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Original Research

The evaluation of antioxidant and anticancer effects of *Lepidium Sativum Subsp Spinescens L.* methanol extract on cancer cells

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Abstract: In recent years, there is an increased research interest for plants which are natural sources of antioxidants. *Lepidium sativum Subsp spinescens L.,* commonly found in South West Asia, is a plant known as a healthy nutritional source containing bio-molecules that carry anti-hypertensive, hypoglycemic, anti-asthmatic, antispasmodic, hepato-protective, chemoprotective, anti-inflammatory and anti-oxidant effects. In this study, we aimed to investigate the antioxidant content and activity *of Lepidium sativum Subsp spinescens L.* methanol extract on cancer cells. Methanol extract of dried *Lepidium sativum Subsp spinescens L.* was prepared. Total amount of phenolic compounds was determined by Slinkard and Singleton method using Folin-Ciocalteu reagent. Total flavonoid amount was determined according to Zhishen method. Antioxidant activity of the extract was evaluated by CUPRAC and ABTS radical scavenging activity assays. Cytotoxic effects of the plant extract on colon and endometrium cancer cells, and human peripheral lymphocyte cells were investigated *in vitro* by MTT and neutral red assays. Furthermore, the plant extract was investigated for necrotic effects by LDH assay; apoptotic activity by DNA ladder fragmentation, ELISA and acridine orange/ethidium bromide staining; and genotoxic effect by comet assay methods. Methanol extract of *Lepidium sativum Subsp spinescens L.* was found to have a high content of phenolic and flavonoid compounds. The extract showed significant antioxidant activity and also cytotoxic activity on colon and endometrium cancer cells in a concentration-dependent manner. Apoptotic activity and genotoxic effects were significantly increased, especially with 200 µg/ml concentrations at 48 hours incubation. In conclusion, it was determined that the extract evaluated in this study could be a natural source of antioxidants. Further molecular studies explaining chemo-preventive and chemotherapeutic effects on cancer cells are required to support anticancer efficacy of the plant.

Key words: *Lepidium sativum Subsp spinescens L.*; Antioxidant activity; Anticancer activity.

Introduction

Cancer is the second leading cause of death worldwide. It is responsible for 17.5 million cases and 8.7 million deaths (1). Oxidative stress is associated with several diseases, especially cancer (2). It is defined as tissue or cell reactive oxygen species (ROS) concentrations exceeding the antioxidant capacity (3). This state causes damage to important biomolecules and cells that have potential effects on the whole organism (4). ROS play a role in inflammation and carcinogenesis processes. It has also been identified in recent studies that inflammation and cancer are related (5). Serious concerns about the toxic effects of synthetic antioxidants, produced to protect humans from oxidative damage, are currently discussed (2, 4). Accordingly, identification of natural and reliable antioxidant sources is important. Natural antioxidants, especially phenolic compounds, flavonoids, tannins and anthocyanidins in plants are biologically active and safe. Thus, there has been a continuous increase in the number of research studies on potential antioxidant effects of plant extracts or plant-derived isolated products in recent years (6).

Lepidium sativum Subsp spinescens L. (LEP) contains vitamins, minerals and numerous anti-carcinogenic subsptances and is used as treatment against various diseases (7, 8). It has been reported that the LEP plant has chemoprotective (9), anti-inflammatory and antioxidant effects (10, 11) and may be an alternative alimentary source for healthy nutrition (12). We aimed to investigate the antioxidant activity of LEP plant and anticancer properties on various cells.

Materials and Methods

The reagents including 1,1-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azinibis 3-ethyl benzothiazoline-6-sulfonic acid (ABTS), gallic acid, Folin-Ciocalteu phenol reagent, ethidium bromide, Dimethyl sulfoxide (DMSO), agarose and Acridine orange were purchased from Sigma; Apoptosis Cell Death Detection ELISA PLUS, Apoptotic DNA Ladder and Cytotoxicity Detection Kit (Lactate Dehydrogenase) kits were purchased from Roche.

Preparation of plant samples and extraction

In our study, methanol: water (80:20) extract of the above-ground parts of dried LEP plant was prepared. The plant was identified by the Department of Biology in Harran University. The plant sample was dried at 40°C and pulverized. Afterwards, the sample was stirred in methanol-water at 40 °C overnight, filtered through Whatman 1 filter paper and the solvent was removed by lyophilization. The samples were then stored at -80 °C for later usage.

Total Phenolic Compound Quantification

Total amount of phenolic compounds was determined with Folin-Ciocalteu reagent according to Slinkard and Singleton's method (13). 1 mg/mL gallic acid stock solution was prepared with the purpose of obtaining a standard curve. Absorbance of standards and samples were then measured at 760 nm against blind. The amount of gallic acid corresponding to absorbance values of the samples was calculated using the standard curve and the results were expressed as gallic acid equivalents.

Total Flavonoid Quantification

Total flavonoid content was determined according to Zhishen method (14). 1.25 mL of distilled water and 75 µL of 5% $NaNO_2$ were added onto 0.25 mL plant extract (100 µg-400 µg/mL), and left for incubation at room temperature for 6 minutes. 150 µL of 10% $AlCl_3$ solution was then added and the mixture was incubated for 5 minutes. After addition of 0.5 mL of 1 M NaOH solution and 275 µL of distilled water, the tubes were thoroughly mixed and absorbance at 510 nm was measured against reagent blind. Quarcetin was used as a standard at concentrations of 20-100 µg/mL. Amount of flavonoids in the extract was expressed as equivalent of milligram quarcetine per gram extract.

ABTS Radical Scavenging Activity Spectrophotometric Method

ABTS (2,2'-Azino-bis (3-ethylbenzenothiazoline-6-sulfonic acid) is a durable radical cation and the assay is based on the determination of ABTS radical scavenging activity of the antioxidants. 7.4 mM ABTS was dissolved in 1 mL of distilled water and 1 mL of 2.6 mM potassium persulfate was then added onto. The mixture was left at room temperature for 12-16 hours in the dark. 60 mL of methanol was added onto 1 mL of this mixture. 150 µL of the plant extract was added onto 2850 µL of the prepared methanolic ABTS solution. The mixture was incubated in the dark for 2 hours. Absorbance at 734 nm was measured on spectrophotometer. Percentage values of the plant extract and standards were calculated and the concentration-percent ABTS radical scavenging activity curve obtained was proportioned to the Trolox curve and the results were expressed as $TEAC_{ABTS.}$ (15).

CUPRAC (Copper (II) Ion Reducing Antioxidant Capacity) Method

The assay is based on the reduction of copper-II neocuproin to copper-I neocuproin by antioxidants (16). 1 mL of $1.0x10^{-2}$ M Cu (II) chloride, 1 mL of 7.5×10^{-3} M neocuproin and 1 mL of 1 M ammonium acetate buffer (pH=7.0) solutions were added in a test tube, respectively. Afterwards, 1 mL of antioxidant standard solutions was added and the final volume was made up to 5 mL by addition of 2 mL distilled water. Sealed tubes were incubated at room temperature for 30 minutes. Absorbance at 450 nm was measured against the reference mixture blind. Concentration-absorbance graphics of plant extracts and standards were obtained. The obtained curve was compared to Trolox curve and the results were expressed as $TEAC_{CUPRAC}$.

Procurement and culture of cell lines

Human colon cancer (DLD-1) and endometrium cancer cell lines (ECC-1) were obtained from Istanbul University, Molecular Medicine Research Laboratory and stored at -80 °C. Cell lines were reproduced in RPMI-1640 medium containing stabilized L-glutamine supplemented with 10% heat-inactivated fetal bovine serum, 10.000 units/ml penicillin and 10 mg/ml streptomycin in a humidified incubator at 37 °C and 5% CO_2. Cell culture procedures were carried out in a sterile laminar airflow working chamber.

Peripheral lymphocyte cells (PML) separation and culture protocol

Mononuclear leukocyte cells were isolated from blood samples collected, into tubes containing heparin, from healthy human subjects. Isolated peripheral lymphocytes and cancer cells were counted with the assistance of specific counting slides called "Thoma slide" before usage in cytotoxicity and genotoxicity studies.

Preparation of plant extract for cell culture

10 mg/ml stock extract solutions were prepared for determination of *in vitro* cytotoxic effects of the extracts on cell lines. DMSO was added (not exceeding 1% of the highest present concentration of the extract) to dissolve the methanol extract weighed. The solvation process was continued by addition of small amounts of cell culture medium to the thawed extract and the stock solution was made up to the required volume.

Measuring cytotoxicity by Neutral Red assay

Neutral Red method is based on the difference in staining of living-healthy and damaged-dead cells (17). The medium applied on the cells was removed after 48 hours of incubation with the Substances subjected to test. Working solution was prepared from the stock solution of neutral red dye. 100 µl of 50 µg/ml working solution was added onto each well of the 96-well plate containing the cells at the end of the incubation period. Plates were left for incubation around 3-4 hours and 150 µl of acetic acid-ethanol mixture, the reading solution, was added to each well at the end of incubation. Absorbance at 540 nm wavelength was measured on ELISA reader, with 8 wells in each group. The experiment was run in 3 replications.

Detection of necrosis by LDH cytotoxicity assay

Measurement of necrosis was performed by determining the amount of lactate dehydrogenase (LDH) released into culture medium from membrane damaged or dead cells. LDH release was performed according to the protocol provided by the manufacturer of Cytotox-

icity Detection (LDH) kit (Roche). LDH "Cytotoxicity Detection Kit" catalyst and staining solutions were used in the experiments. 50 µL of catalyst and staining solutions mixture was added onto each well of the 96-well plates containing the media, without delay. The plates were left for incubation without exposure to light. Absorbance at 492/630 nm was measured by ELISA Plate Reader (Spectra max M5 Microplate Reader) at 30-60th minutes as recommended in the protocol. The mean absorbance values of each condition, applied six times, were calculated. The cytotoxicity value of each condition was then calculated.

Morphological examination of apoptosis by Acridine orange/Ethidium bromide fluorescence staining method

Determination of the apoptotic index was performed using Acridine Orange (AO), staining cell DNA; and Ethidium Bromide (EB), staining only late apoptotic or necrotic cells with impaired membrane integrity. 1x10^6 cells/ml of the cell suspension was transferred to glass petri dishes containing sterile slides. Determined concentrations of phenolic compound and cisplatin were added onto peripheral lymphocyte cells and incubated for 48 hours at 37 °C. Following incubation, the cells were washed with 1× phosphate buffered saline (PBS) and fixed in 70% ethanol for 5 minutes at room temperature. After excess ethanol was removed by distilled water, the cells were incubated at room temperature for 5 minutes with 200 µl of acridine orange (100 mg/ml) and ethidium bromide (100 mg/ml) mixture solution. Morphological changes indicating apoptosis in cells were examined under fluorescent microscope.

Detection of apoptosis by apoptotic DNA ladder method

For this experiment, a 24-well plate was seeded containing 2x10^6 cells, according to the Apoptotic DNA Ladder Kit (Roche) kit procedure. Apoptosis was determined according to Apoptotic DNA Ladder Kit (Roche) procedure.

Determination of apoptosis by ELISA

Apoptotic activities of 100 µg/ml and 200 µg/ml doses of plant extracts and 100 µg/ml of chemotherapeutic agents were performed according to the protocol provided by the manufacturer of Cell Death Detection ELISA Plus Kit (Roche Applied Science, Germany). The CDD (Cell Death Detection ELISA Plus) kit allows the evaluation of proportional apoptosis among samples by comparing the mono- and oligo-nucleosomal contents of cytoplasmic lysates of cells (18).

Determination of genotoxicity by Comet assay

This technique is based on the principle that the damaged DNA is released from the nucleus by electrophoresis. In this study, comet assay method developed by Singh *et al.* was performed with modifications (19). 1.0% normal melting point (NMP) agarose gel was prepared and 100 µl gel drops were placed onto frosted edge slides. The slides were covered with coverslips and left in the refrigerator (2-4 °C) for 5 minutes, afterwards the coverslips were removed. Prepared slides were kept in humidified storage boxes. 10 µl of mononuclear cells diluted to 10^4 cells/mm^3 in PBS was mixed with 80 µl of 0.7% low melting point (LMP) agarose gel (37 °C) and layered as the first layer on the slide and covered with coverslips and left in the refrigerator for 5 minutes to solidify. After the agarose gel is solidified, the slides were incubated in cold lysis buffer (100 mM EDTA, 2.5 M NaCl, 10 mM Trisma base) containing salt and detergents in high concentrations for approximately one hour; to allow cellular and nuclear lysis. 1% Triton X-100 and 10% DMSO were added into the lysis buffer right before working and the buffer was used after cooling. Slides were left to incubate for 30 minutes in alkaline electrophoresis buffer (1 mM EDTA, 300 mM NaOH and pH=13) to allow separation of the DNA strands prior to running on electrophoresis for 30 min at 300 mA, 17 volts electrical field and 5-25 ° C. After electrophoresis was completed, the slides were washed 3 times with neutralization buffer (0.4 M Tris-HCl, pH 7.5) for 3 min to remove the alkaline buffer solution from the medium. Staining procedure was performed following the neutralization process. Ethidium bromide (5 µg/ml), a fluorescent stain, was used for staining. 50 DNA images were evaluated under 20x magnification on fluorescence microscope (Nikon, Tokyo, Japan) (Excitation DB: 546 nm, Emission DB: 580 nm) after 80 µL of the staining solution was dropped onto each slide and covered with coverslips.

Results

Evaluation of antioxidant content and activities of plant extract

Total Phenolic and Total Flavonoid Compound Quantification

Total phenolic compounds of plant samples were calculated as mg gallic acid equivalents (µg GAE/mg extract) by using standard graphics. The result was 184.14 ± 2.5 µg GAE/mg. Total flavonoid amounts of the extract were expressed as µg/mL quercetin equivalent. The result was 12.63 ± 1.5 µg quercetin/mg.

ABTS Radical Scavenging Activity

The ABTS radical scavenging activities of plant extracts and standards are shown in Figure 1. It was observed that the LEP plant extract exhibited activity close to the butylated hydroxytoluene (BHT) standard when the ABTS radical scavenging activity of plant extract

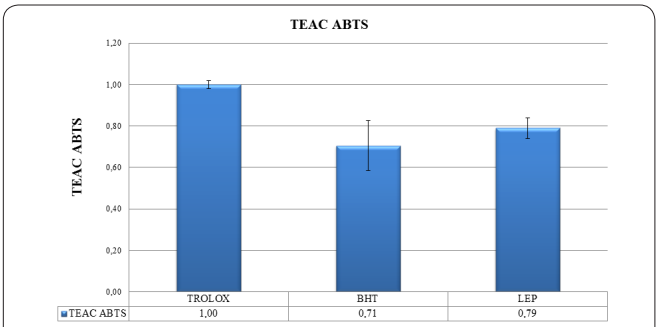

Figure 1. Antioxidant activities of plant extract by ABTS radical scavenging method. ABTS radical scavenging activities of plant extracts and standards are shown. ABTS: 2,2'-Azino-bis (3-ethylbenzenothiazoline-6-sulfonic acid); LEP: Lepidium sativum subsp spinescens L.; BHT: butylated hydroxytoluene.

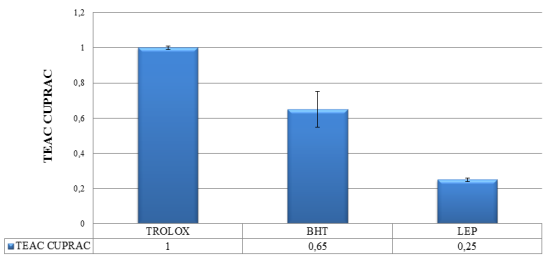

Figure 2. Antioxidant capacities of plant extract evaluated by CUPRAC method. The standards had higher antioxidant activity than the extract. CUPRAC: Copper (II) Ion Reducing Antioxidant Capacity; LEP: Lepidium sativum subsp spinescens L.; BHT: butylated hydroxytoluene.

was compared with the standards.

Examination of antioxidant capacities of plant extract by CUPRAC method

It was observed that the standards had higher antioxidant activity than the extracts according to the CUPRAC analyses performed in the study (Figure 2).

Cytotoxic effects of plant extract on DLD-1, ECC-1 and PML cell lines

MTT assay results

Anti-proliferation effects of methanol extract of the over-ground parts LEP and two chemotherapeutic agents (cisplatin (CIS), 5-fluorouracil (5-FU)) on two tumor cell lines (DLD-1 and ECC-1) and one normal cell line (PML) are presented in Figures 3, 4 and 5, respectively. IC_{50} (µg/ml) values of plant extracts and chemotherapeutic agents (5-fluorouracil, cisplatin) on cells were as follows; 110.42; 48.327; 22.8 for DLD-1 cells; 353.58; 82.02; 12.55, for ECC-1 cells, and 1462.2; 30.87; 19.06 for PML cells, respectively. It was observed that IC_{50} values of LEP plant extract and chemotherapeutic agents were ordered as CIS> 5-FU> LEP indicating cytotoxic effects on DLD-1 cells. The cytotoxic effects of LEP plant extract on ECC-1 cells were observed to be CIS> 5-FU> LEP. Plant extracts used in the study showed cytotoxic effects when IC_{50} values on PML cells were examined.

Neutral Red assay results

According to the results of lysosomal activation by neutral red assay, plant extract was shown to have concentration-dependent cytotoxic effects on DLD-1 cells. It was determined that the highest cytotoxic effect was

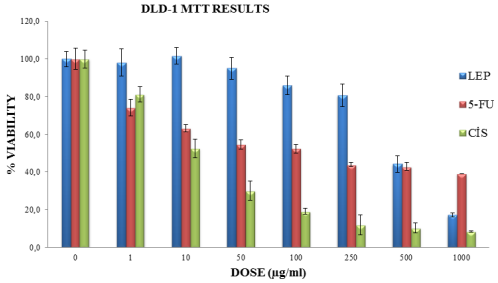

Figure 3. Assessment of cytotoxic effect of plant extract on DLD-1 cell line evaluated by mitochondrial activity (% Mean ± SD). DLD-1: the human colon cancer cell line; LEP: Lepidium sativum subs spinescens L.; Cis: Cisplatin; 5-FU: 5-Fluorouracil.

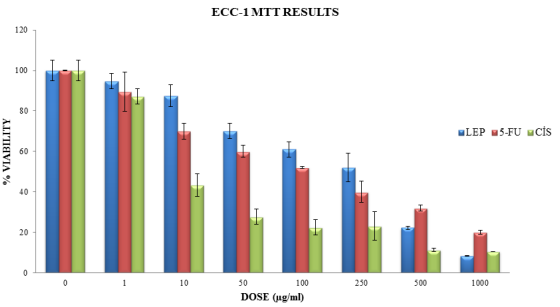

Figure 4. Assessment of cytotoxic effect of plant extract on ECC-1 cell line evaluated by mitochondrial activity (% Mean ± SD). ECC1: endometrium cancer cell line; LEP: Lepidium sativum subs spinescens L.; Cis: Cisplatin; 5-FU: 5-Fluorouracil.

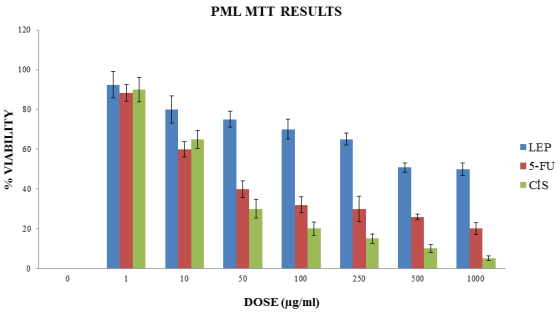

Figure 5. Assessment of cytotoxic effect of plant extract on PML cell line evaluated by mitochondrial activity (% Mean ± SD). PML: Peripheral lymphocyte cells; LEP: Lepidium sativum subs spinescens L.; Cis: Cisplatin; 5-FU: 5-Fluorouracil.

at concentrations of 500 µg/ml and 1000 µg/ml. It was observed that the cytotoxic effect of the highest concentration of both chemotherapeutic Subsptances and the plant extract was close to each other (Figure 6).

It was determined that the cytotoxic effect of plant extract on ECC-1 cells was increased in a dose-dependent manner. The highest cytotoxic effect was observed with 500 µg/ml dosage. Chemotherapeutic agents were found to have a higher cytotoxic effect than plant extracts on this cell line (Figure 7).

The cytotoxic effect of plant extract on peripheral lymphocyte cells (PML) was observed to be very low compared to chemotherapeutic agents. LEP plant extract was found to have cytotoxic effects at very high doses (500-1000 µg/ml) (Figure 8).

Examination of necrotic effects of plant extract

It was observed that plant extract caused necrotic effects at very high doses (1000 µg/ml) on cancer cells,

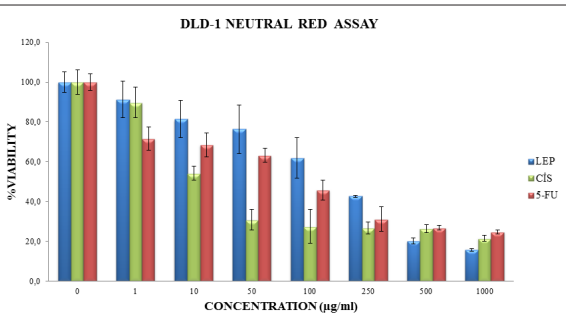

Figure 6. Evaluation of the cytotoxic effect of plant extract on DLD-1 cell line evaluated by lysosomal activity (% Mean ± SD). DLD-1: colon cancer cell line; LEP: Lepidium sativum subs spinescens L.; Cis: Cisplatin; 5-FU: 5-Fluorouracil.

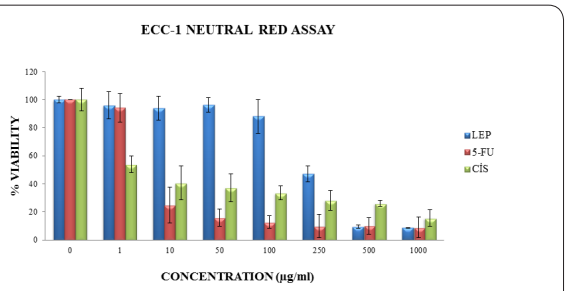

Figure 7. Assessment of cytotoxic effect of plant extract on ECC-1 cell line evaluated by lysosomal activity (% Mean ± SD). The highest cytotoxic effect was observed with 500µg/ml dosage. In this cell line, chemotherapeutic agents were found to have a higher cytotoxic effect than plant extracts. ECC1: endometrium cancer cell line; LEP: Lepidium sativum subs spinescens L.; Cis: Cisplatin; 5-FU: 5-Fluorouracil.

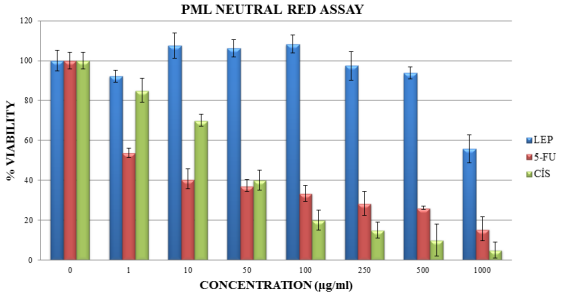

Figure 8. Assessment of cytotoxic effect of plant extract on PML cell line evaluated by lysosomal activity (% Mean ± SD). Cytotoxic effect of the plant extract on peripheral lymphocyte cells (PML) was observed to be very low compared to chemotherapeutic agents. LEP plant extract was found to have cytotoxic effects at very high doses (500-1000µg/ml). PML: Peripheral lymphocyte cells; LEP: Lepidium sativum subs spinescens L.; Cis: Cisplatin; 5-FU: 5-Fluorouracil.

whereas chemotherapeutic agents caused necrotic effects at relatively lower doses (Figures 9 and 10).

Determination of apoptotic effects of plant extract on cancer cells by ELISA

According to the results obtained in this study, plant extract shows apoptotic effects on ECC-1 cells. It was observed that the apoptosis rate was increased with increased doses of the plant extract, while the chemotherapeutic agents had a higher apoptotic effect than the plant extract (Figure 11).

The apoptotic effect of plant extract on DLD-1 cells

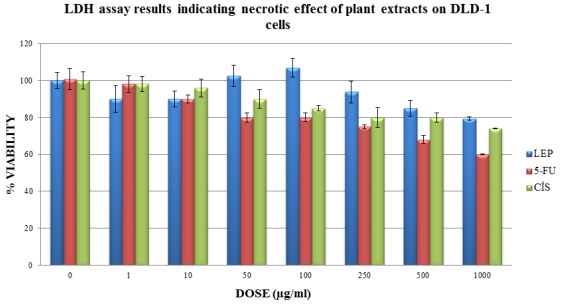

Figure 9. Assessment of necrotic effect of plant extract on DLD-1 cell line by LDH method (% Mean ± SD). It was observed that plant extract caused necrotic effect at very high doses (1000µg/ml) on cancer cells. LDH assay: lactate dehydrogenase assay; DLD-1: colon cancer cell line; LEP: Lepidium sativum subs spinescens L.; Cis: Cisplatin; 5-FU: 5-Fluorouracil.

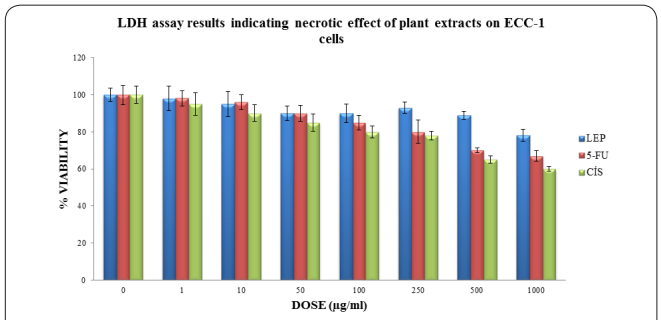

Figure 10. Evaluation of LDH assay indicating the necrotic effect of plant extract on ECC-1 cell line (% Mean ± SD). Chemotherapeutic agents caused necrotic effect at relatively lower doses. LDH: lactate dehydrogenase; ECC1: endometrium cancer cell line; LEP: Lepidium sativum subs spinescens L.; Cis: Cisplatin; 5-FU: 5-Fluorouracil.

was observed to be higher compared to the effects on ECC-1 cells. It was seen that the increased doses of the plant extract caused higher apoptotic effect as observed in ECC-1 cells (Figure 12).

Determination of apoptotic effects of plant extract morphologically by AO/EB fluorescence staining method

Live cells were observed as homogeneously green-stained chromatin; necrotic cells as homogeneously orange-stained chromatin; early apoptotic cells as condensed and fragmented green stained chromatin; and late apoptotic cells as condensed and fragmented orange

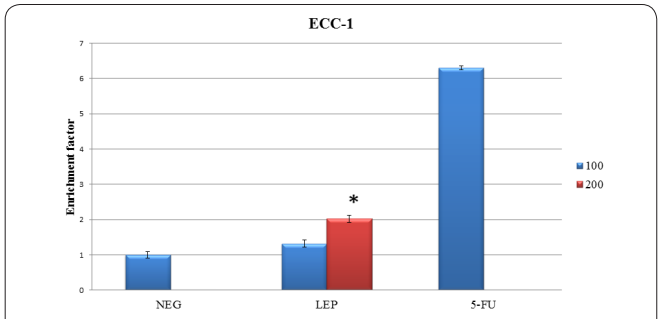

Figure 11. Evaluation of apoptotic effect of plant extract on ECC-1 cell line by ELISA (Mean ± SD). It was observed that the apoptosis rate was increased with increasing doses of the plant extract and it was found that the chemotherapeutic agents had a higher apoptotic effect than the plant extract. ECC1: endometrium cancer cell line; LEP: Lepidium sativum subs spinescens L.; Cis: Cisplatin; 5-FU: 5-Fluorouracil.

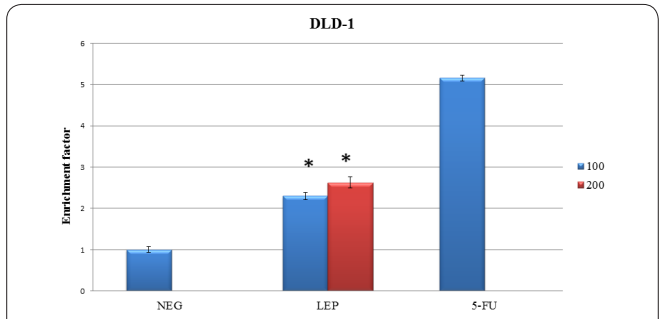

Figure 12. Evaluation of Apoptotic effect of plant extract on DLD-1 cell line by ELISA (% Mean ± SD). It was seen that the dose increase of plant extract resulted in higher apoptotic effect on DLD-1 cells, as seen in ECC-1 cells. DLD-1: colon cancer cell line; LEP: Lepidium sativum subs spinescens L.; Cis: Cisplatin; 5-FU: 5-Fluorouracil.

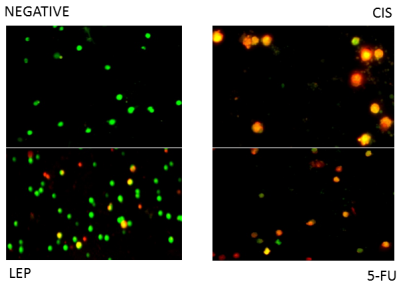

Figure 13. Fluorescence microscope images showing apoptotic effect of plant extract on ECC-1 cell line. The apoptotic effect of the plant extract on the cell line was visualized by fluorescence microscopy ECC1: endometrium cancer cell line; Negative: control; LEP: Lepidium sativum subs spinescens L.; Cis: Cisplatin; 5-FU: 5-Fluorouracil.

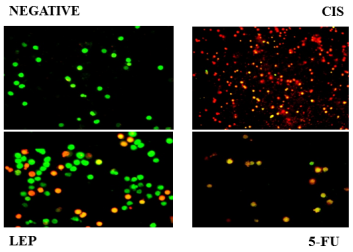

Figure 14. Fluorescence microscope images showing apoptotic effect of plant extract on DLD-1 cell line. The apoptotic effect of the plant extract on the cell line was visualized by fluorescence microscopy DLD-1: colon cancer cell line; Negative: control; LEP: Lepidium sativum subs spinescens L.; Cis: Cisplatin; 5-FU: 5-Fluorouracil.

stained chromatin (Figure 13 and 14). The apoptotic effect of the plant extract on the cell line was visualized by fluorescence microscopy (Figure 15 and 16).

It was observed that 200 µg/ml of plant extract destroy DLD-1 cells at a higher level. It was also observed that the plant extract showed lower necrotic effects on DLD-1 colon cancer cells compared to chemotherapeutic agents (Cisplatin, 5-Fluorouracil) and it kills DLD-1 colon cancer cells with apoptotic effect rather than necrotic effect. Plant extract was observed to exhibit higher level of apoptotic effect on ECC-1 cells rather than the necrotic effect.

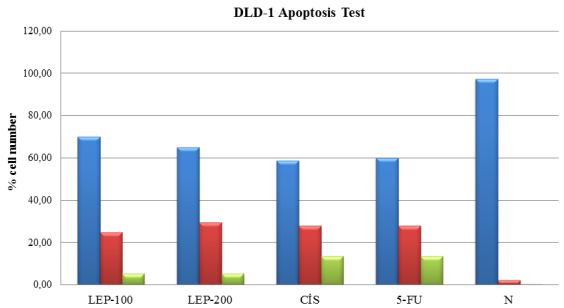

Figure 15. Morphological evaluation of apoptotic effect of plant extract on DLD-1 cell line (% Mean ± SD). It was observed that the plant extract killed DLD-1 colon cancer cells with apoptotic effect while the plant extract had a lower necrotic effect on the DLD-1 cells than the chemotherapeutic agent (Cis, 5-Fu) groups. DLD-1: colon cancer cell line; blue bar: living cells; red bar: apoptotic cells; green bar: necrotic cell; LEP: Lepidium sativum subs spinescens L.; Cis: Cisplatin; 5-FU: 5-Fluorouracil; N: Necrotic.

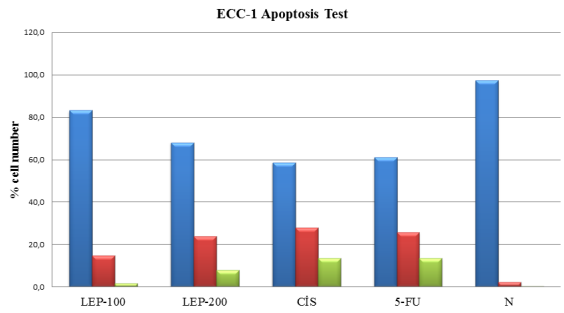

Figure 16. Morphological evaluation of plant apoptotic effect on ECC-1 cell line (% Mean ± SD). It was observed that plant extract showed apoptotic effect at a higher level than the necrotic effect of on ECC-1 cells. ECC1: endometrium cancer cell line; LEP: Lepidium sativum subs spinescens L.; blue bar: living cells; red bar: apoptotic cells; green bar: necrotic cell; Cis: Cisplatin; 5-FU: 5-Fluorouracil; N: Necrotic.

Evaluation of apoptotic effects of plant extract on cancer cells by DNA ladder agarose gel electrophoresis

Apoptotic DNA fragments were shown to appear in ECC-1 cells treated with plant extract and chemotherapeutic agent (Figure 17). Since only apoptotic DNA fragments were isolated in this method, no bands were observed in negative control group. Apoptotic band density observed with 200 µg/ml of plant extracts is higher than apoptotic band density of 100 µg/ml groups, indicating that apoptosis is more prominent. It was observed that the highest band density was in the group applied 200 µg/ml cisplatin.

Apoptosis was shown to occur in all groups of plant extract-treated DLD-1 cells (Figure 18). When the agarose gel image was examined, it was observed that apoptosis was increased with increasing doses in DLD-1 cells as well as in all ECC-1 cells. The DNA ladder agarose gel imaging technique yields a qualitative result of apoptosis occurring in cells. Quantitative analysis of apoptosis was determined by ELISA. There was a positive correlation between ELISA and agarose gel images.

Determination of genotoxic effects of plant extract on cancer cells by Comet assay method

It was shown that plant extract caused DNA damage to ECC-1 cells at different rates (Figure 19). It was ob-

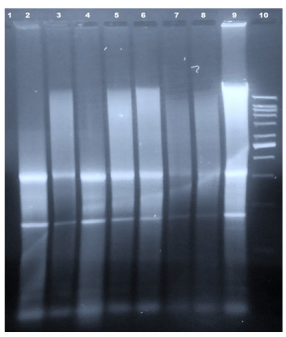

Figure 17. Agarose gel image of ECC1 cells treated with plant extracts: 1st band: Negative, 2nd band: LEP-200µg / ml, 3rd band: LEP-100µg / ml, 8th band: 5-FU, 9th Band: CIS, 10th band: Marker. Apoptotic DNA fragments appeared in ECC-1 cells treated with plant extract and chemotherapeutic agents. ECC1: endometrium cancer cell line; LEP: Lepidium sativum subs spinescens L.; Cis: Cisplatin; 5-FU: 5-Fluorouracil.

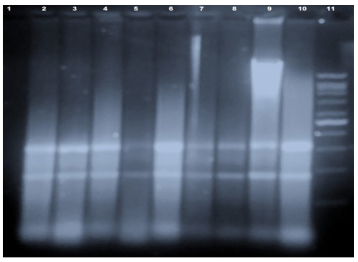

Figure 18. Agarose gel image of plant extract-treated DLD-1 cells: 1st band: Negative, 2nd band: LEP-200µg / ml, 3rd band: LEP-100µg / ml, 8th band: 5-FU, 9th Band: CIS, 10th band: Marker. Apoptosis was shown to occur in all groups of plant extract-treated DLD-1 cells. DLD-1: colon cancer cell line; LEP: Lepidium sativum subs spinescens L.; Cis: Cisplatin; 5-FU: 5-Fluorouracil.

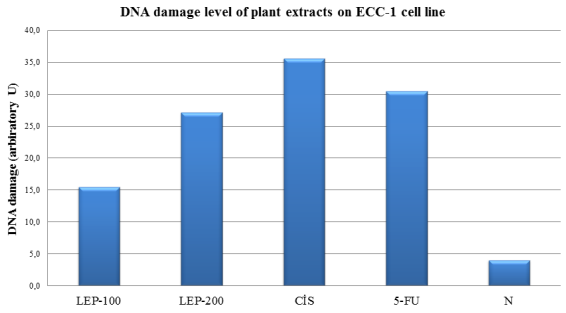

Figure 19. Comet assay results indicating DNA damage effect of plant extract on ECC-1 cell line. All bars represent DNA damage. It was shown that plant extract caused DNA damage to ECC-1 cells at different rates. ECC1: endometrium cancer cell line; LEP: Lepidium sativum subs spinescens L.; Cis: Cisplatin; 5-FU: 5-Fluorouracil; N: Negative.

served that the DNA damage caused by the plant extract in the ECC-1 cells was increased in a dose-dependent manner. The damage caused by LEP-200 plant extract on ECC-1 cells was close to the 5-FU group. The level of DNA damage on the DLD-1 cells of the plant extract was found to be increased with increasing doses (Figure 20).

Discussion

Oxidative stress is being studied increasingly to determine cellular damage due to geriatric and various morbidity factors (3). Free radicals and ROS have important effects on biological cell damage and gene expression (20). Identification of plant-derived antioxidants that protect cells from free radicals and ROS effects is essential for preventing and treating cellular oxidation (21, 22). Several restrictions and prohibitions have been applied to the use of synthetic antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) as a result of the studies showing that they constitute toxic and carcinogenic effects. Thus, the interest in natural antioxidant sources has increased (23).

Plant-derived antioxidants function as free radical scavengers, peroxide disintegrators, enzyme inhibitors and synergists (2, 24). Phenolic compounds exhibit a wide range of physiological properties such as anti-allergenic, anti-atherogenic, anti-inflammatory, anti-microbial, anti-thrombotic, vasodilator and cardioprotective. These compounds are also classified as antioxidants due to free radical scavenging effects (25).

This study investigates the antioxidant and anticancer properties of methanol extract of LEP plant. In the first stage of the study, total phenolic and flavonoid contents of methanol extract of the plant were determined. In addition, antioxidant activities of plant extracts were examined by CUPRAC and ABTS methods.

According to the results obtained from the study, it was determined that the amount of phenolic compounds in LEP extract was high and it was also found that the flavonoid content increased with increasing concentrations. It was observed that the study was consistent with the study by Muhammad *et al.* (26). It was observed that ABTS activity was very high and CUPRAC method had less activity than standards. As a result, it was concluded that the extract exhibited antioxidant activity in all assays performed in this study and it could be a natural antioxidant source.

Today, the fight against cancer has not achieved much success. In addition to chemotherapy and radiotherapy, patients also prefer phytotherapy. Chemopreventive agents that are found in the nature with anticancer properties do not exhibit toxic properties, their action mechanisms are known partly and they are easy to obtain; and for all these reasons, can be used as food supplements (27). It was also shown in various studies that phenolic compounds found in plants exhibit anticancer properties (2, 28, 29).

The second stage of the study constituted Human DLD-1 colon and ECC-1 endometrium cancer cells for evaluation of cytotoxic and apoptotic effects of methanol extract of LEP plant, in order to show its therapeutic potential. The results showed that plant extract was cytotoxic and apoptotic, in a concentration-dependent manner, on DLD-1 and ECC-1 cancer cells. Mitochondrial and lysosomal activation absorbance values decreased significantly, especially at extract concentrations of 100, 200 and 300µg/ml. The highest increase in apoptotic and genotoxic effects was determined at 48th hour at 200µg/ml plant extract concentration.

Laith *et al.* examined the cytotoxic, genotoxic and apoptotic effects of LEP extract on the tongue cancer cell line CAL-27. The study showed that LEP extract had cytotoxic effects at 150µg/ml concentrations on CAL-27 cells, increased intracellular free radicals and caused DNA damage (30). Sawsan *et al.* studied the aqueous extract of LEP seeds for their cytotoxic and apoptotic effects on MCF-7 cancer cells and normal HFS cells;

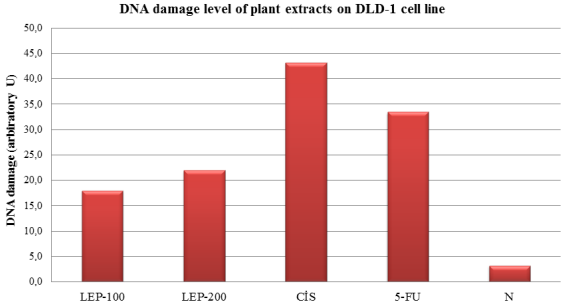

Figure 20. Comet assay results of the plant extract indicating DNA damage level on DLD-1 cell line. All bars represent DNA damage. The level of DNA damage of the plant extract on DLD-1 cells was found to increase with increasing doses. DLD-1: colon cancer cell line; LEP: Lepidium sativum subs spinescens L.; Cis: Cisplatin; 5-FU: 5-Fluorouracil; N: Negative.

the cytotoxic effect of MCF-7 cells was higher than normal cells with application of 50% extract concentration, and this cytotoxic effect was observed to be caused by apoptotic effect. At 75% concentration, the cytotoxic effect was detected with necrosis (31).

In our study, it was determined that the LEP methanol extract had an IC_{50} of 100µg/ml on the colon cancer (DLD-1) cells, an IC_{50} of 353µg/ml on endometrium cancer cells (ECC-1) and an IC_{50} of 1462µg/ml on peripheral lymphocyte cells. In the light of this data, it was observed that LEP extract showed cytotoxic effect mostly on cancer cells while exhibiting cytotoxic effects on normal cells at very low levels. The cytotoxic effect was observed as a result of apoptotic pathway activation as seen with ELISA and DNA fragmentation assays and morphological examination.

Our results show that *Lepidium sativum Subspspinescens L* plant extract inhibits cell proliferation by acting with cytotoxic and apoptotic effects, in a concentration-dependent manner, on DLD-1 and ECC-1 cell lines.

For further evaluation of the plant, extraction of different fractions of the plant, examination of antioxidant and anticancer properties of the obtained extracts and selection of the fraction exhibiting the highest activity and content analyses could be performed. In addition, further molecular studies are required to support presented findings, such as determination of the apoptotic pathways causing the observed effects by the fraction showing the highest cytotoxicity, and to explain the chemopreventive and chemotherapeutic effects of the plant extract. Subspequent studies investigating the active Substance found in the extract and its biological effects are suggested.

Disclosure

There is no any conflict of interest.

Author's contribution

Hypothesis of work is planned by Sahabettin Selek and Ismail Koyuncu. Experiments were set up by Enes Akyuz, Hifa Gulru Caglar, Ibrahim Bektas, Mustafa Abdullah Yilmaz and Ataman Gonel. Results are collected by Hifa Gulru Caglar and Enes Akyuz. The paper is written by Sahabettin Selek.

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