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Original article

Antioxidant and chemotherapeutic synergy: Triphala enhances doxorubicin cytotoxicity in breast cancer cells and reduces toxicity in non-tumorigenic cells

Supamas Charucharana[a, b], Poonlarp Cheepsunthorn[c], Chalisa Louicharoen Cheepsunthorn[d, *]

[a]*Medical Sciences Program, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand*

[b]*Department of Applied Thai Traditional Medicine, Faculty of Sciences and Technology, Phranakhon Rajabhat University, Bangkok, Thailand*

[c]*Department of Anatomy, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand*

[d]*Department of Biochemistry, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand*

Abstract

Background: The combination of antioxidants with chemotherapy is increasingly being explored to minimize toxicity in noncancerous cells during breast cancer treatment. Triphala (TPL), a Thai herbal compound rich in antioxidants, shows potential as a complementary candidate for use in breast cancer chemotherapy.
Objective: This study investigated the combined effects of TPL and low-dose doxorubicin (DOX) on human breast cancer and non-tumorigenic mammary epithelial cells.
Methods: TPL's bioactive compounds were analyzed using high-performance liquid chromatography. Cell viability and the levels of reactive oxygen species (ROS) were assessed in breast cancer (MDA-MB-231, MCF-7) and epithelial (MCF-10A) cells using MTT and chloromethyl 22 ,72 -dichlorodihydrofluorescein diacetate assays performed in triplicate. The combination index (CI) values were determined by CompuSyn. Furthermore, the mRNA expression of apoptosis- and antioxidant-related genes was evaluated using quantitative polymerase chain reaction (qPCR).
Results: Gallic acid (11.9%) was identified as the major component in TPL. The combination of TPL and low-dose DOX synergistically enhanced the cytotoxicity in MCF-7 and MDA-MB-231 cells. This combination significantly reduced the expression of the antioxidant genes superoxide dismutase 1 (*SOD1)* and glutathione peroxidase 1 *(GPX1)* in MDA-MB-231 (*SOD1:* $P < 0.001$, *GPX1:* $P = 0.035$) and MCF-7 (*SOD1:* $P = 0.035$, *GPX1:* $P = 0.036$) cells, which resulted in increased ROS levels in MDA-MB-231 ($P = 0.005$) and MCF-7 ($P = 0.008$) cells. Elevated ROS triggered apoptosis via the increased Bcl-2 associated X-protein *(BAX)*/B-cell lymphoma 2 (*BCL2)* ratio in MDA-MB-231 ($P < 0.001$) and MCF-7 ($P = 0.02$) cells. Conversely, TPL displayed protective effects in nontumorigenic MCF-10A cells by upregulating *SOD1* ($P = 0.031$) and *GPX1* ($P < 0.001$), reducing ROS ($P = 0.002$), and lowering the *BAX*/*BCL2* ratio ($P < 0.001$), thereby promoting cell survival.
Conclusion: TPL, in combination with low-dose DOX, effectively induces cytotoxicity in breast cancer cells while protecting non-tumorigenic cells, which suggests its potential as complementary therapy in breast cancer treatment.

Keywords: Antioxidant therapy, breast cancer, chemotherapy, doxorubicin, gallic acid, reactive oxygen species ,synergistic effect, triphala.

***Correspondence to: Chalisa Louicharoen Cheepsunthorn,** Department of Biochemistry, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand.
E-mail: Chalisa.l@chula.ac.th
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Redox homeostasis refers to the dynamic balance between the production of reactive oxygen species (ROS) by mitochondria and the activity of the cellular antioxidant systems that remove excess ROS [1], a balance that is essential for cell survival and normal physiological processes. However, in cancer cells, maintaining redox homeostasis is equally crucial for their survival and proliferation due to their altered metabolic states; thus, the cell viability of both non-tumorigenic and cancer cells necessitates that the redox balance be tightly regulated. [2] A hallmark of cancer is the presence of increased oxidative stress, driven by elevated ROS levels, which can activate oncogenes and thereby promote tumor progression. [3] In non-tumorigenic cells, basal ROS levels are kept low to maintain homeostasis and prevent oxidative damage; however, cancer cells exhibit substantially higher levels of ROS due to mitochondrial dysfunction and increased metabolism. While these elevated ROS levels can support cancer cell survival and proliferation, the excessive generation of ROS can lead to cellular damage. Thus, cancer cells enhance their antioxidant defenses to mitigate this oxidative stress and maintain redox balance under these challenging conditions. [4] Breast cancer is the most prevalent cancer in women and the leading cause of cancer-related mortality worldwide, with an estimated 2.3 million new cases diagnosed annually. [5] It is clinically classified based on the tumor location, metastatic stage, and molecular markers, including estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2). Among the breast cancer subtypes, triple-negative breast cancer (TNBC) is characterized by the absence of ER, PR, and HER2 expression, which makes it unresponsive to hormone and HER2-targeted therapies. As a result, chemotherapy remains the primary treatment option for TNBC, which has a poorer prognosis compared with the other breast cancer subtypes due to its aggressive nature and high rate of recurrence. [6] Notably, TNBC cells exhibit the highest basal ROS levels among the breast cancer subtypes, which suggests that ROS could serve as both a biomarker and therapeutic target for TNBC. [7] Chemotherapy, by increasing oxidative stress to harmful levels, can induce cancer cell death, thus highlighting the dual role of ROS in both promoting and suppressing cancer progression. [8]

Doxorubicin (DOX), a commonly used anthracycline chemotherapeutic agent, is effective in treating breast cancer by inhibiting cell proliferation and inducing oxidative stress, which leads to apoptosis. [9] However, the use of chemotherapy is limited by its associated toxicity, particularly the detrimental effects of ROS on non-tumorigenic tissues. [10] Therefore, strategies that minimize ROS-induced damage to non-tumorigenic cells during chemotherapy while maintaining the drug's efficacy against cancer cells are highly desirable.

Recent evidence has suggested that cancer cells can modulate their antioxidant capacity to maintain a redox balance separately from that of non-tumorigenic cells. Disrupting this balance by modulating the levels of ROS in tumor cells could suppress oncogenic signaling and reduce cancer cell survival. [11] Several studies have shown that antioxidant treatment can decrease the proliferation and induce cell death in TNBC cells, which suggests that antioxidants may play a therapeutic role by reducing ROS levels in cancer therapy. [12, 13] Importantly, the use of natural antioxidants in combination with chemotherapy has garnered attention for its potential to enhance the cytotoxic effects of chemotherapy while protecting non-tumorigenic tissues from ROS-induced damage. [14]

Modern medicine is increasingly focusing on the use of natural substances for cancer prevention and treatment, particularly antioxidant-rich herbal remedies. Among these, triphala (TPL), a traditional Ayurvedic herbal formulation comprising equal parts of *Terminalia chebula*, *Terminalia bellerica*, and *Phyllanthus emblica*, is recognized for its strong antioxidant properties due to polyphenolic compounds, such as gallic acid, ellagic acid, and tannins, which are abundantly present. [15] Compared to other herbal remedies, TPL offers a unique advantage because of its combined bioactive compounds that act synergistically to exert potent antioxidant and anticancer effects. Unlike single-herb formulations, which may have limited efficacy, TPL's multi-component nature enhances its bioavailability and thereby its therapeutic potency. [16] In addition, TPL has demonstrated broad-spectrum anticancer activity against various types of cancer cells, including breast cancer, by modulating the redox balance and inducing apoptosis while minimizing non-tumorigenic cell toxicity. [17-19] Several polyphenols have been shown to exert considerable anticancer effects by targeting redox homeostasis in breast cancer cells. [20] Gallic acid has been reported to inhibit breast cancer cell

proliferation and induce apoptosis by increasing the generation of ROS. [21] Ellagic acid has been found to modulate oxidative stress and suppress tumor progression, thus making it a promising compound for breast cancer therapy. [22] These findings suggest that TPL's polyphenol-rich composition could provide a multifaceted approach to disrupt the redox homeostasis in breast cancer cells while protecting normal cells from oxidative damage.

Given these antioxidant and anticancer properties of TPL, it is a promising candidate for combination therapy with chemotherapeutic agents such as DOX. Combining TPL with DOX may enhance cancer cell apoptosis via increased oxidative stress while simultaneously mitigating ROS-induced damage in non-tumorigenic cells. This study aimed to investigate the combined effect of an ethanolic extract of TPL and DOX on breast cancer cell survival and apoptosis. In addition, we evaluated the role of TPL in modulating the antioxidant response and redox homeostasis to protect non-tumorigenic breast epithelial cells from DOX-induced cytotoxicity.

Materials and methods

The study was reviewed and approved by the Institute Ethics Committee of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand (IRB no. 511/64).

TPL extraction process

Dried powders of *T. chebula*, *T. bellerica,* and *P. emblica* were purchased from Vejpong Pharmacy Co., Ltd. (Bangkok, Thailand). The TPL ethanolic extract was prepared according to the method described by Jantarach PC, *et al*., and Nutsuda B BS, *et al*., with slight modifications. [23, 24] Briefly, 5 g of TPL powder was macerated in 100 mL of 95.0% ethanol (Merck, USA) in a foil-covered flask. The mixture was gently shaken at room temperature for 6 h and allowed to stand for a further 18 h. The extract was then filtered through 11-µm Whatman filter paper, and the supernatant was dried at 105°C until it reached a constant weight. The dried extract was protected from light and stored at –20°C for future use.

Preparation of TPL and DOX

The stock solution of TPL was prepared by dissolving the dried extract in 5.0% dimethyl sulfoxide (DMSO) (D2660, Sigma-Aldrich, MO, USA) to a concentration of 10 mg/mL. Working solutions of TPL were prepared by diluting the stock solution in the cell culture medium and filtering through a 0.2-µm sterile syringe filter (Johnson Test Papers Ltd., Oldbury, UK) before treatment. DOX (D5220, Sigma-Aldrich) was dissolved in DMSO at a concentration of 10 mM and stored at –20°C and protected from light. DOX was diluted in the cell culture medium directly before use, and the DMSO concentration did not exceed 0.1%, a level that does not induce DMSO-related cytotoxicity. [25] All appropriate working dilutions in the cell culture medium were prepared immediately before testing.

Determination of scavenging activity

The 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay was used to assess the antioxidant activity of the TPL extract, according to a previously described method. [26] Various concentrations of TPL (10, 25, 50, 75, 100, 150, 200, 300, 400, and 500 µg/mL) were mixed with 100 µL of 0.2 mM DPPH (D9132, Sigma-Aldrich) in methanol. After vortexing, the reaction mixtures were incubated in the dark at 37°C for 30 min. The absorbance was measured at 515 nm using a microplate reader (Biotek Synergy, CA, USA). Gallic acid (27645, Sigma-Aldrich) was used as a positive control, and phosphate-buffered saline was used as the blank. The scavenging activity was calculated using the following equation:

$$\text{DPPH scavenging activity (\%)} = (A_0 - A_1) / A_0 \times 100,$$

where A_0 is the absorbance of the control and A_1 is the absorbance of the sample.

High-performance liquid chromatography quantification

The ethanolic extract of TPL was analyzed by high-performance liquid chromatography (HPLC; Agilent 1260 series, Agilent Technologies, CA, USA) using a photodiode array detector. The separation was performed on a Poroshell 120 EC-C18 column (2.7 µm, 4.6 × 150 mm) at 26°C, with a mobile phase consisting of 0.1% acetic acid (solvent A) and acetonitrile (solvent B). [27] The flow rate was set at 1 mL/min, and the injection volume was 20 µL. Detection was performed at 270 nm, with gallic acid serving as the reference standard. The TPL extract (50 µg/mL) and gallic acid standards (25–100 µg/mL) were analyzed in triplicate. Data were processed using the Agilent ChemStation software (C.01.10, Agilent Technologies).

Cell culture

The human breast cancer cell lines MDA-MB-231 (hormone-independent breast cancer) and MCF-7 (epithelial luminal cell line positive for ER and PR) were obtained from the American Type Culture Collection (ATCC, VA, USA). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; HyClone, UT, USA) supplemented with 5.0% heat-inactivated fetal bovine serum (Gibco, MA, USA), 100 U/mL penicillin/streptomycin (Gibco), and 100 U/mL HEPES (Gibco). Human mammary epithelial cells (MCF-10A) were cultured in DMEM/F12 (HyClone) supplemented with 20 ng/mL epidermal growth factor (PeproTech, NJ, USA), 100 ng/mL cholera toxin (Sigma, USA), 10 µg/mL insulin (Sigma-Aldrich), 500 ng/mL hydrocortisone (Sigma-Aldrich), and 5.0% horse serum (Gibco). All cells were maintained at 37°C in a 5.0% CO_2 and 95.0% humidity incubator and passaged twice weekly (BINDER, Tuttlingen, Germany). Cells at 5–20 passages were used in the experiments.

Cytotoxicity and combination index determination

To investigate the optimal concentrations of TPL and DOX, cells were seeded in 96-well plates (Corning, NY, USA) at a density of 4×10^4 cells/well and incubated for 24 h. The cells were then treated with different concentrations of TPL (25–2000 µg/mL) and DOX (0.1–10 µM) for 48 h to determine the half-maximal inhibitory concentration (IC_{50}) values. Cell viability was assessed using the MTT assay (M5655, Sigma-Aldrich). After the treatment period, the cells were incubated with 2 mg/mL MTT reagent for 4 h at 37°C, and the resulting formazan crystals were solubilized in DMSO. Absorbance (OD) was measured at 570 nm, and cell viability was calculated using the following equation:

$$\text{Cell viability (\%)} = (OD_{treatment} / OD_{control}) \times 100$$

For the combination studies, non-toxic doses of TPL (50–200 µg/mL) were combined with DOX (2.5 µM), and the combination index (CI) was calculated using the Chou-Talalay method equation via CompuSyn software:

$$\text{Combination index (CI)} = [(D)_1/(Dx)_1] + [(D)_2/(Dx)_2],$$

where $(D)_1$ and $(D)_2$ are the concentrations of TPL and DOX, respectively, which were used together to achieve 50.0% of the drug effect. $(D_x)_1$ is the concentration of TPL alone, and $(Dx)_2$ is the concentration of DOX as a single chemotherapeutic agent to achieve the same effect. The CI values were interpreted as follows: CI < 1 indicates synergism, CI = 1 indicates an additive effect, and CI > 1 indicates antagonism. [28, 29]

Measurement of intracellular ROS levels

Intracellular ROS levels were measured using the CM-H_2DCFDA assay (Invitrogen, MA, USA). Cells were seeded in a black, clear-bottomed 96-well plate at a density of 4×10^4 cells/well (Corning), treated as indicated, and then incubated with Hanks' balanced salt solution (HBSS; Gibco) containing 10 µM H_2DCFDA for 30 min at 37°C. After the H_2DCFDA was removed, the cells were washed and incubated in HBSS in the dark at 37°C for 6 h. Fluorescence was measured at an excitation of 495 nm and emission of 525 nm using a microplate reader. The ROS levels were normalized to cell viability and expressed relative to the untreated controls.

Reverse Transcription PCR

Total RNA was extracted using Trizol reagent (Thermo Fisher Scientific, MA, USA), and cDNA synthesis was performed using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). Gene expression was quantified using SYBR Green-based qPCR with the primers listed in **Table 1**. The qPCR master mix was 5 µL of 2× Maxima SYBR Green, 2 µL of nuclease-free water, and 2 µM of the forward/reverse primers. The cDNA template was added to reach a final volume of 10 µL. Reactions were run in a thermocycler (Thermo Fisher Scientific) under the following conditions: 95°C for 15 min, followed by 40 cycles of 95°C for 15 s, primer-specific annealing temperatures for 30 s, and 72°C for 30 s. Gene expression was normalized to β-actin and analyzed using the $^{\Delta\Delta}$Ct method.

Statistical analysis

Data were analyzed using SPSS 29.0 software. All experiments were performed in triplicate (n = 3). Data are presented as the mean ± standard error of the mean (SEM). One-way analysis of variance was used to assess differences between the means of the study groups. Tukey's multiple comparison test was employed to compare differences between the control, single-drug, and combination groups. $P < 0.05$ was considered statistically significant.

Table 1. Primer sequences used for qPCR reactions.

Gene	Sequence 5' →3'	Annealing temperature (°C)
β-*actin*	Forward primer: ACTCTTCCAGCCTTCCTTC	55°C
	Reverse primer: ATCTCCTTCTGCATCCTGTC	
CAT	Forward primer: TGGAGCTGGTAACCCAGTAG	55°C
	Reverse primer: CCTTTGCCTTGGAGTATTTGG	
SOD1	Forward primer: GGTGGGCCAAAGGATGAAGA	52°C
	Reverse primer: CCACAAGCCAAACGACTTCC	
GPX1	Forward primer: CAGTCGGTGTATGCCTTCTCG	56°C
	Reverse primer: GAGGGACGCCACATTCTCG	
BAX	Forward primer: AACATGGAGCTGCAGAGGAT	57°C
	Reverse primer: CAGCCCATGATGGTTCTGAT	
BCL2	Forward primer: CGGTTCAGGTACTCAGTCATC	57°C
	Reverse primer: GGTGGGGTCATGTGTGTGG	

BAX, Bcl-2 associated X-protein; B-cell lymphoma 2; CAT, catalase; GPX1, glutathione peroxidase 1; SOD1, superoxide dismutase 1.

Results

Free radical scavenging activity

The free radical scavenging activities of the TPL ethanolic extract were compared with the standard antioxidant, gallic acid, using the DPPH assay (**Figure 1A**). The free radical scavenging activity increased with increasing concentrations of both the TPL extract and gallic acid. At their highest concentrations (200 µg/mL for TPL and 75 µg/mL for gallic acid), the scavenging activities were 92.7 ± 0.3 and 94.3 ± 1.1, respectively, indicating that the extract displayed potent antioxidant activity comparable to that of gallic acid.

HPLC quantification of TPL

HPLC analysis was used to identify the major bioactive compounds in the TPL extract, with gallic acid as the reference standard. The results confirmed that gallic acid was the predominant phenolic compound, with a retention time of 2.8 min and an area of 3,062 ± 10.8 mAU × s (33.3 ± 0.3%) (**Figure 1B**). The gallic acid concentration in the TPL ethanolic extract was determined to be 129.1 ± 0.6 µg/mL, thus establishing its considerable presence in the extract.

Basal ROS levels in breast cancer cell lines

The ROS levels were measured in the breast cancer cell lines MCF-7 and MDA-MB-231 and compared with that of the MCF-10A non-tumorigenic mammary epithelial cells. The results revealed significantly higher levels of ROS in both cancer cell lines relative to that of MCF-10A, with the MCF-7 and MDA-MB-231 cell lines exhibiting a 1.9-and 2.4-fold increase, respectively ($P < 0.001$). MDA-MB-231 displayed the highest basal ROS levels (**Figure 1C**), which suggests that elevated ROS levels may contribute to cancer cell survival.

Cytotoxicity of TPL on breast cancer and epithelial cell lines

To evaluate the cytotoxic effects of TPL, MDA-MB-231, MCF-7, and MCF-10A cells were treated with increasing concentrations of TPL extract for 48 h. The viability of all cell lines decreased in a concentration-dependent manner (**Figure 2A-C**). The IC_{50} values for the TPL ethanolic extract were 0.7 mg/mL for MDA-MB-231, 0.7 mg/mL for MCF-7, and 1.8 mg/mL for MCF-10A, with non-toxic concentrations (50–200 µg/mL) being selected for further combination treatments.

Cytotoxicity of DOX on breast cancer cells

MDA-MB-231 and MCF-7 cells treated with DOX for 48 h exhibited reduced cell viability in a dose-dependent manner. The IC_{50} values were determined to be 5.0 µM for MDA-MB-231 and 4.6 µM for MCF-7 (**Figure 2D-E**). Based on these results, a 2.5 µM concentration of DOX was selected for further combination treatment studies.

Combination effect of TPL and DOX on breast cancer and epithelial cell lines

Low-dose DOX (2.5 µM) significantly reduced the cell viability of MDA-MB-231, MCF-7, and MCF-10A cells by 75.3%, 70.4%, and 44.1%, respectively ($P < 0.001$) (**Figure 3A, C, E**). The TPL ethanolic extract at concentrations of 100–200 µg/mL significantly reduced the viability in both MDA-MB-231 (76.6–81.4%, $P = 0.006$) and MCF-7 (74.4–86.3%, $P = 0.008$) cells. Combination treatment of TPL and DOX resulted in a synergistic effect **(Figure 3B, D, Table 2)**, with cell viability in MDA-MB-231 reduced to 27.9–38.8% ($P < 0.001$) and MCF-7 to 35.5–41.4% ($P < 0.001$). Notably, TPL did not affect

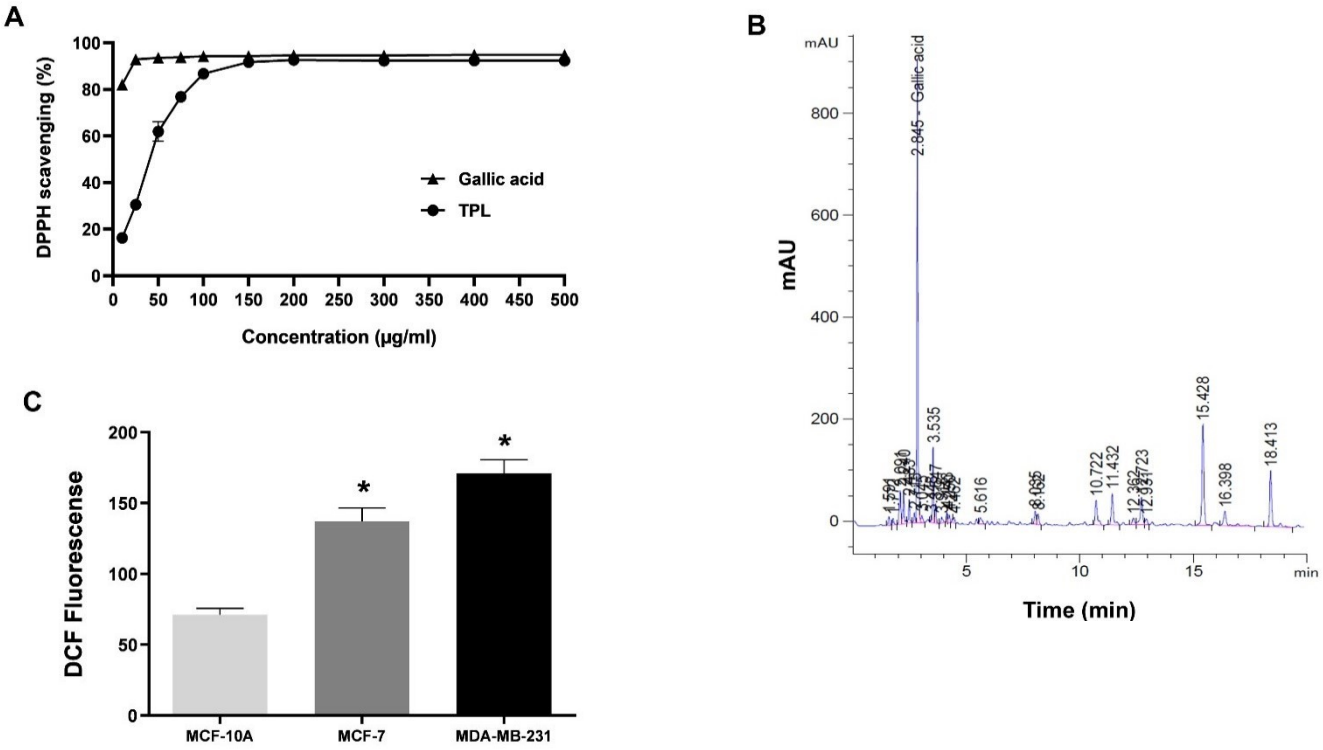

Figure 1. (**A**) DPPH free radical scavenging activity of TPL ethanolic extract compared to gallic acid; (**B**) HPLC chromatogram representing the composition of TPL ethanolic extract; (**C**) Basal ROS levels in MDA-MB-231, MCF-7, and MCF-10A cells. Data are presented as mean ± SEM from three independent experiments performed in triplicate. (*$P < 0.05$ compared to MCF-10A). DPPH , 1, 1-diphenyl-2-picrylhydrazyl; HPLC, high-performance liquid chromatography; ROS, reactive oxygen species, SEM, standard error of the mean; TPL, Triphala.

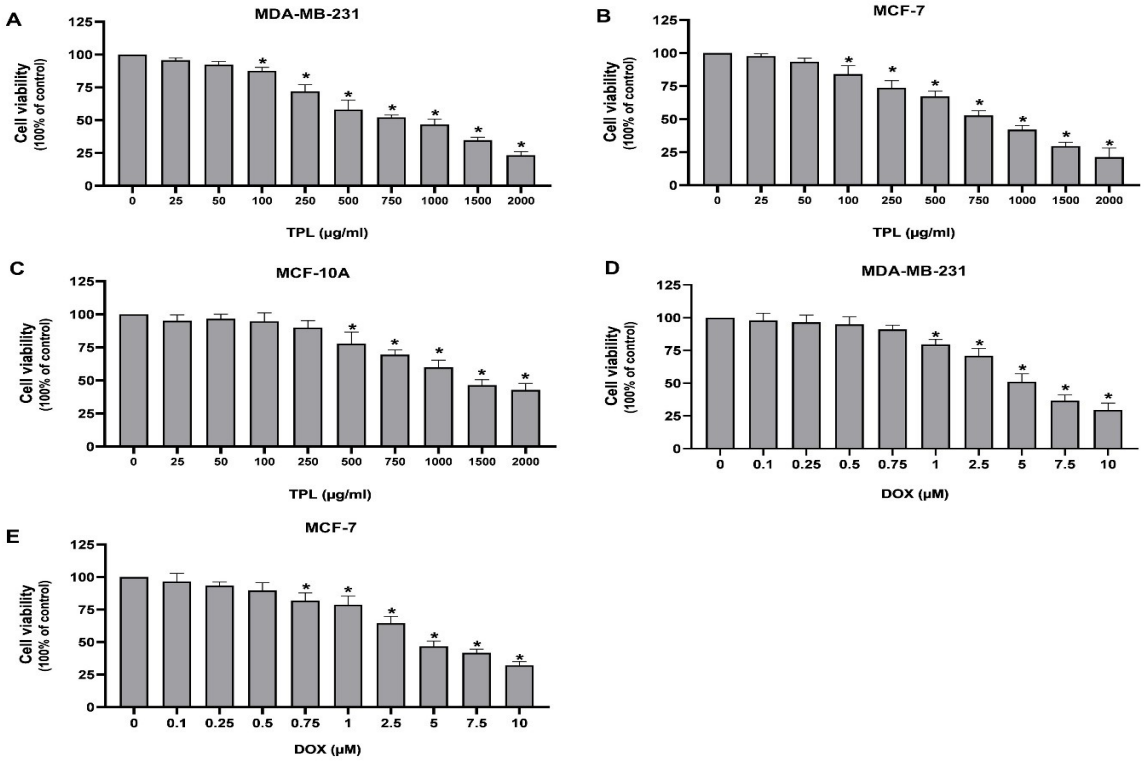

Figure 2. Effect of TPL on cell viability in MDA-MB-231 **(A)**; MCF-7 **(B)**; and MCF-10A **(C)** cells; and DOX on MDA-MB-231 **(D)**; and MCF-7 **(E)**. Data are from three independent experiments. (*$P < 0.05$ value compared to untreated cells). DOX, doxorubicin; TPL, Triphala.

Table 2. CI values of low-dose DOX and TPL in MDA-MB-231, MCF-7, and MCF-10A cells.

2.5 µM DOX + TPL (µg/ml)	CI value (MDA-MB-231)	CI value (MCF-7)	CI value (MCF-10A)
50	0.8	0.9	1.3
75	0.7	0.9	1.6
100	0.4	0.5	2.0
200	0.3	0.4	2.4

CI, combination index; DOX, doxorubicin; TPL, Triphala.

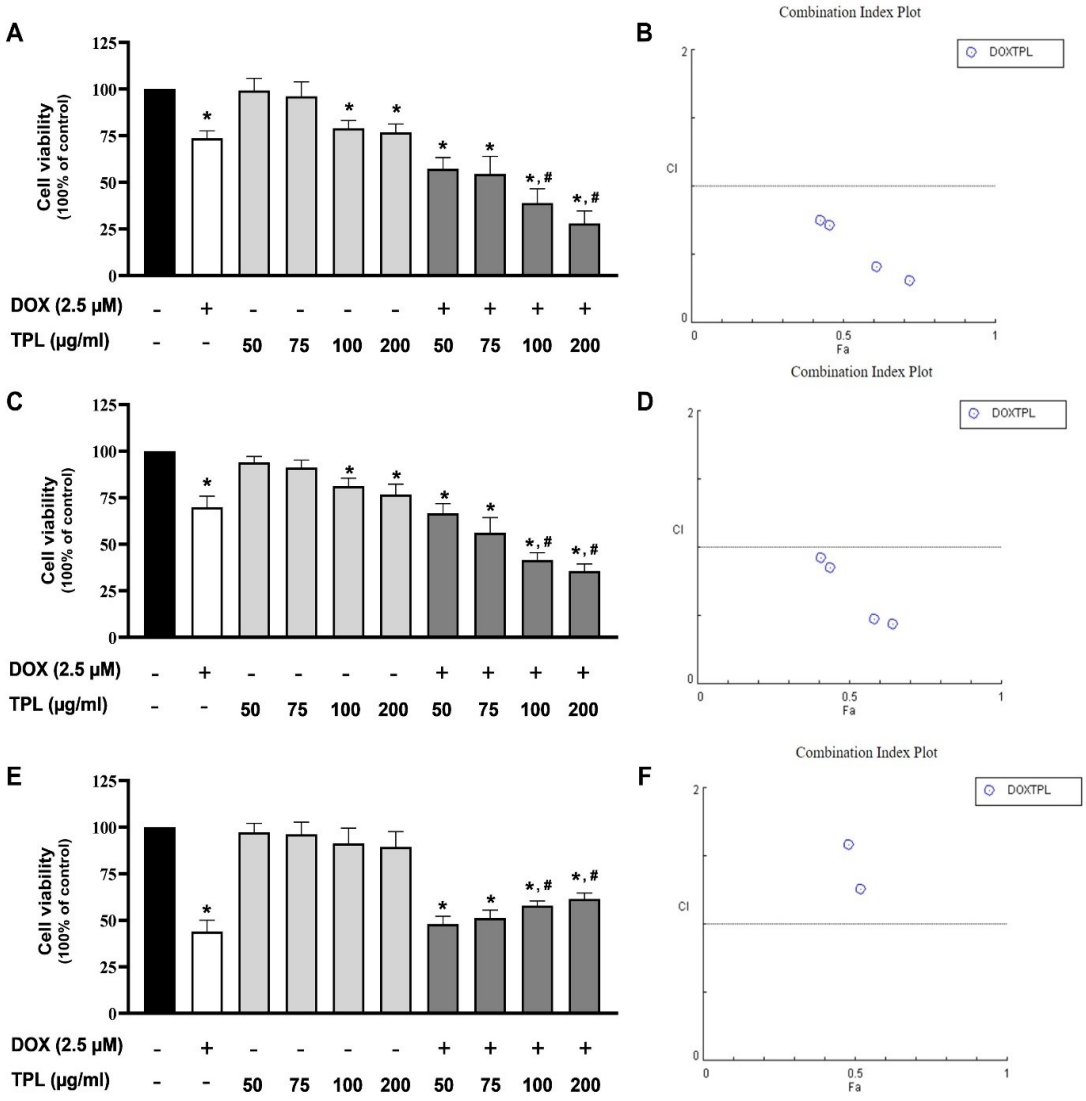

Figure 3. Combination effects of TPL and DOX on cell viability in MDA-MB-231 **(A)**; MCF-7 **(C)**; and MCF-10A **(E)**; with combination index plots **(B, D, F)**.
*$P < 0.05$ compared to untreated cells; and $^{\#}P < 0.05$ compared to DOX-treated cells.
DOX, doxorubicin; TPL, Triphala.

the viability of MCF-10A cells (**Figure 3E**), and the combination of TPL with DOX led to increased viability in MCF-10A cells, indicating that the combination thereof induced a protective effect (**Figure 3F**).

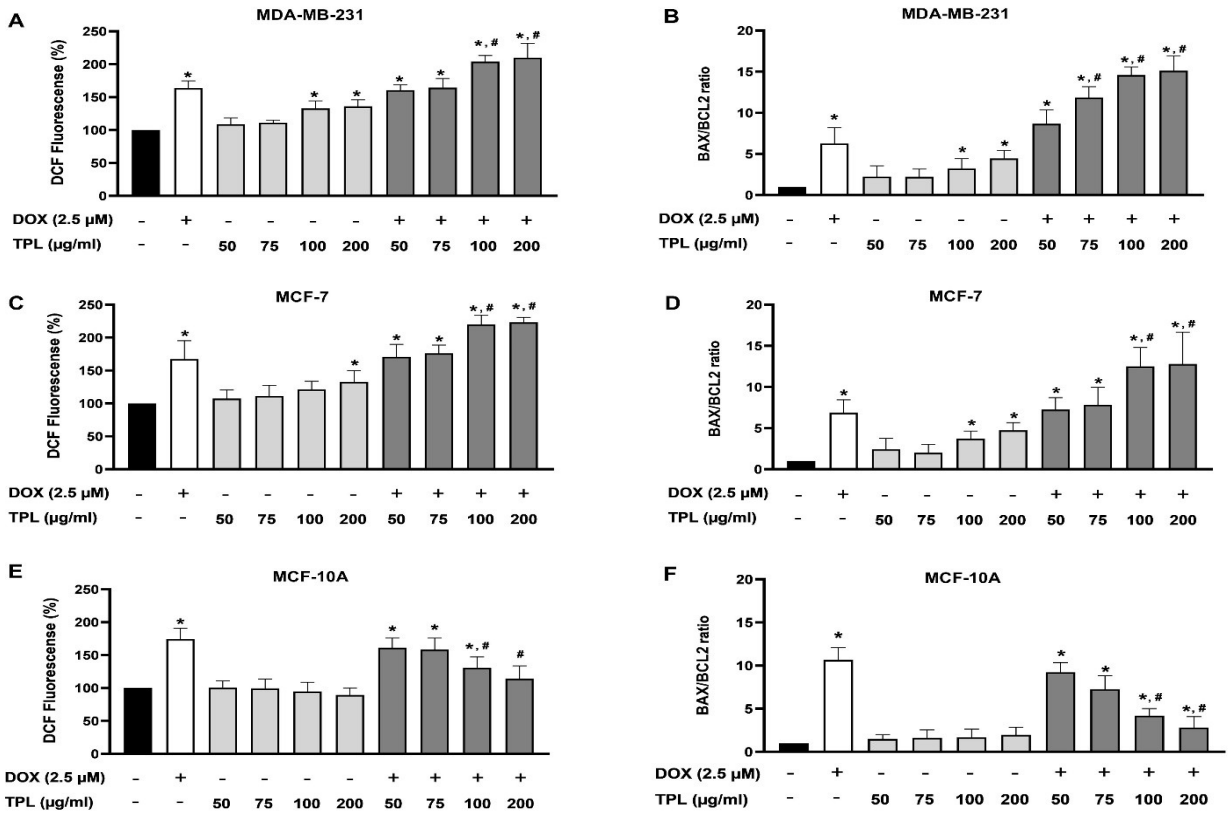

Figure 4. Effects of TPL and low-dose DOX on intracellular ROS levels **(A, C, E)** and *Bcl-2/Bax* gene expression ratio **(B, D, F)** in MDA-MB-231 **(A, B)**, MCF-7 **(C, D)**, and MCF-10A **(E, F)**. mRNA expression levels were normalized to β-actin. Data represent the mean ± SEM from three independent experiments performed in triplicate. Statistical significance was defined as $P < 0.05$. *indicates significance compared to untreated cells, and #indicates significance compared to DOX-alone treated cells. BAX, Bcl-2 associated X-protein; BCL2, B-cell lymphoma 2; DOX, doxorubicin; ROS, reactive oxygen species; SEM, standard error of the mean; TPL, Triphala.

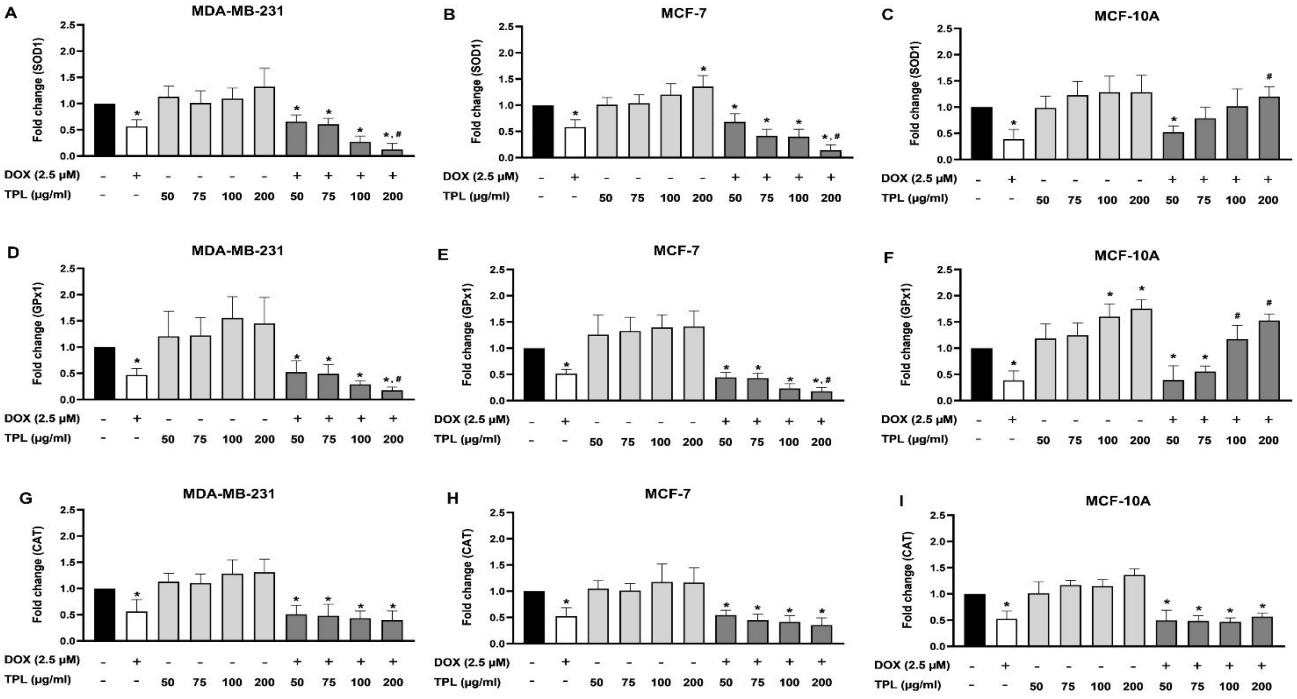

Figure 5. Effects of TPL and low-dose DOX on *SOD1* **(A-C)**, *GPX1* **(D-F)**, and *CAT* **(G-I)** mRNA expression in MDA-MB-231 (**A, D, G**), MCF-7 (**B, E, H**), and MCF-10A cells (**C, F, I**), respectively. Expression levels were normalized to ß-actin. Data are shown as mean ± SEM from three independent experiments performed in triplicate. Statistical significance was defined as $P < 0.05$.

*indicated significance compared to untreated cells and #indicates significance compared to DOX-alone treated cells. CAT, catalase; DOX, doxorubicin; GPX1, glutathione peroxidase 1; SEM, standard error of the mean; SOD1, superoxide dismutase 1; TPL, Triphala.

Combination effect of TPL and DOX on ROS production in breast cancer and epithelial cell lines

Given that antioxidants are a potential target for cancer treatment, particularly in cancer cells that exhibit elevated basal ROS levels, this study evaluated the ROS levels after combination treatment with TPL and DOX. The percentage of intracellular ROS was normalized by cell viability to account for the internal control. As shown in **Figure 4A, C**, **and E**, treatment with low-dose DOX significantly increased the ROS levels in MDA-MB-231 (1.7-fold, $P < 0.001$), MCF-7 (1.7-fold, $P < 0.001$), and MCF-10A (1.7-fold, $P < 0.001$) compared with the control. Treatment with 200 μg/mL of TPL ethanolic extract led to a significant increase in the ROS levels in MDA-MB-231 (1.3-fold, $P = 0.5$) and MCF-7 (1.4-fold, $P = 0.39$) cells compared with the control. Furthermore, the combination treatment of low-dose DOX and TPL significantly increased the ROS levels in MDA-MB-231 and MCF-7 cells, compared to the control. Moreover, combination treatment with higher concentrations of TPL (100–200 μg/mL) resulted in a further increase in ROS levels in the MDA-MB-231 (1.2- to 1.9-fold, $P = 0.005$) and MCF-7 (1.2- to 1.4-fold, $P = 0.008$) groups compared with the groups treated with DOX alone. These results suggest that TPL combined with low-dose DOX synergistically enhances ROS production in breast cancer cell lines.

In contrast, the effect of the combination treatment on the non-tumorigenic cells, represented by MCF-10A, differed. Increased levels of ROS in non-tumorigenic cells can be a side effect of chemotherapy. Our results revealed that TPL did not elevate the levels of intracellular ROS in MCF-10A. Despite the significant increase in ROS levels in cancer cell lines, this effect was absent in MCF-10A cells. Combination treatment with TPL at a concentration of 100–200 μg/mL resulted in a significant reduction in ROS levels (1.3- to 1.4-fold, $P = 0.002$) compared with cells treated with DOX alone. These findings indicate that TPL may protect non-tumorigenic mammary epithelial cells by reducing their production of ROS during low-dose DOX treatment.

Combination effect of TPL and DOX on apoptosis-related gene expression in breast cancer and epithelial cell lines

The expression ratio of *BAX*/*BCL2* was analyzed as an apoptosis marker. The results showed that the mRNA expression levels of *BAX*/*BCL2* were significantly increased in MDA-MB-231 (6.6-fold, $P = 0.028$), MCF-7 (5.7-fold, $P = 0.025$), and MCF-10A (8.9-fold, $P < 0.001$) cells compared with the control (**Figure 4B, D, F**). Treatment with 100–200 μg/mL of TPL significantly increased the *BAX*/*BCL2* expression ratio in MDA-MB-231 (3.2- to 4.6-fold, $P < 0.001$) and MCF-7 (3.7- to 4.7-fold, $P < 0.001$) cells. Compared with the DOX-alone treated group, combination treatment with TPL at concentrations of 75–200 μg/mL significantly enhanced the *BAX/BCL2* expression ratio in MDA-MB-231 (1.9- to 2.4-fold, $P < 0.001$) and MCF-7 (1.8- to 1.9-fold, $P = 0.020$) cells. This indicates that TPL used in combination with DOX synergistically induces apoptosis in MDA-MB-231 and MCF-7 cells.

Interestingly, the combination treatment of TPL and DOX decreased the *BAX*/*BCL2* expression ratio in MCF-10A cells in a concentration-dependent manner. Compared with the DOX-alone treated cells, a significant reduction was observed in the *BAX/BCL2* ratio with the combination treatment of DOX and 100–200 μg/mL of TPL (1.6- to 1.7-fold, $P < 0.001$). These findings suggest that combining TPL with DOX may protect non-tumorigenic epithelial cells from DOX-induced apoptosis by reducing the *BAX/BCL2* ratio.

Combination effect of TPL and DOX on antioxidant-related gene expression in breast cancer and epithelial cell lines

To explore the mechanisms underlying the cytotoxicity of TPL and DOX, we investigated the mRNA expression of antioxidant enzymes, including superoxide dismutase (SOD), glutathione peroxidase (GPX), and catalase (CAT), after the combination treatment. The DOX-alone treatment significantly downregulated the expression of *SOD1* in MDA-MB-231 (1.5-fold, $P = 0.039$), MCF-7 (1.5-fold, $P = 0.037$), and MCF-10A (1.3-fold, $P = 0.044$) cells. In contrast, combination treatment with 200 μg/mL of TPL further reduced *SOD1* expression in MDA-MB-231 (1.8-fold, $P < 0.001$) and MCF-7 (1.7-fold, $P = 0.035$) cells. Interestingly, the combination treatment increased the *SOD1* expression in MCF-10A (1.2-fold, $P = 0.031$) cells compared with the DOX-alone treatment group (**Figure 5A-C**).

Similarly, *GPX1* expression was reduced in the DOX-alone-treated groups in MDA-MB-231 (1.47-fold, $P = 0.049$), MCF-7 (1.5-fold, $P = 0.045$), and MCF-10A (1.6-fold, $P = 0.035$) cells. The combination treatment further decreased the expression of *GPX1*

in MDA-MB-231 (1.6-fold, $P = 0.045$) and MCF-7 (1.7-fold, $P = 0.036$) cells, while the combination treatment significantly increased the *GPX1* expression in MCF-10A cells at both 100 µg/mL (1.2-fold, $P = 0.005$) and 200 µg/mL (1.5-fold, $P < 0.001$) TPL concentrations (**Figure 5D-F**).

For *CAT* expression, the DOX-alone treatment caused a significant reduction in MDA-MB-231 (1.5-fold, $P = 0.047$), MCF-7 (1.5-fold, $P = 0.049$), and MCF-10A (1.5-fold, $P = 0.047$) cells. However, the combination treatment of TPL and DOX did not exhibit any significant effect on *CAT* expression in any of the cell lines compared with the DOX-alone treatment (**Figure 5G-I**).

Discussion

Numerous studies have demonstrated that TPL has substantial antioxidant capacity, which is attributed to the presence of polyphenols, including gallic acid, ellagic acid, chebulagic acid, and chebulinic acid. [16] These compounds exhibit free radical scavenging activity by donating electrons or hydrogen atoms, forming less toxic molecules, and preventing cellular damage. [30] Consistent with these findings, our HPLC results revealed that gallic acid was the main compound in the TPL ethanol extract (11.9%). This aligns with previous literature where gallic acid was identified as the major active compound in TPL extracts. [31] In addition, previous research has demonstrated that the profile of the aqueous TPL extract contains 18 compounds, with gallic acid as the main component (30.0%), in addition to other polyphenolic compounds. [32] The presence of ellagic acid, chebulagic acid, and chebulinic acid suggests that multiple active components contribute to the biological activity of TPL. However, our HPLC analysis also indicated the presence of several unidentified compounds in the TPL ethanolic extract, which suggests the need for further investigation to identify these peaks through additional chromatographic and spectroscopic analyses.

Polyphenols play a key role in increasing ROS levels in various cancers, including breast cancer, by inducing pro-oxidant damage and promoting apoptosis. Gallic acid, as a major component of TPL, has been reported to induce apoptosis in breast cancer cells by elevating the production of ROS. [21] Similarly, TPL has demonstrated pro-oxidant activity by inducing apoptosis through ROS generation. [17] Our study confirmed the significant increase in intracellular ROS levels in breast cancer cells treated with ethanolic TPL extract, thus highlighting its potential as a pro-oxidant therapeutic agent. In addition to gallic acid, other polyphenols, such as ellagic acid, may contribute to TPL's anticancer properties. Ellagic acid has been shown to selectively reduce cell viability in chronic lymphocytic leukemia at 10 µmol/L, whereas a similar effect in healthy B-lymphocytes occurred at a far higher concentration (200 µmol/L). This selective effect was accompanied by increased ROS production, reduced levels of metalloproteinases (MMP), and enhanced apoptosis. [22] These findings suggest that multiple bioactive compounds in TPL may synergistically enhance its antioxidant and pro-oxidant properties, thereby making it a promising candidate for further study.

The mechanism of gallic acid in modulating the redox status is multifaceted. It can function as either an antioxidant or a pro-oxidant depending on the cellular context. Under physiological conditions, gallic acid scavenges free radicals and enhances antioxidant defenses; however, at higher concentrations, it can induce oxidative stress, which leads to apoptosis in cancer cells. [33] This dual function is mediated through its interaction with key redox-sensitive signaling pathways, including the nuclear factor erythroid 2-related factor 2 (NRF2)/Kelch-like ECH-associated protein 1 (KEAP1) and mitochondrial apoptotic pathways. [33] In response, cancer cells upregulate their antioxidant capacity to sustain oncogenic ROS signaling, which allows disease progression while preventing cell death. [4] In agreement with previous studies, our results demonstrated that breast cancer cell lines have higher basal ROS levels than the non-tumorigenic MCF-10A cells, with the highest ROS levels observed in the MDA-MB-231 TNBC cell line. Salinas S, *et al.* reported that aggressive TNBC cells have elevated levels of ROS compared with estrogen-positive and non-tumorigenic cells, and reducing the ROS levels inhibited TNBC proliferation and induced cell death. [7] These findings suggest that targeting ROS may present a therapeutic strategy for breast cancer treatment.

In recent years, natural compounds and herbal medicines with antioxidant properties have garnered increasing attention in cancer treatment. This study evaluated the combined effect of TPL, an antioxidant-rich herbal formulation, and low-dose DOX on breast cancer cell lines (MCF-7 and MDA-MB-231), and

MCF-10A cells were included as a model of non-tumorigenic mammary epithelial cells. Our findings revealed that TPL exhibited concentration-dependent cytotoxic effects on both cancerous (MCF-7 and MDA-MB-231) and non-tumorigenic (MCF-10A) cells. The IC_{50} values of TPL were higher in MCF-10A than in MCF-7 and MDA-MB-231 cells, which indicates that TPL is less toxic to non-tumorigenic cells. These results are consistent with previous reports that TPL and its primary constituent, gallic acid, promote antiproliferative activity and apoptosis in breast cancer cells without affecting non-tumorigenic mammary epithelial cells. [21] Therefore, TPL may hold promise as a potential cancer treatment with reduced cytotoxicity to non-tumorigenic tissues.

DOX, a widely used chemotherapeutic agent, increases the production of ROS in cancer cells by inducing oxidative stress and disrupting mitochondrial respiration. [34] The challenge lies in reducing ROS-induced damage to non-tumorigenic tissues without compromising the drug's efficacy. Antioxidants can play a role in minimizing ROS-induced damage, potentially by reducing the amount of chemotherapy required. In this study, 100–200 µg/mL of TPL increased the cytotoxicity of low-dose DOX in breast cancer cells, as demonstrated by the decreased cell viability in the combined treatment groups. The CI values for the breast cancer cells were less than 1, thus indicating the TPL and DOX exhibited a synergistic effect. In contrast, TPL reduced the cytotoxicity of DOX in MCF-10A cells, as demonstrated by a CI value greater than 1, which suggests an antagonistic effect in non-tumorigenic cells. This finding is novel, indicating that TPL may protect non-tumorigenic tissues while enhancing the anticancer effects of chemotherapy.

Chemotherapy-induced ROS overproduction primarily occurs within the mitochondria and is mediated by NADPH oxidase activity, which leads to the loss of mitochondrial membrane potential and release of superoxide into the cytoplasm. [35] Our study demonstrated that treatment with DOX alone increased the ROS levels in both breast cancer and non-tumorigenic cells. Combination treatment with TPL and low-dose DOX further increased ROS production in MDA-MB-231 and MCF-7 cells but not in MCF-10A cells, suggesting that TPL selectively enhances ROS production in cancer cells. Previous studies have suggested that polyphenols, including those present in TPL, may target the aberrant redox systems of cancer cells, promoting cell death while sparing non-tumorigenic cells. [20, 36]

Cellular antioxidants, such as SOD, GPX, and CAT, are essential for maintaining redox homeostasis by neutralizing ROS. [37] However, chemotherapy often reduces antioxidant levels to exacerbate oxidative damage in both tumor and non-tumorigenic cells. [38, 39] Consistent with previous studies, we found that low-dose DOX decreased the expression of antioxidant-related genes (*SOD1* and *GPX1*) in MCF-10A, MDA-MB-231, and MCF-7 cells. Interestingly, when used in combination with TPL, the antioxidant gene expression was restored in MCF-10A cells, indicating that TPL has a protective effect against ROS-induced damage in non-tumorigenic cells. This protective effect was not observed in cancer cells, where the combination treatment further disrupted the redox balance, as demonstrated by the reduced expression of SOD1 and GPX1.

The selective induction of ROS by TPL in cancer cells can be attributed to their distinct metabolic and redox environments. Cancer cells inherently exhibit higher basal ROS levels because of increased metabolic activity, which brings them closer to the threshold for oxidative damage. This study demonstrated that TPL pushes cancer cells beyond this threshold by further elevating the ROS levels. Moreover, cancer cells often have compromised antioxidant systems compared with normal cells, making them more vulnerable to oxidative stress. By targeting antioxidant pathways, TPL exploits this vulnerability, as demonstrated by the downregulated expression of *SOD1* and *GPX1*, leading to the accumulation of lethal ROS levels. In contrast, the potential restoration of these markers in non-tumorigenic cells suggests the selective protective effects of TPL. While CAT is a key antioxidant enzyme, we did not observe a significant change in its expression following TPL and DOX treatment. This may be due to the low expression of *CAT* in many cancers, including breast cancer, and its lower affinity for H_2O_2 compared to GPX. [40, 41] These findings suggest that TPL primarily targets alternative antioxidant mechanisms, such as SOD1 and GPX1, while CAT's minimal role in this context reflects its reduced activity in breast cancer cells. These findings highlight TPL's potential in modulating oxidative stress via both antioxidant and pro-oxidant mechanisms. The identification of additional bioactive compounds in TPL extracts and their specific roles in redox regulation should be a focus of future research to further elucidate its potential as a therapeutic agent.

The disruption of redox homeostasis by TPL amplifies the ROS levels and activates the downstream apoptotic pathways, as reflected in the *BAX/BCL2* ratio changes. Apoptosis is a critical mechanism for cancer treatment [42], and our results demonstrated that low-dose DOX stimulated an increased *BAX/BCL2* ratio in breast cancer cell lines, which is indicative of enhanced apoptosis. TPL further increased this ratio when used in combination with DOX, suggesting a synergistic effect in promoting apoptosis in MDA-MB-231 and MCF-7 cells. In contrast, TPL reduced the *BAX/BCL2* ratio in MCF-10 cells, indicating a protective effect against chemotherapy-induced cytotoxicity in non-tumorigenic cells **(Figure 6)**. This is in alignment with previous reports, which revealed that herbal medicines, including gallic acid derivatives, can enhance cancer cell death while protecting non-tumorigenic cells. [21, 43, 44] However, caution is necessary, as low-dose chemotherapy has been associated with increased metastatic risks in some cancers. [45]

Conclusion

The combination of TPL and low-dose DOX offers a promising strategy for selective cancer therapy, enhancing DOX's efficacy in breast cancer cells while protecting non-tumorigenic cells. TPL's potential extends to other cancers with high ROS levels, but further studies are needed to optimize treatment, evaluate systemic effects, and ensure safety.

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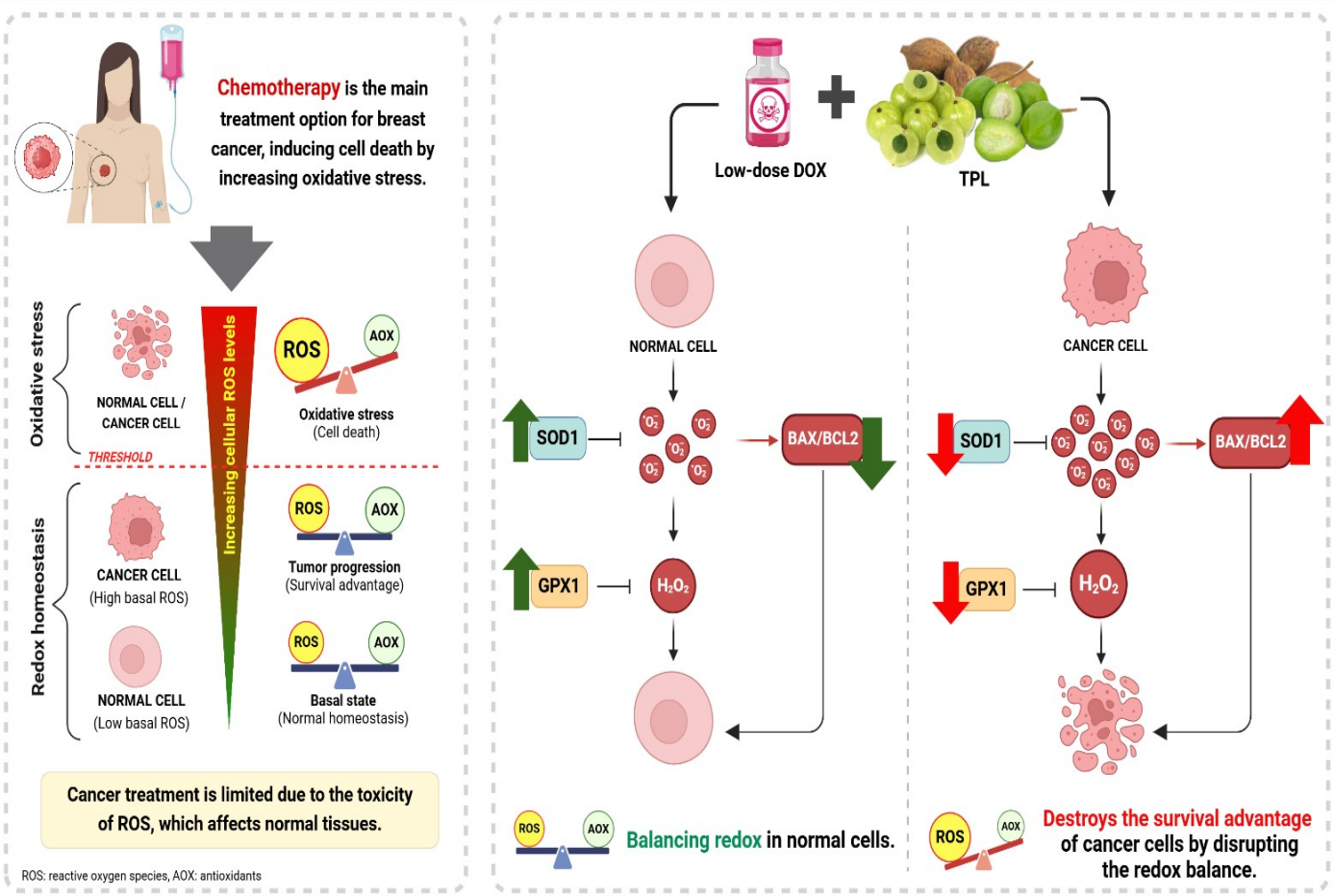

Figure 6. Graphical abstract illustrating the main concept and results of this study.

Conflict of interest statement

Each of the authors has completed an ICMJE disclosure form. The authors declare that they do not have any potential or actual relationship, activity, or conflict of interest related to the content of this article.

Data sharing statement

All data generated or analyzed during the present study are included in this published article. Further details are available for noncommercial purposes from the corresponding author upon reasonable request.

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