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Article

Euphorbia bicolor Diterpene Extract Induces Mitochondrial and Endoplasmic Reticulum Stress-Mediated Apoptotic Pathways in MDA-MB-231 and T47D Cells

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Abstract

Breast cancer is a significant cause of death worldwide. Recent research has focused on identifying natural compounds for developing effective cancer treatments. Resiniferatoxin, a TRPV1 agonist, is a common diterpene in *Euphorbia bicolor*, a plant native to the southern United States that has not been studied before. We investigated the antiproliferative activities and mechanisms of action of *E. bicolor* diterpene extract in estrogen receptor-positive T47D and triple-negative MDA-MB-231 cell lines. The extract significantly reduced the viability of T47D and MDA-MB-231 cells in a dose-dependent manner. In MDA-MB-231 cells, the extract induced apoptosis via intracellular calcium overload, triggered by TRPV1 activation. This effect was diminished by the TRPV1 antagonist capsazepine and the calcium chelator BAPTA-AM. Intracellular calcium influx was confirmed through Fura-2 AM staining, revealing that *E. bicolor* phytochemicals activated TRPV1 in MDA-MB-231 cells. Treatment of T47D cells with *E. bicolor* diterpene extract resulted in apoptosis associated with ROS generation (10-fold higher in T47D cells than in MDA-MB-231 cells) and mitochondrial calcium overload. These effects were significantly blocked when cells were pretreated with NAC (N-acetyl-L-cysteine), a ROS inhibitor. Both cell lines initiated apoptosis through multiple mitochondrial and endoplasmic reticulum-stress-mediated apoptotic pathways, demonstrated by the expression of activated caspase 3, caspase 9, caspase 8, FAS, XBP1s, CHOP, overexpression of BAX proteins and reduction of BCL-2 levels. In addition, PI3K, AKT, and pAKT protein expressions were also reduced in both cell lines, indicating downregulation of PI3K/Akt signaling pathway. Phytochemicals in *E. bicolor* diterpene extract could become promising ingredients for developing breast cancer therapeutics.

Keywords: breast cancer; resiniferatoxin; *Euphorbia bicolor*; TRPV1; ER-positive; triple-negative; ROS; apoptosis; mitochondrial intrinsic and extrinsic apoptosis; endoplasmic reticulum stress-dependent apoptosis

1. Introduction

Breast cancer has emerged as a significant concern for women globally, with 2.26 million reported cases in 2020, surpassing all other forms of cancers and becoming the leading cause of cancer-related deaths in women [1,2]. The number of new cases in the United States had increased by 31% in 2023 [3]. Despite advancements in cancer diagnosis and treatment, the development of chemotherapeutic drugs continues to be intensively researched [4,5]. Plants have been used for medicinal purposes for centuries, and many plant species contain chemical compounds that have exhibited potent effects in cancer treatment [6]. Plant secondary metabolites with anticancer properties, such as alkaloids, terpenoids, and phenolics may provide a broad range of therapeutic benefits [7–9]. Recent research suggests that phytochemicals could have various therapeutic effects,

such as preventing tumor growth by activating several cellular signaling pathways and targeting specific receptors [10–13]. One of the well-known receptors that can be activated by phytochemicals is the transient receptor potential vanilloid 1 (TRPV1) receptor. TRPV1 receptors are ion channels belonging to the TRP channel superfamily, modulated by several phytochemicals and activating several cell death signaling pathways, thus exhibiting antiproliferative effects [14–18]. The TRPV1 channel is a non-selective cation channel primarily linked to pain and temperature sensation [19]. However, recent data suggests that TRPV1 is associated with various physiological and pathological processes, including cancer [14,20–22], and is expressed in different carcinoma tissues, including all types of breast cancers [23]. Various plant phytochemicals, including capsaicin, gingerol, piperine, and resiniferatoxin (RTX), are the commonly known activators of the TRPV1 channel [17,20]. In cancer cells, TRPV1 activation leads to an influx of calcium, initiating subsequent signaling cascades and triggering antiproliferative effects [14,24,25]. Further study is required to fully understand the potential of phytochemicals targeting TRPV1 in cancer therapy.

One genus known to have antiproliferative effects is *Euphorbia* (*Euphorbiaceae*). Many species of *Euphorbia* are used in traditional folk medicine to treat different diseases [26] and have been extensively studied because of their wide range of biological activities, including antiproliferative, anti-inflammatory, and analgesic properties [27–29]. *Euphorbia* species, such as *E. helioscopia*, and *E. macroclada*, have been found to possess anticancer properties by inducing apoptosis and cell cycle arrest, inhibiting cell proliferation, and reversing the multidrug resistance of different breast cancer cell lines [30–33]. *Euphorbia bicolor*, also known as Snow-on-the-prairie, is native to south-central USA. No scientific research has been done on this species. Our previous research found that the latex extract of *E. bicolor* and its phytochemicals showed antiproliferative properties in ER-positive MCF-7 and T47D, as well as triple-negative MDA-MB-231 and MDA-MB-469 breast carcinomas, but the mechanisms of action were not determined [32].

The present investigation aims to determine the antiproliferative mechanisms of action of *E. bicolor* diterpene extract on ER-positive T47D and triple-negative MDA-MB-231 cell lines. Resiniferatoxin, a common diterpene present in *E. bicolor* [29], was reported to activate the TRPV1 channel in neurons and cancer cells [34,35]. We hypothesized that *E. bicolor* diterpene extract would activate TRPV1 and induce TRPV1-dependent antiproliferative mechanisms of action in the breast cancer cell lines. We report that *E. bicolor* diterpene extract possesses antiproliferative properties in both breast cancer cell lines under study and induces apoptosis through multiple cell death pathways. To our knowledge, this is the first study reporting the antiproliferative mechanisms of action of *E. bicolor* diterpene extract on ER-positive T47D and triple-negative MDA-MB-231 breast cancer cell lines.

2. Results

2.1. Antiproliferative Activity of *E. bicolor* Extracts in T47D and MDA-MB-231 Cells

ER-positive T47D and triple-negative MDA-MB-231 cell lines were treated with increasing concentrations of *E. bicolor* ethanol, diterpene extracts, or capsaicin. *E. bicolor* ethanol extract significantly inhibited the cell viability of ER-positive T47D cell lines at 500 µg/ml (Figure 1A). However, *E. bicolor* ethanol extract did not show a reduction of cell viability of triple-negative MDA-MB-231 cells (Figure 1 B).

E. bicolor diterpene extract dose-dependently inhibited the proliferation of ER-positive T47D and triple-negative MDA-MB-231 cell lines (Figure 1C, D). The diterpene extract significantly inhibited cell viability, starting at 2 µg/ml in T47D and 8 µg/ml in MDA-MB-231 cell lines. At 500 µg/ml, *E. bicolor* diterpene extract significantly inhibited T47D and MDA-MB-231 cell viability by more than 95% (Figure 1C, D). Since the diterpene extract is significantly more potent than the ethanol extract in inducing antiproliferative effects, all follow-up experiments were performed with *E. bicolor* diterpene extract.

The proliferation of T47D cells was significantly inhibited by capsaicin treatment at 250 µg/ml and 500 µg/ml concentrations (Figure 1E), while MDA-MB-231 cell viability was significantly reduced at 500 µg/ml capsaicin (Figure 1F). In contrast to *E. bicolor* ethanol extract, the diterpene extract at higher concentrations (125 µg/ml- 500 µg/ml) inhibited the growth of human mammary epithelial cells (HMEC) (Figures 1G and H). Therefore, the rest of the experiments were set up to use 62.5 µg/ml diterpene extract to determine the antiproliferative mechanisms.

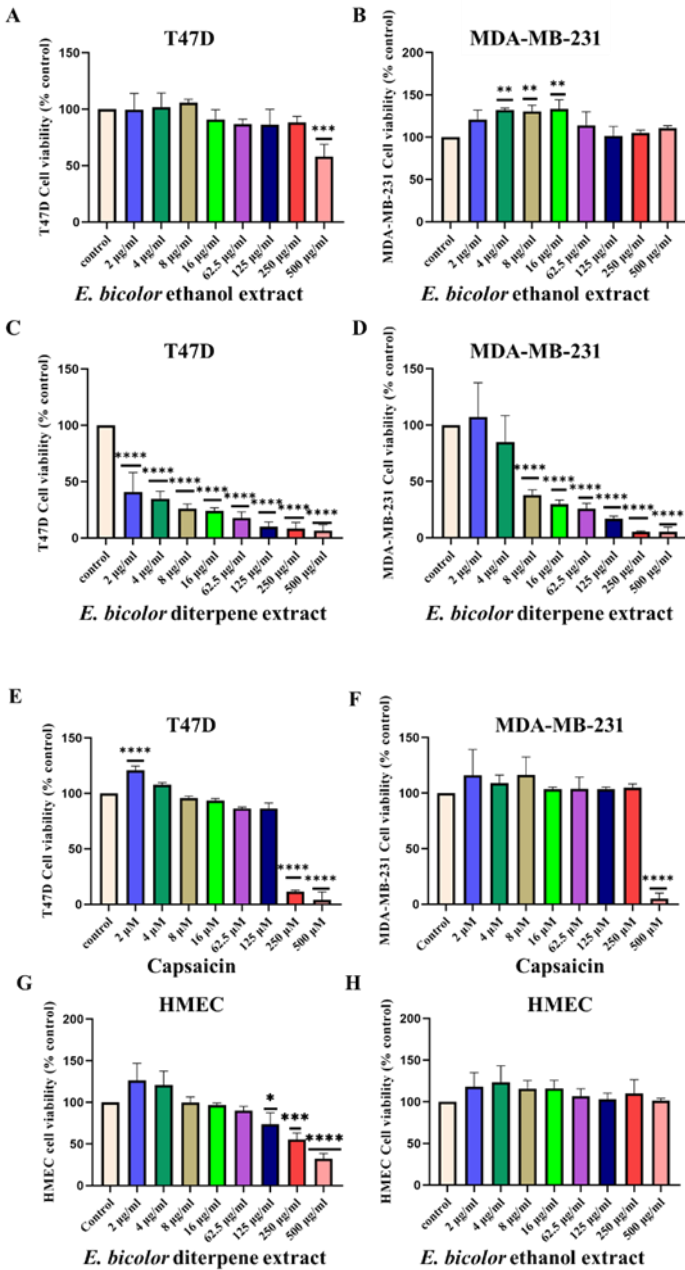


Figure 1. Antiproliferative activities of *E. bicolor* extracts on ER-positive T47D and triple-negative MDA-MB-231 breast cancer cell lines. (A, B) Effect of *E. bicolor* ethanol extract on T47D and MDA-MB-231 cells; (C, D) Effect of *E. bicolor* diterpene extract on T47D and MDA-MB-231 cells; (E, F) Effect of capsaicin (+ control) on T47D and MDA-MB-231 cell lines; (G, H) Effect of *E. bicolor* diterpene and ethanol extracts on the growth of human mammary epithelial cells (HMEC). Values represent the mean ± SEM of three independent experiments. A one-way ANOVA was performed, followed by Tukey's post hoc test. *p<0.05, ***p<0.001 and ****p<0.0001 vs. DMSO control set.

The half-maximal inhibitory concentration (IC50) of *E. bicolor* diterpene extract for T47D cells was 0.7834 µg/ml (Figure 2A), and for MDA-MB-231 cells was 9.341 µg/ml (Figure 2B). The IC50 of

E. bicolor diterpene extract for HMEC cells was 288.6 µg/ml (Figure 2C). The IC₅₀ of capsaicin for T47D cells was 173.4 µg/ml (Figure 2D), and MDA-MB-231 cells were 439.3 µg/ml (Figure 2E).

Cell morphological changes were observed after *E. bicolor* diterpene extract treatment (62.5 µg/ml) in both cell lines. Three days after treatment, *E. bicolor* diterpene extract treatments resulted in a noticeable cell loss, cell shrinkage, cell rounding, and partial or complete detachment from the bottom of the wells, confirming the cytotoxic effects of *E. bicolor* diterpene extract on T47D and MDA-MB-231 cells (Figure 2F).

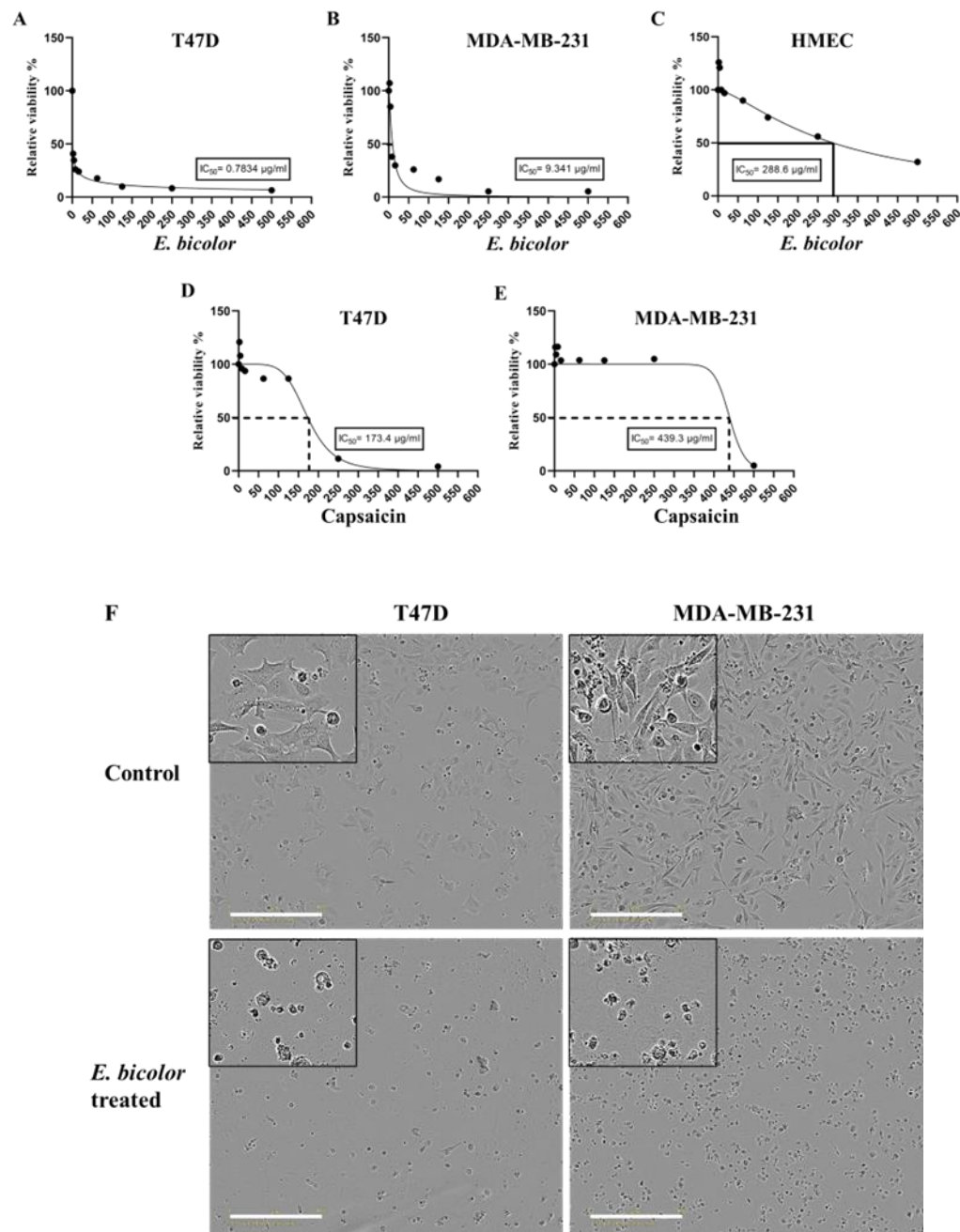


Figure 2. Half-maximal inhibitory concentration (IC₅₀) of *E. bicolor* diterpene extract and capsaicin in ER-positive T47D and triple-negative MDA-MB-231 breast cancer cell lines. (A) T47-D; (B) MDA-MB-231; (C) HMEC; (D, E) IC₅₀ of capsaicin in T47D and MDA-MB-231 cell lines, respectively. (F) T47D and MDA-MB-231 cell morphology 72 h after *E. bicolor* diterpene extract treatment (62.5 µg/ml). Cell morphological changes were observed for seven days using the live cell imaging system IncuCyte. Representative pictures of cell morphologies three days after treatment. Scale bar: 400 µm.

2.2. *E. bicolor* Diterpene Extract Induces Apoptosis in T47D and MDA-MB-231 Cells

T47D and MDA-MB-231 cells were treated with *E. bicolor* diterpene extract (62.5 µg/ml) for 24 hours. TUNEL assays were employed to detect apoptosis. Typical DNA fragmentation in T47D and MDA-MB-231 cell lines was observed compared to the DMSO control cells (Figure 3A). The number of apoptotic T47D and MDA-MB-231 cells significantly increased with *E. bicolor* diterpene extract treatments, as indicated by the relative fluorescence intensity of Alexa 594 (red fluorescence) (Figure 3B).

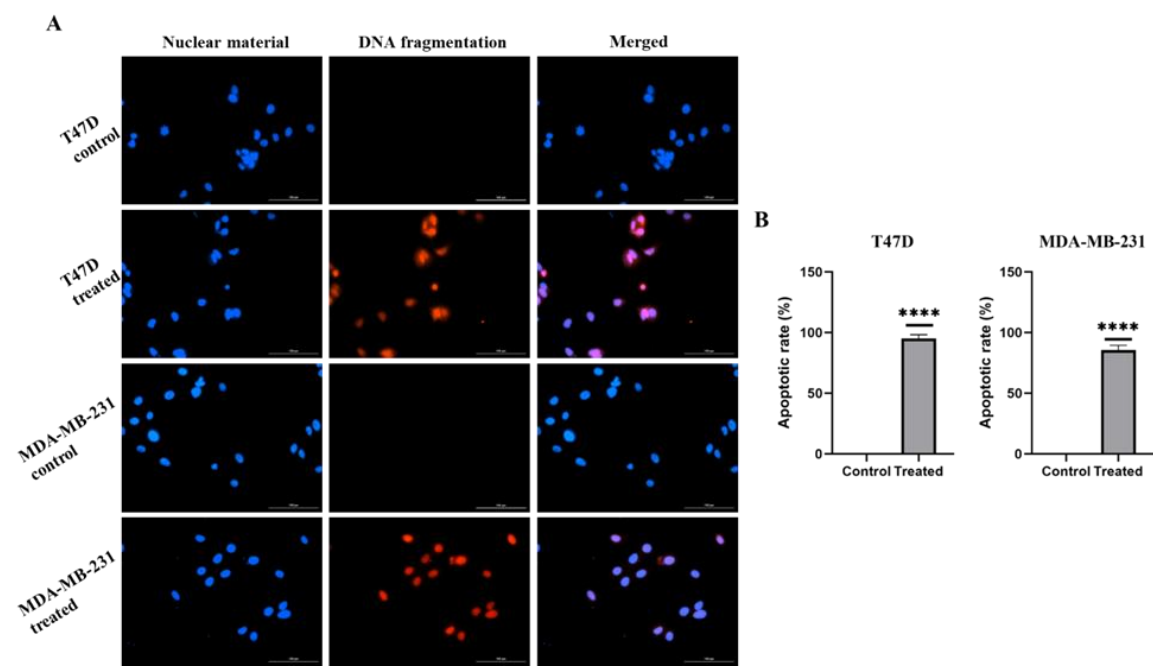


Figure 3. Apoptosis detected by TUNEL assay. (A) T47D and MDA-MB-231 cells treated with *E. bicolor* diterpene extract. Red fluorescence indicates that the modified dUTPs from the TUNEL test kit bind to the apoptotic broken DNA. Hoechst 33342 was used to stain nuclear material (blue fluorescence). Cells were visualized with LionHeart FX microscope, scale bar: 100 µm (B) Apoptotic rate (%) of TUNEL assays. The number of apoptotic cells were counted using LionHeart BioTek Gen5.0 software. Unpaired Welch's t-test, ****p<0.0001 vs. DMSO control set.

2.3. ROS Production Activated *E. bicolor*-Induced Apoptosis in T47D Cells

T47D and MDA-MB-231 cells were treated with *E. bicolor* diterpene extract, and reactive oxygen species (ROS) production was monitored to investigate whether *E. bicolor* treatment could generate ROS accumulation in T47D and MDA-MB-231 cells. The intracellular ROS level was measured using the cell-permeant reagent 2',7'-dichlorofluorescein diacetate (DCFDA). The DCFDA fluorescence intensity was increased in a dose-dependent manner with increasing time of *E. bicolor* diterpene extract treatment in both T47D and MDA-MB-231 cells (Figure 4A, B). *E. bicolor* diterpene extract treatment significantly and dose-dependently triggered intracellular ROS accumulation in T47D and MDA-MB-231 cells compared to the negative control and 20X and 30X higher than the positive control (Figure 4C, D). At higher doses of *E. bicolor* diterpene extract treatments, intracellular ROS accumulation was found to be ten times greater in T47D cells than in MDA-MB-231 cells (Figure 4C). To elucidate whether ROS generation was associated with *E. bicolor*-induced apoptosis, T47D and MDA-MB-231 cells were pretreated with N-Acetyl-L-cysteine (NAC), a ROS inhibitor, for 1 hour and then treated with *E. bicolor* diterpene extract. Pretreatment with NAC significantly ameliorated the effect of *E. bicolor* in T47D cells (Figure 4E), suggesting that ROS generation is involved in *E. bicolor* diterpene extract-induced apoptosis in T47D cells. However, NAC could not inhibit the effect of *E.*

bicolor diterpene extracts in MDA-MB-231 cells (Figure 4F), suggesting that *E. bicolor* diterpene extract induced apoptosis in a TRPV1-dependent manner and only partially through ROS generation.

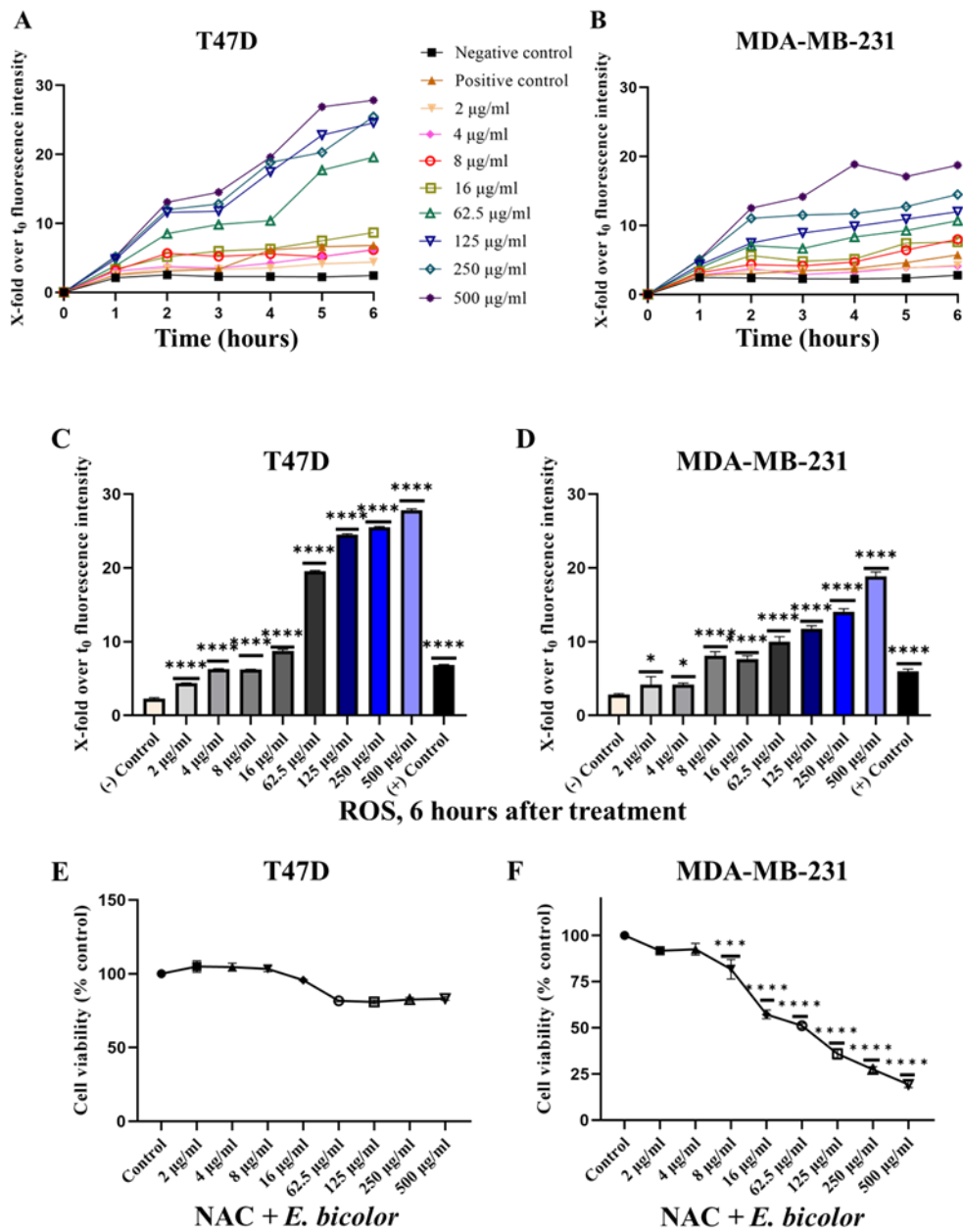


Figure 4. ROS level detection after *E. bicolor* diterpene extract treatment. (A, B) Increased ROS levels with time in T47D and MDA-MB-231 cells. (C, D) ROS accumulation at 6 hr of treatment. Tert-butyl hydroperoxide (TBHP, 250 μ M) was used as the positive control, and DMSO (0.1%) as the negative control. (E, F) Cell viability after inhibition of ROS formation with NAC in T47D and MDA-MB-231 cells. Cells were pretreated with NAC (5 mM), a ROS inhibitor, for 1 h, then treated with or without *E. bicolor* diterpene extract for 72 h. Cell viability was measured using MTS assay. Values represent the mean $\pm$ SEM of three independent experiments. One-way ANOVA, * p <0.05, *** p <0.001, **** p <0.0001 vs. DMSO control set.

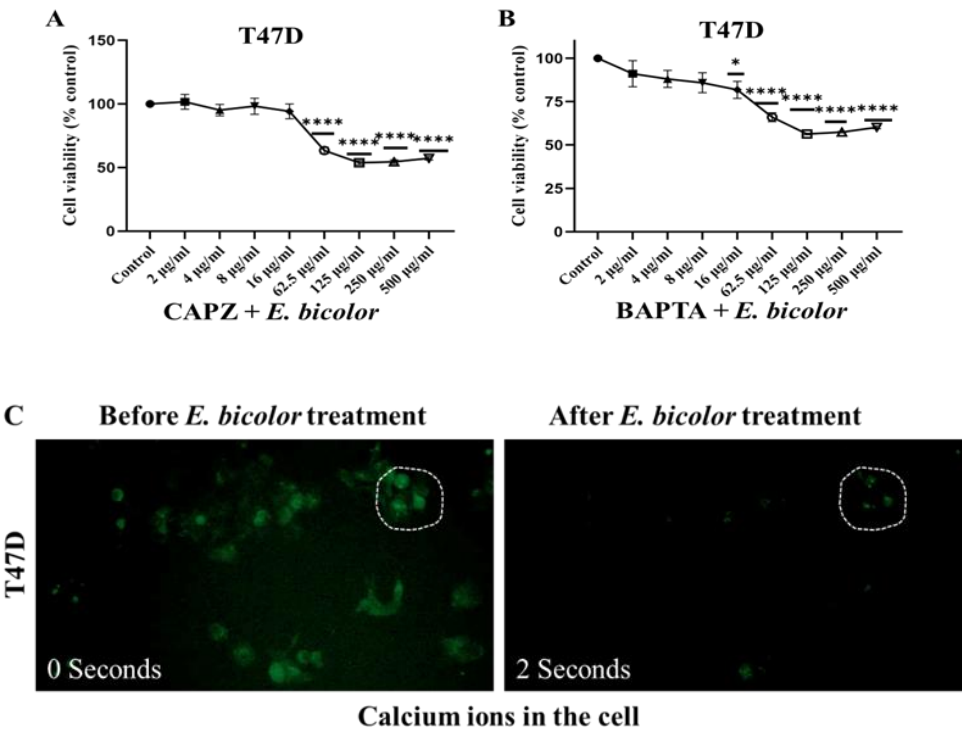
2.4. Blocking TRPV1 and Chelating Calcium Could Not Completely Inhibit the Antiproliferative Activity of *E. bicolor* Extract in T47D

Resiniferatoxin, commonly known as a TRPV1 agonist, is present in *E. bicolor* [29,34]. Therefore, we hypothesized that TRPV1 would be activated by *E. bicolor* diterpene extract, causing an influx of calcium that would lead to cell death. The T47D cells were treated with *E. bicolor* diterpene extract

after pretreating them with 10 μ M of capsazepine (CAPZ), a TRPV1 antagonist. TRPV1 inhibition increased the cell proliferation only at *E. bicolor* diterpene extract concentrations of 2-16 μ g/ml. Capsazepine could not completely block the effect of higher concentrations of *E. bicolor* diterpene extract (Figure 5A). T47D cells were pretreated with 1 μ M of the calcium chelator BAPTA-AM and then treated with *E. bicolor* diterpene extract to determine the involvement of calcium in apoptosis. Chelating calcium increased the cell proliferation only at concentrations of 2-8 μ g/ml of *E. bicolor* diterpene extract. Chelating calcium could not completely block the effect of higher concentrations of *E. bicolor* extract (Figure 5B). To check the activation of TRPV1, intracellular calcium concentrations were evaluated by Fura2-AM staining. Fluorescence followed a downward trend immediately after the *E. bicolor* treatment (Figure 5C), revealing that TRPV1 may not be involved in the antiproliferative mechanism of action.

In search of calcium-regulated apoptotic mechanism of action, an endoplasmic reticulum-targeted low-affinity GCaMP6-210 plasmid variant, a fluorescent reporter for ER calcium signaling, was used to visualize ER Ca^{2+} dynamics. T47D cells were transfected with the GCaMP6-210 variant, and immediately after *E. bicolor* treatment, calcium concentration (green fluorescence) was observed. GCaMP6-210 fluorescence followed a downward trend (Figure 5D), suggesting that *E. bicolor* treatment in T47D cells triggers the release of calcium from ER. This suggests that TRPV1 might not be involved in the cell death of *E. bicolor*-treated T47D cells.

Rhod2-AM was used to monitor mitochondrial calcium. Twenty-seven seconds after *E. bicolor* diterpene extract treatment, Rhod2-AM was localized within the mitochondria of T47D cells, evidenced by the merged staining pattern in Figure 5E.



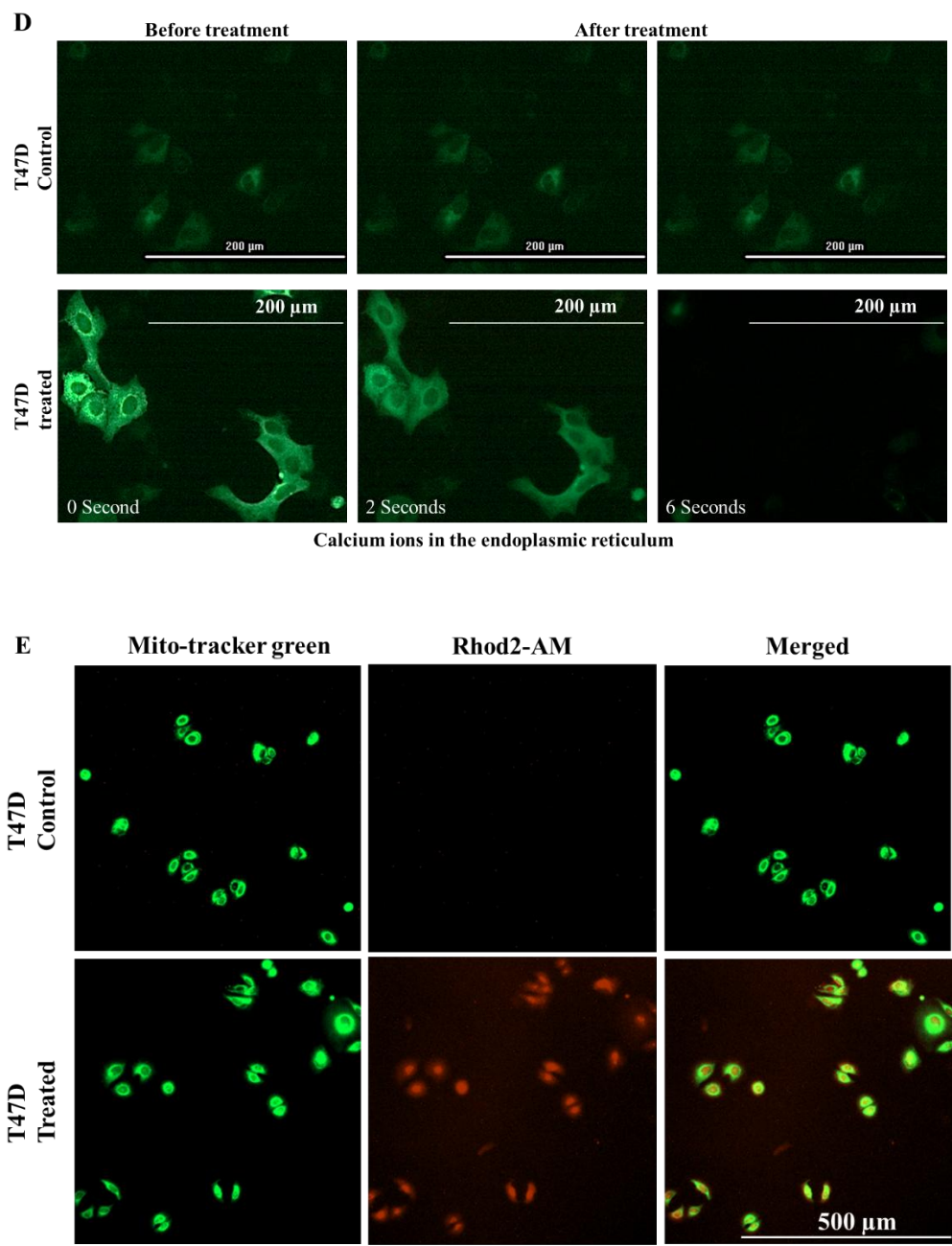


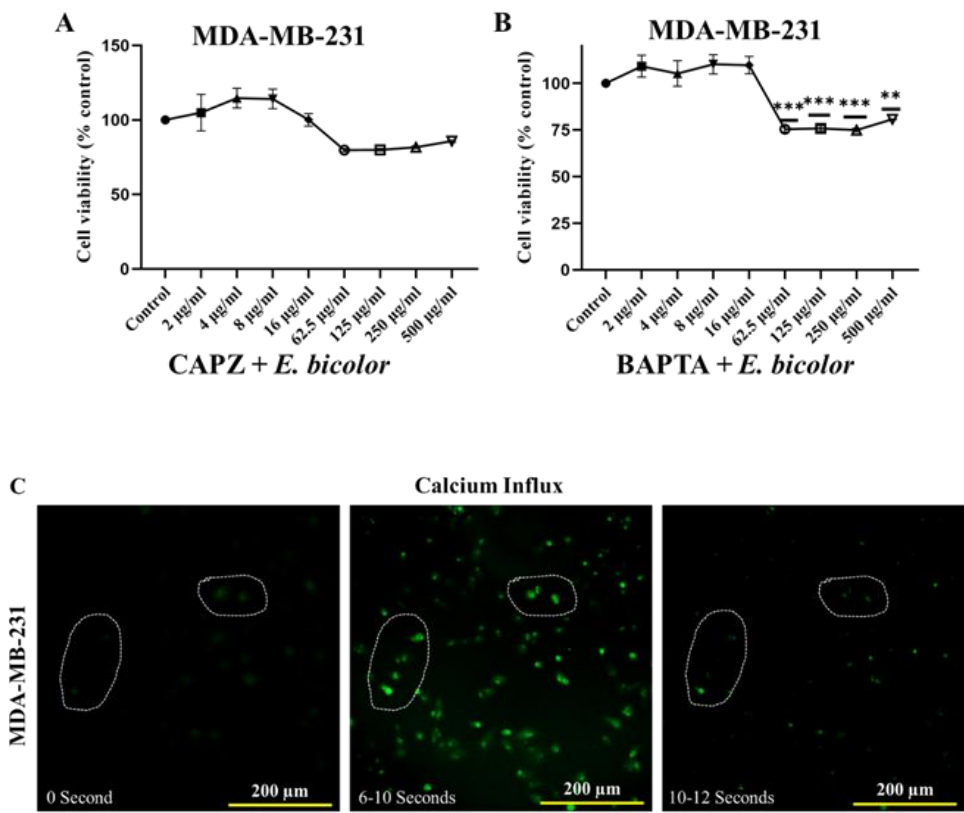
Figure 5. Calcium dynamics in T47D induced by *E. bicolor* diterpene extract treatment. (A) Cell viability after blocking TRPV1 with capsazepine (CAPZ), a TRPV1 antagonist. Cells were pretreated with 10 µM CAPZ, and then treated with *E. bicolor* diterpene extract. (B) Cell viability after chelating calcium with BAPTA-AM. Cells were pretreated with 1 µM of BAPTA-AM, and then treated with *E. bicolor* extract (62.5 µg/ml). Cell viability was measured by MTS assay. Values represent the mean ± SEM of three independent experiments. One-way ANOVA, * $p < 0.05$, *** $p < 0.0001$ vs. DMSO control set. (C) Visualization of calcium dynamics in T47D cells. Cells were loaded with 5 µM of Ca^{2+} sensitive fluorescent probe Fura2-AM and then exposed to 62.5 µg/ml of *E. bicolor* in HBSS buffer (with Ca^{2+} and Mg^{2+}). The fluorescent images were captured every 2 seconds and recorded for 10 min. Representative Ca^{2+} fluorescence images at indicated time points are shown. The region of interest was drawn with a dotted circle. (D) Visualization of ER calcium dynamics with a fluorescent reporter for ER calcium signaling in GCaMP6-210 plasmid variant. T47D cells were grown in a 24-well plate for 24 hours and transfected with GCaMP6-210 plasmid variant. Twelve hours after incubation, cells were exposed to 62.5 µg/ml of *E. bicolor* in HBSS buffer (with Ca^{2+} and Mg^{2+}). The fluorescent images were captured every 2 seconds and recorded for 10 min. (E) visualization of mitochondrial calcium with MitoTracker Green. T47D cells were simultaneously loaded

with 10 μ M of MitoTracker Green and 5 μ M of Rhod2-AM and then exposed to 62.5 μ g/ml of *E. bicolor* diterpene extract. A LionHeart FX microscope, 4X magnification, monitored the corresponding fluorescence signal.

2.5. TRPV1 Activation Triggered *E. bicolor*-Induced Apoptosis in MDA-MB-231 Cells

MDA-MB-231 cells were pretreated with 10 μ M of CAPZ and treated with *E. bicolor* diterpene extract. TRPV1 inactivation by CAPZ significantly increased the cell viability of extract-treated cells (Figure 6A), indicating that *E. bicolor* extract reduces the viability of MDA-MB-231 cells in a TRPV1-dependent manner. To see the effect of calcium chelation, which could oppose the above scenario and increase cell viability, MDA-MB-231 cells were pretreated with 1 μ M of the calcium chelator BAPTA-AM and then treated with *E. bicolor* diterpene extract. At low extract concentrations (2-16 μ g/ml), chelating calcium with BAPTA-AM increases the cell viability by blocking the effect of *E. bicolor* extract (Figure 6B). However, at high extract concentrations (62.5-500 μ g/ml), MDA-MB-231 cells showed a significant reduction in cell viability, possibly due to the cytotoxic effects of *E. bicolor* at high concentrations.

To check the calcium dynamics induced by the activation of TRPV1, Fura2-AM staining was used and an increase in intracellular calcium was observed. Fluorescence intensity followed an upward trend immediately after the *E. bicolor* diterpene extract treatment, which lasted for 6-10 seconds (Figure 6C). To determine if this activation could lead to the accumulation of calcium in mitochondria, Rhod2-AM was used to monitor mitochondrial calcium. An immediate accumulation of calcium in the mitochondria was observed. Within 8 seconds after *E. bicolor* treatment, Rhod2-AM was predominantly localized in the mitochondria of MDA-MB-231 cells, evidenced by the merged staining pattern in Figure 6D.



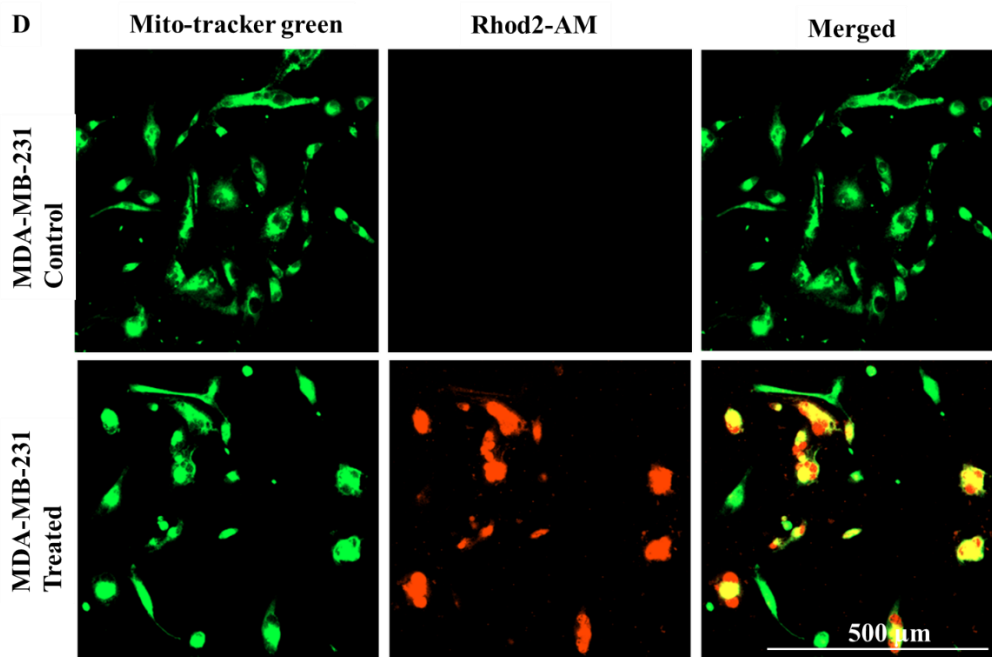


Figure 6. Calcium dynamics in MDA-MB-231 cells induced by *E. bicolor* diterpene extract. (A) Cell viability after blocking TRPV1 with CAPZ. Cells were pretreated with 10 μ M CAPZ and then treated with *E. bicolor* diterpene extract (62.5 μ g/ml). (B) Cell viability after chelating calcium with BAPTA-AM. Cells were pretreated with 1 μ M of BAPTA-AM and then treated with *E. bicolor* diterpene extract (62.5 μ g/ml). Cell viability was measured by MTS assay. Values represent the mean $\pm$ SEM of three independent experiments. One-way ANOVA, ** p <0.01, *** p <0.0001 vs. DMSO control set. (C) Visualization of calcium dynamics in MDA-MB-231 cells. Cells were loaded with 5 μ M of Ca^{2+} sensitive fluorescent probe Fura2-AM and then exposed to *E. bicolor* diterpene extract (62.5 μ g/ml) in HBSS buffer (with Ca^{2+} and Mg^{2+}). The fluorescent images were captured every 2 seconds and recorded for 10 min. Representative Ca^{2+} fluorescence images at indicated time points were shown, and regions of interest were drawn with dotted circles. (D) Visualization of mitochondrial calcium with MitoTracker Green. MDA-MB-231 cells were simultaneously loaded with 10 μ M of MitoTracker Green and 5 μ M of Rhod2-AM and then exposed to *E. bicolor* extract (62.5 μ g/ml). The corresponding fluorescence signal was monitored by a LionHeart FX microscope at 4X magnification.

2.6. *E. bicolor* Diterpene Extract Induced Mitochondrial Intrinsic and Extrinsic Apoptotic Signaling Pathways in T47D and MDA-MB-231 Cells

Mitochondria play a vital role in the process of apoptosis. Activated/cleaved caspase 3 (green fluorescence) was detected in both *E. bicolor* extract (62.5 μ g/ml) treated T47D and MDA-MB-231 cells, and activation of caspase 3 was significantly higher in MDA-MB-231 cells compared with T47D (Figure 7A,B). Capsaicin (positive control) and *E. bicolor* extract treatments led to the expression of caspase 8 and FAS, indicating induction of mitochondrial extrinsic apoptosis (Figure 7C). Capsaicin and *E. bicolor* treatments led to the expression of caspase 9 and reduction of anti-apoptotic BCL-2 protein compared to the DMSO control (Figure 7D). However, the expression of BCL-2 was significantly lower in *E. bicolor* extract treated cells than those of control and capsaicin (Figure 7E). The proapoptotic BAX protein showed a higher molecular weight band (47 KDa) in capsaicin and *E. bicolor* extract treated cells, compared to its known average molecular weight (21 KDa) (Figure 7F). The fold activation of BAX is significantly higher than that of DMSO control (Figure 7G), indicating that *E. bicolor* extract treatment also led to mitochondrial intrinsic apoptotic pathway.

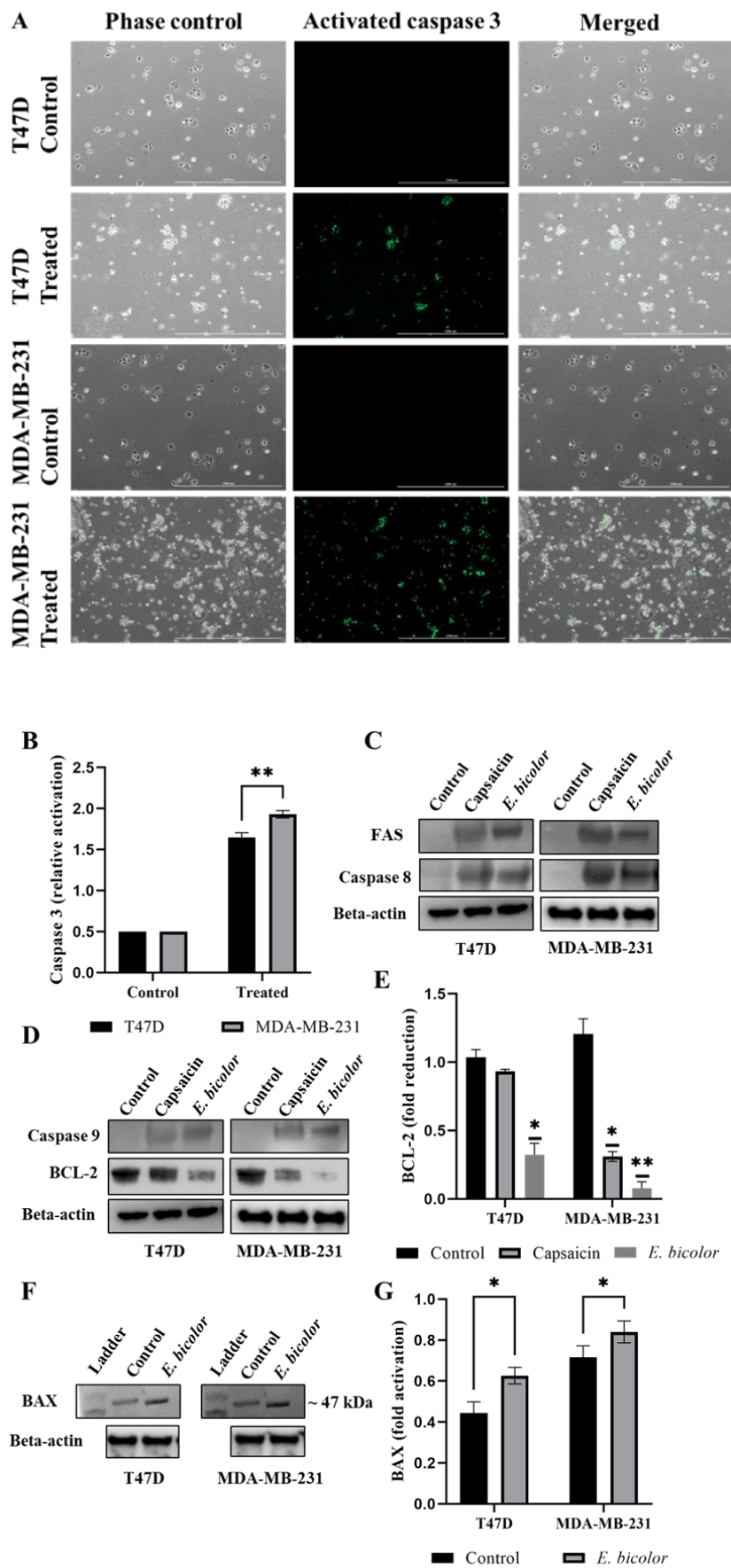


Figure 7. *E. bicolor* diterpene extract induces mitochondrial intrinsic and extrinsic apoptosis in T47D and MDA-MB-231 cells. (A) Visualization of activated caspase 3 after *E. bicolor* diterpene extract treatment. T47D and

MDA-MB-231 cells were treated with *E. bicolor* (62.5 µg/ml) for 24 h, one drop of caspase 3/7 reagent was added, and the corresponding fluorescence signal was monitored by a LionHeart FX microscope; scale bar, 200 µm. (B) Relative activation of caspase 3 measured with LionHeart BioTek Gen5.0 software. Two-way ANOVA, ** $p < 0.001$. (C) Western blot analyses of mitochondrial extrinsic apoptotic proteins FAS and caspase 8. T47D and MDA-MB-231 cells were treated with *E. bicolor* (62.5 µg/mL) or capsaicin (500 µM) for 24 h; cell lysates were isolated and immunoblot analyses of proteins FAS and caspase 8 were performed. (D) Western blot analyses of mitochondrial intrinsic apoptotic protein caspase 9 and antiapoptotic BCL-2. T47D and MDA-MB-231 cells were treated with *E. bicolor* (62.5 µg/mL) or capsaicin (500 µM) for 24 h, and cell lysates were isolated, and immunoblot analyses of proteins caspase 9 and BCL-2 were performed. (E) Fold reduction of BCL-2. One-way ANOVA, * $p < 0.05$, ** $p < 0.01$. (F) Western blot analyses of proapoptotic BAX protein. (G) Fold activation of BAX. Unpaired t-test, * $p < 0.05$. Representative Western blots, beta-actin served as loading control. Control, non-treated cells (DMSO).

2.7. *E. bicolor* Diterpene Extract Downregulates the PI3K/AKT Signaling Pathway in T47D and MDA-MB-231 Cells

The protein kinase B (AKT) regulates cellular signaling and plays essential roles in cell survival, growth, and proliferation [36]. To check for the inhibition of the PI3K/AKT signaling pathway as one of the antiproliferative mechanisms of action of *E. bicolor* diterpene extract, expression of PI3K, phosphorylated AKT, and non-phosphorylated proteins was evaluated. Reduced or no expression of PI3K, AKT, and pAKT proteins were observed in both *E. bicolor* extract treated T47D and MDA-MB-231 cell lines (Figure 8), suggesting inhibition of the PI3K/AKT signaling pathway by *E. bicolor* diterpene extract.

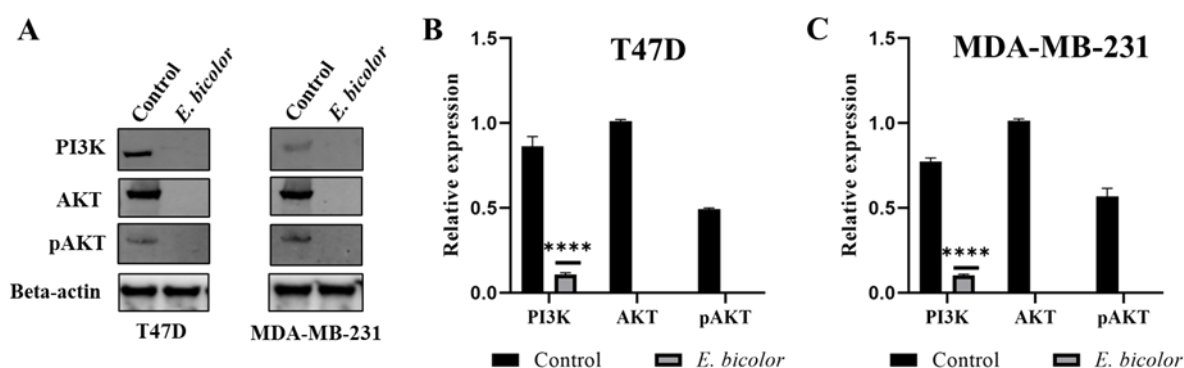


Figure 8. *E. bicolor* diterpene extract inhibits the PI3K/AKT signaling pathway in T47D and MDA-MB-231 cells. (A) Western blot analyses. Cells were treated with *E. bicolor* extract (62.5 µg/ml) for 24 h, cell lysates were isolated, and immunoblot analyses of proteins PI3K, AKT, and pAKT were performed. Representative Western blot, control – untreated cells (DMSO); beta-actin served as loading control. (B, C) Relative expressions of PI3K, AKT, and pAKT in T47D and MDA-MB-231 cells, respectively. Unpaired t-test, *** $p < 0.0001$ vs. DMSO control set. Control, untreated cells (DMSO).

2.8. *E. bicolor* Diterpene Extract Induces ER-Dependent Apoptosis in T47D and MDA-MB-231 Cells

Disruption of cytosolic calcium homeostasis and generation of excessive ROS induces ER stress [37]. To assess the effect of *E. bicolor* diterpene extract on ER stress-dependent apoptotic protein kinase RNA-like ER kinase (PERK) - activating transcription factor 4 (ATF4) - C/EBP homologous protein (CHOP) (PERK/ATF4/CHOP) and activating transcription factor 6 (ATF6) - spliced X-box binding protein 1 - C/EBP homologous protein (CHOP) (ATF6/XBP1s/CHOP) pathways was further investigated. *E. bicolor* extract treatment led to the expression of PERK and CHOP (endoplasmic apoptotic protein) (Figure 9A). However, expression of phosphorylated PERK and ATF4 proteins was absent in capsaicin and *E. bicolor* extract treated T47D and MDA-MB-231 cells (Figure 9A), indicating the absence of PERK/ATF4/CHOP pathway. We further checked the expression of XBP1s protein, which activates CHOP and can be induced by ER stress leading to the ER-stress mediated

apoptotic ATF6/XBP1s/CHOP pathway. Expression of XBP1s was found in both capsaicin and *E. bicolor* extract treated cells, suggesting the presence of the ATF6/XBP1s/CHOP pathway.

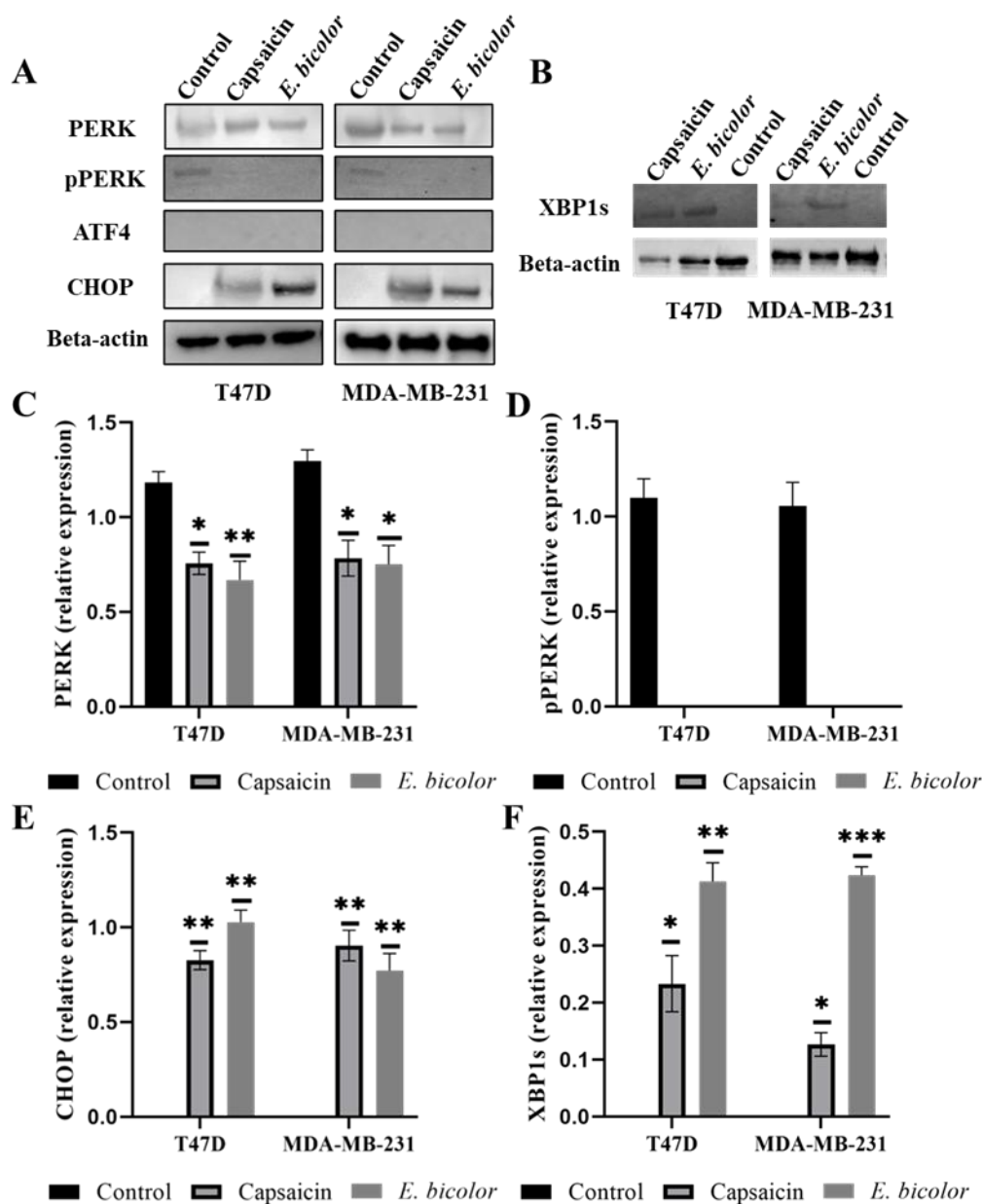


Figure 9. *E. bicolor* induces ER stress-dependent apoptosis in T47D and MDA-MB-231 cells. (A, B) Western blot analysis of ER-stress mediated apoptotic proteins. T47D and MDA-MB-231 cells were treated with *E. bicolor* (62.5 μ g/mL) or capsaicin (500 μ M) for 24 h. Western blot analyses of ER stress-dependent apoptotic proteins PERK, pPERK, CHOP, and ATF4, XBP1s were performed. Representative Western blot, beta-actin served as the loading control. (C, D, E) Relative expression of PERK, pPERK, and CHOP in T47D and MDA-MB-231 cells. (F) Relative expression of XBP1s. One-way ANOVA, ** $p < 0.01$, *** $p < 0.0001$ vs. DMSO control set. Control, cells in DMSO medium. ImageJ software was used to determine fold protein expression.

3. Discussion

Worldwide, breast cancer is the most commonly diagnosed cancer in women [38]. To reduce the global death rate from breast cancer, advances in diagnosis and treatment must be widened. Numerous plant species contain natural substances with anti-cancer properties [39]. Conducting focused research on bioactive plant chemicals, scientists could develop novel therapeutic options for

breast cancer treatment. The present study tested the antiproliferative activities of *E. bicolor* diterpene extract on ER-positive T47D and triple-negative MDA-MB-231 breast cancer cell lines and found that the extract significantly reduced the proliferation of both breast cancer cell lines (Figures 1–3). It was previously reported that *E. bicolor* latex extract possesses antiproliferative activity on T47D and MDA-MB-231 breast cancer cell lines [32], but no mechanisms of action were provided. This study proposes the mechanisms of action of *E. bicolor* diterpene extract in the two cell lines under study presented in Figures 10 and 11.

Plants of *Euphorbia* genus are well-known for their antiproliferative effects. *E. hirta* methanolic extract suppressed MCF-7 cell growth at 24 h with a GI50 value of 25.26 $\mu\text{g/mL}$ [40]. A study on the effect of *E. macroclada* acetone extract made from leaves and flowers reported significant cytotoxicity in MCF-7 breast cancer cells [41]. Another study showed that dichloromethane and ethyl acetate extracts of *E. macroclada* exhibited cytotoxic effects on MDA-MB-468 cells. In contrast, the methanol and latex extracts in DMSO were not cytotoxic at the concentrations used [33]. Butanol, hexane, and methanol extracts of *E. tirucalli* stem inhibited the proliferation of MCF-7 and MDA-MB-231 in a concentration-dependent manner [42]. Our study found that *E. bicolor* ethanol extract possesses antiproliferative effects only at higher concentrations in ER-positive T47D cells. However, no antiproliferative effect was observed on the triple-negative MDA-MB-231 cells (Figure 1). Different solvent extracts of *Euphorbia* species, as well as extract concentration used, show a wide range of antiproliferative effects in breast cancer cell lines.

Compared to *E. bicolor* ethanol extract, the diterpene extract was toxic to HMEC at high concentrations. Previous research showed that the diterpene RTX was not toxic to normal cells [32,43,44]. However, *E. bicolor* diterpene extract contains other diterpenes besides RTX. Biochemical identification results from our lab, previously published, showed that *E. bicolor* latex extract contains the diterpenes RTX, and abietic acid [29]. The HMEC cytotoxicity may be a combined effect of RTX, abietic acid, and other diterpenes in the extract. Therefore, future research should focus on chemical identification and isolation of other diterpenes in *E. bicolor* extracts, to individually be used to study antiproliferative effects, thus selecting the nontoxic ones for possible drug development.

In our attempt to understand the antiproliferative mechanisms of action of *E. bicolor* diterpene extract in T47D and MDA-MB-231 cells, we considered the activation of TRPV1, calcium influx, and apoptotic markers. As calcium ions are secondary messengers in cells, maintaining the balance of Ca^{2+} is crucial for regulating many cellular functions, including processes relevant to tumor growth and development, such as apoptosis and metastasis [43,44]. TRPV superfamily of plasma membrane ion channels is one of the most active calcium-permeable superfamily in regulating Ca^{2+} influx [14,25]. Experimental evidence shows that capsaicin can activate TRPV1 in cancer cells and have potent anticancer activity against certain cancer types through both Ca^{2+} -dependent and -independent mechanisms [44,45]. In our study, capsaicin showed antiproliferative activity in T47D and MDA-MB-231 cells at much higher concentrations than *E. bicolor* diterpene extract, which may be because the extract contains various diterpenes, and they may synergistically work to induce the observed antiproliferative effects.

Blocking TRPV1 and chelating calcium increased the T47D and MDA-MB-231 cell viability but did not completely counteract the effect of *E. bicolor* diterpene extract. On the other hand, inhibiting ROS formation with NAC completely blocked the effect of *E. bicolor* diterpene extract resulting in increased cell viability of only T47D cells, suggesting that increased ROS level is the underlying mechanism of cell death in T47D cells. Other antiproliferative studies employing *Euphorbia* species, such as *E. lathyris*, *E. antiquorum*, and *E. fischeriana*, have also found that increased ROS levels induced apoptosis [46–48]. Our results also revealed an immediate (2 seconds after treatment) release of calcium from the ER and mitochondrial calcium overload (27 seconds after treatment) in T47D cells, indicating that increased ROS levels trigger the accumulation of calcium in mitochondria. Other studies also revealed crosstalk between ROS levels and calcium signaling in different diseases, including cardiac physiology, pathologies, and neurodegeneration [49,50]. Blocking TRPV1 using capsazepine and chelating calcium with BAPTA-AM led to increased cell viability of MDA-MB-231

cells, suggesting that the activation of TRPV1 by *E. bicolor* diterpene extract is the primary mechanism responsible for cell death in that cell line. Consequently, activation of TRPV1 causes an excessive influx of calcium in the cells, ultimately leading to apoptosis in MDA-MB-231 cells. Therefore, a potential approach for developing new drug therapies for triple-negative breast cancer could involve channel activators to induce calcium influx and cell death [16]. In T47D cells, blocking TRPV1 with CAPZ inhibited the effect of *E. bicolor* diterpene extracts but only at small concentrations of extract (2-16 µg/mL), suggesting that TRPV1 may still be involved in reducing cell viability.

Experimental evidence indicates that when calcium homeostasis is disrupted, it leads to the breakdown of the mitochondrial membrane potential. This disruption also increases ROS levels and the release of proapoptotic proteins, resulting in apoptosis [13,51–53]. In our study, mitochondrial Ca²⁺ overload was observed after *E. bicolor* diterpene extract treatments in both T47D and MDA-MB-231 cells (Figure 6). To investigate the molecular mechanisms underlying *E. bicolor* induced cell death, we examined the expression of apoptotic protein markers in both the mitochondrial intrinsic and extrinsic apoptotic pathways. It is known that mitochondrial depolarization induces cytochrome c release into the cytoplasm, activating caspase-3 via caspase-9 [54]. Activated caspase-3 is one of the hallmarks of apoptosis induced by several *Euphorbia* plant extracts such as *E. tirucalli* and *E. esula* [9,11,55–57]. *E. bicolor* diterpene extract activated downstream caspases-3/9 (Figure 7), indicating the activation of the apoptotic pathways, as presented in the proposed models of apoptotic mechanisms in Figures 10 and 11. Anti-apoptotic BCL-2 protein protects cancer cells by preventing apoptosis [58]. We observed reduced expression of BCL-2 protein in *E. bicolor* diterpene extract-treated T47D and MDA-MB-231 cells, suggesting that *E. bicolor* diterpenes could be excellent candidates for targeting BCL-2 proteins in different cancers. Our results indicate that the BAX protein is expressed at a high molecular weight on Western blot. Studies indicated that BAX forms oligomers, necessary for its proapoptotic activity. The high molecular weight BAX oligomers bind to the mitochondrial membrane, leading to the mitochondrial intrinsic apoptotic pathway [16,59].

E. bicolor diterpene extract activated the extrinsic apoptotic pathway, as observed in the expression of FAS and caspase 8 proteins (Figures 7, 11, and 12). Previous studies support our findings and moreover, they indicate that the TRPV1 N-terminus can bind to proapoptotic FAS-associated proteins, activating extrinsic apoptotic pathways [16,60,61]. TRPV1 is also found in the ER membrane [62]. Its activation can lead to calcium disruption and cause ER stress [53], which can be an active source of calcium release in the cytoplasm. ER stress and compromised mitochondria can subsequently release apoptotic signals [16,54]. Our results show that *E. bicolor* diterpene extract induced ER stress and ultimately triggered apoptosis in MDA-MB-231 breast cancer cells. In T47D cells, increased ROS levels may trigger ER stress and activate mitochondrial apoptosis as a result of *E. bicolor* diterpene extract treatment. Expression of XBP1s, an activated (by splicing) transcription factor that facilitates ER stress [63], was observed in both *E. bicolor* extract treated T47D and MDA-MB-231 cells as an indication of ER-mediated stress, which stimulated the expression of CHOP, a transcription factor that increases the expression of other apoptotic factors [64] (Figures 9, 11 and 12).

Our results also suggest that *E. bicolor* diterpene extract targets the PI3K/AKT signal transduction pathway, precisely downregulating it (Figures 8, 11, and 12). The serine/threonine kinase AKT, also known as protein kinase B (PKB), plays a crucial role in regulating many cellular processes, including cell survival, growth, proliferation, cell cycle, and metabolism. The balance between loss or gain of AKT activation is fundamental to the pathophysiological properties of cancer [65,66]. We observed significantly low total and phosphorylated AKT and PI3K protein expressions in both *E. bicolor* diterpene extract-treated T47D and MDA-MB-231 cells (Figure 8). Studies have shown that activation of the PI3K/AKT pathway contributes to tumorigenesis, and inhibition of PI3K and AKT can decrease cellular proliferation and increase cell death [53]. Therefore, our results suggest that *E. bicolor* diterpenes could become potential candidates for targeting the PI3K/AKT signaling pathways in breast cancers.

The current study revealed two different antiproliferative mechanisms for the two breast cancer types, ER-positive and triple-negative, which most likely are because of their distinct molecular

profiles. T47D cancer cells express estrogen and progesterone hormone receptors, the primary targets for current hormone therapies. MDA-MB-231 cancer cells lack hormone receptors, and therefore, they are not responsive to hormone therapies [67,68]. It is known that natural products have multiple molecular targets [69]. *E. bicolor* diterpene extract, containing a variety of diterpenes, may affect different targets depending on the cancer cells molecular characteristics resulting in distinct antiproliferative molecular mechanisms.

In conclusion, our study presents for the first time the ROS-mediated antiproliferative mechanisms of action of *E. bicolor* diterpene extract in T47D cells (Figure 11) and TRPV1-dependent mechanisms of action in MDA-MB-231 cells (Figure 12). *E. bicolor* diterpene extract generates high ROS levels in T47D cells and triggers several apoptotic pathways. In MDA-MB-231 cells, *E. bicolor* diterpene extract activates TRPV1 and induces mitochondrial and ER stress-mediated apoptotic pathways. In addition, *E. bicolor* diterpene extract downregulates the PI3K/AKT signaling pathway in both T47D and MDA-MB-231 cells. Our findings suggest that *E. bicolor* diterpene extract could be used to design specific therapeutics for cancer cell types. For ER-positive cell types, therapeutic agents could target the ROS-inducing apoptotic mechanism. For MDA-MB-231 cell types, the therapeutic mechanism could target the TRPV1-dependent apoptotic pathways.

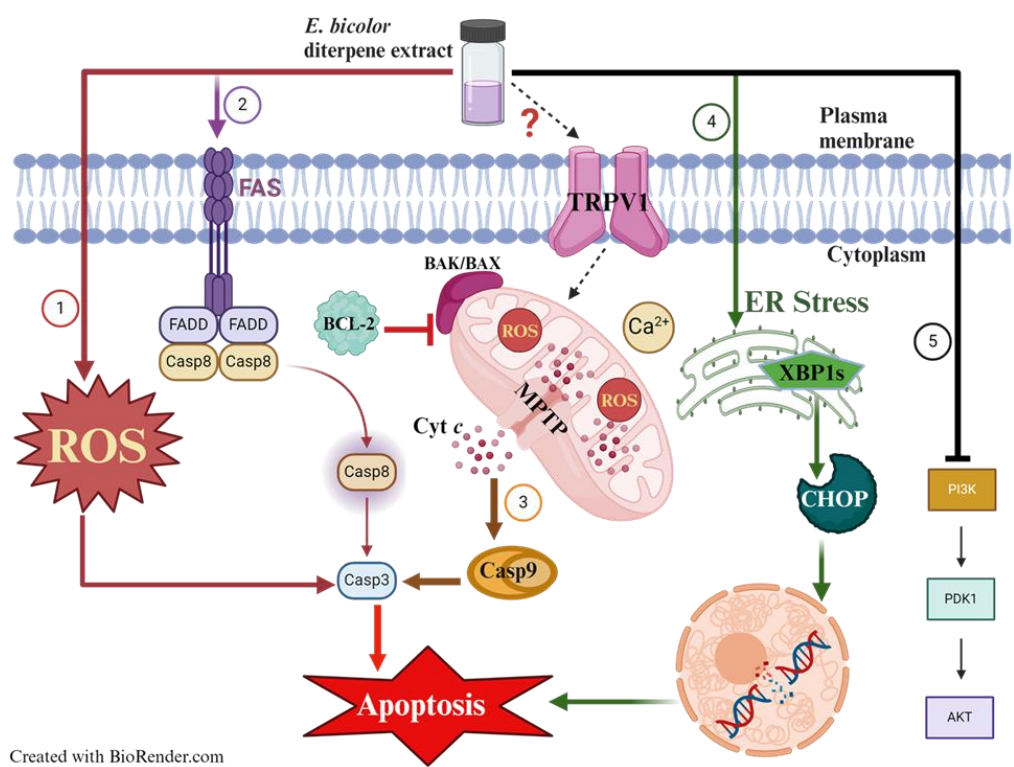


Figure 10. Proposed model of the mechanisms of action of *E. bicolor* diterpene extract on ER-positive T47D breast cancer cells. (1) Plant extract increases ROS level. The elevated ROS level could trigger several apoptotic pathways. Increasing levels of ROS led to caspase 3 activation, increasing proapoptotic proteins BAK/BAX, decreasing anti-apoptotic BCL-2, and leading to mitochondrial intrinsic (3) and extrinsic (2) apoptotic pathway by activating FAS, caspase 8, and caspase 3. It may be that plant extract activates the FAS-caspase 8 pathway directly (2). (4) ER-stress mediated apoptosis pathway. Increasing ROS level and/or *E. bicolor* plant extract led to ER stress. ER-stress activates XBP1s, which activates CHOP leading to XBP1s-CHOP-mediated apoptosis. (5) *E. bicolor* diterpene extract downregulates the PI3K-AKT signaling pathway. The question mark (?) signifies that TRPV1 may or may not be involved in triggering apoptotic pathways depending on *E. bicolor* diterpene extract concentrations. Created with BioRender.com.

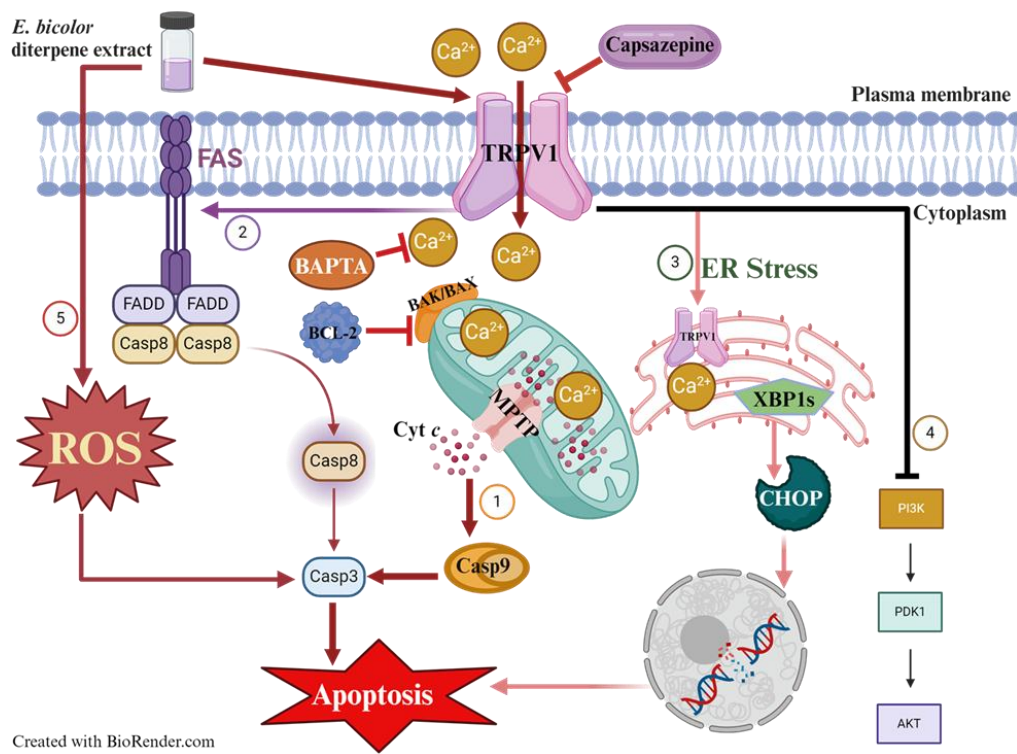


Figure 11. Proposed model of the mechanisms of action of *E. bicolor* diterpene extract on triple-negative MDA-MB-231 breast cancer cells. Plant extract activates TRPV1, inducing calcium influx into cells. The elevated intracellular calcium triggers several apoptotic pathways, such as the mitochondria mediated intrinsic, extrinsic, and ER-stress mediated pathways. (1) Accumulation of Ca^{2+} in mitochondria causes the transient depolarization of mitochondrial membrane potential. As a result, the mitochondrial permeability transition pores (MPTPs) open and release cytochrome c (cyt c), leading to caspase activation, increasing proapoptotic proteins BAK/BAX, decreasing anti-apoptotic BCL-2, thus leading to apoptosis. (2) Calcium influx through TRPV1 triggers the extrinsic pathway by activating FAS, caspase 8, and caspase 3, leading to apoptosis. (3) Plasma membrane TRPV1 activation and calcium influx lead to ER stress. ER TRPV1 becomes another source of calcium release in the cell. ER stress activates XBP1s, which activates CHOP, leading to XBP1s-CHOP-mediated apoptosis. (4) *E. bicolor* diterpene extract downregulates the PI3K-AKT signaling pathway. (5) Plant extract increases cellular ROS levels triggering several apoptotic pathways.

4. Materials and Methods

4.1. Plant Extracts

E. bicolor plants were collected from fields around Denton, Texas, USA, during September and October 2023. Aerial plant tissues (50 g) were extracted in 95% ethanol (1:4 w/v) at room temperature for two days. Supernatants were filtered through Whatman filter paper #54 (Thomas Scientific, Swedesboro, NJ, USA). To extract diterpenes from *E. bicolor*, the protocol from Tidgewell (2007) was used with slight modifications [70]. Plant aerial tissues (50 g) were extracted in 200 proof xylene (Fisher Scientific, NH, USA) (1:4 w/v) at room temperature for one day, and supernatants were filtered through Whatman filter paper. The filtrates were transferred to a pre-weighted vial and evaporated to dryness under nitrogen gas flow. Dried extract was dissolved in dimethyl sulfoxide (DMSO) (Fisher Scientific, NH, USA) in a 1:1 ratio and stored at -20 °C until use. The extract collected using ethanol solvent is named ethanol extract, and the extract collected using xylene solvent is named diterpene extract.

4.2. Cell Lines and Cell Culture Conditions

Estrogen receptor-positive T47D, triple-negative MDA-MB-231 breast cancer cell lines, and adult human mammary epithelial cells (HMEC) were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). T47D and MDA-MB-231 breast cancer cell lines were cultured and maintained in Dulbecco's Modified Eagle's Medium (DMEM; ThermoFisher Scientific, Township, NJ, USA), as previously reported [32]. Adult human mammary epithelial cells (HMEC) were grown in mammary epithelial basal medium (ATCC) supplemented with mammary epithelial cell growth kit (ATCC). All cell lines were incubated under a humid atmosphere of 5% (v/v) CO₂ at 37 °C.

4.3. Cell Culture Treatments

All cells were seeded into 96-well cell culture plates at 10,000 cells/well in a phenol-red-free medium and incubated for 24 h at 37 °C. After 24 h, the cells were treated with different concentrations of *E. bicolor* ethanol, diterpene extracts in DMSO (2, 4, 8, 16, 62.5, 125, 250, 500 µg/ml) or capsaicin at 0.5, 1, 5, 10, 50, 100, 250, and 500 µM concentrations. The final concentration of DMSO was <0.1%.

4.4. Antiproliferative Assays

Antiproliferative activity was evaluated by performing MTS [3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay (Abcam, USA) [32]. Briefly, cells were seeded into a 96-well plate (10,000 cells/well) and exposed to various concentrations of *E. bicolor* ethanol, diterpene extracts, or capsaicin. After 72 h of treatments, 10 µl of MTS reagent was added to each well, and plates were incubated at 37 °C for 2.5 h, and absorbance was read at 490 nm using a Biotek's Synergy HT plate reader (Agilent, Santa Clara, CA, USA) [32]. At least three separate experiments, each containing three replicates, were performed. HMEC cells showed reduction of cell viability starting at 125 µg/ml concentration using *E. bicolor* diterpene extract. Therefore, the rest of the experiments were set up to use 62.5 µg/ml concentration to determine the antiproliferative mechanisms.

4.5. IC₅₀ Estimation

The MTS assay results were used to calculate the 50% inhibitory concentration (IC₅₀) using the GraphPad Prism 9.4 software. A dose-response curve was fitted using nonlinear regression.

4.6. Visualization of Cytotoxic Effects

The morphological changes of the cells were observed using the live cell imaging system IncuCyte (Sartorius, Ann Arbor, MI, USA) for several days. Cells were seeded into a 96-well plate (10,000 cells/well) and exposed to 62.5 µg/ml of *E. bicolor* diterpene extract, before being placed into the IncuCyte live cell imaging system. The system was programmed to capture high-resolution, bright field photos every 4 hours.

4.7. Detection of Apoptosis

Cell apoptosis was observed by employing the Click-iT™ Plus TUNEL (the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling) assay kit (ThermoFisher Scientific, USA). Cells were grown on coverslips inside the 6-well plate and treated with *E. bicolor* diterpene extract at a concentration of 62.5 µg/mL. Twenty-four hours after treatments, T47D and MDA-MB-231 cells were fixed with 4% formaldehyde. TUNEL assays were performed according to the manufacturer's instructions, and nuclear fluorescence of cells was detected and analyzed using a LionHeart FX microscope (Agilent, Santa Clara, CA, USA).

4.8. Capsazepine Treatments to Block TRPV1

Cells were seeded into 96-well cell culture plates at 10,000 cells/well and incubated for 24 h at 37 °C. After 24 h, cells were pretreated with 10 µM of capsazepine (Abcam, USA), a TRPV1 antagonist, for 30 minutes. Afterward, cells were treated with *E. bicolor* diterpene extract (62.5 µg/mL). The plates were incubated for 72 h at 37 °C. Cell viability was measured using MTS assays. Three independent experiments were conducted.

4.9. Calcium Chelation

Cells were seeded into 96-well cell culture plates at 10,000 cells/well and incubated for 24 h at 37 °C. After 24 h, cells were pretreated with 1 µM of BAPTA-AM (Abcam, USA), a calcium chelator, for 30 minutes, and then treated with *E. bicolor* diterpene extract (62.5 µg/mL). The plates were incubated for 72 h at 37 °C. Then, cell viability was measured using MTS assays. Three independent experiments were conducted.

4.10. Visualization of TRPV1 Activation

The cytoplasmic calcium dynamics as the result of TRPV1 activation were observed using Fura2-AM staining. T47D and MDA-MB-231 cells were seeded into a 6-well plate and cultured overnight. After 24 h, cells were loaded with 5 µM of Fura2-AM for 30 min. Following *E. bicolor* diterpene treatment, the cytosolic Ca²⁺ signal was monitored continuously for 20 min using a LionHeart FX microscope.

4.11. ROS Detection

The intracellular ROS levels were determined using cell-permeant reagent 2',7'-dichlorofluorescein diacetate (DCFDA). Tert-butyl hydroperoxide (TBHP; 250 µM) was used as positive control. The fluorescence intensity values at each time point were calculated as the ratio of the value at a specific t-time point to the value at point zero time (t-time point/t₀); t₀ = first measurement). Briefly, cells were plated in FBS-supplemented medium without phenol red onto 96-well plates. After 24 hours, the cells were washed once with 1X buffer provided in the kit, then the cells were incubated with 10 µM of DCFDA for 30 min at 37 °C, protected from light. Following incubation, the wells were washed with PBS, and treated with *E. bicolor* diterpene extract. ROS production was determined immediately by measuring the formation of fluorescent dichlorofluorescein (DCF), using a Synergy microplate reader, at 485 nm excitation and 535 nm emission. Measurements were taken every 60 min for six hours. Three independent experiments were conducted.

4.12. N-acetyl-L-cysteine (NAC) Treatments to Block ROS Generation

Cells were seeded into 96-well cell culture plates at 10,000 cells/well and incubated for 24 h at 37 °C. After 24 h, cells were pretreated with NAC (5 mM) for 1 h followed by treatment with or without *E. bicolor* for another 72 h, and cell viability was measured using MTS assay. Three independent experiments were conducted.

4.13. Visualization of ER Calcium

ER-targeted low-affinity GCaMP6-210 variant, a fluorescent reporter for ER calcium signaling, was used to observe endoplasmic reticulum (ER) Ca²⁺ dynamics. T47D cells were seeded into a 24-well plate at 10.4 × 10⁴ cells in 500 µL phenol red free growth medium. After 24 h, cells were transfected with the GCaMP6-210 variant using Invitrogen™ Lipofectamine™ 3000 Transfection Reagent (ThermoFisher Scientific, USA). Twelve hours after incubation, immediately after *E. bicolor* diterpene extract treatment (62.5 µg/mL), calcium influx was observed with the LionHeart FX microscope.

4.14. Visualization of Mitochondrial Calcium

The mitochondrial calcium dynamics were observed using Rhod2-AM (Abcam, USA). T47D and MDA-MB-231 cells were grown in 6-well plates and simultaneously loaded with 5 μ M of Rhod2-AM, 10 μ M of MitoTracker Green (ThermoFisher Scientific, USA) and then exposed to *E. bicolor* diterpene extracts (62.5 μ g/mL). The corresponding fluorescence signals were monitored for 10 minutes with the LionHeart FX microscope in red and green fluorescence channels.

4.15. Caspase 3 Activation Assay

All cells were seeded into 6-well cell culture plates at 2×10^5 cells/well and incubated for 24 h at 37 °C. After 24 h, cells were treated with *E. bicolor* diterpene extract (62.5 μ g/mL) and incubated at 37 °C for 24 h. One drop of the CellEvent™ Caspase-3/7 Green ReadyProbes™ reagent (ThermoFisher Scientific, USA) was added in each well. After a 45-minute incubation, cell fluorescence was detected and analyzed with a LionHeart FX microscope.

4.16. Western Blotting

As previously reported [16], Western blotting was carried out with a few changes. Proapoptotic and anti-apoptotic proteins were examined using total cellular protein. The cell lysates were prepared using RIPA lysis and extraction buffer (ThermoFisher Scientific, USA) supplemented with Halt™ protease and phosphatase inhibitor cocktail (ThermoFisher Scientific, USA). Cell lysates were centrifuged at 12,000xg at 4 °C for 5 min, the supernatant was collected, and the total protein concentrations were estimated using Pierce™ 660nm Protein Assay Kit (ThermoFisher Scientific). Protein samples were subjected to 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and were transferred onto polyvinylidene difluoride (PVDF) membranes (BioRad, USA). The membranes were immersed in blocking buffer (0.1% (v/v) Tween 20 in Tris-buffered saline, pH 7.4, with 5% (w/v) skim milk) at room temperature for 2 hours. They were then probed using antibodies in the same blocking buffer overnight at 4 °C.

Antibodies used were as follows: anti-beta actin, anti-caspase 9, anti-caspase 8, anti-CHOP, anti-ATF4, anti-FAS, anti-PERK (mouse monoclonal antibody conjugated with HRP; 1:1000, v/v) (Santa Cruz Biotechnology, USA), anti-AKT, anti-pAKT (mouse monoclonal antibody conjugated with Alexa 488; 1:1000, v/v) (Santa Cruz Biotechnology, USA). Membranes were washed three times with Tris-buffered saline containing 0.1% (v/v) Tween 20 (TBST) for 5 minutes each, incubated with enhanced chemiluminescence substrate solution (BioRad, USA) for 5 min, according to the manufacturer's instructions, and visualized with a ChemiDoc system. For anti-Bcl-2, anti-BAX (rabbit monoclonal antibody; 1:1000, v/v) (Abcam, USA), anti-pPERK, anti-PI3K, anti-XBP1s (rabbit monoclonal antibody; 1:1000, v/v) (Cell Signaling Technology, USA), following the overnight incubation, the membranes were incubated with the secondary antibody (Goat Anti-Rabbit IgG H&L, Alexa Fluor® 488; 1:500 v/v) for five minutes each after being rinsed three times in Tris-buffered saline containing 0.1% (v/v) Tween 20 (TBST) for 1 h. Following incubation, the membranes underwent three rounds of washing with TBST buffer and were immediately visualized using a ChemiDoc system.

4.17. Statistical Analyses

Means and standard errors of the mean of at least three experiments were calculated. One-way ANOVA was performed, followed by Tukey's posthoc test to determine significant differences among the means for the antiproliferative (MTS) assays and fold expression analysis of Western blot (to compare DMSO control and *E. bicolor* diterpene extract treatment) using GraphPad Prism 9.4. A p-value < 0.05 was considered statistically significant. Unpaired Welch's t-test, to compare treated to DMSO control set, was used for TUNEL assays. A two-way ANOVA was performed, followed by the Dunnett test to determine significant differences among the means of Western blot fold expression (to compare DMSO control, *E. bicolor* diterpene extract, and capsaicin treatment). ImageJ software

(<https://imagej.net/ij/download.html>) was used to determine fold protein expression on Western blots.

5. Conclusions

Our study presents for the first time the ROS-mediated antiproliferative activity of *E. bicolor* in T47D cells and TRPV1-dependent antiproliferative activity of *E. bicolor* in MDA-MB-231 cells and their mechanisms of action. *E. bicolor* diterpene extract generates high ROS levels in T47D cells and triggers several apoptotic pathways. In contrast, *E. bicolor* diterpene extract activates TRPV1 and induces mitochondrial and endoplasmic reticulum stress-mediated apoptotic pathways in MDA-MB-231 cells. In addition, *E. bicolor* diterpene extract downregulates the PI3K/AKT signaling pathway in both T47D and MDA-MB-231 cells. Our findings suggest that *E. bicolor* could be used to design cancer cell type-specific therapeutics.

Author Contributions: CM and MMR conceived and designed the methodology and experiments. MMR and NN (Figure 1) performed the experiments. CM and MMR analyzed the data. MMR writing and original draft preparation. CM review and editing, project administration, funding acquisition.

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Conflicts of Interest: The authors have declared no conflicts of interest.

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