

Research Article

How to cite this article:

Mahmoudzadeh V, Molavand M, Asadi A, Valizadeh A, Shekarchi A, Vaghari-Tabari M, Yousefi B. Linarin Reverses Doxorubicin Resistance by Modulating PTEN and MDR1 Expression in MCF-7 Breast Cancer Cells. *Advanced Pharmaceutical Bulletin*, doi: 10.34172/apb.46962

Linarin Reverses Doxorubicin Resistance by Modulating PTEN and MDR1 Expression in MCF-7 Breast Cancer Cells

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ARTICLE INFO

Keywords:

Linarin,
Drug resistance,
P-gp,
PTEN,
MCF-7/DOX

Article History:

Submitted: December 19, 2025

Revised: July 11, 2026

Accepted: August 14, 2026

ePublished: September 30, 2026

ABSTRACT

Purpose: Chemoresistance in breast cancer remains a major obstacle that limits the effectiveness of current therapeutic options for patients. Therefore, the identification or development of adjuvant compounds with minimal side effects to enhance the efficacy of standard treatments continues to be an important goal in cancer therapy. In this study, we investigated the impact and molecular mechanisms of linarin to overcome drug resistance in the doxorubicin-resistant MCF-7 breast cancer cell line (MCF-7/DOX).

Methods: The cytotoxic effects of linarin were assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. MDR-1 and PTEN mRNA/protein expression levels were quantified by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and western blotting. Rhodamine 123 uptake was measured after linarin treatment to assess P-glycoprotein (P-gp) activity, and flow cytometry was performed to evaluate cell death.

Results: Our findings indicate that linarin significantly enhanced the cytotoxicity of doxorubicin and reduced the resistance index in MCF-7/DOX cells ($p < 0.05$). Linarin treatment also led to a marked downregulation of MDR1 mRNA expression and a decrease in P-gp levels. Increased intracellular accumulation of Rhodamine 123 was observed, suggesting inhibition of P-gp activity and reduced drug efflux. Notably, linarin upregulated both gene expression and protein levels of PTEN in MCF-7/DOX cells, which are typically characterized by low PTEN levels.

Conclusion: Our findings suggest that linarin may represent a promising adjunct therapeutic agent for overcoming resistance to conventional chemotherapeutic drugs. This effect may be mediated through PTEN upregulation, which appears to play a role in suppressing P-gp-associated resistance mechanisms.

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1. Introduction

Breast cancer remains the leading cause of cancer-related mortality among women aged 20-59 worldwide. Notably, 58% of global breast cancer deaths occur in less-developed countries. In 2022, breast cancer was the most frequently reported malignancy among women in 157 out of 185 countries, contributing to approximately 670,000 deaths globally^{1,2}. Current therapeutic strategies, including mastectomy, radiotherapy, and chemotherapy, are most effective in early-stage disease but lose efficacy in advanced or recurrent cases^{3,4}. Chemotherapeutic agents such as doxorubicin and cyclophosphamide are commonly combined as adjuvant therapy after surgery, particularly in patients with lymph node involvement. They are also employed as part of multidrug regimens for the treatment of metastatic breast cancer⁵. Doxorubicin exerts its anticancer effects through multiple mechanisms, including the inhibition of cell proliferation, induction of DNA damage, and activation of apoptotic pathways⁶. Despite its broad efficacy, the therapeutic benefit of doxorubicin is often limited by the development of multidrug resistance (MDR). Approximately 30% of patients with metastatic or recurrent breast cancer develop MDR, significantly limiting treatment success^{7,8}.

Drug resistance arises through mechanisms such as drug inactivation, target alteration, enhanced DNA repair, apoptosis evasion, tumor plasticity, autophagy, epigenetic changes, and increased drug efflux⁹⁻¹¹. Among these mechanisms, the reduction of intracellular drug concentration through active efflux is a prominent survival-preserving cellular reprogramming in cancer cells. This process is primarily mediated by ATP-binding cassette (ABC) transporters such as P-glycoprotein (encoded by MDR1), MRP1, and BCRP, which expel chemotherapeutic agents from cancer cells and contribute to poor treatment outcomes¹¹⁻¹³.

Multiple studies have identified the overexpression of P-gp as a central factor in resistance to various chemotherapeutic agents, including taxanes, vinca alkaloids, anthracyclines (such as doxorubicin and epirubicin), epipodophyllotoxins, and platinum compounds like cisplatin¹⁴⁻¹⁷. Notably, molecular analyses have documented that P-gp expression is 2.72-fold higher in drug-resistant breast cancer cell lines compared to their drug-sensitive counterparts¹⁸. Due to its chemoresistance-related role, considerable efforts have been directed toward developing P-gp inhibitors through advanced strategies, including direct inhibition, gene silencing, nanocarrier systems, natural compound modulation, and miRNA-based interventions. However, the clinical application of these strategies remains limited due to several challenges, including low specificity, toxicity, off-target effects associated with gene or miRNA silencing, formulation issues, and the suboptimal biocompatibility of nanocarriers¹⁹⁻²¹. Consequently, the identification of effective compounds capable of restoring drug sensitivity with minimal side effects remains a compelling and active area of research in this field.

Downstream effectors of the phosphoinositide 3-kinase (PI3K)/Akt signaling pathway represent a reasonable therapeutic target in cancer treatment, as this oncogenic axis plays a pivotal role in regulating key oncogenic processes such as uncontrolled proliferation, evasion of apoptosis, angiogenesis, metastasis, and immune escape²²⁻²⁵. Studies have demonstrated that the doxorubicin-resistant phenotype in breast cancer cell lines is associated with enhanced activity of the PI3K/Akt signaling pathway. Further investigations have primarily attributed this hyperactivation to the functional loss of PTEN, the key negative regulator of this pathway. In support of this finding, inhibition of this pathway has been shown to restore drug sensitivity²⁶⁻²⁸.

Recent research has focused on natural phytochemicals to overcome chemoresistance. Among these compounds is linarin, whose anti-cancer properties have been shown by some studies^{29,30}. For example, a recent study has shown that linarin can up-regulate proapoptotic proteins including Bax and Caspase3/7 and promote apoptosis in breast cancer cells²⁹. Another recent study has also shown that linarin can attenuate proliferation, migration, and invasion

and promote apoptosis in colorectal cancer cells by suppressing the HIF-1 α /PD-L1 axis³⁰. Linarin, a glycosylated flavonoid derived from the flavone apigenin, is abundantly found in species of *Chrysanthemum indicum* (Indian Chrysanthemum), *Buddleja davidii* (Scrophulariaceae), and *Micromeria* (Lamiaceae). Emerging evidence highlights linarin as a promising therapeutic candidate for various human malignancies. Its anticancer activity is attributed to multiple mechanisms. For instance, linarin suppresses inflammatory mediators such as nitric oxide (NO), prostaglandin E2 (PGE2), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF- α). It also induces apoptosis by upregulating Bax and enhancing the expression of caspase-3/7. Moreover, linarin inhibits metastasis-promoting factors such as matrix metalloproteinase-9 (MMP-9)³¹⁻³³.

Studies have shown that resistance to doxorubicin is closely associated with drug efflux-mediated resistance mechanisms regulated by the PI3K/Akt axis. In this regard, substantial evidence indicates that inhibition of the PI3K/Akt signaling pathway by agents, such as resveratrol, thymoquinone, and piperine markedly enhances doxorubicin sensitivity³⁴⁻³⁷. Additionally, Zhang et al. demonstrated that inhibiting the PI3K/Akt/NF- κ B signaling pathway with LY294002 significantly reversed doxorubicin resistance in MDA-MB-231/ADR cells. This was achieved through downregulation of P-gp, p-Akt, and p-I κ B, confirming the pathway's key role in P-gp-mediated multidrug resistance³⁸.

Furthermore, Shengwei Liu et al. showed that PD-L1 promotes doxorubicin resistance in breast cancer by upregulating MDR1/P-gp via activation of PI3K/AKT and MAPK/ERK pathways³⁹.

Collectively, these findings led us to hypothesize that the antitumor agent linarin may reverse P-gp-mediated resistance in cancer cells by modulating PTEN expression. In this study, we aimed to investigate the effect of linarin on overcoming doxorubicin resistance in MCF-7/DOX breast cancer cells. We assessed its impact on cell viability, apoptosis, and the restoration of doxorubicin sensitivity. Additionally, we explored the molecular mechanisms involved, focusing on the expression of multidrug resistance proteins such as P-gp and the regulation of PTEN.

2. Materials and methods

2.1. Cell culture

Human breast cancer cells MCF-7 were procured from the Pasteur Institute of Iran Cell Bank (Tehran, Iran). The cells were cultured in DMEM medium (Gibco; Cat. No. 11-965-092) supplemented with 10% fetal bovine serum (FBS; Gibco; Cat. No. A5669801) and 1% penicillin-streptomycin (Sigma-Aldrich; Cat. No. P4333) (100 IU/mL and 100 μ g/mL, respectively) and maintained under standard conditions (37°C, 5% CO₂). Upon reaching 70-80% confluency, the cells were subcultured using standard passaging techniques.

2.2. Cell viability assay

The MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma-Aldrich; Cat. No. CT01) was employed to assess cell viability⁴⁰. In this assay, the yellow tetrazolium MTT is reduced by metabolically active cells, primarily via dehydrogenase enzymes, to purple formazan crystals, which can be quantified spectrophotometrically. For the MTT assay, 10,000 cells (sensitive and resistant cell lines) were seeded in 200 μ L culture medium per well of a 96-well plate. After 24 hours (when cell confluency reached 60-70%), the cells were treated with varying concentrations of doxorubicin (Sigma-Aldrich; Cat. No. PHR1789), a fixed concentration of 200 μ M linarin (Sigma-Aldrich; Cat. No. PHL80822), and a combination of both for 48 hours. Control wells (untreated) were also included. Following incubation, the culture medium was removed, and 200 μ L of fresh medium containing 50 μ L MTT solution (5 mg/mL in PBS) was added to each well and incubated in the dark for 4

hours. Then, the MTT-containing medium was carefully removed, and the wells were washed once with PBS. Subsequently, 200 μ L of DMSO was added to each well to solubilize the formazan crystals. Finally, after 15 minutes of gentle shaking, the optical density (OD) of each well was measured at 570 nm with a reference wavelength of 630 nm using a microplate reader (Biotek ELx800, USA).

2.3. Combination Index Analysis

To formally evaluate the pharmacological interaction between linarin and doxorubicin, combination index (CI) analysis was performed using the Chou–Talalay method and CompuSyn software (ComboSyn Inc., Paramus, NJ, USA). MCF-7/DOX cells were treated with linarin and doxorubicin at a fixed molar ratio based on their individual IC₅₀ values (constant-ratio combination design) across a range of concentrations. Cell viability was determined by the MTT assay after 48 hours, and the fraction affected (Fa) was calculated for each concentration. The CI values were calculated by the median-effect equation:

$$CI = (D)_1/(Dx)_1 + (D)_2/(Dx)_2$$

where (D)₁ and (D)₂ are the doses of drug 1 and drug 2, respectively, in combination that produce x% effect, and (Dx)₁ and (Dx)₂ are the doses of drug 1 and drug 2, respectively, alone that produce the same effect. CI < 1 indicates synergism, CI = 1 indicates additivity, and CI > 1 indicates antagonism. CI values were calculated across Fa values of 0.50, 0.75, and 0.90, and a Fa–CI plot (combination index plot) was generated to visualize the interaction across the full dose-response range.

2.3. RNA extraction and quantitative reverse transcription polymerase chain reaction (qRT-PCR)

To investigate the potential mechanism of action of the DOX-LIN combination, changes in MDR1 and PTEN gene expression were analyzed across the following five experimental groups: (1) sensitive MCF-7 cell line, (2) control group (drug-resistant MCF-7/DOX without treatment), (3) DOX-treated group (41 μ M), (4) LIN-treated group (200 μ M), and (5) combination treatment group (DOX + LIN). Total RNA was isolated from cell samples using TRIzol reagent (Thermo Fisher Scientific; Cat. No. 15596026) following the manufacturer's recommended protocol. Subsequently, cDNA synthesis was performed using a cDNA synthesis kit (Thermo Fisher Scientific; Cat. No. K1671). The synthesized cDNA was then subjected to qRT-PCR amplification using SYBR Green master mix and a Mic qPCR Cycler instrument (Bio Molecular Systems, Australia). β -actin was utilized as the housekeeping gene to normalize mRNA expression levels. The qRT-PCR results were calculated using the 2^{−ΔΔCT} method⁴¹. The primer sequences designed for amplifying PTEN, MDR1, and β -actin genes are listed in Table 1.

Table 1. List of primers

Gene	Forward Primer	Reverse Primer
PTEN	5'-CGAACTGGTGTAATGATATGT-3'	5'-CATGAACCTGTCTTCCCGTCA-3'
MDR1	5'-CCCATCATTGCAATAGCAGG-3'	5'-GTTCAAACCTTCTGCTCCTGA-3'
β -actin	5'-TCGTGCGTGACATTAAGGAG-3'	5'-AGGAAGGAAGGCTGGAAGAG-3'

2.4. Apoptosis detection by flow cytometry

MCF-7/DOX cells were seeded at a density of 300,000 cells per well in 6-well plates and cultured until they reached approximately 70% confluency. The cells were then divided into four experimental groups: (1) control group (untreated), (2) doxorubicin (DOX)-treated group, (3) linarin (LIN)-treated group, and (4) combination treatment group (LIN + DOX). After 48 hours, the cells were trypsinized, washed twice with PBS, and once with binding buffer after centrifugation. The cell concentration was adjusted to 500,000–600,000 cells in 100 μ L binding buffer per well. Subsequently, 5 μ L Annexin V-FITC was added to each sample, followed by incubation at room temperature for 10–15 minutes in the dark. Without washing, 5 μ L propidium iodide (PI) staining solution was added directly to each sample. The cells were kept on ice and analyzed within 1 hour using a flow cytometer (BD FACSCalibur)⁴².

2.5. Rhodamine 123 uptake assay by fluorometry

To assess MDR1 efflux pump activity, the uptake of rhodamine 123 (a known substrate for MDR1) in the presence of linarin was measured in 6-well plates⁴³. Cells were treated with doxorubicin, linarin, or their combination for 48 hours, followed by incubation with 10 μ M rhodamine 123 at 37°C for 30 minutes. After washing with PBS, the cells were lysed in 0.1% Triton X-100. Fluorescence intensity was measured using a fluorometer and normalized to total protein content determined by Bradford assay. Data were collected and analyzed for each of the following experimental groups: (1) control group (untreated), (2) doxorubicin (DOX)-treated group, (3) linarin (LIN)-treated group, and (4) combination treatment group (LIN + DOX).

2.6. Western blotting

MCF-7 cells were cultured in 6-well plates containing DMEM medium supplemented with 10% FBS. Following experimental treatments, cellular proteins were extracted and quantified using the Bradford assay. 20 μ L of the extracted proteins were mixed with sample buffer and heated at 95°C for 5 minutes. The samples were subjected to SDS-PAGE (10% polyacrylamide gel, 100 V). Subsequently, proteins were transferred to a PVDF membrane using a semi-dry transfer apparatus (Bio-Rad Trans-Blot SD) with transfer buffer. After transfer, the membrane was blocked with 5% non-fat dry milk in TBST buffer at room temperature for 1 hour, followed by three washes (5 minutes each) with TBST buffer. The membrane was then incubated overnight at 4°C with primary antibodies against PTEN (Cat. #9552S; 1:1000), MDR1 (Cat. # 12273; 1:1000), and β -actin (Cat. #4970T; 1:1000). After three TBST washes, the membrane was incubated with HRP-conjugated anti-mouse IgG secondary antibody at room temperature for 2 hours.

Immunoblots were visualized using an enhanced chemiluminescence (ECL) detection kit according to the manufacturer's protocol. The membrane was incubated in ECL reagents for 5 minutes, and signals were captured using a chemiluminescence imaging system. A broad-range molecular weight marker was used as a reference⁴².

2.7. Statistical analysis

Data are presented as mean $\pm$ standard deviation (SD) from at least three independent experiments. Statistical analyses were performed using SPSS (v26) and GraphPad Prism (v6) software (San Diego, CA, USA). Comparisons between two groups were performed using Student's t-test, while multiple group comparisons were analyzed by one-way ANOVA followed by Tukey's post-hoc test. A p-value < 0.05 was considered statistically significant⁴⁰.

3. Results

3.1. Effect of Doxorubicin (DOX) on the proliferation of MCF-7 and MCF-7/DOX cells

The cytotoxic effects of doxorubicin (DOX) on MCF-7 and MCF-7/DOX cells were evaluated using the MTT assay. Following treatment with varying concentrations of DOX and incubation for 48 hours, cell toxicity was calculated based on the standard formula. As shown in Figure 1A, DOX toxicity increased in a dose-dependent manner in both cell lines. The IC₅₀ values after 48 hours of treatment were calculated as 24 ± 1 μ M for MCF-7 cells and 41 ± 1 μ M for the drug-resistant MCF-7/DOX cells ($p < 0.05$), indicating a significantly higher resistance to DOX in MCF-7/DOX cells compared to parental MCF-7 cells.

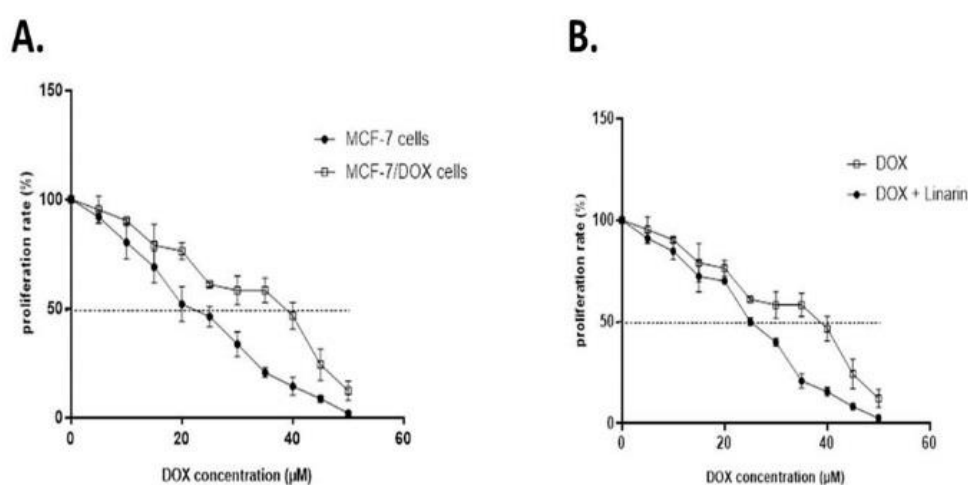


Figure 1. A. Dose-dependent cytotoxic effect of doxorubicin (DOX) on MCF-7 and MCF-7/DOX cells after 48 hours of treatment. B. Effect of 200 μ M linarin on MCF-7/DOX cell proliferation and doxorubicin sensitivity after 48 hours. Data represent mean $\pm$ SD ($n \geq 3$).

3.2. Effect of linarin (LIN) on the proliferation of MCF-7/DOX cell lines

The anti-proliferative effect of linarin (LIN) on MCF-7/DOX cells was assessed using the MTT assay. Prior to combination experiments, the dose-response relationship of linarin in MCF-7/DOX cells was characterized across concentrations ranging from 25 to 800 μ M over 48 hours using the MTT assay. Linarin reduced cell viability in a concentration-dependent manner, with a calculated IC₅₀ of 387 ± 14 μ M (Figure 2). The working concentration of 200 μ M selected for subsequent experiments corresponds to approximately 52% of the IC₅₀, representing a sub-cytotoxic concentration at which direct linarin-mediated killing contributes minimally to the observed sensitization effect, thereby permitting attribution of enhanced doxorubicin cytotoxicity to modulation of resistance mechanisms. As shown in Figure 1B, in the presence of 200 μ M LIN, the IC₅₀ value of DOX was reduced from 41 ± 1 μ M to 25 ± 1 μ M ($p < 0.05$), indicating enhanced sensitivity of MCF-7/DOX cells to DOX when co-treated with linarin. To formally evaluate the nature of the drug interaction, combination index (CI) analysis was conducted using the Chou–Talalay method via CompuSyn software. CI values derived from MTT-based dose-response data

ranged from 0.61 to 0.78 across varying Fa levels in MCF-7/DOX cells, confirming a synergistic interaction ($CI < 1$) between linarin and doxorubicin (Table 2).

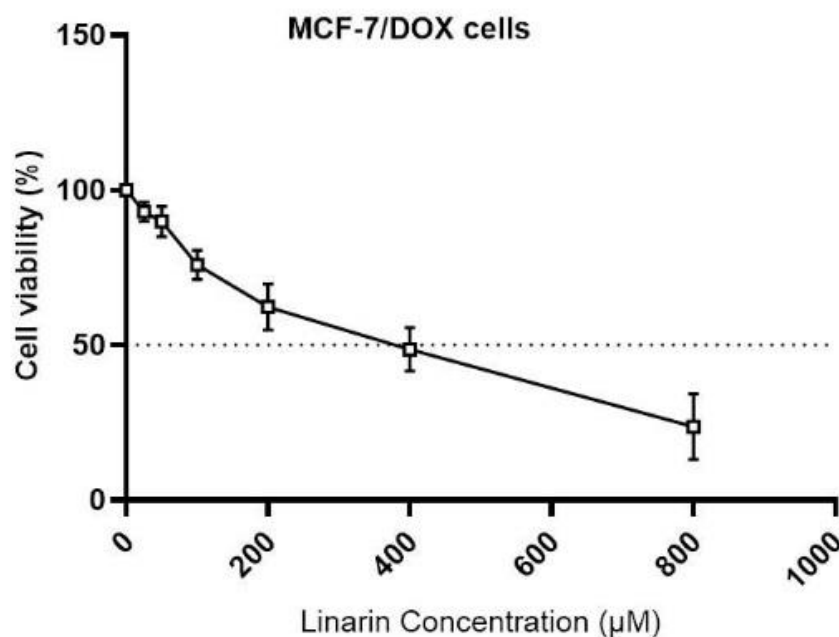


Figure 2. Dose-response data for linarin (LIN) in MCF-7/DOX cells after 48 hours of treatment, as assessed by the MTT assay ($n = 3$, mean $\pm$ SD).

Table 2. Combination index (CI) analysis of linarin and doxorubicin in mcf-7/dox cells using the Chou–Talalay method.

DOX Concentration (μM)	Linarin Concentration (μM)	Fraction Affected (Fa)	CI Value	Interaction
5	50	0.118	0.78	Synergism
10	100	0.254	0.74	Synergism
20	150	0.417	0.71	Synergism
40	200	0.613	0.66	Synergism
60	250	0.776	0.63	Synergism
80	300	0.879	0.61	Synergism

CI Interpretation (Chou–Talalay): $CI < 1$ = Synergism; $CI = 1$ = Additivity; $CI > 1$ = Antagonism. Data are presented as mean $\pm$ SD ($n = 3$). CI values were calculated using CompuSyn software. Fa = fraction of cells affected ($1 - \text{cell viability fraction}$). All CI values were statistically consistent across replicates.

3.3. Effect of DOX and LIN combination on MDR1 and PTEN gene expression

Gene Expression Results: For PTEN expression, all treatment groups exhibited a significant increase relative to the control. Specifically, as shown in Figure 3, PTEN expression was significantly higher in MCF-7 cells compared to the control ($t = 9.10$, $p = 0.0008$), and both linarin ($t = 5.50$, $p = 0.0053$) and doxorubicin (DOX) treatment ($t = 6.28$, $p = 0.0033$) led to a significant elevation. Notably, the combination treatment (DOX + linarin) induced the

greatest increase in PTEN expression ($t = 36.94$, $p < 0.00001$), showing a highly significant difference versus the control. For MDR1 expression, MCF-7 cells showed a significant decrease relative to the control ($t = -11.30$, $p = 0.0003$), indicating lower baseline MDR1 expression in sensitive cells.

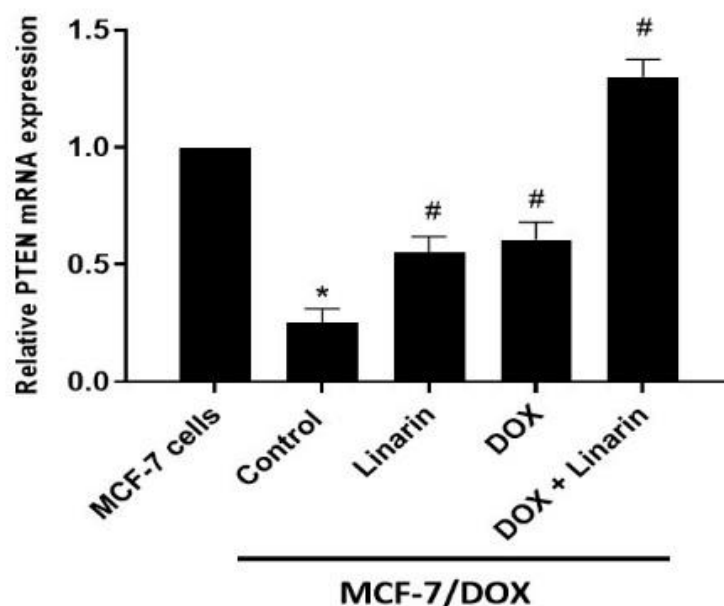


Figure 3. Relative PTEN mRNA expression ($2^{-\Delta\Delta CT}$, normalized to β -actin) in MCF-7/Dox cells after treatment with doxorubicin (DOX), linarin (LIN), and their combination for 48 hours. Data are presented as mean $\pm$ SD ($n=3$); * $p < 0.05$ vs MCF-7 cells, # $p < 0.05$ vs control.

As shown in Figure 4, treatment with linarin resulted in a significant reduction in MDR1 expression ($t = -3.66$, $p = 0.0215$), whereas DOX alone produced a non-significant decrease ($t = -2.57$, $p = 0.0618$). Importantly, the combination of DOX + linarin significantly decreased MDR1 expression compared to the control ($t = -8.57$, $p = 0.0010$), approaching the levels observed in non-resistant MCF-7 cells.

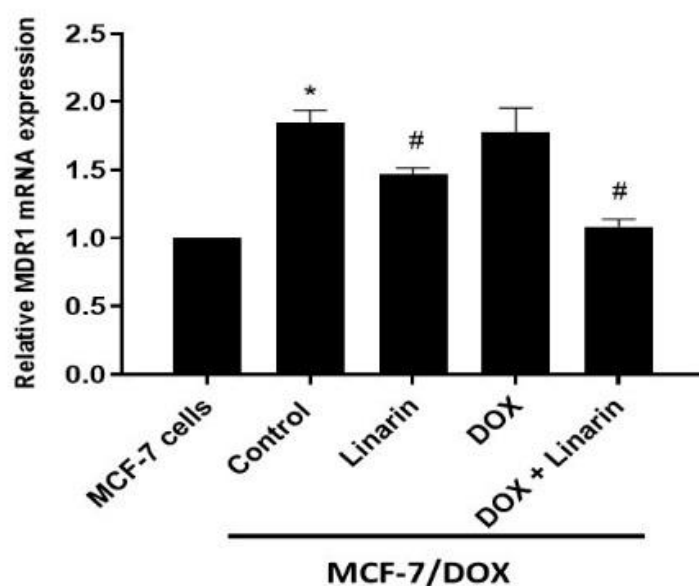


Figure 4. Relative MDR1 mRNA expression in MCF-7/Dox cells after treatment with doxorubicin (DOX), linarin (LIN), and their combination for 48 hours. Data are presented as mean $\pm$ SD ($n=3$); * $p < 0.05$ vs MCF-7 cells, # $p < 0.05$ vs control.

3.4. Apoptosis analysis results

In the control group, the baseline apoptosis rate was 14.3%, reflecting spontaneous cell death under untreated conditions. Treatment with linarin (LIN) increased apoptosis to 35%, representing a 244% elevation compared to the control ($p < 0.05$). Doxorubicin (DOX) treatment resulted in an apoptosis rate of 46.7%, corresponding to a 326% increase over the control ($p < 0.05$). Notably, the combination of DOX and LIN induced the highest apoptosis rate at 80%, marking a 559% increase relative to the control ($p < 0.001$) (as shown in Figure 5). Direct comparison between the treatments revealed that while both LIN and DOX independently promoted apoptosis, DOX exhibited a significantly stronger pro-apoptotic effect than LIN alone ($p < 0.01$). Moreover, the DOX + LIN combination exerted a significantly greater apoptotic effect than DOX monotherapy ($p < 0.0001$), suggesting a synergistic interaction between the two agents. This synergistic effect was further supported by Bliss independence analysis, which predicted an additive apoptotic rate of 65.3% for the combination, whereas the observed apoptosis rate was 80%, exceeding the expected effect by 14.7%.

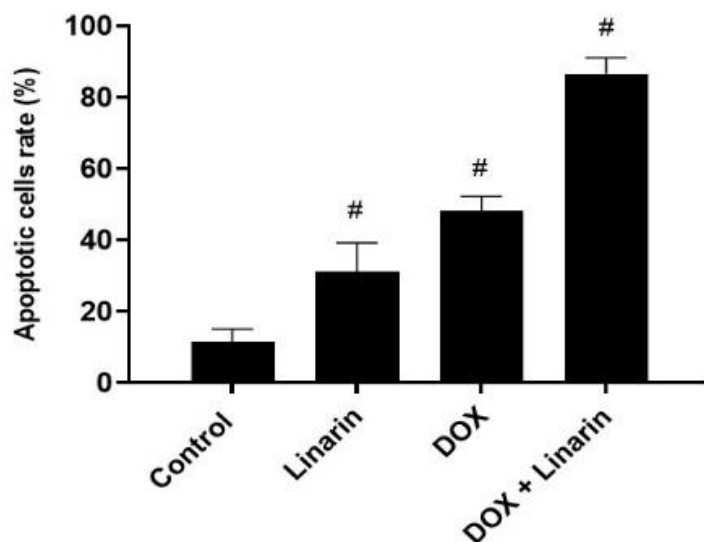


Figure 5. Apoptosis rates in MCF-7/DOX cells after treatment with doxorubicin (DOX), linarin (LIN), or their combination for 48 hours, as assessed by Annexin V/PI flow cytometry. Data are mean $\pm$ SD ($n=3$); # $p < 0.05$ vs control.

3.5. Effect of drug treatments on rhodamine 123 uptake

Both doxorubicin (DOX) and linarin treatment resulted in significant increases in mean fluorescence intensity (MFI) compared to the control. DOX treatment led to a significant increase ($t = 5.19$, $p = 0.0066$), and linarin treatment also produced a significant increase ($t = 6.57$, $p = 0.0028$). Notably, the combination treatment (DOX + linarin) induced the highest increase in MFI, which was highly significant compared to the control ($t = 12.45$, $p < 0.001$) (as shown in Figure 6).

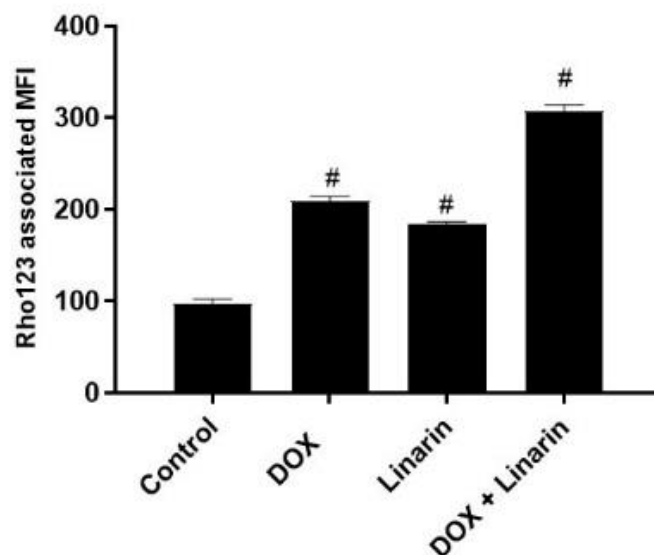


Figure 6. Mean fluorescence intensity (MFI) (arbitrary units, normalized to protein content) of rhodamine 123 in MCF-7/Dox cells after treatment with doxorubicin (DOX), linarin (LIN), or their combination. Data are mean $\pm$ SD (n=3); # $p < 0.05$ vs control. The fluorescence readout was normalized to total protein concentration determined by Bradford assay.

3.6. Effect of DOX and LIN on PTEN and MDR1 protein expression in MCF-7 and MCF-7/Dox cells

Combination treatment (DOX + LIN) induced a statistically significant upregulation of PTEN protein expression ($p < 0.05$, as shown in Figure 7). Linarin alone did not exhibit a significant effect on PTEN protein levels in the doxorubicin-resistant cell line (MCF-7/Dox). A significant downregulation of MDR1 protein expression was observed in the combination group (DOX + LIN) ($p < 0.05$, as shown in Figure 8). Linarin monotherapy also significantly reduced MDR1 protein expression ($p < 0.05$).

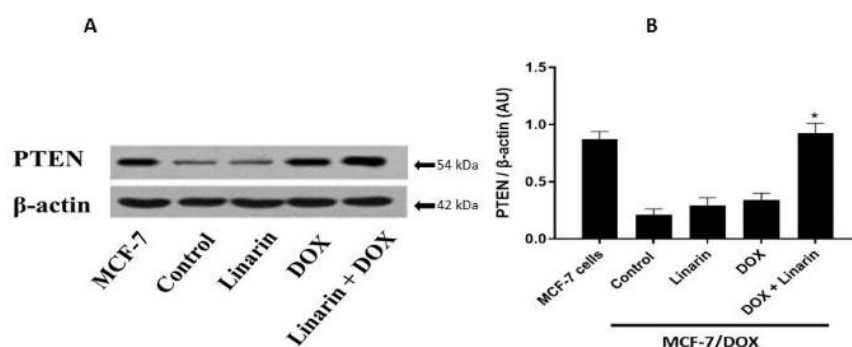


Figure 7. A: PTEN protein expression in MCF-7/Dox cells after treatment with doxorubicin (DOX), linarin (LIN), or their combination, assessed by western blot. Data are mean $\pm$ SD (n=3); $p < 0.05$ vs. control. **B:** Densitometric quantification of PTEN protein expression by western blot in MCF-7/Dox cells across experimental treatment groups (n = 3, mean $\pm$ SD,

normalized to β -actin). AU = arbitrary units. Values represent band intensities of the target protein normalized to the corresponding β -actin band intensity within each lane, quantified using ImageJ software (version 1.54, NIH). Statistical significance was determined by one-way ANOVA followed by Tukey's post-hoc test. * $p < 0.05$ versus MCF-7/DOX control. Data represent mean $\pm$ SD from three independent biological replicates.

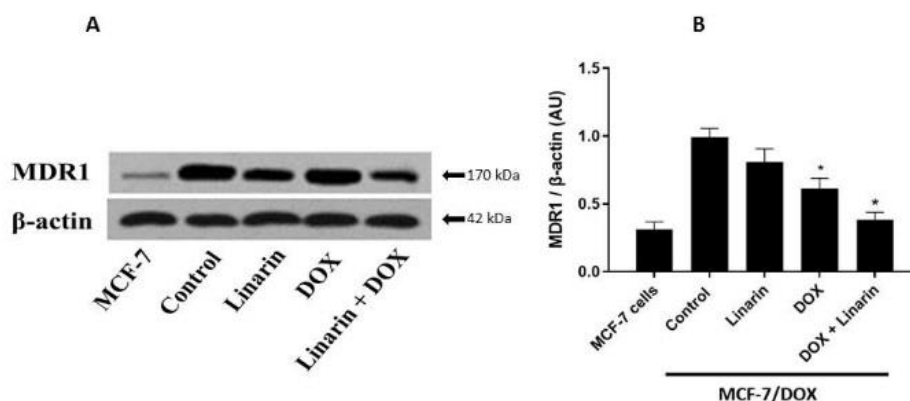


Figure 8. A: MDR1 protein expression in MCF-7/DOX cells after treatment with doxorubicin (DOX), linarin (LIN), or their combination, assessed by western blot. Data are mean $\pm$ SD ($n=3$); $p < 0.05$ vs. control. **B:** Densitometric quantification of MDR1 protein expression by western blot in MCF-7/DOX cells across experimental treatment groups ($n = 3$, mean $\pm$ SD, normalized to β -actin). AU = arbitrary units. Values represent band intensities of the target protein normalized to the corresponding β -actin band intensity within each lane, quantified using ImageJ software (version 1.54, NIH). Statistical significance was determined by one-way ANOVA followed by Tukey's post-hoc test. * $p < 0.05$ versus MCF-7/DOX control. Data represent mean $\pm$ SD from three independent biological replicates.

4. Discussion

Drug resistance in cancer, particularly in metastatic breast cancer, poses a major impediment to chemotherapy, often driven by compensatory signaling, target mutations, and enhanced drug efflux defining the MDR phenotype^{44,45}. In MDR, tumor cells simultaneously resist structurally diverse agents such as doxorubicin, paclitaxel, and vinorelbine^{46,47}. Central to this process is overexpression of ATP-binding cassette transporters, most notably P-gp, which actively extrude chemotherapeutics from cells^{48,49}. Alarming, nearly 90% of patients with metastatic breast cancer develop MDR, underscoring its profound impact on treatment outcomes⁵⁰.

Doxorubicin is a cornerstone of breast cancer therapy, owing to its potent inhibition of cell proliferation, induction of DNA damage, and activation of apoptotic pathways. In early stage disease, response rates often exceed 60–70%; however, in advanced and metastatic settings, intrinsic or acquired resistance sharply diminishes its clinical benefit. Biganzoli et al. reported that in patients receiving doxorubicin based regimens for metastatic breast cancer, the incidence of treatment failure due to resistance rose from under 20% at initial relapse to over 50% in later lines of therapy, underscoring the progressive loss of efficacy and the urgent need for strategies to overcome doxorubicin resistance⁵¹. In recent years, various approaches such as the use of CRISPR/Cas9 technology and nanoparticles have been proposed to overcome chemoresistance in cancer cells^{52,53}. The use of natural bioactive compounds as adjuvants has also been among the proposed approaches for this purpose, which has attracted the attention of some

researchers. Among these compounds are flavonoids. Plant derived flavonoids have emerged as attractive agents for overcoming chemoresistance, owing to their low toxicity and ability to target multiple cellular pathways⁵⁴. By inhibiting drug efflux pumps such as P-gp, inducing mitochondrial mediated apoptosis, modulating pro survival signaling cascades (e.g., PI3K/Akt, NF- κ B), and altering epigenetic marks, flavonoids can effectively restore tumor cell sensitivity to a broad spectrum of chemotherapeutics⁵⁵. Although compounds like quercetin and fisetin have been extensively characterized, linarin remains underexplored. This flavone glycoside is distinguished by its rutinose moiety and enhanced aqueous solubility. Early reports suggest that linarin can synergize with doxorubicin to reduce IC₅₀ values and upregulate tumor suppressors such as PTEN, yet its full potential against multidrug-resistant cancer phenotypes has not been systematically evaluated. This gap highlights linarin as a promising candidate for adjuvant therapy in resistant breast cancers. Findings in this research indicate that Linarin effectively overcomes doxorubicin resistance in MCF-7/DOX cells by simultaneously upregulating PTEN and downregulating MDR1 expression. As a result, P-gp-mediated drug efflux is diminished, enhancing intracellular retention of chemotherapeutics.

Experimental results from the Rhodamine 123 uptake assay, followed by detection of cellular apoptosis, confirm Linarin's efficacy in reducing resistance to doxorubicin in the resistant cell line. This functional overlap with other flavonoids reinforces Linarin's role as a complementary agent in combination therapies. Our findings are consistent with previous reports demonstrating the MDR reversing effects of flavonoids. Costea et al. showed that various flavonoids can inhibit P-gp activity, while Li et al. demonstrated that quercetin not only suppresses P-gp-mediated drug efflux in breast cancer cells but also synergizes with agents such as doxorubicin, paclitaxel, and vincristine to enhance their cytotoxicity^{56,57}.

Although downregulation of P-gp in breast cancer cells may be a promising approach to overcome chemoresistance, the functions of P-gp in normal tissues should also be considered. P-gp is abundantly expressed in the intestinal epithelium, where it serves as an efflux transporter that limits the absorption of xenobiotics and toxic compounds^{58,59}. P-gp plays a protective role in intestinal mucus. It has been shown that pathological events such as increased crypt length, goblet cell loss, and inflammatory thickening of the mucosa occur in colon tissue of MDR1A KO mice⁶⁰. Therefore, systemic inhibition of P-gp may impair these protective mechanisms and increase susceptibility to impairment of mucosal integrity and tissue damage. Therefore, in-vivo studies are needed to evaluate the effect of the used dose of linarin on intestinal mucus.

Furthermore, treatment with 200 μ M linarin markedly lowered the doxorubicin IC₅₀ in resistant MCF-7/DOX cells, reflecting restored drug sensitivity. Importantly, formal combination index analysis using the Chou–Talalay method confirmed a synergistic, rather than merely additive, interaction between linarin and doxorubicin in resistant MCF-7/DOX cells. CI values consistently below 1.0 across multiple effect levels provide robust quantitative evidence for synergism, strengthening the interpretation that linarin acts through complementary mechanisms to enhance doxorubicin efficacy rather than simply producing independent cytotoxic effects. While 200 μ M of linarin was effective in vitro, its clinical relevance remains uncertain without pharmacokinetic validation in vivo. Comparable findings have been reported by Niazvand et al., where quercetin-loaded nanoparticles similarly reduced the IC₅₀ and triggered apoptosis in MCF-7 cells⁶¹. Further analysis revealed that PTEN mRNA levels were markedly lower in MCF-7/DOX cells than in drug-sensitive counterparts. Combined treatment with linarin and doxorubicin restored PTEN expression at both transcript and protein levels to exceed those of sensitive cells, whereas linarin alone had no significant effect.

The observed synergy appears to stem from modulation of the PTEN/Akt/mTOR axis and inhibition of cell proliferation. These findings align with prior reports; Jiang et al. showed that quercetin upregulates PTEN and

downregulates AKT/mTOR signaling to inhibit proliferation and promote apoptosis in MCF-7 cells, and Li et al. found that fisetin elevates PTEN levels and impedes metastasis via AKT/GSK3 β pathway inhibition^{62,63}. Collectively, this study highlights the multi-mechanistic role of linarin in reversing chemoresistance by simultaneously downregulating MDR1/P-gp, restoring PTEN expression, and enhancing doxorubicin-induced apoptosis, while also demonstrating a synergistic interaction with doxorubicin. However, our study has some limitations. One of these limitations is that our study lacks an in-vivo component, so we cannot speak with complete certainty about the therapeutic effect of linarin in weakening doxorubicin resistance, and animal model studies are needed to evaluate and confirm the results of our study. These studies should also analyze the pharmacokinetics and bioavailability of linarin. On the other hand, despite the fact that our study showed the effect of linarin in up-regulation of PTEN, we were unable to analyze downstream signaling, namely AKT/mTOR signaling, due to financial limitations. Therefore, future studies should focus on analyzing this pathway by examining the levels of factors such as phospho-Akt and mTOR.

5-Conclusion

Our findings demonstrate that linarin effectively reverses chemoresistance in doxorubicin-resistant MCF-7/DOX breast cancer cells through multiple complementary mechanisms: it downregulates MDR1 and inhibits P-gp activity to increase intracellular drug accumulation, restores PTEN expression to suppress pro-survival PI3K/AKT signaling, and promotes apoptosis via synergistic interactions with doxorubicin. Together, these results highlight linarin's potential as a promising adjuvant to enhance the efficacy of doxorubicin in resistant breast cancers. In summary, linarin demonstrated a sensitizing effect on doxorubicin-resistant MCF-7/DOX cells by reducing the IC₅₀ of doxorubicin. Formal combination index analysis confirmed a synergistic interaction between linarin and doxorubicin (CI = 0.61–0.78), providing quantitative support for linarin's potential as an adjuvant sensitizer in doxorubicin-resistant breast cancer. However, comprehensive pharmacokinetic and in vivo studies, are warranted to confirm these effects and fully elucidate linarin's therapeutic potential.

Acknowledgements

The authors would like to thank Shohada Clinical Research Development Unit, Shohada Hospital, Tabriz University of Medical Science, Tabriz, Iran.

Ethical issues

This paper is a part of the Master's Degree thesis of Vahid Mahmoudzadeh approved by the Research Ethics Committees of Islamic Azad University -Shahrood Branch (IR.IAU.SHAHROOD.REC.1402.118).

Competing interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used ChatGPT (OpenAI) solely to improve the readability and language of the manuscript. After using this tool, the authors carefully reviewed and edited the content, and take full responsibility for the final version of the manuscript.

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