ANADOLU UNIVERSITY
FACULTY OF PHARMACY

European Journal of Life Sciences 2024; 3: 21-30

Research Article

Comparison of apoptotic effects of lupeol on A549 and C6 cell lines

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https://doi.org/10.55971/EJLS.1466309

Received: 07.04.2024
Accepted: 22.04.2024
Available online: 30.04.2024

ABSTRACT

Lupeol compound is a cyclic triterpene alcohol that is widely found in plants. The compound lupeol has been reported to exhibit antitumor, anti-inflammatory, anxiolytic, neuroprotective and hepatoprotective effects. Recent research shows that lupeol could be a potential medicine for various diseases and also an adjuvant for intractable diseases. Cancer poses a health threat that is increasingly common around the world. Among new cancer cases, lung cancer is one of the most common and deadly cancers worldwide. In this study, the anticancer efficiency of lupeol on human lung adenocarcinoma A549 and glioma C6 cell lines were examined. Various concentrations of lupeol (500, 250, 125, 62.5, 31.25, 15.62, 7.81, 3.90 µM) effectually reduced cell viability in the A549 and C6 cell lines in a dose-dependent manner. These results showed that lupeol had selective anticancer activity against A549 and C6 cell lines.

Keywords: Adenocarcinoma, Cytotoxicity, Glioma, Lung cancer, Lupeol

1. INTRODUCTION

Cancer poses a health threat that is increasingly common around the World [1]. According to actual cancer epidemiological statistics collected from the official sources of the World Health Organization (WHO) and the American Cancer Society (ACS), cancers constitute the largest burden worldwide (244.6 million). It is prevalent in both men (137.4 million) and women (107.1 million), ensued by ischemic heart disease (203.7 million) and stroke (137.9 million) [2].

Lung cancer maintains the leading cause of cancer-related deaths around the world, driven by increased cigarette consumption [3]. Lung cancer is divided into two according to pathological types: non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC), and NSCLC constitutes 85% of all lung cancer cases [4]. Current lung cancer therapeutic agents are expensive, ineffective, and have serious adverse effects and toxicity on non-cancerous tissue [5].

Gliomas are tumors originating compose of glial cells, which are the most extensive tumors of the central nervous system and constitute 81% of malignancies. They generally arise from glial or pioneer cells and evolve into astrocytoma, oligodendroglioma, ependymoma, or oligoastrocytoma, respectively. As regards to the WHO gradation, gliomas are segreated into four grades. Grade 1 and grade 2 gliomas describe low-grade ones, while grade 3 and grade 4 gliomas remark high-grade gliomas. In general, a relatively

high grade is associated with bad prognosis. While the average survival time for low-grade glioma is 11.6 years, the average survivance time for grade 3 glioma patients is approximately 3 years and for grade 4 glioma is 15 months. Glioblastoma is the most common type of grade 4 glioma [6].

In recent times, researchers have been increasingly interested in natural products, particularly compounds derived from plants, due to their potential as anti-tumor agents. The herb *Portulaca oleracea* L., used in traditional Chinese medicine to treat various types of inflammation and tumors in the digestive tract, contains lupeol terpenoid and has been proven effective like a therapeutic agent in the treatment of colitis associated cancer [7]. Lupeol compound is a cyclic triterpene alcohol (Figure 1) that is widely found in plants [8]. *S. surattense*, a Solanaceae plant, contains the phytochemical lupeol, and the phytochemicals in members of this family are known to generally have anticancer properties [9]. Lupeol is a pentacyclic triterpenoid found in *Ziziphi Spinosae Semen* oil, and it has been studied that the lupeol compound is effective in the plant's sleep-enhancing effect through neurotransmitter regulation and anti-oxidative stress mechanisms [10]. Lupeol is also found in pitaya (*Hylocereus undatus*) fruits and is the wax component of the fruit [11]. The compound lupeol has been reported to exhibit antitumor, anti-inflamatory, anxiolytic, neuroprotective and hepatoprotective effects [12]. The wound amelioration occasion of the ethanolic extract of the bark of *Thespesia populnea* L. was investigated and further phytochemical searches of the bioactive fractions caused the isolation of many compounds belonging to various chemical classes, with the compound lupeol acetate isolated and evaluated as potent wound healers through their antioxidant and anti-inflammatory activities as well as stimulating collagen synthesis [13]. Despite the limited water solubility and bioavailability of lupeol have restricted its therapeutic use, lupeol-chitosan nanoparticles encapsulated in cellulose acetate membranes were synthesized to triying their potential in treating diabetes and cancer. Not only did the *in vitro* assay using A431 human skin cancer cells reveal anticancer efficiency with an IC_{50} value as low as 54.56 mg/mL, the membrane also showed significant antidiabetic potential, effectively inhibiting carbonhyrate-digesting enzymes [14]. The synergistic effect and mechanism of a nanoliposome carrier combined with FDA-approved indocyanine green (ICG)-mediated optical therapy and the natural molecule lupeol in enhancing natural killer (NK) cell activity for colon cancer cell inactivation were investigated. This nanoliposome shows that it reduces colon cancer cell activity to 59.6% after 20 minutes of irradiation and to 43.4% after 20 minutes + 10 minutes of irradiation. When NK cells were added after 20 minutes + 10 minutes of irradiation, the activity decreased to 16.7%, providing a new approach in the treatment of colon cancer, proving its anti-cancer effect [15]. According to these data, we planned to examine the anticancer activity and apoptotic effect of lupeol on A549 and C6 cells.

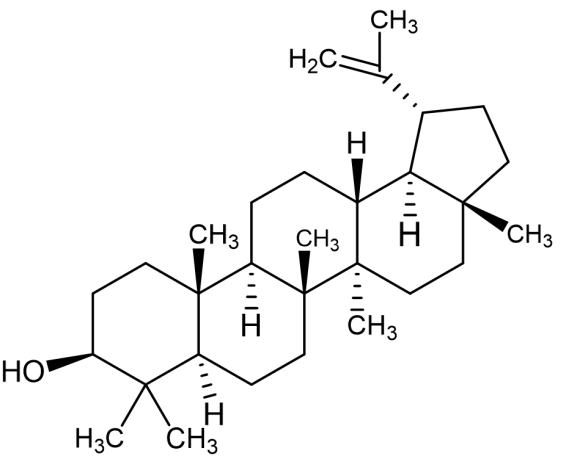

Figure 1. Chemical structure of lupeol.

2. MATERIALS AND METHODS

2.1. Materials

The A549 and C6 cell lines were bought from American Type Culture Collection. Lupeol (S957712) and Cisplatin were acquired from Sigma-Aldrich. Dulbecco's Modified Eagle Medium, penicillin streptomycin and fetal bovine serum were from Gibco, Phosphate-buffered saline tablets were from Sigma-Aldrich, MTT was from Alfa Aesar, and dimethylsulfoxide (DMSO) was from Sigma-Aldrich The Annexin -V FITC/Propidium iodide (PI) apoptosis detection kit was bought from BD Biosciences.

2.2. Methods

2.2.1. Cell Line Model

A549 and C6 cells were keeped in Dulbecco's Modified Eagle's Medium (DMEM) and was added with antibiotics (100 µg/ml streptomycin and 100 U/ml penicillin G) and 10% fetal bovine serum (FBS) in a incubator at 37°C (5% CO_2 and 95% air).

2.2.2 MTT Assay for Cytotoxicity

One of the most popular techniques for assessing cell viability, cytotoxicity, and proliferation is MTT [16]. Metabolically active cells reduce yellow tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, or MTT) to purple formazan crystals, which forms the basis of this colorimetric assay. The A549 and C6 cells were cultivated for 24 hours at 37°C in a humidified environment with 5% CO_2 in the air at a density of $2x10^4$ cells per well on flat-bottomed 96-well plates with different concentrations (500, 250, 125, 62.5, 31.25, 15.62, 7.81, 3.90 µM) of lupeol and cisplatin.

Lupeol was applied at various concentrations (500, 250, 125, 62.5, 31.25, 15.62, 7.81, 3.90 µM) to a 96-well plate with $2x10^4$ L929, A549 and C6 seeded into each well. Following the incubation period, 20 µL of phosphate-buffered saline (PBS) was added to each well to dissolve the MTT powder (5 mg/mL). The cells were then incubated at 37°C for 2-4 hours. After the waiting period, 100 µl of DMSO was administered to each well after the medium was removed. Cells were measured with a Bio-Tek microplate reader at 540 nm. Cell viability was measured as a percentage relative to control cells for each concentration and this was repeated in three wells. The IC_{50} concentration that provided 50% inhibition of the cell population was found based on the computation findings.

2.2.3. Flow cytometric analysis: Annexin-V for early/late apoptosis

In a 6-well plate, $2x10^5$ cells/mL of A549 and C6 cells were seeded. It was incubated in a moisturized environment with 5% CO_2 in the air for 24 hours at 37°C. Next, cisplatin and lupeol at their IC_{50} doses were appended to the cells and incubated for a further 24 hours. At the end of this period, cells were collected from the plate and placed in flow tubes and then centrifuged at 1200 rpm for four minutes. Following centrifugation, the supernatant was removed with care, twice more centrifuged, and then cleaned with 1 milliliter of cold 1x PBS. After centrifugation, the supernatant was collected and 100 µl of the assay buffer (1x) was pipetted gently into each flow tube. Subsequently, 5 microliters of Annexin-V and 5 microliters of PI dyes were added, and the mixture was left in the dark for 15 minutes.

After this time, 400 µl of assay buffer was added, and the data was examined using FACS Diva Version 6.1.1 software and flow cytometry (CytoFLEX Beckman Coulter).

2.2.4. Statistical analysis

IC_{50} doses and percent inhibition were calculated compared to the control group. All data taken into account were indicated as the standard deviation (SD) of the mean from three repetition of the experiments. Microsoft Excel software and GraphPad Prism 8 were used to calculate the data.

3. RESULTS AND DISCUSSION

3.1. MTT assay for cytotoxicity

In our study, the cytotoxic impacts of lupeol on A549 and C6 cells were enquired using the *in vitro* MTT test On the other part, we used L929 cells to analyze the selectivity of lupeol. Cytotoxicity and IC_{50} values for lupeol and cisplatin in A549 and C6 cells were designated by MTT analysis. The dose and percent viability values used in the analysis are as shown in Figure 2, 3. This cell line was treated with a wide range of lupeol and cisplatin concentrations (500, 250, 125, 62.5, 31.25, 15.62, 7.81, 3.90 µM) for 24 hours. Lupeol suppressed A549 cell viability in a dose-dependent manner (Figure 2).

IC_{50} values of lupeol and cisplatin were determined in A549 cells as 483.24 µM and 49.78 µM for 24h respectively (Table 1). Although there are results supporting our findings, lupeol has been observed to exhibit different sensitivity with different cells. In a recent study, results supporting our findings were acquired. In the study directed by Aborehab et al. (2023), the IC_{50} value of lupeol in A549 cells was 344.8 µg/mL [17]. For example, in a study directed by Li et al. in 2013, the cytotoxicity of lupeol was examined in various cell lines such as A549, LAC, HepG2 and HeLa. Lupeol showed various cytotoxicity in these cell lines [18].

IC_{50} values of cisplatin and lupeol were specified in C6 cells as 287.23 µM and 29.84 µM for 24h respectively (Table 2). Also, IC_{50} value of lupeol was specified in L929 cells as 248.30 µM for 24h (Table 3). The IC_{50} value of lupeol was 248.30 µM for 24 h. in L929 cell line (Table 3 and Figure 4E). The dose-dependent percentage viability of lupeol in A549, C6 and L929 cell lines is shown in Figure 5.

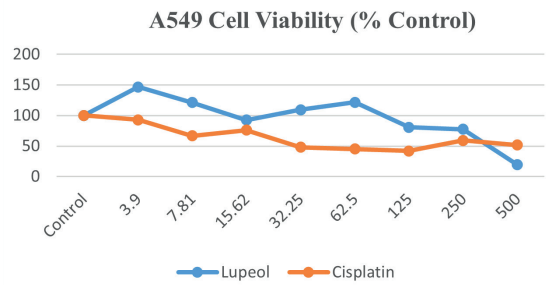

Figure 2. Dose-dependent percent viability graph of lupeol and cisplatin on A549 cells after 24 hours incubation.

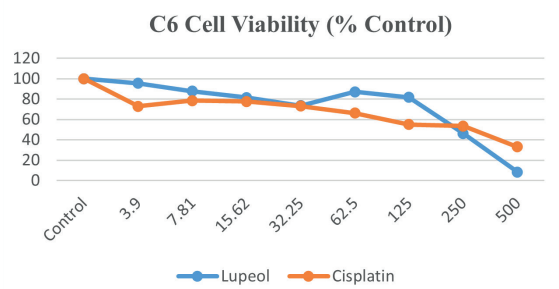

Figure 3. Dose-dependent percent viability graph of lupeol and cisplatin on C6 cells after 24 hours incubation.

Table 1. IC_{50} (µg/mL)[a] values of lupeol and cisplatin against A549 cell line

	IC_{50} (µM) A549	IC_{50}/2 (µM) A549
Lupeol	483.24 ± 148.93	241.62 ± 74.46
Cisplatin	49.78 ± 15.43	24.89 ± 7.71

[a]Cytotoxicity of lupeol and cisplatin. Incubation for 24 hours. The values represent mean ± standard deviation of triplicate determinations.

Table 2. IC_{50} (µg/mL)[a] values of lupeol and cisplatin against C6 cell line

	IC_{50} (µM) C6	IC_{50}/2 (µM) C6
Lupeol	287.23 ± 2.46	143.62 ± 1.23
Cisplatin	29.85 ± 4.05	14.92 ± 2.64

[a]Cytotoxicity of lupeol and cisplatin. Incubation for 24 hours. The values represent mean ± standard deviation of triplicate determinations.

Table 3. IC_{50} (µg/mL)[a] value of lupeol for L929 cell line

	IC_{50} (µM) L929
Lupeol	248.30 ± 2.64

[a]Cytotoxicity of lupeol. Incubation for 24 hours. The values represent mean ± standard deviation of triplicate determinations.

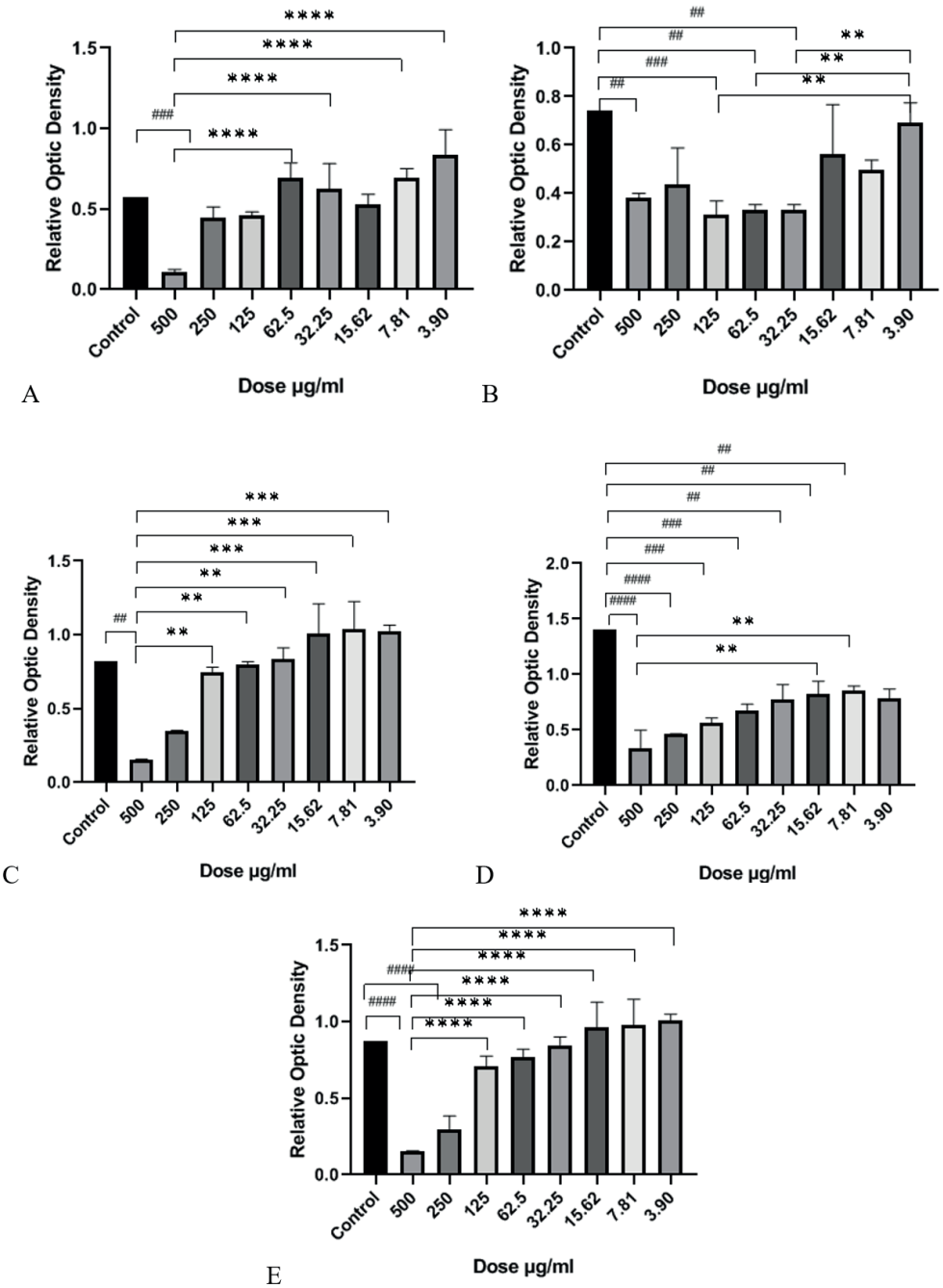

Figure 4. A) After MTT analysis relative optic density of lupeol on A549 cell line after 24 hours of incubation. ### p <0.005 significantly from control , ****p<0.0001 significantly different from 500 µg/mL dose **B)** Relative optic density of cisplatin on A549 cell line after 24 hours of incubation. ### p <0.005 significantly from control, ** p<0.005 significance values between groups **C)** After MTT analysis relative optic density of lupeol on C6 cell line after 24 hours of incubation. ## p <0.005 significantly from control, **, ***p<0.005 significantly different from 500 µg/mL dose **D)** Relative optic density of cisplatin on C6 cell line after 24 hours of incubation. ##, ### p <0.005, ####<0.0001 significantly from control, ** p<0.005 significantly different from 500 µg/mL dose. **E)** Relative optic density of lupeol on L929 cell line after 24 hours of incubation. The experiment was repeated three times and the data are presented as mean± S.D. #### p<0.0001 significantly different from control. ****p<0.0001 significantly different from 500 µg/mL dose.

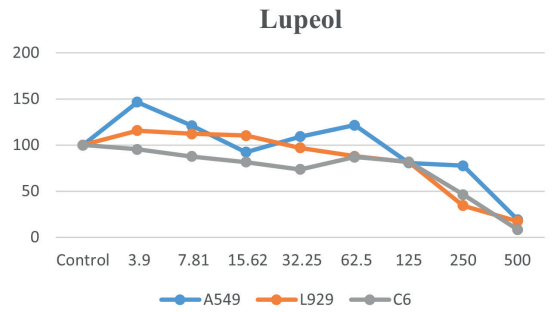

Figure 5. Dose-dependent percent viability graph of lupeol on A549, C6 and L929 cells. Lupeol was also used on L929 murine fibroblast cells and compared with A459 cells to establish a control group while determining the reliable IC_{50} dose. Data were obtained as a result of MTT analysis.

In 2019, Lemes et al. showed that the triterpene lupeol, one of the isoflavonoids they isolated from the *Deguelia costata* plant, was ineffective in the 48 hours cytotoxicity test on C6 glioma cells. However, in our study, activity was found after 24 hours of incubation. This suggests that the effect of lupeol varies over time [19]. The findings of our study indicate that lupeol has selective anticancer activity against A549 and C6 cell lines.

3.2. Flow cytometric analyses by annexinV-FITC

After 24 hours of incubation with IC_{50} and $IC_{50}/2$ doses of lupeol and cisplatin in A549 and C6 cells, readings were made using the kit procedure (BD, Pharmingen) on a flow cytometry device (Beckman Coulter) using Cytoflex Software.

According to the MTT result, we examined the mechanism of action of cell death caused by lupeol in A549 and C6 cells. IC_{50} and $IC_{50}/2$ values of lupeol were used to determine the apoptotic effects of lupeol (Figure 6 and Table 4). Annexin-V binding capacity was examined by flow cytometry by applying lupeol to A549 and C6 cell lines.

After incubation of A549 cells, lupeol and cisplatin at IC_{50} and $IC_{50}/2$ doses, respectively, early apoptotic cell percentages were determined as 13.21 %, 4.39 % and 7.21 %, 5.76 %. Lupeol is identified as an effective apoptosis-inducing agent in various cell lines. However, the mechanism of action varies depending on cell line types. Additionally, the doses of lupeol used to increase apoptosis percentages vary in different studies. For example, in the study of Jin et al., SMMC-7721 cells were evaluated in terms of untreated control cells. In mentioned study, the

Table 4. Percents of typical quadrant analysis of annexin V-FITC/propidium iodide flow cytometry of A549 cells treated with the lupeol and cisplatin

Groups	% Early	% Late	% Viable
Control (untreated)	3.37	3.91	90.66
Lupeol IC_{50} dose treated cells	13.21	21.70	59.46
Lupeol $IC_{50}/2$ dose treated cells	4.39	3.99	91.05
Cisplatin IC_{50} dose treated cells	7.21	4.42	87.96
Cisplatin $IC_{50}/2$ dose treated cells	5.76	3.37	90.68

Table 5. Percents of typical quadrant analysis of annexin V-FITC/propidium iodide flow cytometry of C6 cells treated with the lupeol and cisplatin

Groups	% Early	% Late	% Viable
Control (untreated)	4.54	1.24	94.21
Lupeol IC_{50} dose treated cells	7.30	1.31	91.38
Lupeol $IC_{50}/2$ dose treated cells	15.96	4.64	79.26
Cisplatin IC_{50} dose treated cells	9.95	1.63	88.41
Cisplatin $IC_{50}/2$ dose treated cells	4.95	0.98	94.06

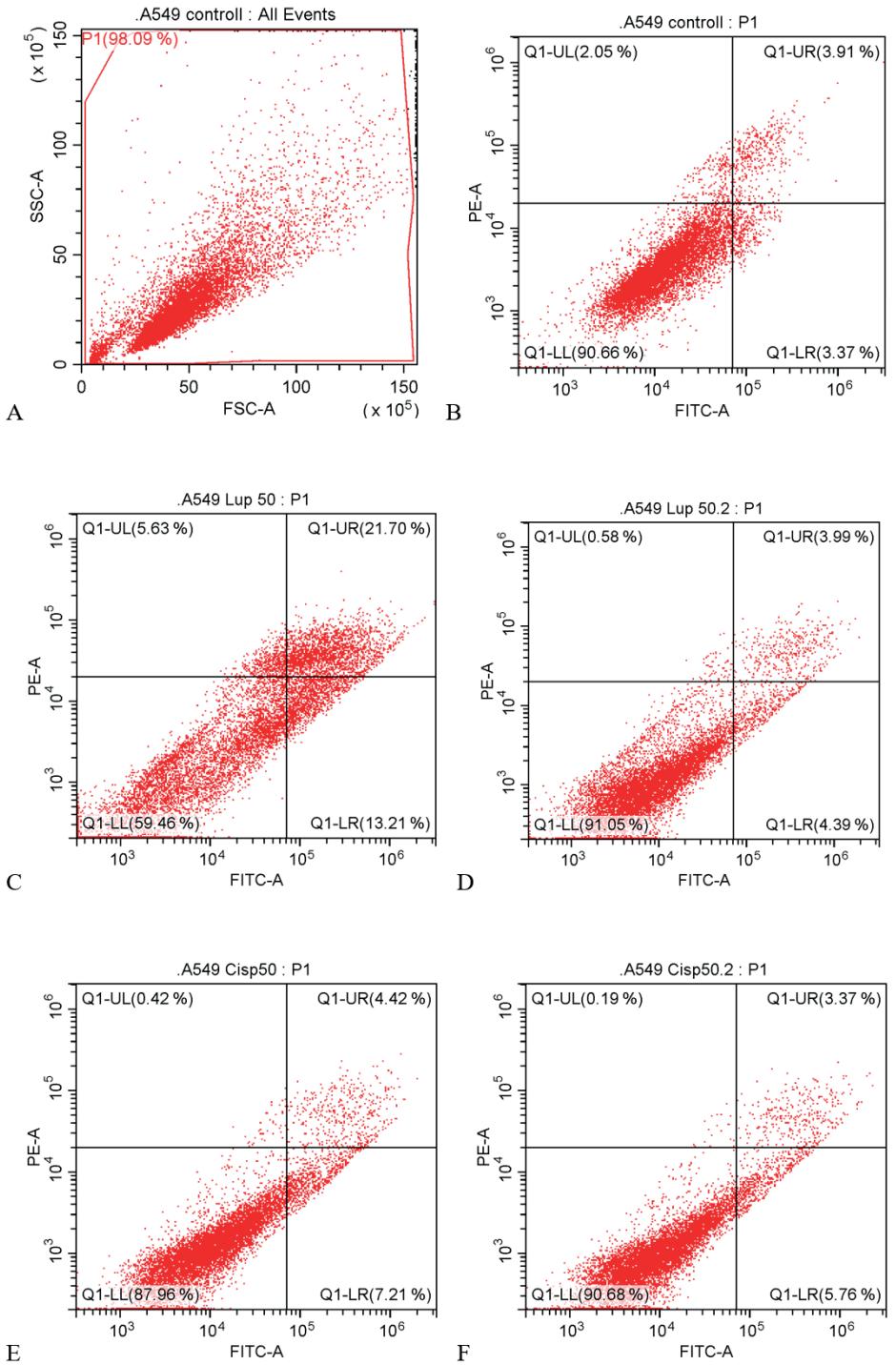

Figure 6. Flow cytometric analysis of A549 cells treated with IC_{50} and $IC_{50}/2$ concentrations of lupeol and cisplatin. A549 cells were cultured for 24 h in medium containing 48.24 and 241.62 µM lupeol and 49.78 and 24.89 µM cisplatin. At least 10,000 cells per sample were analyzed and quadrant analysis was performed. Q1-LR: Early apoptotic cell percentages, Q1-UR: late apoptotic cell percentages, Q1-UL: Necrotic cell percentages, Q1-LL: viable cell percentages. A) Main gate selected from the cell population, B) Control group, C) Apoptotic effect of IC_{50} dose of lupeol, D) Apoptotic effect of $IC_{50}/2$ dose of lupeol, E) Apoptotic effect of IC_{50} dose of cisplatin, F) Apoptotic effect of $IC_{50}/2$ dose of cisplatin.

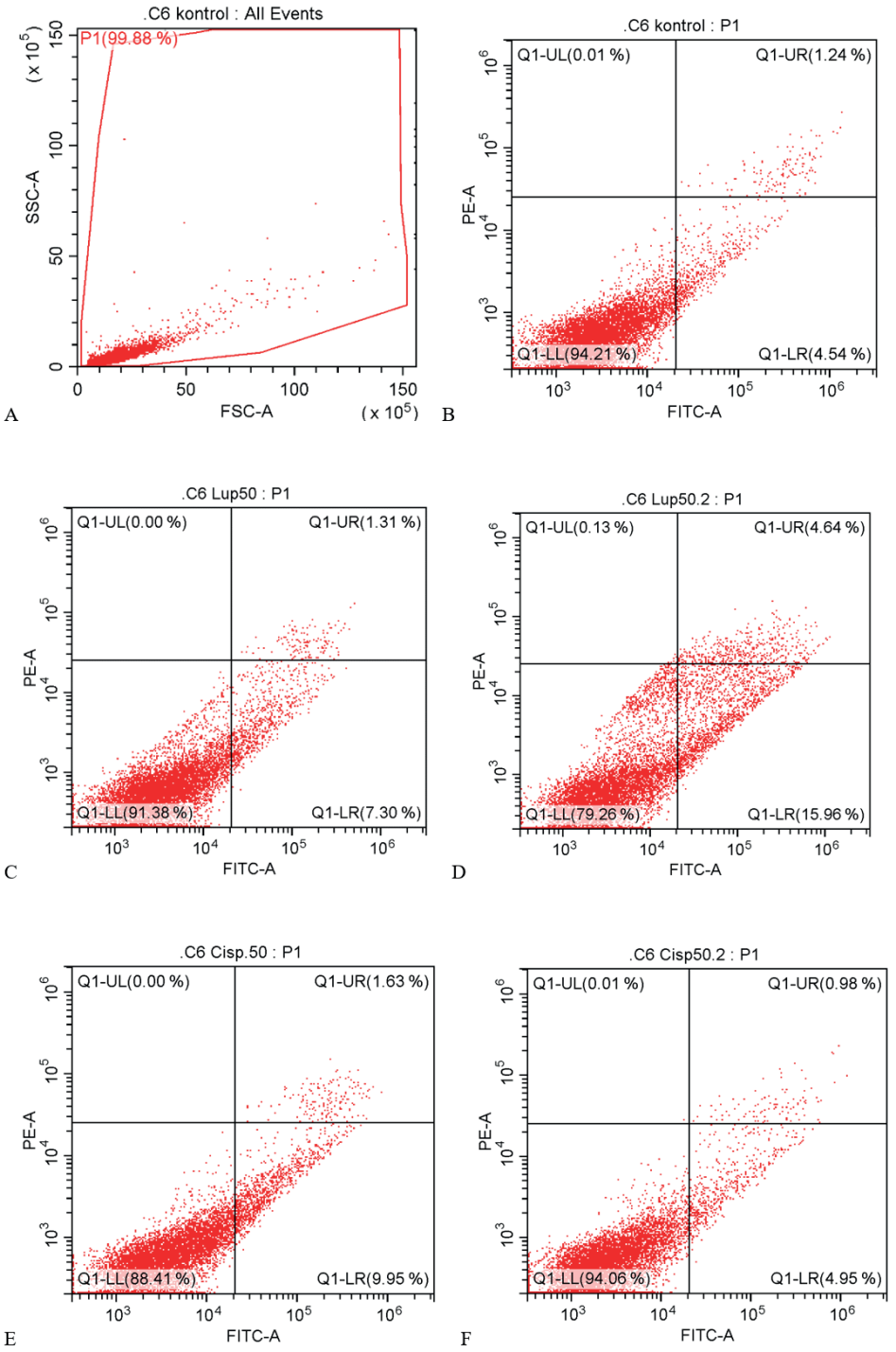

Figure 7. Flow cytometric analysis of C6 cells treated with IC_{50} and $IC_{50}/2$ concentrations of lupeol and cisplatin. C6 cells were cultured for 24 h in medium containing 287.23 and 143.62 and 241.62 µM lupeol and 29.85 and 14.92 µM cisplatin. At least 10,000 cells per sample were analyzed and quadrant analysis was performed. Q1-LR: Early apoptotic cell percentages, Q1-UR: late apoptotic cell percentages, Q1-UL: Necrotic cell percentages, Q1-LL: viable cell percentages. A) Main gate selected from the cell population, B) Control group, C) Apoptotic effect of IC_{50} dose of lupeol, D) Apoptotic effect of $IC_{50}/2$ dose of lupeol, E) Apoptotic effect of IC_{50} dose of cisplatin, F) Apoptotic effect of $IC_{50}/2$ dose of cisplatin.

researchers aimed to induce apoptosis in SMMC-7721 cells by combining lupeol treatment and radiation. As a result of lupeol treatment combined with radiation, an increase in apoptotic cells was observed (48.48% for 4 Gy plus lupeol, 10.65% for control) [20]. At the same time, Yuan et al. showed that lupeol can cause apoptosis in A431 and LNCaP cells via mitochondrial cell death [21].

After incubation of C6 cells, lupeol and cisplatin at IC_{50} and $IC_{50}/2$ doses, respectively, early apoptotic cell percentages were determined as 4.54 %, 15.96 % and 9.95 %, 4.95 % (Figure 7, Table 5). In line with all this information, it was observed that although lupeol triggered apoptosis dose-dependently in the A549 cell line, it triggered dose-dependent apoptosis in the C6 cell line.

4. CONCLUSION

According to our data, the lupeol compound is thought to have dose-dependent anticancer properties on A549 cell line. In addition to these results, it was determined that lupeol caused apoptotic cell death in A549 and C6 cells. The apoptotic mechanism of this cell death was determined by the interaction of the Annexin V protein that a calcium-binding protein, with the released phosphotidylserine, and the resulting necrotic and living cells can be distinguished by PI stain. When we evaluate our findings according to our literature review, lupeol shows a dose-dependent effect in the A549 cell line, but not in the C6 cell line. This suggests that this difference varies depending on the cell type. Although lupeol is thought to be a key agent with anticancer and antiproliferative properties in the light of this study, more studies are needed on this subject.

Acknowledgements

Figure 1 was created with ChemDraw19.0 software.

Ethical approval

Not applicable. Because in this article only cell lines were used that were obtained from ATCC.

Author contribution

Concept: İE, ŞKA, HET; Design: İE, ŞKA, HET; Supervision: İE, ŞKA, HET; Materials: İE, ŞKA, HET; Data Collection and/or Processing: İE, ŞKA, HET; Analysis and/or Interpretation: İE, ŞKA, HET; Literature Search: İE, ŞKA, HET; Writing: İE, ŞKA, HET; Critical Reviews: İE, ŞKA, HET.

Source of funding

This research received no grant from any funding agency/sector.

Conflict of interest

The authors declared that there is no conflict of interest.

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