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Determination of anticancer effects of *Urospermum picroides* against human cancer cell lines

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Abstract: Plants continue to be a good source for developing effective anticancer agents. In this study, *in vitro* various biological effects of crude ethanolic extract from flowering parts of *Urospermum picroides* collected from the Muğla province of Turkey were investigated for the first time. Daudi, A549 and HeLa cancer cell lines and BEAS-2B normal cell line were used to identify the cytotoxic effect of the extract using MTT assay. The effect of the extract on cell cycle progression was detected by flow cytometric analysis. The level of VEGF, IL-1α, IL-6 and TNF-α secretion in the cells treated with the extract were measured using ELISA The extract caused a higher cytotoxic effect on Daudi cells with an IC_{50} value of 85.64 µg/mL than the other cells tested. The IC_{50} values in HeLa and A549 cells were determined to be 135.35 and 234.8 µg/ mL, respectively. The selective cytotoxicity was considered between Daudi and BEAS-2B (109.80 µg/mL) cell lines. In addition, the effect of the extract on cell cycle progression changes according to cell line used. Moreover, the extract decreased the level of secreted VEGF in treated A549 cells by 31%. In addition, the extract resulted in a significant decrease in the secretion of IL-1α, IL-6 and TNF-α cytokines in A549 and Daudi cells compared to the untreated cells. These findings suggest that the flowering parts of *U. picroides* may be a potential source for anticancer agents.

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1. INTRODUCTION

Cancer is an important health problem around the globe and significantly contributes to human deaths. The annual number of new cancer cases are increasing estimated to be 21.6 million by 2030 [1].

Because the drugs used for the treatment of cancer can cause serious side effects [2], it is important to develop new-selective anticancer agents. Plants have been used for treating various diseases throughout the ages and they continue to be a critical source of potent anticancer agents

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due to their contents [3]. Some of the plant derived compounds such as vincristine, vinblastine and paclitaxel are still used as chemotherapeutic agents in clinical cancer treatment [4]. Therefore, the anticancer potentials of the plants are being investigated all over the world to obtain new agents.

Urospermum picroides (L.) SCOP. ex. F. W. Schmidt is a species of the Asteraceae family [5] and also known as prickly golden fleece [6]. It belongs to the traditional Mediterranean diet and has anti-inflammatory activity [7]. The consumption of this plant was reported to reduce postprandial platelet aggregation in metabolic syndrome patients [8]. Previous studies demonstrated the antioxidant and antimicrobial activity [9,10] of this species as well as its phytochemical content [11,12] with potential biological properties. However, to our knowledge, there is no detailed study in the literature about the different biological activities of *U. picroides*. So, for the first time we aimed to determine the various biological effects of ethanolic extract from flowering parts of *U. picroides* on different cancer cell lines.

2. MATERIALS AND METHODS

2.1. Collection and extraction of plant material

U. picroides were harvested from Muğla province, Turkey, in the between June and July 2014. The plant species was identified by Dr. Fatma Güneş at the Department of Pharmaceutical Botany, Trakya University, Edirne. The voucher specimen was kept in their herbarium.

The fresh flowering parts of plant were firstly washed with the deionized water and air dried at room temperature under shade for about 15 days. The dried flowers were powdered and each 10 grams powder were extracted with 100 mL absolute ethanol (Merck, USA) using soxhlet apparatus for 10 h. The extract was then filtered through Whatman filter paper no: 1 and solvent was separated from the extract using a rotary evaporator (IKA, RV 10, USA). The ethanolic crude extract of *U. picroides* was stored at -20^{o}C in polyethylene tubes protected from light-until needed.

2.2. Cell culture conditions

A549 (lung adenocarcinoma), HeLa (cervix adenocarcinoma), Daudi (Burkitt's lymphoma) and BEAS-2b (normal bronchial epithelium) human cell lines were obtained from ATCC. The cell lines were grown in RPMI 1640 (Biochrom, Germany) medium with stable L-glutamine (Biochrom, Germany) contained 10% heat inactivated fetal bovine serum (FBS) (Biochrom, Germany) supplemented with 100 units/mL penicillin and 100 mg/mL streptomycin (Biochrom, Germany). All cell cultures were incubated at 37^{o}C in a humidified atmosphere with 5% CO_2 and 95% air.

2.3. Cytotoxic assay

MTT [3 (4, 5 Dimethyl-2-thiazolyl)-2, 5 diphenyl-2H-tetrazolium bromide)] (Applichem, USA) assay [13] was used for determination of cytotoxicity of the extract on cell lines used in present study. The assay is based on the reduction of yellow tetrazolium salt MTT to purple formazan crystal by mitochondrial dehydrogenase in the viable cells [14]. Briefly, the exponentially growing cells were seeded at a density of $4x10^3$ cells per well in 180 µL of growth medium into 96-well plates (Greiner, Germany) as triplicate for each tested extract concentration. After 24 h of incubation, the cells were treated with the extract at different final concentrations (15.625 to 1000 µg/mL) for 72 h. The stock solution of the extract was prepared in 10% DMSO (Applichem, USA) in growth medium and diluted with growth medium to obtain serial dilutions. So, the cells were exposed to DMSO at less than 0.1% of the final concentration. The untreated cells were used as a control. Later, the medium was removed and 100 µL of fresh growth medium was added into each well. Then, the cells in each well were incubated with 10 µL of 5 mg/mL MTT in phosphate-buffered saline (PBS) for 4 h at 37^{o}C. At the end of 4 h, the

medium with MTT was gently discarded from the wells and formazan crystals formed in the cells were solubilized with 100 µL of DMSO by shaking at 150 rpm for 5 min. The absorbance (Abs) measurement of reduced MTT in each well was made at 540 nm using a microplate reader (ThermoScientific, Multiscan FC, USA). The following formula was used to calculate the percentage of cell viability for each concentration.

Cell viability %= (Mean Abs of treated cells/Mean Abs of untreated cells) x 100

2.4. Cell cycle analysis

Cell cycle detection in A549 and HeLa cells was performed by propidium iodide (PI) (Sigma-Aldrich, USA) staining using flow cytometry. Firstly, the cells were seeded at $5x10^5$ cells/well in 6-well plates and incubated for 24 h. Then, the cells were exposed to 500 and 200 µg/mL extract for 24 h. DMSO at 1% final concentration was used as a control. After treatment, cells were harvested by trypsinization, washed with cold PBS twice, fixed gently in absolute ethanol and stored at -20°C for 48 h. After centrifugation at 1200 rpm for 10 min at 4°C, cell pellets were washed with cold PBS and resuspended in 1 mL 0.1% (v/v) Triton X-100 (Amresco, USA) in PBS. Then, cell suspensions were treated with 100 µL of RNase A (200 µg/mL) (Applichem, USA) and incubated at 37°C for 30 min. Finally, 100 µL of PI (1 mg/mL) was added to each cell suspensions and cells were incubated for additional 15 min at room temperature in the dark. After staining, cells were analysed for cell cycle phases by BD FACSCanto flow cytometry (BD Biosciences, San Jose, CA) using ModFit LT 3.0 software.

2.5. Enzyme-linked immune sorbent assay (ELISA)

The supernatants of the cell cultures were used for quantification of Vascular endothelial growth factor (VEGF) in A549 and for quantification of cytokines (IL-1α, IL-6 and TNF-α) in both A549 and Daudi by using commercial human ELISA kits (Boster Biological Technology, USA). To obtain supernatants, cells were seeded in a 6 well-plate at a density of $2x10^5$ cells/well. After 1 h incubation at 37°C in CO_2 incubator, cells were treated with extract at 200 µg/mL for 6 h. The untreated cells were used as a control. Later, supernatants were collected from treated and untreated cells and centrifuged at 14000 rpm for 30 seconds. The obtained supernatants were aliquoted and stored at -20°C until use. A 100 µL of supernatant was analysed for VEGF or inflammatory each cytokine production via ELISA kits according to the manufacturer instructions. The absorbance in each well was read at a wavelength of 450 nm using a microplate reader. The level of secreted VEGF or each cytokine was interpolated from the standard curve prepared separately for each assay.

2.6. Statistical analysis

The results obtained from in this study were expressed as mean ± standard error (SE). Statistical analysis and data processing were performed by using GraphPad Prism 7.0 (GraphPad Software, Inc., San Diego, CA).

3. RESULTS

3.1. Cytotoxic effect of the extract on different cell lines

The effect of the extract on cell viability was investigated on A549, HeLa, Daudi and BEAS-2B cell lines for 72 h by MTT assay. The extract was prepared at seven serial concentrations from 1000 to 15.625 µg/mL and tested against the cell lines used. The IC_{50} values (µg/mL) that causes 50% cell death were then calculated for each cell lines. As shown in Figure 1, cell viability, particularly in cancer cell lines was significantly inhibited by the extract in a concentration-dependent manner. However, the results showed that the extract at 15.625-62.5 µg/mL concentrations were less cytotoxic on normal BEAS-2B cells when compared to cancer cells tested. The extract exhibited the higher cytotoxicity against Daudi

cells with an IC_{50} value of 85. 64 µg/mL when compared to normal BEAS-2B cells (IC_{50} value= 109.8 µg/mL) and other cancer cells. The calculated IC_{50} values were 234.8 and 135.35 µg/mL for A549 and HeLa, respectively. So, Daudi cell lines were found to be the most sensitive line to the extract.

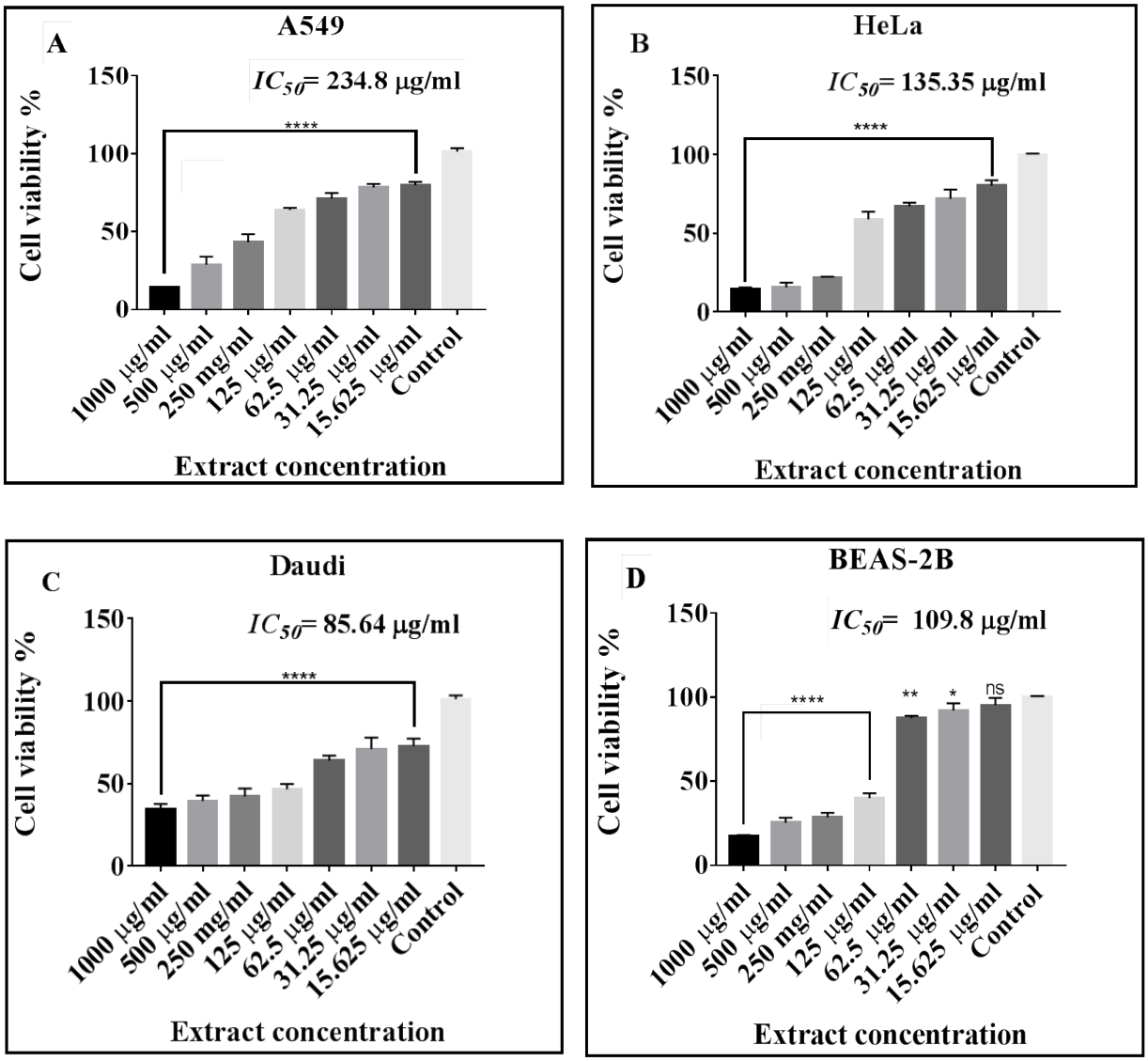
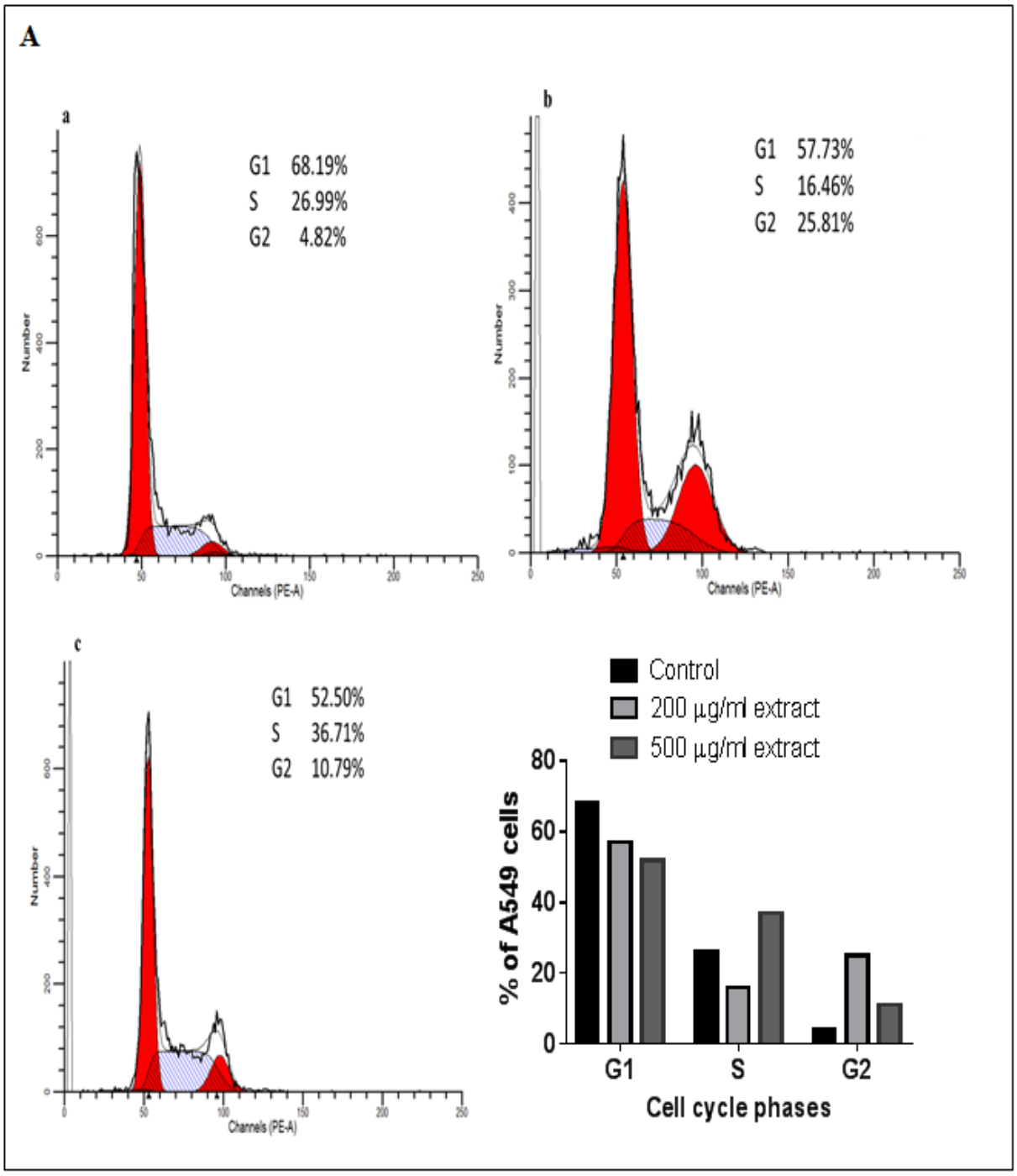


Figure 1. Cytotoxic effects of ethanol extract of flowering parts of *U. picroides* against A549 (A), HeLa (B), Daudi (C) and BEAS-2B (D) cell lines after an exposure time of 72 h. Cell viability was determined using MTT assay. The data represent mean ± SE of three independent experiments for each concentration. ****P< 0.0001, **P< 0.01, *P< 0.05 and ns: non-significant (P> 0.05) compared to control (ANOVA and Tukey's multiple comparison test).

3.2. Effect of the extract on cell cycle progression in A549 and HeLa cell lines

The flow cytometry analysis was performed to investigate cell cycle progression in A549 and HeLa cancer cells after treatment with extracts at 500 and 200 µg/mL for 24 h (Figure 2). The treatment with 500 µg/mL extract showed 5.97% and 9.72% increases of A549 cells in G2 and S phase compared to control, respectively and there was a concomitant decrease in the percentage of A549 cells in the G1 phase. The percentage of A549 cells in G2 phase after treatment with extract at 200 µg/mL significantly increased from 4.82 to 25.81 and it was accompanied by a decrease in the percentage of cells in G1 and S phase. In addition, the percentage of HeLa cells in G1 phase increased from 48.97 to 59.00 after treatment with the

extract at 500 µg/mL and there was a sharp decrease in the percentage of G2 and S phase. On the other hand, the treatment of HeLa cells with 200 µg/mL extract resulted in 4.19% and 5.06% increase of cells in S and G2 phase, respectively. These data demonstrated that the plant extract affect the cell cycle progression in the different phases according to the type of cell line tested.



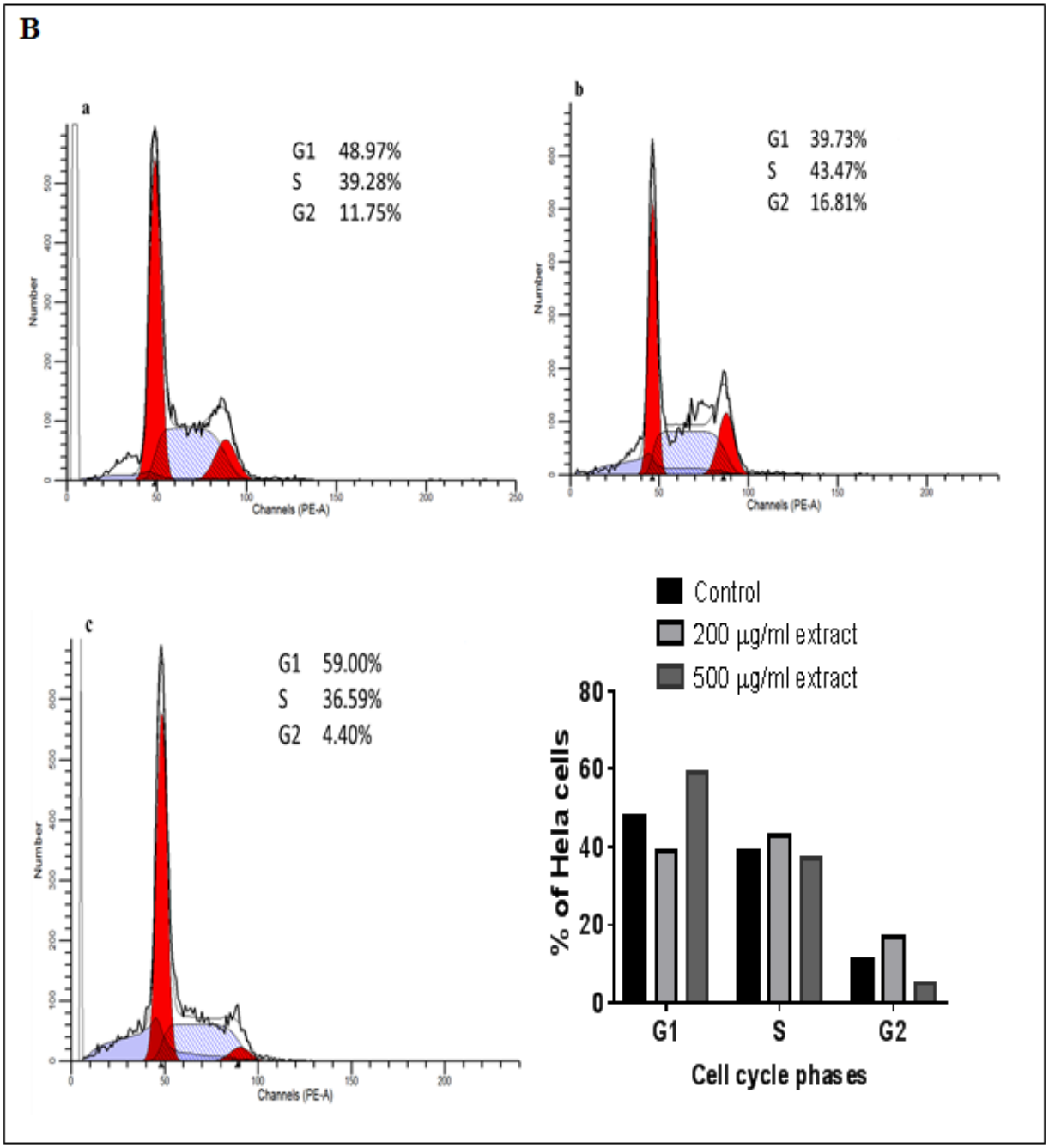


Figure 2. Effects of the extract on cell cycle distribution in A549 and HeLa cells. A549 (**A**) and HeLa (**B**) cells treated with 1% DMSO as control (a), 200 (b) and 500 (c) µg/mL extract for 24 h. Bar graph with percentages of cells at different cell cycle phases are shown (d).

3.3 Effect of the extract on VEGF secretion

It was reported that A549 cells released VEGF [15]. In this study, we investigated the VEGF levels in supernatants of A549 cell culture using human VEGF ELISA assay. Our findings showed that VEGF secretion was reduced by 31% in A549 cells treated with 200 µg/mL extract compared to untreated cells (Figure 3). This result indicate that the extract has antiangiogenic potential against A549 cells because the VEGF is an angiogenic factor [16].

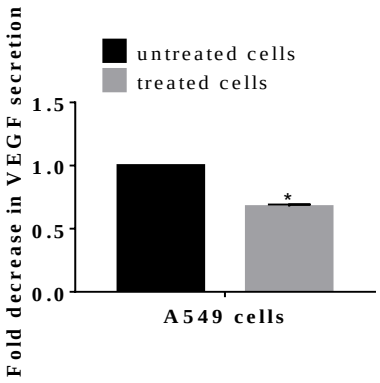


Figure 3. Effects of the extract on VEGF secretion of A549 cells. The VEGF concentration in supernatants of A549 cells treated with the extract at 200 µg/mL for 6 h was determined by ELISA. The values represent the mean of three independent experiments ± SE. The concentration of VEGF in control (untreated) cells is taken as 1-fold. The asterisks indicate statistical significance when compared to untreated cells. **P*< 0.05

3.4 Effect of the extract on secretion of IL-1α, IL-6 and TNF-α cytokines

In this study, we also investigated the effect of the plant extract at 200 µg/mL on IL-1α, IL-6 and TNF-α secretion in A549 and Daudi cells by using ELISA. The results were graphically shown in Figure 4. The extract did not cause any increase in concentrations of IL-1α, IL-6 and TNF-α cytokines in treated cells compared to untreated cells. The highest inhibition effect of the extract was found in A549 for IL-6 secretion (12%) and Daudi for IL-1α secretion (15%). As a result, the plant extract does not induce secretion of cytokine that can promote cancer development and progression.

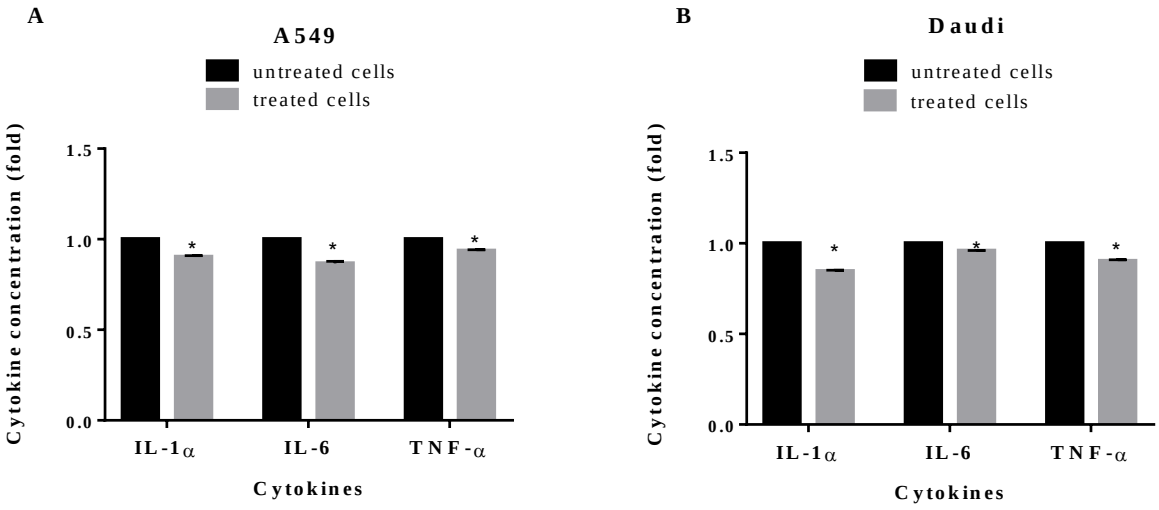


Figure 4. Effects of the extract on IL-1α, IL-6 and TNF-α secretion of A549 (A) and Daudi (B) cells. The cytokines concentration in supernatants of the cancer cells treated with the extract at 200 µg/mL for 6 h was determined by ELISA. The values represent the mean of three independent experiments ± SE. Results presented as fold of change in relation to the control (untreated) cells. The asterisks indicate statistical significance when compared to untreated cells. **P*< 0.05.

4. DISCUSSION and CONCLUSION

Cancer is one of the most important diseases that can be fatal worldwide [2]. Because cancer cells can develop drug resistance in traditional therapies [17], the discovery of a new drug with anti-cancer mechanism is of great importance. For centuries, natural products especially plant-derived compounds have an important role in the development of drugs used in the treatment of cancer [18].

It was reported that *U. picroides* was commonly used in the traditional Mediterranean diet [7] and sold in the Dalmatia markets [6]. Interestingly, a significant information was not found that it was a common edible plant in the world.

There are various research studies on the chemical composition of *U. picroides* in the literature [11,12,19,20]. Also, the anti-inflammatory [7], antioxidant and antimicrobial activity [9,10] of *U. picroides* were reported. In addition, Fragpouli et al. [8] stated that the consumption of *U. picroides* meals significantly reduced the *ex vivo* platelet activating factor-induced platelet aggregation postprandially. However, to our knowledge, the cytotoxic and anti-inflammatory and antiangiogenic activity of ethanolic extract of flowering parts of *U. picroides* collected from Turkey were investigated for the first time on different cancer cell lines.

We first aimed to assess the cytotoxic activity of the extract on different cancer cell lines after treatment for 72 h. The extract was found to exhibit cytotoxicity at the different level according to the type of cell line tested and inhibit the viability of these cells in a dose-dependent manner. Daudi cells with an IC_{50} value of 85.64 µg/mLwas the most sensitive cell lines to the extract. However, the IC_{50} value of the extract against BEAS-2B normal cell line was calculated as 109.80 µg/mL. In addition, IC_{50} values in HeLa and A549 cells were found to be 135.35 and 234.8 µg/mL, respectively. In Egypt, El-Nabawy et al. [9] assessed the cytotoxic activity of different fractions of aerial parts and seeds of *U. picroides* on different cell lines. They reported that the seeds butanol fraction and the aerial parts ethyl acetate fraction were very cytotoxic to MCF-7 (IC_{50} value= 9.4±0.37 and 8.8±0.47 µg/mL, respectively), and to HePG-2 (IC_{50} value= 14.7±0.85 and 10.1±0.88 µg/mL, respectively) cells. So, it can be suggested that the different part of *U. picroides* and the type solvent used for extraction may cause cytotoxicity at the different level according to the cell type examined.

One of the hallmarks of cancer is uncontrolled cell division [21]. Because the normal cell regulation process in the human cancer cells are altered [22], it is important to investigate new anti-cancer drugs which can inhibit the different steps of the cell cycle. We examined the effect of the extract on cell cycle for 24 h. Our results showed that the extract caused cell cycle arrest for A549 cells and HeLa cells at different phase of cell cycle. These results indicate that the effect of the plant extract on cancer cell proliferation may occur by arresting the cell cycle.

The development of new blood vessels from pre-existing ones is called angiogenesis and this process is important in the growth and metastasis of cancer tissue [23]. VEGF is known as a key regulator of angiogenesis in cancer [24] so that VEGF is considered as a rational target for anticancer therapy. In the present study, we tested the effect of the extract on the secretion of VEGF in A549 cells after treatment for 6 h. Our results show that the treatment with the extract at 200 µg/mL reduced the level of VEGF secretion by 31% in A549 cells compared to untreated cells, suggesting the antiangiogenic potential of the extract against A549 cells.

It is known that the inflammatory cytokines play a critical role in tumor development [25]. The pro-inflammatory cytokines such as TNF-α, IL-1 and IL-6 may contribute the growth and the metastasis of cancer cells [26]. We finally investigated whether the extract altered the TNF-α, IL-1-α and IL-6 secretion in the A549 and HeLa cells after treatment for 6 h. The treatment with the extract at 200 µg/mL did not cause any increase in the secretion of pro-inflammatory cytokines examined in cancer cell lines used. Strzelecka et al. [7] investigated the

anti-inflammatory activity of the extracts of some traditional Mediterranean diet plants and reported that the extracts of plants including *U. picroides* showed promising anti-inflammatory properties. Thus, our results suggest that the plant extract may not promote the development of cancer associated with the pro-inflammatory cytokines.

As a result, this study demonstrated for the first time that the ethanolic extract from the flowering parts of *U. picroides* showed significant cytotoxic activity against different cancer cell lines. Daudi cells were the most sensitive to the extract than the other cells used. The extract resulted in an arrest of A549 and HeLa cells at different phases of cell cycle. In addition, the extract caused a significant decrease in the secretion of cytokines after 6 h treatment in comparison with the untreated cells. Therefore, these findings may provide an important contribution to obtain a new bioactive compound with anti-cancer potential from the flowering part of *U. picroides*. Future studies should evaluate the determination of the potential of novel agents for cancer therapy via in vivo and in vitro analysis.

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