

ORIGINAL RESEARCH

Phillyrin restrains breast cancer cell growth by inducing ferroptosis

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Abstract

Background: Breast cancer is one of the most common malignant tumors worldwide, predominantly affecting women and associated with high mortality. Phillyrin (PHN), a bioactive compound isolated from *Forsythia suspensa* (Thunb.) Vahl has been confirmed to possess diverse biological activities, including anti-inflammatory, antioxidant, and anti-tumor effects. Nevertheless, its regulatory role in breast cancer progression remains unclear. **Methods:** Cell viability was assessed using the Cell Counting Kit-8 (CCK-8) assay, while proliferative capacity was evaluated through colony formation assay. Apoptosis was determined by flow cytometry. The level of reactive oxygen species (ROS) was measured by 2',7'-dichlorofluorescein diacetate (DCFH-DA) staining. The concentrations of malondialdehyde (MDA), glutathione (GSH), and Fe²⁺ were quantified using the corresponding detection kits. Protein expression levels were analyzed by western blotting. **Results:** PHN significantly inhibited breast cancer cell growth and induced ferroptosis. Treatment with 50 μ mol/L PHN reduced cell proliferation, an effect that was reversed by Ferrostatin-1 (Fer-1), a ferroptosis inhibitor. Furthermore, PHN was shown to suppress activation of the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) signaling pathway. **Conclusions:** PHN inhibited breast cancer cell growth by inducing ferroptosis and suppressing the PI3K/Akt pathway. These findings suggest that PHN may represent a potential therapeutic agent for the treatment of breast cancer.

Keywords

Phillyrin; Ferroptosis; PI3K/Akt pathway; Breast cancer

1. Introduction

Breast cancer is one of the most common malignant tumors in women, and its incidence continues to increase annually, posing a significant threat to public health [1, 2]. Current treatment options for breast cancer primarily include surgery, postoperative radiotherapy, and chemotherapy [3], while hormone therapy and targeted therapy are also widely recognized as standard approaches [4, 5]. Nevertheless, recurrence and metastasis remain major clinical issues, making breast cancer treatment highly challenging [6]. Therefore, elucidating the molecular mechanisms underlying tumor growth and identifying effective therapeutic agents remains essential for improving breast cancer management.

Phillyrin (PHN), a bioactive compound isolated from *Forsythia suspensa* (Thunb.) Vahl, has been demonstrated to exert diverse biological functions, including anti-inflammatory, antioxidant, and anti-tumor activities [7]. For instance, PHN inhibits activation of the nucleotide-binding oligomerization domain-like receptor family pyrin domain-containing 3 (NLRP3) inflammasome, thereby alleviating pulmonary inflammation induced by influenza A virus [8].

It also antagonizes endothelin-1 signaling to protect against myocardial remodeling [9]. In sepsis-induced acute lung injury, PHN mitigates damage by suppressing the pyroptosis pathway [10]. In addition, PHN inhibits cell proliferation and angiogenesis in colorectal cancer by targeting cluster of differentiation 147 (CD147) [11]. In hepatocellular carcinoma, PHN downregulates topoisomerase II α (TOP2A), thereby modulating the Janus kinase 2 (JAK2)/signal transducer and activator of transcription 3 (STAT3) pathway to reduce tumorigenesis [12]. Despite these findings, the precise regulatory effects of PHN and its associated pathways in breast cancer progression have not yet been clarified.

Therefore, the aim of this study was to investigate the role of PHN in breast cancer progression and explore its underlying molecular mechanisms, with particular attention to ferroptosis and the PI3K/Akt signaling pathway. Our findings demonstrated that PHN significantly inhibited breast cancer cell growth by inducing ferroptosis and suppressing PI3K/Akt pathway activation, supporting PHN as a potential therapeutic candidate breast cancer treatment.

2. Materials and methods

2.1 Cell culture and treatment

Breast cancer cell lines (MDA-MB-231 and BT549) were purchased from the American Type Culture Collection (ATCC, USA) and cultured in Dulbecco's Modified Eagle's Medium (DMEM; 11965084, Gibco, Carlsbad, CA, USA) supplemented as recommended. Cells were maintained in a humidified incubator at 37 °C with 5% CO₂. Phillyrin (PHN; S392602, Shanghai Selleck Chemicals Co., Ltd., Shanghai, China) was used at concentrations of 0, 2.5, 5, 10, 25, 50, 100, and 200 µmol/L. Based on preliminary results, 25 and 50 µmol/L PHN were selected for subsequent experiments [12].

2.2 Cell counting kit-8 (CCK-8) assay

MDA-MB-231 and BT549 cells were seeded into 96-well plates and treated accordingly. CCK-8 solution (10 µL/well; CK04, Dojindo Laboratories, Kumamoto, Japan) was added and incubated for 2 h. Absorbance was measured using a spectrophotometer (ND-ONE-W, Thermo Fisher Scientific, Waltham, MA, USA) to determine cell viability.

2.3 Colony formation assay

MDA-MB-231 and BT549 cells were seeded in 6-well plates and incubated for 14 days. Colonies were fixed with 4% paraformaldehyde, stained with 0.1% crystal violet, and imaged. Colony numbers were subsequently counted for analysis.

2.4 Flow cytometry

MDA-MB-231 and BT549 cells were harvested and resuspended in binding buffer. The cells were stained in the dark with 5 µL FITC Annexin V (556420, BD Biosciences, Franklin Lakes, NJ, USA) and 10 µL propidium iodide. Then, the apoptotic cells were quantified using a flow cytometer (FACSCanto II, BD Biosciences, San Jose, CA, USA).

2.5 ROS examination

MDA-MB-231 and BT549 cells were mixed with 2',7'-dichlorofluorescein diacetate (DCFH-DA; E004-1-1, Nanjing Jiancheng Technology Co., Ltd., Nanjing, China), and the corresponding fluorescence signals were captured using a fluorescence microscope (BX41, Olympus, Tokyo, Japan).

2.6 Detection of MDA, ROS and Fe²⁺

The levels of malondialdehyde (MDA; ab118970, Abcam, Shanghai, China), glutathione (GSH; ab112132, Abcam, Shanghai, China), and Fe²⁺ (ab83366, Abcam, Shanghai, China) were quantified using the corresponding commercial kits, following the manufacturers' protocols.

2.7 Western blot

Proteins were extracted from MDA-MB-231 and BT549 cells and separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). They were subsequently transferred onto polyvinylidene fluoride (PVDF) membranes

(Beyotime, Shanghai, China). After blocking, membranes were incubated with primary antibodies for 12 h, followed by incubation with a horseradish peroxidase-conjugated secondary antibody (1:1000; ab7090, Abcam, Shanghai, China) for 2 h. Protein bands were visualized using a chemiluminescence detection kit (89880, Thermo Fisher Scientific, Inc., Waltham, MA, USA).

The primary antibodies used includes: p-PI3K (1:1000; ab235266), PI3K (1:1000; ab40755), p-Akt (1:1000; ab8933), Akt (1:2000; ab8805), and β-actin (1:1000; ab8226).

2.8 Statistical analysis

All data were expressed as mean ± standard deviation (SD). Statistical analyses were performed using GraphPad Prism 9 software (GraphPad Software, San Diego, CA, USA). Differences between groups were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test. A *p*-value < 0.05 was considered statistically significant.

3. Results

3.1 PHN suppressed cell growth in breast cancer

Cell viability was significantly reduced following treatment with 100 and 200 µmol/L PHN, whereas no changes were observed at concentrations of 0, 2.5, 5, 10, 25, and 50 µmol/L (Fig. 1a), indicating that PHN exhibited no cytotoxicity at or below 50 µmol/L, and therefore 25 and 50 µmol/L were selected for subsequent experiments. Consistently, colony formation assays revealed that cell proliferative capacity was significantly reduced after treatment with 25 and 50 µmol/L PHN (Fig. 1b). Furthermore, flow cytometry analysis demonstrated that apoptosis was significantly increased under the same treatment conditions (Fig. 1c). These findings suggest that PHN may effectively suppress breast cancer cell growth.

3.2 PHN triggered ferroptosis in breast cancer

DCFH-DA staining revealed that intracellular ROS levels were significantly elevated following treatment with 25 and 50 µmol/L PHN (Fig. 2a). Consistently, MDA levels were also increased in response to PHN treatment (Fig. 2b). In contrast, GSH levels were markedly reduced after PHN exposure (Fig. 2c), and Fe²⁺ levels were significantly increased in PHN-treated cells (Fig. 2d). Together, these findings indicate that PHN induced ferroptosis in breast cancer cells.

3.3 PHN restrained breast cancer cell growth by inducing ferroptosis

Colony formation assays demonstrated that cell proliferation was markedly reduced following treatment with 50 µmol/L PHN; however, this inhibitory effect was reversed by the ferroptosis inhibitor Fer-1 (Fig. 3a). Similarly, flow cytometry revealed that apoptosis was significantly increased after 50 µmol/L PHN treatment, whereas co-treatment with Fer-1 attenuated this effect (Fig. 3b). In addition, western blot analysis showed that the protein expression levels of

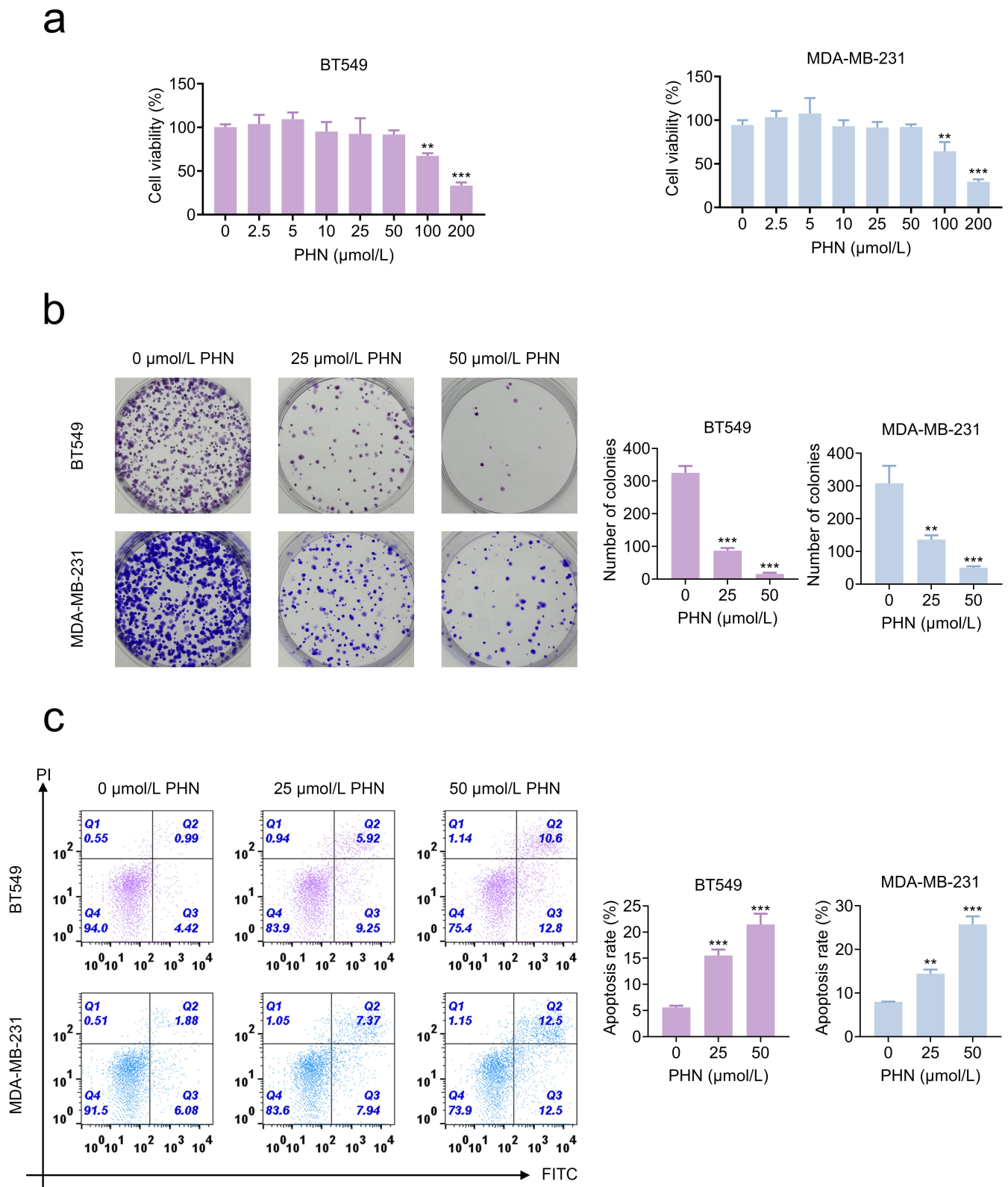


FIGURE 1. PHN suppressed cell growth in breast cancer. (a) Cell viability was assessed by CCK-8 assay following treatment with PHN at concentrations of 0, 2.5, 5, 10, 25, 50, 100, and 200 $\mu\text{mol/L}$. (b) Cell proliferation was evaluated by colony formation assay in the 0, 25, and 50 $\mu\text{mol/L}$ PHN groups. (c) Cell apoptosis was analyzed by flow cytometry in the 0, 25, and 50 $\mu\text{mol/L}$ PHN groups. ** $p < 0.01$, *** $p < 0.001$. Abbreviations: PHN, Phillyrin; PI, propidium iodide; FITC, fluorescein isothiocyanate.

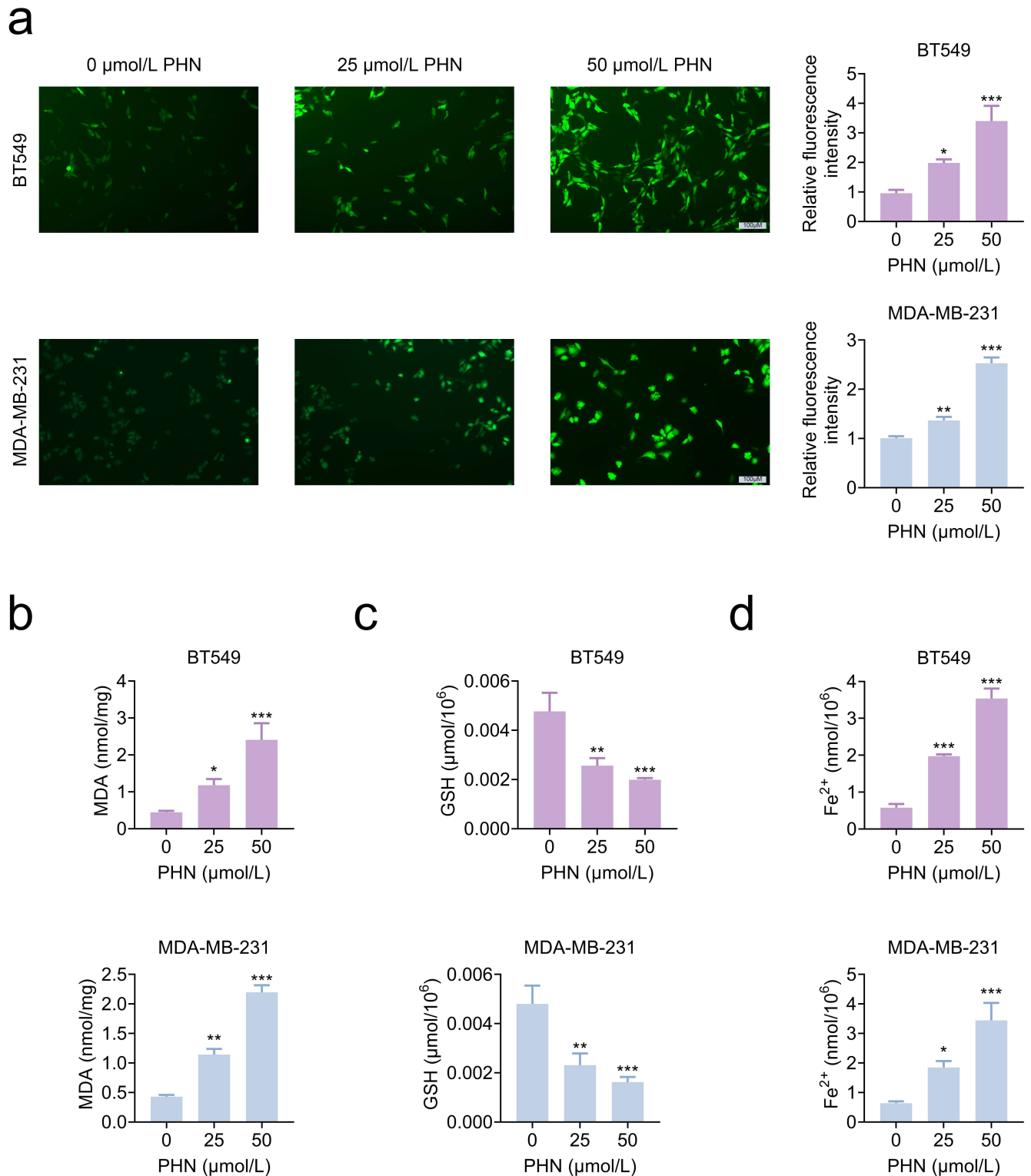


FIGURE 2. PHN triggered ferroptosis in breast cancer. (a) Intracellular ROS levels were evaluated by DCFH-DA staining in the 0, 25, and 50 $\mu\text{mol/L}$ PHN groups. (b) MDA levels were measured using the MDA assay kit. (c) GSH levels were determined with the GSH assay kit. (d) Fe^{2+} levels were quantified using the Fe^{2+} assay kit. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Abbreviations: PHN, Phillyrin; GSH, glutathione.

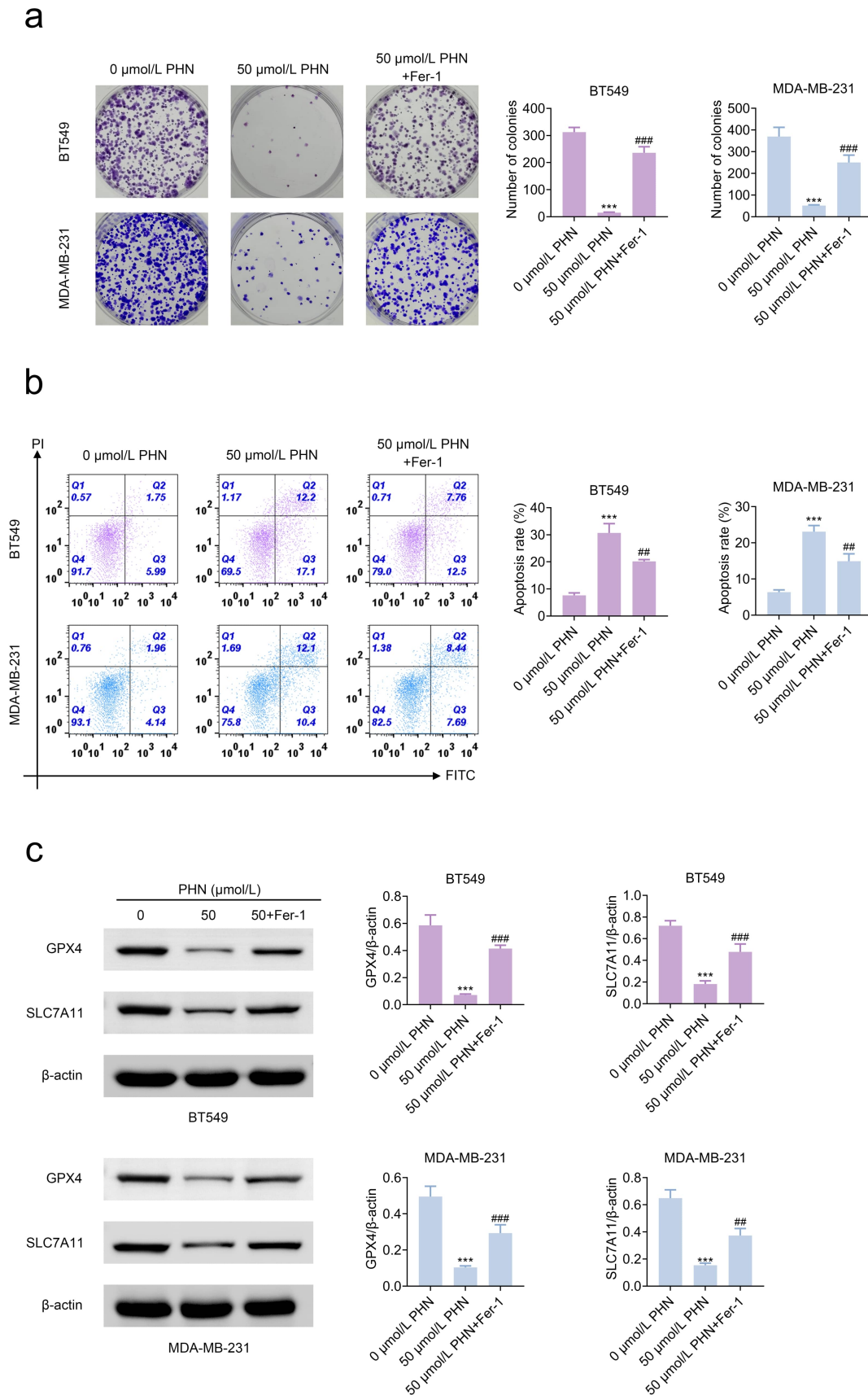


FIGURE 3. PHN restrained breast cancer cell growth by inducing ferroptosis. (a) Cell proliferation was evaluated by colony formation assay in the 0 $\mu\text{mol/L}$ PHN, 50 $\mu\text{mol/L}$ PHN, and 50 $\mu\text{mol/L}$ PHN + Fer-1 groups. (b) Cell apoptosis was analyzed by flow cytometry under the same treatment conditions. (c) The protein expression levels of GPX4 and SLC7A11 were determined by western blot analysis. *** $p < 0.001$ vs. 0 $\mu\text{mol/L}$ PHN group; ## $p < 0.01$, ### $p < 0.001$ vs. 50 $\mu\text{mol/L}$ PHN group. Abbreviations: PHN, Phillyrin; Fer-1, Ferrostatin-1; GPX4, glutathione peroxidase 4; SLC7A11, solute carrier family 7 member 11.

glutathione peroxidase 4 (GPX4) and solute carrier family 7 member 11 (SLC7A11) were reduced after PHN exposure, but this reduction was reversed by Fer-1 treatment (Fig. 3c). Collectively, these findings indicate that PHN inhibited breast cancer cell growth by inducing ferroptosis.

3.4 PHN retarded the PI3K/Akt pathway

Western blot analysis revealed that the ratios of p-PI3K/PI3K and p-Akt/Akt were significantly reduced following PHN treatment at 25 and 50 $\mu\text{mol/L}$ (Fig. 4a). Furthermore, the downregulation of p-PI3K/PI3K and p-Akt/Akt induced by 50 $\mu\text{mol/L}$ PHN was reversed by co-treatment with Fer-1 (Fig. 4b). These findings indicate that PHN inhibited activation of the PI3K/Akt signaling pathway, and that this effect was dependent on ferroptosis.

4. Discussion

PHN has been reported to exert anti-inflammatory, antioxidant, and anti-tumor activities [8–12]. However, its regulatory role in breast cancer has remained unclear. In the present study, we demonstrated that PHN suppressed breast cancer cell growth, providing new insights into its potential therapeutic value.

Ferroptosis is a recently identified form of iron-dependent programmed cell death that has attracted considerable attention in cancer research in recent years [13]. Significant progress has been made in understanding its role in breast

cancer. For example, neutrophils can regulate aconitate decarboxylase 1 to suppress ferroptosis, thereby promoting breast cancer metastasis [14]. Metformin has been shown to inhibit ubiquitin-fold modifier 1 (UFMylation) of SLC7A11, leading to ferroptosis induction in breast cancer [15]. In contrast, DnaJ/Hsp40 homolog subfamily C, member 12 (DNAJC12) can inhibit doxorubicin-induced ferroptosis, resulting in chemotherapy resistance [16]. Furthermore, curcumenol enhances ferroptosis in triple-negative breast cancer by regulating the SLC7A11/NF- κ B (nuclear factor kappa-light-chain-enhancer of activated B cells)/TGF- β (transforming growth factor beta) pathway [17]. Notably, PHN has been reported to increase tumor cell sensitivity to ferroptosis in lung cancer [18]. Consistent with these findings, our results demonstrated that PHN induced ferroptosis in breast cancer cells. In particular, treatment with 50 $\mu\text{mol/L}$ PHN significantly reduced cell proliferation, whereas this effect was reversed by the ferroptosis inhibitor Fer-1, confirming the involvement of ferroptosis in the anti-tumor action of PHN.

The PI3K/Akt pathway plays a pivotal role in tumorigenesis [19], and increasing evidence indicates that it is critically involved in breast cancer progression. For example, baicalein suppresses breast cancer growth by blocking the PI3K/Akt pathway and enhancing autophagy [20]. Similarly, curcumenol inhibits breast cancer development by modulating this signaling cascade [21], while polyphyllin II restrains tumorigenesis by regulating the same pathway [22]. Beyond cancer, PHN

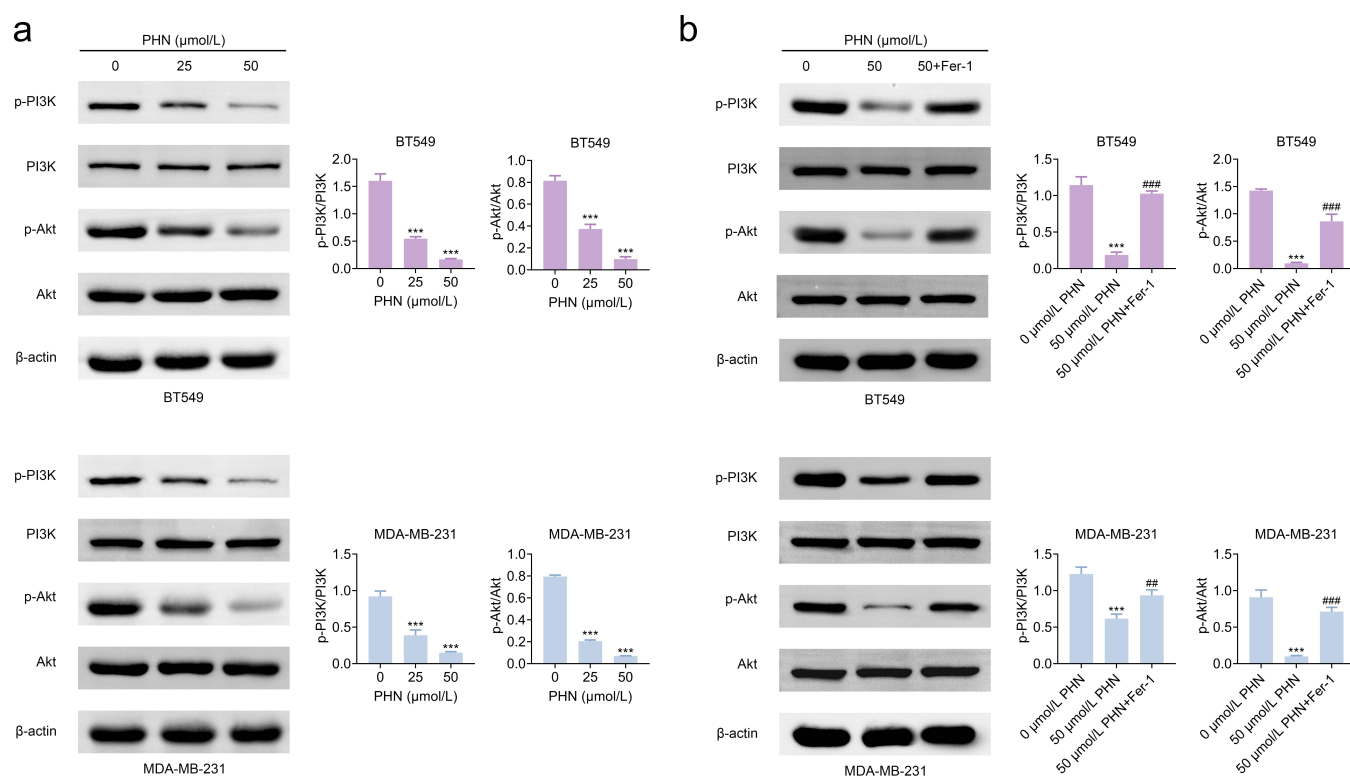


FIGURE 4. PHN inhibited the PI3K/Akt pathway. (a) Protein expression levels of p-PI3K, PI3K, p-Akt, and Akt were analyzed by western blot in the 0, 25, and 50 $\mu\text{mol/L}$ PHN groups. *** $p < 0.001$. (b) Protein expression levels of p-PI3K, PI3K, p-Akt, and Akt were determined by western blot in the 0 $\mu\text{mol/L}$ PHN, 50 $\mu\text{mol/L}$ PHN, and 50 $\mu\text{mol/L}$ PHN + Fer-1 groups. *** $p < 0.001$ vs. 0 $\mu\text{mol/L}$ PHN group; ### $p < 0.01$, #### $p < 0.001$ vs. 50 $\mu\text{mol/L}$ PHN group. Abbreviations: PHN, Phillyrin; Fer-1, Ferrostatin-1; p-PI3K, phosphorylated phosphatidylinositol 3-kinase; p-Akt, phosphorylated protein kinase B.

has also been reported to inhibit the PI3K/Akt pathway in non-malignant conditions, such as diabetic nephropathy, where it alleviates pathological changes [23] and reduces oxidative stress and inflammation [24]. In line with these observations, our study demonstrated that PHN inhibited the PI3K/Akt pathway in breast cancer cells, and this effect was closely associated with ferroptosis induction.

5. Conclusions

This study demonstrated that PHN inhibited breast cancer cell growth by inducing ferroptosis and suppressing the PI3K/Akt signaling pathway. However, several limitations should be acknowledged: (1) the absence of clinical studies; (2) the lack of human samples; (3) the absence of *in vivo* animal experiments; and (4) the limited evaluation of PHN concentrations. Future investigations could address these limitations by including lower PHN concentrations, expanding the range of experimental groups, and incorporating both animal models and clinical samples, for a more comprehensive understanding of the specific effects of PHN in breast cancer.

AVAILABILITY OF DATA AND MATERIALS

The authors declare that all data supporting the findings of this study are available within the paper and any raw data can be obtained from the corresponding author upon request.

AUTHOR CONTRIBUTIONS

LBY—designed the study and carried them out. LBY, FFY, ZLY—supervised the data collection; analyzed the data; interpreted the data; prepared the manuscript for publication and reviewed the draft of the manuscript. All authors have read and approved the manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This article does not contain any studies with human participants or animals performed by any of the authors.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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