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Anti-cancer activities of curcumin and propolis extracts on MCF-7 breast cancer cell line model

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Abstract

It is evident that chemotherapy, which is one of the most preferred methods in cancer treatment, have several disadvantages and decrease the success rate of treatment. Therefore, identification and development of natural anti-cancer agents with less toxicity and side effects has recently become one of the areas of interest. In the present study, we reveal the potential anticancer activities of propolis and curcumin extracts and cisplatin on the breast cancer cell line (MCF-7). Individual and combinatorial treatments of propolis and curcumin was performed. MTT cell viability assay was used to determine the anti-proliferative activities of extracts, and Annexin V/PI double staining flow cytometric method was used to determine induction of apoptosis in breast cancer cells. In our study,the most significant reduction in MCF-7 cell viability was found to be 100 µg/ml for cisplatin, 5 µg/ml for curcumin and 160 µg/ml for propolis. Apoptotic cell ratios were also found to be consistent with MTT findings. The highest proportion of apoptotic cells in the combinatorial study was found to be in the presence of cisplatin + propolis. In conclusion, here we show that combinatorial cisplatin + propolis treatments have significant anti-cancer activities on MCF-7 breast cancer cells in vitro. Studies involving natural products might be a new hope for complementary and alternative medicine by paving the way for clinical studies.

Keywords: Breast cancer, curcumin, propolis, phytotherapy, chemotherapy, antitumor

Introduction

Cancer is a pathological condition resulting from the loss of control on the regular cell division process, the cell cycle, and/or it may result from reduced apoptosis [1]. According to the International Cancer Research Agency, GLOBOCAN 2018 cancer incidence and mortality estimates; breast cancer mortality rate in women is %11.6 [2]. Breast cancer, which is one of the most common cancers in women, has a high incidence worldwide [3]. The pathophysiology of breast cancer is multidimensional and still not fully understood. However, it is known that some factors (advanced age, gender, genetic predisposition etc.) may be a risk for breast cancer [4,5]. Today, the main treatment methods used in the fight against cancer include surgical intervention, chemotherapy, radiotherapy, hormone replacement therapy, and immunotherapy. Among these modes of treatments, chemotherapy is a method frequently used in the fight against cancer. Disadvantages of chemotherapy (failure to respond to treatment, severe toxicity, and multiple drug resistance, etc.) reduce the success rates [6]. Efficient results can be obtained in the treatment depending on the stage and type of cancer and the use of either monotherapy or combination therapy [7].

In recent years, the number of studies has been on the rise, investigating natural remedies that act on apoptosis and signalling pathways in cancer cells resistant to chemical medications [8]. As one of the natural treatment methods that remains current today, phytotherapy is defined as a mode of treatment by the use of extracts prepared from different plant parts such as roots, seeds, pollens, shells, and fruits [9]. For example, Vinka alkaloids obtained from Vinca rosea (*Catharanthus roseus*), are among the first plant alkaloids to be used as anti-cancer agents. It is known that Vinca alkaloids that are generally used with chemotherapeutic agents enhance the anti-cancer activity by reducing the toxicity [10]. Cinnamon is a natural plant ingredient that shows a wide range of

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pharmacological functions, including anti-oxidant, anti-microbial and anti-cancer activities. Cinnamon as a therapeutic agent has been shown to have anti-cancer effects by affecting the pathway associated with apoptosis [11]. Especially, there is a growing number of studies about curcumin, investigating its several roles as an antioxidant, anti-inflammatory, antimicrobial, and anticancer agent. Curcumin is obtained from the rhizomes of the *Curcuma longa* plant, of the Zingiberaceae family, whose homeland is South Asia. Curcumin and propolis, which have been used in the treatment of various diseases from ancient times, are widely used also today to prevent inflammatory diseases [12]. Curcumin is known to suppress transformation and proliferation, induce apoptosis and inhibit tumor metastasis through the regulation of various transcription and growth factors, inflammatory cytokines, protein kinases and other enzymes. In addition, some studies show that propolis, which contains various flavonoids and is known as a powerful antioxidant, shows apoptotic effects in cancer cells by stimulating molecules in signal pathways [13, 14]. Accordingly, the aim of present study was to determine the anti-cancer activities of propolis and curcumin extracts on the breast cancer cell line (MCF-7) and to investigate the antagonistic and/or synergistic effects of cisplatin in combination with these extracts.

Material and Methods

Preparation of the Extracts and the Chemical Agents

A 200 µg/ml stock solution of cisplatin (50 mg/100 ml; Koçak Farma, Turkey) was prepared in a 0.9% isotonic sodium chloride solution (Biofleks, Turkey). Different concentrations of cisplatin (1.56, 3.12, 6.24, 12.5, 25, 50, 100 µg/ml) were used in the study.

A 0.1814 g/ml sample of curcumin powder of Indian origin was added to 200 µl of 0.5 M NaOH + 800 µl of PBS (Gibco, Life Technologies, USA); vortexed, and dissolved. The obtained solution was filtered by using a filter with a pore size of 0.22 µm (Minisart® NML Syringe Filter, Germany). Fresh extracts of curcumin were prepared before their use at each phase of the study. Different concentrations of curcumin (0.08, 0.16, 0.31, 0.63, 1.25, 2.5, and 5µg/ml) were used in the study.

Propolis collected from Apis mellifera carnica bee colony (Edirne, Türkiye) was dried and crushed by using a pestle and a mortar. The obtained propolis powder was added to 70% ethanol (Merck, USA) and left for 3 days at room temperature in a container, which was covered not to allow light to reach the solution. Then, it was left to dissolve in a shaking incubator. After the solution was filtered with a Whatman® grade 1 qualitative filter paper (Sigma-Aldrich, Germany), the solution was subjected to drying in a laboratory freeze dryer (Alpha 2-4 LD Plus Christ/18573) and pulverized. After dissolving the propolis extract in 70% ethanol, we filtered the solution using a filter of 0.22 µM pore size (Minisart® NML Syringe Filter, Germany) to obtain a stock solution of 320 µg/ml concentration. Different concentrations of propolis (2.5, 5, 10, 20, 40, 80, and 160 µg/ml) were freshly prepared before the study.

Cell Culture

In this study, a human breast adenocarcinoma (MCF-7) cell line obtained from the immunology department of Gazi University School of Medicine (Ankara, Turkey) was used. For the cells; 10% heat-inactivated fetal bovine serum (FBS), 1% penicillin/streptomycin, and 1%L-Glutamine-containing Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Life Technologies, USA) were prepared. The cells were transferred to a 75 cm^2-flask (VWR/SPC, Canada) and cultivated for 48 hours at 37°C in an incubator (Healforce, China) containing 5% CO_2.

Cell Viability Assays
Trypan Blue Staining of the Cells

After reaching the adequate density, the MCF-7 cells cultivated at the 75 cm^2-flask were removed with 0.25% Trypsin-EDTA (Gibco, Life Technologies, USA). A cell suspension was prepared and the cells stained with 0.4% trypan blue (Sigma-Aldrich, Germany) were counted with the Neubauer chamber (CE).

MTT Assay

In this study, a commercial MTT kit (Vybrant® İnvitrogen, USA) was used. From the MCF-7 cell suspension, a volume of 100 µl was pipetted in a 96-well plate so that each well would contain $3x10^4$ cells. The plate was incubated at 37°C in a 5% CO_2 containing incubator (Healforce, China) for 48 hours. Then, the cells were exposed to different concentrations of propolis (2.5-160µg/ml), curcumin (0.08-5µg/ml), and cisplatin (1.56-100µg/ml) for 48 hours. The cells cultured in the absence of propolis, curcumin, and cisplatin preparations were prepared as negative control. After 48 hours of incubation, the medium was removed from the wells and replaced with 100 µl phenol red-free Roswell Park Memorial Institute (RPMI) (Gibco, USA). Then, a volume of 10 µl MTT reagent was added into each well and pipetted. Following this step, the plate was left for incubation for 4 hours at 37°C in a 5% CO_2 containing incubator. Finally, a 100 µl volume of the SDS-HCI solution obtained from the kit was added into each well and the well content was mixed by pipetting. At the end of the incubation period, the optical density (OD) of the plates was measured with a Multiskan GO microplate reader (Thermo Scientific, Waltham, MA USA) at a 570 nm wavelength. The experiment was done in triplicate and was repeated five times. The percentage of viable cells was determined with the below formula [15].

Cell viability % = (OD_{570}treated cells/OD_{570} control)× 100

The concentrations closest to the inhibitor concentration (IC_{50}) that killed 50% of the cells were determined. The percentages of cell viability were determined 48 hours later after the cells were treated with different combinations of propolis, curcumin, and cisplatin (propolis + curcumin, cisplatin + propolis, cisplatin + curcumin, cisplatin + curcumin + propolis), each at the closest concentrations to IC_{50} doses.

Flow cytometry with Annexin V-FITC/ Propidium Iodide Staining

Using the Alexa Fluor ™ 488 kit (Thermo Fisher Scientific, Waltham, MA USA), apoptosis in MCF-7 cells, induced by propolis and curcumin extracts and cisplatin, was determined by flow cytometry. The cultured MCF-7 cells were detached from the base of the flask by using 0.25% Trypsin/EDTA (Thermo Fisher Scientific, Waltham, MA USA). A 2 ml volume of the cell suspension was pipetted into sterile 6-well cell culture plates to have $3x10^4$ cells/well. Then, the plates were left for incubation at 37°C in a 5% CO_2 containing incubator for 48 hours. Then, the cells were exposed to different concentrations of cisplatin (3.125-100 µg/ml), curcumin (0.156-5 µg/ml), and propolis (5-160 µg/ml) for 48 hours. Only MCF-7 cells were added to a well, which was prepared as the control well. After the incubation, the cells and the medium were removed from the base of the plate by using a cell scraper and the cells were transferred to the flow cytometry tubes. Then, 100 µl 1X annexin binding buffer was added and resuspended. After this procedure, 5 µl Annexin V-FITC and 1 µl PI were added to the tubes and pipetting was performed. The tubes were covered not to allow light to reach inside and they were left for 15 minutes at room temperature. At the end of the incubation period, 400 µl 1X annexin binding buffer was added into the tubes taken on ice, then, pipetting was performed. Then, the tubes were read with a flow cytometer (FACS Calibur, BD Bioscience) and the obtained images were interpreted. Finally, apoptotic effects were determined 48 hours after the MCF-7 cells were treated with combinations of propolis, curcumin, and cisplatin doses closest to IC_{50} doses.

Statistical Analysis

The study data were analyzed by using SPSS 18.0 software (Statistics Program for Social and Science IBN, USA). The results of MTT and apoptosis tests were compared with ANOVA analysis. Subgroups were compared with the Duncan test. A p-value of <0.001 was considered statistically significant.

Results

Results of the Trypan Blue Staining Assay

The number of cells ($3x10^4$) planned for the study was calculated according to the formula below:

Total cell count (ml) = Live cell count x Dilution factor x 10^4

$= 104x2x10^4$

$= 208x10^4$ cells/ml

According to the above formula, the number of cells was calculated as $208x10^4$ ml cells per 1 ml cell suspension. For MTT, the wells were diluted in triplicate and the process was repeated five times so that each well would contain $3x10^4$ cells.

MTT assay results

To reveal anti-proliferative activities of either curcumin andpropolis alone or in combination with cisplatin we used MMT cell viability assay. Compared to the control, there was a statistically significant decrease in the MCF-7 cell viability 48 hours after either of cisplatin, curcumin, or propolis alone treatments (p = 0.00). It was determined that the most significant decrease in cell viability compared to the control occurred at a 5 µg/ml dose of curcumin, 100 µg/ml dose of cisplatin, and 80 µg/ml dose of propolis. The highest cell viability was found at a 1.56 µg/ml dose of cisplatin, 0.16 µg/ml dose of curcumin, and 40 µg/ml dose of propolis. Analyzing the rates of cell death, which were determined in comparison to the control; the closest doses to IC_{50} , showing the 50% viability rate, were calculated as 3.12 µg/ml for cisplatin, 0.31 µg/ml for curcumin, and 160 µg/ml for propolis (Figure 1).

Compared to the control, a statistically significant decrease was found in the MCF-7 cell viability 48 hours after applying different combinations of cisplatin (3.12 µg/ml) and curcumin (0.31 µg/ml) and propolis (160 µg/ml) extracts at the closest doses to the respective IC50 doses (p<0.001). Compared to the control, the lowest cell viability was observed with the triple treatment with cisplatin+curcumin+propolis doses (61.37 ± 2.00), and the highest cell viability was observed with the dual treatment with the use of propolis + curcumin doses (80.39 ± 5.20) (Figure 2).

Apoptosis Assay with Annexin V-FITC

In this study; the live, dead, and apoptotic cell percentages were analyzed by flow cytometry in order to determine the effects of cisplatin, curcumin, or propolis alone or in combination on MCF-7 cells.

Compared to the control, an increase was determined in the MCF-7 cell apoptosis rates 48 hours after applying either of cisplatin, curcumin, or propolis alone. Accordingly, the highest rates of apoptotic cells compared to the control occurred at a 100 µg/ml dose of cisplatin, 5 µg/ml dose of curcumin, and 160 µg/ml dose of propolis. The lowest rate of apoptotic cells was found at a 3.125 µg/ml dose of cisplatin, 0.156 µg/ml dose of curcumin, and 5 µg/ml dose of propolis (Figure 3-A, B).

Compared to the control, an increase was determined in the MCF-7 cell apoptosis rates after applying different combinations of cisplatin and curcumin and propolis extracts at the closest concentrations to the respective IC_{50} doses. Accordingly, the highest rate of apoptotic cells compared to the control was found with the cisplatin+propolis combination and the lowest rate of apoptotic cells was found with the cisplatin+curcumin combination (Figure 4-A, B)

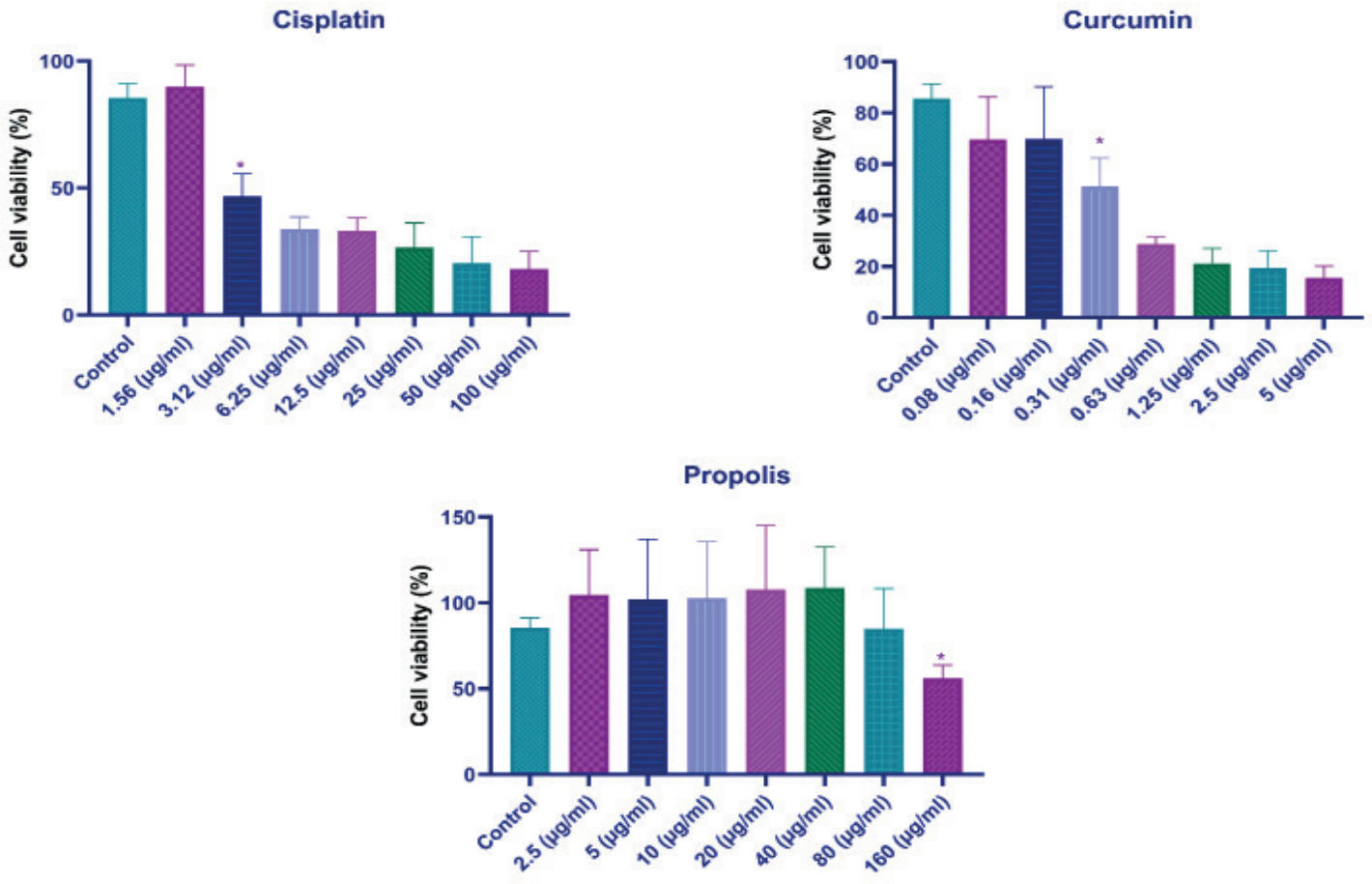

Figure1. Viability percentages of MCF-7 cells in the MTT cell viability assay for cisplatin, curcumin, and propolis concentrations (n = 5). The mean values were found to be statistically different from the control group in the groups indicated by different letters (ANOVA-Duncan test*p <0.001).

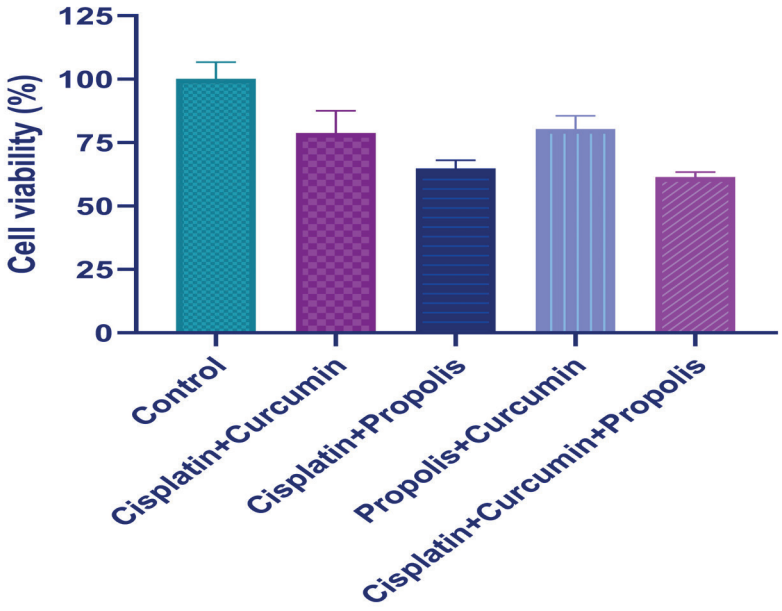

Figure 2. Viability percentages of MCF-7 cells in the MTT cell viability assay after being treated with combinations of cisplatin, curcumin, and propolis (n=5). The mean values were found to be statistically different from the control group in the groups indicated by different letters (ANOVA-Duncan test *p <0.001).

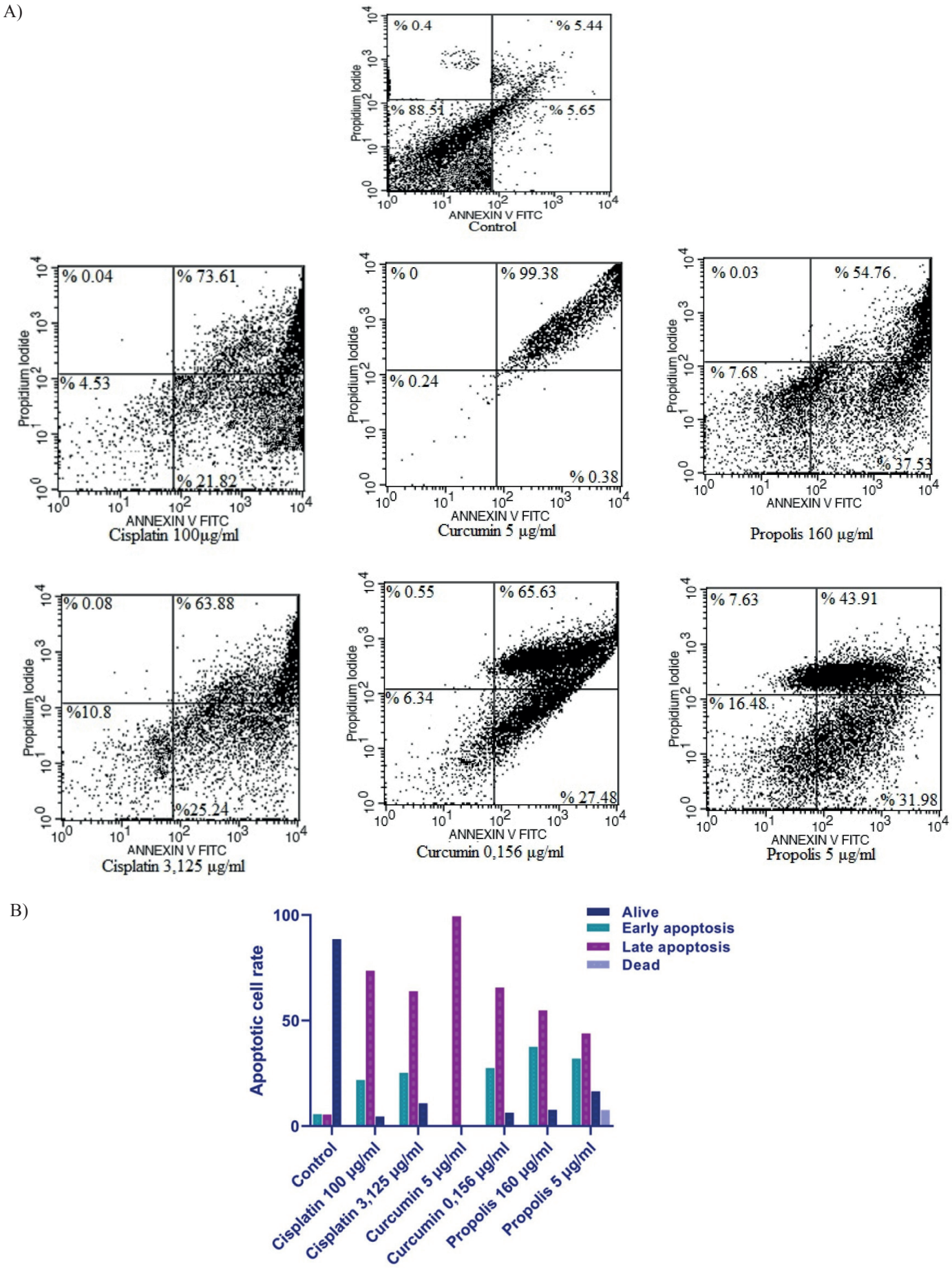

Figure 3. The highest and the lowest percentages of the live, dead, and apoptotic MCF-7 cells by the concentrations of cisplatin, curcumin and propoliscompared to the control (A,B).

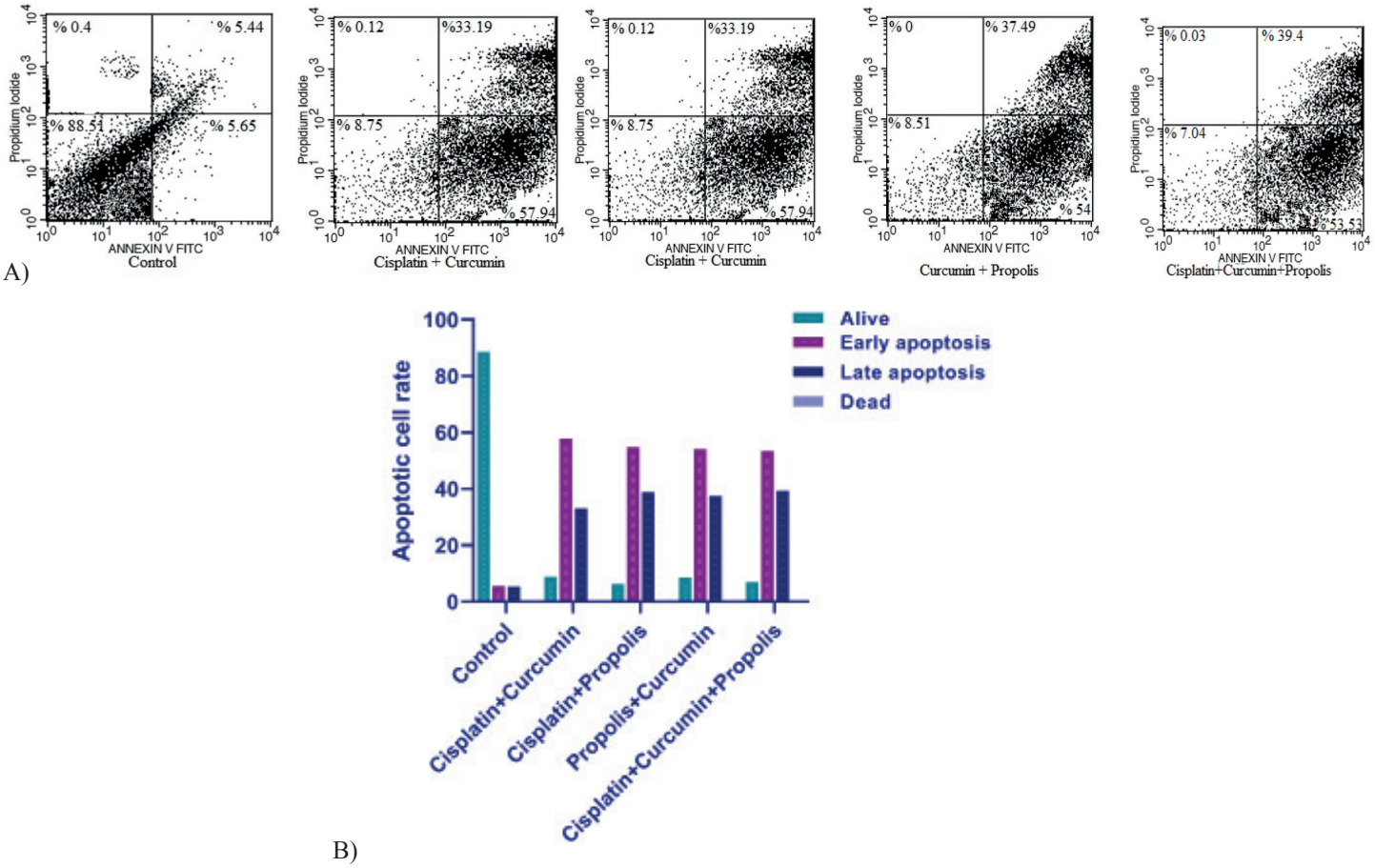

Figure 4. The highest apoptotic MCF-7 cell percentages compared to the control after the treatment with combinations of cisplatin, curcumin, and propolis (A, B).

Discussion

In this study, MTT viability assay was performed in MCF-7 human breast cancer cells incubated with either curcumin and propolis alone or in combination with cisplatin. Afterwards, the closest doses to IC_{50} were determined for each of them combinations were prepared, and their synergistic effects were evaluated by the apoptosis assay.

In the study in 2016, Nurcahyanti et al. incubated cervix and colon cancer cells (HeLa and Caco-2) for 24 hours with cisplatin and determined the IC50 values of cisplatin with the MTT method as 54.07 µM and 96.38 µM, respectively. They found the IC_{50} values in the hepatocellular carcinoma cells (Hep-G2 and SK-HEP-1) as 14.87 µM and 77.89 µM, respectively [16]. In another study performed by Becit in 2017, the phenolic compounds of pycnogenol and curcumin were evaluated for their anticancer effects and the effects on cell viability on Chinese hamster lung fibroblast (V79), human liver cancer (Hep-G2), and human cervical cancer (HeLa) cells against cisplatin toxicity by using the MTT method. A single dose of 500 µM pycnogenol and curcumin was demonstrated to reduce the cisplatin-induced toxic effect values in HepG2, V79, and HeLa cells [17].

In the literature; studies are available, investigating the effects of curcumin on MCF-7, HeLa, and HepG2 cells [18-20]. In their study, Ding et al (2015) incubated MCF-7, HeLa, and HepG2 cells with curcumin for 72 hours and found out the IC50 values as 9.40 µg/ml, 17.67 µg/ml, and 22.88 µg/ml, respectively [21]. In a study by Abdel-Lateef et al. (2016), which investigated the anticancer effects of curcumin on HepG2 cells, the IC_{50} value was determined to be 41.5 µg/ml (~113 µM) after a 72-hour incubation period [22].

In this present study, statistically significant results compared to the control were obtained in the MCF-7 cell viability and apoptosis rates after applying cisplatin (1.56-100 µg/ml) and curcumin (0.08-5 µg/ml) at different dose ranges ($p<0.001$). These results are consistent with the previous observations, strongly indicating that combinatorial therapy of cisplatin and curcumin increases the apoptotic cell rate.

In vivo and in vitro studies have demonstrated that the active ingredients in propolis inhibit the growth of cancer cells and increase apoptosis and antitumoral efficacy [23-25]. In an in vitro study on Hep-2 cells (2010), Bufalo et al. reported that propolis extract concentrations of 25, 50, and 100 µg/µL reduced cell viability but propolis concentrations of 5 and 10 µg/µL had no effects on cell viability [26]. In the study, where Kamiya et al. (2012) investigated the effect of the Brazilian red propolis ethanol extract on apoptosis via the endoplasmic reticulum pathway on MCF-7 and fibroblast cells, propolis significantly reduced MCF-7 cell viability via the caspase-3 activity but had no effects on

the viability of fibroblasts. That study concluded that propolis increased apoptosis in MCF-7 cells [27]. In this present study, it is possible to argue that a limited significance compared to the control was obtained in cell viability and apoptosis rates in MCF-7 cells at the end of 48 hours after applying propolis at a dose range of 2.5-160 µg/ml ($p<0.001$). Further studies are needed to comprehensively explain the cytotoxic and apoptotic effects of propolis on MCF-7 cells.

In the literature, there are few studies investigating the effects of different concentrations of curcumin and propolis on MCF-7 cell viability in combination with anticancer drugs with known pharmacological efficacy. Being a subject matter that attracted the attention of researchers in recent years, the synergistic and/or antagonistic effects of combinations of curcumin and propolis extracts with cisplatin were examined on the viability of MCF-7 cells in this study.

In the combined study of cisplatin + curcumin by Ueki et al. (2013) on mice, curcumin has been shown to reduce cisplatin-induced nephrotoxicity. Ueki et al. assigned mice to four groups (control, curcumin, cisplatin, and cisplatin + curcumin) and they examined the effects of treatment 72 hours after injecting curcumin and cisplatin intraperitoneally into the mice. At the end of the study, it was demonstrated that curcumin reduced cisplatin-induced toxic effects, prevented necrosis, and elevated serum levels of tumour necrosis factor-alpha (TNF-α) [28].

In an in vivo study by Waseem et al. (2014), 200 mg/kg curcumin was given to rats orally and 6 mg/kg cisplatin was administered intraperitoneally. After 24 hours, rats were sacrificed. At the end of the study, it was observed that cisplatin compromised the immune system and caused structural and functional damage in mitochondria but curcumin abolished those damages. Furthermore, it was reported that even a single dose of cisplatin was enough to cause hepatic injury. The results of that study demonstrated that cisplatin was indicative of systemic toxicity and that curcumin reduced that toxic effect [29]. In this present study, the lowest cell viability compared to the control was observed with the triple combination of cisplatin+curcumin+propolis doses and the highest cell viability was observed with the dual combination of propolis + curcumin doses. Because no studies are available in the literature, investigating the effects of either a dual combination of curcumin+propolis or a triple combination of cisplatin+curcumin+propolis on cell viability and apoptosis in MCF-7 cells, we could not compare our study results with any information in the literature. The limitation of this study is that the combination results could not be compared with other studies and no breast cancer cell line was used in the study other than MCF-7.

Conclusion

We are of the opinion that the use of propolis and curcumin adjunctive to the treatment with cisplatin may bring a new perspective to anticancer therapy with further contributions to be provided by the results of similar future studies. However, further in vitro and in vivo studies are needed in to evaluate the long-term toxicity of these compounds. The results obtained from these studies will pave the way for the use of herbal products such as curcumin and propolis in complementary and alternative medicine.

Conflict of interests

The authors declare that they have no competing interests.

Financial Disclosure

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Ethical approval

We made in-vitro study. We worked only with tumor ATCC cell line. We did not work with the patient sample.

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