

Synergistic NF- κ B suppression by magnolol and honokiol enhances epirubicin efficacy in triple-negative breast cancer

Reza zareei

Arak University of Medical Sciences

Seyed Mohammad Jafar Haeri

Arak University of Medical Sciences

Malihe Bagheri

Arak University of Medical Sciences

Hadi Karami

hadimolmed@gmail.com

Arak University of Medical Sciences

Research Article

Keywords: Epirubicin, Honokiol, Magnolol, NF-kappa B, Triple-Negative Breast Neoplasms

Posted Date: October 6th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-11096781/v1>

License:   This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Additional Declarations: No competing interests reported.

Abstract

Objective

Triple-negative breast cancer (TNBC) is aggressive and chemoresistant, largely due to constitutive NF- κ B activation. Magnolol and honokiol, bioactive lignans from *Magnolia officinalis*, individually inhibit NF- κ B, but their combined ability to enhance epirubicin efficacy in TNBC remains unexplored. This study evaluated synergistic effects of magnolol, honokiol, and epirubicin in MDA-MB-231 cells.

Materials and Methods

MDA-MB-231 cells were treated with magnolol, honokiol, and epirubicin alone or combined at IC₅₀ doses. Viability was assessed by MTT and combination index (CI) analysis. Proliferation was measured via trypan blue exclusion and colony formation. Migration was evaluated by wound healing. Apoptosis was quantified using Hoechst staining, ELISA-based nucleosome detection, and caspase-3 activity. Gene expression (Bax, p53, Survivin, MMP-9) was analyzed by qRT-PCR, and NF- κ B proteins (p65, p-p65, I κ B α , p-I κ B α) by Western blot.

Results

All combinations showed synergy (CI < 1). The triple combination yielded the lowest IC₅₀ (2.71 μ M) and maximally suppressed migration (27.8%) and colony formation (27.5%). It upregulated Bax (2.67 fold) and p53 (2.3 fold), downregulated Survivin and MMP-9, and inhibited NF- κ B by reducing phospho-p65 (34.5%) and phospho-I κ B α (33.7%) while increasing total I κ B α (2.36-fold). Caspase-3 activity rose 3.38-fold.

Conclusion

Magnolol and honokiol synergistically enhance epirubicin efficacy in TNBC via dual-level NF- κ B blockade, offering a promising dose-reduction strategy to overcome chemoresistance.

Introduction

Breast cancer remains the most frequently diagnosed malignancy and the leading cause of cancer-related mortality among women worldwide, accounting for over 2.3 million new cases and 685,000 deaths in 2020 [1]. The global burden is projected to reach 3 million new cases and 1 million deaths annually by 2040 [2]. Despite advances in early detection and therapy, triple-negative breast cancer (TNBC) presents substantial clinical challenges due to its aggressive nature, high metastatic potential, and chemoresistance [3]. TNBC accounts for 15–20% of all breast cancers, lacks estrogen receptor,

progesterone receptor, and HER2 expression, and is associated with poor prognosis [3]. The MDA-MB-231 cell line serves as a valuable TNBC model for investigating novel therapeutic approaches [3].

Epirubicin, an anthracycline chemotherapeutic agent, remains a cornerstone in breast cancer treatment due to its potent antitumor activity through DNA intercalation, topoisomerase II inhibition, and free radical generation that induces apoptosis [4]. However, clinical utility is hampered by frequent development of drug resistance [5]. Resistance mechanisms include enhanced drug efflux via ATP-binding cassette transporters (e.g., BCRP/ABCG2), intracellular detoxification through glutathione S-transferase, alterations in topoisomerase II, DNA repair changes, and apoptotic pathway dysregulation [5]. These complex mechanisms underscore the urgent need for novel therapeutic strategies to circumvent chemoresistance [6].

The nuclear factor-kappa B (NF- κ B) signaling pathway is a critical regulator of cancer progression and therapy resistance in breast cancer [7]. NF- κ B promotes tumor cell survival, proliferation, invasion, metastasis, and angiogenesis through transcriptional regulation of target genes including Bcl-2, survivin, cyclins, COX-2, MMP-9, and VEGF [7]. Constitutive NF- κ B activation is frequently observed in breast cancer and correlates with aggressive behavior, poor prognosis, and chemoresistance [8]. Both canonical and noncanonical NF- κ B pathways have been implicated, with the noncanonical RelB-mediated pathway promoting proliferation, migration, and metastasis via Cyclin D1, Bcl-2, and MMP-9 upregulation [8]. Thus, NF- κ B represents an attractive molecular target for therapeutic intervention [7, 8].

Natural products from medicinal plants have gained attention as promising anticancer agents capable of modulating key signaling pathways [9]. *Magnolia officinalis*, a traditional Chinese and Japanese medicinal plant, yields two major bioactive biphenolic compounds, magnolol and honokiol, which demonstrate remarkable anticancer properties through multiple molecular targets [9, 10].

Magnolol, a hydroxylated biphenyl, induces G2/M phase arrest and caspase-dependent intrinsic apoptosis in breast cancer cells through ROS generation, upregulation of pro-apoptotic proteins (Bax, p21, p53), and downregulation of anti-apoptotic proteins (Bcl-2, cyclin-B1, CDK-1) [10]. It inhibits cancer cell migration and invasion by suppressing matrix metalloproteinases essential for extracellular matrix degradation [10]. Importantly, magnolol inhibits NF- κ B activation and reduces nuclear NF- κ B levels, suppressing NF- κ B-regulated gene products involved in proliferation, survival, angiogenesis, and metastasis [11]. Magnolol also enhances TRAIL therapeutic effects through DR5 upregulation and c-FLIP/Mcl-1 downregulation [10].

Honokiol, another bioactive neolignan, induces apoptosis through Bcl-2 family modulation, increasing pro-apoptotic proteins (Bax, Bak, Bad) while decreasing anti-apoptotic proteins (Bcl-xL, Mcl-1), leading to caspase-3, -8, -9 activation and PARP cleavage [9]. Honokiol inhibits breast cancer cell migration and invasion by blocking epithelial-to-mesenchymal transition through Snail and Slug downregulation [12]. It inhibits EGFR signaling and potently suppresses NF- κ B activation [9]. Honokiol augments chemotherapeutic agent effects through synergistic apoptosis enhancement, suggesting potential to overcome multidrug resistance [13]. Both magnolol and honokiol inhibit BCRP function and expression

via EGFR/PI3K signaling, positioning them as promising candidates for reversing chemotherapy resistance [11]. Hyaluronic acid-modified liposomal honokiol nanocarriers have been developed to enhance anti-metastasis and antitumor efficacy against breast cancer [14].

Despite well-established individual anticancer activities of magnolol and honokiol, the potential synergistic effects of their combination on breast cancer cell growth, migration, colony formation, and chemosensitivity to epirubicin, particularly through NF- κ B modulation, remain poorly understood. Given NF- κ B's critical role in breast cancer progression and chemoresistance [15], the combination may offer a novel strategy to suppress tumor growth and invasion while sensitizing cells to epirubicin. Therefore, this study aims to comprehensively investigate individual and combined effects of magnolol and honokiol on cell viability, migration, colony formation, proliferation, apoptosis, caspase-3 activation, NF- κ B signaling, and expression of key regulatory genes (survivin, p53, MMP-9 and Bax) in MDA-MB-231 breast cancer cells. We also seek to evaluate whether combination treatment enhances epirubicin sensitivity, potentially providing rationale for novel combination therapeutic strategies for TNBC management.

Material and methods

Cell culture

The MDA-MB-231 human breast adenocarcinoma cell line was procured from the Pasteur Institute in Tehran, Iran. Cells were routinely cultured in RPMI-1640 medium (Gibco, USA) enriched with 15% heat-inactivated fetal bovine serum (FBS, Gibco), a penicillin-streptomycin solution (100 U/mL and 100 μ g/mL, respectively) (Gibco), and 2 mM L-glutamine. The cultures were sustained in a humidified incubator set to 95% relative humidity, with an internal atmosphere composed of 5% CO₂ and 95% air.

MTT assay

The MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was used to evaluate cell survival across nine groups: blank, solvent, epirubicin (0–8 μ M), honokiol (0–40 μ M), magnolol (0–40 μ M), epirubicin+honokiol (0–8 μ M), magnolol+epirubicin (0–8 μ M), magnolol+honokiol (0–40 μ M), and magnolol+epirubicin+honokiol (0–8 μ M). MDA-MB-231 cells were plated at 5×10^3 /well in 96-well plates, adhered for 24 h, and exposed to treatments for 24 h. Then, 10 μ L of MTT (5 mg/mL; Sigma-Aldrich, St. Louis, MO, USA) was added per well for 4 h. After removing the medium, formazan precipitates were dissolved in 150 μ L DMSO per well, and absorbance was read at 490 nm on a plate reader (Awareness Technology, Palm City, FL, USA). Cell survival curves and IC₅₀ values were derived using GraphPad Prism 6.01 (GraphPad, San Diego, CA, USA). In all subsequent experiments, we used the compounds at their IC₅₀ concentrations.

Combination index (CI) analysis

The potential interaction between honokiol, magnolol, and epirubicin was assessed via CI analysis using the Chou-Talalay method [16, 17]. MTT data were transformed into fractional affected (Fa) values (0 = 100% viability; 1 = 0% viability) and processed with CompuSyn software (Combosyn, Paramus, NJ, USA). In this framework, $CI < 1$, $CI = 1$, and $CI > 1$ denote synergistic, additive, and antagonistic effects, respectively.

Cell Proliferation assay

To evaluate the anti-proliferative activity of magnolol, honokiol, and epirubicin, alone or in combination, we employed the trypan blue dye exclusion method. MDA-MB-231 breast cancer cells were plated in 6-well plates at 4×10^4 cells/well and exposed to the IC_{50} doses of each compound, singly or combined, for 24–120 h. After incubation, the cells were trypsinized and mixed with an equal volume of 0.4% trypan blue (Merck KGaA, Darmstadt, Germany) for 3 min. Cell counts were performed using a hemocytometer and an inverted microscope (Nikon Instrument Inc., Melville, NY, USA), and viability was calculated as the percentage of unstained (viable) cells out of the total number of cells counted.

Quantitative Real-Time PCR (qRT-PCR) Analysis

The effect of the experimental treatments on mRNA transcript levels was evaluated using quantitative real-time PCR. Total cellular RNA was isolated after a 24 h incubation period with an RNA purification kit (Parstous, Tehran, Iran), and first-strand cDNA synthesis was performed from the resulting RNA template utilizing oligo-dT primers and the Easy cDNA Synthesis Kit (Parstous). Real-time monitoring of amplification was executed on a Roche LightCycler 96 instrument with SYBR Green chemistry (Parstous). The master mix for each well was prepared with 10 μ L of SYBR Green Mix, 7 μ L of nuclease-free water, 1 μ L of each gene-specific primer, and 1 μ L of cDNA. The amplification protocol included an initial denaturation step at 95°C for 10 min, succeeded by 35 cycles of denaturation at 95°C for 15 sec, primer annealing at 57°C for 40 sec, and elongation at 72°C for 35 sec. Quantification of relative expression was performed using the standard $2^{-\Delta\Delta C_t}$ comparative threshold cycle method [18, 19], with β -actin serving as the internal reference gene to correct for sample-to-sample variability. The primer pairs employed are detailed in Table 1.

Table 1
Primer sequences used for qRT-PCR

Genes	Sequence (5'→3')	Primer length (base)	Tm (° C)
β-actin FW	GACATCCGCAAAGACCTGTA	20	57.62
β-actin RV	GGAGCAATGATCTTGATCTTCA	22	56.22
Survivin FW	AGAACTGGCCCTTCTTGGAG	24	59.30
Survivin RV	CTTTTATGTTCTCTATGGGGTC	19	57.06
Bax FW	GATGCGTCCACCAAGAAGC	19	59.20
Bax RV	CCAATGTCCAGCCCATGA	18	56.91
MMP-9 FW	AGACCTGGGCAGATTCCAAAC	21	62.04
MMP-9 RV	CGGCAAGTCTTCCGAGTAGT	20	61.34
P53 FW	CAGCACATGACGGAGGTTGT	20	60.60
P53 RV	TCATCCAAATACTCCACACGC	21	58.37
FW: Forward, RV: Reverse			

Western blotting

Following cell lysis, protein concentrations were measured with a BCA protein assay kit (Pierce Biotechnology, Inc., Rockford, IL, USA). Aliquots containing 30–50 µg of protein per lane were resolved on 10–15% SDS-polyacrylamide gels and then electroblotted onto nitrocellulose membranes (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA). After blocking for 1 hour with 5% (w/v) nonfat dried milk, the blots were probed overnight at 4°C with primary antibodies targeting the following: monoclonal mouse anti-human IκBα (1:1000), monoclonal rabbit anti-human p-IκBα (1:1000), monoclonal rabbit anti-human p65 (1:1000), monoclonal rabbit anti-human p-p65 (1:1000), and monoclonal rabbit anti-human β-actin (1:1000). Subsequently, the membranes were treated for 1 hour with secondary antibodies, goat anti-rabbit IgG (1:3000) or horse anti-mouse IgG (1:4000). Protein bands were visualized using the SuperSignal™ West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Waltham, MA, USA).

Wound healing assay

To evaluate migration, we subjected confluent MDA-MB-231 monolayers in 6-well plates to a wound-healing assay. Using a 200-µL pipette tip, we created a consistent scratch across each well, cleared cellular debris with PBS, and added 500 µL of RPMI-1640 supplemented with IC₅₀ levels of honokiol, magnolol, and epirubicin, as single agents or in combination, for 24 h. At 24 h following treatment

initiation, we photographed three random fields per well. Migratory activity was assessed by measuring wound closure (reduction in scratch width) and comparing each treatment group against the control.

Colony formation assay

To evaluate the long-term proliferative capacity of breast cancer cells following drug exposure, a clonogenic (colony formation) assay was performed. MDA-MB-231 cells were seeded at a density of 1×10^4 cells per well in 6-well plates and subsequently treated with IC_{50} concentrations of honokiol, magnolol, and epirubicin for 24 h. Following treatment, the culture medium was replaced with fresh growth medium, and the plates were incubated for an additional two weeks to allow colony outgrowth. At the end of the incubation period, the resulting colonies were fixed and stained with 0.4% crystal violet. Colony formation was quantified by counting stained colonies using ImageJ software (National Institutes of Health, USA), and the treatment groups were compared against untreated controls.

Hoechst staining

The ability of honokiol, magnolol, and epirubicin, both as single agents and in combination, to trigger apoptosis in MDA-MB-231 cells was investigated using Hoechst nuclear staining. Briefly, cells were seeded at a density of 1×10^5 per well in 12-well plates and cultured for 24 h prior to treatment. Following a 24 h exposure to IC_{50} concentrations of the test compounds, the cells were rinsed twice with PBS, fixed in 3.7% formaldehyde, and incubated with Hoechst 33342 (5 μ g/mL) for 30 minutes. Apoptotic cell death was then assessed by visualizing characteristic nuclear morphological changes, including chromatin condensation, pyknosis, and fragmentation, using fluorescence microscopy.

ELISA cell death assay

Apoptosis was quantified using a commercial ELISA-based cell death detection kit (Roche Diagnostics GmbH), which specifically measures cytoplasmic mono- and oligonucleosomes as hallmarks of apoptotic cell death. Briefly, breast cancer cells were seeded at a density of 1×10^5 cells per well in 24-well plates and treated with honokiol, magnolol, and epirubicin for 24 h. Following cell lysis and centrifugation, 20 μ L of the resulting supernatant was incubated with 80 μ L of immunoreagent (containing anti-DNA-POD and anti-histone-biotin) in streptavidin-coated microtiter plates. After 2-hour incubation at 25°C, the plates were thoroughly washed and 100 μ L of ABTS substrate was added to each well. The enzymatic reaction was terminated with stop solution, and absorbance was measured at 405 nm using a microplate reader (Awareness Technology). The extent of apoptosis was expressed as the fold-change in absorbance relative to untreated control cells.

Caspase-3 activity assay

Caspase-3 activity in breast cancer cells was assessed calorimetrically using a commercial kit (Abnova, Taipei, Taiwan) as per the supplied instructions. Cells were resuspended in 50 μ L of lysis buffer and centrifuged at 10,000 rpm to eliminate insoluble debris. Aliquots of the cleared cell lysates were

subsequently reacted with a substrate mixture comprising 50 μ L of 2X reaction buffer (supplemented with 10 mM DTT) and 5 μ L of the chromogenic substrate DEVD-pNA (4 mM). The reaction mixtures were incubated for 2 h at 37°C, after which the release of p-nitroaniline was monitored by measuring the absorbance at 405 nm using a microplate absorbance reader, providing a quantitative measure of active caspase-3 levels.

Statistical Analysis

Data are expressed as mean $\pm$ SD from at least three independent experiments, each performed in triplicate. Differences between two groups were analyzed by unpaired Student's t-test, while multiple group comparisons were assessed using one- or two-way ANOVA with Tukey's or Dunnett's post-hoc test, as appropriate. A *p*-value of < 0.05 was considered statistically significant.

Results

Honokiol and magnolol potentiate the cytotoxic efficacy of epirubicin in triple-negative MDA-MB-231 breast cancer cells

MTT assay results revealed that honokiol, magnolol, and epirubicin reduced MDA-MB-231 cell survival in a concentration-dependent manner (Fig. 1a), with epirubicin showing the lowest single-agent IC_{50} (6.56 μ M), followed by honokiol (17.20 μ M) and magnolol (25.18 μ M). The combination of honokiol and magnolol significantly enhanced anti-proliferative activity ($IC_{50} = 12.07 \mu$ M) compared to their individual treatments ($p < 0.05$), indicating a favorable interaction between the two natural compounds. All binary combinations with epirubicin further amplified this effect, particularly honokiol + epirubicin ($IC_{50} = 3.54 \mu$ M) and magnolol + epirubicin ($IC_{50} = 4.26 \mu$ M). Importantly, the triple combination yielded the most robust inhibition of cell proliferation ($IC_{50} = 2.71 \mu$ M), which was significantly more effective than any double combination (Table 2, $p < 0.05$). These data indicate that honokiol and magnolol synergistically augment the chemosensitivity of breast cancer cells to epirubicin, suggesting that integrating these natural compounds into conventional regimens could improve therapeutic outcomes while potentially lowering the required dosage of the chemotherapeutic agent.

Table 2

In vitro cytotoxicity (IC₅₀) of honokiol, magnolol, and epirubicin as single agents and in combination against MDA-MB-231 breast cancer cells.

Treatment	IC ₅₀ (μM)
Honokiol	17.20
Magnolol	25.18
Epirubicin	6.56
Honokiol+ Magnolol	12.07*
Honokiol + Epirubicin	3.54*
Magnolol + Epirubicin	4.26*
Honokiol + Magnolol + Epirubicin	2.71 [#]
* <i>p</i> < 0.05 relative to the respective single treatment; [#] <i>p</i> < .05 relative to the respective double treatment.	

Combination index analysis reveals synergy among honokiol, magnolol, and epirubicin

CI analysis demonstrated synergistic antiproliferative interactions (CI < 1) across all tested dual and triple combinations of honokiol, magnolol, and epirubicin, with CI values ranging from 0.67 to 0.92 (Fig. 1b). The most potent synergy was observed with the magnolol + epirubicin combination at Fa = 0.72 (CI = 0.67), followed closely by the triple combination at Fa = 0.56 (CI = 0.71) and the honokiol + magnolol combination at Fa = 0.84 (CI = 0.72). The honokiol + epirubicin combination yielded a CI of 0.73 at Fa = 0.48. Collectively, these findings indicate consistent synergism across all combinations, supporting the potential for dose reduction and reduced chemotherapeutic toxicity.

Enhanced proliferation inhibition of MDA-MB-231 cells by honokiol and mMagnolol in combination with epirubicin

The anti-proliferative activity of honokiol, magnolol, and epirubicin was evaluated in MDA-MB-231 cells over 120 hours using the trypan blue exclusion assay. As illustrated in Fig. 1, cell viability in the blank control and solvent control groups remained near 100% throughout the experiment. Conversely, all treatment groups demonstrated time-dependent reductions in viability (Fig. 1c). At 24 hours, single-agent treatments with honokiol, magnolol, and epirubicin reduced viability to 83.48%, 81.21%, and 73.87%, respectively. By 120 hours, these values declined further to 40.69%, 44.97%, and 24.84%. Notably, the combination treatments produced enhanced cytotoxic effects. The honokiol + epirubicin and magnolol + epirubicin combinations decreased viability to 62.58% and 58.49% at 24 hours, and to 20.56% and 28.18% at 120 hours. The triple combination of honokiol + magnolol + epirubicin was the most effective, achieving 56.75% viability at 24 hours and 23.49% at 120 hours (*p* < 0.05). These results

demonstrate that honokiol and magnolol significantly sensitize MDA-MB-231 cells to epirubicin, with the triple combination exhibiting the most potent anti-proliferative activity.

Pro-apoptotic and anti-invasive gene modulation by honokiol and magnolol

The effects of honokiol and magnolol, alone and in combination, on mRNA transcript levels were evaluated using quantitative real-time PCR with β -actin as the internal reference gene. Compared to the control and solvent groups (baseline 1.0), treatment with honokiol significantly upregulated the pro-apoptotic genes Bax (to 1.8 fold) and p53 (to 2.1 fold), while magnolol alone significantly increased p53 (to 1.7 fold) but only slightly increased Bax (to 1.2 fold). Notably, the combined honokiol + magnolol treatment produced the most robust upregulation of both Bax (2.67 fold) and p53 (2.3 fold), which was significantly higher than the monotherapy treatments ($p < 0.05$). In contrast, the anti-apoptotic and invasive genes Survivin and MMP-9 were significantly downregulated by both honokiol (reducing expression to 63.5% and 67.1% of control, respectively) and magnolol (to 74.9% and 54.5% of control, respectively) compared to the control groups. The honokiol + magnolol combination exhibited the strongest inhibitory effect, significantly decreasing Survivin and MMP-9 expression to 57.3% and 42.4% of control, respectively, which was significantly lower than either single-agent treatment ($p < 0.05$). Collectively, these data indicate that the honokiol and magnolol combination synergistically modulates gene expression by promoting apoptosis while concurrently suppressing cell survival and invasion pathways (Fig. 2).

Honokiol and magnolol cooperatively upregulate I κ B α to block NF- κ B

Western blotting was utilized to examine the effects of honokiol and magnolol on the NF- κ B pathway, with protein levels normalized to β -actin. We observed that the total p65 levels were uniform across all groups, confirming the treatments did not alter basal p65 expression (Fig. 2). However, the phosphorylated forms were markedly affected. The relative expression levels of phospho-p65 were significantly suppressed by honokiol and magnolol to 66.38% and 57.78% of the control, respectively ($p < 0.05$). The combined treatment of honokiol + magnolol resulted in a more pronounced suppression, reaching 34.51%, which was significantly lower than either monotherapy ($p < 0.05$). In line with these results, the phospho-I κ B α expression was also downregulated by honokiol (59.46%) and magnolol (77.83%) compared to the control. The combination treatment further significantly reduced phospho-I κ B α to 33.70% ($p < 0.05$). Conversely, total I κ B α expression was notably elevated. Honokiol and magnolol alone significantly increased total I κ B α to 1.95 fold and 1.41 fold of the control, respectively. The honokiol + magnolol group exhibited the highest upregulation, reaching 2.36 fold ($p < 0.05$ vs. controls and monotherapies). The solvent control group showed no statistical difference from the untreated control. Overall, these results indicate that co-treatment with honokiol and magnolol acts synergistically to inhibit NF- κ B activation by blocking the phosphorylation of p65 and I κ B α and

enhancing the expression of the inhibitory I κ B α protein, thereby potentially attenuating downstream inflammatory responses and providing a stronger therapeutic effect than either drug alone.

Triple combination of honokiol, magnolol, and epirubicin potently suppresses breast cancer cell migration

To assess the anti-migratory effects of honokiol, magnolol, and epirubicin, a wound healing assay was performed on MDA-MB-231 cells over 24 hours. As shown in the representative images and quantified data, the control and solvent groups exhibited near-complete wound closure (Fig. 3a and 3b). Treatment with monotherapies (honokiol, magnolol, and epirubicin) resulted in a significant, dose-dependent inhibition of cell migration, with migration rates of 81.47%, 74.69%, and 65.73%, respectively, compared to the control (100%). Notably, dual combinations significantly enhanced this inhibitory effect, reducing migration rates to 33.80%–37.11%. Furthermore, the triple combination exhibited the most potent anti-migratory activity, reducing the migration rate to 27.84% (* $p < 0.05$ vs. control; # $p < 0.05$ vs. monotherapy). These results indicate that combining these agents synergistically suppresses MDA-MB-231 cell migration.

Combined honokiol, magnolol, and epirubicin significantly reduces colony formation in MDA-MB-231 cells

To evaluate the long-term proliferative capacity of MDA-MB-231 cells following drug exposure, a colony formation assay was performed. As shown in the Fig. 3, treatment with honokiol, magnolol, and epirubicin at their respective IC₅₀ concentrations significantly suppressed colony formation compared to the untreated and solvent controls ($p < 0.05$). Specifically, single-agent treatment with honokiol, magnolol, and epirubicin reduced colony formation to 56.6%, 44.9%, and 35.3% of the control, respectively. The double combination treatments exhibited relative colony counts of 47.8% (honokiol + magnolol), 39.2% (honokiol + epirubicin), and 42.7% (magnolol + epirubicin). Notably, the triple combination exerted the most pronounced inhibitory effect, drastically reducing colony formation to 27.5% of the control. These data indicate that the triple combination exhibits the strongest anti-proliferative and clonogenic suppression against MDA-MB-231 cells.

Pro-apoptotic potency of combined phytochemicals and epirubicin at reduced effective doses

Apoptosis was assessed using Hoechst staining, ELISA, and caspase-3 activity assays; all performed at their respective IC₅₀ concentrations (Figs. 3 and 4). Hoechst staining revealed condensation and fragmentation in combination groups, particularly the triple combination, whereas control and solvent groups showed normal round nuclei, indicating apoptosis. In the ELISA, combinations significantly increased nucleosome enrichment compared to honokiol and magnolol alone ($p < 0.05$), but not compared to epirubicin, nor was the triple combination significantly higher than doubles (fold changes:

honokiol 1.61, magnolol 1.40, epirubicin 1.95, doubles 1.80–2.16, triple 1.93). Caspase-3 activity was significantly elevated in all combination groups versus magnolol, with the triple combination reaching the highest level (3.38 fold; $p < 0.05$ vs. doubles and controls), while double combinations ranged from 1.93 to 2.46 fold. Critically, despite the lack of statistical significance in the magnitude of apoptosis for some groups, prior MTT data confirmed these effects were achieved at substantially lower concentrations (reduced IC_{50} values). Therefore, the combinations exhibited a more potent and synergistic pro-apoptotic profile, achieving comparable or superior apoptotic responses at significantly reduced doses, indicating that these combinations provide a significant therapeutic advantage overall.

Discussion

The present study investigated the synergistic anti-cancer effects of magnolol and honokiol, alone and in combination with epirubicin, against MDA-MB-231 triple-negative breast cancer (TNBC) cells [1]. Our findings demonstrate that these phytochemicals potentiate epirubicin cytotoxicity through NF- κ B pathway inhibition, pro-apoptotic gene upregulation, and suppression of migratory and clonogenic capacity, providing a promising strategy to overcome chemoresistance in TNBC [9].

Our results reveal that honokiol, magnolol, and epirubicin each reduced MDA-MB-231 cell viability in a concentration-dependent manner, with epirubicin demonstrating the lowest single-agent IC_{50} (6.56 μ M), followed by honokiol (17.20 μ M) and magnolol (25.18 μ M) [20]. This differential sensitivity aligns with established literature documenting the potent cytotoxic activity of anthracyclines in TNBC models, while the relatively higher IC_{50} values for the phytochemicals reflect their moderate potency as single agents [5]. Notably, the combination of honokiol and magnolol significantly enhanced anti-proliferative activity (IC_{50} = 12.07 μ M), supporting previous reports of synergistic interactions between these structurally related biphenolic compounds [11]. The most striking finding was the substantial sensitization of MDA-MB-231 cells to epirubicin when combined with the phytochemicals. The honokiol and epirubicin combination reduced the IC_{50} to 3.54 μ M, while magnolol in combination with epirubicin achieved 4.26 μ M. Critically, the triple combination yielded the most robust inhibition (IC_{50} = 2.71 μ M), significantly outperforming all dual combinations. These findings corroborate recent work by Cheng et al. [21], who demonstrated that honokiol enhances the therapeutic efficacy of conventional agents by suppressing NF- κ B-mediated survival signals. Similarly, a 2022 study reported that honokiol synergizes with chemotherapy drugs in multidrug-resistant breast cancer cells through enhanced apoptosis and necrotic cell death pathways [22].

Combination index analysis confirmed synergistic interactions ($CI < 1$) across all tested combinations, with magnolol and epirubicin showing the strongest synergy (CI = 0.67 at Fa = 0.72). This is consistent with the Chou-Talalay mathematical model, which defines synergy as CI values below unity. The observed synergy between magnolol and epirubicin aligns with studies showing magnolol's ability to inhibit P-glycoprotein function and drug efflux transporters, potentially enhancing intracellular drug accumulation [23]. The triple combination also demonstrated potent synergy (CI = 0.71), supporting the

rationale for multi-agent approaches in TNBC management. The time-dependent proliferation assays revealed that combination treatments produced sustained growth inhibition over 120 hours. By 120 hours, the triple combination achieved 23.49% viability compared to 40.69% for honokiol alone and 24.84% for epirubicin alone. This persistent effect suggests that the phytochemicals may induce long-term transcriptomic changes, consistent with reports that honokiol modulates AMPK signaling pathways and inhibits breast carcinogenesis through metabolic reprogramming [24].

Gene expression analysis demonstrated that honokiol and magnolol significantly upregulated pro-apoptotic Bax and p53 while downregulating anti-apoptotic survivin and pro-invasive MMP-9. The combination produced the most robust modulation (Bax: 2.67 fold; survivin: 57.3% of control; MMP-9: 42.4% of control). These findings are consistent with recent studies documenting that magnolol targets multiple apoptosis pathways, including death receptor-mediated and mitochondria-mediated cascades through Bax upregulation and p53 activation [14]. Importantly, MDA-MB-231 cells harbor mutant p53, yet our data show p53 upregulation, suggesting a p53-independent apoptotic mechanism, as previously reported for honokiol in various cancer cell lines [25]. Our Western blot analyses revealed that combination treatment significantly suppressed phospho-p65 (34.51% of control) and phospho-I κ B α (33.70% of control) while elevating total I κ B α (2.36 fold). This pattern demonstrates effective NF- κ B pathway blockade, corroborating recent findings showing that magnolol inhibits I κ B kinase activation, thereby preventing I κ B α phosphorylation and degradation [26]. The enhanced I κ B α upregulation observed in our combination group suggests a cooperative mechanism that stabilizes the NF- κ B inhibitory complex, potentially through dual targeting of IKK and alternative p65 kinases such as RSK1 or MSK1 [27].

The wound healing and colony formation assays demonstrated that combination treatments potently suppressed migration and clonogenic survival, with the triple combination reducing migration to 27.84% and colony formation to 27.5% of control. These results parallel recent reports showing that magnolol inhibits cancer cell invasion by suppressing MMP-9 expression through NF- κ B inhibition and STAT3 inactivation [8]. Our data further demonstrate that the addition of epirubicin to the phytochemical combination amplifies anti-migratory effects, suggesting broad-spectrum suppression of metastatic phenotypes. The apoptosis assays revealed that combinations significantly increased nucleosome enrichment and caspase-3 activity, with the triple combination achieving the highest caspase-3 activation (3.38 fold). Notably, these pro-apoptotic effects were achieved at substantially reduced effective doses, as confirmed by the lowered IC₅₀ values. This pharmacological advantage supports the concept of dose-reduction strategies to minimize chemotherapy-related toxicity while maintaining therapeutic efficacy [28].

The mechanistic basis for the observed synergistic effects lies primarily in the NF- κ B signaling pathway, a central regulator of TNBC progression and chemoresistance [27]. Our results demonstrate that honokiol and magnolol cooperatively inhibit NF- κ B activation through multiple interconnected mechanisms. The canonical NF- κ B pathway is typically activated when IKK phosphorylates I κ B α at Ser32/36, triggering ubiquitination and proteasomal degradation, thereby releasing p65/p50 for nuclear

translocation [7]. Our Western blot data showing suppressed phospho-I κ B α levels and elevated total I κ B α confirm that the phytochemicals interfere with this "release" step. This is consistent with recent studies demonstrating that both magnolol and honokiol inhibit IKK activation, preventing I κ B α phosphorylation and subsequent degradation [26]. The stabilization of I κ B α traps NF- κ B in the cytoplasm, preventing nuclear translocation and target gene transcription. Beyond the cytoplasmic sequestration, our data reveal that honokiol and magnolol also suppress phospho-p65 levels, indicating inhibition of the "transcriptional boost" phase. As outlined in the mechanistic framework of NF- κ B regulation, p65 phosphorylation at Ser536 enhances its interaction with transcriptional co-activators such as CBP/p300, enabling robust target gene expression [7]. The observed reduction in phospho-p65 (34.51% of control in the combination group) suggests that the phytochemicals interfere with this nuclear amplification step, potentially through inhibition of IKK β or alternative p65 kinases such as RSK1 or MSK1 [27]. This dual-level inhibition, blocking both I κ B α degradation and p65 transactivation, explains the profound suppression of NF- κ B target genes observed in our study.

The downstream consequences of NF- κ B inhibition manifest in the modulation of key regulatory genes. Survivin, a direct NF- κ B target was significantly downregulated (57.3% of control). Survivin functions as an inhibitor of apoptosis protein (IAP) that blocks caspase activation. Its downregulation removes this apoptotic blockade, enabling the observed caspase-3 activation (3.38 fold in the triple combination) and subsequent apoptosis [14]. Similarly, MMP-9 downregulation (42.4% of control) reflects NF- κ B's role in transcriptionally regulating matrix metalloproteinases, which are essential for extracellular matrix degradation and tumor invasion [8]. The suppression of MMP-9 directly explains the reduced migratory capacity observed in wound healing assays; consistent with findings that honokiol inhibits breast cancer cell metastasis by blocking epithelial-to-mesenchymal transition [14].

The upregulation of pro-apoptotic Bax and p53 further amplifies the apoptotic response. Honokiol and magnolol have been shown to induce apoptosis through Bcl-2 family modulation, increasing Bax and Bak while decreasing Bcl-xL and Mcl-1 [14]. Our data showing robust Bax upregulation (2.67 fold in the combination group) support this mechanism. The elevated Bax/Bcl-2 ratio promotes mitochondrial outer membrane permeabilization, releasing cytochrome c and activating the intrinsic apoptotic cascade. Notably, although MDA-MB-231 cells express mutant p53, the observed p53 upregulation may activate alternative apoptotic pathways through reactive oxygen species generation and stress response signaling [25].

Epirubicin's contribution to the synergy lies in its DNA-intercalating activity and topoisomerase II inhibition, which generate DNA damage and free radicals. The phytochemicals enhance epirubicin efficacy by blocking NF- κ B-mediated survival signals that would otherwise counteract DNA-damage-induced apoptosis. This is particularly relevant in TNBC, where constitutive NF- κ B activation drives resistance to anthracyclines through upregulation of anti-apoptotic genes and drug efflux transporters [27]. Recent studies have shown that honokiol inhibits BCRP function and expression via EGFR/PI3K signaling, potentially enhancing intracellular epirubicin accumulation and overcoming multidrug resistance mechanisms [26].

This study's limitations include the use of a single TNBC cell line (MDA-MB-231) lacking in vivo validation, 24-hour exposure windows that may not capture long-term effects, absence of normal cell toxicity assessment to confirm therapeutic selectivity, and the lack of protein-level confirmation for all genes beyond Western blots. These constraints limit the generalizability of our findings [9].

Conclusion

Our findings demonstrate that honokiol and magnolol synergistically suppress TNBC cell growth, migration, and colony formation while enhancing epirubicin chemosensitivity through NF- κ B pathway inhibition, pro-apoptotic gene modulation, and MMP-9 suppression. The triple combination offers a promising strategy to reduce epirubicin dosage and overcome chemoresistance in triple-negative breast cancer [1]. Future studies should validate these findings in in vivo xenograft models to assess bioavailability, toxicity, and therapeutic efficacy. Additionally, molecular docking studies could elucidate precise binding interactions between phytochemicals and NF- κ B pathway components. Clinical investigations exploring combination regimens in TNBC patients with epirubicin-based chemotherapy are warranted [20].

Abbreviations

ABCB1: ATP binding cassette subfamily B member 1, **ABCC1**: ATP binding cassette subfamily C member 1, **ABCG2**: ATP binding cassette subfamily G member 2, **ABC**: ATP binding cassette, **ABTS**: 2,2' azino bis(3 ethylbenzothiazoline 6 sulfonic acid), **Akt**: Protein kinase B, **AP 1**: Activator protein 1, **Bax**: Bcl 2 associated X protein, **Bcl 2**: B cell lymphoma 2, **Bcl xL**: B cell lymphoma extra large, **BCRP**: Breast cancer resistance protein, **CI**: Combination index, **COX 2**: Cyclooxygenase 2, **DMSO**: Dimethyl sulfoxide, **DTT**: Dithiothreitol, **ECL**: Enhanced chemiluminescence, **EDTA**: Ethylenediaminetetraacetic acid, **EGFR**: Epidermal growth factor receptor, **ELISA**: Enzyme linked immunosorbent assay, **ER**: Estrogen receptor, **ERK**: Extracellular signal regulated kinase, **Fa**: Fraction affected, **FBS**: Fetal bovine serum, **HER2**: Human epidermal growth factor receptor 2, **HNK**: Honokiol, **HRP**: Horseradish peroxidase, **IC₅₀**: Half maximal inhibitory concentration, **I κ B α** : Inhibitor of nuclear factor kappa B alpha, **JNK**: c Jun N terminal kinase, **MAG**: Magnolol, **MAPK**: Mitogen activated protein kinase, **MDR1**: Multidrug resistance protein 1, **MDR**: Multidrug resistance, **MMP 9**: Matrix metalloproteinase 9, **MTT**: 3 (4,5 dimethylthiazol 2 yl) 2,5 diphenyltetrazolium bromide, **mTOR**: Mammalian target of rapamycin, **NF κ B**: Nuclear factor kappa light chain enhancer of activated B cells, **PARP**: Poly(ADP ribose) polymerase, **PI3K**: Phosphoinositide 3 kinase, **PR**: Progesterone receptor, **PVDF**: Polyvinylidene fluoride, **qRT PCR**: Quantitative real time polymerase chain reaction, **ROS**: Reactive oxygen species, **SDS**: Sodium dodecyl sulfate, **TNBC**: Triple negative breast cancer, **TRAIL**: TNF related apoptosis inducing ligand

Declarations

Ethical approval

This study did not involve human participants or animal experimentation. The research utilized the commercially available human MDA-MB-231 breast cancer cell line. All experimental protocols were conducted in accordance with relevant institutional guidelines and regulations. The study design and ethical considerations were reviewed and approved by the Ethics Committee of AUMS, Arak, Iran (Approval Code: IR.ARAKMU.REC.1398.234).

Conflict of Interest

There is no conflict of interest in this study.

Author Contribution

R.Z., S.M.J.H., M.B., H.K.; Conceptualization, Investigation, Methodology, Formal analysis and writing original draft preparation. H.K., S.M.J.H.; Resources, Supervision, Editing, and funding acquisition. All authors read and approved the final manuscript.

Acknowledgement

The authors thank the Traditional and Complementary Medicine Research Center, Arak University of Medical Sciences (AUMS), for providing the necessary equipment for this work. This manuscript was supported by a grant from Students research committee from AUMS of Iran [Grant number 3472].

Data availability

The data presented in this study are available on request from the corresponding author.

References

1. Sung H et al (2021) Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA Cancer J Clin* 71(3):209–249
2. Arnold M et al (2022) Current and future burden of breast cancer: Global statistics for 2020 and 2040. *Breast* 66:15–23
3. Medina MA et al (2020) Triple-Negative Breast Cancer: A Review of Conventional and Advanced Therapeutic Strategies. *Int J Environ Res Public Health*, 17(6)
4. Minotti G et al (2004) Anthracyclines: molecular advances and pharmacologic developments in antitumor activity and cardiotoxicity. *Pharmacol Rev* 56(2):185–229
5. Nedeljković M, Damjanović A (2019) Mechanisms of Chemotherapy Resistance in Triple-Negative Breast Cancer-How We Can Rise to the Challenge. *Cells*, 8(9)

6. Derakhshan F, Reis-Filho JS (2022) Pathogenesis of Triple-Negative Breast Cancer. *Annu Rev Pathol* 17:181–204
7. Wang X et al (2020) Endocrinotherapy resistance of prostate and breast cancer: Importance of the NF- κ B pathway (Review). *Int J Oncol* 56(5):1064–1074
8. Simplicio-Revoredo CM et al (2020) Expression of Nrf2 and NF- κ B transcription factors in breast cancer and breast fibroadenoma: Insights for a new therapeutic approach. *Oncotarget* 11(18):1629–1636
9. Mottaghi S, Abbaszadeh H (2022) Natural Lignans Honokiol and Magnolol as Potential Anticarcinogenic and Anticancer Agents. A Comprehensive Mechanistic Review. *Nutr Cancer* 74(3):761–778
10. Woo SM, Min KJ, Kwon TK (2020) *Magnolol Enhances the Therapeutic Effects of TRAIL through DR5 Upregulation and Downregulation of c-FLIP and Mcl-1 Proteins in Cancer Cells*. *Molecules*, 25(19)
11. Yu CP et al (2021) Magnolol and Honokiol Inhibited the Function and Expression of BCRP with Mechanism Exploration. *Molecules*, 26(23)
12. Wang WD et al (2019) Honokiol inhibits breast cancer cell metastasis by blocking EMT through modulation of Snail/Slug protein translation. *Acta Pharmacol Sin* 40(9):1219–1227
13. Tian W et al (2013) Honokiol synergizes chemotherapy drugs in multidrug resistant breast cancer cells via enhanced apoptosis and additional programmed necrotic death. *Int J Oncol* 42(2):721–732
14. Wang J et al (2020) Hyaluronic acid-modified liposomal honokiol nanocarrier: Enhance anti-metastasis and antitumor efficacy against breast cancer. *Carbohydr Polym* 235:115981
15. Kumar S et al (2021) Dll1(+) quiescent tumor stem cells drive chemoresistance in breast cancer through NF- κ B survival pathway. *Nat Commun* 12(1):432
16. Nazmabadi R et al (2024) Dihydroartemisinin Enhances the Therapeutic Efficacy of BH3 Mimetic Inhibitor in Acute Lymphoblastic Leukemia Cells via Inhibition of Mcl-1. *Asian Pac J Cancer Prev* 25(1):325–332
17. Abbasi Y et al (2024) Formononetin and Dihydroartemisinin Act Synergistically to Induce Apoptosis in Human Acute Myeloid Leukemia Cell Lines. *Cell J* 26(2):121–129
18. Amini R, Karami H, Bayat M (2021) Combination Therapy with PIK3R3-siRNA and EGFR-TKI Erlotinib Synergistically Suppresses Glioblastoma Cell Growth In Vitro. *Asian Pac J Cancer Prev* 22(12):3993–4000
19. Ashofteh N et al (2021) MiRNA-Mediated Knock-Down of Bcl-2 and Mcl-1 Increases Fludarabine-Sensitivity in CLL-CII Cells. *Asian Pac J Cancer Prev* 22(7):2191–2198
20. Wang X et al (2022) Magnolol as a Potential Anticancer Agent: A Proposed Mechanistic Insight. *Molecules*, 27(19)
21. Cheng X et al (2023) Honokiol inhibits interleukin-induced angiogenesis in the NSCLC microenvironment through the NF- κ B signaling pathway. *Chem Biol Interact* 370:110295

22. Skolastika S et al (2022) *Comprehensive Computational Analysis of Honokiol Targets for Cell Cycle Inhibition and Immunotherapy in Metastatic Breast Cancer Stem Cells*. Evid Based Complement Alternat Med, 2022: p. 4172531
23. Lee YC et al (2022) Magnolol Induces Apoptosis Through Extrinsic/intrinsic Pathways and Attenuates NF- κ B/STAT3 Signaling in Non-small-cell Lung Cancer Cells. Anticancer Res 42(8):3825–3833
24. Sengupta S et al (2017) *Activation of tumor suppressor LKB1 by honokiol abrogates cancer stem-like phenotype in breast cancer via inhibition of oncogenic Stat3*. Oncogene, 36(41): pp. 5709–5721
25. Naghashpour M et al (2023) Evaluating the Magnolol Anticancer Potential in MKN-45 Gastric Cancer Cells. Med (Kaunas), 59(2)
26. Yang J et al (2023) Nanotechnology-Based Drug Delivery Systems for Honokiol: Enhancing Therapeutic Potential and Overcoming Limitations. Int J Nanomed 18:6639–6665
27. Drain AP et al (2021) Matrix compliance permits NF- κ B activation to drive therapy resistance in breast cancer. J Exp Med, 218(5)
28. Hua H et al (2013) Honokiol augments the anti-cancer effects of oxaliplatin in colon cancer cells. Acta Biochim Biophys Sin (Shanghai) 45(9):773–779

Figures

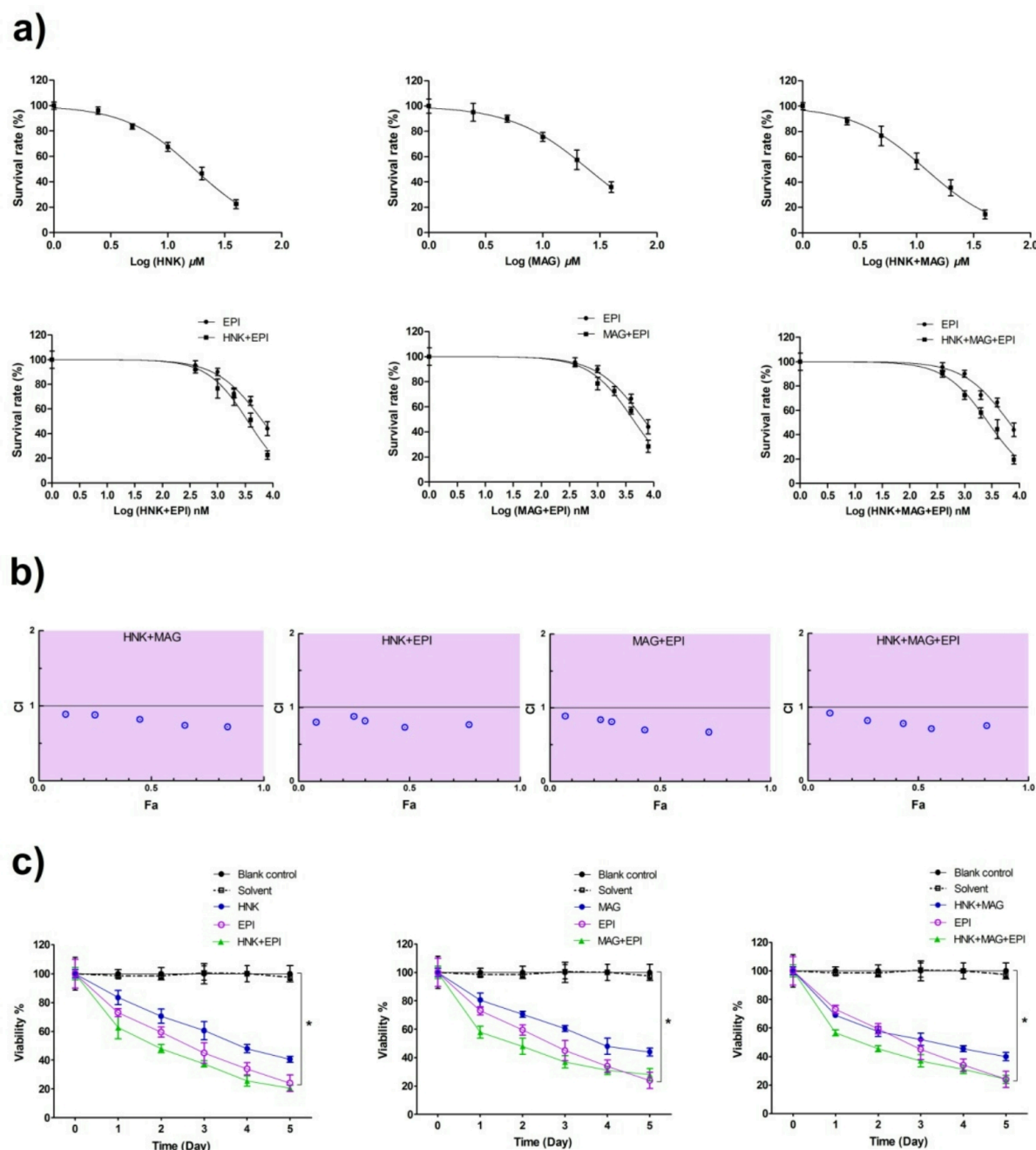


Figure 1

Synergistic cytotoxic and anti-proliferative effects of Honokiol (HNK), Magnolol (MAG), and Epirubicin (EPI) in MDA-MB-231 triple-negative breast cancer cells. (a) Dose-response cell survival curves and corresponding IC_{50} values determined by MTT assay after 24 h of treatment with HNK, MAG, EPI, and their dual or triple combinations. Data are presented as mean $\pm$ SD of three independent experiments. (b) Combination index (CI) analysis using the Chou-Talalay method. CI values < 1 indicate synergistic

interactions across various fractional affected (Fa) levels for all dual and triple combinations. **(c)** Time-dependent anti-proliferative effects assessed by trypan blue exclusion assay. * $p < 0.05$ vs. control group.

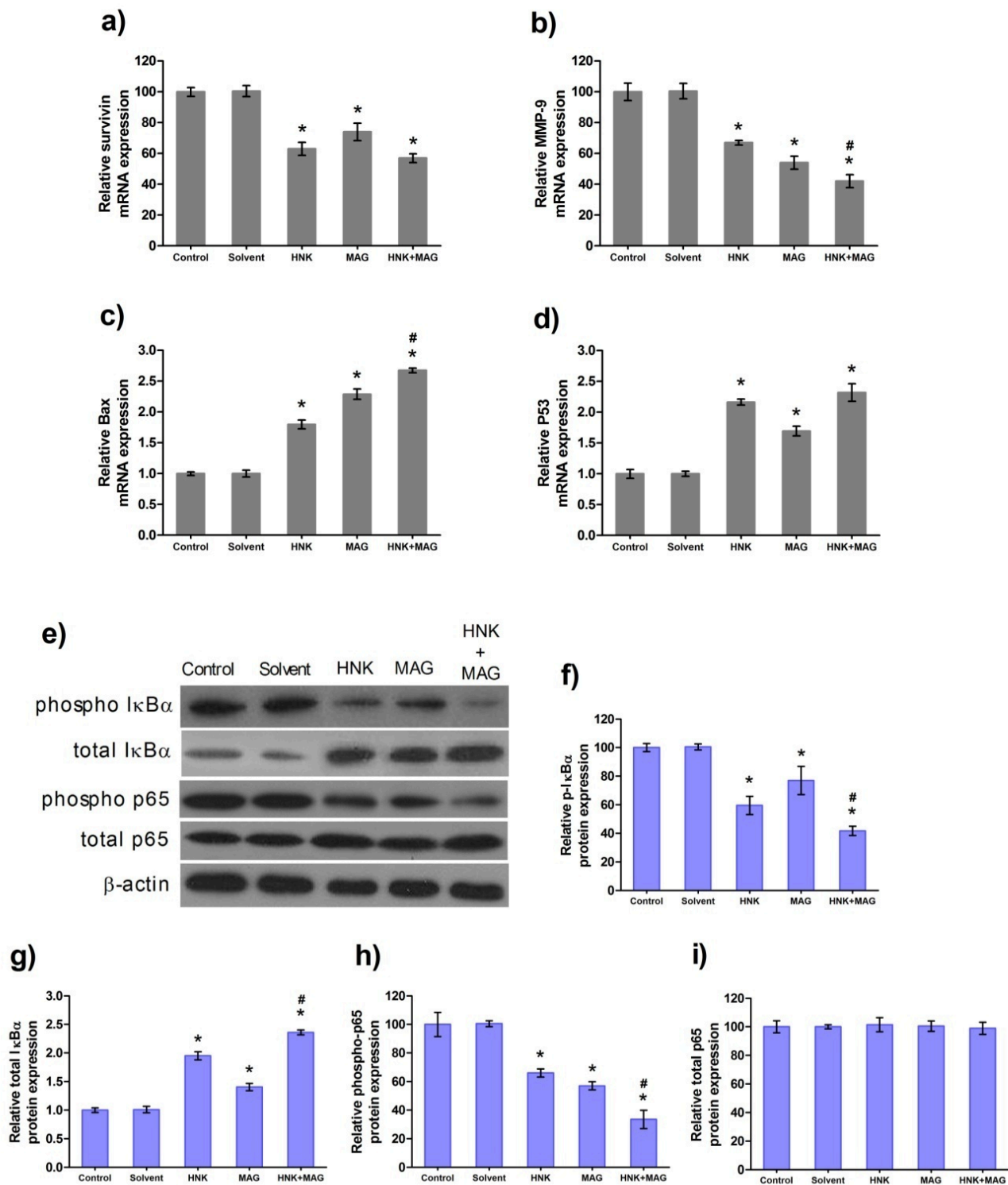


Figure 2 Modulation of apoptosis-related gene expression and inhibition of the NF-κB signaling pathway by Honokiol (HNK), Magnolol (MAG), and their combination in MDA-MB-231 cells. **(a–d)** Quantitative real-

time PCR (qRT-PCR) analysis of mRNA expression levels of Survivin (a), MMP-9 (b), Bax (c), and p53 (d). β -actin was used as the internal reference gene. (e) Representative Western blot images showing the protein expression levels in MDA-MB-231 cells treated with HNK, MAG, or HNK+MAG for 24 h. (f–i) Densitometric quantification of relative protein expression of phospho-IkBa (f), total IkBa (g), phospho-p65 (h), and total p65 (i), normalized to β -actin. Data are presented as mean $\pm$ SD of at least three independent experiments. * p <0.05 vs. control group; # p <0.05 vs. single-agent treatment groups.

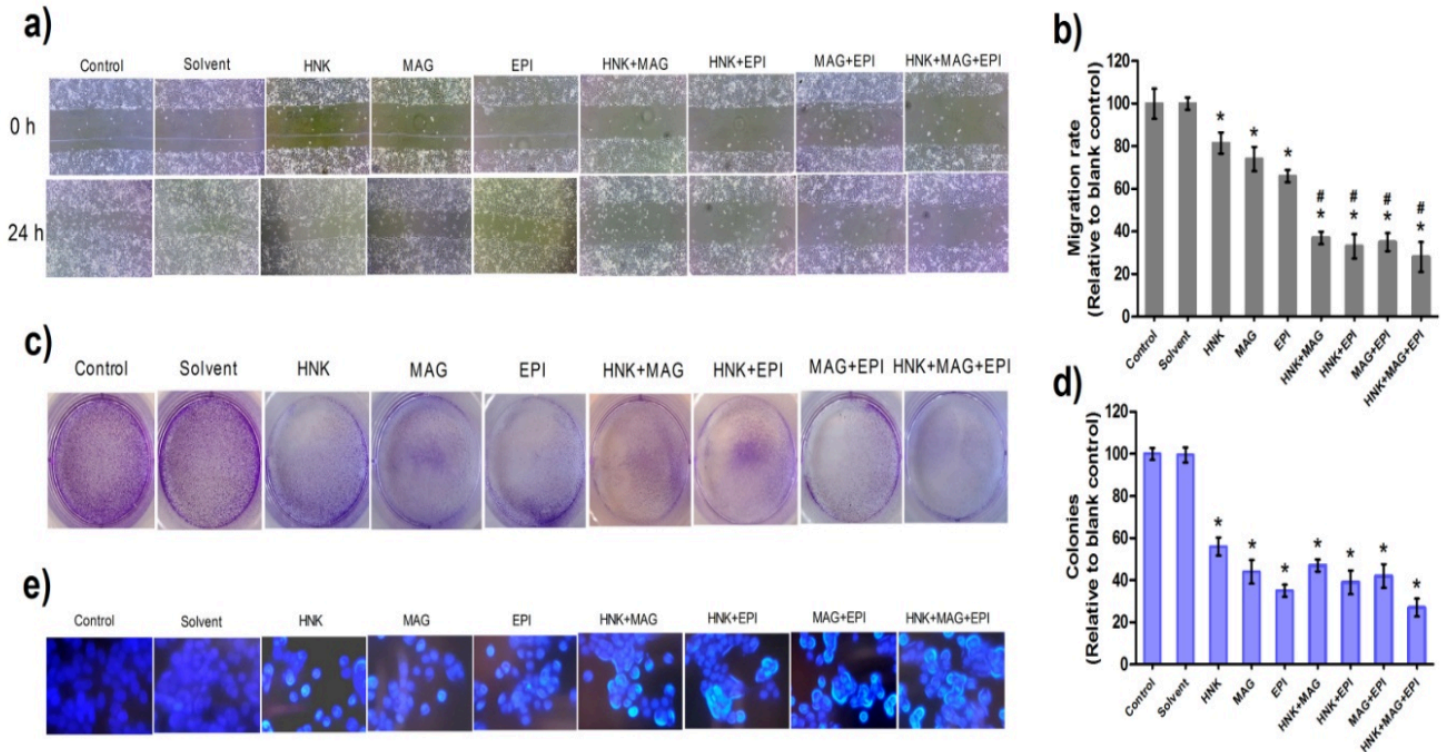


Figure 3

Suppression of migration, clonogenic survival, and induction of apoptosis by Honokiol (HNK), Magnolol (MAG), and Epirubicin (EPI) in MDA-MB-231 triple-negative breast cancer cells. (a) Representative images of the wound healing assay captured at 0 h and 24 h post-treatment. (b) Quantification of the cell migration rate, expressed as a percentage relative to the blank control. (c) Representative images of the colony formation assay stained with crystal violet after 14 days of incubation. (d) Quantification of colony numbers, expressed as a percentage relative to the blank control. (e) Representative fluorescence microscopy images of Hoechst 33342-stained cells. Data are presented as mean $\pm$ SD from at least three independent experiments. * p <0.05 vs. control group; # p <0.05 vs. single-agent treatment groups.

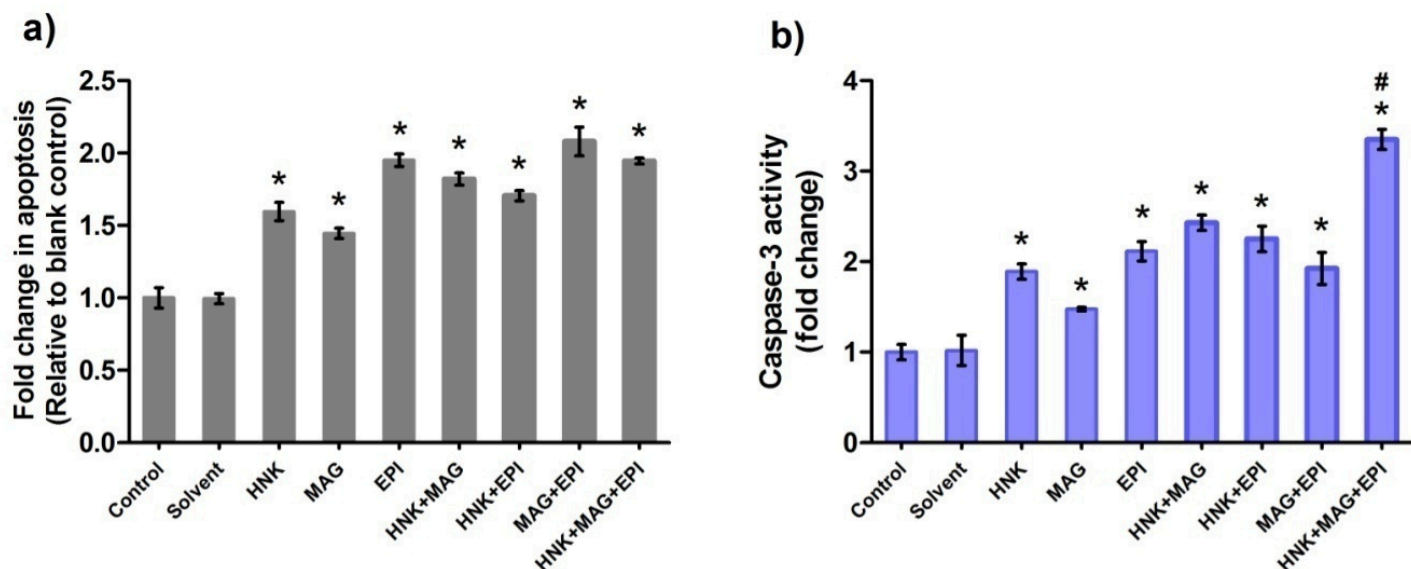


Figure 4

Quantitative analysis of apoptosis and caspase-3 activation by Honokiol (HNK), Magnolol (MAG), and Epirubicin (EPI) in MDA-MB-231 triple-negative breast cancer cells. **(a)** Apoptosis was quantified using an ELISA-based cell death detection kit, measuring the fold change in cytoplasmic mono- and oligonucleosomes relative to the blank control after 24 h of treatment. **(b)** Caspase-3 activity was evaluated using a colorimetric assay (DEVD-pNA cleavage) and is presented as the fold change relative to the blank control.

Data are presented as mean $\pm$ SD from at least three independent experiments. * p <0.05 vs. control group; # p <0.05 vs. single-agent treatment groups.