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THE CYTOTOXIC AND APOPTOTIC EFFECTS OF *Abies nordmanniana* subsp. *bornmülleriana Mattf* RESIN EXTRACT ON PROSTATE CANCER CELLS

MUHAMMET BURAK BATIR[1], SEVİNÇ BATIR[1], FEYZAN ÖZDAL KURT[1], FETHİ SIRRI ÇAM[2]

[1] Faculty of Arts and Sciences, Department of Biology, Manisa Celal Bayar University, Manisa, TÜRKİYE
[2] Faculty of Medicine, Department of Medical Genetics, Manisa Celal Bayar University, Manisa, TÜRKİYE

ABSTRACT. Cancer has become one of the most critical health issues, with an increasing incidence and mortality in recent years. Meanwhile, many studies are carried out on discovering new compounds which reflects effective results on cancer cells. Therefore, this study aimed to examine the cytotoxic, anti-proliferative and apoptotic effects on cancer cell lines by using the extract obtained from Uludağ Fir (*Abies nordmanniana* subsp. *bornmülleriana Mattf*) resin. Uludağ Fir resin extract was obtained by applying the methanol extraction method. In our study, fibroblast cell L-929, human metastatic prostate cancer PC-3 and human metastatic prostate cancer DU-145 cell lines were cultured in RPMI-1640 medium. The effects of the resin extract concentrations on the viability of the cells were determined with the Muse™ Cell Count & Viability test. In addition, apoptosis and cell cycle phases of the cells were determined using the Muse™ cell analyzer. Finally, RT-qPCR analysis was performed to determine the resin extract effect on pro-apoptotic and anti-apoptotic gene expression. The apoptotic effect of Uludağ Fir resin extract on cancer cell lines was significantly higher in contrast to the healthy normal cells. In addition, it was determined that Uludağ Fir extract caused G0/G1 cell cycle arrest in cancer cells. In RT-qPCR analysis, the pro-apoptotic gene expression levels were apparently increased in cancer cells, which was followed by a decrease in the anti-apoptotic gene expression levels. All taken together, these results indicate that Uludağ Fir resin extract exerts two prong effects as it induces a cell cycle arrest and apoptotic pathway activation on human prostate cancer cell lines.

1. INTRODUCTION

Cancer, caused by the uncontrolled proliferation of cells, is one of our age's most important health problems, and the mortality rate among patients is relatively high [1]. One of these cancer type is prostate cancer and it is a type of cancer that develops in the prostate gland, which is a small walnut-shaped gland that produces seminal fluid in males. It is one of the most common types of cancer in men, and its incidence increases with age [2]. Also, In underdeveloped countries, the problem is more severe due to the lack of diagnostic techniques, standard

Keywords. Prostate cancer, Uludağ Fir, cell viability, apoptosis, cell cycle arrest

buraks1@hotmail.com- Corresponding author; 0000-0002-8722-5055
soylevsevinc@gmail.com- 0000-0003-1817-3113
feyzanozdalkurt@yahoo.com- 0000-0001-6070-3742
sirricam@gmail.com- 0000-0002-0972-8896

treatment methods and higher treatment costs [3]. These countries, which have problems in the diagnosis and treatment of cancer, overcome these problems using synthetic drugs. These drugs target the rapidly growing and dividing cells of various tumors [4]. However, although these synthetic drugs can cause some acute and irreversible side effects also effect rapidly dividing normal cells in our body [5]. In addition, it has been reported by various studies that chemotherapeutic drugs used in cancer treatment cause multi-drug resistance [6,7]. For this reason, the high cost, increasing drug resistance, and side effects of current therapeutic approaches are forcing scientists to explore alternative medicines known as conventional medicine as an option to find new chemicals for cancer treatment.

Herbal agents are currently being researched for different uses in many laboratories worldwide. For example, from the point of view of cancer treatment, many researchers worldwide are working on treatment by testing extract samples obtained from different species, such as algae and plants, by different methods in different cancer cell types [8-10]. In some recent studies, it has been determined that essential oils obtained from some Pinus, Citrus, Lavandula and Melaleuca species have cytotoxic effects against cervical (HeLa), breast (MCF-7), kidney (HEK-293), colon (HT-29), livers (HepG2, BEL-7402) and many other cancer cell lines [11-14]. In addition, it has been reported that resin extract obtained from Abies balsamea (L.) Mill. (Canadian Fir) has anti-cancer effects in studies on various cancer cell lines. Also, it was observed that healthy cells were affected less than cancer cells [15]. In a study on the determination of the cytotoxic activity of the resin obtained from Taurus Fir, the resin extract exhibited cytotoxic activity on cancer cells [16]. Also, a compound derived from Abies sibirica was able to inhibit the growth of cancer cells. The researchers suggested that this effect may be due to the compound's ability to induce cell death in the cancer cells [17]. However, there is some evidence to suggest that Fir resin may have an effect on cancer cells, but the research is still in the early stages and more studies are needed to fully understand its potential benefits.

As mentioned above, many different species of Fir resins were experimentally tested on different cancer cell lines. However, the different species of Fir resins' extracts constituents could differ. One of these species is Uludağ Fir (Abies nordmanniana subsp. bornmülleriana Mattf), an endemic species for Turkey and the subject of our study. This species locates in the Western Black Sea Region, between Kızılırmak and Uludağ [18]. Therefore, this study aimed to unveil the cytotoxic, anti-proliferative and apoptotic effects of Uludağ Fir resin extract on the mouse fibroblast cell line L-929 and prostate cancer cell lines (PC-3 and DU-145).

2. MATERIALS AND METHODS

2.1. Preparation and application of Uludağ Fir resin extract

1 g of Uludağ Fir resin was initially dissolved in 4 ml pure methanol in a 15 ml centrifuge tube. The resin solution dissolved in methanol was passed through a 40 µm nylon filter to remove plant residues from it and passed through a 20 µm polyethersulfone (PES) filter to prevent bacterial contamination. Then, the cap of the tube containing the extract solution was loosened and incubated at 37 °C for two days to evaporate the methanol in the tube. As a result, 700 mg of pure resin was obtained after all operations and dissolved in 7 ml of Dimethyl sulfoxide (DMSO). Thus, a resin extract solution was obtained with a final concentration of 100 mg/ml. The extract obtained at the end of all processes was stored at -20 °C for further processing. Then, different amounts as 1500, 1000, 750, 500, 300, and 100 µg/ml, doses of resin extract from prepared stock solution were simultaneously applied to healthy normal cells and cancer cell lines for treatment.

2.2. Cell line and culture conditions

Mouse fibroblast cell line L-929, human prostate cancer cell lines PC-3 and DU-145 were purchased from ATCC (American Type Cell Culture). L-929, PC-3 and DU-145 cells were cultured in a medium containing RPMI 1640 (Gibco, 11875093), 10% fetal bovine serum (FBS) (Gibco, 10270098), %1 penicillin/streptomycin (Gibco, 15070063) and %1 L-Glutamine (Gibco, 25030081) at 37° C in a 5% CO_2 incubator. The medium was changed in every second day. When the cells became confluent, they were passaged to ensure growth. Cells (45000 cells/ml/well) were cultured in 24-well plates for 24 hours, then were incubated with the extracts at different dilutions for 24 and 48hours. All experiments were performed in triplicate for each extract. After the last incubation time, the cells were collected for viability and proliferation assays.

2.3. Cell viability analysis in cell lines treated with Uludağ Fir resin extract

Cell viability assays were carried out with flow cytometry analysis. Flow cytometry analysis, the attached cells in the 24 well plates were washed with Dulbecco's phosphate-buffered saline 1X (DPBS) (Gibco, 14190144), and washed cells were then trypsinized (Gibco, 25200056). Following trypsinization, centrifuged cells were suspended and diluted in a related complete medium. After dilution of the cells, the total cell concentrations and viability of the cells were determined with Muse Count & Viability reagent (Merck Millipore, Billerica, MA, USA, MCH100102) by following the manufacturer's instructions with using the automated Muse® Cell Analyzer (Merck Millipore, Billerica, MA, USA) which stains viable and dead cells based on their permeability to two different DNA binding dyes.

2.4. Apoptosis analysis in cell lines treated with Uludağ Fir resin extract

The apoptotic effects in L-929, PC-3 and DU-145 cells, which were grown in 6-well plates and then applied Uludağ Fir resin extract for 24 and 48 hours at IC_{50} and IC_{25} values, was determined using the Muse Annexin V and Dead Cell kit. For this reason, the viability and cell number of the cells were determined in the MUSE device with the Merck Muse® Count & Viability Assay kit. After cell counting, 250.000 cells in 2 ml RPMI-1640 complete medium were divided into groups of 3 for each cell type (as control, IC_{50} and IC_{25}) added into 6-well culture plate for 24 and 48 hours extract applications. In addition, three 6-well culture plate were used for these analyses. Cells transferred to 6-well culture plate were placed in a humidified incubator at 37°C and 5% CO_2 for 16-18 hours to ensure 70-80% cell confluency in the wells before applying the extract. After the incubation process, Uludağ Fir resin extract concentrations determined for IC_{50} and IC_{25} values in 2 ml RPMI-1640 complete medium were added to the wells, excluding the control well, respectively the culture plate were placed to the incubator at 37°C and 5% CO_2 humidified environment. At the end of 24 hours, the first group cells were taken from the incubator and the cells removed from the wells by trypsinization as centrifuged at 1000 rpm for 5 minutes in 15 ml tubes. At the end of centrifugation, the supernatant was removed, and the cells at the bottom of the tube were suspended with 1 ml of culture medium. The suspended cells were transferred to different 1.5 ml centrifuge tubes as 100 µl and then 100 µl of Muse Annexin V and Dead Cell solution was added into each tube. After incubating the cells for at least 60 minutes at room temperature in the dark, the cells were analyzed on the Muse flow cytometry instrument to determine the apoptosis rate. Similar procedures were performed within the 48-hour experiment group [19].

2.5. RT-qPCR analysis of mRNA expression levels of anti-apoptotic Bcl-2, Bcl-xL and pro-apoptotic Caspase-3, Bax and Cytochrome C target genes in cell lines treated with Uludağ Fir resin extract

Isolation of RNA from L-929, PC-3 and DU-145 cells, which were grown in 6-well plates and Uludağ Fir resin extract applied for 24 and 48 hours at IC_{50} and IC_{25} values, was carried out with PureLink™ RNA Mini (Thermo Fischer Scientific) according to the manufacturer's procedure. cDNA synthesis was performed with a High-Capacity cDNA Reverse Transcription kit from the samples isolated total RNAs in the SensoQuest thermal cycler according to the manufacturer's procedure. After this, to perform mRNA expression analyses of target genes on cDNAs Bcl-2 (BCL2F1 5'-CTTCGCCGAGATGTCCAGC-3', BCL2R1 5'-CTCTCCACACACATGACCCC-3'), Bcl-xL (BCLxLF1 5'-TCCCCATGGCAGCAGTAAAG-3', BCLxLR1 5'-TCCCCATGGCAGCAGTAAAG-3') Caspase-3 (CASP3F15'-TGAGATCAAGCCCCACGATG-3', CASP3R1 5'-ACAGCAGATGAAGCAGTCCA-3'), Bax (BAXF1 5'-ACGGCAACTTCAACTGGGG-3', BAXRCATGTCAGCCCAAT-3') and

Cytochrome C (COX1F1 5'-CCTCTTCGTCTGATCCGTCC-3', COX1R1 5'-TGAGGGTTGCGGTCTGTTAGT-3') primer pairs were designed. Also, expression analysis of target genes was performed with a 25 µl total reaction mixture of 2 µl cDNA, 1.25 µl EvaGreen, 0.3 µl 10 pmol Primer Forward, 0.3 µl 10 pmol Primary Reverse, 12.5 µl 2X Hot-start master mix and 8.65 µl ddH2O in a Rotor-Gene Real-Time PCR (Qiagen, USA) device. Normalization of target genes was carried out according to the most stable expression values of the Hypoxanthine-guanine phosphoribosyltransferase 1 (HPRT1) housekeeping gene. Quantification of normalized expression values of target genes was determined according to the $2^{-\Delta\Delta Ct}$ method [20].

2.6. Analysis of cell cycle state in cell lines treated with Uludağ Fir resin extract

Cell cycle analysis of L-929, PC-3 and DU-145 cells, which were grown in 6-well plates and then applied Uludağ Fir resin extract for 24 and 48 hours at IC_{50} and IC_{25} values, was carried out in the MUSE device with the Merck Muse® Muse Cell Cycle Kit. Cell cycle analysis of L-929, PC-3 and DU-145 cells, which were grown in 6-well plates and then Uludağ Fir resin extract applied for 24 and 48 hours at IC_{50} and IC_{25} values and carried out in the MUSE device with the Merck Muse® Cell Cycle Assay kit. First, the incubated cells at 37°C in 95% humidity and 5% CO_2 environment were fixed in 70% ethanol (EtOH) and incubated at -20°C for 3 hours. Then, the fixed cells were washed with cold PBS and collected by centrifugation at 1000 rpm for 5 minutes. Finally, the cells were analyzed in the MUSE flow cytometry device after staining with the Muse Cell Cycle Kit (Millipore, Germany) in the dark for 30 minutes.

2.7. Statistical analysis

The SPSS (statistical package software, Windows 23.0) was used to analyze the significances of differences observed between the groups. The obtained data were tested by performing One-way ANOVA and Tukey post hoc test. The p-values smaller than 0.05 were considered and all data represented the triplicate experiments. Finally, the data were presented as a mean ± standard deviation (SD).

3. RESULTS

3.1. Detection of IC_{50} and IC_{25} values for Uludağ Fir resin extract

In vitro anti-cancer activity of the extract of Uludağ Fir resin extract was assessed through the MUSE flow cytometry assays against prostate cancer cell lines. Uludağ Fir resin extract was added onto L-929, PC-3 and DU-145 cells at concentrations of 1500, 1000, 750, 500, 300, and 100 µg/mL for 24 and 48 hours. Cell viability analyses performed with the MUSE flow cytometry device; comparative data were obtained in cell viability increases in accordance with the decreasing extract concentration. The analysis performed with MUSE flow

cytometry determined that the cell viability value was 49.0% in L-929 cells at a concentration of 1500 µg/mL for 24 hours (Figure 1). Cell viability values for PC-3 and DU-145 cells were determined as 4.9% and 5.8%, respectively. The 24 hours IC_{50} values of L-929, PC-3, and DU-145 cells, obtained from the flow cytometry analysis were found as 1520 µg/mL, 1040 µg/mL, and 1065 µg/mL, respectively. IC_{25} values of L-929, PC-3, and DU-145 cells were 750 µg/mL, 500 µg/mL, and 520 µg/mL, respectively.

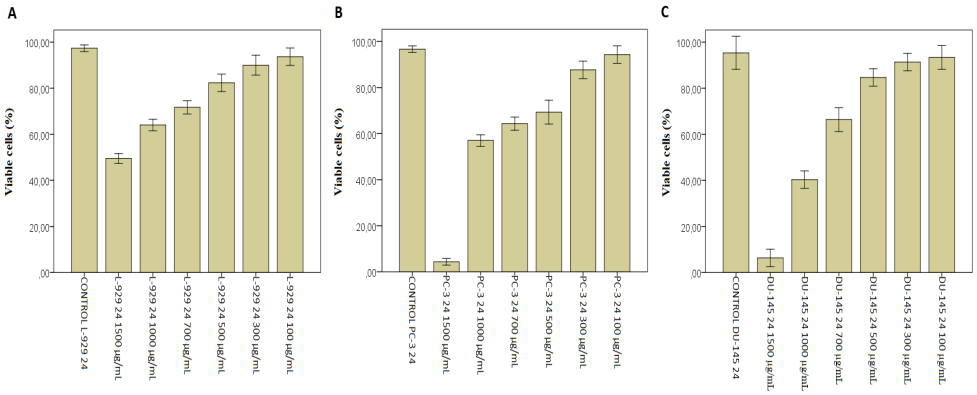

FIGURE 1. Muse cell viability analysis of 24-hours Uludağ Fir resin extracts treated. A L-929, B PC-3, C DU-145 cell lines.

As a result of 48 hours of extract applications at 1500 µg/mL concentration, it was determined that the viability decreased below 7.0% in all cell line groups (Figure 2). Also, IC_{50} and IC_{25} values of the cell lines were determined according to the timeline due to detection of the longitude effect of Uludağ Fir resin extract. The IC_{50} values of L-929, PC-3, and DU-145 cells, obtained from the flow cytometry analysis were found as 1110 µg/mL, 930 µg/mL, and 940 µg/mL, respectively. IC_{25} values of L-929, PC-3, and DU-145 cells were 550 µg/mL, 450 µg/mL, and 460 µg/mL, respectively.

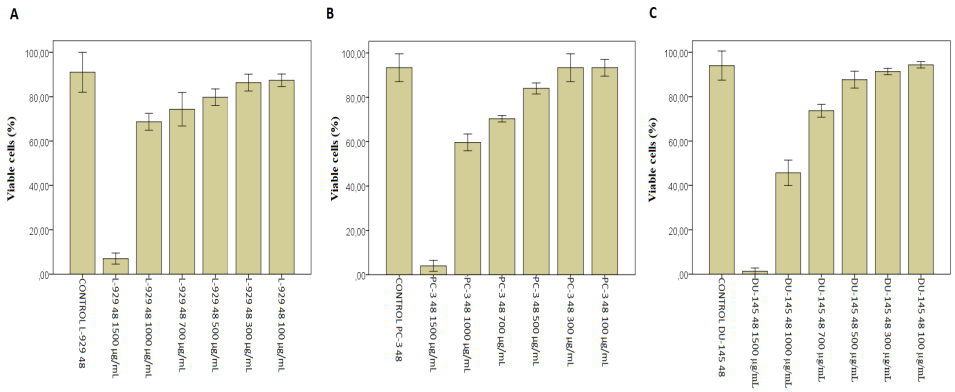

FIGURE 2. Muse cell viability analysis of 48-hours Uludağ Fir resin extracts treated. A L-929, B PC-3, C DU-145 cell lines.

3.2. Evaluation of the antitumor activity of Uludağ Fir resin

The apoptotic potential of the Uludağ Fir resin extract was measured by flow cytometry, which allows the identification of viable (AnnV-/PI-), early apoptotic (AnnV+/PI-), late apoptotic (AnnV+/PI+) and necrotic (AnnV-/PI+) cells. According to the data obtained from cell viability, IC_{50} and IC_{25} extract ratios were applied to L-929, PC-3, and DU-145 cell lines in 6-well cell culture dishes for 24 and 48 hours, respectively. In addition, the apoptotic process occurring in the cell lines was determined in the Muse flow cytometry device, using the Muse Annexin V and Dead Cell kit (Figure 3).

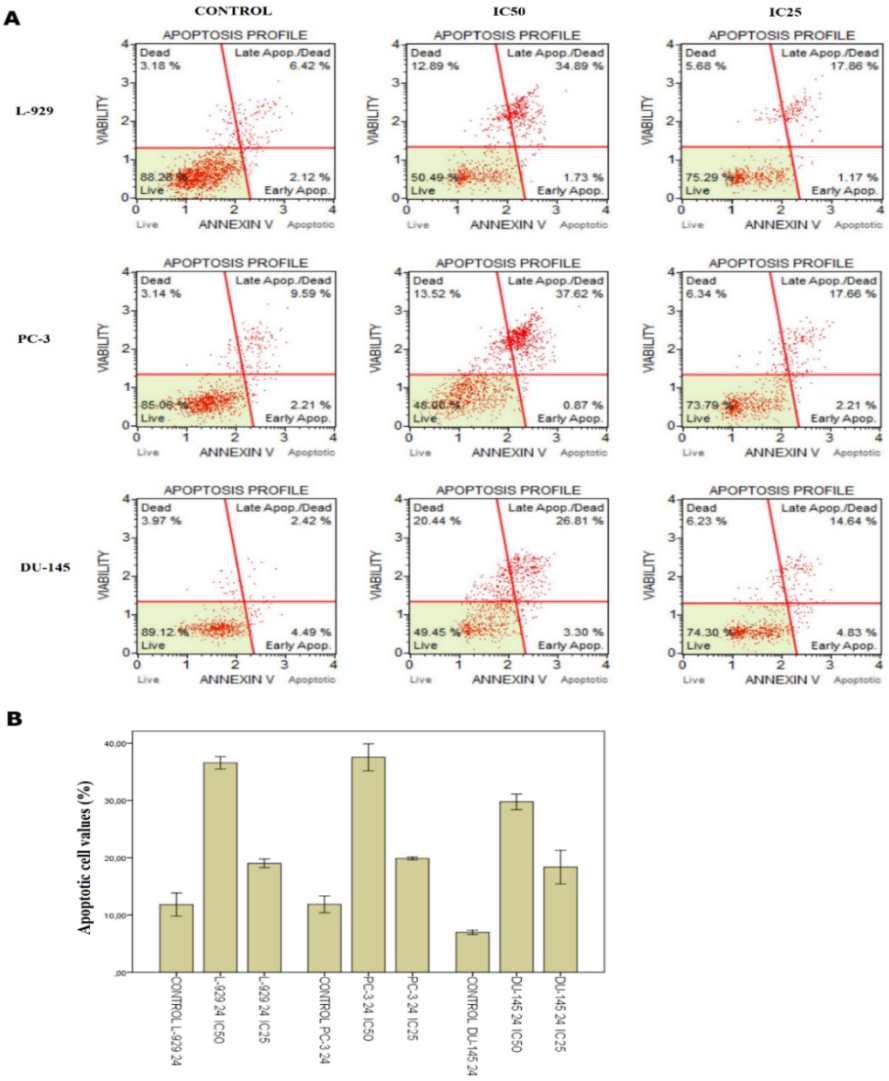

FIGURE 3. 24-hours apoptosis values of L-929, PC-3, DU-145 cell lines treated with IC_{50} and IC_{25} Uludağ Fir resin extract. A graphic of flow cytometry analysis, B % ratio of apoptotic cell values of control and treated cell lines.

Cells were treated with IC_{25} (concentration causing 25% growth inhibition) and IC_{50} (concentration causing 50% growth inhibition) of Uludağ Fir resin extract to demonstrate the concentration-dependent apoptotic effect. All cancer cell lines tested with IC_{25} and IC_{50} concentrations, consistent with flow cytometry cell viability analysis (p=0.001), showed reduced viability and markedly increased apoptotic cells at lower concentrations than healthy normal cells.

As shown in Figure 3, the total number of apoptotic (early and late apoptosis) cells at 24 hours in the control group of L-929 cells was 11.86±0.60%. In L-929 cell groups, which were applied extract during 24 hours at concentrations of 550 µg/mL (IC_{25}) and 1110 µg/mL (IC_{50}), the total number of apoptotic cells increased by 19.03±0.30% ($p<0.0001$) and 36.62±0.50% ($p<0.0001$) according to the control group, respectively. Compared to PC-3 control cells with a total apoptotic cell count of 11.86±0.60% at 24 hours, the application of the extract at concentrations of 450 µg/ml (IC_{25}) and 930 µg/ml (IC_{50}) resulted in the increasing number of total PC-3 apoptotic cell count as a 19.87±0.10% (p=0.0001) and 37.58±0.90% (p=0.0001), respectively. Compared to DU-145 control cells with a total apoptotic cell count of 6.97±0.11% at 24 hours, the application of the extract at concentrations of 460 µg/mL (IC_{25}) and 940 µg/mL (IC_{50}) resulted in the increasing number of total DU-145 apoptotic cell count as an 18.87±1.21% (p=0.0001) and 29.77±0.58% (p=0.0001), respectively. These results showed that Uludağ Fir resin extract could induce apoptosis in all tested cancer cells to a significant extent in a concentration-dependent manner.

As shown in Figure 4, the total number of apoptotic (early and late apoptosis) cells at 48 hours in the control group of L-929 cells was 6.24±0.30%. In L-929 cell groups, which were applied extract for 48 hours at concentrations of 550 µg/mL (IC_{25}) and 1110 µg/mL (IC_{50}), the total number of apoptotic cells increased by 28.89±0.70% ($p<0.0001$) and 41.48±1.2% ($p<0.0001$) according to the control group, respectively. Compared to untreated PC-3 control cells with a total apoptotic cell count of %6.22±0.51 at 48 hours, the application of the extract at concentrations of 450 µg/mL (IC_{25}) and 930 µg/mL (IC_{50}) resulted in the increasing number of total PC-3 apoptotic cell count as a %29.80±0.61 (p=0.0001) and %48.64±0.51 (p=0.0001), respectively. Also, compared to DU-145 control cells with a total apoptotic cell count of %4.88±1.65 at 48 hours, the application of the extract at concentrations of 460 µg/mL (IC_{25}) and 940 µg/mL (IC_{50}) resulted in the increasing number of total DU-145 apoptotic cell count as an %12.27±0.40 (p=0.0001) and %40.41±1.10 (p=0.0001), respectively. Unlike the 24-hour applications, the early apoptosis rate in L-929 cells was higher in contrast to PC-3 and DU-145 cancer cells in 48-hour applications. In comparison, the late apoptotic or dead cell rate was found to be higher in PC-3 and DU-145 cancer cells according to the healthy normal L-929 cells in 48-hour applications.

These results show that healthy normal L-929 cells that survived 24 hours of extract application were affected much later in contrast to PC-3 and DU-145 cancer cells. The cell viability and apoptosis studies revealed that the **extract** has minor effects on normal cell lines and can be qualified for additional applications.

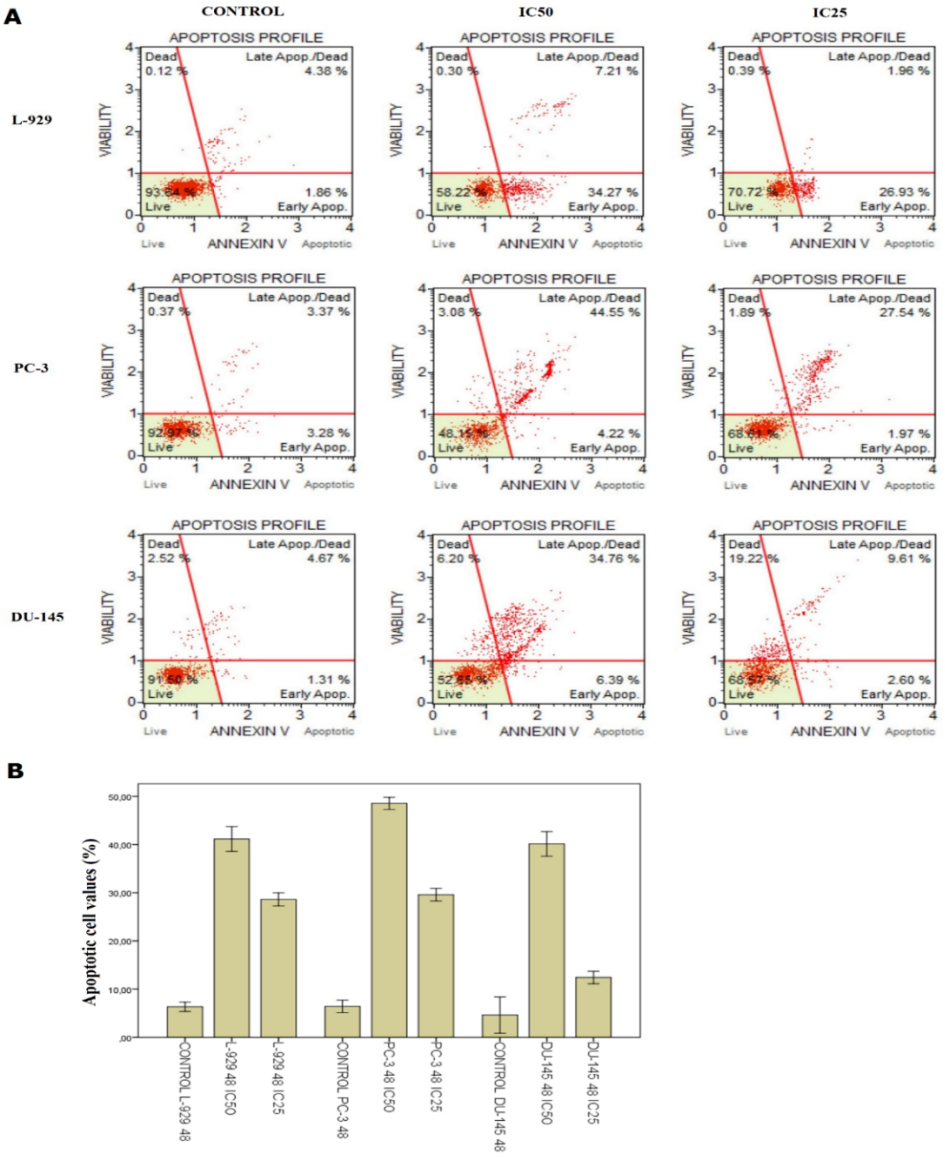

FIGURE 4. 48-hours apoptosis values of L-929, PC-3, DU-145 cell lines treated with IC_{50} and IC_{25} Uludağ Fir resin extract. A graphic of flow cytometry analysis, B % ratio of apoptotic cell values of control and treated cell lines.

3.3. Evaluation of cell cycle status of Uludağ Fir resin extract treated cells

Cell viability analysis revealed that Fir resin extract had a significant cytotoxic effect on all tested cancer cell lines. For this reason, the effect of extract on cell distribution in different phases of the cell cycle was also investigated. Therefore, cancer cells were treated with the calculated IC_{25}, and IC_{50} values of Uludağ Fir resin extract for 24 and 48 hours and analyzed by flow cytometry. According to the analysis results, the extract caused an increase in cell populations that remained in the G0/G1 stage. In contrast, this increase was accompanied by a decrease in the cell population in the G2/M stage. As shown in Figure 5, the following data were obtained according to cell cycle analyses from 24-hour extract applications. There was no significant difference between the G0/G1 population (61.7±0.57) of the L-929 IC_{25} treatment group ($p=0.507$) and the G0/G1 population (59.3±0.36%) of the untreated L-929 control group. However, a significant difference was found between the G0/G1 population 66.9±1.53% of the L-929 IC_{50} treatment group ($p=0.001$) and the G0/G1 population (59.3±0.36%) of the untreated L-929 control group. Furthermore, a major difference was found between the G0/G1 population of the PC-3 IC_{25} treatment group (73.30±0.85%) ($p<0.0001$) and the G0/G1 population of the PC-3 IC_{50} treatment group (78.70±1.44%) ($p<0.0001$) compared to the G0/G1 population (%64.56±1.28) of untreated PC-3 control cells. Meanwhile, there was a significant difference between the G0/G1 population of the IC_{25} treatment group (53.23±2.05%) ($p<0.0001$) and the G0/G1 population of the IC_{50} treatment group (55.73%±1.04%) ($p<0.0001$) compared to the G0/G1 population (44.30±0.50) of DU-145 control cells.

As shown in Figure 6, the following data were obtained according to cell cycle analyses resulting from 48-hour extract applications. Although, there was a slight difference between the G0/G1 population of the untreated L-929 control group (51.70±0.26%) and the G0/G1 population of the IC_{25} treatment group (55.20±1.25%), which was significant ($p=0.041$). However, a significant difference was also found between the G0/G1 population of the IC_{50} treatment group (62.23±0.51) compared to the G0/G1 population of the L-929 control group ($p=0.001$). On the other hand, a significant difference was found between the G0/G1 population of the PC-3 IC_{25} treatment group (78.30±0.60%) ($p<0.0001$) and the G0/G1 population of the PC-3 IC_{50} treatment group (82.10±0.87) ($p<0.0001$) compared to the G0/G1 population of the untreated PC-3 control group (55.17±1.02%). Also, there was a major difference between the G0/G1 population of the IC_{25} treatment group (%65.03±0.61) ($p<0.0001$) and the G0/G1 population of the IC_{50} treatment group (%67.86±0.72) ($p<0.0001$) compared to the G0/G1 population of the DU-145 control group (%52.80±0.75).

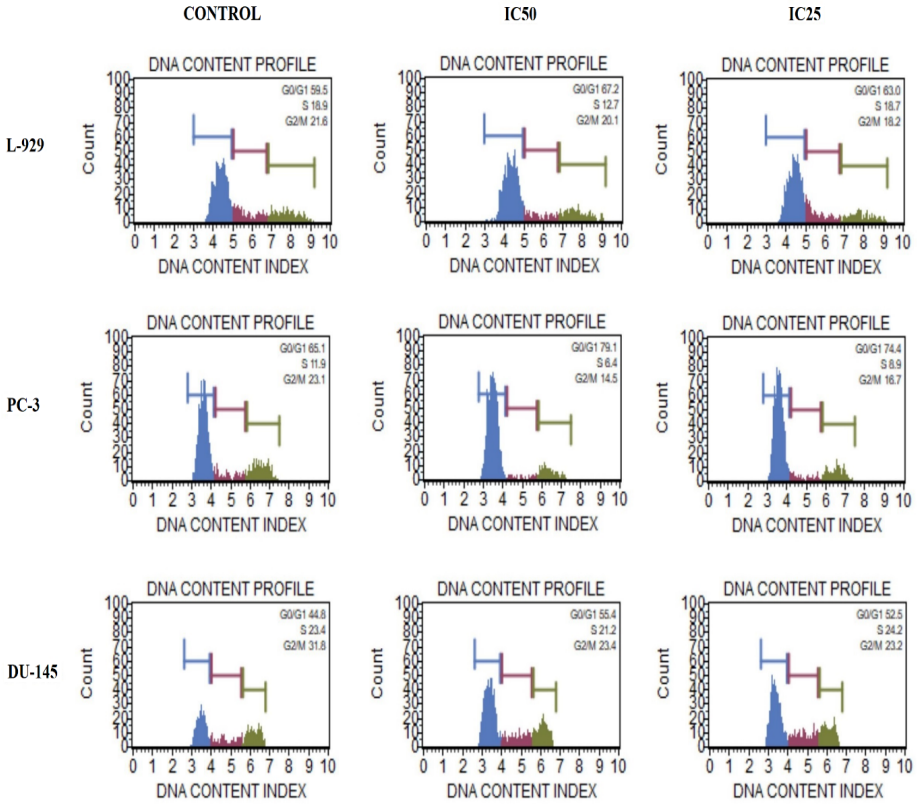

FIGURE 5. 24-hours cell cycle analysis values of L-929, PC-3, DU-145 cell lines treated with IC_{50} and IC_{25} Uludağ Fir resin extract

3.4 RT-qPCR analysis of mRNA expression levels of anti-apoptotic and pro-apoptotic target genes in cell lines treated with Uludağ Fir extract

The data on the changes in the expression levels of target genes were obtained according to the RT-qPCR analysis of Anti-apoptotic Bcl-2, Bcl-xL and Pro-apoptotic Caspase-3, Bax and Cytochrome C genes in cells treated with Uludağ Fir resin extract for 24 and 48 hours. Figure 7 shows an important decrease in the Bcl-2 gene mRNA expression level of all cell types treated with extract at IC_{50} value for 24 hours compared to untreated controls ($p<0.0001$). However, the decrease in Bcl-2 gene expression in L-929 cells was lesser than in other cancer cells. In addition, there was no valuable distinction between the decrease in the Bcl-xL gene expression level of the IC_{50} treated group of L-929 cells according to the untreated control group ($p=0.295$). On the other hand, a significant difference was observed between the decrease in Bcl-xL gene expression level of all other cancer cell IC_{50} extract treated groups and untreated controls ($p<0.0001$).

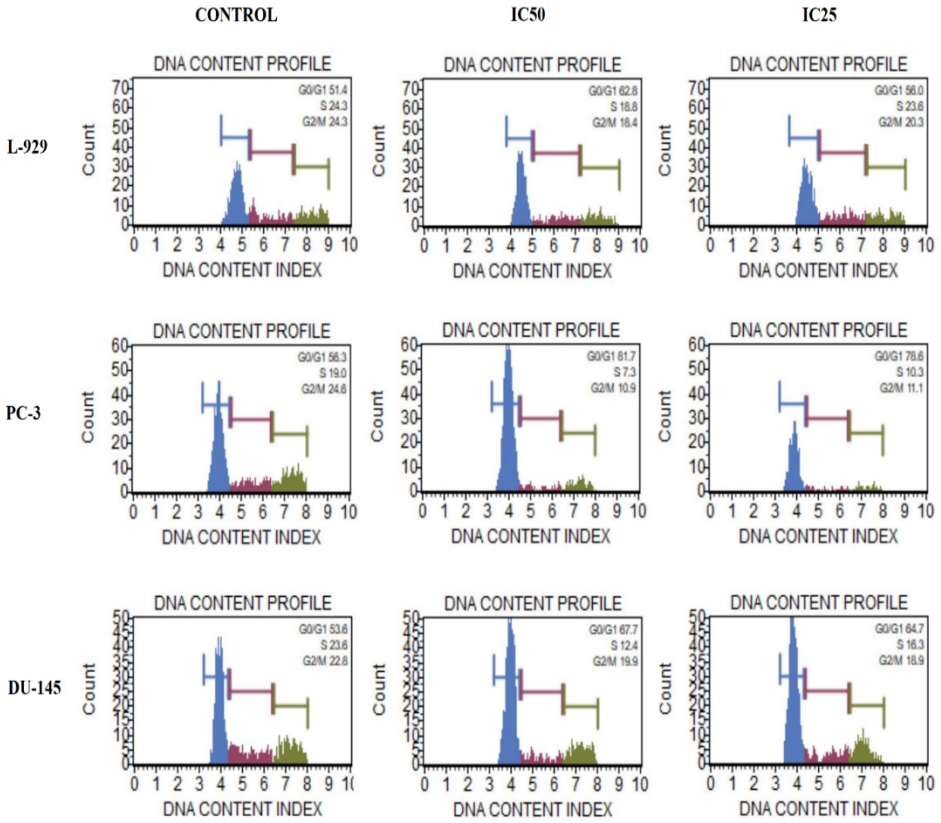

FIGURE 6. 48-hour cell cycle analysis values of L-929, PC-3, DU-145 cell lines treated with IC_{50} and IC_{25} Uludağ Fir resin extract

In addition, there was a significant increase in Caspase-3 and Cytochrome C gene expression levels of L-929, PC-3 and DU-145 treated at IC_{50} value for 24 hours compared to controls ($p<0.0001$). There was no significant difference between the increase in the Bax gene expression level of the IC_{50} extract treated group of L-929 cells and the Bax gene expression level of the control group ($p=0.060$). However, there was an significant difference between the increase in the Bax gene expression level of all IC_{50} extract treated cancer cell groups according to the untreated controls ($p<0.0001$).

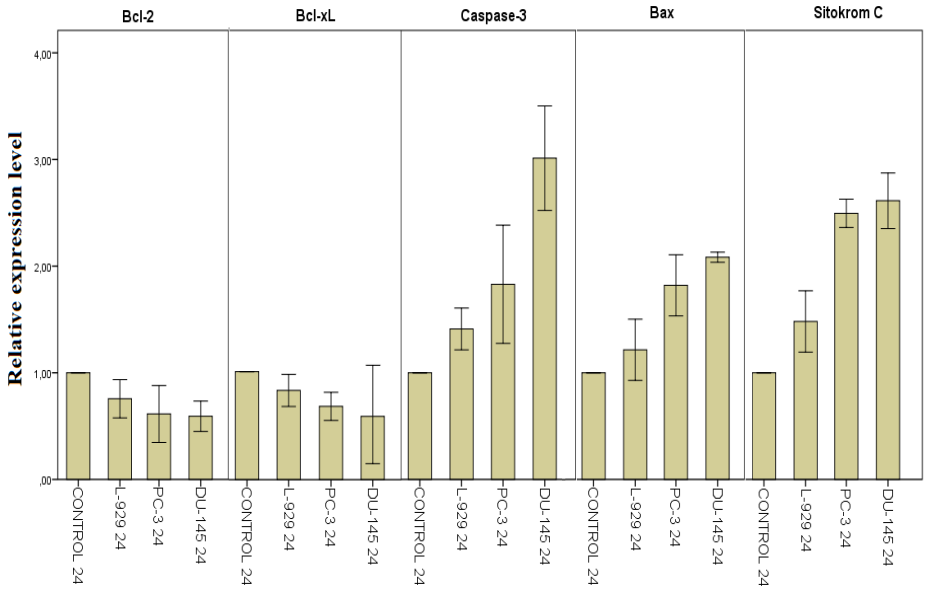

FIGURE 7. 24-hour relative gene expression values of L-929, PC-3, DU-145 cell lines treated with IC_{50} Uludağ Fir resin extract

Figure 8 shows a main decrease in mRNA expression levels of Bcl-2 and Bcl-xL genes in all cell types treated with extract at an IC_{50} value for 48 hours compared to untreated controls ($p<0.0001$), depending on the increased application time. Conversely, there was a significant increase in Caspase-3, Bcl-xL and Cytochrome C gene expression levels of all cell types treated with extract at IC_{50} value for 48 hours compared to untreated controls ($p<0.0001$). However, this increase in Caspase-3, Bcl-xL, and Cytochrome C genes of L-929 cells was more limited compared to PC-3 and DU-145 cancer cells.

Fir resins revealed high variation in composition even in closely related species, which agrees with previous reports on Balsam Fir and Cilician Fir [15,16]. In the current study, Uludağ Fir resin extract exhibited chemo preventive activities, such as inducing apoptosis and cell cycle arrest. Interestingly, Uludağ Fir resin extract revealed a less toxic effect on healthy normal L-929 control cells in contrast to PC-3 and DU-145 prostate cancer cells. Also, there are few studies about to anti-cancer effect of Fir resins extract.

Compared to the earlier studies, their findings revealed differences in means of IC_{50} values on healthy normal and cancer cells. Furthermore, according to Legault et al., the study on the cytotoxic activity of Balsam Fir resin extract was evaluated against different cancer and healthy normal cell lines.

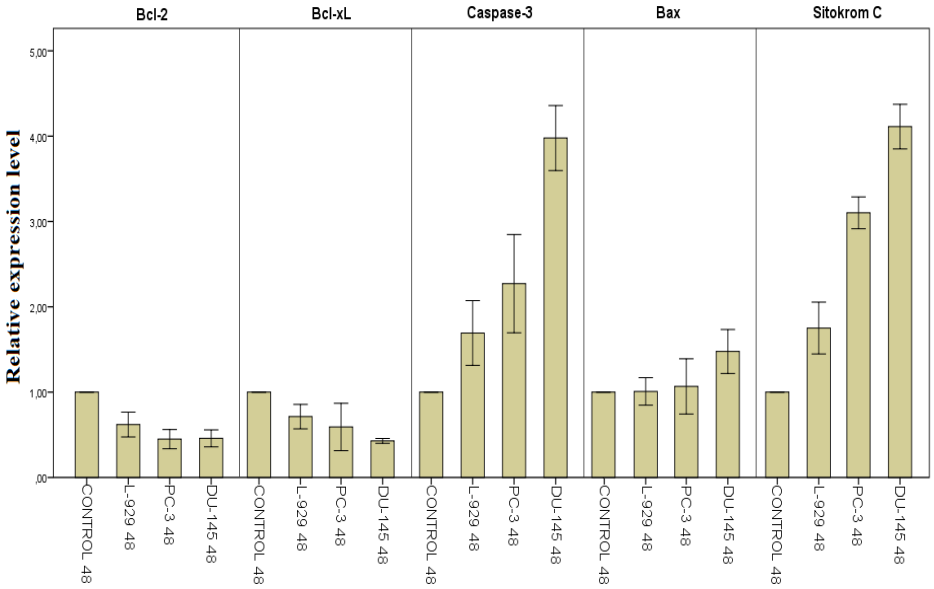

FIGURE 8. 48-hour relative gene expression values of L-929, PC-3, DU-145 cell lines treated with IC_{50} Uludağ Fir resin extract

As a result, they found a less toxic effect on L-929 fibroblast healthy normal cells (IC_{50}, 3100 µg/mL) and a higher toxic effect according to the IC_{50} level on different kinds of cancer cell lines ranging from 760 to 1700 µg/mL during the 24 hours experimental timeline [15]. On the other hand, the other study about the cytotoxicity of Cilician Fir resin extract revealed different results according to the above-mentioned study. They found a higher cytotoxic activity of the resin on WI-38 fibroblast healthy normal cells with lower concentration (IC_{50}, 18.32 µg/mL) and a lower toxic effect according to the IC_{50} level on different cancer cell lines ranging from 23 to 430.1 µg/mL during the 48 hours experimental timeline [16]. Meanwhile, our study results for Uludağ Fir resin extract exhibited lower cytotoxic activity on L-929 healthy normal cells (IC_{50}, 1110 µg/mL) and a higher toxic effect according to the IC_{50} level on different kinds of prostate cancer cell lines ranging from 930 to 940 µg/mL during the 48 hours experimental timeline, which is similar to the Legault et al. study results.

Determining the different types of programmed cell death (PCD) in the agent-treated cell group is essential [21]. PCD includes death mechanisms such as apoptosis, autophagy, necroptosis, and pyroptosis [22,23]. Therefore, determining the different types of cell death mechanisms in the agent-treated cell group is essential. In agent applications, the most common death mechanism is apoptosis. Furthermore, the mechanism of apoptosis was first investigated in cells to which Uludağ Fir resin extract was applied. Also, there are no reports about PCD type of Fir resin-treated cancer cells. According to this study result, apoptosis-related cell death mechanism was apparently determined in the Uludağ

Fir resin extract-treated cell lines during the 24 and 48 hours of extract treatment. Apoptosis detection occurred at higher concentrations in healthy normal cells, while it was observed in prostate cancer cell lines at lower concentrations, which can be clearly seen in Figures 3 and 4. On the other hand, cell cycle arrest is closely associated with apoptosis; that is, cell cycle arrest guides apoptosis via outcomes on different signaling molecules and regulatory proteins. There are many studies on cell cycle arrest and herbal extracts treatment association. Most of the herbal extract treatments on cancer cells result in the G0/G1 cell cycle arrest of cancer cells [24-26]. Similarly, our results demonstrated that Uludağ Fir resin extract induces cell cycle arrest at the G0/G1 phase in human prostate cancer cell lines. Besides, in both 24 and 48 hours IC_{50} and IC_{25} extract applications, the increase in the cell populations that arrested in the G0/G1 stage in healthy normal L-929 cells was found to be significantly less than in all other prostate cancer cell lines ($p<0.0001$).

The capacity of an agent to induce apoptosis in cancer cells demonstrates its potential for use as an anti-cancer agent. Therefore, the apoptosis-inducing potential of Uludağ Fir resin extracts was analyzed by examining the mRNA expression profile of apoptosis-related genes. Furthermore, determining the programmed cell death type makes it necessary to determine which gene-related signaling pathway effectively participates in the cell death process. Previous studies demonstrated that Compounds α/β-pinene and limonene revealed high in Fir resin in previous studies, are promising anticancer agents in recent years due to their ability to induce apoptosis and modulate various signaling pathways [15,16]. In addition, many studies have reported anti-cancer activity for α/β-pinene and limonene against various cancer types [27-30]. The apoptotic level is raised after the induction of nucleases, while the induction of caspase-3 is essential for heterochromatin aggregation and DNA fragmentation in apoptotic cells. Hence, to identify the involvement of caspase-3 in Uludağ Fir resin extract-mediated apoptosis, caspase-3 activity was estimated at the mRNA level. In the previous study, the activity of caspase-3 was found to be enhanced in α-pinene-treated human ovarian cancer cells (PA-1) [31]. Similarly, the result of this study revealed an increased level of caspase-3 expression in parallel with the increasing level of cytochrome-C and Bax expressions. Also, this increases in the expression level of pro-apoptotic caspase-3, cytochrome-C and Bax genes was accompanied by a decrease in the expression level of anti-apoptotic Bcl-2 and Bcl-xL genes. However, the pro-apoptotic and anti-apoptotic mRNA expression changes were lesser in healthy normal L-929 cells according to the human prostate cancer cell lines. According to this, to identify the involvement of caspase-3 in Uludağ Fir resin extract-mediated apoptosis, caspase-3 activity was estimated at the mRNA level.

4. CONCLUSION

Overall, in this study, the anti-cancer effect of the Uludağ Fir resin was analyzed by using flow cytometry and RT-qPCR. The tested resin extract demonstrates

anti-cancer effects in different prostate cancer cell lines by promoting cytotoxicity, halting cell cycle progression and the activation of apoptotic cell death. All these from the present study suggest that the extract of Uludağ Fir resin has the potential for cancer prevention.

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Declaration of Competing Interests The authors declare no conflict of interest.

REFERENCES

[1] Blagosklonny, M.V., Hallmarks of cancer and hallmarks of aging. *Aging*, 14 (9) (2022), 4176-4187. https://doi.org/10.18632/aging.204082.

[2] Chang, A.J., Autio, K.A., Roach, M., Scher, H.I., High-risk prostate cancer-classification and therapy. *Nature Reviews Clinical Oncology*, 11 (6) (2014), 308–323. https://doi.org/10.1038/nrclinonc.2014.68.

[3] Di Martile, M., Garzoli, S., Ragno, R., Del Bufalo, D., Essential oils and their main chemical components: The past 20 years of preclinical studies in melanoma. *Cancers*, 12 (9) (2020), 2650. https://doi.org/10.3390/cancers12092650.

[4] Cao, Y., Li, H., Harmony between humanity and nature: natural vs. synthetic drug preference in Chinese atheists and taoists. *Journal of Religion and Health*, 61 (4) (2022), 2743–2752. https://doi.org/10.1007/s10943-021-01314-6.

[5] Luethi, D., Liechti, M.E., Designer drugs: mechanism of action and adverse effects. *Archives of Toxicology*, 94 (4) (2020), 1085–1133. https://doi.org/10.1007/s00204-020-02693-7.

[6] Vaghari-Tabari, M., Hassanpour, P., Sadeghsoltani, F., Malakoti, F., Alemi, F., Qujeq, D., Asemi, Z., Yousefi, B., CRISPR/Cas9 gene editing: a new approach for overcoming drug resistance in cancer. *Cellular & Molecular Biology Letters*, 27 (1) (2022), 49. https://doi.org/10.1186/s11658-022-00348-2.

[7] Xavier, C.P.R., Caires, H.R., Barbosa, M.A.G., Bergantim, R., Guimarães, J.E., & Vasconcelos, M.H., The role of extracellular vesicles in the hallmarks of cancer and drug resistance. *Cells*, 9 (5) (2020), 1141. https://doi.org/10.3390/cells9051141.

[8] Martínez Andrade, K. A., Lauritano, C., Romano, G., Ianora, A., Marine microalgae with anti-cancer properties. *Marine Drugs*, 16 (5) (2018), 165. https://doi.org/10.3390/md16050165.

[9] Saadaoui, I., Rasheed, R., Abdulrahman, N., Bounnit, T., Cherif, M., Al Jabri, H., Mraiche, F., Algae-derived bioactive compounds with anti-lung cancer potential. *Marine Drugs*, 18 (4) (2020), 197. https://doi.org/10.3390/md18040197.

[10] Fitsiou, E., Pappa, A., Anticancer activity of essential oils and other extracts from aromatic plants grown in Greece. *Antioxidants*, 8 (8) (2019), 290. https://doi.org/10.3390/antiox8080290.

[11] Elansary, H. O., Abdelgaleil, S. A. M., Mahmoud, E. A., Yessoufou, K., Elhindi, K., El-Hendawy, S., Effective antioxidant, antimicrobial and anticancer activities of essential oils of horticultural aromatic crops in northern Egypt. *BMC Complementary and Alternative Medicine*, 18 (1) (2018), 214. https://doi.org/10.1186/s12906-018-2262-1.

[12] Zuzarte, M., Francisco, V., Neves, B., Liberal, J., Cavaleiro, C., Canhoto, J., Salgueiro, L., Cruz, M.T., *Lavandula viridis* L´Hér. Essential oil inhibits the inflammatory response in macrophages through blockade of NF-KB signaling cascade. *Frontiers in Pharmacology*, 12 (2022), 695911. https://doi.org/10.3389/fphar.2021.695911.

[13] Walia, M., Mann, T.S., Kumar, D., Agnihotri, V.K., Singh, B., Chemical composition and in vitro cytotoxic activity of essential oil of leaves of *Malus domestica* growing in Western Himalaya (India). *Evidence-based Complementary and Alternative Medicine*, eCAM, 2012, 649727. https://doi.org/10.1155/2012/649727.

[14] Thalappil, M.A., Butturini, E., Carcereri de Prati, A., Bettin, I., Antonini, L., Sapienza, F.U., Garzoli, S., Ragno, R., Mariotto, S., *Pinus mugo* essential oil impairs STAT3 activation through oxidative stress and induces apoptosis in prostate cancer cells. *Molecules*, 27 (15) (2022), 4834. https://doi.org/10.3390/molecules27154834.

[15] Legault, J., Dahl, W., Debiton, E., Pichette, A., Madelmont, J.C., Antitumor activity of balsam fir oil: production of reactive oxygen species induced by alpha-humulene as possible mechanism of action. *Planta Medica*, 69 (5) (2003), 402–407. https://doi.org/10.1055/s-2003-39695.

[16] Uçar, S.E, Şahin, B.S., Ulu, M., Akpolat, A., The determination of in vitro antioxidant and cytotoxic activities of resin obtained from Cilician Fir (Abies cilicica (Antoine & Kotschy) Carrière). *Kahramanmaraş Sütçü İmam Üniversitesi Tarım ve Doğa Dergisi,* 23 (6) (2020), 1503-1509. https://doi.org/10.18016/ksutarimdoga.vi.720675.

[17] Kudryavtseva, A., Krasnov, G., Lipatova, A., Alekseev, B., Maganova, F., Shaposhnikov, M., Fedorova, M., Snezhkina, A., Moskalev, A., Effects of *Abies sibirica* terpenes on cancer- and aging-associated pathways in human cells. *Oncotarget*, 7 (50) (2016), 83744–83754. https://doi.org/10.18632/oncotarget.13467.

[18] Anşin, R., Özkan, Z., Abies Mill. Göknarlar, Tohumlu Bitkiler (Spermatophyta), Odunsu Taksonlar, Karadeniz Teknik Üniversitesi, Orman Fakültesi. Trabzon. 167, 19 (1997), 66-72.

[19] Oztatlici, H., Oztatlici, M., Daglı, S. N., Karadeniz Saygili, S., Bir sefalosporin olan sefepim, nb2a nöroblastoma hücrelerinde apoptozu ve oksidatif stresi indükler. *Euroasia Journal of Mathematics, Engineering, Natural & Medical Sciences*, 9 (21) (2022), 79–86. https://doi.org/10.38065/euroasiaorg.962.

[20] Livak, K. J., Schmittgen, T. D., Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*, 25 (4) (2001), 402–408. https://doi.org/10.1006/meth.2001.1262.

[21] Lin, L., Zhang, M. X., Zhang, L., Zhang, D., Li, C., Li, Y. L., Autophagy, pyroptosis, and ferroptosis: New regulatory mechanisms for atherosclerosis. *Frontiers in Cell and Developmental Biology*, 9 (2022), 809955. https://doi.org/10.3389/fcell.2021.809955.

[22] Zhao, J., Jiang, P., Guo, S., Schrodi, S. J., He, D., Apoptosis, autophagy, NETosis, necroptosis, and pyroptosis mediated programmed cell death as targets for innovative therapy in Rheumatoid Arthritis. *Frontiers in Immunology*, 12 (2021), 809806. https://doi.org/10.3389/fimmu.2021.809806.

[23] Bertheloot, D., Latz, E., Franklin, B.S., Necroptosis, pyroptosis and apoptosis: an intricate game of cell death. *Cellular & Molecular Immunology*, 18 (5) (2021), 106-1121. https://doi.org/10.1038/s41423-020-00630-3.

[24] Trang, D.T., Hoang, T.K.V., Nguyen, T.T.M., Van Cuong, P., Dang, N.H., Dang, H.D., Nguyen Quang, T., Dat, N.T., Essential oils of lemongrass (Cymbopogon citratus Stapf) induces apoptosis and cell cycle arrest in A549 lung cancer cells. *BioMed research international*, (2020), 5924856. https://doi.org/10.1155/2020/5924856.

[25] Chung, K. S., Hong, J. Y., Lee, J. H., Lee, H. J., Park, J. Y., Choi, J. H., Park, H. J., Hong, J., Lee, K. T., β-Caryophyllene in the essential oil from Chrysanthemum Boreale induces G1 phase cell cycle arrest in human lung cancer cells. *Molecules*, 24 (20) (2019), 3754. https://doi.org/10.3390/molecules24203754.

[26] Seal, S., Chatterjee, P., Bhattacharya, S., Pal, D., Dasgupta, S., Kundu, R., Mukherjee, S., Bhattacharya, S., Bhuyan, M., Bhattacharyya, P. R., Baishya, G., Barua, N. C., Baruah, P. K., Rao, P. G., Bhattacharya, S., Vapor of volatile oils from Litsea cubeba seed induces apoptosis and causes cell cycle arrest in lung cancer cells. *PloS One*, 7 (10) (2012), e47014.4 https://doi.org/10.1371/journal.pone.0047014.

[27] Salehi, B., Upadhyay, S., Erdogan Orhan, I., Kumar Jugran, A., L D Jayaweera, S., A Dias, D., Sharopov, F., Taheri, Y., Martins, N., Baghalpour, N., Cho, W. C., Sharifi-Rad, J., Therapeutic potential of α- and β-Pinene: A miracle gift of nature. *Biomolecules*, 9 (11) (2019), 738. https://doi.org/10.3390/biom9110738.

[28] Li Y, Gao Y, Deng L, Lian H, Guo W, Wu W, Xue B, Li B, Su Y, Zhang H. Volatile profiling and transcriptome sequencing provide insights into the biosynthesis of α-pinene and β-pinene in Liquidambar formosana hance leaves. *Genes*, 14 (1) 163. https://doi.org/10.3390/genes14010163.

[29] Chebet, J. J., Ehiri, J. E., McClelland, D. J., Taren, D., Hakim, I. A., Effect of d-limonene and its derivatives on breast cancer in human trials: a scoping review and narrative synthesis. *BMC cancer*, 21 (1) (2021) 902. https://doi.org/10.1186/s12885-021-08639-1.

[30] Alipanah, H., Farjam, M., Zarenezhad, E., Roozitalab, G., Osanloo, M., Chitosan nanoparticles containing limonene and limonene-rich essential oils: potential phytotherapy agents for the treatment of melanoma and breast cancers. *BMC Complementary Medicine and Therapies*, 21 (1) (2021), 186. https://doi.org/10.1186/s12906-021-03362-7.

[31] Hou, J., Zhang, Y., Zhu, Y., Zhou, B., Ren, C., Liang, S., Guo, Y., α-Pinene induces apoptotic cell death via caspase activation in human ovarian cancer cells. *Medical Science Monitor*, 25 (2019), 6631–6638. https://doi.org/10.12659/MSM.916419.