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Immunostimulant/Cytotoxic Effect of Cardamom Extract with Adjuvant Combination on Breast Cancer Cell Line

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Keywords:

Elettaria cardamomum, Breast cancer, MCF-7, THP-1, Cytotoxicity, Immunostimulatory effect

Abstract

Breast cancer is the most common type of cancer among women and is the second most common cause of cancer-related deaths. It is caused by the uncontrolled proliferation of cells in the breast tissue as a result of various factors. For the treatment of breast cancer, chemical drugs are utilized generally. However, chemical cancer drugs have a cytotoxic effect on healthy cells. That causes dose limitation and a decrease in the effectiveness of the treatment. For this reason, herbal-based studies in the treatment of breast cancer become popular day by day. In the literature, the anticancer, anti-inflammatory, and antimicrobial properties of many plants have been analyzed and their effectiveness has been proven. Diindolylmethane (DIM) and indole-3-carbinol (I3C) molecules found in Elettaria cardamomum (*E. cardamomum*, Cardamom) plant, reduce metastasis, tumorigenesis, immunomodulation. It has been shown in the literature that they play a role in preventing the development of breast cancer via inducing apoptotic pathways in the breast cancer cell. Herein, it is aimed to prevent breast cancer development by applying cardamom extract and cardamom Extract-Freund's adjuvant combination to THP-1 human macrophage cell line and MCF-7 breast cancer cell line and to increase the efficiency of cardamom extract with the use of an adjuvant. As a result, it was determined that the cytotoxic effect of the cardamom Extract-Freund's adjuvant combination on breast cancer cells was higher than the alone cardamom extract application. At the same time, it was determined that the toxic effect of Freund's adjuvant could be reduced by increasing the plant extract concentration.

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1 INTRODUCTION

Breast cancer is the most common type of cancer among women in developed and underdeveloped countries and it is the second most common cause of death among cancer-related deaths. Breast cancer occurs when cells in the breast begin to grow and divide abnormally. While damage in normal cells results in cell death, cancer cells continue to proliferate without stop signals in the cell cycle due to DNA mutations [1]. Breast cancer treatment usually includes breast-conserving surgery or mastectomy, depending on the characteristics of the tumor. However, the treatment methods generally affect the quality of life of patients due to side effects [2]. Therefore, herbal treatments methods become popular to prevent cancer and to improve the quality of life of patients by reducing the side effects of the treatments as a complementary therapy. Herbal treatments create an alternative solution to eliminate the cytotoxic effects of drugs [3]. In literature, anticancer effects of various herbs indicated such as black cumin seeds, echinacea, curcumin, ginger, ginseng, and cardamom.

Cardamom is a plant with an anti-cancer effect used in cancer research. Also, it has antioxidant, cytotoxic, and anti-inflammatory properties that can inhibit cancer growth and promote cancer cell death. At the same time, the immunomodulatory effects of cardamom oil, as well as its antimicrobial and anti-inflammatory properties, make cardamom an effective alternative for breast cancer treatment [3]. Phytochemicals such as Diindolylmethane (DIM) and indole-3-carbinol (I3C) obtained from plants affect cancer pathways. In addition, these phytochemicals may regulate hormone activities in breast cancer. The I3C is found in the cardamom plant and it reduces metastasis, tumorigenesis, immunomodulation, and is involved in the prevention of breast cancer development via triggering the apoptotic pathway [4]. In a study, it was stated that I3C stimulated the apoptotic pathway in the MCF-7 cell line [5]. Due to the anti-cancer, antimicrobial, and antiinflammatory effects of the cardamom plant, researches show that cardamom can reduce tumor size and its therapeutic use will be effective in the treatment of breast cancer.

Adjuvants are used to increase the effectiveness of formulations applied in vaccine studies. In this way, a higher immune response can be obtained. Adjuvants do not create an antibody response when they are used alone but increase the immunogenicity in the organism ensuring a strong immune response. Freund's adjuvant is an oil adjuvant that aggregates in the injection site. It provides a slow release of the agent and stimulates antibody production in plasma cells [6]. The immunostimulant effect should be considered before in vivo studies of the prepared agent and adjuvant combinations. For this purpose, the immunostimulant effect is determined by analysis of nitric oxide (NO) levels in macrophages.

The overall goal of this study is to apply the combination of cardamom extract and cardamom Extract-Freund's adjuvant to THP-1 human macrophage cell line and MCF-7 breast cancer cell line for the first time. The combination of the cardamom and Freund’s adjuvant is expected to prevent the development of breast cancer. Also, it is aimed to increase the effectiveness of cardamom by using a combination of cardamom plant and adjuvant.

2 MATERIAL AND METHOD

2.1 Preparation of *E. cardamomum* Extract

Firstly, the cardamom was peeled, and the seeds were removed. The seeds were crushed in a mortar. 8 g of crushed cardamom seeds were taken, and 50 ml methanol was added to it. It was then room temperature incubated in the dark for 6 days. The obtained extract was filtered on Whatman paper and taken into a beaker. The methanol in the mixture was removed in the fume hood with the heat application [7]. The obtained extract was mixed with the medium and applied to the cells.

2.2 Cell Culture Studies

In the study, the human macrophage cell line (THP-1) and breast cancer cell line (MCF-7) in the cryobank was used. RPMI-1640 (Gibco, New York, USA) medium was used for proliferation of cells. The stock medium was prepared by adding 1% penicillin-streptomycin and 1% L-glutamine. Fetal bovine serum (FBS) (Sigma Aldrich, Milan, Italy) was added to the media at the experimental stage [8]. The number of passages of the cell line used in the study is between 10 and 15.

The culture of THP-1 cell lines was carried out in RPMI-1640 (Gibco) medium containing 10% FBS. Cells were incubated at 37^{o}C and 5% CO_2.After the cells reached the required confluency, they were collected and centrifuged

at 25ºC, 1000 rpm for 5 minutes [8]. Then, $1x10^5$ cells ml^{-1} per well were seeded into 96-well plates. Cells inoculated into the culture medium created were kept under the required incubation conditions for 24 hours.

2.3 Nitric Oxide Analysis

To analyze the immunostimulatory activity of the prepared extract and Freund's adjuvant combinations at different concentrations, the amount of nitric oxide (NO) produced by the THP-1 cell was determined by the Griess method [9]. The prepared extract and extract-adjuvant combinations were applied to the cells incubated for 24 hours under incubation conditions. Cells were treated at certain concentrations (5 µg/ml, 10 µg/ml, 20 µg/ml, 40 µg/ml, 100 µg/ml extract and 10 µg/ml adjuvant combination) for 48 hours. After 48 hours, supernatants were collected. It was reacted with Griess reagent (2.5 ml phosphoric acid, 0.1 g N-(1-Naphthyl) Ethylenediamine, and 1 g Sulfanilamide). To measure NO production, 50 µl of the culture medium was taken and 50 µl of Griess reagent was added to it. After 10 minutes of incubation at room temperature, absorbances were measured in an ELISA reader at 540 nm.

2.4 MTT Analysis

MTT method was used to determine the cytotoxic effects of the prepared extract and extract-adjuvant formulations. The viability of the cell lines was evaluated with the MTT salt, 3-(4.5-dimethylthiazol-2-yl)-2.5-Diphenyltatrazilium bromide. Extract and extract-adjuvant combinations were added to cells seeded in 96-well plates. Cells were incubated for 48 hours in a 37ºC incubator containing 5% CO_2. Then, 10 µl of MTT solution was added to each well. It was incubated for 3 hours at 37°C in an oven containing 5% CO_2. After incubation, 100 µl of dimethylsulfoxide (DMSO) was added to each well. Plates were kept room temperature and in the dark for 30 minutes. Cell viability was measured at a wavelength of 570 nm [10]. Cell viability analysis data were obtained using equation 1 and data plots were generated.

$$Cell\ viability\ (\%) = \frac{\text{The absorbance of sample} * 100}{\text{Absorbance of control}} \quad (1)$$

2.5 Statistical Analysis

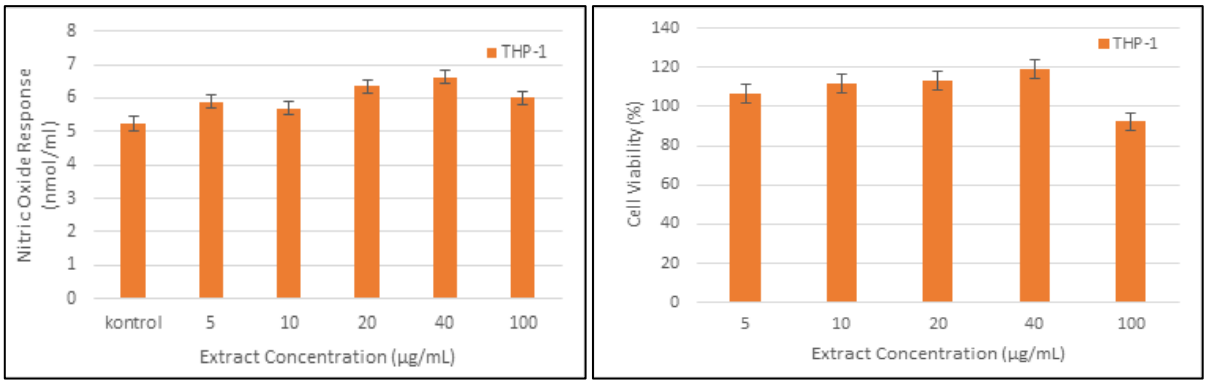
The data obtained from the study were analyzed in the IBM SPSS 25.0 (IBM Corporation, Armonk, NY, USA) package program. Comparisons between groups were made with a one-way analysis of variance and a one-way ANOVA test. Results were given as mean±standard deviation (Mean±SD) and statistical significance was accepted as $p<0.05$.

3 RESULTS

Many active compounds such as secondary metabolites, phenolic acids, flavonoids, tannins, quinones, and anthocyanins obtained from plants suppress cancer pathways. They are also effective in preventing cancer [11]. One of the plants used in these studies is *E. cardamomum*. In this study, immunostimulatory activity and cytotoxic effect on THP-1 human macrophage cell line were investigated for the first time to determine the therapeutic efficacy of the extract prepared by maceration method from E. Cardamom plant alone or in combination with Freund's adjuvant. *c*As a result of this study, it was determined that the most appropriate extract-adjuvant combination that can be used in the treatment of breast cancer can be used in future in vivo studies.

The percentage of viability of the cells shown in the figures is the percentage obtained by simple ratio calculation in the other groups when the value of the positive control group is accepted as 100% and indicates the percentage of viable cells. According to the results of the MTT test, cell viability was evaluated over the % cell viability among all groups at the end of the 48th hour.

The immunostimulatory activity and viability analysis of THP-1 human macrophage cell lines treated with the extract are shown in Figure 1. The highest immunostimulant activity of the extract obtained from the cardamom plant is at a concentration of 40 µg ml^{-1}. İmmunostimulatory activity obtained from the treatment of cardamom extract to THP-1 cells at a concentration of 40 µg ml^{-1} was 6.628 nmol ml^{-1} ($p<0.05$).In the study, in the cytotoxicity analysis of the cardamom extract at a concentration of 40 µg ml^{-1} alone, 119.16% viability was detected in the THP-1 cell line.



(a) (b)

Figure 1. Analysis of the immunostimulant and cytotoxic effect of the extract in the THP-1 cell line (a) Nitric oxide analysis of extract, (b) Cytotoxic analysis of extract

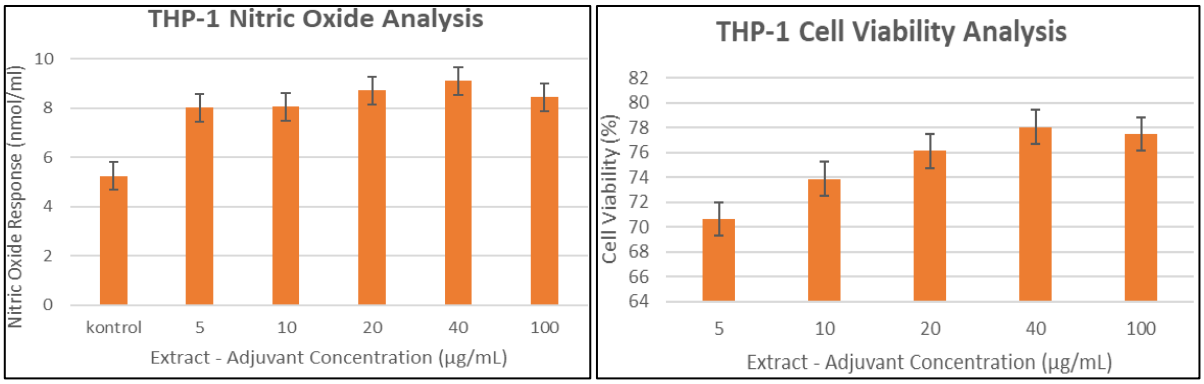


Figure 2. Analysis of the immunostimulant and cytotoxic effect of the extract and adjuvant combination in THP-1 cell line

Immunostimulatory activity and viability analysis of THP-1 human macrophage cell lines treated with the extract and Freund's adjuvant are shown in Figure 2. The highest immunostimulant efficiency of the pure extract obtained from the *E. cardamomum* extract and its combination with Freund's adjuvant is at a concentration of 40 µg ml^{-1}. The highest immunostimulatory activity obtained from the treatment of pure *E. cardamomum* extract and Freund's adjuvant combination to THP-1 cells at a concentration of 40 µg ml^{-1} was 9.109 nmol ml^{-1} ($p<0.05$). In the study performed, in the cytotoxicity analysis of the combination of 40 µg ml^{-1} *E. cardamomum* extract with Freund's adjuvant, 78.06% viability was detected in the THP-1 cell line.

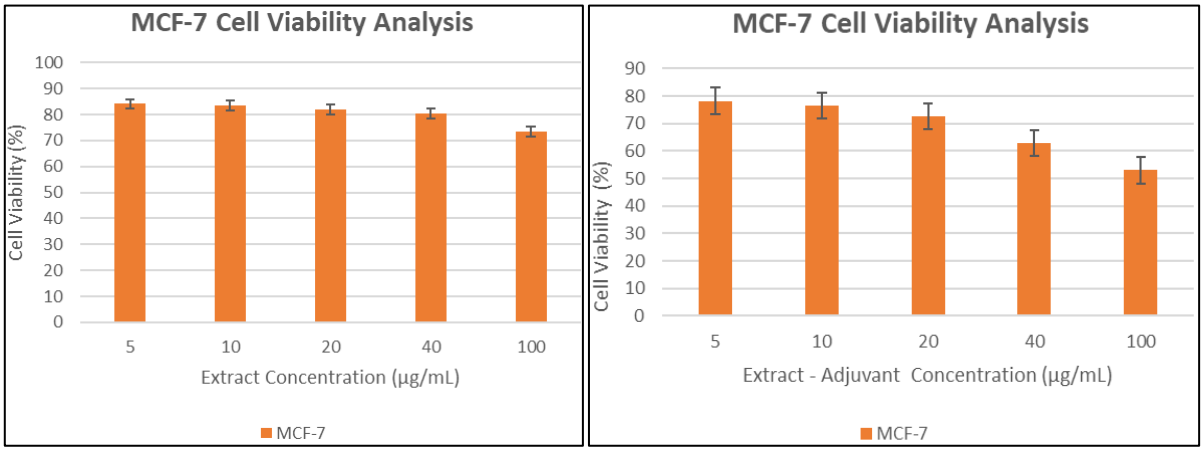


Figure 3. Analysis of the cytotoxic effect of the *E. cardamomum* extract and adjuvant in MCF-7 breast cancer cell line

The viability analysis of the MCF-7 breast cancer cell line treated with *E. cardamomum* extract and *E. cardamomum* Extract-Freund's adjuvant due to their cytotoxicity is shown in Figure 3. The highest killing efficiency of the extract obtained from the cardamom plant is at a concentration of 100 µg ml^{-1}. In the study, in the

cytotoxicity analysis of cardamom extract at a concentration of 100 μg ml^{-1}, 73.52% viability was detected in the MCF-7 cell line. The highest killing efficiency of the extract and adjuvant combination obtained from the cardamom plant is at a concentration of 100 μg ml^{-1}. In the study, in the cytotoxicity analysis of cardamom extract at a concentration of 100 μg ml^{-1} in combination with Freund's adjuvant, 52.94% viability was detected in the MCF-7 cell line.

Oncological treatment studies have a large percentage in the literature. Cancer treatment strategies are developing more and more every day. Studies show that the use of chemical drugs is the most frequently used treatment method. However, the cytotoxic effect of chemical drugs on healthy tissue cause dose limitation. Also, chemical drugs reduce the quality of life. This leads to a decrease in treatment efficacy. All these disadvantages make plant sources attractive.

Herbal supplementary treatment approaches have been an option for the human race since before Christ. Today, the anticancer, anti-inflammatory and antimicrobial contents of many plants have been analyzed and their effectiveness has been proven in the literature. At the same time, herbal-based treatment approaches were observed to be more reliable in terms of low cytotoxicity on healthy cells compared to chemical and radioactive treatment approaches. The most critical problem of herbal treatment approaches in the literature is the low effectiveness.

In literature, the cytotoxic effect of *E. cardamomum* extract on breast cancer cells has been proven [12]. In this study, the anticancer effect of the *E. cardamomum* plant and its combination with Freund's adjuvant on breast cancer was investigated. *E. cardamomum* extract obtained by maceration method. Then, the extract was applied to the MCF-7 breast cancer cell line and THP-1 human macrophage cell line. While MTT was applied for cytotoxicity analysis, nitric oxide analysis was performed to examine the immunostimulant effect.

The extract has increased cell viability of the THP-1 cell line at a concentration of 40 μg ml^{-1}, and 119.16% cell viability was observed. Therefore, it was determined that the *E. cardamomum* extract did not have a cytotoxic effect on the THP-1 cell line, and even increased the cell viability. When the *E. cardamomum* extract- Freund's adjuvant combination was applied at a concentration of 40 μg ml^{-1} on the THP-1 cell line, 78.06% cell viability was detected.

When an *E. cardamomum* extract at a concentration of 40 μg ml^{-1} was applied to the MCF-7 breast cancer cell line, 80% cell viability was observed, while 73% cell viability was observed at 100 μg ml^{-1} concentration. 40 μg ml^{-1} *E. cardamomum* extract-Freund's adjuvant combination decreased cell viability up to 62.89% in the MCF-7 cell line. *E. cardamomum* extract-Freund's adjuvant combination at 100 μg ml^{-1} concentration results in 52.94% cell viability in the MCF-7 cell line. All in all, cardamomum extract-Freund's adjuvant combination at 100 μg ml^{-1} was the most appropriate concentration because of the higher cytotoxic effect on MCF-7, a favorable immunostimulant effect on the THP-1 cell line.

In this study, the immunostimulant effect and cytotoxic effect of *E. cardamomum* extract and *E. cardamomum* extract-Freund's adjuvant combination on healthy cells were investigated by using a THP-1 cell line. In addition, the cytotoxic effect of *E. cardamomum* extract-Freund's adjuvant combination on the MCF-7 breast cancer cell line was investigated. In the literature, as the extract concentration increases, the killing effects of the extract on cancer cells increase [13]. As the given concentration increases in *E. cardamomum* extract, the cytotoxic effect of the *E. cardamomum* extracts on breast cancer cells increases. Therefore, pure *E. cardamomum* extract applied to the MCF-7 breast cancer cell line showed the highest cytotoxic effect at a concentration of 100 μg ml^{-1}.

4 CONCLUSION

Freund's adjuvant has cytotoxic effects at high concentrations [14]. Therefore, it is desired to reduce the toxic effect of Freund's adjuvant by using plant extracts. In addition, it was aimed to increase the effectiveness of the plant extract with adjuvant [15]. In the study, it was determined that the Freund's adjuvant increased the cytotoxic effect of *E. cardamomum* extract on MCF-7 breast cancer cell lines and decrease the toxic effects on THP-1 cell line as the concentrations of the plant extract combined with the adjuvant increased, consistent with the information in the literature.

As a result, it was determined that when *E. cardamomum* extract was used alone, it has a low cytotoxic effect on breast cancer cells. Due to the low killing efficiency of *E. cardamomum* extract, the combination of *E. cardamomum* extract with Freund's adjuvant was utilized and showed a high cytotoxic effect on breast cancer. At the same time, it was determined that the toxic effect of Freund's adjuvant could be reduced by increasing the plant extract concentration.

Author Contributions

Yağmur ZENGİN: Methodology, Validation, Formal analysis, Investigation, Resources, Data Curation, Writing - Review & Editing
Murat IHLAMUR: Conceptualization, Validation, Formal analysis, Investigation, Resources, Data Curation, Writing - Original Draft, Writing - Review & Editing, Visualization
Hümeyra BAŞARI: Methodology, Validation, Formal analysis, Investigation, Resources, Writing - Review & Editing
All authors read and approved the final manuscript.

Conflict of interest

No conflict of interest was declared by the authors.

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