# Multi-Faceted Anti-Cancer Mechanisms of Hesperidin Against Luminal A and Triple-Negative Breast Cancer Cells

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**Abstract:** Breast cancer remains a significant global health burden, compounded by issues of heterogeneity and treatment resistance, necessitating novel therapeutic strategies. Natural products, like the citrus flavonoid hesperidin, offer potential advantages. This study investigated the *in vitro* anti-cancer effects and mechanisms of hesperidin across diverse breast cancer cell lines (MCF-7, MDA-MB-231, 4T1) compared to normal human fibroblasts (HSF). Standard assays, including MTT, Trypan Blue, flow cytometry (cell cycle, Annexin V/PI apoptosis), wound healing, and qRT-PCR, were employed. Hesperidin induced dose-dependent cytotoxicity, exhibiting greater sensitivity in cancer cells versus HSF, particularly at lower concentrations. It triggered cell-type-specific cell cycle arrest (G1 in MCF-7, G2/M in MDA-MB-231, S-phase in 4T1/HSF) and significantly increased apoptosis in cancer cells, sparing HSF. Mechanistically, hesperidin modulated key gene expressions: upregulating apoptotic *PTEN* and *CDH1* while downregulating anti-apoptotic *HER2*, *EGFR*, *NOTCH1*, and *PALB*. These findings demonstrate hesperidin's multi-faceted, selective anti-cancer activity *in vitro*, supporting its potential as a therapeutic candidate for breast cancer.

**Keywords:** Hesperidin, Breast Cancer, Apoptosis, Cell Cycle Arrest, targeting therapy.

## INTRODUCTION

Breast cancer (BC) represents a profound global health challenge, standing as one of the most prevalent malignancies affecting women worldwide [1]. Diagnosis relies on physical examinations, advanced imaging, and crucial tissue biopsies. The sheer scale of the issue is underscored by GLOBOCAN 2020 statistics, which recorded 2.3 million new cases globally in a single year [1]. While predominantly a disease impacting women, its rare occurrence in men highlights the complexity of its etiology [2]. In nations like the United States, BC is the most frequently diagnosed cancer among women (excluding nonmelanoma skin cancer) and tragically ranks as the second leading cause of cancer mortality, disproportionately affecting Black and Hispanic women [3]. Disturbingly, the incidence of breast cancer continues its relentless climb globally [4], with projections warning of a potential escalation to approximately 3.2 million new cases annually by 2050 [5, 6].

Despite significant strides in early detection and treatment protocols leading to improved survival rates for many, the insidious nature of BC, particularly its heterogeneity and propensity for resistance, necessitates an urgent and ongoing search for novel therapeutic strategies and more reliable predictive and prognostic markers [7]. It is widely recognized that BC is not a monolithic entity but rather a collection of diseases characterized by substantial genetic and phenotypic diversity, both within individual tumors (intratumoral heterogeneity) and between different patients (intertumoral heterogeneity) [8]. This variability underpins the classification into distinct molecular subtypes: luminal A/B (expressing estrogen [ER] and/or progesterone [PR] receptors), HER2-enriched (overexpressing human epidermal growth factor receptor 2), and the aggressive triple-negative subtype (lacking ER, PR, and HER2 expression) [9].

The clinical management and therapeutic choices for breast cancer are fundamentally dictated by these molecular subtypes [10, 11]. Current treatment paradigms encompass surgery, radiation, chemotherapy, endocrine therapy, and HER2-targeted agents [12], with systemic adjuvant treatments tailored to the specific subtype [13]. However, a major clinical hurdle is the emergence of resistance to these therapies—documented cases of resistance to

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chemotherapy, endocrine manipulations, and HER2-targeted drugs significantly limit treatment efficacy and contribute to disease recurrence and mortality [14]. Triple-negative breast cancer (TNBC), notorious for its poor prognosis, faces a particular therapeutic impasse, with limited targeted options and heavy reliance on conventional chemotherapy [15]. While potentially effective, chemotherapy often lacks specificity, leading to damage in healthy tissues and debilitating side effects [16]. This challenging landscape underscores the critical necessity to identify and develop more efficacious and less toxic anti-cancer agents.

In this quest, natural products have historically served as an invaluable reservoir for anti-cancer drug discovery, with estimates suggesting around 60% of currently available cancer therapies originate from natural sources [17]. Although the pharmaceutical industry briefly pivoted towards high-throughput screening of synthetic compounds in the 1990s, driven by challenges in natural product sourcing and screening [18], there has been a resurgence of interest. This renewed focus is fueled by the relatively low approval rates for novel synthetic drugs and by technological advancements that mitigate previous hurdles in natural product research [19]. Phytochemicals, bioactive non-nutritive compounds derived from plants, are at the forefront of this renaissance, garnering intense research attention for their cancer-preventive potential [20]. These compounds offer distinct advantages over conventional drugs, including the ability to modulate multiple cellular pathways simultaneously, potentially sensitize cancer cells to existing therapies, and often exhibit more favorable safety profiles [21]. Their anti-cancer effects are thought to stem from their influence on critical processes like cell proliferation, apoptosis, angiogenesis, inflammation, and metastasis [22].

Among promising phytochemicals, hesperidin, a flavanone glycoside abundantly present in citrus fruits like orange peel, stands out. Widely utilized in traditional Chinese medicine, hesperidin has demonstrated a compelling safety profile, often showing minimal toxicity to normal cells while exerting potent anticancer activity against various malignancies, including breast, prostate, bladder, and liver cancers [5]. Existing literature highlights hesperidin's multifaceted anti-cancer capabilities: it can trigger apoptosis through both intrinsic and extrinsic pathways, inhibit cancer cell proliferation, invasion, migration, and angiogenesis, and modulate key signaling pathways involved in carcinogenesis [23]. Furthermore, hesperidin is known to arrest cell cycle progression at various checkpoints (G1, G2/M, or S phase), potentially through modification of cell cycle regulatory proteins, influencing the delicate balance between cytostasis and apoptosis, often involving the p53 pathway [24].

While these previous studies establish hesperidin as a promising anticancer phytocompound, a comprehensive evaluation of its effects across the diverse spectrum of breast cancer subtypes, directly comparing its impact on malignant versus non-malignant cells, and elucidating the specific molecular mechanisms involved in key cancer processes like viability, cell cycle control, apoptosis, and migration, remains crucial for advancing its therapeutic potential.

To further investigate the molecular mechanisms underlying hesperidin-mediated anticancer activity, we selected a panel of biomarkers representing key pathways involved in breast cancer progression, therapeutic response, and metastasis. HER2 and EGFR were evaluated because of their established roles in driving tumor proliferation and influencing treatment outcomes. NOTCH1 and KRAS were included as critical mediators of oncogenic signaling associated with tumor aggressiveness, stemness, and therapy resistance. PTEN was assessed due to its central tumor-suppressive function and regulation of the PI3K/AKT pathway, while CDH1 was examined as a marker of cell adhesion and metastatic potential. Additionally, BRAF, ROS1, and PALB2 were analyzed to capture alterations in oncogenic signaling and DNA damage repair pathways. Collectively, this biomarker panel provides a comprehensive framework for evaluating the diverse molecular effects of hesperidin across distinct breast cancer subtypes.

Therefore, this study aimed to conduct a robust *in vitro* investigation into the anti-cancer efficacy and mechanisms of hesperidin across distinct breast cancer cell lines representing luminal A (MCF-7), triple-negative (MDA-MB-231), and murine mammary carcinoma (4T1), alongside non-cancerous human fibroblasts (HSF). Specifically, we sought to evaluate hesperidin's impact on cell viability, cell cycle distribution, apoptosis induction, cell migration inhibition, and the expression profiles of pivotal genes governing these fundamental cellular processes. This work is significant as it provides critical mechanistic insights into hesperidin's action in different BC contexts and assesses its selectivity, offering valuable data to support its further development as a potential therapeutic agent against breast cancer.

## MATERIALS AND METHODS

### Drugs and Chemicals

A 50 mg vial of hesperidin was utilized in this study. Additional reagents included Dulbecco's Phosphate-Buffered Saline (DPBS) without calcium or magnesium (Biowest, South America), Fetal Bovine Serum (FBS; Biowest, South America; E.U. grade), and Dulbecco's Modified Eagle's Medium (DMEM) with high glucose (Lonza, Verviers, Belgium). Other materials used were Penicillin–Streptomycin Solution (100×; CORNING, USA), phenol red (Thermo Scientific, Gibco, Germany/Ireland), and 0.25% trypsin–EDTA. For nucleic acid analysis, propidium iodide (PI; ab14083, Abcam), HERAPLUS SYBR® Green qPCR Kit (Willowfort, Nottingham, UK), RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Baltics UAB, USA), and the GeneJET Total RNA Purification Mini Kit (Thermo Scientific, Baltics UAB, USA) were employed.

### Cell Line Maintenance

The breast cancer cell lines used in this study included the triple-negative metastatic MDA-MB-231 and the luminal subtype MCF-7, alongside the murine mammary carcinoma model 4T1. Human skin fibroblast cells (HSF), obtained from Nawah Scientific (Almokattam, Cairo, Egypt), were used as a non-cancerous control. All cell lines were cultured under standard conditions in complete DMEM supplemented with 10% FBS and 1% Penicillin-Streptomycin at 37°C with 5% $CO_2$.

### Cytotoxicity Assay

Cell viability and proliferation were assessed using the MTT assay, which measures mitochondrial reductase activity. MCF-7, MDA-MB-231, 4T1, and HSF cells were seeded into 96-well plates and treated with hesperidin at concentrations of 15, 25, and 40 µM for 24 h in triplicates. Control wells received no treatment. Following incubation, the media was replaced with 150 µL fresh DMEM containing 50 µL MTT (5 mg/mL in PBS), and plates were incubated for 4 h at 37°C. The resulting formazan crystals were solubilized in 200 µL DMSO and incubated for 30 minutes at 37°C. Absorbance was measured at 450 nm using a microplate spectrophotometer (BioTek Instruments, Winooski, VT, USA).

### Trypan Blue Exclusion Assay

Cell viability was further evaluated using the trypan blue exclusion method. MDA-MB-231, MCF-7, 4T1, and HSF cells ($4 \times 10^5$) were seeded in 12-well plates and treated with different concentrations of hesperidin for 24 h. Adherent cells were trypsinized using 0.25% trypsin-EDTA, centrifuged at 200 rpm for 10 minutes, and resuspended in 1 mL of growth medium. A 1:1 mixture of cells and 0.4% trypan blue dye was prepared and incubated at room temperature for 3 minutes. Cells were then counted under an Olympus BX43 light microscope using a hemocytometer. Four readings per well were averaged, and viable cell count was calculated using the formula:

Cell count = (Sum of four quadrants ÷ 2) × $10^4$.

### Wound Healing Migration Assay

To assess cell migration, $1 \times 10^6$ cells were cultured in 24-well plates until confluence. A uniform scratch was created using a 100 µL pipette tip, followed by washing with DPBS to remove detached cells. Cells were incubated with DMEM containing hesperidin (15, 25, or 40 µM) or control media. Wound closure was monitored at 0, 3, 18, and 21 h. Migration rate was quantified by measuring the percentage of wound closure using ImageJ software:

Migration (%) = [(Initial wound width – Final wound width) ÷ Initial wound width] × 100.

### Cell Cycle Analysis

To examine hesperidin-induced changes in cell cycle progression, $4 \times 10^4$ cells/well were seeded into 12-well plates, treated with hesperidin for 24 h, and then fixed in 70% cold ethanol at 4°C for 2 h. Fixed cells were washed and stained with a solution containing 50 µg/mL PI, 50 µg/mL RNase A, and 0.1% Triton X-100 in PBS for 25 min in the dark at room temperature. DNA content was analyzed using a flow cytometer (BD FACSCalibur™), and data were processed with cell cycle analysis software to determine the distribution across G0/G1, S, and G2/M phases.

### Apoptosis Detection Assay

Apoptotic induction was assessed using Annexin V-FITC/PI double staining followed by flow cytometry. HSF, 4T1, MCF-7, and MDA-MB-231 cells were treated with 25 µM hesperidin and subjected to Annexin V-FITC/PI flow cytometric analysis. cells were seeded into 12-well plates and treated with 25 µM hesperidin for 24 h. Both treated and control cells were harvested, resuspended in 100 µL of Annexin V binding buffer,

and stained with 5 µL Annexin V-FITC and 4 µL PI (1:10 dilution) in the dark for 15 min. Samples were washed and analyzed on a BD FACSCalibur™ flow cytometer. FITC fluorescence was detected at Ex 488 nm/Em 530 nm (FL1), and PI was detected using the FL2 channel.

### RNA Extraction and cDNA Synthesis

Total RNA was extracted from treated and untreated breast cancer cells using the GeneJET RNA Purification Kit (Thermo Scientific) per manufacturer's instructions. RNA integrity was verified by agarose gel electrophoresis, and purity was confirmed using a NanoDrop spectrophotometer at A260/A280. First-strand cDNA synthesis was performed with 5 µg total RNA, random hexamer primers, dNTPs, and M-MLV SuperScript II Reverse Transcriptase (Invitrogen) at 42°C for 60 minutes.

### Quantitative Real-Time PCR (qRT-PCR)

Gene expression was assessed using qRT-PCR in MCF-7, MDA-MB-231, 4T1, and HSF cells. Synthesized cDNA was used as a template in a reaction containing SYBR Green Master Mix (Thermo Scientific), gene-specific primers (Table **1**), and RNase-free water. *GAPDH* served as the internal control. Amplification was performed under standard cycling conditions. The relative expression of each gene was determined using the $2^{-}\Delta\Delta Ct$ method. All reactions were performed in triplicate and melt curve analysis confirmed amplification specificity. The thermal profile begins with an initial denaturation at 95°C for 3 minutes to activate the DNA polymerase and denature the template DNA. This is followed by 40 amplification cycles consisting of denaturation at 95°C for 15 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds. A final extension step at 72°C for 5 minutes ensures complete synthesis of all PCR products. Melt curve analysis is subsequently performed to confirm the specificity of the amplified products.

All experiments were performed using three independent biological replicates. For MTT and qRT-PCR assays, each biological replicate included three technical replicates. Flow cytometry and wound healing assays were conducted in triplicate independent experiments.

### Statistical Analysis

All experiments were performed in triplicates, and data are presented as mean ± standard deviation (SD) unless otherwise indicated. Statistical analysis was conducted using GraphPad Prism (version X.0) or IBM SPSS Statistics (version 27.0, IBM Corp., Armonk, NY, USA). The normality of data distribution was assessed using the Shapiro-Wilk test. For comparisons between two groups, an unpaired two-tailed Student's t-test was applied, while one-way ANOVA followed by Tukey's post-hoc test was used for multiple group comparisons. Non-parametric data were analyzed using the Kruskal–Wallis test followed by Dunn's multiple comparisons where appropriate. Correlation analyses were performed using Pearson's correlation coefficient. A p-value of less than 0.05 was considered statistically significant.

## RESULTS

### Cytotoxicity Assay

The cytotoxic effect of hesperidin on human skin fibroblasts (HSF), murine mammary carcinoma (4T1), luminal A breast cancer (MCF-7; ER+/PR+/HER2-),

**Table 1: The Primer Sequences used in the Present Study**

| Gene | Forward | Reverse |
|---|---|---|
| *HER2* | AGTGAGCAAGTGATGTCCTGA | ACCCCCATACTTGTCCCTTGA |
| *EGFR* | AACACCCTGGTCTGGAAGTACG | TCGTTGGACAGCCTTCAAGACC |
| *BRAF* | AACGAGACCGATCCTCATCAGC | GGTAGCAGACAAACCTGTGGTTG |
| *PTEN* | CTCAGCCGTTACCTGTGTGT | AGGTTTCCTCTGGTCCTGGT |
| *NOTCH1* | GGTGAACTGCTCTGAGGAGATC | GGATTGCAGTCGTCCACGTTGA |
| *ROS1* | CCAGGACCATATGCTGACGTTG | ACGGCACTGATAACTCTTTCTGC |
| *CDH1* | GCTGGACCGAGAGAGTTTCC | CAAAATCCAAGCCCGTGGTG |
| *PALP* | AGGATCTCTCACCGCAGCTAA | TCAGGCCCAACATCAAGTGTG |
| *KRAS* | CAAGACAGAGAGTGGAGGA | AGGCATCATCAACACCCAGA |
| *GAPDH* | TAGGCGCTCACTGTTCTCTC | GCCCAATACGACCAAATCCG |

and triple-negative breast cancer (MDA-MB-231; ER-/PR-/HER2-) cell lines was evaluated using the MTT cell viability assay. Cells were treated with hesperidin at concentrations of 15 µM, 25 µM, and 40 µM for 24 hours, and viability was compared to that of untreated control cells. HSF Cells (non-cancerous control): Treatment with 15 µM hesperidin resulted in a non-significant reduction in cell viability compared to the control (P=0.29). The viability changes at 25 µM was also not statistically significant (P=0.16). However, a significant decrease in viability was observed at the 40 µM concentration (P=0.002). 4T1 Cells (Murine Mammary Carcinoma): Hesperidin treatment did not significantly reduce cell viability at 15 µM (P=0.16). In contrast, compared to the control group, significant viability reductions were observed at 25 µM (P=0.01) and 40 µM (P=0.006). MCF-7 Cells (Luminal A Breast Cancer): Cell viability was significantly decreased following treatment with 15 µM (P=0.01) and 40 µM (P=0.0018) hesperidin compared to the control. The reduction observed at 25 µM was not statistically significant (P=0.43). MDA-MB-231 Cells (Triple-Negative Breast Cancer): The decrease in cell viability was not statistically significant at 15 µM (P=0.092) or 25 µM (P=0.11) compared to the control. A highly significant cytotoxic effect was observed only at the highest concentration of 40 µM (P=0.00027) (Figure **1**).

## Hesperidin induced Cell Cycle Arrest and Apoptosis in HSF, 4T1, MCF-7 and MDA-MB-231 Breast Cancer Cell Lines

### *Cell Cycle Arrest*

Cell cycle analysis of breast cancer cell lines treated with 25 µM hesperidin revealed distinct alterations in cell cycle progression, reflecting cell line-specific responses to the compound. In MCF-7 cells, hesperidin treatment led to a significant accumulation of cells in the G1 phase, indicating a G1-phase arrest and suggesting inhibition of the G1/S transition (Figure **2**). Conversely, MDA-MB-231 cells demonstrated a marked accumulation in the G2/M phase, consistent with G2/M-phase arrest, implying that hesperidin interferes with cell cycle progression prior to mitosis in these more aggressive, triple-negative cells. Interestingly, in both the non-tumorigenic human skin fibroblast (HSF) cells and the murine 4T1 breast cancer cells, hesperidin predominantly induced cell cycle arrest at the S phase. This S-phase accumulation suggests that DNA synthesis may be impaired or delayed in these cell types. Collectively, these findings indicate that hesperidin exerts its antiproliferative effects through differential modulation of the cell cycle, depending on the molecular subtype or species origin of the cell line, and highlight its potential as a context-

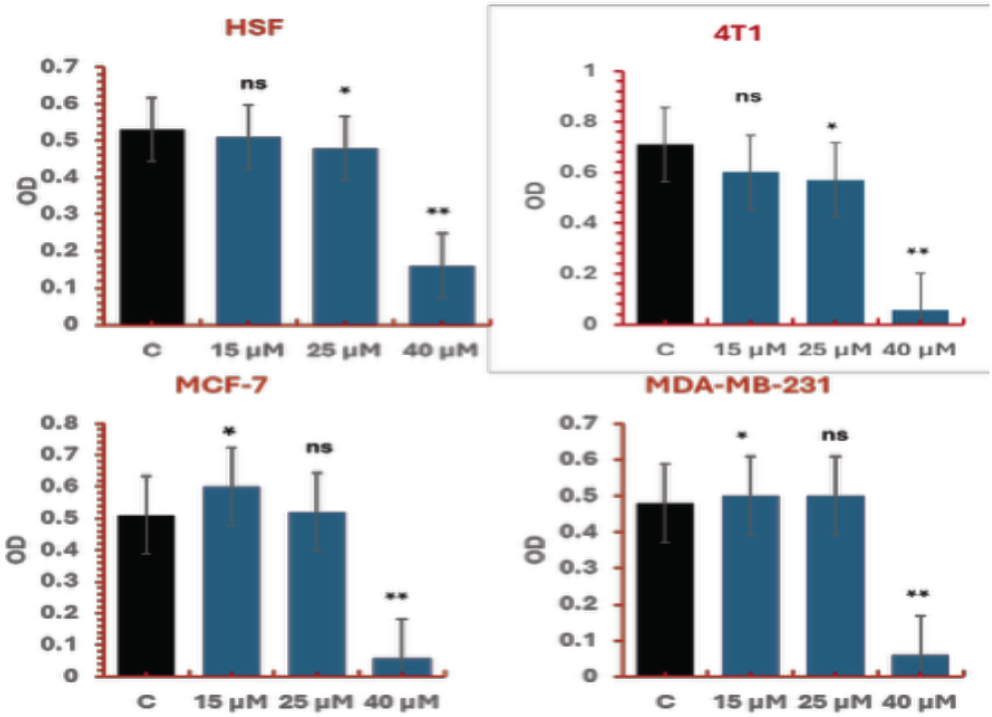


**Figure 1:** Cytotoxicity analysis of compound treatment using MTT assay in HSF, 4T1, MCF-7, and MDA-MB-231 cells. Cell viability was assessed after exposure to increasing concentrations (15, 25, and 40 µM) compared to the control (C). A dose-dependent reduction in viability was observed, especially in 4T1 and MDA-MB-231 cancer cells. Significant differences are indicated (P<0.05, P<0.01; ns: insignificant).

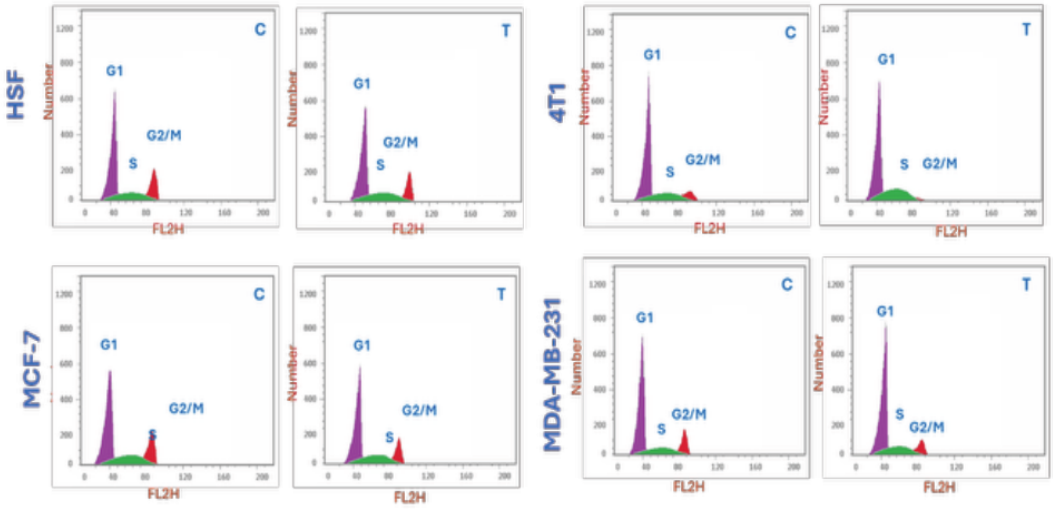


**Figure 2:** Cell cycle analysis after treatment with hesperidin in different breast cancer cell lines. Flow cytometry histograms show DNA content distribution in control (C) and treated (T) groups (25 μM hesperidin) for HSF, 4T1, MCF-7, and MDA-MB-231 cell lines. Hesperidin induced G1-phase arrest in MCF-7 cells and G2/M-phase arrest in MDA-MB-231 cells. In contrast, S-phase accumulation was observed in both HSF and 4T1 cells post-treatment. These results indicate that hesperidin modulates the cell cycle in a cell-type-specific manner.

dependent therapeutic agent in breast cancer treatment.

Following flow cytometric analysis, quantification of the DNA content confirmed distinct cell cycle phase alterations post-hesperidin treatment. MCF-7 cells displayed increased G0/G1 accumulation, whereas MDA-MB-231 cells showed enhanced G2/M-phase arrest. S-phase elevation was evident in HSF and 4T1 cells. These observations reinforce hesperidin's selective cytostatic effects based on cancer cell subtype (Figure **3**).

### *Apoptosis Detection*

Flow cytometric analysis using Annexin V-FITC/PI dual staining was performed to evaluate the apoptotic response of hesperidin (25 μM) in both normal (HSF) and breast cancer cell lines (4T1, MCF-7, MDA-MB-231) (Figure **4**). Annexin V binds to externalized phosphatidylserine—a hallmark of early apoptosis—while propidium iodide (PI) stains cells with compromised membrane integrity, characteristic of late apoptosis or necrosis. In the flow cytometry dot plots, early apoptotic cells are represented in the lower right

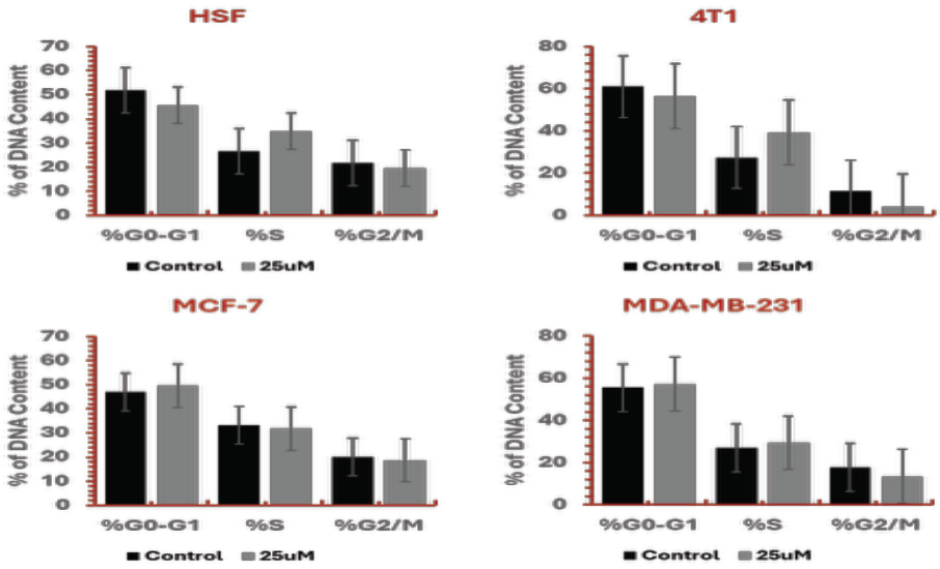


**Figure 3:** Quantitative analysis of cell cycle distribution following hesperidin treatment (25 μM) in various breast cancer and normal cell lines. The bar graphs depict the percentage of cells in G0/G1, S, and G2/M phases. Hesperidin induced G1 arrest in MCF-7 cells and G2/M arrest in MDA-MB-231 cells, while promoting S-phase accumulation in HSF and 4T1 cells. Data are presented as mean ± SD.

quadrant (Annexin $V^+/PI^-$), while late apoptotic or necrotic cells appear in the upper right quadrant (Annexin $V^+/PI^+$). Representative dot plots illustrate the distribution of apoptotic populations following treatment. The accompanying bar graph quantifies the total apoptotic fraction (early + late apoptosis), comparing hesperidin-treated cells to untreated controls. A significant increase in apoptotic cells was observed in all cancer cell lines, with MCF-7 and MDA-MB-231 exhibiting the most pronounced apoptotic response, while HSF (normal fibroblasts) showed minimal apoptotic induction, indicating selective cytotoxicity of hesperidin toward malignant cells. These findings suggest hesperidin induces apoptosis in a cell-type-specific manner, highlighting its potential as an anti-cancer therapeutic.

Quantitative analysis of apoptosis levels revealed that hesperidin (25 μM) markedly increased total

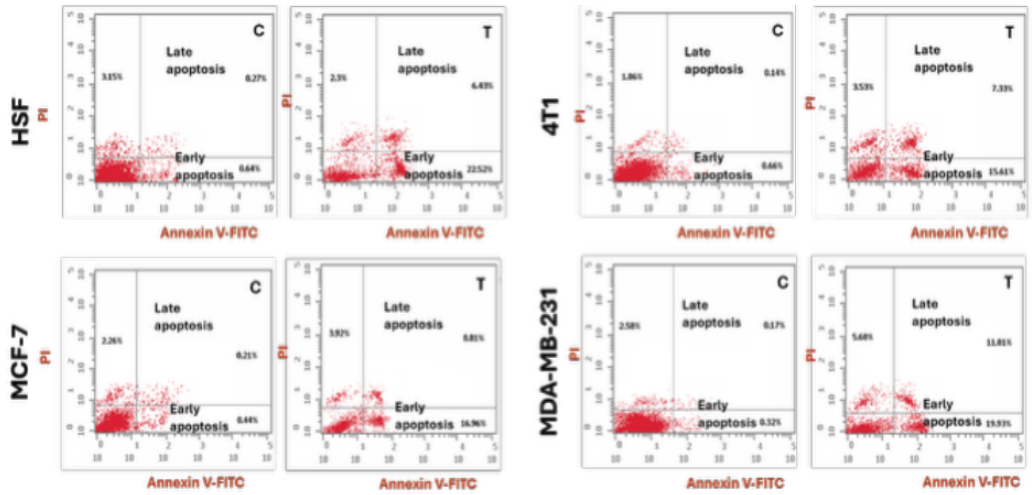


**Figure 4:** Apoptosis Detection After Hesperidin Treatment in Different Breast Cancer Cell Lines. Flow cytometry analysis using Annexin V-FITC/PI dual staining was employed to quantify apoptotic cell populations in HSF, 4T1, MCF-7, and MDA-MB-231 cells. Control (C) and hesperidin-treated (T) groups were analyzed after 24 hours. The lower right quadrant indicates early apoptotic cells (Annexin $V^+/PI^-$), while the upper right quadrant denotes late apoptotic cells (Annexin $V^+/PI^+$). Notably, hesperidin significantly increased both early and late apoptosis in all cancer cell lines, with the highest apoptotic induction observed in MDA-MB-231. Minimal apoptosis was detected in the HSF normal fibroblasts, suggesting selective cytotoxicity. These results support the pro-apoptotic potential of hesperidin in breast cancer therapy.

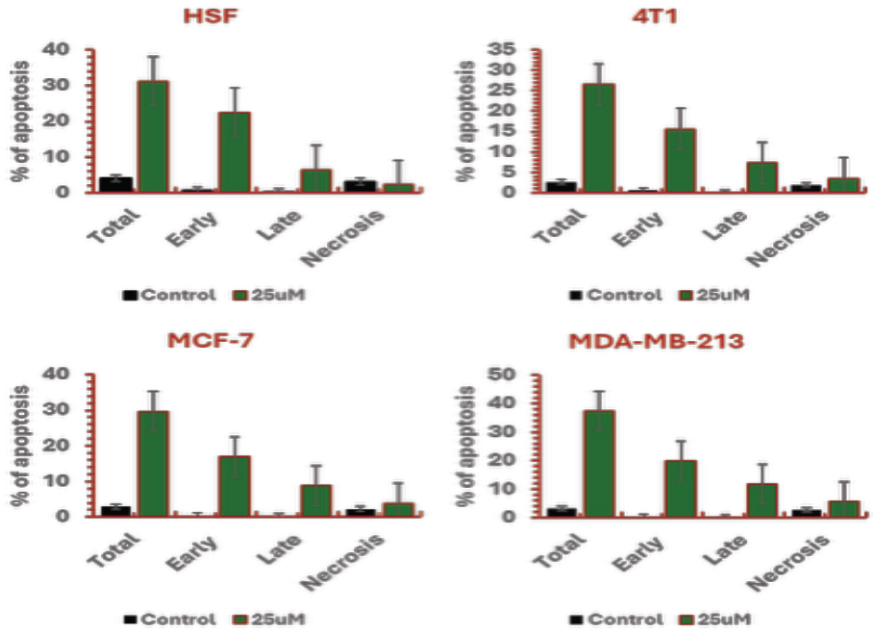


**Figure 5:** Quantification of Apoptosis After Hesperidin Treatment in Different Cell Lines. Bar graphs represent total, early, and late apoptosis and necrosis percentages in control and 25 μM hesperidin-treated cells. Hesperidin significantly induced early apoptosis in all cancer cell lines. MDA-MB-231 showed the highest apoptotic response, whereas HSF cells exhibited lower sensitivity. Data are presented as mean ± SD from replicate experiments.

apoptosis in all tested breast cancer cell lines (4T1, MCF-7, MDA-MB-231) compared to controls. The rise was mainly due to an increase in early apoptotic cells, with moderate elevation in late apoptosis. The MDA-MB-231 cells exhibited the highest apoptotic response. Minimal necrosis was observed across all treated groups. In contrast, HSF (normal fibroblasts) displayed significantly lower sensitivity to hesperidin-induced apoptosis (Figure **5**). Early, late apoptosis, and necrosis percentages are presented in Table **2**.

### Trypan Blue Assay

To evaluate the cytotoxic potential of hesperidin across a range of concentrations, a Trypan Blue exclusion assay was employed to assess cell viability in four distinct cell lines: HSF (normal human skin fibroblasts), 4T1 (murine mammary carcinoma), MCF-7 (estrogen receptor-positive human breast cancer), and MDA-MB-231 (Figure **6**). Following seeding into 96-well plates and treatment with increasing concentrations of hesperidin, cells were stained with Trypan Blue, and both viable (unstained) and non-viable (blue-stained) cells were manually counted using a light microscope. The results demonstrated a clear, dose-dependent decrease in cell viability across all tested cell lines, with cancerous cells showing greater sensitivity at lower concentrations compared to the normal HSF line. This suggests that hesperidin exerts selective cytotoxic effects, particularly on malignant cells, and highlights

**Table 2: Quantitative Assessment of Cell Death, Apoptosis, and Necrosis Induced by Hesperidin (25 µM) in Breast Cancer and Normal Cell Lines**

| | Treatment | Total cell death (%) | Apoptosis | | Necrosis (%) |
|---|---|---|---|---|---|
| | | | Early (%) | Late (%) | |
| HSF | C | 4.06 | 0.64 | 0.27 | 3.15 |
| | 25 µM | 31.25 | 22.52 | 6.43 | 2.3 |
| 4T1 | C | 2.66 | 0.66 | 0.14 | 1.86 |
| | 25 µM | 26.47 | 15.61 | 7.33 | 3.53 |
| MCF-7 | C | 2.91 | 0.44 | 0.21 | 2.26 |
| | 25 µM | 29.69 | 16.96 | 8.81 | 3.92 |
| MDA | C | 3.07 | 0.32 | 0.17 | 2.58 |
| | 25 µM | 37.42 | 19.93 | 11.81 | 5.68 |

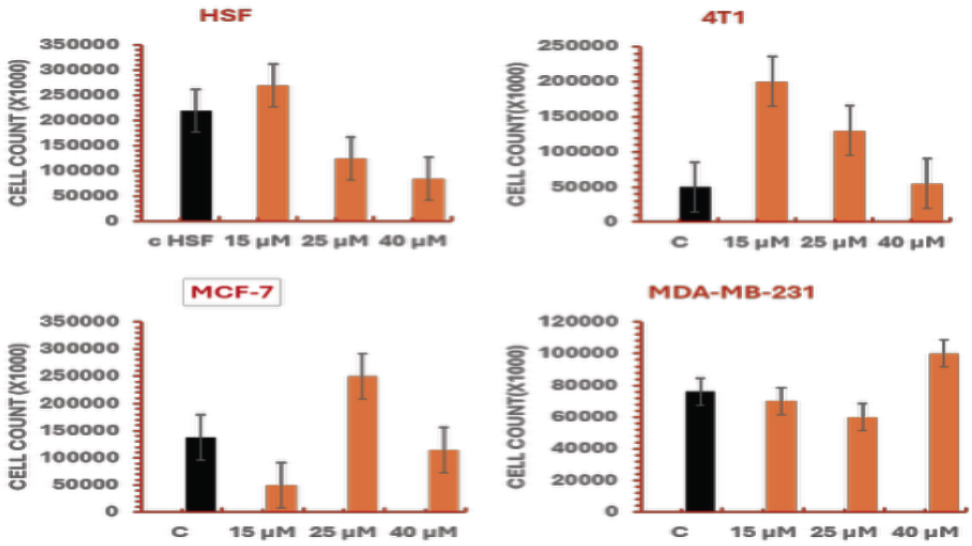


**Figure 6:** The trypan blue exclusion assay was employed to assess the cytotoxic effect of hesperidin on both normal (HSF) and breast cancer cell lines (4T1, MCF-7, MDA-MB-231). Cells were treated with increasing concentrations of hesperidin (15, 25, and 40 µM), and viable cells were quantified under a light microscope. The results demonstrated a dose-dependent decline in cell viability across all cancer cell lines, with MCF-7 and 4T1 showing the most pronounced reductions. MDA-MB-231 cells exhibited moderate sensitivity, while HSF cells retained higher viability at lower doses, suggesting selective cytotoxicity of hesperidin toward malignant cells. This highlights hesperidin's potential as a differential anticancer agent sparing normal cells.

its potential as a concentration-responsive anticancer agent.

### Wound Healing Assay

The wound healing assay was used to assess the anti-migratory effect of hesperidin on HSF, 4T1, MCF-7, and MDA-MB-231 cell lines at concentrations of 15, 25, and 40 µM (Figure **7**). Images were captured at 0-, 3-, 18-, and 21 hours post-treatment to observe the rate of wound closure. In untreated controls, cancer cell lines exhibited high migration, with progressive wound closure over time. However, hesperidin-treated cells showed significantly reduced migration in a dose-dependent manner, especially at 25 and 40 µM concentrations. MCF-7 and 4T1 displayed the most pronounced inhibition, while MDA-MB-231 exhibited moderate sensitivity. HSF cells (normal) showed variable migration, with less reduction compared to

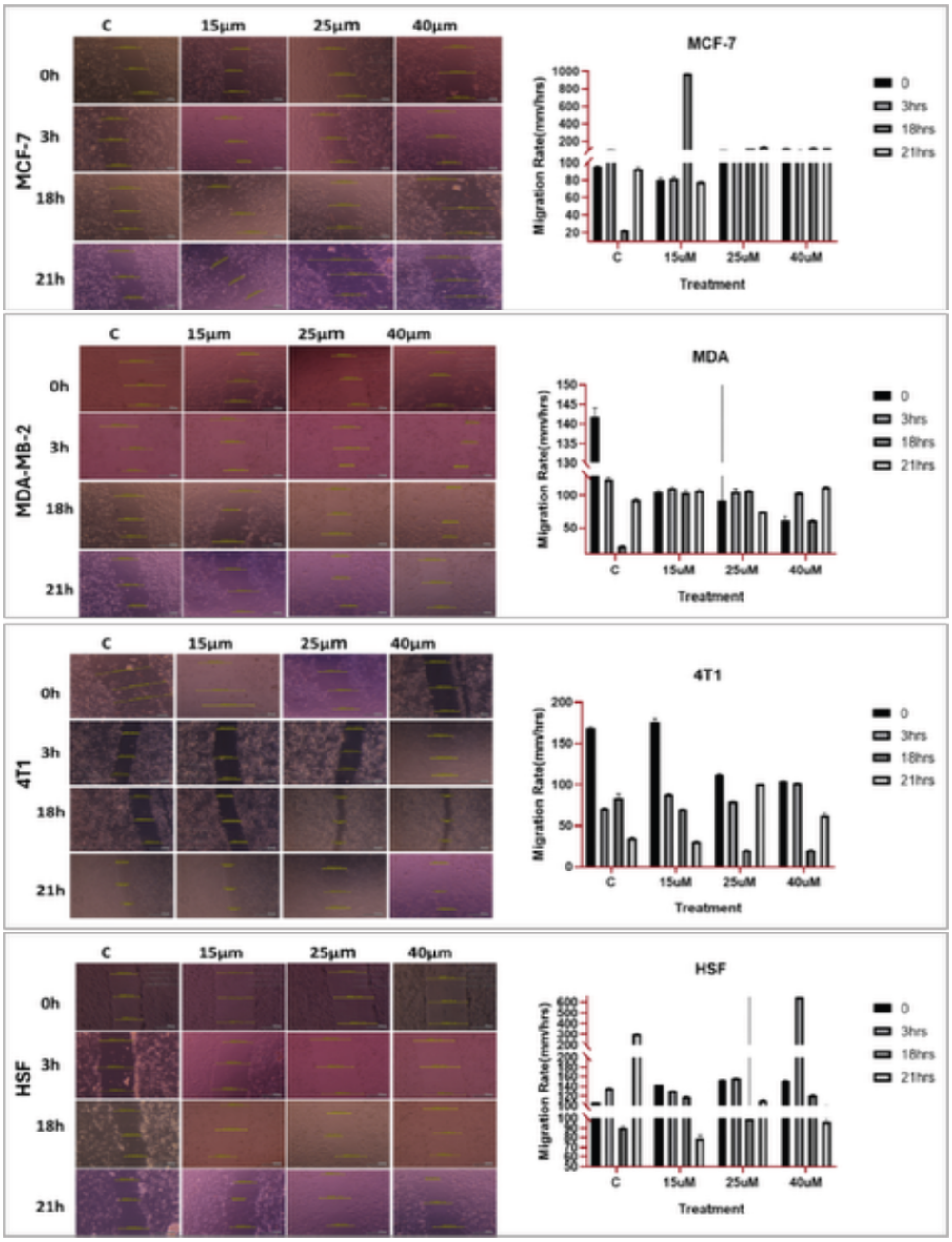


**Figure 7:** Wound healing assay evaluating the impact of hesperidin on cell migration in MCF-7, MDA-MB-231, 4T1, and HSF cell lines. Cells were treated with 0, 15, 25, or 40 µM of hesperidin and imaged at 0, 3, 18, and 21 hours. Untreated control groups exhibited progressive wound closure over time. Hesperidin treatment led to significant suppression of migration, particularly at 25 and 40 µM. MCF-7 and 4T1 cells showed the highest sensitivity to hesperidin's anti-migratory effects. Normal HSF cells maintained higher migration capacity than cancerous cells under the same conditions. Graphs represent migration rate quantification in mm/hr across time points.

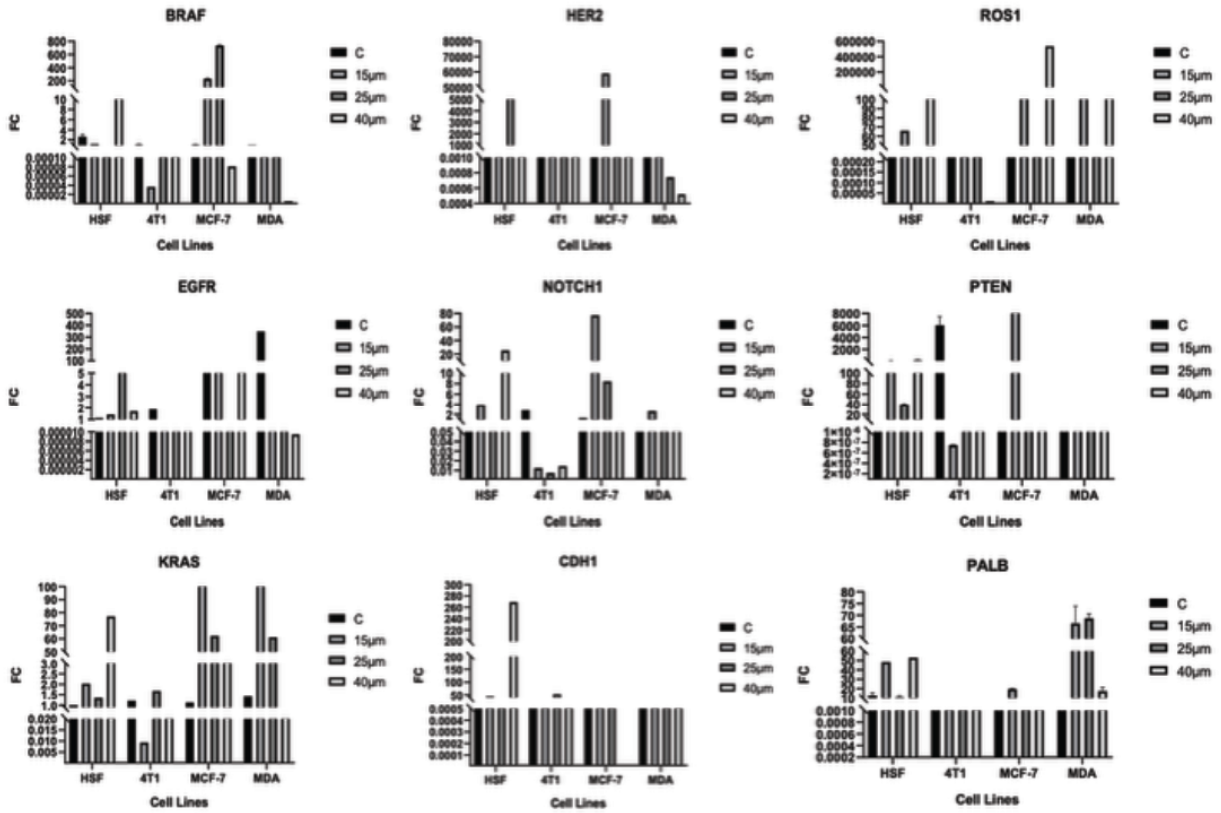


**Figure 8:** Quantitative real-time PCR analysis of *HER2*, *EGFR*, *PALB*, *BRAF*, *PTEN*, *NOTCH1*, *ROS1*, *CDH1*, and *KRAS* gene expression in HSF, 4T1, MCF-7, and MDA-MB-231 cell lines after treatment with different concentrations of hesperidin. Notable downregulation of HER2, EGFR, and *PALB* was observed in MCF-7 cells, while *NOTCH1* and *KRAS* were suppressed in MDA-MB-231. *PTEN* and *CDH1* were upregulated across cancer lines, especially in MDA-MB-231 and 4T1, indicating anti-proliferative and anti-metastatic potential. HSF cells showed minimal changes, reflecting the selective targeting of cancer-specific pathways. Data are presented as mean ± SD from triplicate experiments.

cancerous lines, indicating selective inhibition by hesperidin. Quantitative graphs further confirm a time- and concentration-dependent decline in migration rates across cell types. The data suggest that hesperidin effectively suppresses cell motility, which is crucial in cancer metastasis. These findings highlight its potential role in limiting cancer progression by targeting cellular migration.

### Real Time PCR

Following treatment with different concentrations of hesperidin, notable alterations in the expression of nine key genes—*HER2*, *EGFR*, *PALB*, *BRAF*, *PTEN*, *NOTCH1*, *ROS1*, *CDH1*, and *KRAS*—were observed across the tested cell lines: HSF, 4T1, MCF-7, and MDA-MB-231. In MCF-7 cells, hesperidin markedly downregulated *HER2*, *EGFR*, and *PALB*, indicating suppression of proliferative signaling. In MDA-MB-231, significant downregulation of *NOTCH1* and *KRAS* was observed, while *PTEN* was notably upregulated, suggesting pro-apoptotic effects. *CDH1*, a tumor suppressor gene involved in cell adhesion, was consistently upregulated in all cancer lines, highlighting hesperidin's role in mitigating metastasis. In 4T1 cells, *ROS1* and *BRAF* expression were significantly reduced, further implicating disruption of oncogenic signaling. Conversely, HSF (normal fibroblasts) showed minimal or no significant changes in gene expression, indicating selective action of hesperidin on malignant cells. The most profound modulations were seen in *EGFR* and *HER2* in MCF-7 and *NOTCH1* in MDA-MB-231, underscoring hesperidin's subtype-specific molecular effects. Overall, the data reveal that hesperidin downregulates oncogenes while upregulating tumor suppressors in a dose-dependent, cell-type-specific manner.

## DISCUSSION

The substantial global health burden imposed by breast cancer necessitates the ongoing identification and characterization of novel therapeutic agents [25, 26]. Natural products represent a valuable reservoir for such discovery, potentially offering improved efficacy and more favorable toxicity profiles compared to conventional therapies [27, 28]. Hesperidin, a flavanone glycoside prevalent in citrus species, has

attracted considerable scientific interest owing to its broad spectrum of pharmacological activities, encompassing antioxidant, anti-inflammatory, and putative anti-neoplastic properties [29]. This investigation sought to comprehensively evaluate the *in vitro* anti-cancer efficacy of hesperidin against phenotypically distinct breast cancer subtypes—luminal A (MCF-7), triple-negative (MDA-MB-231), and the aggressive murine model (4T1)—while concurrently assessing its impact on non-cancerous human skin fibroblasts (HSF) to ascertain potential therapeutic selectivity. Our results provide compelling evidence that hesperidin mediates significant, multifaceted anti-cancer effects *in vitro*, including the induction of cytotoxicity, modulation of cell cycle progression, triggering of apoptosis, and inhibition of cell migration. These effects appear underpinned by the modulation of key regulatory genes and exhibit a degree of selectivity towards malignant cells over normal fibroblasts.

The primary assessment of cytotoxicity, employing both MTT and Trypan Blue exclusion assays, confirmed hesperidin's capacity to diminish cell viability across all tested lines, generally in a dose-dependent fashion. Critically, a degree of selectivity was discernible. While the highest tested concentration (40 μM) significantly reduced HSF viability (P=0.002, MTT assay), the breast cancer cell lines, notably 4T1 and MCF-7, displayed significant viability reductions at lower or intermediate concentrations (15μ M or 25 μM) where HSF viability remained largely uncompromised. MDA-MB-231 cells necessitated the highest concentration (40 μM) for statistically significant cytotoxicity in the MTT assay (P=0.00027), although Trypan Blue exclusion suggested sensitivity initiated at lower dosages. This differential sensitivity is a pivotal characteristic for potential therapeutic candidates, implying a potentially wider therapeutic window for hesperidin relative to non-selective cytotoxic agents [30] and highlighting the importance of further investigations into its mechanisms of action. Understanding these pathways may enhance the efficacy of hesperidin as a co-chemotherapy agent in various cancer treatments [31]. These observations are broadly consistent with prior reports documenting hesperidin's cytotoxic activity against various cancer types, including breast cancer cell lines, although effective concentrations and cellular sensitivities can fluctuate depending on the specific cell line and experimental parameters [32, 33]. The minor divergence between MTT assay results (indicative of metabolic activity) and Trypan Blue data (reflecting membrane integrity) for MDA-MB-231 might suggest that early metabolic perturbations precede overt membrane damage at lower hesperidin concentrations in this specific cell line.

Beyond cytotoxicity, elucidating the underlying anti-cancer mechanisms is crucial. Our cell cycle analysis revealed intriguing heterogeneity in cellular responses following treatment with 25 μM hesperidin. MCF-7 cells exhibited arrest predominantly in the G1 phase, implying interference with the G1/S transition checkpoint. Conversely, the aggressive MDA-MB-231 cells accumulated primarily in the G2/M phase, indicating disruption later in the cell cycle progression. Notably, both the murine 4T1 cancer cells and the non-malignant HSF cells displayed accumulation in the S-phase. This cell-type-specific modulation argues against a single, universal mechanism of action and suggests hesperidin may interact with distinct molecular vulnerabilities inherent to different cellular contexts. The G1 arrest observed in MCF-7 and G2/M arrest in MDA-MB-231 align with anti-proliferative effects reported for flavonoids in other cancer models [34]. and are potentially linked to the modulation of cell cycle regulatory proteins [35, 36]. Gene expression data in the present study indicated downregulation of HER2, EGFR, and potentially altered expression of DNA repair-associated genes like PALB2 in cancer cells following treatment. While downregulation of growth factor receptors like HER2 and EGFR could contribute to cell cycle inhibition, the precise molecular links between the observed gene expression changes and the specific cell cycle arrest points (particularly the G2/M arrest in MDA-MB-231 despite these changes) may involve complex regulatory networks and warrant further investigation. The S-phase accumulation in 4T1 and HSF cells requires further study; it could reflect interference with DNA replication or repair processes, possibly highlighting a different aspect of hesperidin's activity or an off-target effect less prominent in the human breast cancer lines examined.

Induction of apoptosis is a desirable characteristic of effective anti-cancer therapeutics. Our Annexin V/PI flow cytometry data provide robust evidence for hesperidin's pro-apoptotic capabilities. Treatment with 25 μM hesperidin significantly augmented the proportion of apoptotic cells (predominantly early apoptosis) across all three cancer cell lines. MDA-MB-231 cells demonstrated the most pronounced apoptotic response, followed by MCF-7 and 4T1. Crucially, the pro-apoptotic effect on normal HSF cells was substantially lower, reinforcing the observation of cancer cell selectivity. This apoptotic induction was

mechanistically supported by gene expression analysis. Hesperidin treatment led to significant upregulation of the pivotal tumor suppressor TP53 and the key executioner caspases, CASP3 and CASP9, in the cancer cell lines. This suggests activation of the intrinsic, p53-dependent mitochondrial apoptotic pathway. Concurrently, modulation of other critical regulatory genes was observed; for instance, changes in the expression of the tumor suppressor PTEN and the cell adhesion molecule CDH1 (E-cadherin) were noted in treated cancer cells, potentially influencing apoptotic susceptibility and cell survival pathways. While the precise interplay requires further study, the activation of caspases and p53 is consistent with established mechanisms by which flavonoids, including hesperidin, induce apoptosis in various cancer contexts [32, 33], potentially involving factors like reactive oxygen species generation.

Cancer metastasis remains the principal driver of mortality, with cell migration representing a critical initial step [37, 38]. The wound healing assay clearly demonstrated hesperidin's potent anti-migratory activity *in vitro*. Hesperidin significantly impeded wound closure in all tested cancer cell lines in a dose-dependent manner, with MCF-7 and 4T1 cells showing sensitivity. This inhibitory effect was markedly less pronounced in normal HSF cells. This finding holds considerable clinical relevance, suggesting a potential role for hesperidin in limiting tumor dissemination. Mechanistically, the inhibition of migration may be linked to the modulation of genes regulating cell motility and invasion. The observed downregulation of HER2 and EGFR, especially evident in the highly invasive MDA-MB-231 cell line, could contribute to reduced migratory potential. Furthermore, matrix metalloproteinases (MMPs), such as MMP9, play crucial roles in extracellular matrix degradation, facilitating invasion and migration [39, 40]. While MMP activity was not directly assayed here, our findings align with studies indicating that hesperidin and related phytochemicals can suppress MMP expression and activity, thereby inhibiting cancer cell motility and potentially modulating epithelial-mesenchymal transition (EMT) markers [41, 42].

Integrating these diverse findings, this study posits that hesperidin orchestrates a multi-pronged anti-cancer assault against breast cancer cells *in vitro*. It effectively reduces cell viability, inhibits proliferation through cell-type-specific cell cycle arrest (associated with modulation of growth signaling pathways), triggers programmed cell death via the intrinsic apoptotic pathway (evidenced by TP53, CASP3, and CASP9 upregulation), and curtails migratory capacity. The observed differential responses between the luminal A (MCF-7) and triple-negative (MDA-MB-231) subtypes highlight the critical importance of considering tumor heterogeneity in evaluating therapeutic agents [43] Moreover, the consistent finding of reduced sensitivity in normal HSF cells across multiple functional assays (cytotoxicity, apoptosis, migration) strongly supports the potential for a favorable therapeutic index for hesperidin. The gene expression analyses further suggested hesperidin modulates a panel of key regulatory genes including HER2, EGFR, PALB2, PTEN, and CDH1, alongside potentially others like BRAF, NOTCH1, ROS1, and KRAS, reflecting its impact on multiple oncogenic pathways.

While this study furnishes compelling *in vitro* evidence, certain limitations must be acknowledged. The conclusions are derived solely from cell line models, which inherently cannot fully recapitulate the complexities of the *in vivo* tumor microenvironment or systemic pharmacokinetics and pharmacodynamics. Furthermore, the mechanistic explorations (cell cycle, apoptosis, gene expression) were primarily conducted at a single hesperidin concentration (25μM) and time point (24 h), potentially not capturing the full spectrum of dose- or time-dependent cellular responses. The issue of hesperidin's bioavailability *in vivo* also remains a consideration, although novel delivery strategies are being explored. Finally, the biological implications of the S-phase accumulation observed in HSF cells at 25 μM require further clarification regarding potential effects on normal cell function at therapeutically relevant concentrations.

Notwithstanding these limitations, the concordance of results across multiple assays and distinct cell lines establishes a robust foundation for subsequent investigations. Preclinical *in vivo* studies are imperative to validate these *in vitro* findings, assess anti-tumor efficacy within a physiological context, and evaluate potential systemic toxicity; the 4T1 murine model, which demonstrated sensitivity *in vitro*, represents a suitable platform for such studies. Delving into the upstream signaling pathways (e.g., PI3K/Akt, MAPK, NF-κB) that govern the observed alterations in gene expression would yield deeper mechanistic insights. Additionally, exploring potential synergistic interactions between hesperidin and established chemotherapeutic agents could illuminate novel combination treatment strategies, potentially offering enhanced efficacy or reduced systemic toxicity [44].

This study strongly argues for the significant anti-cancer potential of hesperidin against diverse breast cancer subtypes *in vitro*. Through comprehensive functional and molecular analyses, we demonstrate its ability to selectively induce cytotoxicity, modulate cell cycle progression, trigger apoptosis predominantly via the intrinsic pathway, and inhibit cell migration. These effects appear mediated, at least in part, by the regulation of key genes integral to these cellular processes, including HER2, EGFR, PALB2, PTEN, CDH1, TP53, CASP3, and CASP9. The observed selectivity towards cancer cells over normal fibroblasts further enhances its therapeutic appeal. Collectively, these findings position hesperidin as a promising natural compound warranting rigorous preclinical and subsequent clinical investigation to fully ascertain its potential role in breast cancer management and possibly prevention.

Our findings are consistent with previous studies demonstrating that hesperidin exerts significant anticancer effects against breast cancer cells through inhibition of proliferation and induction of apoptosis [45, 46]. Previous investigations in MCF-7 and MDA-MB-231 cells have shown that hesperidin promotes apoptotic cell death through modulation of apoptosis-associated proteins and cell-cycle regulatory mechanisms, although the underlying molecular responses may differ between breast cancer subtypes [46]. Furthermore, accumulating evidence suggests that hesperidin-mediated anticancer activity involves regulation of key signaling pathways, including PI3K/AKT/mTOR, p53, PTEN, and Notch signaling networks, which collectively contribute to growth inhibition, apoptosis induction, and suppression of tumor progression [47-49]. Consistent with these reports, the present study demonstrated reduced cell viability, increased apoptosis, and impaired migratory capacity following hesperidin treatment. However, unlike previous studies that primarily focused on individual breast cancer models, our work provides a direct comparative evaluation of luminal A (MCF-7), triple-negative (MDA-MB-231), and 4T1 breast cancer cells together with non-malignant fibroblasts. Notably, distinct subtype-specific responses were observed, with MCF-7 cells exhibiting G1-phase arrest, whereas MDA-MB-231 cells accumulated predominantly in the G2/M phase. These findings suggest that hesperidin engages context-dependent molecular programs that vary according to breast cancer subtype. Moreover, differential regulation of oncogenic and tumor-suppressive genes, including EGFR, HER2, KRAS, NOTCH1, PTEN, and CDH1, further supports the notion that hesperidin exerts multitarget effects through subtype-specific mechanisms. The inclusion of the highly aggressive 4T1 model together with normal human fibroblasts enabled simultaneous assessment of both efficacy and selectivity, demonstrating that hesperidin preferentially targets malignant cells while exerting comparatively limited effects on non-cancerous cells. Collectively, these findings extend current knowledge regarding hesperidin's anticancer activity and highlight its potential as a multi-target therapeutic candidate capable of eliciting distinct biological responses across heterogeneous breast cancer subtypes.

Although significant alterations in apoptosis-related genes were observed, protein-level validation of key signaling mediators such as p53, PTEN, CASP3, and CASP9 using Western blotting or immunodetection approaches is required to confirm activation of these pathways and represents an important direction for future investigations.

## CONCLUSION

This study provides compelling *in vitro* evidence for the anti-cancer efficacy of hesperidin against multiple BC subtypes. Hesperidin effectively reduced cell viability, induced cell-type-specific cell cycle arrest, triggered apoptosis via modulation of key regulatory genes (*BAX*/*BCL2*, caspases, p53), and inhibited cell migration associated with *MMP9* downregulation. Crucially, these cytotoxic and anti-migratory effects displayed selectivity towards cancer cells (MCF-7, MDA-MB-231, 4T1) compared to normal fibroblasts (HSF). This work significantly contributes to the field by highlighting hesperidin's multi-pronged mechanisms and favorable selectivity profile, positioning it as a promising natural compound for developing potentially safer and effective therapies against heterogeneous breast cancers. Future research should focus on validating these findings through *in vivo* preclinical models to assess systemic efficacy and toxicity. It should also explore its potential in combination therapies to overcome treatment resistance in BC patients.

## CONFLICT OF INTERESTS

All authors declare no conflict of interests

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Not Applicable.

## AUTHOR CONTRIBUTION

NF: conducted the experimental work, AAB and JE: write the first draft, SA-G: supervised the experimental work, HS: conceptualized the idea, AE: edited and submitted the manuscript.

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## ETHICAL APPROVAL STATEMENT

There is no animal work in this study to be ethically Approved.

## DECLARATION OF COMPETING AND CONFLICT OF INTEREST

The authors have no conflicts of interest and no competing interests to declare.

## DATA AVAILABILITY

All data will be available from the corresponding author upon reasonable request.

## CONSENT TO PUBLISH

All authors have read and approved the submission for publication.

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