating. As a result of epidemiological studies, it has been determined that the consumption of coffee containing a high rate of chlorogenic acid could be helpful in peventing various chronic diseases, primarily type 2 diabetes, Parkinson's disease and various liver diseases (59, 60).

The antioxidant activity of the phenolic and flavonoid content of the methanol extract of the AR plant was also examined with the ABTS and CUPRAC methods. As a result of the tests applied in this study, it was determined that the AR plant showed antioxidant activity. These results demonstrated that AR extract could be used as a natural antioxidant source. Due to the phenolic and flavonoid content of this natural antioxidant source, there is thought to be a free radical cleaning effect.

In studies conducted on the relationship between cancer and antioxidants, when fruit and vegetables rich in antioxidants are consumed, the risk of cancer development in several organs has been shown to be significantly reduced. Recent studies have focussed on the cell cycle regulatory effects and the effects on the apoptotic pathways of plant-origin compounds (61, 62). Polyphenols found in plants demonstrate anti-cancer properties by antiproliferation, halting the cell cycle, inhibiting metastasis and angiogenesis and providing apoptosis induction (63).

When the studies are examined that have been conducted related to the anti-cancer properties of the *Artemisia absinthium L*. (AR) plant, Shafi et al examined the cytotoxic and apoptotic effect of the ethanolic extract of the AR plant on MCF-7 and MDA-MB-231 breast cancer cell lines. The results of the study determined that the AR plant showed a cytotoxic and apoptotic effect on breast cancer cell lines. It was suggested that this effect of the plant extract occurred through the effect on the Bcl-2 family proteins and the MEK/ERK pathway of the breast cancer cells (32).

In the current study, it was investigated whether or not AR extract had a therapeutic property by evaluating the cytotoxic, genotoxic and apoptotic effects on human colon adenocarcinoma (DLD-1) and human endometrium adenocarinoma (ECC-1) cancer cells. When the cytotoxic effect of AR extract on cancer (DLD-1, ECC-1) and HEK-293 cells was examined, the cytotoxic effect values (IC_{50}) on DLD-1, ECC-1 and HEK-293

cells were determined as 3.92, 480.25, and 652.70 µg/ml, respectively and the IC_{50} values of the 5-FU chemotherapeutic substance used as positive control on the same cells were 3.87, 0.871 and 0.929 µg/ml, respectively (Figures 2-4). According to these results, while the AR extract showed a very high cytotoxic effect on colon cancer (DLD-1), as the cytotoxic effect on the normal HEK-293 cells was lower compared to the cancer cells, sensitivity to the normal cells was determined to be very high compared to the 5-FU substance.

To demonstrate whether or not there was an apoptotic effect of the AR which showed a cytotoxic effect on cancer cells, morphological examination was made with the ELISA and acridin orange/ethidium bromide staining methods. There was observed to be an apoptotic effect of the AR extract on cancer cells. While it was observed that the apoptosis ratio increased related to a dose increase in the AR extract, the apoptotic effect on DLD-1 cells was seeen to be greater than the effect on the ECC-1 cells. In both the ELISA and acridin orange/ethidium bromide morphological examinations, it was determined that AR extract triggered apoptosis in cancer cells (Figures 5,6).

Various mechanisms lead to apoptosis of cells. One of the most important of these causes is DNA damage. If DNA damage that occurs in cells is not repaired, the cell is oriented to apoptosis. The DNA damage created in the DLD-1 and ECC-1 cancer cells by AR extract was measured using the comet assay method. A dose-related increase was seen in the level of the DNA damage created in the DLD-1 and ECC-1 cancer cells by the plant extract (Figure 7).

Increasing oxidative stress in the cells is one of the most important factors leading to DNA damage. There is a specific intracellular oxidant/antioxidant balance and when this balance is disrupted with the effect of various factors, an increase in intracellular oxidative stress occurs. Increased intracellular oxidants lead to damage in the lipid and protein biomolecules, primarily DNA. In this study, it was determined that by the AR plant extract decreasing the TAS levels in the DLD-1 and ECC-1 cancer cells and increasing the TOS level, the intracellular oxidative stress level was increased (Figures 8-10).

In conclusion, the results of the study showed that the AR plant extract caused apoptosis of the cancer cells (DLD-1, ECC-1) by increasing the amount of intracellular free radicals, thereby leading to DNA damage. This effect is thought to be caused by Chlorogenic acid, which is the most abundant phenolic compound in the content of AR extract. These results show that AR plant extract is an effective inhibitor of cell proliferation through cytotoxic and apoptotic effects associated with the concentration in the cell line of colon cancer.

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Author Contributions
Ismail KOYUNCU participated in the experimental design and completion, as well as interpretation, manuscript design and preparation.

Conflicts of Interest
The authors declare no conflict of interest.

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