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RESEARCH

Apoptotic effects of Lycopodium clavatum extract on SKBR-3 human breast cancer cells

Lycopodium clavatum ekstraktının SKBR-3 insan meme kanseri hücreleri üzerindeki apoptotik etkileri

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Abstract

Purpose: Breast cancer is an important public health problem worldwide. Natural compounds derived from plants have emerged as promising candidates for fighting cancer due to their safety, minimal toxicity, and potential effectiveness. This study investigated the apoptotic effect of the ethanol extract of Lycopodium clavatum on SKBR-3 human breast cancer cells.

Materials and Methods: The effect of applying Lycopodium clavatum ethanol extract at different doses (100, 200, and 300 µg/mL) and duration (12, 24, and 48 hours) to evaluate the viability of human breast cancer cells was investigated using the WST-1 cytotoxicity test. Also, the mechanism of apoptosis of Lycopodium clavatum ethanol extract was investigated by intrinsic (BAX and Caspase-9) and extrinsic (Caspase-8 and Caspase-3) pathways.

Results: The application of Lycopodium clavatum ethanol extract had a cytotoxic effect on SKBR-3 cells and this effect was dependent on the dose and duration of treatment. After 12 hours of incubation with LC-EE, 10%, 25%, and 40% cell death were observed in the 100, 200, and 300 µg/mL groups, respectively, compared to the control group. Additionally, our findings demonstrate that Lycopodium clavatum treatment induces the stimulation of apoptotic proteins, including BAX, Caspase-9, Caspase-8, and Caspase-3.

Conclusion: The anti-cancer effect of Lycopodium clavatum ethanol extract in SKBR-3 cells was determined by activating intrinsic and extrinsic apoptotic pathways. These findings suggest that Lycopodium clavatum may assist in the development of new therapeutic strategies as an effective anti-cancer agent against human breast cancer.

Keywords: Lycopodium clavatum extract, SKBR-3, human breast cancer, cytotoxicity, apoptosis

Öz

Amaç: Meme kanseri dünya çapında önemli bir halk sağlığı sorunudur. Bitkisel kaynaklı doğal bileşikler, düşük toksisiteleri, güvenilirlikleri ve potansiyel etkinlikleri nedeniyle kanserle mücadelede umut verici adaylar olarak kabul edilmektedir. Bu çalışmada, Lycopodium clavatum'un etanol ekstraktının SKBR-3 insan meme kanseri hücreleri üzerindeki apoptotik etkisi araştırılmıştır.

Gereç ve Yöntem: İnsan meme kanseri hücrelerinin canlılığını değerlendirmek için farklı dozlarda (100, 200 ve 300 µg/mL) ve sürelerde (12, 24 ve 48 saat) Lycopodium clavatum etanol ekstraktı uygulamasının etkisi, WST-1 sitotoksisite testi kullanılarak incelenmiştir. Ayrıca, Lycopodium clavatum etanol ekstraktının apoptoz mekanizması, içsel (BAX ve Kaspaz-9) ve dışsal (Kaspaz-8 ve Kaspaz-3) yolaklar ile araştırılmıştır.

Bulgular: Lycopodium clavatum etanol ekstraktı uygulaması SKBR-3 hücrelerinde bu etkinin uygulanan doz ve tedavi süresine bağlı olarak sitotoksik bir etki göstermiştir. LC-EE ile 12 saatlik inkübasyondan sonra kontrol grubuna kıyasla; 100 µg/mL verilen grupta %10, 200 µg/mL verilen grupta %25, 300 µg/mL verilen grupta %40'lık bir hücre ölümü gözlenmiştir. Ayrıca, yapılan gözlemler Lycopodium clavatum tedavisinin BAX, Kaspaz-9, Kaspaz-8 ve Kaspaz-3 gibi apoptozla ilişkili proteinlerin aktivasyonunu tetiklediğini göstermektedir.

Sonuç: Lycopodium clavatum etanol ekstraktının SKBR-3 hücrelerinde anti-kanser etkisini içsel ve dışsal apoptotik yolları aktive ederek gerçekleştirebileceğini ortaya koymuştur. Bu bulgular, Lycopodium clavatum'un insan meme kanserine karşı etkili bir anti-kanser ajanı olarak yeni terapötik stratejilerin geliştirilmesine yardımcı olabileceğini göstermektedir.

Anahtar kelimeler: Lycopodium clavatum ekstraktı, SKBR-3, insan meme kanseri, sitotoksisite, apoptoz

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INTRODUCTION

Breast cancer is one of the most prevalent forms of cancer affecting women worldwide, and there is a continuous need for the development of practical therapeutic approaches. While it also affects men, most cases occur in women. Breast cancer is the first or second cause of death for women[1]. It has become a global health problem due to the increasing number of cases. There are different treatment options for breast cancer treatment such as radiotherapy, surgery, chemotherapy, and aromatase inhibitors. The focus is on improving the quality of life for breast cancer patients by aiming for treatments that minimize side effects while maximizing therapeutic benefits[2].

Natural products have long been explored as potential sources of anticancer agents due to their diverse chemical compositions and therapeutic properties. Lycopodium clavatum extract (LC), a plant commonly known as clubmoss, has gained attention in recent years for its potential anticancer properties[3,4].

The LC plant has long been used in traditional medicine for the treatment of various liver ailments and Alzheimer's disease. Also, anti-cancer activity has been demonstrated in mice with liver carcinogens [5]. In the study investigating the effects of combined administration of LC and Quercetin on colon cancer cells (Colo-320), it was found that they exhibited a cytotoxic effect on Colo-320 cells by influencing the mRNA expression of Catalase, Bcl2, Cyclin D1, Caspase 3, Bax, Wnt 1, and p53 genes.

It has been emphasized that it may have anti-cancer potential by reducing the proliferation of colon cancer cells. In the research investigating its antimicrobial activity in addition to its cytotoxic effect, LC has only shown antimicrobial activity against Escherichia coli and Pseudomonas aeruginosa [6]. With this study, the combination of LC with other cancer drugs may create a synergistic effect, and its anti-cancer properties can be increased with combined treatments in future studies. The effects of homeopathic diluted LC forms (LC-5C and LC-15C) on normal blood cells and HeLa cells were evaluated. Results indicated that the homeopathic treatment had minimal or no negative effects on normal cells, but it induced an apoptosis pathway in HeLa cells. Elevated mRNA levels of Bax, Cas-3, and protein were detected, accompanied by a decline in the expression of Apaf, and Bcl-2. These findings suggest the potential of LC as a targeted therapy for HeLa cells while sparing normal cells, warranting further investigation for its therapeutic applications. [7].

The use of plant extracts in cancer research has shown promising results, and LC has been documented to exhibit diverse bioactive properties and compounds with potential anticancer effects. Apoptosis, a process of programmed cell death, is essential for maintaining tissue balance and is frequently disrupted in cancer cells. Identifying natural compounds that can induce apoptosis in cancer cells holds great promise for the development of novel anticancer therapies. Previous studies have indicated that LC-EE can affect different cancer types. However, there is currently a research void regarding the precise mechanisms by which LC influences breast cancer cells and their apoptotic pathway at the molecular level. This study pursued a two-fold objective: firstly, to investigate the potential cytotoxic effects of LC on breast cancer cells, and secondly, to inquire whether this cytotoxicity is associated with modifications in the apoptotic pathway resulting from specific molecules. The underlying hypothesis driving this research proposes that LC's influence on breast cancer involves both intrinsic and extrinsic apoptotic pathways.

Various concentrations of LC-EE (100, 200, and 300 μg/mL) were assessed over 12, 24, and 48-hour incubation periods to assess its impact on SKBR-3 cells. The WST-1 method was employed to measure the cytotoxic impact of the ethanol extract of Lycopodium clavatum on human cancer cells (SKBR-3). Additionally, immunostaining techniques were used to analyze the apoptotic process of the LC-EE on SKBR-3 cells. This analysis focused on measuring the synthesis of specific proteins (Caspase-9, BAX, Caspase-3, and Caspase-8) known to be involved in the apoptotic pathway. Our research reveals that LC-EE induces apoptosis in the SKBR-3 cell line through both intrinsic and extrinsic pathways. These findings provide valuable insights for future researchers in this area.

MATERIALS AND METHODS

Lycopodium clavatum ethanol extraction (LC-EE)

Ethanol solvents were utilized to extract plant compounds in the experiments. For the ethanol-based solvent tests, the alcohol concentration was set at 60%. To extract the plant compounds, a 10 g

sample of the Arifoglu plant (obtained from a local distributor) was mixed with 200 mL of solvent and stirred at 400 rpm for 24 hours. The examples underwent filtration using sterile filters, which had a pore size of 0.22 μL. The resulting extract was then subjected to drying using a lyophilizer under a vacuum of 3000 mT at a temperature of -49 ℃. Finally, the dried extract was stored at a temperature of 4 °C [8].

Lyophilization and preparation of dry matter

LC-EE was created by dissolving it in dimethyl sulfoxide (DMSO) at a concentration of 100 mg/mL. The stock solution of LC-EE was subsequently preserved by storing it at a temperature of -20 °C. The DMSO used for preparation was sourced from Calbiochem (product number 317275-500).

Cell culture

The SKBR-3 cell line (ATCC) was cultured in DMEM high glucose medium supplemented with 10% fetal bovine serum, L-glutamine (1%), and penicillin-streptomycin (10,000 U/mL-10,000 μg/mL). The cells were kept in a humidified 5% CO_2 incubator at 37 °C. Cell viability and growth rates were monitored through microscopic observation. The cell culture materials, including DMEM (Sigma-D6429-500ML), fetal bovine serum (Biowest, S181H-500), L-glutamine (Gibco, 25030081) and penicillin-streptomycin (Gibco, 10378016).

Microscopic monitoring

SKBR-3 cells were evenly distributed into each well of a 6-well plate at a uniform density. Following a 24-hour incubation period, the existing media was discarded, and a new medium was added, containing three distinct concentrations of LC-EE (100, 200, and 300 μg/mL). Then, the cells were placed in the incubator (5 % CO2, 37 °C) and cultured for 12, 24, or 48 hours. Differential Interference Contrast (DIC) microscopy was used to observe the cell cultures for three days, allowing for the tracking of alters in proliferation structures and cell morphology.

Cytotoxicity assay - Cell viability (WST-1)

To evaluate the anticancer activity of LC-EE, we employed the WST-1 assay (Abbkine, KTA1020) following the provided instructions. In this assay, the conversion of tetrazolium salt WST-1 to formazan is dependent on cellular mitochondrial dehydrogenases. As a result, the quantity of formazan dye produced is related to the number of viable cells in the culture. SKBR-3 cells were distributed into 96-well plates at a density of 5,000 cells per well. Afterward, the plates were placed in a humidified incubator at 37 °C with 5% CO_2 for incubation. The initial concentrations of the tested compounds were adjusted by diluting their stock solutions in a complete medium to achieve final concentrations of 100, 200, and 300 μg/mL. Cell cultures were subjected to varying concentrations of the compound or received the highest dose of the vehicle solution (0.1% DMSO). After 12, 24, and 48 hours of incubation, the viability of the cells was evaluated. To assess viability, WST-1 was introduced to the SKBR-3 cells, and a microplate reader (Tecan Infinite 200 pro) measured the absorbance at 450 nm after a 4-hour incubation at 37 °C.

Immunostaining

Cells on the coverslips were treated with 3.5X paraformaldehyde (Sigma-158127). Thus, the cells were fixed on the coverslips. After rinsing with PBS, the cells were preserved in a PBS azide solution (Chemcruz, SC-296028). All fixed cells were exposed to primary antibodies and incubated at +4 °C for 24 hours, including Caspase-9 (Abcam, ab202068, rabbit), Caspase-3 (Abcam, ab13847, rabbit), Caspase-8 (Abcam, ab25901, rabbit), and BAX (Abcam, ab32503, rabbit). Cells were washed twice with PBS (Gibco, 14190-094) to eliminate unbound antibodies. After two time washes with PBS FITC-labeled secondary antibody (Sigma, F9887) was used to stain process. This incubation occurred for 2 hours (5% CO2, 37 °C). Next, cells were washed with PBS and then incubated with 7-amino actinomycin D (7-AAD) for half an hour (37 °C). Following three additional PBS washes, the samples were coated with a mounting medium and stored at -20 °C till examination under a fluorescent microscope. To eliminate non-specific interactions, negative control staining using only the secondary antibody was performed. To exclude non-specific interactions, we conducted a negative control staining using solely the secondary antibody.

Statistical analysis

Cytotoxicity experiments were conducted, involving one control group and three experimental groups (100, 200, and 300 μg/mL), with six replicates. Furthermore, test results were collected at three

different time points: 12 hours, 24 hours, and 48 hours of incubation with LC-EE. The cytotoxicity data obtained in this study underwent statistical analysis utilizing the GraphPad Prism 9 software. The one-way analysis of variance (ANOVA) method was utilized to compare the different groups followed by Tukey's test for multiple comparisons. To determine the normal distribution of the data, the Shapiro-Wilk and Kolmogorov-Smirnov normality tests were employed. Data were assessed for normality and then compared using one-way ANOVA. A significance level of $p < 0.05$ was employed to assess the statistical significance of the findings.

RESULTS

Cytotoxic effect of LC-EE extract on SKBR-3

Morphological monitoring

The experiment's results indicated that after exposing cells to LC-EE for 12, 24, and 48 hours, we conducted a comparative analysis of cell density and morphology between the treatment group (exposed to LC-EE) and the control group (not exposed to LC-EE). The findings revealed a significant decrease in cell density within the treatment group compared to the control group, suggesting that exposure to a concentration of 300 µg/mL of LC-EE led to a decrease in cell count in the treated samples.

To assess cell morphology, which encompasses the overall structure, shape, and appearance of cells, we employed a Differential Interference Contrast (DIC) imaging system, facilitating the visualization of cellular structures with enhanced contrast.

Cell viability results

The WST-1 assay is a commonly utilized method for evaluating cell viability and cytotoxicity. It measures the activity of mitochondrial dehydrogenases in viable cells, which can be indicative of their metabolic activity and overall health.

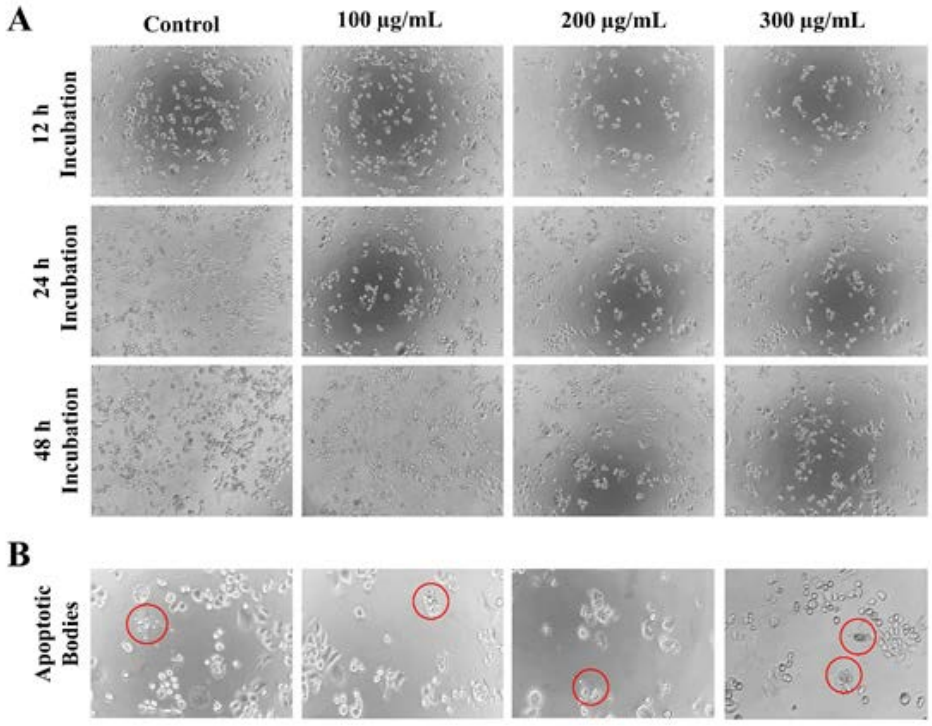

Figure 1 A). Differential Interference Contrast (DIC) microscopy observation of LC-EE treated and untreated SKBR-3 cells and B) apoptotic cells in treated samples with 100, 200, and 300 µg/mL concentrations, during 12-, 24-, and 48-hour incubation times (Magnification scale in LC-EE 10X and Apoptotic Cells 20X).

By measuring the reduction of the WST-1 reagent, which is converted to a colored formazan product, researchers can determine and measure the number of viable cells present in a sample. The study utilized the WST-1 assay to assess the viability of human breast cancer cells in response to different doses and durations of LC-EE administration. By measuring the color intensity generated by the WST-1 assay, we could determine the relative viability of the human breast cancer cells after exposure to LC-EE. This assay allows them to quantify the cytotoxic effects of LC-EE treatment and evaluate how different doses and durations of administration impact the viability of the cells. The viability of the cells under various conditions was evaluated using the WST-1 assay (with IC50 values of approximately 1188.3472 ug/ml).

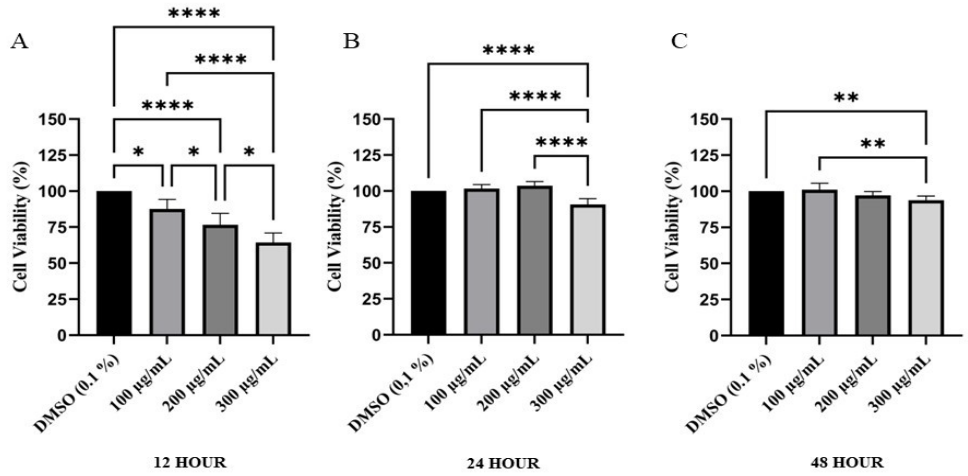

Figure 2. Cytotoxic impact of LC-EE on SKBR-3 cells after A) 12 hours B) 24 hours and C) 48 hours incubation (*p <0.05).

In the 12-hour LC-EE application, the mortality rate of cancer cells demonstrated a dose-dependent escalation. The enhancement in the death rate in cancer cells is statistically significant compared to the control group. In the 24 and 48-hour application, a statistically significant decrease was observed only at 300 µg/mL compared to the control group.

Induction of apoptosis by LC-EE extract promotes cell death in SKBR-3

Immunostaining is a widely used technique in biological research that relies on antigen-antibody complexes. It involves labeling specific cellular components (such as proteins or organelles) with antibodies that recognize and bind to specific target antigens. This allows for the visualization and localization of the target antigen within the cells or tissues of interest. The specific antigens targeted for immunostaining in this study would be proteins or markers that are known to be involved in apoptotic pathways. These antigens could include cleaved caspases, Bcl-2 family proteins, cytochrome c, or other relevant apoptotic markers. In the study, we examined the synthesis of BAX and Caspase-9, which are intrinsic pathways, as well as Caspase-3 and Caspase-8, which are extrinsic pathways, in SKBR-3 cells following LC-EE application. For immunostaining experiments, cells were administered with LC-EE (300 µg/mL) for 24 hours, corresponding to the time required for protein synthesis to occur.

The FITC-labeled secondary antibodies were used to bind to the primary antibodies targeting Caspase-8, BAX, Caspase-3, and Caspase-9. This allowed for the detection and localization of these proteins within the SKBR-3 cells. Additionally, 7-AAD (7-amino actinomycin D) was employed for nuclear immunostaining. 7-AAD binds to DNA and enables the visualization and localization of cell nuclei. The amount of BAX and Caspase-9 proteins, which are

extrinsic apoptotic pathways, synthesized in SKBR-3 cells with and without LC-EE application. The amount of these proteins synthesized with and without applying LC-EE to SKBR-3 cells is shown in Fig 3 and Fig 4. The amount of protein of Caspase-3 and Caspase-8, which are intrinsic apoptotic pathways, synthesized in SKBR-3 cells with and without LC-EE application. The amount of these proteins synthesized with and without applying LC-EE to SKBR-3 cells is shown in Fig 5 and Fig 6.

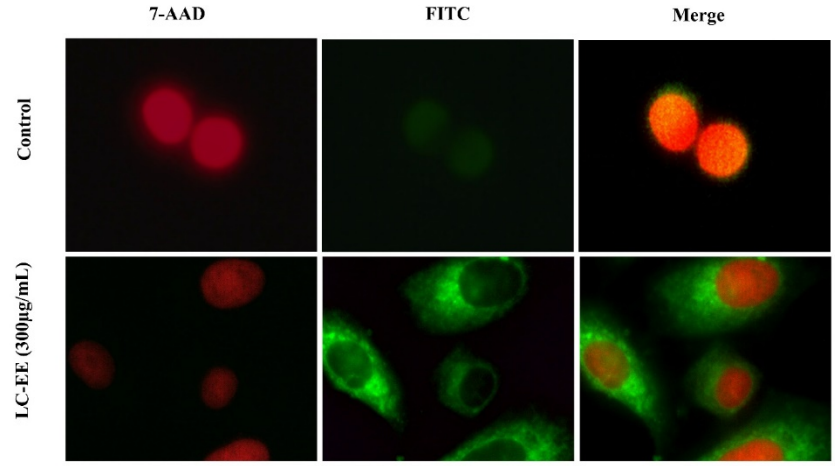

Figure 3. The amount of BAX protein synthesized in the control group and LC-EE (300 µg/mL) treated for 24 hours were shown. Cell nuclei are marked with 7-AAD (red signal) dye. BAX was detected with the antibody labeled with FITC (green signal). (Magnification 100x).

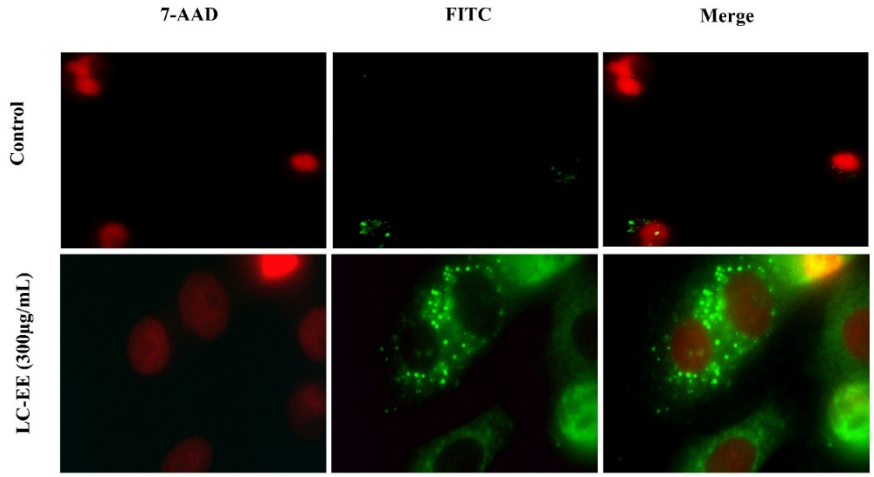

Figure 4. The amount of Caspase-9 protein synthesized in the control group and LC-EE (300 µg/mL) treated for 24 hours were shown. Cell nuclei are marked with 7-AAD (red signal) dye. BAX was detected with the antibody labeled with FITC (green signal). (Magnification 100x).

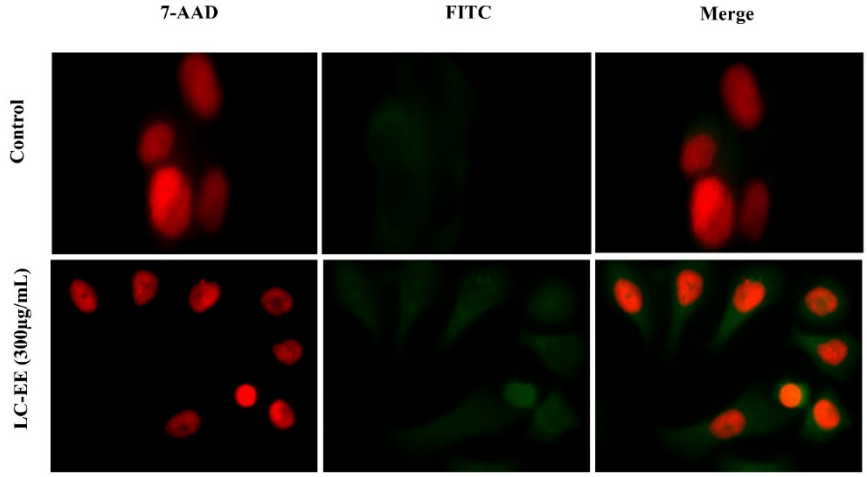

Figure 5. Shows the amount of Caspase-3 protein synthesized in the control group and LC-EE (300 µg/mL) treated for 24 hours. Cell nuclei are marked with 7-AAD (red signal) dye. BAX was detected with the antibody labeled with FITC (green signal). (Magnification 100x).

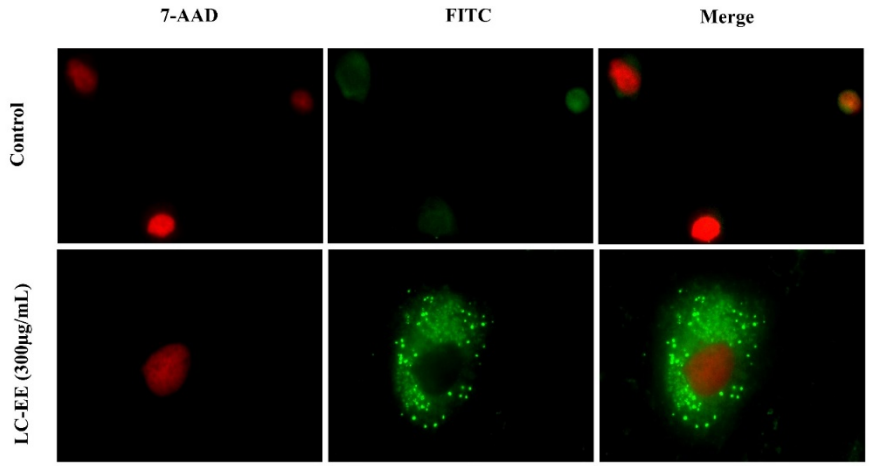

Figure 6. shows the amount of Caspase-8 protein synthesized in the control group and LC-EE (300 µg/mL) treated for 24 hours. Cell nuclei are marked with 7-AAD (red signal) dye. BAX was detected with the antibody labeled with FITC (green signal). (Magnification 100x).

The immunostaining experiments revealed that the synthesis of BAX protein was significantly augmented in SKBR-3 cells compared to the control group. Similarly, the synthesis of Caspase-3, Caspase-8, and Caspase-9 proteins was also higher in the treatment groups compared to the control group.

These results indicate that LC-EE treatment affected the synthesis of Casp-8, BAX, Casp-9, and Casp-3 proteins in the SKBR-3 cell line. The increased synthesis of these proteins indicates their potential involvement in the apoptotic mechanism triggered by LC-EE treatment. The provided information indicates that specific figures (Figures 3, 4, 5, 6) are available in the study, which presents the immunostaining results, demonstrating the higher synthesis of Caspase-8, BAX, Caspase-9, and Caspase-3 proteins in LC-EE treated cells compared

to the control group. In this experiment, we utilized immunostaining to investigate the apoptotic mechanism activated by the administration of LC-EE in SKBR-3 cells. One of our study aims is to clarify the mechanism of cell death related to apoptosis during LC-EE treatment

DISCUSSION

Raw plant extracts have been used for traditional treatments in many parts of the world for many years. With modern approaches, it is possible to produce drugs with specific chemical compounds from plants. Plants that provide sources of terpenoids, steroids, polysaccharides, alkaloids, flavonoids, and antibiotics can be used in the treatment of many diseases, including cancer treatments[9]. In our study, the deadly impact of LC-EE extract on SKBR-3 was tested depending on various doses and times. The potentiality anti-cancer efficacy of LC-EE at 100 μg/mL, 200 μg/mL, and 300 μg/mL concentrations, during 12, 24, and 48 hours incubation times were evaluated by cytotoxicity test and immunostaining method.

In 2010, Mandal et al. showed that lycodin from LC extract in the Hela cell line increased chromatin condensation and nucleosomal DNA fragmentation. It has also been shown to increase Caspase-3 activation and cytochrome-c release. Lycopodin has been shown to inhibit the proliferation of HeLa cells by inducing apoptosis through caspase-3 activation. Thus, it has been demonstrated that Lycopodine has potential for use in chemotherapy [5]. In the study where apigenin A375 and A549 anticancer activity isolated from the ethanolic extract of LC were assessed, the expression level of Bax in A375 and A549 cells elevated proportionally with the increase of apigenin level, while the expression level of Bcl-2 decreased with the rise of apigenin concentration. Apigenin enhanced the expression levels of PARP, Caspase-9, and Caspase-3 in the A375 cell line. In A549 cells, Casp-9 and Casp-3 protein levels increased proportionally with the apigenin dose[3]. In 2013, Bishayee et al. it has been shown that lycopodin can interact with cellular DNA, thereby inhibiting proliferation. Lycopodin administration down-regulated the expression of the epidermal growth factor (EGF) receptor, 5-lipoxygenase, and 5-oxo-ETE receptor (OXE receptor1). It resulted in depolarization of mitochondrial inner membrane potential and up-regulation of cytochrome c synthesis without change in p53 activity. The treatment led to a notable decrease in the growth and division of cancer cells, potentially achieved by specifically targeting the mitochondria through the modulation of 5-lipoxygenase, which triggered the process of apoptosis in the cells[10]. The cytotoxic effect of α-onocerin isolated from LC in MOLT-3 (acute lymphoblastic leukemia), HuCCA-1 (human cholangiocarcinoma), HepG2 (hepatocarcinoma), and A-549 (lung carcinoma) cancer cell lines were evaluated. It showed a significant cytotoxic effect in the HepG2 cancer cell line[11]. In a study investigating the effects of serratane triterpenoids isolated from LC on inflammatory bowel disease (IBD), which is linked to colorectal cancer, serratane triterpenoids reduced the expression of NF-κB and pERK 1/2 in macrophage cells (RAW 264.7). Due to the anti-inflammatory effect of serrate triterpenoids, it is shown as a potential natural material for the treatment of IBD[12]. In addition to the direct use of LC and LC components to fight cancer, it has been shown to supply a regulated and gradual release of 5-fluorouracil (5-FU), a chemotherapeutic agent loaded on LC spores, to aid in the treatment of gastrointestinal cancer. The controlled release of 5-FU in the gastrointestinal system proves highly beneficial for colon, stomach, and breast cancer treatment, potentially eliminating the need for frequent administration of doses. Therefore, LC spores show promise as a natural candidate for encapsulating 5-FU 13. In our results, like the literature, we demonstrated the cytotoxic effect of LC-EE on SKBR-3 by the WST-1 test. The most effective cytotoxic dose and duration of cancer cells was seen in the 12-hour application of LC-EE 300 μg/mL. The decrease in the lethal effect in the 24 and 48-hour application may have been related to the decreased active ingredient quantity dependent on time. Our recommendation is to repeat the application of LC-EE every 12 hours so that the lethal effect can be observed more clearly.

A critical feature of cancer cells is their resistance to apoptosis induction. Activation of the caspase cysteine family of proteases subsequently leads to morphological alterations, including cell shrinkage and the breakdown of cells into apoptotic bodies. Apoptosis can activate the mitochondrial-dependent (intrinsic: Caspase-9 and BAX) pathway and the death receptor (extrinsic: Caspase-3 and Caspase-8) pathway[14]. Apoptosis, the programmed cell death process, involves two main pathways within the cell: the cytoplasmic pathway and the mitochondrial pathway. These pathways are regulated by various

genes and proteins. Among the crucial molecules involved in these pathways is the caspase family. Caspases can be categorized into three groups: terminator effector caspases (Caspase-3, Caspase-6, Caspase-7), inflammatory caspases (Caspase-1, Caspase-4, Caspase-5), and initiator caspases (Caspase-2, Caspase-8, Caspase-9, Caspase-10)[15]. There is no sufficient research article in the literature on the apoptotic effects of LC on cancer cells. In this study, the apoptosis mechanism of LC-EE was investigated by intrinsic and extrinsic pathways. In comparison to the control group, there was a notable rise in protein synthesis for the intrinsic pathways Caspase-9 and BAX. Caspase-8, which is one of the extrinsic pathways, and Caspase-3, which is responsible for both the intrinsic and extrinsic pathways, have significantly increased protein synthesis compared to the control, like the intrinsic pathways. The results show that LC-EE can exert its cytotoxic effect by activating intrinsic and extrinsic pathways.

Although our study has yielded promising findings, it is important to consider several limitations. Our study primarily focused on examining the cytotoxic effects and potential mechanisms of LC-EE on breast cancer cells. However, cancer is a multifaceted disease, and additional research is needed to assess the broader impact of LC-EE on various aspects of cancer progression, including metastasis and resistance to treatment. Finally, our study did not address the possible side effects or toxicity profiles of LC-EE, which is a critical consideration in the development of any therapeutic agent. Future studies should address these limitations to provide a more comprehensive understanding of the potential of LC-EE in cancer treatment. In addition, we recommend using different groups and broader spectrum LC-EE concentrations in healthy breast cell lines as well as breast cancer cell lines in future studies. Also, our recommendation is to repeat the application of LC-EE every 12 hours during cell culture conditions, so that the lethal effect can be observed more clearly.

Employing techniques such as qRT-PCR for gene expression analysis and western blotting for protein-level investigations can provide valuable insights. Secondly, understanding the precise mechanisms through which LC-EE extracts induce apoptosis in breast cancer cells is crucial. To achieve this, future studies may consider conducting in vivo experiments to assess the efficacy of LC-EE extracts as potential anti-cancer agents. Furthermore, evaluating the safety profiles of LC-EE extracts through animal model experiments holds crucial importance for translating the findings into practical and effective therapies. Additionally, investigating the potential synergistic effects of LC-EE and various agents with anticancer properties could be a promising avenue for enhancing their anticancer properties. This may involve exploring different dosages and treatment regimens to optimize treatment outcomes. In conclusion, our study opens doors to a range of exciting research opportunities aimed at advancing cancer treatment strategies. These suggestions offer a roadmap for future investigations that can further unravel the potential of LC-EE extracts as valuable tools in the fight against cancer. Understanding the apoptotic outcome of LC-EE on SKBR-3 cancer cells can provide valuable insights into its therapeutic potential as an alternative or adjuvant therapy for breast cancer.

In summary lant extracts contain a blend of structurally diverse components. In this investigation, we observed that the LC-EE extract affected SKBR-3 cells. Our study demonstrated that LC-EE induces an anti-cancer effect in SKBR-3 cells by activating intrinsic and extrinsic apoptotic pathways. Our research offers promising evidence suggesting that LC-EE from this plant could potentially serve as a novel treatment option for combating cancer. However, the exact mechanism through which LC-EE acts on cancer cells remains incompletely understood. Further research is essential to explore the potential therapeutic utility of LC-EE. Subsequent studies may recommend utilizing various molecular techniques such as Western Blot and qRT-PCR, varying the doses and durations of LC-EE exposure, to gain a more comprehensive understanding of the underlying molecular mechanisms.

Author Contributions: Concept/Design : MD, YK; Data acquisition: YK, MD; Data analysis and interpretation: YK, MD; Drafting manuscript: YK, MD; Critical revision of manuscript: YK, MD; Final approval and accountability: MD, YK; Technical or material support: MD, YK; Supervision: MD, YK; Securing funding (if available): n/a.
Ethical Approval: Since this research is a cell culture study and the SKBR-3 cell line is used, ethics committee approval is not required. The human breast cancer cell line (SKBR-3) used in this study was obtained from Assoc. Dr. Bala Gür Dedeoğlu in Ankara University Biotechnology Institute.
Peer-review: Externally peer-reviewed.
Conflict of Interest: Authors declared no conflict of interest
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