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Erica arborea L. induces apoptosis and G2/M cell cycle arrest by regulating the CDK signaling pathway through the ROS generation in breast cancer cells

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Abstract

Aim: The anticancer effect of any *Erica* L. genus member has not yet been investigated. Hence, this study investigated the anticancer effect of *Erica arborea*, a high-level flavanone producer, in mouse breast cancer 67NR and 4T1 cells.

Materials and Methods: Healthy mouse fibroblast L929, mouse primary breast cancer 67NR and mouse metastatic breast cancer 4T1 cells were treated with *Erica arborea* methanol and ethanol extracts to investigate the cellular toxicity, apoptosis, gene expression level and oxidative stress effect on cells.

Results: According to our data, the methanol and ethanol extracts of *Erica arborea* caused apoptosis and G2/M cell cycle arrest. Also, the methanol and ethanol extracts of *Erica arborea* enhanced the level of reactive oxygen species (ROS), especially higher levels in mouse breast cancer 67NR and 4T1 cells, according to the healthy mouse fibroblast L929 cells. The treatment of the cells with Erica arborea extracts causes up-regulation of the pro-apoptotic Caspase-3, Caspase-9, Bax, and Bak genes and down-regulation of the anti-apoptotic Bcl-2 and Bcl-xL genes. Moreover, treatment with *Erica arborea* extract up-regulated the expression levels of cyclin-dependent kinase (CDK) inhibitor P21 and P27 genes while down-regulating the expression levels of cyclin B1 and cyclin A genes in cells. However, the mRNA expression level changes of these genes are significantly higher in breast cancer 67NR and 4T1 cells, according to the healthy mouse fibroblast L929 cells.

Conclusion: The result of the present study indicated that *Erica arborea* stimulated apoptosis induction in mouse breast cancer 67NR and 4T1 cells, which was associated with G2/M cell cycle arrest by regulating the CDK signaling pathway through ROS generation.

Introduction

Breast cancer is the most commonly diagnosed cancer in women globally and is a major cause of cancer-related deaths [1,2]. General treatment methods include surgery, chemotherapy, radiotherapy, and hormone therapy. However, these treatment methods often have serious side effects and carry the risk of developing resistance to treatment in patients [3]. Therefore, it is of great importance to develop alternative treatment approaches that are more effective and have reduced side effects in the treatment of breast cancer [4].

Herbal extracts are among the natural substances examined for their potential in cancer treatment. These extracts typically have low toxicity and contain bioactive components that display various biological activities [5,6]. In particular, herbal compounds such as polyphenols, flavonoids, alkaloids, and terpenoids are known for their anti-cancer properties [7,8]. In this context, the Erica spp. plants are also noteworthy [9-11].

The Ericaceae family comprises 100 genera and 3,000 species and is found in various regions, including the Himalayas, New Guinea, and South Africa [12]. In Turkey, the family comprises 12 genera and approximately 30 species. The genus *Erica* L. (Ericaceae) is predominantly present in South Africa, West Europe, and the Mediterranean, with over 700 species. In the flora of Turkey, there are five species belonging to this genus, with one of them, *Erica arborea* L. (Hereafter referred to as *E. arborea*), commonly found in the coastal areas of İzmir, Turkey [13]. The traditional use of leaves from *E. arborea* includes the treatment of various diseases such as antimicrobial, anti-inflammatory, and antilithic properties. However, the anti-

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cancer effect of *E. arborea* as a high-level flavanone, which is a strong reactive oxygen species (ROS) inducer in cancer cells, producer has not been studied well [13, 14].

ROS are generally defined as chemically reactive oxygen-containing molecules produced during cellular metabolism [15,16]. A moderate amount of ROS is essential for cellular signaling, which regulates cell proliferation and survival [17-19]. However, excessive levels of ROS can damage cellular components such as lipids, proteins, and DNA, resulting in an imbalance in redox conditions and disrupting cellular homeostasis [20-23]. Numerous studies on ROS-related anticancer therapies have been developed, focusing on inducing oxidative damage by increasing ROS levels in cancer cells. Approaches that induce ROS depend on the fact that raising the level of ROS beyond the cytotoxic threshold can specifically eliminate cancer cells [24-26]. The increased ROS level disrupts the redox balance and leads to cancer cell death [27,28]. When exogenous agents trigger the generation of ROS, the redox-imbalanced cancer cells become more susceptible than normal cells, which results in Cyclin-dependent kinases (CDKs) signaling pathway regulation-induced cell cycle arrest and subsequent apoptosis induction-related cell death [29-31]. Therefore, understanding the effects of herbal extracts on ROS production and CDKs is important in comprehending their anticancer mechanisms.

However, our understanding is that the *E. arborea* extract's anticancer properties have not yet been investigated. Therefore, this study investigates the toxicity of methanol and ethanol extracts from *E. arborea* on cell lines, including the L929 healthy mouse fibroblast cells, 67NR mouse primary breast cancer cells and 4T1 mouse metastatic breast cancer cells. The focus is evaluating cell viability and apoptosis to understand the cellular mechanisms involved. The ultimate goal is to provide valuable insights into the potential use of *E. arborea* as an adjuvant in cancer treatment.

Materials and Methods

Preparation and application of E. arborea sample extract

15 grams of *E. arborea* leave sample were ground into a fine powder using a household grinder, and then the sample was weighed 10g and dissolved in pure methanol and ethanol. The dissolved methanol and ethanol samples were incubated at room temperature on the stirrer for 24 hours. After the incubation period, the samples were filtered to eliminate residues and prevent bacterial contamination before being poured into the 15 ml conical tubes. The solutions tubes' cap of methanol and ethanol extracts was loosened and then incubated at 37°C for 48 hours to allow the evaporation of methanol and ethanol. 320 mg of crude methanol and 240 mg of crude ethanol extracts were obtained. They were then dissolved in 1 ml of Dimethyl sulfoxide (DMSO). Concentrations of methanol (1,000, 800, 600, 400, 200, and 100 µg/ml) and ethanol extracts (10, 8, 6, 4, 2, and 1 µg/ml) applied to healthy L929 cells and breast cancer 67NR and 4T1 cells for treatment.

Culturing conditions of cell lines

The cultured L929, 67NR, and 4T1 cells were grown in a RPMI 1640 medium, which is containing 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 1% L-Glutamine at 37°C in a humidified 5% CO_2 incubator. When the cells reached confluence, they were subcultured to ensure growth. Cells were grown with the cell density of 60,000 cells in each well of 24-well plates, followed by incubation with the *E. arborea* extracts at related concentrations for 24 and 48 hours.

Apoptosis analysis

The apoptotic effects in L929, 67NR, and 4T1 cells were evaluated. These cells were grown in 24-well plates and then treated with *E. arborea* methanol and ethanol extracts at their half-maximal inhibitory concentration (IC_{50}) values. Following the post-incubation period, the cells were labelled using the Muse Apoptosis kit according to the manufacturer's guidelines. The Muse flow cytometer instrument was used to analyze the cells and calculate the rate of apoptosis [32].

Cell cycle state analysis

Cell cycle analysis was conducted on L929, 67NR, and 4T1 cells, which were grown in 24-well plates (60,000 cells/well). Subsequently, the cells were exposed to *E. arborea* methanol and ethanol extracts at the IC_{50} value. The cells were subsequently analyzed with the Muse Cell Cycle Kit [32].

Oxidative stress analysis

L929, 67NR, and 4T1 were seeded in 24-well plates (60,000 cells/well) and incubated for 16-18 hours. After incubation, these cells were treated with *E. arborea* methanol and ethanol extracts at their IC_{50} values. At the end of the post-incubation time, the cells were stained with the Muse Oxidative Stress Kit according to the manufacturer's instructions. The cells were then analyzed using the Muse Oxidative Stress Kit to determine the ROS-positive and ROS-negative cell rate.

Gene expression analysis

RNA was extracted from L929, 67NR, and 4T1 cells cultured in 24-well plates and exposed to *E. arborea* methanol and ethanol extracts at IC_{50} concentrations for 24 and 48 hours using the Quick-RNA Miniprep Kit (ZymoResearch) according to the manufacturer's instructions. The High-Capacity cDNA Reverse Transcription kit was used to conduct cDNA synthesis, following the protocol provided by the manufacturer. The process took place in the SensoQuest thermal cycler using isolated total RNAs as samples. After this, the target Bcl-2, Bcl-xL, Caspase-3, Caspase-9, Bax, Bad, Cyclin A, Cyclin B1, Cdk1, Cdk2, p21 and p27 genes' primer pairs were designed (Supplementary Table 1). Target gene expression analysis was conducted using a 20 µl total reaction mixture containing cDNA, EvaGreen, Primer Forward, Primer Reverse, 2X master mix, and ddH_2O. The analysis was carried out in a Rotor-Gene device. The housekeeping gene Hypoxanthine-guanine phosphoribosyltransferase 1 (HPRT1) was used for the normalization of target genes, as it exhibited the most stable expression values. The $2^{-\Delta\Delta Ct}$ method was employed to calculate the normalized expression values of the target genes.

Statistical analysis

The differences observed between the groups were analyzed using SPSS (Statistical Package for the Social Sciences, Windows 23.0). The data obtained underwent testing through One-way ANOVA and a Tukey post hoc test. If the relevant experimental data of the IC_{50} extract-applied group differed from the control group data or if the relevant experimental data of the IC_{50} extract-treated groups of different cell types differed, $p \leq 0.05$ was considered statistically significant and the notations of * ($p \leq 0.05$), ** ($p \leq 0.01$), and *** ($p \leq 0.001$) were used for the comparison versus the control group. The findings were then expressed as the mean ± standard deviation (SD). Also, IC_{50} values after the flow cytometer viability assay were calculated with Graphpad Prism 9. All experiments were performed in duplicate.

Results

At the beginning of the cytotoxicity experiments, methanol and ethanol *E. arborea* extracts were applied to L929, 67NR, and 4T1 cells at 1,000, 800, 600, 400, 200, and 100 µg/ml concentrations. However, concentrations of *E. arborea* ethanol extract above 10 µg/ml caused high death rates in all cell types. For this reason, ethanol extract was applied to the cells at concentrations of 10 µg/ml and below. As a result, it was determined that the viability of L929, 67NR, and 4T1 cells in methanol and ethanol extract-treated groups decreased in a dose-dependent manner at the 24 and 48 hour of cytotoxicity experiments (Figure 1).

According to the viability values shown in Figure 1, the IC_{50} (The half-maximal inhibitory concentration (IC_{50})) values of L929, 67NR, and 4T1 cells, which were treated with methanol extract for 24 hours, were found to be 227.7 µg/ml, 79.77 µg/ml and 105.3 µg/ml, respectively. Also, the IC_{50} values of L929, 67NR, and 4T1 cells, treated with ethanol extract for 24 hours, were found to be 6.43 µg/ml, 4.42 µg/ml and 4.85 µg/ml, respectively. On the other hand, the IC_{50} values of L929, 67NR, and 4T1 cells, which were treated with methanol extract for 48 hours, were 304.5 µg/ml, 126.9 µg/ml and 143.9 µg/ml, respectively. Also, the IC_{50} values of L929, 67NR, and 4T1 cells, treated with ethanol extract for 48 hours, were 6.56 µg/ml, 3.90 µg/ml and 5.21 µg/ml, respectively. The microscopic analysis results of the 24 and 48 hours IC_{50} values of methanol and ethanol extract treatment are also shown in Figure 2. Microscopy analysis also shows high particle density in the plate well containing healthy L929 fibroblast cells applied with E. arborea extract at higher IC_{50} concentrations. In this case, healthy L929 fibroblast cells continue their vital processes even at this high IC_{50} concentration.

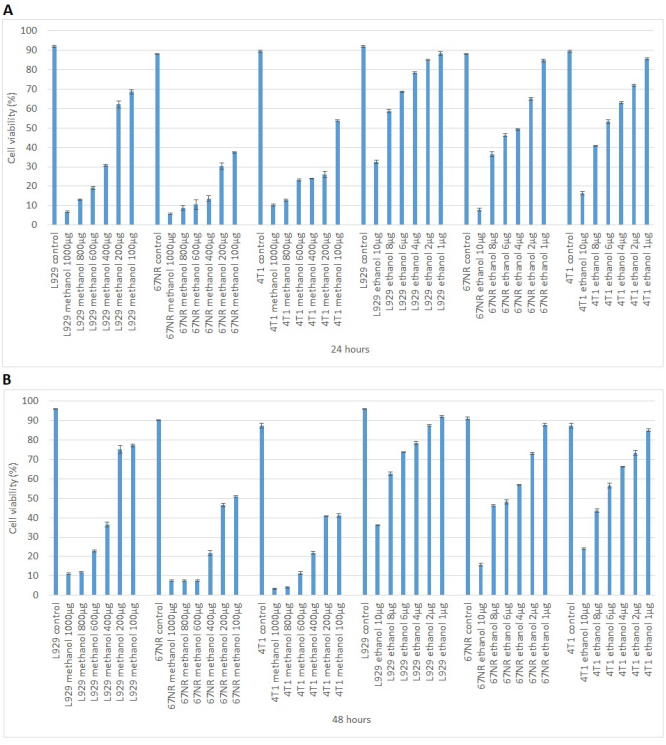

Figure 1. The viability values of L929, 67NR, and 4T1 cells treated with different concentrations of *E. arborea* methanol and ethanol extract for 24 (A) and 48 (B) hours.

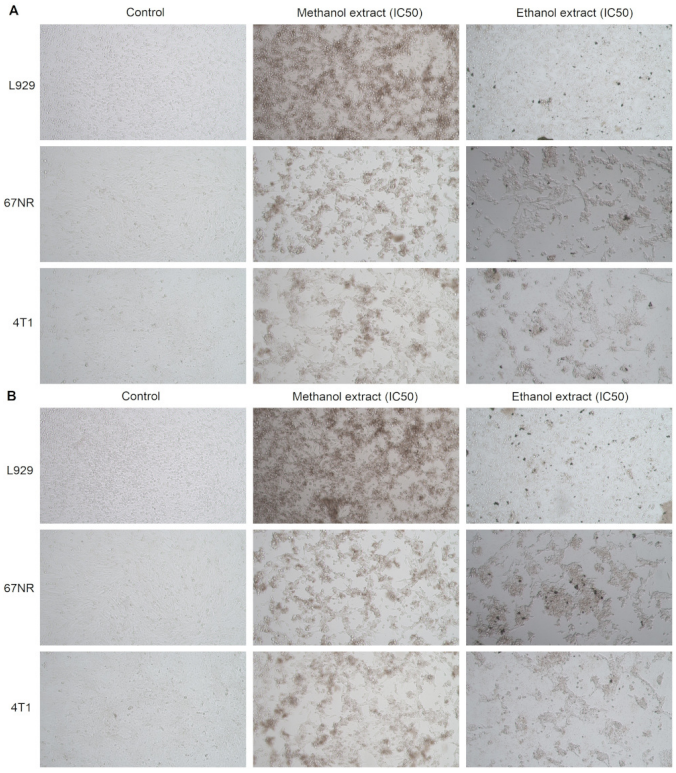

Figure 2. Value of IC_{50} of methanol and ethanol extract treatment of L929, 67NR, and 4T1 cells for 24 (A) and 48 (B) hours (x10 magnification).

The flow cytometry method measured the apoptotic potential of *E. arborea* methanol and ethanol extract. The information from cell viability data indicated that *E. arborea* methanol and ethanol extracts were administered to L929, 67NR, and 4T1 cells at IC_{50} concentrations for durations of 24 and 48 hours. It has been found that the viability rate decreased, and apoptotic cell number increased significantly for 24 and 48 hours in 67NR and 4T1 cancer cells, which were treated with methanol and ethanol IC_{50} concentrations, according to the healthy L929 fibroblast cells ($p<0.0001$). As seen in Figure 3, at the end of 24 hours of methanol extract treatment, the number of apop-

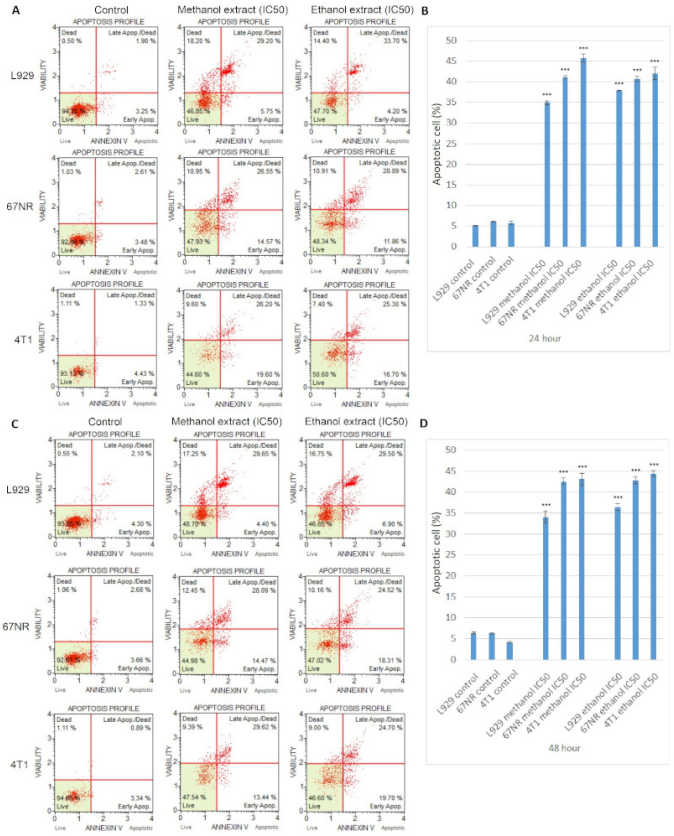

Figure 3. Apoptosis values of L929, 67NR and 4T1 cells treated with IC_{50} *E. arborea* methanol and ethanol extract concentrations. Apoptosis flow cytometer graphs of methanol and ethanol extract treated cells for 24 hours (A), apoptosis bar graph results of methanol and ethanol extract treated cells for 24 hours (B), apoptosis flow cytometer graphs of methanol and ethanol extract treated cells for 48 hours (C), and apoptosis bar graph results of methanol and ethanol extract treated cells for 48 hours (D).

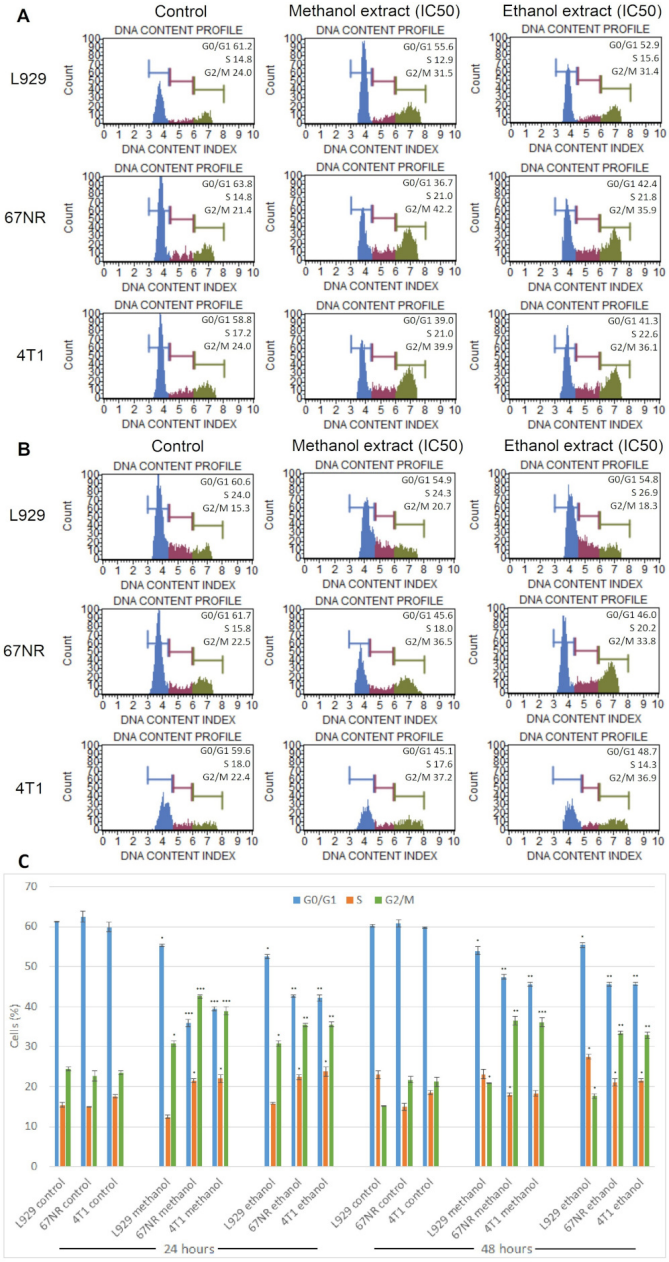

Figure 4. Effect of IC_{50} *E. arborea* methanol and ethanol extract concentrations on Cell Cycle Distribution in L929, 67NR, and 4T1 cells. The percentages of G1, S, and G2/M population distribution after treatment with IC_{50} *E. arborea* methanol and ethanol extract concentrations for 24 hours (A) and 48 hours (B). The G1, S, and G2/M population distribution percentages were plotted in the histograms (C).

totic (early and late apoptosis) cells was found for L929, 67NR and 4T1 cells as 34.95±0.48%, 41.12±0.44% and 45.8±0.9%, respectively. At the end of 24 hours of ethanol extract treatment, the number of apoptotic (early and late apoptosis) cells was found for L929, 67NR and 4T1 cells as 37.9±0.05%, 40.75±0.63% and 42.0±1.5%, respectively. Also, at the end of 48 hours of methanol extract treatment, the number of apoptotic (early and late apoptosis) cells was found for L929, 67NR and 4T1 cells as 34.05±1.48%, 42.57±0.72% and 43.06±1.47%, respectively. At the end of 48 hours of ethanol extract treatment, the number of apoptotic (early and late apoptosis) cells was found for L929, 67NR and 4T1 cells as 36.4±0.9%, 42.82±0.91% and 44.4±0.7%, respectively.

The distribution profile of the cell cycle was evaluated to investigate how the *E. arborea* extracts induce an antiproliferative effect in L929, 67NR, and 4T1 cells. Figure 4 A and B illustrate that the treatment of the IC_{50} *E. arborea* methanol and ethanol extract concentrations increased the frequency of arrested L929, 67NR, and 4T1 cells at the S and G2/M phases while decreasing the cell population in the G1 phase according to their control. In particular, treatment of L929, 67NR, and 4T1 cells with *E. arborea* methanol and ethanol extract for 24 hours resulted in a higher number of cells in the G2/M phase compared to 48 hours (see Figure 4C). However, the increase in the number of cells arrested in the S and G2/M phases in healthy L929 cells treated with IC_{50} *E. arborea* methanol and ethanol extract concentrations remained lower than in 67NR and

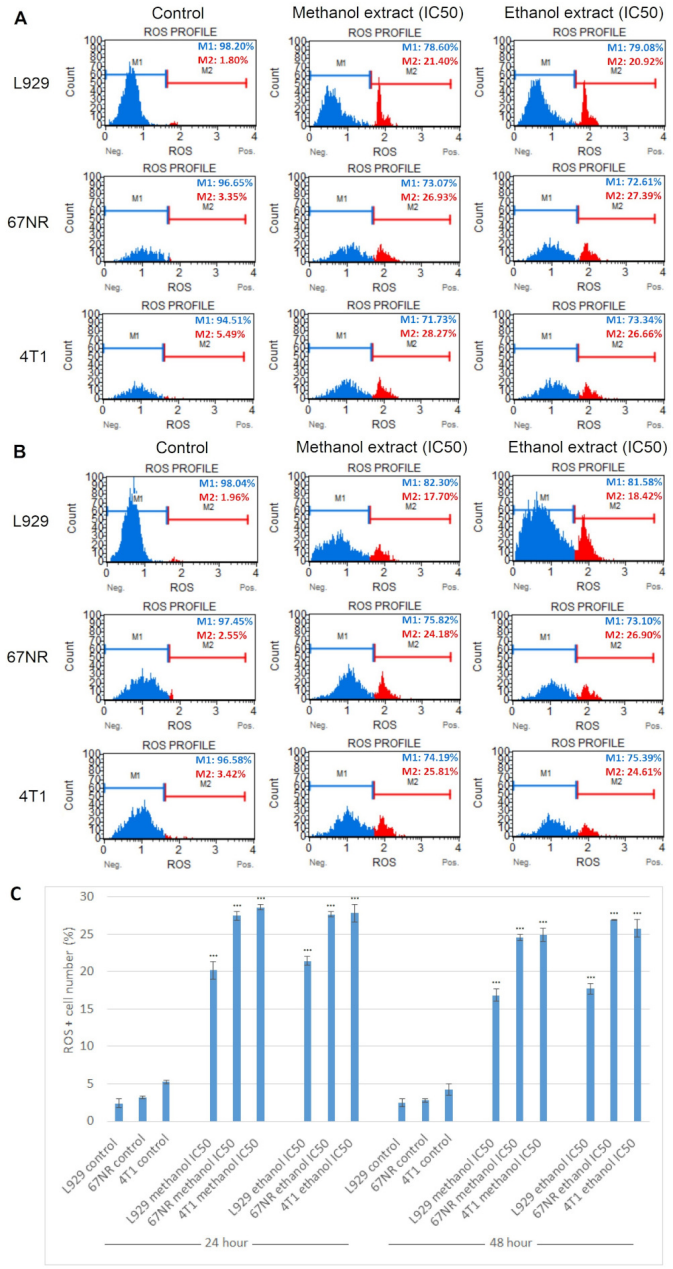

Figure 5. Effect of IC_{50} *E. arborea* methanol and ethanol extract concentrations on the percentage of ROS-positive L929, 67NR, and 4T1 cell numbers. The percentages of ROS-positive cell population after treatment with IC_{50} *E. arborea* methanol and ethanol extract concentrations for 24 hours (A) and 48 hours (B). The ROS-positive cell population distribution percentages were plotted in the histograms (C).

4T1 cancer cells. Also, the increase rate in 67NR and 4T1 cells arrested in the S and G2/M phases were significantly higher according to the L929 cells ($p<0.0001$) after *E. arborea* methanol and ethanol extract treatment.

Our next aim was to determine if the induction of apoptosis by the *E. arborea* methanol and ethanol extracts was associated with the production of intracellular ROS. For this reason, the intracellular ROS level induced by extracts was measured using Muse™ Oxidative Stress Kit. Figures

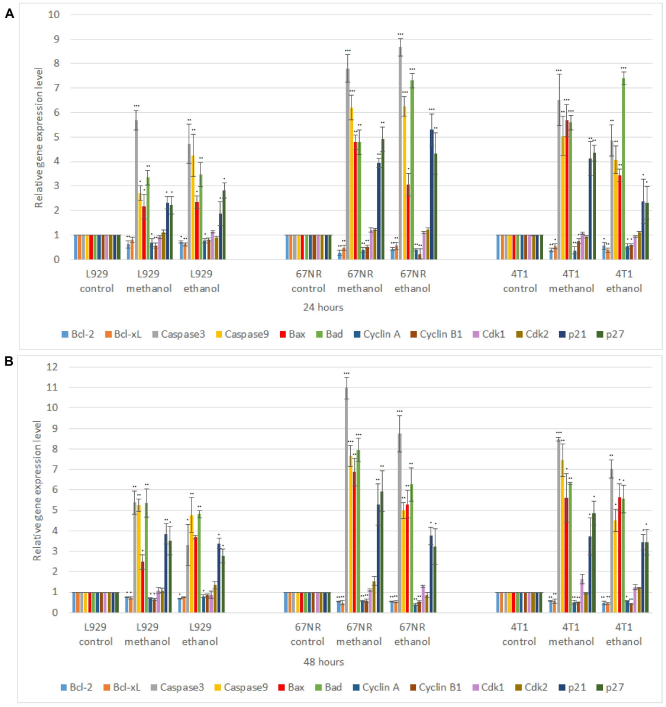

Figure 6. RT-qPCR analysis results of anti-apoptotic, pro-apoptotic, and G2/M phase-associated gene expression levels of L929, 67NR and 4T1 cells treated with *E. arborea* methanol and ethanol extract at IC_{50} concentrations for 24 hours (A) and 48 (B) hours.

5 A and B illustrate that the treatment of the IC_{50} concentrations of *E. arborea* methanol and ethanol extract increased the percentage of ROS-positive L929, 67NR, and 4T1 cell numbers according to their control. However, after the methanol or ethanol extract treatment, the increase in the number of ROS-positive L929 cells significantly remained lower than in 67NR and 4T1 cancer cells as in cell cycle analysis ($p<0.001$). Together, these results indicated that ROS induced by *E. arborea* methanol or ethanol extract could mediate apoptotic processes in L929, 67NR, and 4T1 cells.

The target gene expression levels of pro-apoptotic, anti-apoptotic, and G2/M phase-associated genes were analyzed with RT-qPCR analyses to investigate the mechanism of the *E. arborea* methanol and ethanol extract-induced apoptosis and cell cycle arrest in L929, 67NR, and 4T1 cells. As seen in Figure 6 A and B, treatment of the cells with IC_{50} concentrations of *E. arborea* methanol and ethanol extracts for 24 hours and 48 hours increased the mRNA expression level of pro-apoptotic Caspase-3, Caspase-9, Bax, and Bad genes in L929, 67NR and 4T1 cells, while decreased the anti-apoptotic Bcl-2, Bcl-xL mRNA expression level according to their non-treated controls. However, it was determined that the change in Bcl-2, Bcl-xL, Caspase-3, Caspase-9, Bax, and Bad gene expression levels was higher in 67NR and 4T1 cells compared to L929 cells ($p<0.01$) for 24 and 48 hours of methanol and ethanol extract treatment. On the other hand, as seen in Figure 6 A and B, the RT-qPCR results showed that treatment of the cells with IC_{50} concentrations of *E. ar-*

borea methanol and ethanol extracts reduced the mRNA expression levels of cyclin A and B1 according to their non-treated controls ($p<0.01$). In contrast, the mRNA expression level of CdK1 and CdK2 remained similar at the control level ($p>0.05$). Nevertheless, the mRNA expression level of a Cdk inhibitor P21 (CDKN1A) and P27 (CDKN1B) genes were significantly up-regulated in response to the extract treatment. Altogether, our results showed that *E. arborea* methanol and ethanol extract treatment induced apoptosis and cell cycle arrest in L929, 67NR, and 4T1 cells. However, the extract-related induction of apoptosis and cell cycle arrest was limited in healthy L929 cells, even at higher IC_{50} concentrations, according to the breast cancer 67NR and 4T1 cells.

Discussion

Medicinal plants have been utilized as traditional remedies for various human ailments for thousands of years [33]. Also, many studies have shown that certain anticancer agents activate the cell cycle arrest checkpoint, leading to apoptotic cell death [34]. An uncontrolled cell cycle is a characteristic of tumor cells and plays a role in cancer development and progression [35]. For this reason, our study investigated the potential of *E. arborea* as a bioactive substance derived from natural sources, examining its pharmacological effectiveness in anticancer properties through in vitro experiments. On the other hand, the literature searches revealed no studies on cancer cells treated with a substance or extract obtained from plants belonging to the Erica genus. For this reason, we compare our results with similar research conducted on plant-derived extract treatment experiments on various cancer cells.

It is important to identify the various forms of programmed cell death (PCD) present in the cell population that has been treated with the agent [5]. PCD encompasses death mechanisms like apoptosis, autophagy, necroptosis, and pyroptosis [36]. Hence, it is important to identify the different types of cell death mechanisms occurring in cells exposed to the substance. In processes involving living organisms, apoptosis is the most common mechanism of cell death [37]. Therefore, this study first investigated whether the cell deaths occurring under the guidance of the results obtained from cytotoxicity tests were caused by apoptosis. As a result of the flow cytometer analysis, it was determined that the cell deaths in both 24 and 48 hours of *E. arborea* extract treatment were caused by apoptosis. Also, it was observed that the total apoptotic cell rate was higher in 67NR and 4T1 cells in contrast to L929 fibroblast cells, both at the 24^{th} and 48^{th} hours. Moreover, it was noteworthy that ethanol extract of *E. arborea* causes apoptotic death at lower than 10 µg/ml concentrations in all cell types, but this death rate of L929 cells was lower according to the 67NR and 4T1 cells. Apoptosis is commonly divided into two main pathways: the extrinsic and the intrinsic pathways, which involve the mitochondria [38-40]. The extrinsic pathway is initiated when death ligands bind to their respective receptors. This interaction leads to the formation and activation of caspase-8, which stimulates effector caspases like caspase-3 [41]. In contrast, the intrinsic pathway begins with the release of cytochrome c from the mitochondria into the cytosol. This release occurs due to increased mitochondrial permeability. Once in the cytosol, cytochrome c activates effector caspases by forming the apoptosome, which consists of Apaf-1, cytochrome c, and caspase-9. The intrinsic pathway is specifically regulated by Bcl-2 family proteins, such as Bax and Bad [40,42]. Parallel to certain studies using different cancer cell lines, which were treated with different extracts or agents obtained from different genera of plants, our results show that *E. arborea* up-regulated caspase-3, caspase-9, Bax, and Bad mRNA expression levels, which was associated with the down-regulation of the Bcl-2 and Bcl-xL mRNA expression levels [43-46]. However, the rate of changes in mRNA expression levels of these genes was more limited in L929 cells than in 67NR and 4T1 cells, as was the apoptosis analysis.

Our results indicate that the methanol and ethanol extracts of *E. arborea* suppressed cell proliferation by inducing arrest in the G2/M cell cycle phase. For this reason, to further understand the molecular machinery of cell cycle arrest induced by *E. arborea*, attention was placed on cyclins and CDKs. The central machinery driving cell cycle progression is regulated by Cdks, which is regulated through interactions with cyclins that are specific to the cell cycle and Cdk inhibitors [47,48]. Cyclin A/B and the Cdk1/2 complex are involved in cell progression through the S and G2 phases. Consistent with the results of other studies, cell cycle analyses using different cancer cell lines, which were treated with different extracts obtained from different genera of plants, our results show that *E. arborea* extracts reduced the mRNA expression level of cyclin B1 and A. In contrast, the expression of CdK1 and Cdk2 remained unchanged according to the control [49-51]. Also, the mRNA expression level of P21 and P27 was significantly up-regulated by *E. arborea* extracts. The up-regulated mRNA expression levels of P21 and P27 inhibit the kinase activity of Cdks and halt the cell cycle progression in cells [52, 53]. In this study, it was found that up-regulated mRNA levels of P21 and P27 resulted in the suppression of their kinase activity, ultimately causing G2/M arrest in cells. Furthermore, the ethanol extract of *E. arborea* significantly inhibits the cell cycle progression of 67NR and 4T1 breast cancer cells at a lower than the concentration of 5 µg/ml, a concentration that has an effect of less than 30 micrograms is suitable for use as a drug according to the U.S. Food and Drug Administration (FDA).

ROS plays a role in various cellular activities, and maintaining ROS at low to moderate levels appears necessary for cell survival [54]. Nevertheless, an overabundance of ROS leads to cell death by triggering DNA damage-related apoptotic pathways [55,56]. Recent studies have announced that various bioactive compounds stimulate apoptosis and cell cycle arrest through ROS generation [43,57]. The results suggested that elevated ROS levels could be a therapeutic approach to combating cancer. For this reason, the oxidative stress level of the *E. arborea* extracts-treated cells was evaluated to determine ROS production in cells. As a result, increased levels of oxidative stress were observed in *E. arborea* extracts-treated cells compared to non-treated cells. Also, consistent with pre-

vious studies [58–60], this study revealed that the high levels of ROS were an upstream regulator of the *E. arborea* extracts induced apoptosis. However, the rate of ROS production in healthy fibroblast cells L929 cells after *E. arborea* extract treatment was lower according to the 67NR and 4T1 breast cancer cells, which revealed that the L929 cells are more resistant to ROS production according to the 67NR and 4T1 cells after extract treatment.

Conclusion

This study found that ROS generation by the *Erica* genus member *E. arborea* methanol and ethanol extracts is responsible for inducing apoptosis and causing an arrest in the G2/M cell cycle phase in healthy mouse fibroblast L929 cells, primary mouse breast cancer 67NR, and metastatic mouse breast 4T1 cancer cells. This is achieved by regulating the CDK signaling pathway. However, *E. arborea* methanol and ethanol extracts related apoptosis and cell cycle arrest rate were higher in 67NR and 4T1 cells, even in lower concentrations according to the L929 cells. In particular, ethanol extract of *E. arborea* shows strong apoptosis and cell cycle arrest effects on cells at very low concentrations according to the methanol extract of *E. arborea*. Based on these findings, *E. arborea* selectively killed breast cancer cells by inducing apoptosis and G2/M cell cycle arrest by regulating the CDK signaling pathway through ROS generation.

Ethical approval

It is a study that does not require ethics committee approval.

References

1. Barzaman, K., Karami, J., Zarei, Z., Hosseinzadeh, A., Kazemi, M. H., Moradi-Kalbolandi, S., Safari, E., & Farahmand, L. (2020). Breast cancer: Biology, biomarkers, and treatments. International immunopharmacology, 84, 106535. https://doi.org/10.1016/j.intimp.2020.106535.
2. Katsura, C., Ogunmwonyi, I., Kankam, H. K., & Saha, S. (2022). Breast cancer: presentation, investigation and management. British journal of hospital medicine (London, England : 2005), 83(2), 1–7. https://doi.org/10.12968/hmed.2021.0459.
3. Valente, S., & Roesch, E. (2024). Breast cancer survivorship. Journal of surgical oncology, 130(1), 8–15. https://doi.org/10.1002/jso.27627.
4. Olsson Möller, U., Beck, I., Rydén, L., & Malmström, M. (2019). A comprehensive approach to rehabilitation interventions following breast cancer treatment - a systematic review of systematic reviews. BMC cancer, 19(1), 472. https://doi.org/10.1186/s12885-019-5648-7.
5. Batir, MB., Batır, S., Ozdal Kurt, F, & Çam, S. The cytotoxic and apoptotic effects of Abies nordmanniana subsp. bornmülleriana Mattf resin extract on prostate cancer cell cells. Communications Faculty of Sciences University of Ankara Series C Biology. 2023;32(2):119-36. https://doi.org/10.53447/communc.1272043.
6. Peng, B., Zhang, S. Y., Chan, K. I., Zhong, Z. F., & Wang, Y. T. (2023). Novel Anti-Cancer Products Targeting AMPK: Natural Herbal Medicine against Breast Cancer. Molecules (Basel, Switzerland), 28(2), 740. https://doi.org/10.3390/molecules28020740.
7. Esmeeta, A., Adhikary, S., Dharshnaa, V., Swarnamughi, P., Ummul Maqsummiya, Z., Banerjee, A., Pathak, S., & Duttaroy, A. K. (2022). Plant-derived bioactive compounds in colon cancer treatment: An updated review. Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie, 153, 113384. https://doi.org/10.1016/j.biopha.2022.113384.
8. Zou, J. Y., Chen, Q. L., Luo, X. C., Damdinjav, D., Abdelmohsen, U. R., Li, H. Y., Battulga, T., Chen, H. B., Wang, Y. Q., & Zhang, J. Y. (2024). Natural products reverse cancer multidrug resistance. Frontiers in pharmacology, 15, 1348076. https://doi.org/10.3389/fphar.2024.1348076.
9. Aires, A., & Carvalho, R. (2017). Profiling of Polyphenol Composition and Antiradical Capacity of Erica cinerea. Antioxidants (Basel, Switzerland), 6(3), 72. https://doi.org/10.3390/antiox6030072.
10. Bekkai, D., Oulad El Majdoub, Y., Bekkai, H., Cacciola, F., Miceli, N., Taviano, M. F., Cavò, E., Errabii, T., Laganà Vinci, R., Mondello, L., & L'Bachir El Kbiach, M. (2022). Determination of the Phenolic Profile by Liquid Chromatography, Evaluation of Antioxidant Activity and Toxicity of Moroccan Erica multiflora, Erica scoparia, and Calluna vulgaris (Ericaceae). Molecules (Basel, Switzerland), 27(13), 3979. https://doi.org/10.3390/molecules27133979.
11. Amroun, D., Hamoudi, M., Khennouf, S., Boutefnouchet, S., Harzallah, D., Amrane, M., & Dahamna, S. (2021). In-vivo anti-inflammatory activity and safety assessment of the aqueous extract of Algerian Erica arborea L. (Ericaceae) aerial parts. Journal of ethnopharmacology, 271, 113881. https://doi.org/10.1016/j.jep.2021.113881.
12. Heywood, VH. (1979). Flowering Plants of the World. Oxford University Press, Oxford.
13. Köroğlu, A., Hürkul, MM., Kendir, G., Küçükboyacı, N. (2019). In vitro antioxidant capacities and phenolic contents of four Erica L. (Ericaceae) taxa native to Turkey. J Res Pharm.; 23(1): 93-100. https://doi.org/10.12991/jrp.2018.113.
14. Raghav, P.K., Mann, Z., Krishnakumar, V., Mohanty, S. (2022). Therapeutic Effect of Natural Compounds in Targeting ROS-Induced Cancer. In: Chakraborti, S., Ray, B.K., Roychoudhury, S. (eds) Handbook of Oxidative Stress in Cancer: Mechanistic Aspects. Springer, Singapore. https://doi.org/10.1007/978-981-15-9411-3_116.
15. Brieger, K., Schiavone, S., Miller, F. J., Jr, & Krause, K. H. (2012). Reactive oxygen species: from health to disease. Swiss medical weekly, 142, w13659. https://doi.org/10.4414/smw.2012.13659.
16. Batir, M. B., Candan, F., Buyuk, I., & Aras, S. (2015). The determination of physiological and DNA changes in seedlings of maize (Zea mays L.) seeds exposed to the waters of the Gediz River and copper heavy metal stress. Environmental monitoring and assessment, 187(4), 169. https://doi.org/10.1007/s10661-015-4389-6.
17. Trachootham, D., Alexandre, J., & Huang, P. (2009). Targeting cancer cells by ROS-mediated mechanisms: a radical therapeutic approach?. Nature reviews. Drug discovery, 8(7), 579–591. https://doi.org/10.1038/nrd2803.
18. Tomasetti, M., Santarelli, L., Alleva, R., Dong, L. F., & Neuzil, J. (2015). Redox-active and redox-silent compounds: synergistic therapeutics in cancer. Current medicinal chemistry, 22(5), 552–568. https://doi.org/10.2174/0929867321666140915142219.
19. Okon, I. S., & Zou, M. H. (2015). Mitochondrial ROS and cancer drug resistance: Implications for therapy. Pharmacological research, 100, 170–174. https://doi.org/10.1016/j.phrs.2015.06.013.
20. Cross, C. E., Halliwell, B., Borish, E. T., Pryor, W. A., Ames, B. N., Saul, R. L., McCord, J. M., & Harman, D. (1987). Oxygen radicals and human disease. Annals of internal medicine, 107(4), 526–545. https://doi.org/10.7326/0003-4819-107-4-526.
21. Vendemiale, G., Grattagliano, I., & Altomare, E. (1999). An update on the role of free radicals and antioxidant defense in human disease. International journal of clinical & laboratory research, 29(2), 49–55. https://doi.org/10.1007/s005990050063.
22. Casado, J. A., Merino, J., Cid, J., Subirá, M. L., & Sánchez-Ibarrola, A. (1996). Oxidantes y radicales libres en biomedicina [Oxidizing agents and free radicals in biomedicine]. Revista de medicina de la Universidad de Navarra, 40(3), 31–40.
23. Samson, F. E., & Nelson, S. R. (2000). The aging brain, metals and oxygen free radicals. Cellular and molecular biology (Noisy-le-Grand, France), 46(4), 699–707.
24. Perillo, B., Di Donato, M., Pezone, A., Di Zazzo, E., Giovannelli, P., Galasso, G., Castoria, G., & Migliaccio, A. (2020). ROS in cancer therapy: the bright side of the moon. Experimental & molecular medicine, 52(2), 192–203. https://doi.org/10.1038/s12276-020-0384-2.

25. Li, X., Chen, Y., Zhao, J., Shi, J., Wang, M., Qiu, S., Hu, Y., Xu, Y., Cui, Y., Liu, C., & Liu, C. (2019). The Specific Inhibition of SOD1 Selectively Promotes Apoptosis of Cancer Cells via Regulation of the ROS Signaling Network. Oxidative medicine and cellular longevity, 2019, 9706792. https://doi.org/10.1155/2019/9706792.
26. Chakrabarti, G., Gerber, D. E., & Boothman, D. A. (2015). Expanding antitumor therapeutic windows by targeting cancer-specific nicotinamide adenine dinucleotide phosphate-biogenesis pathways. Clinical pharmacology : advances and applications, 7, 57–68. https://doi.org/10.2147/CPAA.S79760.
27. Li, T., & Gao, S. J. (2023). KSHV hijacks FoxO1 to promote cell proliferation and cellular transformation by antagonizing oxidative stress. Journal of medical virology, 95(3), e28676. https://doi.org/10.1002/jmv.28676.
28. Han, E., Kwon, B., Yoo, D., Kang, C., Khang, G., & Lee, D. (2017). Dual Stimuli-Activatable Oxidative Stress Amplifying Agent as a Hybrid Anticancer Prodrug. Bioconjugate chemistry, 28(4), 968–978. https://doi.org/10.1021/acs.bioconjchem.6b00683.
29. Chen, X. X., Xie, F. F., Zhu, X. J., Lin, F., Pan, S. S., Gong, L. H., Qiu, J. G., Zhang, W. J., Jiang, Q. W., Mei, X. L., Xue, Y. Q., Qin, W. M., Shi, Z., & Yan, X. J. (2015). Cyclin-dependent kinase inhibitor dinaciclib potently synergizes with cisplatin in preclinical models of ovarian cancer. Oncotarget, 6(17), 14926–14939. https://doi.org/10.18632/oncotarget.3717.
30. Kapoor, S., & Padwad, Y. S. (2023). Phloretin induces G2/M arrest and apoptosis by suppressing the β-catenin signaling pathway in colorectal carcinoma cells. Apoptosis : an international journal on programmed cell death, 28(5-6), 810–829. https://doi.org/10.1007/s10495-023-01826-4.
31. Feldmann, G., Mishra, A., Bisht, S., Karikari, C., Garrido-Laguna, I., Rasheed, Z., Ottenhof, N. A., Dadon, T., Alvarez, H., Fendrich, V., Rajeshkumar, N. V., Matsui, W., Brossart, P., Hidalgo, M., Bannerji, R., Maitra, A., & Nelkin, B. D. (2011). Cyclin-dependent kinase inhibitor Dinaciclib (SCH727965) inhibits pancreatic cancer growth and progression in murine xenograft models. Cancer biology & therapy, 12(7), 598–609. https://doi.org/10.4161/cbt.12.7.16475.
32. Batır, M. B., Şahin, E., & Çam, F. S. (2019). Evaluation of the CRISPR/Cas9 directed mutant TP53 gene repairing effect in human prostate cancer cell line PC-3. Molecular biology reports, 46(6), 6471–6484. https://doi.org/10.1007/s11033-019-05093-y.
33. Sidhu, P., Shankargouda, S., Rath, A., Hesarghatta Ramamurthy, P., Fernandes, B., & Kumar Singh, A. (2020). Therapeutic benefits of liquorice in dentistry. Journal of Ayurveda and integrative medicine, 11(1), 82–88. https://doi.org/10.1016/j.jaim.2017.12.004.
34. Xie, C., Zhou, X., Liang, C., Li, X., Ge, M., Chen, Y., Yin, J., Zhu, J., & Zhong, C. (2021). Apatinib triggers autophagic and apoptotic cell death via VEGFR2/STAT3/PD-L1 and ROS/Nrf2/p62 signaling in lung cancer. Journal of experimental & clinical cancer research : CR, 40(1), 266. https://doi.org/10.1186/s13046-021-02069-4.
35. van de Donk, N. W., & Dhimolea, E. (2012). Brentuximab vedotin. mAbs, 4(4), 458–465. https://doi.org/10.4161/mabs.20230.
36. Ketelut-Carneiro, N., & Fitzgerald, K. A. (2022). Apoptosis, Pyroptosis, and Necroptosis-Oh My! The Many Ways a Cell Can Die. Journal of molecular biology, 434(4), 167378. https://doi.org/10.1016/j.jmb.2021.167378.
37. Bertheloot, D., Latz, E., & Franklin, B. S. (2021). Necroptosis, pyroptosis and apoptosis: an intricate game of cell death. Cellular & molecular immunology, 18(5), 1106–1121. https://doi.org/10.1038/s41423-020-00630-3.
38. Yanumula, A., & Cusick, J. K. (2023). Biochemistry, Extrinsic Pathway of Apoptosis. In StatPearls. StatPearls Publishing.
39. Kashyap, D., Garg, V. K., & Goel, N. (2021). Intrinsic and extrinsic pathways of apoptosis: Role in cancer development and prognosis. Advances in protein chemistry and structural biology, 125, 73–120. https://doi.org/10.1016/bs.apcsb.2021.01.003.
40. Carneiro, B. A., & El-Deiry, W. S. (2020). Targeting apoptosis in cancer therapy. Nature reviews. Clinical oncology, 17(7), 395–417. https://doi.org/10.1038/s41571-020-0341-y.
41. Tian, X., Srinivasan, P. R., Tajiknia, V., Sanchez Sevilla Uruchurtu, A. F., Seyhan, A. A., Carneiro, B. A., De La Cruz, A., Pinho-Schwermann, M., George, A., Zhao, S., Strandberg, J., Di Cristofano, F., Zhang, S., Zhou, L., Raufi, A. G., Navaraj, A., Zhang, Y., Verovkina, N., Ghandali, M., Ryspayeva, D., ... El-Deiry, W. S. (2024). Targeting apoptotic pathways for cancer therapy. The Journal of clinical investigation, 134(14), e179570. https://doi.org/10.1172/JCI179570.
42. Carpenter, R., & Brady, M. F. (2023). BAX Gene. In StatPearls. StatPearls Publishing.
43. Park, C., Cha, H. J., Lee, H., Hwang-Bo, H., Ji, S. Y., Kim, M. Y., Hong, S. H., Jeong, J. W., Han, M. H., Choi, S. H., Jin, C. Y., Kim, G. Y., & Choi, Y. H. (2019). Induction of G2/M Cell Cycle Arrest and Apoptosis by Genistein in Human Bladder Cancer T24 Cells through Inhibition of the ROS-Dependent PI3k/Akt Signal Transduction Pathway. Antioxidants (Basel, Switzerland), 8(9), 327. https://doi.org/10.3390/antiox8090327.
44. Kim, S. O., Cha, H. J., Park, C., Lee, H., Hong, S. H., Jeong, S. J., Park, S. H., Kim, G. Y., Leem, S. H., Jin, C. Y., Hwang, E. J., & Choi, Y. H. (2019). Cordycepin induces apoptosis in human bladder cancer T24 cells through ROS-dependent inhibition of the PI3K/Akt signaling pathway. Bioscience trends, 13(4), 324–333. https://doi.org/10.5582/bst.2019.01214.
45. Park, C., Choi, E. O., Hwangbo, H., Lee, H., Jeong, J. W., Han, M. H., Moon, S. K., Yun, S. J., Kim, W. J., Kim, G. Y., Hwang, H. J., & Choi, Y. H. (2022). Induction of apoptotic cell death in human bladder cancer cells by ethanol extract of Zanthoxylum schinifolium leaf, through ROS-dependent inactivation of the PI3K/Akt signaling pathway. Nutrition research and practice, 16(3), 330–343. https://doi.org/10.4162/nrp.2022.16.3.330.
46. Hong, S. H., Cha, H. J., Hwang-Bo, H., Kim, M. Y., Kim, S. Y., Ji, S. Y., Cheong, J., Park, C., Lee, H., Kim, G. Y., Moon, S. K., Yun, S. J., Chang, Y. C., Kim, W. J., & Choi, Y. H. (2019). Anti-Proliferative and Pro-Apoptotic Effects of Licochalcone A through ROS-Mediated Cell Cycle Arrest and Apoptosis in Human Bladder Cancer Cells. International journal of molecular sciences, 20(15), 3820. https://doi.org/10.3390/ijms20153820.
47. Kang, S. H., Bak, D. H., Chung, B. Y., & Bai, H. W. (2021). Centipedegrass extract enhances radiosensitivity in melanoma cells by inducing G2/M cell cycle phase arrest. Molecular biology reports, 48(2), 1081–1091. https://doi.org/10.1007/s11033-021-06156-9.
48. Kumar, S., Mulchandani, V., & Das Sarma, J. (2022). Methanolic neem (Azadirachta indica) stem bark extract induces cell cycle arrest, apoptosis and inhibits the migration of cervical cancer cells in vitro. BMC complementary medicine and therapies, 22(1), 239. https://doi.org/10.1186/s12906-022-03718-7.
49. Zhu, L., Wang, Y., Lv, W., Wu, X., Sheng, H., He, C., & Hu, J. (2021). Schizandrin A can inhibit non-small cell lung cancer cell proliferation by inducing cell cycle arrest, apoptosis and autophagy. International journal of molecular medicine, 48(6), 214. https://doi.org/10.3892/ijmm.2021.5047.
50. Hsu, T. I., Chen, Y. J., Hung, C. Y., Wang, Y. C., Lin, S. J., Su, W. C., Lai, M. D., Kim, S. Y., Wang, Q., Qian, K., Goto, M., Zhao, Y., Kashiwada, Y., Lee, K. H., Chang, W. C., & Hung, J. J. (2015). A novel derivative of betulinic acid, SYK023, suppresses lung cancer growth and malignancy. Oncotarget, 6(15), 13671–13687. https://doi.org/10.18632/oncotarget.3701.
51. Zhang, L., Zheng, Y., Deng, H., Liang, L., & Peng, J. (2014). Aloperine induces G2/M phase cell cycle arrest and apoptosis in HCT116 human colon cancer cells. International journal of molecular medicine, 33(6), 1613–1620. https://doi.org/10.3892/ijmm.2014.1718.
52. Ahn, M. Y., Lee, J., Na, Y. J., Choi, W. S., Lee, B. M., Kang, K. W., & Kim, H. S. (2009). Mechanism of apicidin-induced cell cycle arrest and apoptosis in Ishikawa human endometrial cancer cells. Chemico-biological interactions, 179(2-3), 169–177. https://doi.org/10.1016/j.cbi.2008.11.011.
53. Nilmani, D'costa, M., Bothe, A., Das, S., Udhaya Kumar, S., Gnanasambandan, R., & George Priya Doss, C. (2023). CDK regulators-Cell cycle progression or apoptosis-Scenarios in normal cells and cancerous cells. Advances in protein chemistry and structural biology, 135, 125–177. https://doi.org/10.1016/bs.apcsb.2022.11.008.
54. Lee, S. O., Joo, S. H., Kwak, A. W., Lee, M. H., Seo, J. H., Cho, S. S., Yoon, G., Chae, J. I., & Shim, J. H. (2021). Podophyllotoxin Induces ROS-Mediated Apoptosis and Cell Cycle Arrest in Human Colorectal Cancer Cells via p38 MAPK Signaling. Biomolecules & therapeutics, 29(6), 658–666. https://doi.org/10.4062/biomolther.2021.143.

55. Li, A. X., Sun, M., & Li, X. (2017). Withaferin-A induces apoptosis in osteosarcoma U2OS cell line via generation of ROS and disruption of mitochondrial membrane potential. European review for medical and pharmacological sciences, 21(6), 1368–1374.
56. Guo, H., Yang, Y., Lou, Y., Zuo, Z., Cui, H., Deng, H., Zhu, Y., & Fang, J. (2023). Apoptosis and DNA damage mediated by ROS involved in male reproductive toxicity in mice induced by Nickel. Ecotoxicology and environmental safety, 268, 115679. https://doi.org/10.1016/j.ecoenv.2023.115679.
57. Park, C., Lee, H., Hwangbo, H., Ji, S. Y., Kim, M. Y., Kim, S. Y., Hong, S. H., Kim, G. Y., & Choi, Y. H. (2020). Ethanol Extract of Hizikia fusiforme Induces Apoptosis in B16F10 Mouse Melanoma Cells through ROS-Dependent Inhibition of the PI3K/Akt Signaling Pathway. Asian Pacific journal of cancer prevention : APJCP, 21(5), 1275–1282. https://doi.org/10.31557/APJCP.2020.21.5.1275.
58. Phan, T. N., Kim, O., Ha, M. T., Hwangbo, C., Min, B. S., & Lee, J. H. (2020). Albanol B from Mulberries Exerts Anti-Cancer Effect through Mitochondria ROS Production in Lung Cancer Cells and Suppresses In Vivo Tumor Growth. International journal of molecular sciences, 21(24), 9502. https://doi.org/10.3390/ijms21249502.
59. Lee, M. H., Hong, S. H., Park, C., Kim, G. Y., Leem, S. H., Choi, S. H., Keum, Y. S., Hyun, J. W., Kwon, T. K., Hong, S. H., & Choi, Y. H. (2016). Hwang-Heuk-San induces apoptosis in HCT116 human colorectal cancer cells through the ROS-mediated activation of caspases and the inactivation of the PI3K/Akt signaling pathway. Oncology reports, 36(1), 205–214. https://doi.org/10.3892/or.2016.4812.
60. Song, J., Shu, L., Zhang, Z., Tan, X., Sun, E., Jin, X., Chen, Y., & Jia, X. (2012). Reactive oxygen species-mediated mitochondrial pathway is involved in Baohuoside I-induced apoptosis in human non-small cell lung cancer. Chemico-biological interactions, 199(1), 9–17. https://doi.org/10.1016/j.cbi.2012.05.005.