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Original article

Investigation of the Anti-Cancer Effects of β-asaron and Etoposide in MCF-7 Breast Cancer Cells

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Abstract

Currently, the options available for the treatment of various cancers including breast cancer, are associated with several limitations such as severe toxicity, drug resistance, poor prognosis, and high risk of recurrence. Therefore, there appears to be an increasing interest and necessity in investigating various phytochemicals from natural sources for a superior and safer alternative treatment strategy. The bioactive phytochemical alpha (α) and beta (β)-asarone from Acorus calamus is a traditional medicine system that has been shown to have anti-tumor and chemo-inhibitory activities in numerous preclinical studies both in vitro and in vivo. Various experimental studies with human malignant cell lines and animal models have also confirmed the anti-tumor and anti-cancer activities of β-asarone. In this study, we aimed to investigate the anti-cancer effects of β-asarone alone or together with etoposide buy measuring cellular responses such as cell viablity, cell cycle arrest and apoptosis using breast cancer cell line MCF-7 cells. In order to get insight in to the mechanism, we also tested the expression of of NF-κB / p65 activity and the expression of Bcl-2 family member pro-apoptotic Bax protein together with p53 and p21 activities in response to β-asarone alone or together with etoposide treatment. As a result, it was concluded that the use of β-asarone alone in breast cancer cells is effective in reducing cell viability, but when used together with Etoposide, it does not cause a synergistic effect. Here we suggest that that in particular activation of NF-kB/p65 may be lead resistance to etoposide treatment.

Keywords: β-asarone, etoposide, MCF-7, MTT, p53, p21, NF-kB/p65

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INTRODUCTION

Cancer is a complex disease that is affected by genetic and environmental conditions, the incidence of which increases every year and there are few treatment options. The success rate of treatments varies from person to person. In addition, it is known that current drug treatments create drug resistance in some patients, which seriously reduces the success of treatment. In general, the options available for the treatment of different types of cancer have several limitations, including severe toxicity, drug resistance, poor outcomes, and high risk of recurrence. Therefore, there has been an increasing interest and requirement for screening various phytochemicals from natural sources for a superior and safer alternative for several decades, and many plant-derived drugs with anti-cancer activity have been used for a long time without any side effects (Smina et al., 2021).

Acorus calamus Linn, which grows in the temperate regions of the world, is a member of the Acoraceae family. The medicinal use of the plant in India and China has a long history. Its fragrant leaves and roots have been used as traditional medicine. Acorus species are known for their traditional value in the treatment of neurological disorders such as respiratory disorders, epilepsy and cognitive deficits. The main components of the plant are monoterpenes, sesquiterpenes, phenylpropanoids, flavonoids, quinine and volatile compounds alpha (α) and beta (β) asarone. β-asarone is the major component in leaves with a ratio of 27.4% to 45.5%. α-asarone and/or β-asarone; they show similar pharmacological properties such as antioxidant, antiepileptic, antidepressant, anxiolytic, neuroprotective, hypolipidemic (Chellian et al., 2017). β-asarone has been also confirmed for its anti-tumor and anti-cancer activities in various experimental studies using human malignant cell lines and animal models. Various experimental studies have also confirmed the anti-tumor and anti-cancer activities of β-asarone with human malignant cell lines and animal models. Studies have shown that β-asarone has anti-cancer functions in colorectal cancer (Liu Wang et al., 2013; Zou et al., 2012), gastric cancer (Wu et al., 2015), lung cancer (Wang Ouyang et al., 2018), lymphoma (Lv et al., 2019) hepatocellular arcinoma (Stegmuller et al., 2018) and glioblastomas (Li L et al., 2018; Qi et al., 2015; Wang et al., 2017). It has been reported that nitro derivatives of β-asarone exert also anti-proliferative effects in prostate cancer cells (PC-3), neuroblastoma (IMR-32) cells, cervical cancer (HeLa) cells, synovial cancer (SW982) cells and breast cancer (MCF-7) cells (Shenvi et al., 2014). Aqueous and ethanolic extracts of A. calamus has been also reported to reduce the cell viability in breast cancer MDA-MB-435S cells and liver carcinoma Hep3B cells (Venkatadri, Guha, Kumar, & Lazar, 2009).

In general, the anticancer effects of β-asarone have been attributed to its role in regulating the levels of proteins involved in the mitochondrial apoptosis pathway and cell death. In addition, β-asarone involved in activation of death receptor proteins such as TNF-related apoptosis-inducing ligand (TRAIL) and Fas ligand (FasL). β-asarone causes induction of apoptosis through activation of caspase-3, 8 and 9, up-regulation of pro-apoptotic proteins Puma, Bax and Bad, and down-regulation of anti-

apoptotic proteins XIAP, Bcl-2 and Survivin proteins. β-asarone triggers cell cycle arrest in the G0/G1 phase through regulation of cell cycle-related proteins p21 and p27 and down-regulation of cyclin D, cyclin E, Cdc25A and CDK2 (Das et al., 2019).

Breast cancer is the most frequently diagnosed type of cancer among women, accounting for 23% of total cancer cases and 14% of cancer-related deaths (Gunduz and Gunduz, 2011). It is the second leading cause of cancer-related death in women (Tang, Wang, Kiani, & Wang, 2016). At the molecular level, breast cancer is a heterogeneous disease. Based on the presence or absence of molecular markers for estrogen or progesterone receptors and human epidermal growth factor 2 (ERBB2/HER2), there are three types of hormone receptor positive/ERBB2 negative tumors, ERBB2 positive tumors, and triple negative (negative for 3 standard molecular markers) tumors. Treatment strategies differ according to the molecular subtype. Breast cancer management is multidisciplinary and includes local (surgery and radiation therapy) and systemic treatment approaches (Harbeck et al., 2019). Currently, the options available for the treatment of various cancers, including breast cancer, are associated with several limitations such as severe toxicity, drug resistance, poor prognosis, and high risk of recurrence. Therefore, it is important and necessary to investigate phytochemicals obtained from various natural sources for a safer alternative treatment strategy.

In this study, the effects of β-asarone either alone or in combination with etoposide on cellular stress responses such as cell cycle arrest and apoptosis were investigated in breast cancer cell line MCF-7 cells. In addition, we also tested the inhibition of NF-κB / p65 activity and the expression of pro-apoptotic Bcl-2 family member Bax protein, together with p53 and p21 activities in order to shed light in to the mechanism of β-asarone.

MATERIALS AND METHODS

Cell Culture

MCF-7 breast cancer cell line was obtained from American Type Culture Collection (ATCC-HTB 22TM). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (Gibco), 1% Penicillin Streptomycin (Gibco), 1% Sodium Pyruvate in a 37°C constant temperature incubator with 5% CO_2 It was cultured using -HA (Gibco) medium.

MTT Test

The MCF-7 cell line was seeded as 8-10x103 cells per well/100 µL using 96-well plates. In order to determine the effective concentration and duration of β-asarone in MCF-7 cells, 3 different time intervals of 24, 48 and 72 hours and different concentrations of β-asarone between 50 µM and 750 µM were used. In addition, different concentrations of Etoposide eitheralone (100, 150 and 200 µM) or in combination with β-asarone (β100, 150, 200, 250 µM) were used to treated with MCF-7 cells for 24 and 48 hours. DMSO or ethanolwere used as solvent controls. Cell lines in the experimental groups were

incubated in a 37°C constant temperature incubator containing 5% CO2 for the specified times. At the end of the experiments, cell viability was determined by adding 10 µl of MTT (3-4,5-dimethyl-thiazolyl-2,5-diphenyltetrazolium bromide) (Cayman Chemical Company) solution to each sample well Absorbance (A) was read at 570-590 nm using a Multiscan spectrum microplate reader. Cell viability was calculated according to the formula: Viability (%) = [(A Sample-A blank)/(A control-A blank)] x100 as indicated in (Eren et al., 2021).

Annexin V/7AAD,

MCF-7 cells were seeded at $4x10^4$ cells per well/200µl in a 48-well plate and treated with Etoposide and β-asarone concentrations for 24 hours. Cells were incubated in a constant temperature incubator of 37°C containing 5% CO_2 till the end of the period. When the incubation period was completed, the medium was removed and 100µl of 1X trypsin-EDTA was added to each well and incubated at 37°C for 2 minutes. Accordingly, cells were collected with growth medium and centrifuged at 300xg for 5 minutes. The supernatant from all samples was discarded and the pellet left with 100 µl of medium on. 100 µl of 'Annexin V' solution was added to each sample pellet and the pellets were suspended by pipetting. After 20 minutes of incubation at room temperature in the dark, apoptosis profiles of cells were determined using Muse® Annexin V and Dead Cell according to the manufacturer's instructions. Quantitative analyzes of total apoptotic cells were performed using the Muse Cell Analyzer (Millipore, Austin, TX, USA) as described in (Eren et al., 2021).

Cell Cycle Analysis

MCF-7 cells were seeded in a 48-well plate with $4x10^4$ cells/well/200µl and treated with Etoposide and β-asarone for 24 hours and incubated in a 37°C constant temperature incubator containing 5% CO_2. At the end of the incubation period, the medium was removed and cells were washed with 1X PBS, and incubated with 1X Trypsin-EDTA at 37°C for 2-3 minutes. At the end of the incubation, cells were collected with 1 ml of growth medium and centrifuged at 300xg for 5 minutes. After centrifugation, the supernatant was discarded from all samples, and pellets were suspended with 1 ml of 1X PBS and washed again. After centrifugation, PBS was removed and the pellet was suspended with 70 % cold ethanol and incubated at -20 °C for at least 3 hours. Then, the ethanol was removed by the centrifugation and the pellet was washed with 1 ml of 1X PBS again. In the last step, PBS was removed and 200 µl of Muse Cell Cycle reagent (Brdu/PI) was added to the samples and incubated for 30 minutes at room temperature in the dark, and the results were measured with Muse Cell Analyzer (Millipore, Austin, TX, USA) (Eren et al., 2021) as described in (Pilevneli and Kilic Eren, 2021).

Protein isolation and Western Blot analysis

MCF-7 cells were treated for 24 hours with the indicated Etoposide and β-asarone concentrations. At the end of the the treatment, cells were lysed in RIPA Buffer with 1 mMNa3VO4 (0.5M TrisHCl,

5M NaCl, 0.5M EDTA, 0.1g SDS and 0.1g $C_{24}H_{39}NaO_4$,) supplemented with protease inhibitor cocktail (Roche Applied Science, Mannheim, Germany). Protein amount were then determined with BCA protein assay according to the manufacturer's instruction. 50 μg of proteins from each sample were then separated by using SDS-PAGE and electrotransferred to a PVDF membrane (polyvinylidine difluoride; Bio-RadHercules, CA, USA) by semi-dry blotting as described in Kilic-Eren et al 2021. The following primary antibodies were used; mouse monoclonal anti-p53 (1:1000 Cell Signaling), rabbit monoclonal anti-p21 (1:1000 Cell Signaling), rabbit monoclonal antibody Bax (1:1000 Cell Signaling), rabbit polyclonal anti-p65 (Nf-κB) (1 :1000 Proteintech) and mouse monoclonal anti-GAPDH antibody (1:10000; ProteinTech). Horse radish peroxidase-coupled secondary antibodies anti-mouse IgG-HRP or anti-rabbit IgG-HRP (1:1500; Santa Cruz Biotechnology, CA, USA) were also used. ECL solution (Bio-Rad, Hercules, CA, USA) was used to visualize the membranes. Membranes were imaged at appropriate times with the ChemiDoc ItR2 Imager (UVP, UK).

Graphics and Statistical Analyses

Preparation of graphics were accomplished by using 'Origin 8.0' program. The mean and standard deviations of the results form at least three independent experiments in 3 replicates were used for analysis. Statistical analysis was performed by One Way Anova test and the values $p \leq 0.05$ were considered as statistically significant.

RESULTS

The efficacy of β-asarone and Etoposide treatment on cell viability of MCF-cells

For the determination of the effect of β-asarone on cell viability of MCF-7 cells, cells were treated with 50 μM-750 μM of β-asarone for 24 hours, and the 'MTT Test' protocol was applied. The absorbance values measured with MTT were normalized with the control group, and the metabolic activity change of different concentrations of β-Asaron on MCF-7 cells was plotted as % mean ± standard deviation as shown in Figure 1A. Effects of different concentrations of β-asarone on cell viability were detected as shown in Figure 1A) The effects of β-asarone on cell viability at 200 μM and above were found to be statistically significant when compared with the control. As a result, increasing concentrations of β-asarone decreased the cell viability. β-asarone was effective in reducing cell viability at the end of the 24-hour period. At concentrations of 200 μM and above, the effect was maximized.

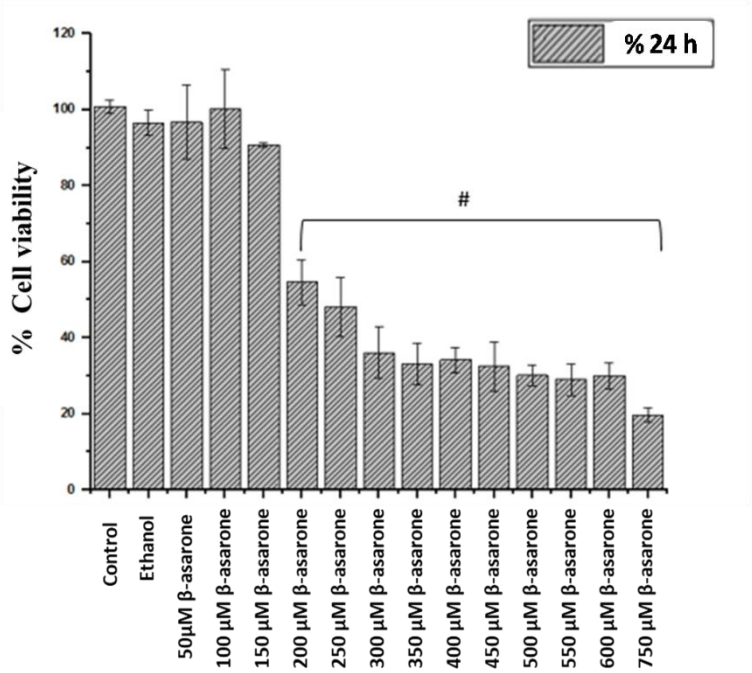

Figure 1. A) Analysis of cell viability in MCF-7 cells after 24 h treatment with β-asarone (#: Represents statistically significant difference at $p < 0.05$ compared to control.)

The effects of β-asarone on cell viability of MCF-7 cells for 48 hours were also measured with the MTT Test (Figure 1B). As shown in Figure 1Bthe effects were found to be statistically significant at concentrations of 200 μM and above. At the end of the 48 hour period, the percentage of the viability of MCF-7 cells was found to be lower compared to 24 hours. The effects of the varying concentrations of β-asarone (50 μM and 750 μM) for 72 hour period in cell viability of MCF-7 cells were also measured with the MTT test (Fig.1C). As shown in Figure 1C, cell viability was determined as following respectively,: At the end of the 72 hour period, the percentage of the viability of MCF-7 cells was found to be significantly lower than that of 48 hours. In particular, starting with 50 μM concentration cell viability is decreased gradually. At 250 μM 53% (±2) of cells were viable, whereas between 350 -450 μM the cell viability is decreased at least % 80 .

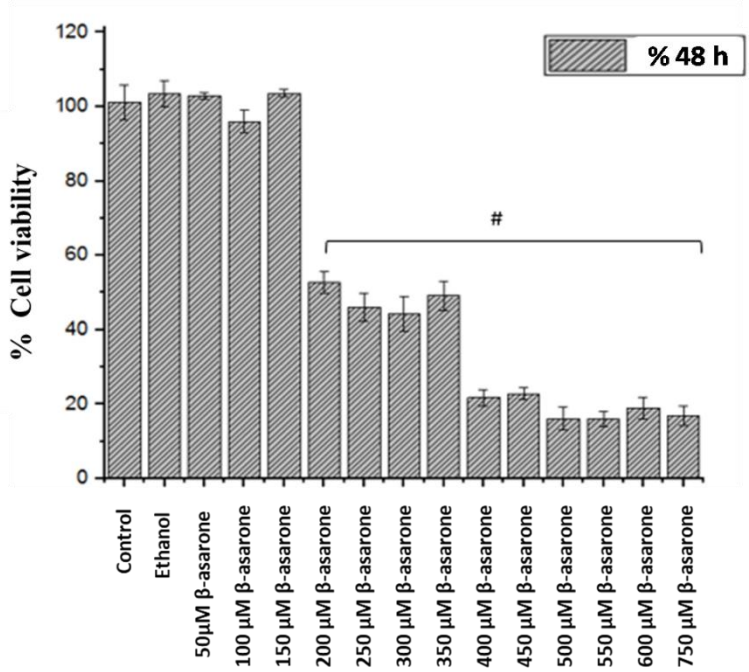

Figure 1. B) Analysis of cell viability in MCF-7 cells after 48 hours of treatment with β-asarone (#: Represents statistically significant difference at $p < 0.05$ compared to control.)

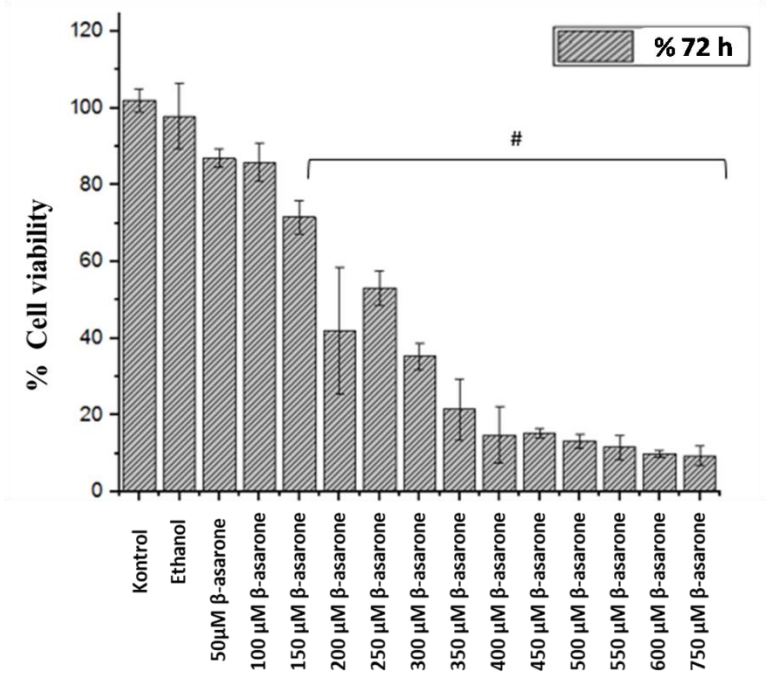

Figure 1. C) Analysis of cell viability in MCF-7 cells after 72 hours of treatment with β-asarone (#: Represents statistically significant difference at $p < 0.05$ compared to control.)

The effect of Etoposide treatment either alone or in combination with β-asarone in cell viability of MCF-7 cells was measured as shown in Figure 2. As seen in Figure 2, the viability rates in etoposide

treated cells were found as higher than that of the treated with β-asarone. In the combination of β-asarone and Etoposide treatment groups, when the Etoposide concentration was kept constant and the β-asarone concentration was increased from 100 µM to 150 µM, the viability rate decreased from 79% to 63%. However, when β-asarone concentration was kept constant at 150 µM and Etoposide was increased from 100 µM to 150 µM, the viability rate decreased from 63% to 58%. The cell viability rates of β-asarone and Etoposide combined treatments E100+β100 µM, E100+β150 µM, E150+β150 µM were found to be 79% (±2), 63% (±2), 58% (±2), respectively. According to these results, β-asarone was found to be more effective than Etoposide in reducing cell viability. Concentrations marked with # in the figure were normalized with the control and were found to be statistically significant. The p value was calculated as $p< 0.05$.

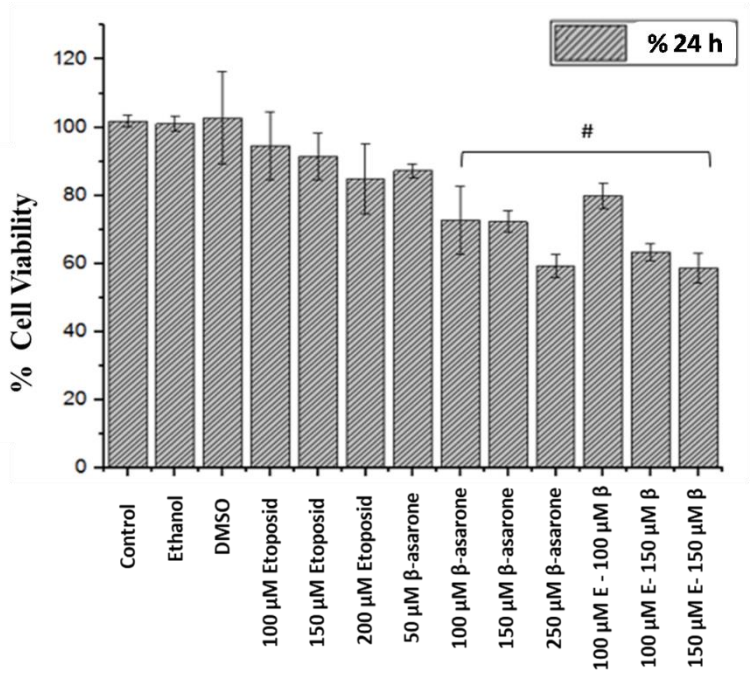

Figure 2. Cell viability analysis after 24 h treatment of low concentrations of β-Asarone and Etoposide in MCF-7 cells (# : Represents the statistically significant difference at $p < 0.05$ compared to control.)

The efficacy of β-Asarone and Etoposide treatment on cell death

As next, MCF-7 cells were treated with 50, 100, 150, 250 µM concentrations of β-Asarone and 100, 150 and 200 µM concentrations of Etoposide for 24 hours and the Annexin-V/7AAD protocol was applied to determine the apoptosis rate in the cells. Untreated MCF-7 cells were used as control, MCF-7 cell lines treated with ethanol and DMSO were used as solvent controls (Figs. 3 and 4). The apoptosis rate was found to be 8.56% (±2) in the MCF-7 control group. There was no significant increase in Etoposide treated groups. The highest concentration of Etoposide treatment (200 µM) induced only 5.74% (±2apoptosis. Apoptosis rates of the other groups treated with β-asarone between100-250 µM

were around 12% and higher than that of etoposide. Interestingly combined treatment of Etoposide and β-asarone did not induce a significantly higher apoptosis than that of either β-asarone or etoposide alone. The results obtained from β-100, β-150, β-250 and E100-β150, E150-β150 groups were found to be statistically significant compared to the control. According to statistical calculation, p value was found as $p < 0.05$. The 24-hour apoptosis rate was found to be higher in cells treated with β-asarone.

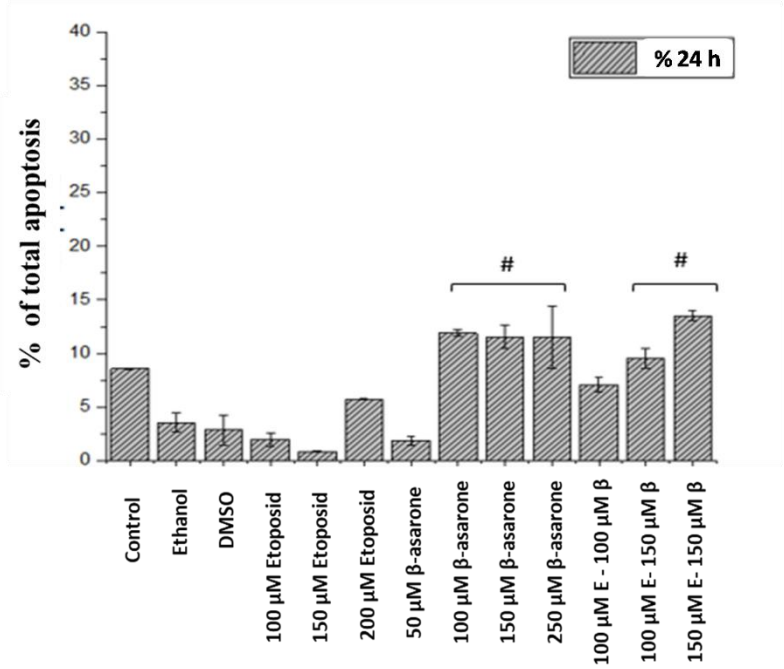

Figure 3. ANNEXIN V/7AAD analysis and total apoptosis rates in MCF-7 cells as a result of 24 Hour Etoposide and β-asarone treatment (#: Represents the statistically significant difference at $p< 0.05$ compared to the control.)

The efficacy of β-Asarone and Etoposide on cell cycle profile

In order to get more in sight in effects β-Asarone and Etoposide treatment in MCF-7 cells the cell cycle analysis was accomplished. The cell cycle analysis results obtained by treatment of MCF-7 cells with either etoposide (100, 150, 200 µM) and/or β-asarone (50-250 µM) for 24 hours are shown in Figure 5 according to the results, 150 and 250 µM of β-asarone treatment induced the cell cycle arrest in G1 phase. Interestingly, etoposide treatment did not induce cell cycle arrest (Figure 5). Increasing concentrations of β-asarone increased the cell cycle arrest in the G0/G1 phase. The combination of 100 µM etoposide and 150 µM β-asarone, induced 50%the G0/G1 phase arrest. At 150 µM etoposide and 150 µM of β-asarone, the G0/G1 phase arrest was 44.3%, the S phase arrest was 5%, and the G2/M phase was 50.5%. When combined and single concentrations were compared, it was observed that β-asarone increased the arrest in the G0/G1 phase and decreased the arrest in the S and G2/M phases. When the S phase ratio of the etoposide and β-asarone treatment group was compared with the control

group, there was also a decrease from 11.9% to 6.5%. Following, in etoposide and β-asarone treatment group the percentage of G2/M phase was increased from 41.8% to 48.8% compared to the control group.

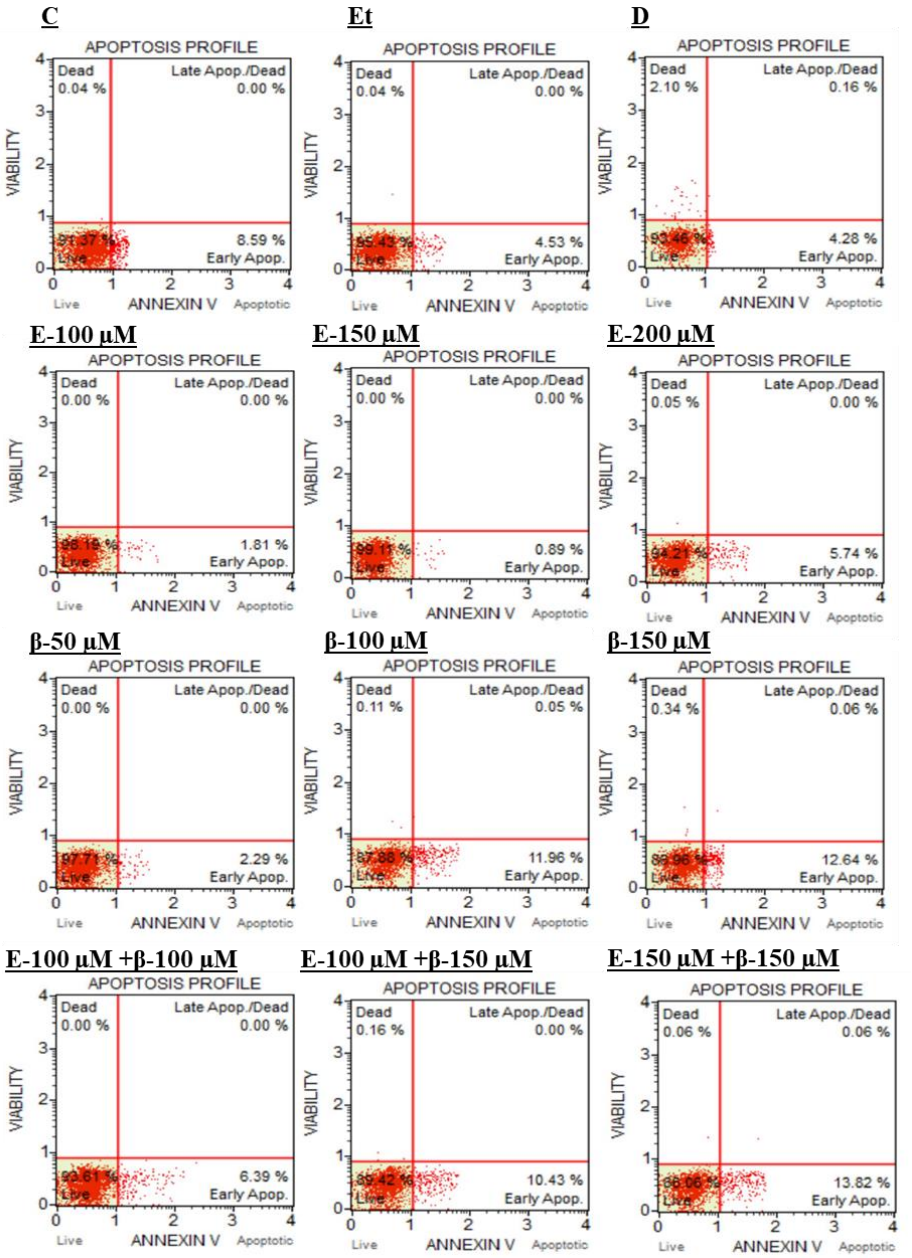

Figure 4. 24 Hour ANNEXIN V/7AAD analysis apoptosis profile in MCF-7 cells (C: control, Et: ethanol, β: β-asarone, D: DMSO, E: Etoposide)

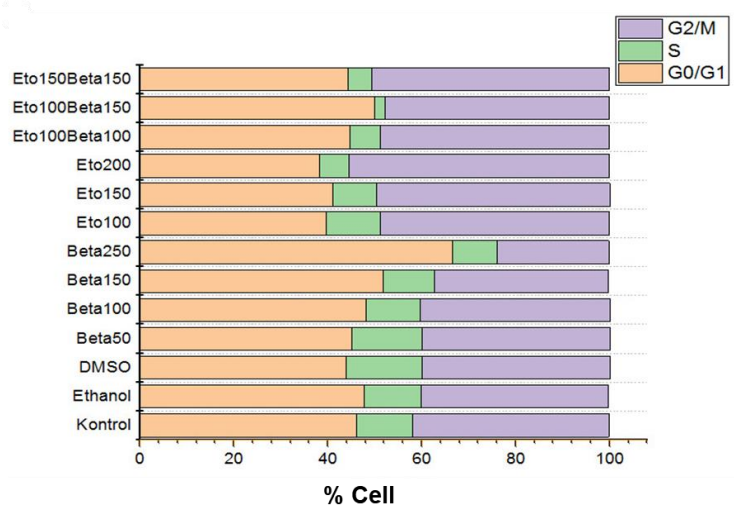

Figure 5. Cell cycle analysis after 24 sour Etoposide and β-asarone treatment in MCF-7 cells

Analysis of p53, p21, Bax and NFκB-P65 expressions

The protein expressions of p53, p21, Bax and NFκB-P65 which are associated with cell cycle regulation, apoptosis and cell survival respectively, were also investigated in MCF-7 cells in response to etoposide (100, 150 µM) and β-asarone (50, 100, 150 µM) treatments for 24 hours. As shown in Figure 6 etoposide or β-asarone treatments did not change the the expression of p53, p21, Bax. However, etoposide treatment either alone or in combination with β-asarone increased the protein levels of NFκB-P65 subunit. β-asarone treatments did not induce any changes in NFκB-P65 subunit. The equal loading was measured with the expresson of housekeeping gene GAPDH.

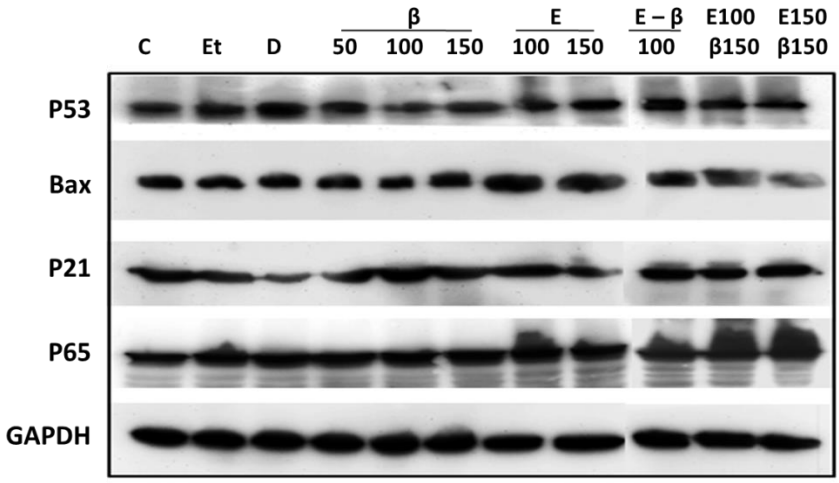

Figure 6. Western blot analysis of P53, Bax, P21 and P65 (NFκB) expressions in MCF-7 cells after treatment with Etoposide and β-asarone for 24 hours (K: control, Et: ethanol, β: β-asarone, E: Etoposide)

DISCUSSION

In this study, the anti-cancer effect of β-asarone (Bhrigu Kumar Das et al., 2021) alone and with etoposide was investigated in MCF-7 breast cancer cells. The effect of β-asarone was measured for 24, 48 and 72 hours in single concentrations, subsequently cell cycle arrest and cell death due to apoptosis were detected in atime and concentration dependent manner.. In the combined administration of β-asarone and Etoposide, only a β-asarone dependent effect leading to decrease in cell viability was observed. β-asarone lead to increase in the effect of Etoposide but did not induce a synergistic effect. Interestingly, among the tested proteins related to apoptosis and survival signaling such as p53, p21, Bax and NFκB/p65, only expression of NFκB-p65 is found to be increased due to etoposide treatment. No increase or decrease was observed in p53, p21 or Bax expressions.

In recent *in vitro* studies with MCF-7 cell line, the IC50 value of β-asarone was found to be higher than >150 μM (Shenvi, Diwakar, & Reddy, 2014). In a previous study conducted with Acorus calamus extract which is known as one of the main sources of β-asarone containing 60% of β-asarone , the IC50 value was found to be 52.07 μg/ml in MCF-7 cells for 24 hours. In this study the cell death induced by β-asarone at 18.75 μg/ml concentrations was 17.04%, however it was found to be 94.50% when the concentration was increased to300 μg/ml (Sreejaya & Santhy, 2013). In our study, when various concentrations of β-asarone which are below and above the IC50 value were tested, the results were in line with the data in the literature. Etoposide (100 μM) treatment was found to have almost no effect on the cell viability of MCF-7 cells, while the co-administration of Etoposide (100 μM) and β-asarone (100 μM) decreased the cell viability. These results showed that the decrease in cell viability is dependent on β-asarone activity. It was also found that induction of apoptosis is not the major reason for the decrease in cell viability.. Because the rate of apoptosis did not exceed over 15 %. as a result of β-asarone treatment for 24 h, On the other hand, β-asarone treatment in increasing concentrations induced the cell cycle arrest in the G1 phase wheras Etoposide treatment either alone or in combination with β-asarone in the G2 phase. The fact that β-asarone induced the cell cycle arrest in the G1 phase; whereas Etoposide in the G2 phase indicates that the mechanism of action and the activated checkpoint kinases are different.

Etoposide is an anti-cancer drug that has been used in the treatment of various cancer types for many years. It creates a triple complex with DNA and topoisomerase II enzyme, causing it to break into DNA chains, triggering an anti-cancer response. It is known from previous studies conducted by us and others that MCF-7 cells are resistant to Etoposide (Pilevneli and Kilic Eren, 2021; Alpsoy et al., 2014). Thus, in this study similar results were also obtained. More importantly, the combined use of β-asarone and Etoposide did not induced a synergistic effect and enhanced induction of cell death in MCF-7 cells. Interestingly while etoposide treatment induced no change in expression of p53, p21 and Bax, expression of NF-kB/p65 is increased and remained also stable with the combined administration of etoposide and

β-asarone combined. NF-κB is an important transcription factor involved in the development and progression of cancer and chemotherapy resistance through the activation of multiple mediators, including anti-apoptotic genes. Preclinical models have shown that many chemotherapy agents, such as platinum-based agents, anthracyclines, and taxanes, promote activation of the NF-κB pathway (Godwin et al., 2013). As in all other cancer types, chemotherapy is an indispensable treatment strategy in breast cancer. As a frequently used anticancer agent in breast cancer Etoposide targets the enzyme topoisomerase II, which is involved in DNA replication and transcription, Although etoposide is frequently used in breast cancer, it also creates resistance to treatment in patients (Alpsoy, Yasa, & Gündüz, 2014). In this study we provide evidence ethat etoposide promote activation of the NF-κB pathway by increasing the expression of the p65 subunit in MCF-7 cells and suggest that activation of the NF-κB pathway may be the cause of the resistance.

It has been reported that β-asarone attenuates pro-inflammatory mediators by blocking IκB degradation to inhibit NF-κB signaling in microglial and lymphoma cells (Lim et al., 2014; Lv et al., 2019). It is also known that β-asarone inhibits the translocation of nuclear factor κB (NFκB) by reducing JNK phosphorylation. The anti-cancer effects of β-asarone are not only related to the inhibition of NF-κB/p65 activity, but also with other proteins involved in apoptosis (Lv et al., 2019). However, in contrat to the previous data our results indicate that the effect β-asarone is not related with the inhibition of NF-kB/p65. In addition, β-asarone has been observed to induce cellular senescence in various cancers. In human colorectal cancer, β-asarone mediated the activation of lamin B1 and promoted the tumor suppressor protein () expressions such as p53a nd p21, thus caused cell cycle arrest and induced cellular senescence (LiuWang et al., 2013). β-asarone-induced activation of senescence or other non-apoptotic cell death programmes in MCF-7 cells will be the subject of our further studies. In this study, the effect of β-asarone on the MCF-7 breast cancer cell line and it's potential to create a synergistic effect with etoposide was investigated for the first time. The use of β-asarone alone in breast cancer cell line MCF-7 is effective in reducing cell viability via cell cycle arrest and induction of apoptosis. However its combined use with Etoposide is not sufficient to suppress the resistance to Etoposide treatment. In particular, increased NF-kB activation may be effective in manifestation of etoposide resistance.

Conclusion

This study suggests that β-asarone may have a potential as a chemotherapeutic agent in breast cancer which requires further confirmation by in vivo studies. Future *in-vitro* and *in-vivo* studies will answer the questions of the mechanism of action of β-asarone in cancer cells and whether or not increasing concentrations exert cytotoxic effects in healthy normal cells

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