The Apoptotic Effect of The Lycopodium Clavatum Extracts on MCF-7 Human Breast Cancer Cells

Yusuf Kucukbagriacik
Department of Biophysics, Health sciences University, Gulhane Faculty of Medicine, Ankara, and Yozgat Bozok University, Medical School, Yozgat, Turkey

Mohammadreza Dastouri (mrdastouri@ankara.edu.tr)
Ankara University Biotechnology Institute and Sisbiyotek Advanced Research Unit, Ankara, Turkey
https://orcid.org/0000-0003-3882-0728

Humeyra Yilmaz
Department of Medical Biology, Institute of Health Sciences, Ankara Yildirim Beyazit University, Ankara, Turkey

Evrim Gunes Altuntas
Ankara University Biotechnology Institute and Sisbiotek Advanced Research Unit, Ankara, Turkey

Research Article

Keywords: Lycopodium Clavatum extract, cytotoxicity, apoptosis, human breast cancer, MCF-7

Posted Date: June 16th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-3029849/v1

Version of Record: A version of this preprint was published at Medical Oncology on September 1st, 2023. See the published version at https://doi.org/10.1007/s12032-023-02159-7.

Abstract

Breast cancer is a significant health problem worldwide, and the search for effective treatments is critical. Side effects of cancer treatments such as surgery, radiotherapy, and chemotherapy reduce the patient's standard of living. Recently, natural compounds from plants have gained attention as potential anticancer agents due to their safety, low toxicity, and potential efficacy. Lycopodium Clavatum (LC) is an herb abundant in tropical regions and Europe and is known for its various medicinal properties. In this study, we investigated the cytotoxic and apoptotic effects of LC Water Extract (LC-WE) and LC Ethanol Extract (LC-EE) plant extracts on MCF-7 human breast cancer cells. Our results showed that LC treatment led to a dose and time-dependent cytotoxic effect on MCF-7 cells, indicating its potential as an anticancer agent against human breast cancer. Additionally, we observed that LC treatment activated apoptosis-related proteins, including BAX, Caspase-3, and Caspase-9. These results suggest that LC may induce apoptosis as a mechanism underlying its cytotoxic effect on MCF-7 human breast cancer cells. Previous studies have shown the anti-cancer potential of LC against different types of cancer. However, the anti-cancer effect of LC on human breast cancer cells has not been investigated to date. Therefore, our study provides novel insights into the potential of LC as an anti-cancer agent against breast cancer. Overall, our results highlight the potential of LC as a promising natural compound for breast cancer treatment.

INTRODUCTION

According to the International Agency for Research on Cancer (IARC), breast cancer has become the most common type of cancer in the world, surpassing lung cancer. The total number of people diagnosed with cancer in 2020 increased to 19.3 million. It is estimated that the number of people diagnosed with cancer in 2040 will increase by about 50% compared to 2020. Today, one in five people worldwide is at risk of developing cancer in their lifetime. More than one in six deaths worldwide is related to cancer [1].

Multidisciplinary approaches and treatment options are used in the treatment of breast cancer, including radiotherapy, and surgery. Treatments with maximum therapeutic effect and minimal side effects are targeted to improve the quality of life of breast cancer patients [2]. Chemotherapy has variable effects on the immune response, depending on the type, dose, and timing of the drug [3]. Cancer is a disease that requires a versatile approach from different disciplines.

Commonly known as "club algae," Lycopodium Clavatum (LC) is a perennial plant found almost all over the world. It is a medicinal plant used in traditional and alternative medicine [4]. LC is also known as club moss, ground pine, clubfoot moss, wolf's claw, and foxtail [5]. The LC's raw ethanolic extract is an infusion of about 201 alkaloids [6]. For the treatment of many liver problems and Alzheimer's disease, the raw ethanolic extract of the LC plant has been utilized in complementary and alternative medicine for a very long time. It has been demonstrated that − 3 oil is shielded from oxidation brought on by ambient light and ultraviolet radiation by sporopollenin exine capsules (SpECs), which are derived from the spores of LC. LC-derived SpECs act as biologically derived antioxidants to protect them from oxidation [7]. In

addition, chronic liver carcinogens have shown potential anti-cancer characteristics in mice fed with p-dimethylamino azobenzene and phenobarbital [8].

LC has been shown to have hepatoprotective and anti-carcinogenic effects on liver cancer in mice [9]. HupA, one of the main alkaloids found in Lycopodium extracts, is beneficial for treating Alzheimer's disease [10]. According to Mandal et al. (2009) found that lycopodynine induces internucleosomal DNA fragmentation, and chromatin condensation, and increases the cell population in the sub G1 region with the rise of reactive oxygen species, inducing cytochrome-c release, and Caspase-3 activation, inducing apoptosis. Lycopodin significantly inhibited the growth of HeLa cells, demonstrating its potential for use in chemotherapy [8].

The anti-cancer potential of apigenin, which was extracted from the ethanolic extract of LC, was examined in both cancer cells and healthy peripheral blood mononuclear cells. Melanoma cell line (A375) and lung carcinoma cell line (A549) led to apoptosis but had no cytotoxic effect on normal peripheral blood mononuclear cells (PBMC). Apigenin has been shown to exhibit anti-cancer potential in A375 and A549 cells through a direct or indirect effect on the mitochondrial oxidative phosphorylation system via DNA interaction and mitochondrial dysfunction [4].

Another study showed that the administration of homeopathically diluted (LC-5C and LC-15C) forms of LC had little or no cytotoxic effect on normal peripheral blood mononuclear cells. However, it has been revealed that it causes cell death by apoptosis in HeLa cells. Protein and mRNA expression levels of Caspase 3 and BAX increased, while expression levels of Bcl-2 and Apaf decreased [11]. To analyze the synergistic effect on colon cancer cells, a combination treatment of quercetin and LC caused a significant downregulation of MMP2 and MMP9 activities. Combined treatment significantly influenced the mRNA expression of p53, Wnt 1, Bcl-2, Caspase-3, Cyclin D1, and BAX genes in colon cancer cells, leading the cancer cells to apoptosis [12].

Apoptosis is carried out by cytoplasmic and mitochondrial pathways in the cell. These pathways also involve many genes and proteins. The caspase family is one of the important molecules involved in these pathways: initiator caspases (Caspase 2, 8, 9, 10), terminator effectors caspases (Caspase 3, 6, 7), and inflammatory caspases (Caspase 1, 4, 5) [13]. Caspase-9 is a member of the caspase cysteine protease family. When the cell receives apoptotic stimuli, it binds to the cytoplasmic protein Apaf-1 with the release of cytochrome-c from mitochondria and procaspase-9 is activated, and this complex structure is called apoptosome. A series of caspase activation begins with the activation of procaspase-9 [14]. Caspase-3 can be activated depending on or independently of mitochondrial cytochrome-c release and Caspase-9. Caspase-3 is a stimulated protease responsible for apoptotic cell death [15]. BAX is an important mediator of mitochondria-dependent programmed cell death. BAX belongs to the Bcl-2 family of proteins, and its activation is regulated by its interaction with other Bcl-2 member proteins [16]. The balance between the proapoptotic gene BAX and the antiapoptotic gene Bcl-2 determines the choice between cell life or death.

There are numerous studies aiming to discover new therapeutic agents with less toxic side effects compared to existing chemotherapeutic agents. Two general approaches are used to investigate the anticancer and antibacterial effects of plant extracts. The first approach involves testing the plant extract as a whole in anticancer and antibacterial studies. If positive results are obtained, individual substances in the plant extract are tested to determine which ones are effective. The second approach involves separating the plant extract into its basic components, followed by testing the effectiveness of each substance individually.

In this study, the water and ethanol extract of LC were investigated as a whole, using the first approach. The LC extract will be tested on the MCF-7 human breast cancer cell line using cytotoxic and apoptotic experiments. The anti-cancer properties of LC at concentrations of 100, 200, and 300 µg/mL will be evaluated during 12, 24, and 48-hour incubation periods. The cytotoxic effect of the LC extract on the MCF-7 human breast cancer cell line will be measured using the WST-1 method. The apoptotic mechanism of the LC extract on human breast cancer cell lines will be analyzed using immunostaining methods to measure the synthesis of caspase-3, caspase-9, and BAX proteins, which play a role in the apoptotic pathway.

MATERIALS AND METHODS

LC Water Extract (LC-WE) and LC Ethanol Extract (LC-EE) obtention

In the experiments, plant extracts were obtained by using both distilled water and ethanol solvents. In the experiments where ethanol was used as a solvent, the alcohol ratio was adjusted to 60%. For the extraction of the plants, 10 g of the plant sample (obtained from, a local herbalist in Ankara-Turkey) was dissolved in 200 mL of solvent and mixed at 400 rpm for 24 hours. The mixed samples were filtered through 0.22 µm sterile filters (Sartorius, Germany), and the obtained extract was dried with a lyophilizer (with 3000 mT vacuum at -49°C) and stored at 4°C [17].

Lyophilization and Preparation of Dry Matter

The stock solution of the tested compounds was prepared in DMSO (Calbiochem, 317275-500) for LC-EE at 100 mg/mL concentration and in sterile ddH_2O for LC-WE at 100 mg/mL. LC-EE stock solution was conserved at -20°C and LC-WE stock solution at + 4°C.

Cell Culture

Human breast adenocarcinoma cell line MCF-7 (ATCC, HTB-22) cells were cultured in DMEM high glucose medium (Sigma-D6429-500ML) supplemented with 10% heat-inactivated fetal bovine serum (Biowest, S181H-500), penicillin-streptomycin (10,000 U/mL-10,000 µg/ml) (Gibco, 10378016) and 1% L-glutamine (Gibco, 25030081). In an incubator with 5% CO_2 that was humidified, cells were maintained at 37°C.

Microscopic Monitoring

MCF-7 human breast cancer cells were cultured and seeded in a 6-well plate at the same density for each well. After 24 hours, the medium was removed and replaced with fresh medium containing different concentrations of LC extract (100, 200, 300 µg/ml) in both LC-EE and LC-WE. Cells were then incubated for 12, 24, or 48 hours. The cultures were observed for two days under Differential Interference Contrast (DIC) microscopy to monitor cell morphology and any changes in growth patterns.

Cell Viability/Cytotoxicity Assay (WST-1)

We used the WST-1 test (Takara, MK400) by the manufacturer's instructions to determine the anticancer activity of LC. The assay is based on the cellular mitochondrial dehydrogenases' cleavage of the tetrazolium salt WST-1 to formazan. As a result, the number of living cells in the culture directly correlates with the amount of formazan dye generated. MCF-7 cells were cultivated at 37°C in an incubator with 5% CO_2 humidity and 8.000 cells per well in 96-well plates. Stock solutions were diluted in a complete medium to give the final concentration of 100µg/mL, 200µg/mL, and 300µg/mL. The maximum vehicle dose (0,1% DMSO) or various doses of the investigated substances were used to treat the cells. After compound incubation for 12 hours, 24 hours, and 48 hours, cell viability was evaluated. Briefly, WST-1 reagent was added to the cells, and after 4 hours of incubation at 37°C, the absorbance at 450 nm was measured using a microplate reader (Tecan Infinite 200pro).

Immunostaining

Cells cultured on the coverslip were fixed with 3,5X Paraformaldehyde (Sigma-158127). After washing with PBS (Gibco,14190-094), they were conserved in PBS azide (Chemcruz, SC-296028). The cells were subjected to a fixed incubation process with primary antibodies, namely Caspase-3 (Abcam, ab13847, rabbit), Caspase-9 (Abcam, ab202068, rabbit), and BAX (Abcam, ab32503, rabbit) for 24 hours at + 4°C. Following this, the coverslip was washed twice with PBS to eliminate any unbound antibodies. Subsequently, the cells were incubated with a FITC (anti-rabbit) labeled secondary antibody (Sigma, F9887) for 2 hours at 37°C. After washing with PBS, the cells were treated with 7-aminoactinomycin D (7-AAD) (Invitrogen, A1310) a DNA dye, for 30 minutes at 37°C. Finally, the samples were washed thrice with PBS and covered with a mounting medium. Slides were conserved at -20°C examined until observation under a fluorescent microscope. Negative control (secondary antibody only) staining was used in the samples to eliminate non-specific interaction.

RESULTS

LC extracts have a cytotoxic effect on MCF-7 human breast cancer cells

a. Morphologically monitoring

The results of the experiment indicated that after 48 hours of incubation with 300 µg/mL LC-WE, the cell density and morphology were compared to the control group (without LC-WE) using the DIC imaging system. The cell density in the treatment group was significantly decreased compared to the control

group. Additionally, similar results were obtained after 24 hours of incubation with 300 µg/mL LC-EE compared to the control group (Fig. 1).

b. Cell viability results

WST-1 is a cytotoxicity test used to measure viable cells. With this method, the lethal effect of different dosing and temporal administration of LC extracts on human breast cancer cells was analyzed.

Figure 2a shows the lethal effect of LC-EE in a dose-dependent manner after 12 hours of application. The 100 µg/mL LC-EE was statistically significantly reduced compared to the control. The 200 µg/mL LC-EE was statistically decreased compared to the control. However, no statistical difference was observed between 100 µg/mL and 200 µg/mL. The highest dose of 300 µg/mL LC-EE is the most effective dose in 12 hours of incubation. As shown in Fig. 2b, the lethal effect of LC-EE increased in a dose-dependent manner as a result of the 24-hour application. The 100 µg/mL LC-EE was statistically significantly reduced compared to the control. The 200 µg/mL LC-EE was statistically decreased compared to the control. 200 µg/mL LC-EE was statistically decreased compared to 100 µg/mL LC-EE. The cytotoxic effect was observed to increase statistically at 300 µg/mL compared to 200 and 100 µg/ml. As shown in Fig. 2c, after 48 hours of application, the lethal effect of LC-EE increased in a dose-dependent manner only at a dose of 300 µg/ml. There is no statistically significant difference between 100 µg/mL LC-EE and the control. There is no statistically significant difference between 200 µg/mL and control. The LC-EE we applied only 300 µg/mL was statistically decreased compared to the control. The 300 µg/mL LC-EE was statistically decreased compared to 200 µg/mL and 100 µg/mL.

In Fig. 3a, it was shown that the lethal effect of LC increased in a dose-dependent manner with 12-hour WE application. The 100 µg/mL LC-WE was statistically significantly reduced compared to the control. The 200 µg/mL LC-WE was statistically decreased compared to the control. The 200 µg/mL LC-WE was statistically decreased compared to 100 µg/mL. The highest dose of 300 µg/mL LC-WE was found to be the most effective dose in 12 hours of incubation. As shown in Fig. 3b, the lethal effect of LC increased in a dose-dependent manner as a result of the 24-hour WE application. The doses of 100 µg/mL, 200 µg/mL, and 300 µg/mL LC-WE were statistically significantly reduced compared to the control. However, there is no statistical significance between 100 µg/mL, 200 µg/mL, and 300 µg/mL LC-WE. In Fig. 3c, the lethal effect of LC increased in a dose-dependent manner as a result of 48 hours of WE application. The 100 µg/mL LC-WE was statistically significantly reduced compared to the control. The 200 µg/mL LC-WE was statistically decreased compared to the control. 200 µg/mL LC-WE was statistically decreased compared to 100 µg/mL. LC-WE with the highest dose of 300 µg/mL is the most effective dose in 48 hours of incubation.

LC extracts stimulate MCF-7 human breast cancer cells death by activating apoptosis

Immunostaining is an immunological method based on antigen-antibody complex. With this method, the apoptosis mechanism of death in MCF-7 cells was tried to be explained after the application of LC extracts. The synthesis of BAX, Caspase-3, and Caspase-9 protein was investigated after LC extracts application to MCF-7 human breast cancer cells. Immunostaining experiments were performed for LC-EE 300 µg/mL for 24 hours and LC-WE 300 µg/mL for 48 hours, when we got the highest yields in the cytotoxicity test. The FITC-labelled secondary (green) was used for the BAX, Caspase-3, and Caspase-9 antibodies. 7-AAD (red) was used to immunostaining the nucleus (DNA). The synthesis of BAX protein was higher in MCF-7 human breast cancer cells treated with LC-EE 300 µg/mL for 24 hours and LC-WE 300 µg/mL for 48 hours compared to the control (Fig. 4). Similar results were obtained for Caspase-3 and Caspase-9 where the synthesis of Caspase protein was higher in MCF-7 human breast cancer cells treated with LC-EE 300 µg/mL for 24 hours and LC-WE 300 µg/mL for 48 hours compared to control (Fig. 5, 6).

DISCUSSION

Human breast cancer is a major global health issue, and finding effective treatments is of utmost importance. In recent years, natural compounds from plants have gained attention as potential anticancer agents due to their safety, low toxicity, and potential efficacy. LC is an abundant herb in tropical regions and many European countries. Many studies in the literature have shown the anti-inflammatory, immunomodulatory, antioxidant, antimicrobial, analgesic, neuroprotective, and hepatoprotective activities of LC. It is also used in the treatment of gastroenteritis, aneurysm, fever, constipation, and chronic lung, and bronchial disorders [5]. Recently, the anti-cancer effect of extracts obtained from LC has started to be investigated on different cancer cell lines.

Our study aimed to investigate, for the first time, the impacts of LC-EE and LC-WE plant extracts on human breast cancer cells in terms of their cytotoxicity and ability to induce apoptosis. Morphologic and cytotoxic analyses revealed a dose-dependent cytotoxic effect of LC on MCF-7 cells, indicating its potential as an anticancer agent against human breast cancer (Fig. 1–3). In addition to the cytotoxic effect, we also observed that LC treatment led to the activation of apoptosis-related proteins, including BAX, Caspase-3, and Caspase-9 (Fig. 4–6). These proteins are known to play critical roles in the intrinsic apoptotic pathway, which is a tightly regulated process of programmed cell death that is often dysregulated in cancer cells. The activation of these proteins suggests that LC may induce apoptosis as one of the mechanisms underlying its cytotoxic effect on MCF-7 human breast cancer cells.

When we look at the literature, extracts of LC have been investigated on many diseases and different types of cancer. In mice fed the spore extract of LC, it can exert a positive effect against the cytotoxicity and genotoxicity induced by carcinogens. It also provides evidence of their anti-cancer potential by reducing the number and size of tumors [18]. In a study conducted by Samadder et al. in 2013, forms of LC caused an increase in the expression levels of Caspase-3 and Bax in HeLa cells. It also decreased expression levels in Bcl-2 and Apaf. By inducing apoptosis in LC cancer cells, it has shown a potential to be used as a support drug for cancer treatment [11]. Nano-formulated LC in colon cancer cells (HCT15)

may have significantly inhibited the growth and apoptosis of colon cancer cells, possibly via Caspase-3, because it was shown that the Bcl2/Bax pathway remained unchanged after nano-formulated drug treatment in colon cancer cells. The potential anti-cancer activity of LC against colon cancer cells has been demonstrated [19]. In 2020, Banerjee et al. LC was applied to colon cancer cells LC led colon cancer cells to apoptosis by upregulating the mRNA expression of Bax and Caspase-3 genes [12]. To date, the anti-cancer effect of LC extract on breast cancer cells has not been investigated. In our study, the cytotoxic and apoptotic effects of LC-EE and LC-WE human breast cancer cells were investigated for the first time. According to the results we obtained, it is in line with the results obtained by other researchers on different types of cancer. The lethal effect of LC has been demonstrated by the increase in protein synthesis of Bax, Caspase-3, and Caspase-9, which it can perform by triggering apoptotic pathways.

The extraction procedures of the plant, the duration, and the measurement methods may affect the active components of the plants. Therefore, appropriate methods should be selected for the most effective biological activity in medicinal plant research [20]. According to the data we obtained, the apoptotic effect on MCF-7 cells increased in proportion to dose and time in LC-WE. However, according to our LC-EE results, this increase in cytotoxic effect is observed at 12 and 24 hours, while the increase in effect decreases at 48 hours. In this experiment, LC extracts were added to the experimental groups at the zero hour and the effect was followed for two days. No medium changes were made during this period. Therefore, the concentration of the active substance in the medium may decrease depending on the uptake of the active substance into the cell over time. To prevent this, we think that the medium containing the LC extract should be changed every 12 hours. In future experiments, we recommend that the active substance be applied in this way. According to the results of LC-applied immunostaining experiments, Bax, Caspase-3 and Caspase-9 protein synthesis increased more in 24 hours LC-EE 300 µg/mL and 48 hours LC-WE 300 µg/mL doses applied to MCF-7 cells compared to control groups. Our results showed that LC exerts its lethal effect on MCF-7 cells by activating apoptotic pathways.

CONCLUSION

Our study demonstrates that LC has a dose-dependent cytotoxic effect on MCF-7 human breast cancer cells. Furthermore, we observed the activation of apoptosis-related proteins, including BAX, Caspase-3, and Caspase-9, following LC treatment. These proteins are known to play critical roles in the intrinsic/extrinsic apoptotic pathway, which is a tightly regulated process of programmed cell death that is often dysregulated in cancer cells. The activation of these proteins suggests that LC may induce apoptosis as one of the mechanisms underlying its cytotoxic effect on MCF-7 human breast cancer cells. Further research is needed to determine the mechanisms of action of LC extracts on breast cancer cells and to evaluate their efficacy in vivo. The findings of this study contribute to the growing body of evidence supporting the use of LC extracts as a potential candidate for therapeutic use as an anti-cancer drug.

Declarations

CONFLICT OF INTEREST

No potential conflict of interest was reported by the authors.

AUTHORS CONTRIBUTION

Conceptualization, Y.K., M.D.; methodology, Y.K., M.D.; investigation, Y.K., M.D., E.G.A., and H.Y; formal analysis, Y.K., M.D., writing-original draft preparation, Y.K., M.D., and H.Y.; writing, review, and editing, Y.K., M.D., E.G.A., and H.Y.; funding acquisition. All authors have read and agreed to the published version of the manuscript.

FUNDING

This study was supported by the Yozgat Bozok University Project Coordination Application and Research Center under grant number TGA-2022-982.

References

1. WHO. "Breast cancer now most common form of cancer: WHO taking action." WHO. https://www.who.int/news/item/03-02-2021-breast-cancer-now-most-common-form-of-cancer-who-taking-action (accessed 3 March 2023, 2023).
2. Fisusi FA, Akala EO. "Drug Combinations in Breast Cancer Therapy," (in eng), *Pharmaceutical nanotechnology*, vol. 7, no. 1, pp. 3–23, 2019, doi: 10.2174/2211738507666190122111224.
3. Emens LA, Middleton G. "The interplay of immunotherapy and chemotherapy: harnessing potential synergies," (in eng), *Cancer Immunol Res*, vol. 3, no. 5, pp. 436 – 43, May 2015, doi: 10.1158/2326-6066.Cir-15-0064.
4. Das S, Das J, Samadder A, Boujedaini N, Khuda-Bukhsh AR. Apigenin-induced apoptosis in A375 and A549 cells through selective action and dysfunction of mitochondria," (in eng). Exp Biol Med (Maywood). Dec 2012;237(12):1433–48. 10.1258/ebm.2012.012148.
5. Banerjee JN, Biswas S, Madhu NR, Karmakar SR, Biswas SJ. A better understanding of pharmacological activities and uses of phytochemicals of Lycopodium clavatum: A review. J Pharmacognosy Phytochemistry. 2014;3:207–10.
6. Ma X, Gang DR. "The Lycopodium alkaloids," (in eng), *Nat Prod Rep*, vol. 21, no. 6, pp. 752 – 72, Dec 2004, doi: 10.1039/b409720n.
7. Thomasson MJ, et al. Sporopollenin exine capsules (SpECs) derived from Lycopodium clavatum provide practical antioxidant properties by retarding rancidification of an ω-3 oil. Ind Crops Prod. 2020;154:112714. 10.1016/j.indcrop.2020.112714.
8. Mandal SK et al. "Lycopodine from Lycopodium clavatum extract inhibits proliferation of HeLa cells through induction of apoptosis via caspase-3 activation," (in eng), Eur J Pharmacol, vol. 626, no. 2–3, pp. 115 – 22, Jan 25 2010, doi: 10.1016/j.ejphar.2009.09.033.

9. Pathak S, Banerjee A, Paul S, Khuda-Bukhsh AR. Protective potentials of a plant extract (Lycopodium clavatum) on mice chronically fed hepato-carcinogens," (in eng). Indian J Exp Biol. Jul 2009;47(7):602–7.
10. Wang H, Tang XC. Anticholinesterase effects of huperzine A, E2020, and tacrine in rats," (in eng). Zhongguo Yao Li Xue Bao. Jan 1998;19(1):27–30.
11. Samadder A, Das S, Das J, Paul A, Boujedaini N, Khuda-Bukhsh AR. The Potentized Homeopathic Drug, Lycopodium clavatum (5C and 15C) Has Anti-cancer Effect on HeLa Cells In Vitro. J Acupunct Meridian Stud. 2013;6(4):180–7. https://doi.org/10.1016/j.jams.2013.04.004. 2013/08/01/.
12. Banerjee A, Pathak S, Jothimani G, Roy S. Antiproliferative effects of combinational therapy of Lycopodium clavatum and quercetin in colon cancer cells," (in eng). J Basic Clin Physiol Pharmacol. May 12 2020;31(4). 10.1515/jbcpp-2019-0193.
13. Rai NK, Tripathi K, Sharma D, Shukla VK. "Apoptosis: A Basic Physiologic Process in Wound Healing," Int J Low Extrem Wounds, vol. 4, no. 3, pp. 138–44, 2005/09/01 2005, doi: 10.1177/1534734605280018.
14. Kuida K. "Caspase-9," (in eng), *Int J Biochem Cell Biol*, vol. 32, no. 2, pp. 121-4, Feb 2000, doi: 10.1016/s1357-2725(99)00024-2.
15. Porter AG, Jänicke RU. "Emerging roles of caspase-3 in apoptosis," (in eng), *Cell Death Differ*, vol. 6, no. 2, pp. 99–104, Feb 1999, doi: 10.1038/sj.cdd.4400476.
16. Jensen K, WuWong DJ, Wong S, Matsuyama M, Matsuyama S. Pharmacological inhibition of Bax-induced cell death: Bax-inhibiting peptides and small compounds inhibiting Bax," (in eng). Exp Biol Med (Maywood). May 2019;244(8):621–9. 10.1177/1535370219833624.
17. Mu S et al. "Transdermal permeation research of ethanol extracts from Lycopodium clavatum," *IOP Conference Series: Earth and Environmental Science*, vol. 559, no. 1, p. 012026, 2020/08/01 2020, doi: 10.1088/1755-1315/559/1/012026.
18. Pathak S, Banerjee A, Khuda-Bukhsh A. Efficacy of ethanolic spore extract of < i > Lycopodium clavatum in reducing induced hepatotoxicity and genotoxicity in mice. Int J Biol Chem Sci. 2010;4(3). 10.4314/ijbcs.v4i3.60510.
19. Paramita P, et al. Evaluation of potential anti-cancer activity of cationic liposomal nanoformulated Lycopodium clavatum in colon cancer cells," (in eng). IET Nanobiotechnol. Sep 2018;12:727–32. 10.1049/iet-nbt.2017.0106.
20. Kar F, Özyurt D, Öztürk B, "THE EFFECTS OF OPTIMIZATION METHODS ON THE DETERMINATION OF TOTAL ANTIOXIDANT CAPACITY IN SOME PLANTS,". Fresenius Environ Bull, vol. 28, pp. 6589–95, 07/02 2019.

Figures

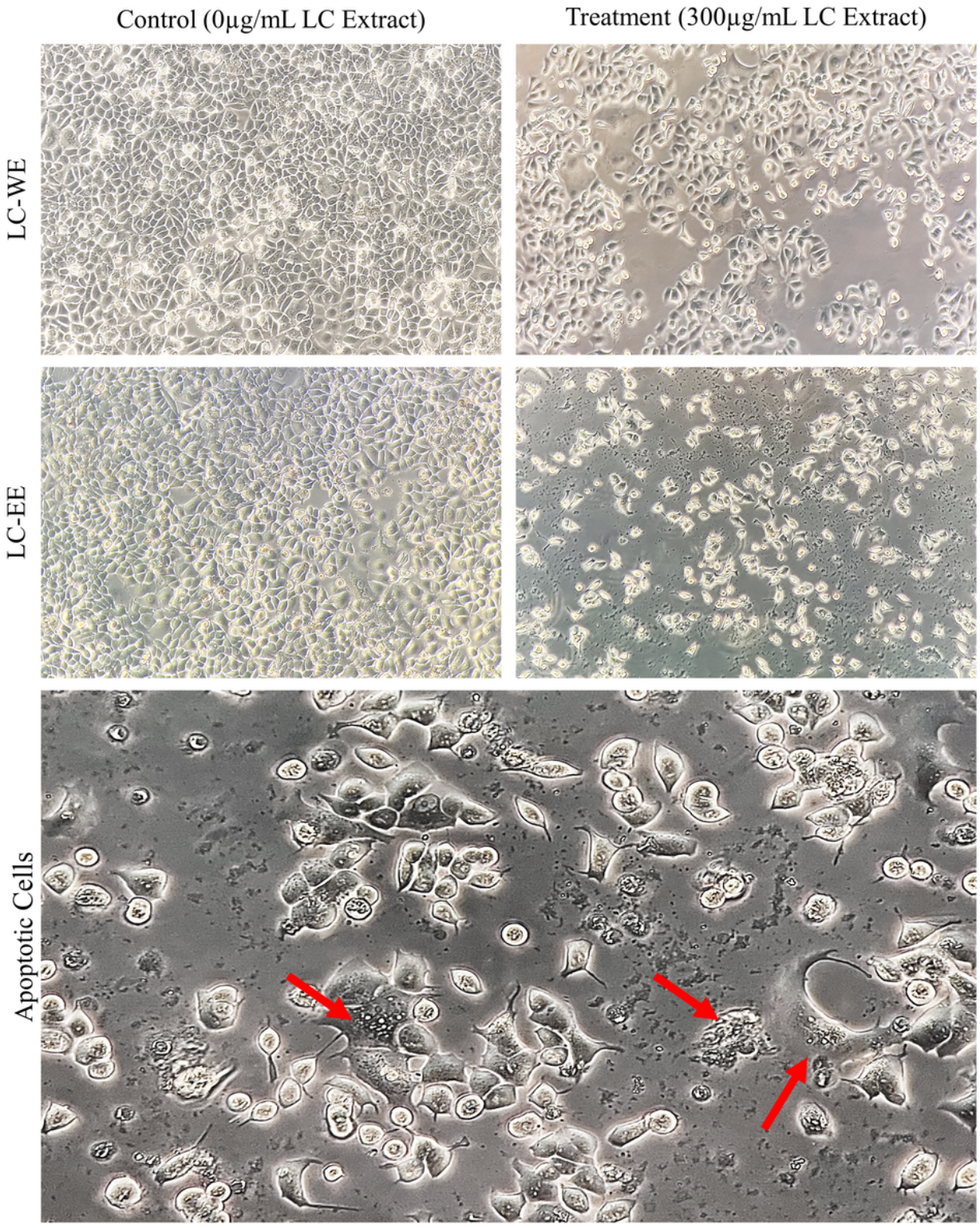

Figure 1

Differential Interference Contrast (DIC) microscopy observation of LC-WE and LC-EE treated and untreated MCF-7 cells and Apoptotic cells in treated samples with 300 µg/mL(Magnification scale in LC-WE, LC-EE 10x and in Apoptotic Cells 20x)

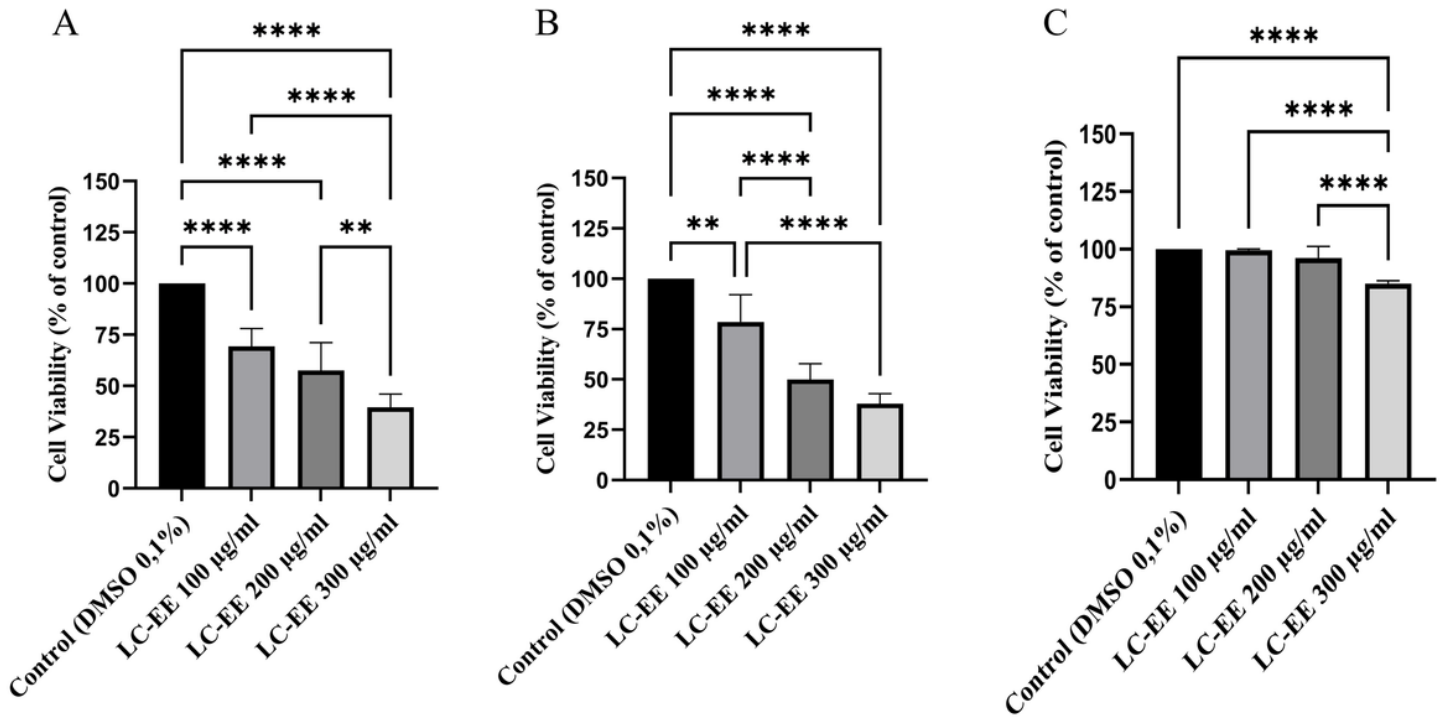

Figure 2

Cytotoxic effect of LC-EE on MCF-7 cells after a)12 hours incubation b) 24 hours and c) 48 hours incubation (*p <0,05).

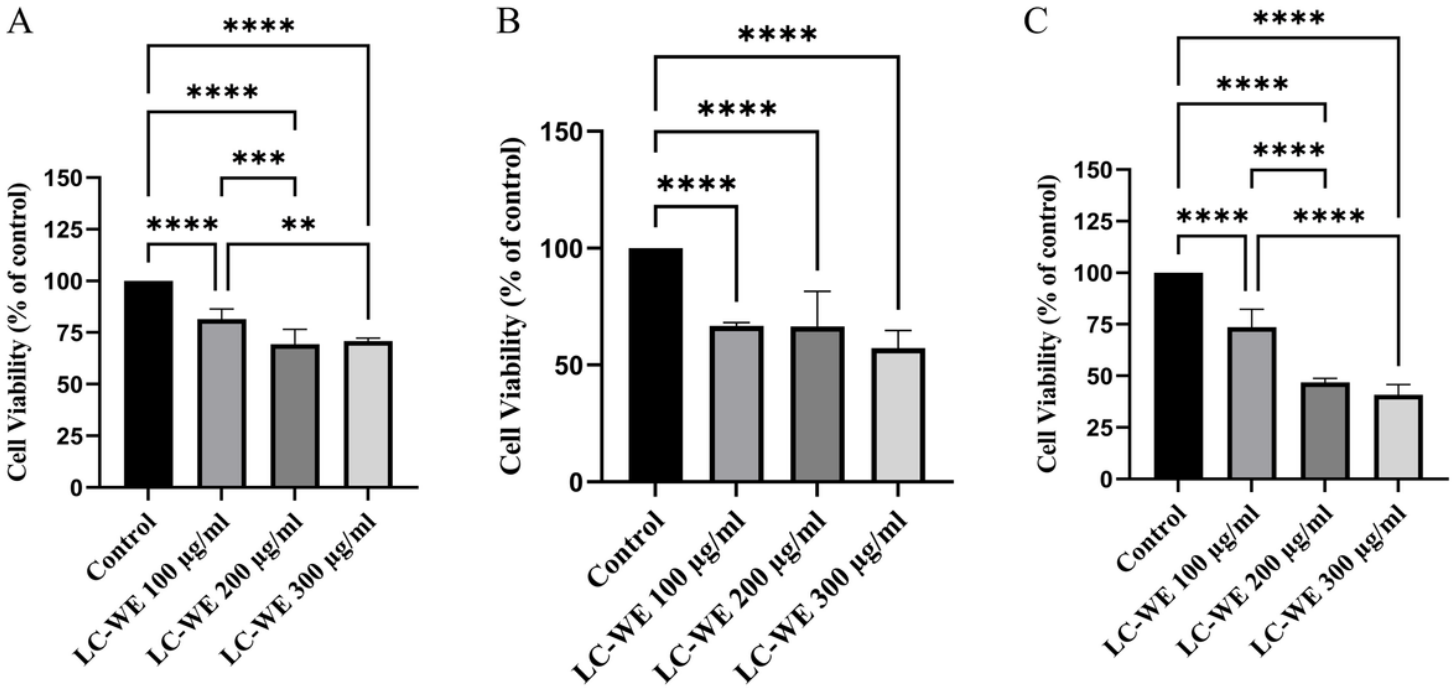

Figure 3

A) Cytotoxic effect of LC-WE on MCF-7 cells after a)12 hours incubation b) 24 hours and c) 48 hours incubation (*p <0,05).

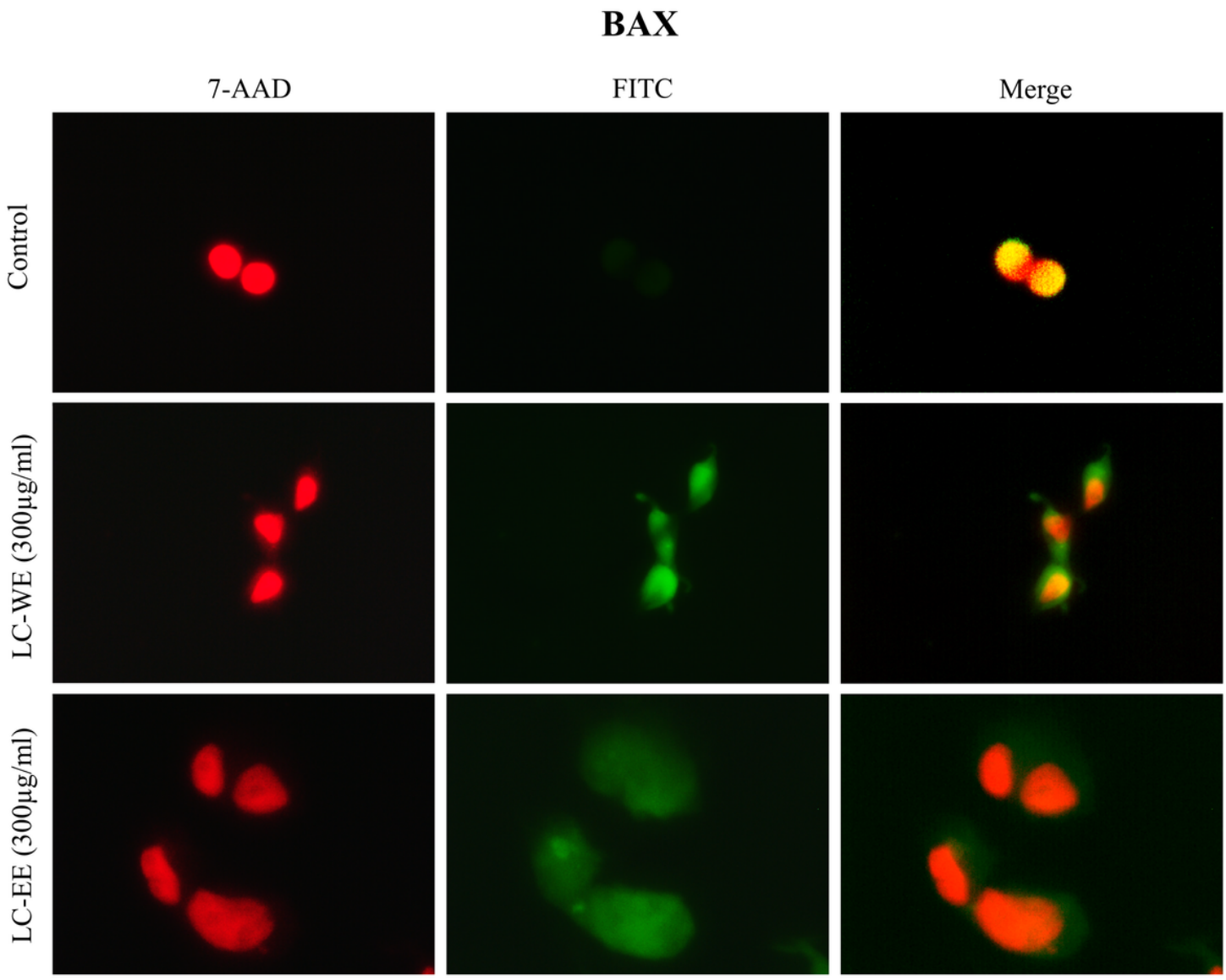

Figure 4

The amount of BAX protein synthesized with and without applying LC to MCF-7 cells. The amount of BAX protein synthesized after incubation of cancer cells with LC-WE (300 µg/mL) for 48 hours and with LC-EE (300 µg/mL) for 24 hours was shown. Cell nuclei are marked with 7-AAD (red signal) dye. BAX was detected with the antibody labeled with FITC (green signal). (Magnification 100x).

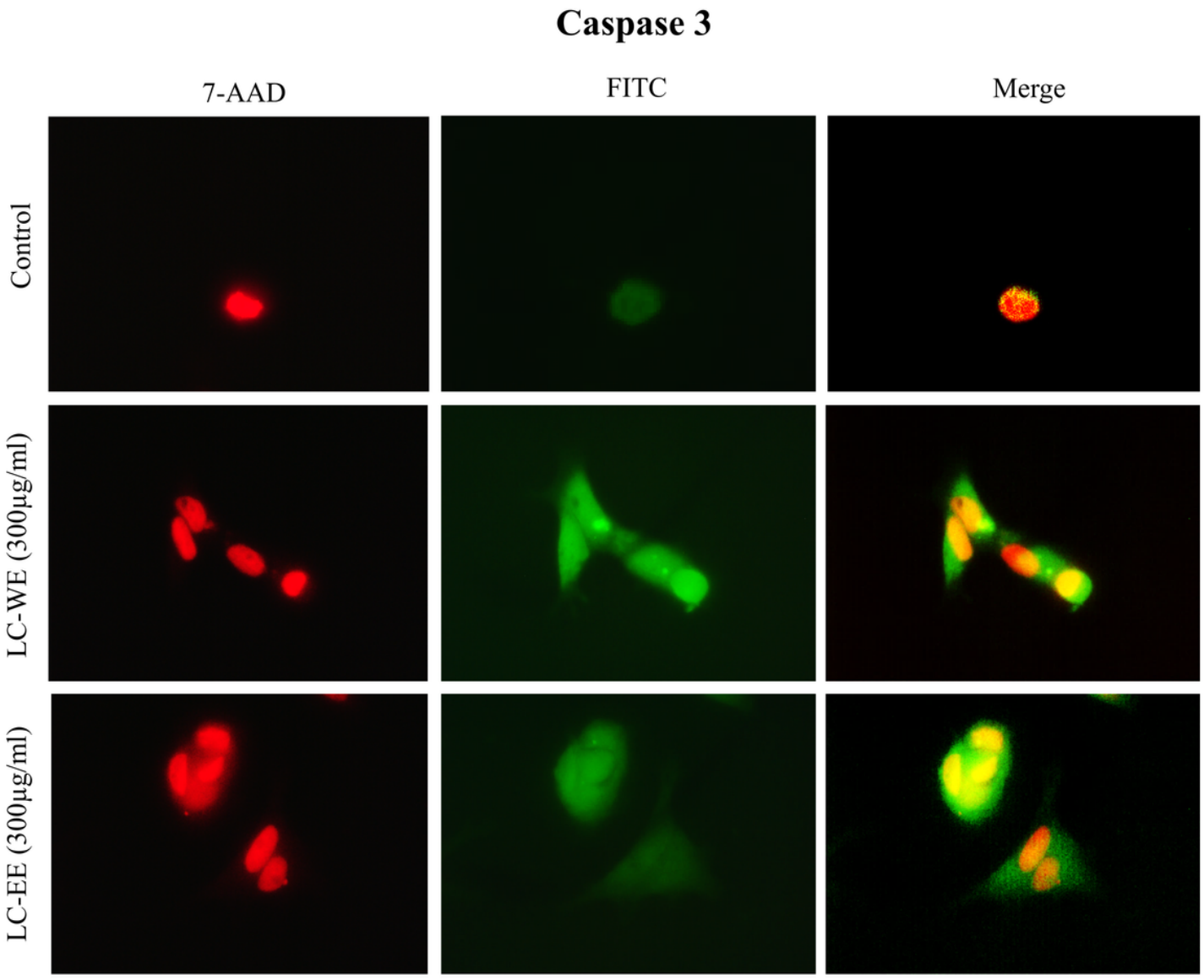

Figure 5

The amount of Caspase-3 protein synthesized with and without applying LC to MCF-7 cells. The amount of Caspase-3 protein synthesized after incubation of cancer cells with LC-WE (300 µg/mL) for 48 hours and with LC-EE (300 µg/mL) for 24 hours was shown. Cell nuclei are marked with 7-AAD (red signal) dye. Caspase-3 was detected with the antibody labeled with FITC (green signal). (Magnification 100x).

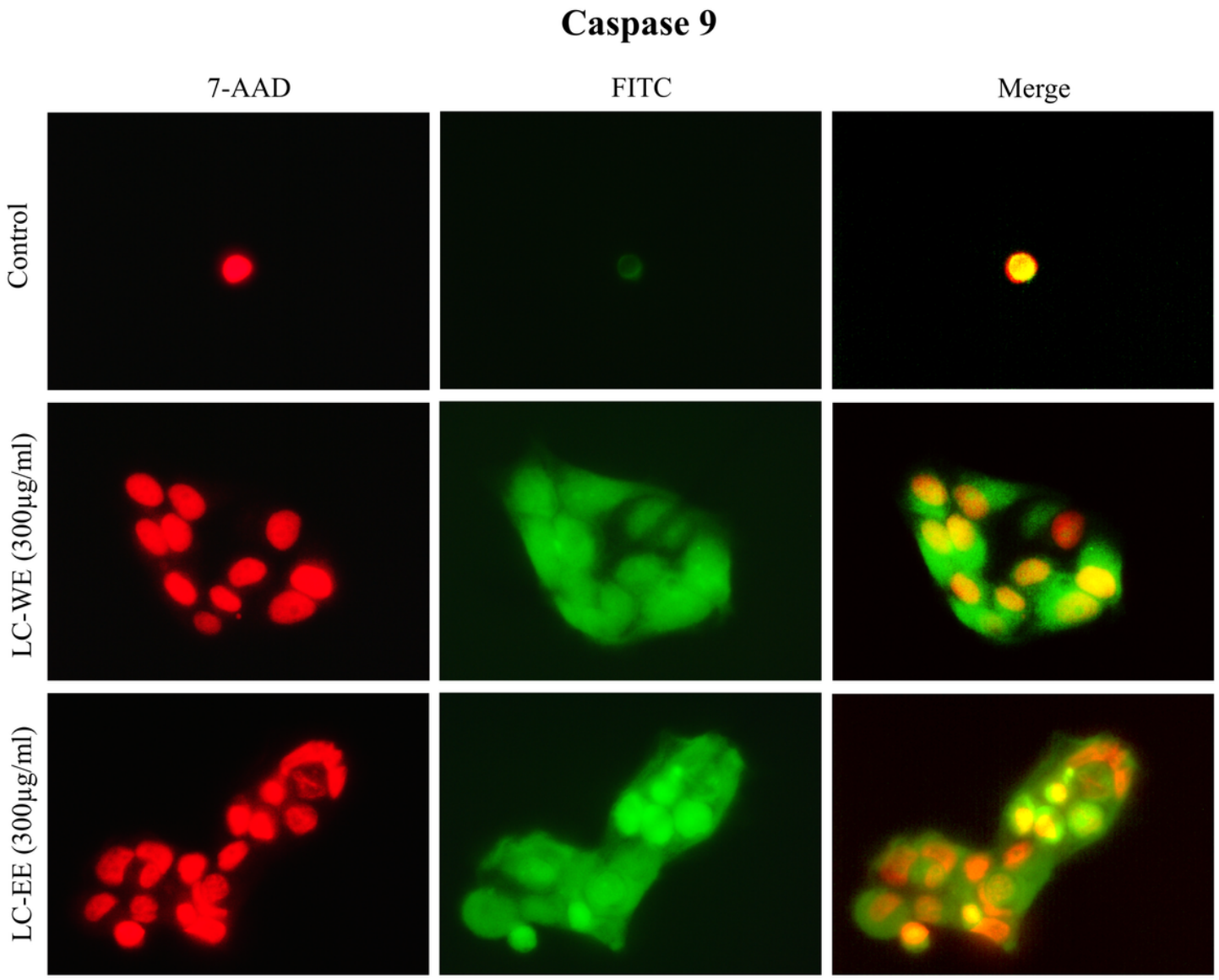

Figure 6

The amount of Caspase-9 protein synthesized with and without applying LC to MCF-7 cells. The amount of Caspase-9 protein synthesized after incubation of cancer cells with LC-WE (300 µg/mL) for 48 hours and with LC-EE (300 µg/mL) for 24 hours was shown. Cell nuclei are marked with 7-AAD (red signal) dye. Caspase-9 was detected with the antibody labeled with FITC (green signal). (Magnification 100x).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- graficalabstract.tif