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Research Article

Capsaicin inhibits proliferation and induces apoptosis in human lung adenocarcinoma A549 cell line

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ABSTRACT

Capsaicin is the main component of many hot peppers, exerts anticancer effects on various cancer cells by inducing apoptotic pathways. In addition, the ineffectiveness of this apoptotic effect of capsaicin on healthy cells provides a wide-ranging use of capsaicin. In recent years, many studies have been carried out to determine the safety of capsaicin use in lung cancer, which is a common cancer type worldwide with high mortality rates. In the present study anticancer effects of capsaicin were studied on A549 human lung adenocarcinoma cell line. Also, citotoxicity of this compound was studied on L929 mouse embryonic fibroblast cell line. Various concentration of capsaicin (3.90625-500 µM) effectively decreased cell viability in A549 cell line in a dose-dependent manner. Also, this antiproliferative effect of capsaicin was selective compared to L929 cell line. On the other hand, apoptosis inducing effects of capsaicin were studied by Annexin V-FITC and Caspase 3 assays by using IC_{50} and $IC_{50/2}$ concentrations on A549 cell by flow cytometric methods. Our results revealed that the anticancer effects induced by capsaicin on A549 cell line involved apoptosis by inducing Caspase 3 pathway.

Keywords: Capsaicin, A549, Apoptosis, Flow cytometry

1. INTRODUCTION

Lung cancer is the most common type of cancer in the globe. The impact of environmental variables is especially essential in the etiology of lung cancer, with cigarette smoke being the single most important causative agent. Other relevant concerns include radiation exposure and a variety of occupational-related hazards, the most well-known of which being asbestos. Lung cancer is classified into several histological categories, the most common of which are adenocarcinoma and squamous cell carcinoma (25-40 percent each), followed by small cell lung cancer (20-25 percent), and large cell carcinoma (20-25 percent). A succession of genetic mutations are necessary for the formation of a malignant lung carcinoma, as is the case for the majority of human cancers. K-ras mutations are thought to be particularly responsible for adenocarcinomas. Small cell lung cancer is linked to the oncogene myc, and several lung malignancies have been linked to mutations in the tumor suppressor gene p53 [1]. Various medications for molecular targeted treatment and immunotherapy have been developed and authorized for clinical use up to this point. Despite the advancement of these treatments, lung cancer remains the greatest cause of cancer-related mortality, accounting for an anticipated 1.8 million deaths in 2020. New treatment approaches are urgently required because the majority of these fatalities ultimately result from medication resistance and poor sensitivity. Recently, it has become clear that controlling the metabolism of cancer offers

great potential for combating treatment resistance and boosting medication effectiveness. The primary barriers to successful treatment include a lack of early diagnosis, a poor prognosis, the development of resistance to chemotherapeutic medicines, and a five-year survival rate of fewer than 15% of the cancer population. [2,3].

8-methyl-N-vanilyl-trans-6-nonenamide, which we know as capsaicin and is a homovalinic acid derivative is the active ingredient of Capsicum annum chili peppers. Although it is known as a spice around the world, it is used as a medicine with its anti-inflammatory, anti-analgesic, analgesic and anti-obesity effects, and it has a wide place in the literature. Cluster headaches, rheumatoid arthritis, post-mastectomy pain syndrome, diabetic neuropathy, and herpes zoster are just a few of the conditions that capsaicin has been used to treat [4]. Particularly lung cancer, have been the subject of in-depth research on the action of capsaicin, which may have anticancer and antiproliferative properties [3-5]. Most often, capsaicin-mediated cytotoxicity is linked to cancer cell apoptosis through the activation of a number of processes, such as the production of reactive oxygen species (ROS), the beginning of endoplasmic reticulum (ER) stress, and changes in protein kinases [6,7].

Both apoptosis and autophagy are tightly controlled processes involved in several crucial cellular activities, however apoptosis often serves as a mechanism for self-destruction as opposed to autophagy's cytoprotective function. Binding to death receptors starts the extrinsic pathway of apoptosis, which then activates initiator caspase-8 and effector caspases [6]. In the intrinsic pathway, the BCL-2 family proteins (Bax and Bak) assemble into oligomers in the mitochondria's outer membrane, causing cytochrome c to leak out into the intermembrane space. When cytochrome c is released, apoptosomes are formed, containing Apaf-1 and caspase-9. Caspase-9 then activates downstream caspases, such caspase-3, by proteolytic cleavage [8].

In the light of the reported data we have planned to study anticancer activity of capsaicin on A549 cell line. Capsaicin was screened to determine it's cytotoxic effects on A549 and L929 cell lines. Also apoptotic properties of it was studied on A549 human lung adenocarcinoma cell lines.

2. MATERIALS AND METHODS

2.1. Materials

The A549 cells were purchased from American Type Culture Collection (ATCC, USA). Cisplatin and capsaicin were from Sigma-Aldrich (USA), Dulbecco's Modified Eagle Medium (DMEM) was from Gibco (Brasil), fetal bovine serum (FBS) and penicillin streptomycin were from Gibco (South America, USA), Dulbecco's phosphate-buffered saline concentrate (10X) was from Biological Industries (Israel), MTT was from Alfa Aesar (Germany), and dimethylsulfoxide (DMSO) was from Sigma-Aldrich. The Annexin-V FITC/ propidium iodide (PI) apoptosis detection kit, caspase-3 kit were purchased from BD Biosciences (USA). The Annexin-V FITC were purchased from Santa Cruz Biotechnology (USA).

2.2. Methods

2.2.1. Cell Line Model

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

2.2.2. MTT Assay for Cytotoxicity

This colorimetric analysis is based on the reduction of yellow tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide or MTT) to pink formazan crystals by metabolically active cells. MTT is one of the most widely used methods work to determine for cell viability, cytotoxicity, and proliferation [9]. The A549 cells were cultured at a density of $5x10^3$ cells per well in flat bottomed 96-well plates with various concentrations (500, 125, 62.50, 31.25, 15.625, 7.813, 3.906 µM) of capsaicin and cisplatin for 24h at 37°C in a humidified atmosphere containing 5% CO_2 in air.

Various concentrations (500, 125, 62.50, 31.25, 15.625, 7.813, 3.906 µM) of capsaicin were applied to L929 cells inoculated with $5x10^3$ cells in each well in a 96-well plate, and the same incubation conditions as A549 cells were provided. At the end of incubation, MTT powder (5 mg/mL) dissolved in phosphate-buffered saline (PBS) for each well (20 µL). The cells were again incubated for 2-4 h at 37◦C. At the end of the waiting period, the media in the wells was removed and 100 µl of DMSO was added to each well. The cells were read at 540 nm using a microplate reader (Bio-Tek). Every concentration was repeated in three wells and cell viability was calculated as a percent ratio relative to control cells. According to the calculation results, the IC_{50} concentration, which provides the inhibition of 50% of the cell population, and the $IC_{50/2}$, which provides half of the 50% inhibition, were determined.

2.2.3. Flow cytometric analysis

Early evaluation of tumor response in intensively applied chemotherapy is important in terms of quality of life. Induction of apoptosis is the primary mechanism in the death of tumor cells. Annexin-V and caspase-3 assays, which have high affinity for apoptotic cells, have been frequently used imaging methods in the detection of apoptosis [10].

2.2.3.1. Annexin-V for early/late apoptosis

A549 cells were seeded in a 6-well plate at 10^5 cells/mL per well. It was incubated for 24 hours at 37°C in a humidified atmosphere containing 5% CO_2 in air. Then, determined with MTT IC_{50} and $IC_{50/2}$ doses of cisplatin and capsaicin were applied to the cells and incubated again for 24 hours. At the end of this period, the cells on the plate were removed and placed in flow tubes, centrifuged at 1200 rpm for 4 minutes. After centrifugation, the supernatant was carefully discarded, washed with 1 ml of cold 1X PBS, centrifuged again, and this was done twice. After centrifugation, the supernatant was removed and 100 µl of the assay buffer prepared as 1X was poured into each flow tube and pipetted slowly. Then 5 µl Annexin-V and 5 µl of PI dyes were added and incubated in the dark for 15 min. At the end of this period, 400 µl of assay buffer was added and analyzed. After centrifugation, the supernatant was discarded and 100 µl of the assay buffer prepared as 1X was poured into each flow tube and pipetted slowly. Then, 5 µl of Annexin-V and 5 µl of PI dyes were added and left in the dark for 15 minutes. At the end of this period, 400 µl of assay buffer was added and analyzed by flow cytometry (CytoFLEX Beckman Coulter) using FACS Diva Version 6.1.1. Software.

2.2.3.2. Analyses for Caspase-3

A549 cells were seeded in a 6-well plate at 10^5 cells/mL per well. It was incubated for 24 hours at 37°C in a humidified atmosphere containing 5% CO_2 in air. Cells were treated with calculated IC_{50} and $IC_{50/2}$ doses of cisplatin and capsaicin for 24 hours. Then, the cells on the plate were removed and placed in flow tubes and centrifuged at 1200 rpm for 4 minutes. After centrifugation, the supernatant was carefully removed and washed with 1 ml of cold 1X PBS, centrifuged again, this procedure was done twice. After centrifugation, the supernatant was discarded and 500 µl of Cytofix/Cytoperm solution was added to each tube and the tubes were kept on ice for 20 min. At the end of this period, it was centrifuged again. The supernatant was discarded, 500 µl of Permwash solution was added to each tube and centrifuged again, this process was repeated twice. The solution prepared by adding 20 µl of antibody into 100 µl of 1X Perm/Wash solution was added to the tubes and the cells were incubated for 30 minutes at room temperature. At the end of this period, it was centrifuged, the supernatant was discarded and 1 ml of Permwash solution was added and centrifuged again. Finally, 0.5 ml of Permwash was added and analyzed by flow cytometry (CytoFLEX Beckman Coulter) using FACS Diva Version 6.1.1. Software.

3. RESULTS AND DISCUSSION

3.1. MTT assay for cytotoxicity

In our study, the cytotoxic and apoptotic effects of capsaicin on A549 lung adenocarcinoma cells were investigated using in vitro MTT, Annexin V-FITC, and Caspase-3 assays. Cytotoxicity and IC_{50} values for cisplatin and capsaicin in A459 and L929 cells were determined by MTT analysis. (Tables 1 and 2). The dose and percent viability values used in the analysis are as shown in the Figures 1 and 2.

Table 1. IC_{50} and $IC_{50/2}$ dose chart for capsaicin and cisplatin for A549 cells

	IC_{50} (µM)	$IC_{50/2}$ (µM)
Cisplatin	59,233 ±	29,617 ±
Capsaicin	183,268 ±	91,634 ±

Table 2. IC_{50} table of capsaicin for L929 and A549 cells

	L929 cells	A549 cells
IC_{50} (µM)	245.569 ±	183.268 ±

These cell lines were treated with a wide range of concentrations of capsaicin and cisplatin (500, 250, 125, 62.50, 31.25, 15.625, 7.8125, 3.9063 µM) for 24 h (Table 1). Capcaicin inhibited A549 cell viabilities in a dose and time dependent manner (Figure 1). IC_{50} values of capsaicin and cisplatin were determined in A549 cells as 183,268 µM and 59,233 µM for 24h respectively (Table 1). Also, IC_{50} value of capcaicin was determined in L929 cells as 245.569 µM for 24h (Table 2). The viability of cells was decreased when the dose of capsaicin was increased (Figures 1 and 2). In the previous studies with different cell lines have obtained results supporting our findings. Liu and his colleagues (2022) discovered that IC_{50} value of capsaicin was 200 µM in A549 cells for 48 h. whereas the need was 100 µM for NCI-H23 cells for obtaining IC_{50} value [9]. The value was established to be approximately 200µM for 24 h for A549 cell lines by Lewinska and her co-workers similar as in our study [10]. Through the MTT assay, we found IC_{50} value of capsaicin as 183,268 µM in A549 cells for 24 h. On the other hand different cancer cell lines exhibit differential sensitivity to capsaicin, suggesting that the capcaisin effects vary based on the cell type. For example, the IC_{50} of capsaicin in human leukemia H60 cell was found to be 100µM for 24 hours [11], while the IC_{50} value of capsaicin was found to be 200µM for 24 hours in human glioblastoma cells [12], while the IC_{50} value of capsaicin in breast cancer cell was found to be 200µM for 72 hours [13]. On the other hand, we used L929 cells to examine selectivity of capsaicin. The IC_{50} value of capsaicin was 245.569 µM for 24 h. in L929 cell line (Table 2 and Figure 2). This result shows that capsaicin had selective antiproliferative activity against A549 cell line.

3.2. Flow cytometric analyses by annexinV-FITC

After the A549 cells were incubated with capsaicin and cisplatin at IC_{50} and $IC_{50/2}$ doses for 24 hours, reading was performed on the flow cytometry device (Beckman Coulter) using Cytoflex Software by applying the kit procedure (BD, Pharmingen). After this MTT result we examined mechanism of action of cell death on A549 cells caused by capsaicin. IC_{50} and $IC_{50/2}$ values of capsaicin were used to determine

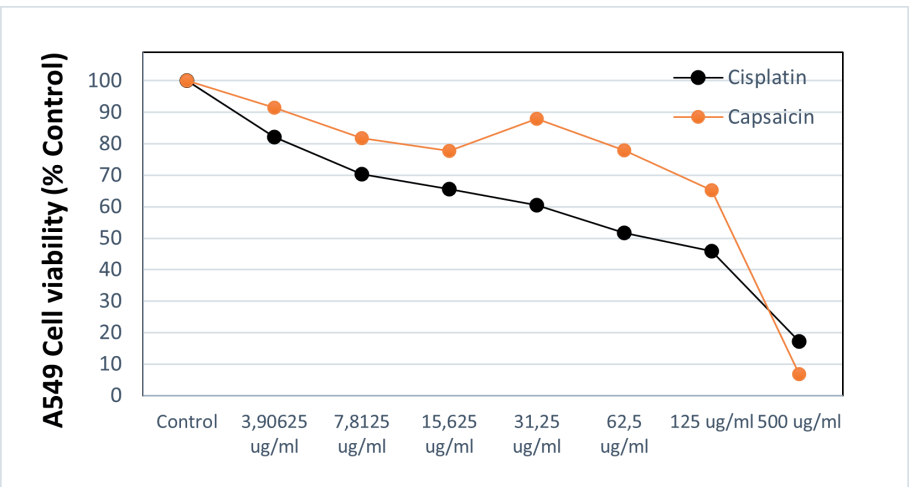

Figure 1. Dose-dependent percent viability graph of capsaicin and cisplatin on A549 cells. Cytotoxic effects of cisplatin and capsaicin on A549 cells are adenocarcinomic human alveolar basal epithelial cells after 24 hours incubation. The dose-percentage viability graph according to the effects of capsaicin and cispilatin was determined by calculating with MTT analysis.

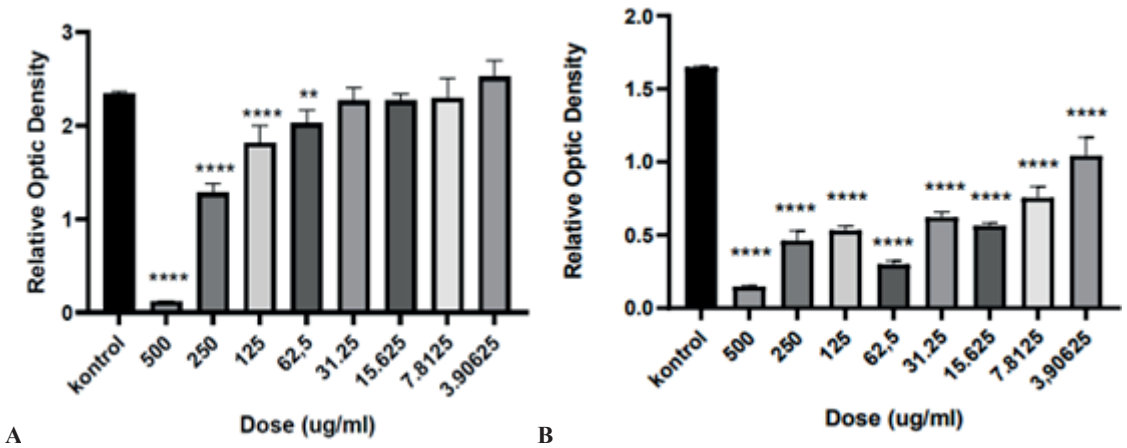

Figure 2. (A) After MTT analysis % viability ratio of capsaicin on A549 cell line after 48 hours of incubation, B) % viability rate of cisplatin on A549 cell line after 48 hours of incubation. (data recorded as mean ± SD). **p < 0.0045, ****p<0.0001 significantly different from control.

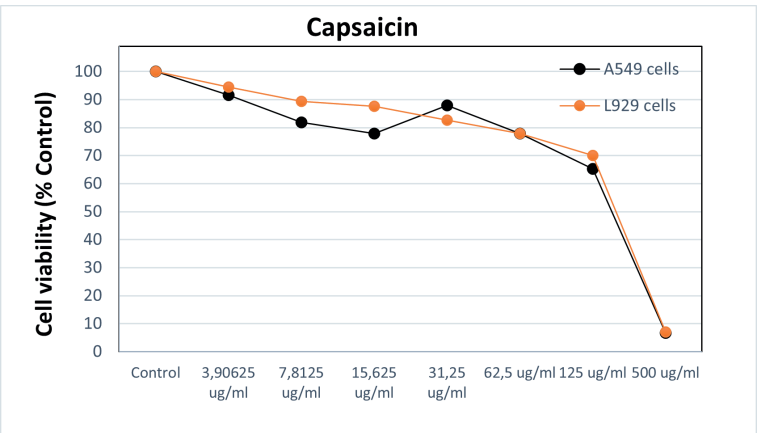

Figure 3. Dose-dependent percent viability graph of capsaicin on L929 and A549 cells. Capsaicin was also used on L929 murine fibroblast cells and compared with A459 cells to establish a control group while determining the reliable IC50 dose. data were obtained as a result of MTT analysis.

apoptotic effects of capsaicin (Figure 3, Table 3). Annexin-V binding capacity was examined by flow cytometry by applying capsaicin on the A549 cell line. After incubation of A549 cells with different concentrations of capsaicin (91.634 ve 183.268 µM), early apoptotic cell percentages were determined as 6.87 and 9.74 (Figure 3, Table 3). In addition, the percentages of late apoptotic cells were detected as %1.27 and %2.58 in incubations with different concentrations of capsaicin (Figure 3, Table 3). These results indicate that increasing capkaicin concentrations on A549 cells induce apoptosis, which includes morphological and biochemical changes. Thus, Capsaicin is identified as an effective

Table 3. Percents of typical quadrant analysis of annexin V-FITC/propidium iodide flow cytometry of A549 cells treated with the capsaicin and cisplatin

Groups	% Early	% Late	% Viable
Control (untreated)	4.39	1.22	94.39
Capsaicin $IC_{50/2}$ dose treated cells	6.87	1.27	91.84
Capsaicin IC_{50} dose treated cells	9.74	2.58	84.0
Cisplatin $IC_{50/2}$ dose treated cells	28.52	25.31	46.15
Cispaltin IC_{50} dose treated cells	50.30	35.24	14.43

apoptosis-inducing agent in different cell lines. But, its mechanism of action varies depending on cell line types. Also, doses of capsaicin used for increasing apoptosis percentages varies in different studies. In human nasopharyngeal carcinoma cells, apoptosis percentages were measured as 60% at 150µM concentration of capsaicin for 24 h [14]. In another study carried out by Leea et al. (2000) the apoptotic cell range of human glioblastoma cells were 57.2% at 200 µM of capsaicin for 24 h [12]. Also, Chou et all. (2009) showed that 200 µM of capsaicin caused 59.2% apoptotic cell percentage rate in MCF-7 cells for 24 hours [15].

3.3. Flow cytometric analyses of caspase-3 activation

The cells that were seeded in 6-plate plates were incubated for 24 hours by applying the active ingredients at the IC_{50} and $IC_{50/2}$ doses we determined. After the incubation period, readings were made with flow cytometer Beckman Coulter using Cytoflex Software by applying the kit procedure (BD, Pharmingen). Successful administration with chemotherapeutic agents is largely dependent on their ability to trigger cell death in tumor cells, and the inducing of apoptosis is mostly involved in the caspase dependent pathway [16]. It was observed that capsaicin had a dose-dependent effect on the A549 cell line, reducing caspase-3 activity (Table 4, Figure 4). Caspases regulates different biochemical pathways and morphological structures such as cell cycle regulation signaling pathways DNA condensation, fragmentation and membrane blebbing [17,18] and Caspase-3 activation was observed by capsaicin on different cell types in A549 cells [19], colon carcinoma cells [20], human renal carcinoma [21].

3.4. Statistical analysis

Percent inhibition and IC50 dosing were calculated based on the control group. All calculated data were expressed as the mean±standart error of mean (SEM) from 3 times repetition within the experiments. Data were calculated on Microsoft Excel software using a sigmoidal dose-response hill curve.

Table 4. Percent of quadrant analysis of active Caspase-3 staining by flow cytometry of A549 cells treated with the compounds

Groups	Caspase 3 positive % (+) cells	Caspase 3 negative % (-) cells
Control (untreated)	98.55	1.45
Capsaicin $IC_{50/2}$ dose treated cells	98.31	1.69
Capsaicin IC_{50} dose treated cells	95.98	4.01
Cisplatin $IC_{50/2}$ dose treated cells	77.22	22.75
Cisplatin IC_{50} dose treated cells	72.18	27.75

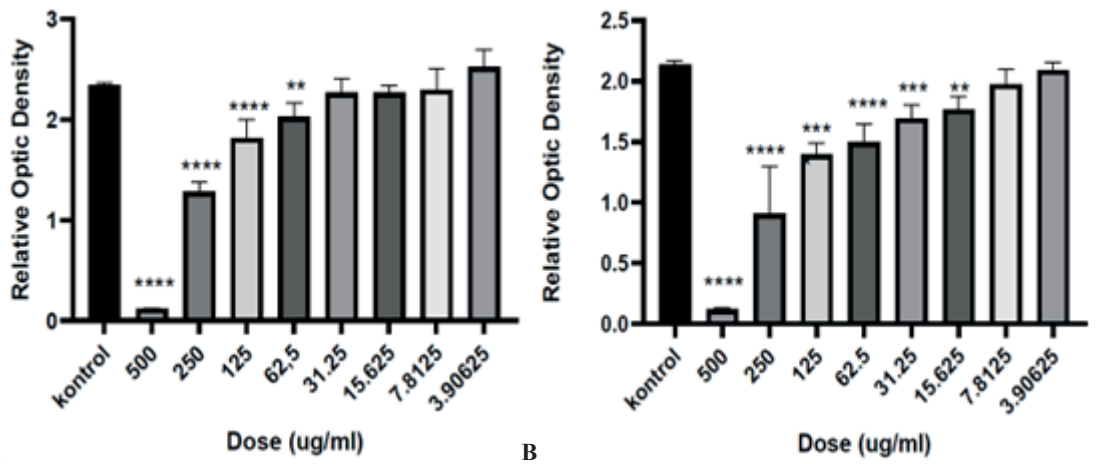

Figure 4. A) After MTT analysis % viability ratio of capsaicin on A549 cell line after 48 hours of incubation, B) % viability rate of capsaicin on L929 cell line after 48 hours of incubation. (data recorded as mean ± SD). **p < 0.0045, ***p<0.0005, ****p<0.0001 significantly different from control.

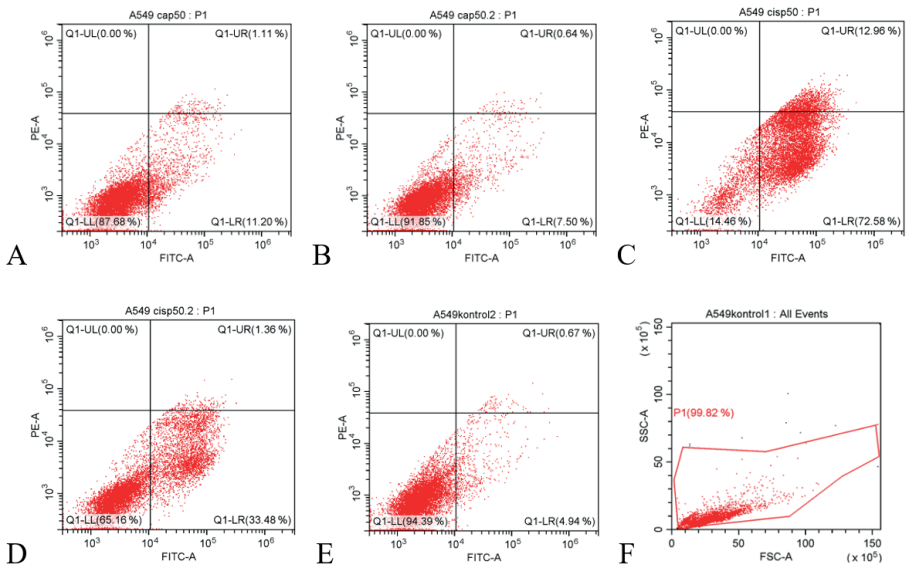

Figure 5. Flow cytometric analysis of A549 cells treated with IC50 and IC50/2 concentrations of the capsaicin and cisplatin. A549 cells were cultured for 24 hours in medium with 183.263 and 91.634 µM of capsaicin and 59.233 and 29.617 µM for cisplatin. At least 10.000 cells were analyzed per sample, and quadrant analysis was performed. Q1-LR: Early apoptotic cell percantages, Q1-UR: late apoptotic cell percentages, Q1-UL: Necrotic cell percentages, Q1-LL: viable cell percentages. A) Master gate selected from the cell population, B) Control group, C) Apoptotic effect of capsaicin IC50 dose, D) Apoptotic effect of capsaicin IC50/2 dose, E) Apoptotic effect of cisplatin IC50 dose, F) Apoptotic effect of cisplatin IC50/2 dose.

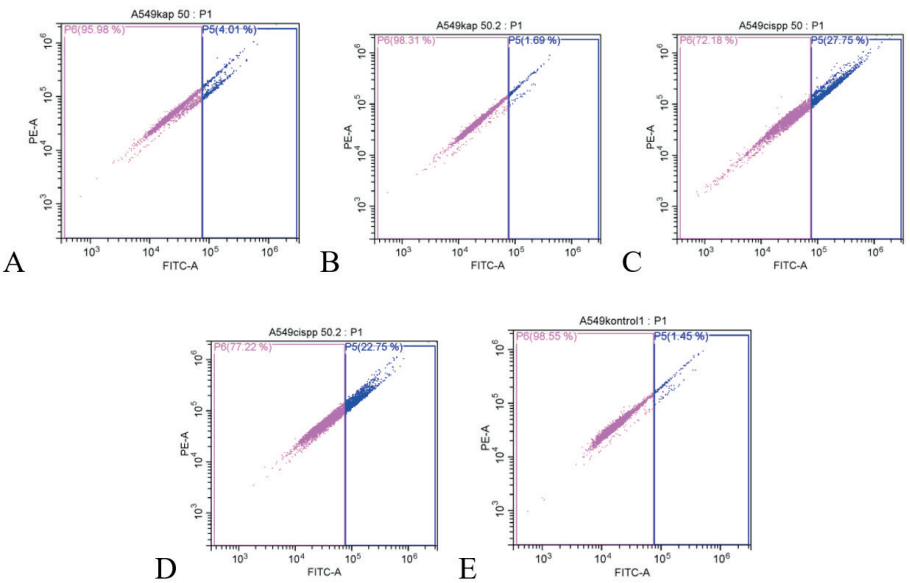

Figure 6. Flow cytometric analysis of caspase-3 activity of A549 cancer cells treated with IC50 and IC50/2 concentrations of the capsaicin and cisplatin. A549 cells were cultured for 24 hours in medium with 183.263 and 91.634 µM values of capsaicin and 59.233 and 29.617 µM for cisplatin. At least 10.000 cells were analyzed per sample, and quadrant analysis was performed. The portion (%) of cell number is shown in each quadrant. P5 shows Caspase 3 negative (-) cell percentages. P6 shows Caspase 3 positive (+) cell percentages. Controls: untreated control cells. A) Caspase-3 activity at IC50 dose of capsaicin, B) Caspase-3 activity at IC50/2 dose of capsaicin, C) Caspase-3 activity at IC50 dose of cisplatin, D) Caspase-3 activity at IC50 dose of cisplatin, E) Control Group.

4. CONCLUSION

According to our results, capsaicin has a high antiproliferative effect on the A549 cell line. These effects were found to be dose dependent. Also, capsaicin caused apoptotic cell death in A549 cells. It was determined that this effect was shown in the caspase-3 pathway, which plays an active role on the apoptotic mechanism. With this study, it is thought that capsaicin may be effective on the tumor. Although we have determined that capsaicin is effective on apoptosis in the light of the data we have obtained, more studies are needed to reveal and interact with other mechanisms.

Ethical approval

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

Author contribution

Concept: MT, AH, GAÇ, İÖA; Design: MT, AH, GAÇ; Materials: MT, AH, GAÇ; Data Collection and/or Processing: MT, AH, GAÇ; Analysis and/or Interpretation: MT, AH, GAÇ; Literature Search: MT, AH, GAÇ; Writing: MT, AH, GAÇ, İÖA; Critical Reviews: MT, AH, GAÇ, İÖA.

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Conflict of interest

The authors declared that there is no conflict of interest.

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