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Hypophyllanthin and Phyllanthin from *Phyllanthus niruri* Synergize Doxorubicin Anticancer Properties against Resistant Breast Cancer Cells

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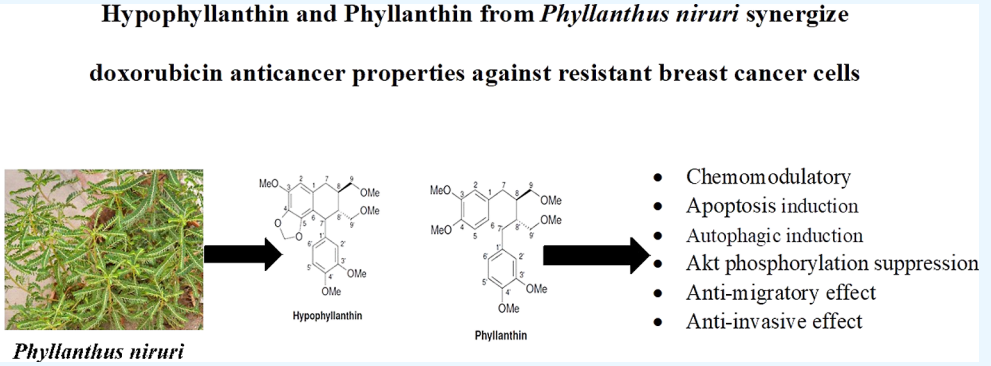

ABSTRACT: Doxorubicin (DOX) is a cornerstone chemotherapeutic agent for the treatment of several malignancies such as breast cancer; however, its activity is ameliorated by the development of a resistant phenotype. *Phyllanthus* species have been studied previously for their potential anticancer properties. The current work is aimed to study the potential cytotoxicity and chemomodulatory effects of hypophyllanthin (**PN4**) and phyllanthin (**PN5**) isolated from *Phyllanthus niruri* to DOX against the adriamycin multidrug-resistant breast cancer cells (MCF-7^{ADR}) and elucidate their mechanism of action. The major compounds of the active methylene chloride fraction were isolated and assessed for their potential cytotoxicity and chemomodulatory effects on DOX against naïve (MCF-7) and resistant breast (MCF-7^{ADR}) cancer cells. The mechanism of action of both compounds in terms of their impacts on programmed/non-programmed cell death (apoptosis and autophagy/necrosis), cell cycle progression/arrest, and tumor cell migration/invasion was investigated. Both compounds **PN4** and **PN5** showed a moderate but similar potency against MCF-7 as well as MCF-7^{ADR} and significantly synergized DOX-induced anticancer properties against MCF-7^{ADR}. The chemomodulatory effect of both compounds to DOX was found to be via potentiating DOX-induced cell cycle interference and apoptosis induction. It was found that **PN4** and **PN5** blocked the apoptosis-escape autophagy pathway in MCF-7^{ADR}. On the molecular level, both compounds interfered with SIRT1 expression and consequently suppressed Akt phosphorylation, and **PN5** blocked apoptosis escape. Furthermore, **PN4** and **PN5** showed promising antimigratory and anti-invasive effects against MCF-7^{ADR}, as confirmed by suppression of N-cadherin/β-catenin expression. In conclusion, for the first time, hypophyllanthin and phyllanthin isolated from *P. niruri* showed promising chemomodulatory effects to the DOX-induced chemotherapeutic activity against MCF-7^{ADR}. Both compounds significantly synergized DOX-induced anticancer properties against MCF-7^{ADR}. This enhanced activity was explained by further promoting DOX-induced apoptosis and suppressing the apoptosis-escape autophagy feature of the resistant breast cancer cells. Both compounds (hypophyllanthin and phyllanthin) interfered with the SIRT1/Akt pathway and suppressed the N-cadherin/β-catenin axis, confirming the observed antiproliferative, cytotoxic, and anti-invasive effects of hypophyllanthin and phyllanthin.

1. INTRODUCTION

Breast cancer is the most common cancer among women worldwide and represents the leading cause of cancer-related deaths.[1] Chemotherapy is a major strategy for the treatment of breast cancer; however, the development of chemoresistance and tumor recurrence remains a common cause of treatment

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failure and poor clinical outcomes.[2,3] Indeed, for a standard chemotherapy drug such as doxorubicin (DOX), patients with breast cancer may develop chemoresistance within only 6−10 months. Moreover, about 30−50% of metastatic breast cancer patients are irresponsive to the DOX treatment. Therefore, targeting drug resistance in breast cancer is crucial.[4]

Mechanisms of DOX resistance are multifaceted and include upregulation of drug-resistant proteins, changes in membrane permeability of cancer cells, impairment of DNA damage repair mechanisms, and autophagy-arbitrated drug resistance.[5] Among these mechanisms, autophagy-mediated chemotherapy resistance has gained increasing attention. Tumor cells can evade apoptosis through autophagy regulation, thereby increasing drug resistance and enhancing tumor survival.[6]

Sirtuin 1 (SIRT1) has been revealed to control diverse physiological and pathological processes, including autoimmunity, cellular metabolism, and tumor development.[7] Studies demonstrated that high expression of SIRT1 significantly promotes breast cancer progression and is associated with DOX resistance via abnormal activation (deacetylation) of several oncogenic pathways such as Akt.[8] Thus, targeting the SIRT1/Akt signaling axis represents a cornerstone in the response of breast cancer patients to DOX treatment.[9]

In addition, SIRT1 induces tumor invasion by targeting epithelial−mesenchymal transition (EMT)-related pathways.[10] Over the past decade, an increasing body of evidence indicated that the EMT might be a key mechanism in tumor progression, invasion, metastasis, and DOX resistance as well.[11,12] In aggressive breast cancer conditions, cell polarity and cell−cell contact are fallen in epithelial cells, and specific epithelial markers (such as E-cadherin and cytokeratin) are diminished, while mesenchymal characteristics (such as increased cell migration, activation of β-catenin signaling, vimentin, and N-cadherin) are expanded, participating in tumor resistance to chemotherapeutic drugs.[13,14] Therefore, targeting EMT is becoming one of the promising strategies for drug resistance reversal in breast cancer.[15]

Plant-derived phytochemicals have been widely investigated for their therapeutic potential in the prevention and treatment of cancer, besides their established role in chemosensitizing tumors to overcome drug resistance.[16] *Phyllanthus niruri* L. (Syn *Phyllanthus amarus* Schum & Thonn, family *Phyllanthaceae*) is a small herb widely distributed in tropical areas worldwide (such as India, China, Java, Southern Florida, and Bahamas), extensively used in ethnopharmacology for the treatment of diabetes, hypertension, and jaundice.[17] Recently, researchers have shown a growing interest in evaluating the anticancer activity of this herb.[18] The whole plant has diverse biological activities such as anti-inflammatory, antitumor, antinociceptive, and antioxidant properties.[19] The aqueous extract of the aerial parts of *P. niruri* showed an antiproliferative effect on human hepatocellular carcinoma cells (HepG2 and Huh-7) and colorectal carcinoma cells (HT-29).[20] However, many questions are still pending about the efficacy of this herb in eliminating cancer cells, especially high resistance and recurrence breast cancer phenotype.

In our previous work, lignan-rich extract from the aerial parts of *P. niruri* was prepared using non-conventional methods to increase the level of lignans calculated as phyllanthin.[19] Recently, we investigated the cytotoxic and chemomodulatory effects of crude methanolic extract and several subfractions of *P. niruri* against MCF-7 and MCF-7^{ADR} breast cancer cells.[21] The methylene chloride fraction (CH_2Cl_2) showed the most potent cytotoxic activity among all tested fractions. Surprisingly, CH_2Cl_2 was more cytotoxic against the adriamycin (ADR) multidrug-resistant breast cancer cells (MCF-7^{ADR}) when compared to the naïve cells (MCF-7). In addition, this fraction at sub-cytotoxic concentrations significantly enhanced the cytotoxicity of DOX against both the naïve (MCF-7) and the resistant (MCF-7^{ADR}) breast cancer cells. Therefore, the major compounds from this fraction were isolated and assessed for their potential cytotoxicity and chemomodulatory effects on DOX against naïve and resistant breast cancer cells. Also, we investigated the mechanism of action of these compounds in terms of their impacts on programmed/non-programmed cell death (apoptosis and autophagy/necrosis), cell cycle progression/arrest, and tumor cell migration/invasion.

2. MATERIALS AND METHODS

2.1. Chemicals. Phyllanthin was purchased from Fluka (Lot #BCBL2476V, product of India). The analytical grade solvents used in extraction and chromatographic separation were purchased from El Gomhouria for Drugs Co. (Cairo, Egypt). Solvents of HPLC grade were purchased from Sigma-Aldrich (Steinheim, Germany). Precoated TLC silica gel F_{254} plates (20 × 20 cm) and RP silica gel were obtained from Sigma-Aldrich chemicals (Darmstadt, Germany). Sulforhodamine-B (SRB) and DOX were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Plant Material. Samples of aerial parts of *P. niruri* L. were collected in August 2018, from Tasek Gelugor, Penang, Malaysia. The plant material was supplied and identified by the staff member of the Malaysian Agricultural Research and Development Institute (MARDI). A voucher specimen (PN-01-082018B) was kept at the herbarium of the Faculty of Pharmacy, Cairo University. The aerial parts of the collected plant were dried and powdered and saved in tightly closed containers until use.

2.3. Extraction and Isolation of Bioactive Compounds. The powdered aerial parts of *P. niruri* (750 g) were exhaustively extracted with MeOH (3 × 1.5 L). The solvent was removed under vacuum at a temperature not exceeding 60 °C to yield 93 g of MeOH extract (**Ext-1**). Part of the MeOH extract (70 g) was suspended in H_2O (600 mL) and extracted with CH_2Cl_2 (3 × 400 mL). The solvent was removed by distillation to yield CH_2Cl_2 fr. (24.5 g). The CH_2Cl_2 fraction (10 g) was applied on a Si gel column (5 × 25 cm, 230−400 mesh size). Elution was started with 10% EtOAc in *n*-hexane (500 mL), followed by increasing the percentage of EtOAc up to 50% EtOAc, and the column was finally washed with 100% EtOAc. Fractions (100 mL each) were collected and monitored by TLC. Fractions with a similar chromatographic pattern were pooled together to give 10 major fractions (Frs **I**−**X**). Based on TLC and HPLC chromatograms, lignans were eluted in fractions **VI**−**X**.[19]

Fraction **III** (0.358 g) on crystallization from MeOH yielded compound **PN-1** as a white amorphous powder (65 mg) and was identified as β-sitosterol by comparing to the reference sample.[22]

Fraction **VI** (0.207 g) was subjected to MPLC on a RP18 column (12 × 2 cm, 40−60 μm, Merck, Germany) using MeOH-0.1% TFA in water (75:25 v/v) as an eluent (flow rate 4 mL/min and 5 mL fractions) to give compound **PN-2** as an amorphous powder (53 mg). Similarly, MPLC of fraction **VII** (230 mg) was treated similarly to give 80 fractions. Frs 30−60

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(75 mg) was subjected to HPLC separation on a RP-18 column (Kromasil 100-5-C18, 10 × 250 mm, 5 μm) using a mobile phase composed of MeOH (solvent A) and 0.1% TFA in H_2O (solvent B) in the following gradient elution mode: 0 min 0% A, 10 min to 50% A, 10 min to 80% A, 5 min to 85% A, 10 min 85% A, and finally 5 min to 100% A. The condition of chromatographic separation is as follows: flow rate of 2 mL/min, detection at UV 320, 254, and 230 nm, and injection volume was 5 mg/50 μL. Compound **PN-3** (t_R 30.5 min) was obtained as an amorphous powder (11 mg).

Fraction **VIII** (144 mg) was subjected to MPLC on a RP18 column (12 × 2 cm) using MeOH-0.05% TFA in water (85:15 v/v, flow rate 5 mL/min), and 30 fractions were collected (4 mL each). Fractions 6−8 yielded compound **PN-4** as an amorphous powder (7.5 mg) at t_R 10.073 min.

Fraction **IX** (526 mg) was chromatographed on normal a Si gel column (20 × 3 cm, mesh size 230−400) using 20% EtOAc in *n*-hexane to give compound **PN-5** as an amorphous powder (90 mg) t_R 11.46 min.

2.4. Cell Culture. The human breast cancer cell line (MCF-7) and the DOX-resistant breast cancer cell line (MCF-7^{ADR}) were obtained from Nawah Scientific Inc. (Mokattam, Cairo, Egypt) and cultured in Dulbecco's modified Eagle's medium, Gibco-Thermo Fisher Scientific, Waltham, MA, USA. The culture media were complemented with 10% FBS (fetal bovine serum) and 100 units/mL PS (penicillin/streptomycin). The cells were passaged at 37 °C in a humidified atmosphere with 5% CO_2.

2.5. Cell Viability Assay. The cell viability was determined by the SRB assay. MCF-7 and MCF-7^{ADR} cells were seeded in 96-well plates, approximately 10^4 cells/well. After treatment for 72 h, the media were replaced by 150 μL of 10% TCA (trichloroacetic acid) (Merck) for 1 h at 4 °C, followed by five times washing with distilled water. Afterward, 70 μL of SRB solution (0.4% w/v) (Sigma-Aldrich) was added for 10 min at room temperature in a dark place. The cells were washed with 1% acetic acid three times and left overnight to air-dry. The protein-bound SRB stain was dissolved by adding 150 μL of 10 mM Tris base (pH 7.4). The O.D. was measured at 540 nm using the microplate reader FluoStar Omega (BMG, Labtec, Ortenberg, Germany). The dose−response curves of drugs under investigation were analyzed using the E_{max} model.[23]

2.6. Data Analysis. The dose−response curves of compounds were investigated by applying the E_{max} model as previously described.[24] The combination index (CI) was calculated as previously described.[25] Drug interactions are classified as an additive if CI is between 0.8 and 1.2, antagonistic if CI is > 1.2, and synergistic if CI is < 0.8.

2.7. Cell Cycle Analysis. DOX-resistant breast cancer cells (MCF-7^{ADR}) were treated with the precalculated IC_{50} of hypophyllanthin (**PN4**) and phyllanthin (**PN5**) alone or in combination with DOX for 48 h. Then, the cells were harvested by trypsinization, twice washed with PBS (phosphate-buffered saline), fixed in ice-cold 60% ethanol at 40 °C, and re-washed in PBS. After that, the cells are resuspended in 500 μL of propidium iodide (PI) with RNase staining buffer, BD (Franklin Lakes, NJ, USA), and incubated for 30 min. Last, FACS analyses were executed utilizing the ACEA Novocyte flow cytometer (ACEA Biosciences Inc., San Diego, USA). For every sample, data from 12,000 cells were collected, and the distribution of cell cycle phases was analyzed using the ACEA Novo Express software (ACEA Biosciences Inc., San Diego, USA).[26]

2.8. Apoptosis Analysis. MCF-7^{ADR} cells were treated with hypophyllanthin (**PN4**) and phyllanthin (**PN5**) alone or in combination with DOX for 48 h and then trypsinized and washed twice with PBS. Apoptosis assessment was performed via the Annexin V-FITC/PI Apoptosis Detection Kit (BD Biosciences, San Diego, USA) following manufacturer's instructions. In brief, the cells were resuspended in 0.5 mL of the binding buffer, and then 5 μL of Annexin V-FITC and 5 μL of PI (staining solution) were added for 15 min at room temperature in a dark place. Finally, the cells were applied, within 1 h of staining, to FACS analysis using an ACEA Novocyte flow cytometer (ACEA Biosciences Inc., San Diego, CA, USA).[27]

2.9. Autophagy Assessment. For autophagic assessment in response to hypophyllanthin (**PN4**) and phyllanthin (**PN5**) alone or in combination with DOX treatment for 48 h, MCF-7^{ADR} cells were trypsinized and washed twice with ice-cold PBS. Then, 0.5 mL of the staining solution (1 μg/mL of acridine orange in PBS) was added and incubated in the dark for 30 min at room temperature. The cells were adjusted at 12,000 events when applied to flow cytometric analysis via the ACEA Novocyte flow cytometer, and fluorescent signals were analyzed via a FL1 signal detector (488 nm excitation/530 nm emission). The net fluorescence intensities (NFI) were quantified.[28]

2.10. Cell Scratch Assay. MCF-7^{ADR} cells were seeded at 90% confluency in 12-well plates in triplicate for each condition. After 24 h, a scratch was introduced across the center of each well using a 1 mm pipette tip. The cells were washed twice with PBS to remove cell debris and replaced with fresh media alone or in combination with **PN4**, **PN5**, and/or DOX. The cells migrated into the wound surface, and the average distance of migrating cells was determined by inverted microscopy at designated time points (0, 24, 48, 72, and 96 h).[29]

2.11. Western Blot Analysis. Cultured cells were harvested and lysed in a readymade RIPA buffer (Beyotime Biotechnology) containing a protease inhibitor cocktail (1 μg/mL aprotinin and leupeptin) to extract proteins from the cells. Proteins in the cell lysate (30 μg) were separated through SDS-PAGE gels and transferred to nitrocellulose membranes (Amersham Pharmacia Biotech, Buckinghamshire, UK). Membranes were blocked with 5% skimmed milk for 3 h; then, the membrane was incubated with the primary antibodies at 4 °C overnight. The membrane was washed with TBS-T buffer and then incubated with the corresponding secondary antibodies at 37 °C for 2 h. Protein signals were analyzed with a luminescent image analyzer (LAS-1000; Fujifilm, Tokyo, Japan) using the Immobilon Western Chemiluminescent HRP Substrate (Millipore, Burlington, MA, USA).[30]

3. RESULTS

From our previous study,[21] only the CH_2Cl_2 fraction at 100 μg/mL exerted cytotoxic activity against both MCF-7 and MCF-7^{ADR} cell lines, and it was relatively more cytotoxic against MCF-7^{ADR}. In addition, the CH_2Cl_2 fraction at 10 and 100 μg/mL improved the cytotoxic effect of DOX against both cell lines. Fractionation of the CH_2Cl_2 fraction using preparative TLC afforded four major fractions: B1, B2, B3, and B4. Among them, only the B2 fraction (at 100 μg/mL) was cytotoxic against MCF-7^{ADR} but not MCF-7. In addition, fraction B2 at a concentration of 10 μg/mL showed an additive effect when combined with DOX. Therefore, the separation of

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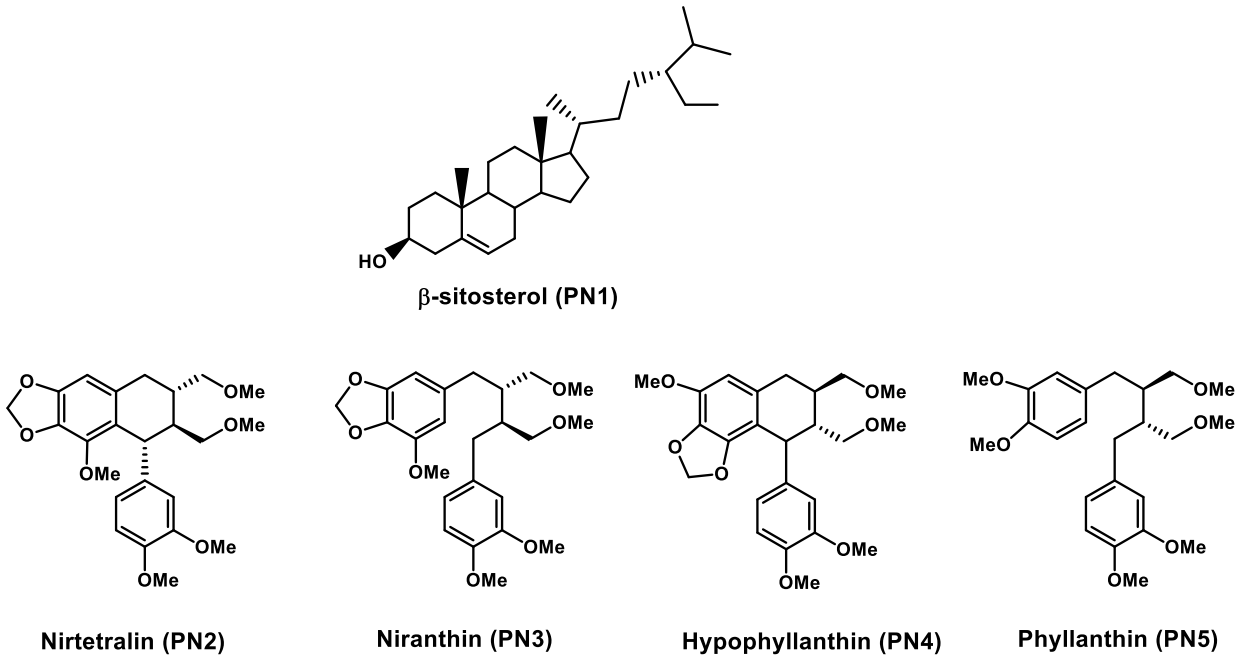

Figure 1. Compound s Isolated from *P. niruri*.

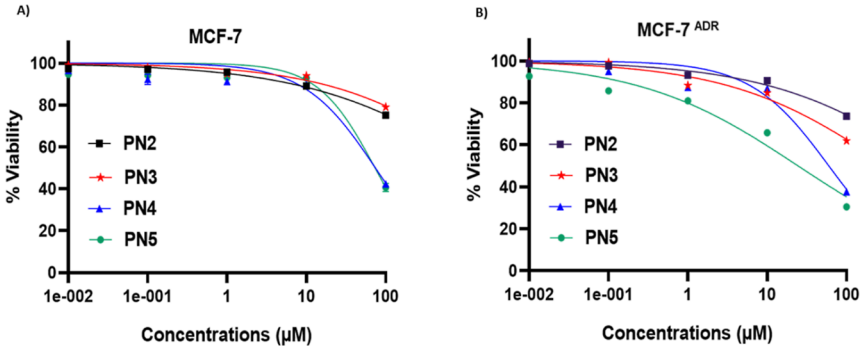

Figure 2. Cytotoxic activity of the isolated compounds on MCF-7 and MCF-7^{ADR} cells. MCF-7 (A) and MCF-7^{ADR} (B) cells were treated with serial dilution of **PN2**−**PN5** for 72 h. Cell viability was evaluated by the SRB assay, and data are expressed as mean ± SD (n = 3).

the major components in the range of R_f of B2 fraction was carried out.

3.1. Isolation of Major Compounds from Bioactive Fraction. Column chromatography of the CH_2Cl_2 fraction afforded five compounds. The identity of the isolated compounds was confirmed by comparing their NMR data with those reported in the literature and by comparison to authentic samples. Spectral analysis of the isolated compounds revealed the identification of sterol (**PN1**) and four lignans (**PN2**−**PN5**) (Figure 1).

Compound **PN1** was identified as β-sitosterol,[22] by comparison with the standard sample (Co-TLC) and ^{1}H NMR spectrum (Figure S1).

The NMR spectra (^{1}H, ^{13}C NMR, MS, and UV) of compounds **PN2** to **PN5** (Figures S2−S17 and Table S1) showed the common feature of aryl lignans. The result of NMR analysis revealed that compounds **PN2** and **PN4** are belonging to the aryltetralin skeleton based on the presence of only one benzylic CH_2 group, while the presence of two benzylic CH_2 groups in **PN3** and **PN5** indicated a dibenzylbutane skeleton.[31,32] In addition, the spectral data of compounds **PN2**, **PN3**, and **PN4** showed the presence of a methylene dioxy group (δ_H 5.80−5.89 and δ_C 100.0−101.0) and five methoxy groups, while compound **PN5** showed the absence of the methylene dioxy group and the presence of six methoxy groups. From the previous discussion and by comparison of the spectral data (^{1}H, ^{13}C NMR, MS, and UV) of the isolated compounds with those reported in the literature, **PN2** was identified as nirtetralin[31,32] (Figures S2−S5), **PN3** as niranthin[31−33] (Figures S6−S9), **PN4** as hypophyllanthin[32−34] (Figures S10−S13), and **PN5** as phyllanthin[32−34] (Figures S14−S17).

3.2. Effect of the Isolated Compounds PN2−5 on the Proliferation of MCF-7 and MCF-7^{ADR}. MCF-7 and MCF-7^{ADR} cell lines were separately treated with the isolated compounds **PN2**, **PN3**, **PN4**, and **PN5** over a concentration range of 0.01−100 μM for 72 h, and the viability was evaluated by the SRB assay (Figure 2A,B). **PN2** and **PN3** did not induce apparent cytotoxicity against both cell lines with IC_{50} values higher than 100 μM. Hypophyllanthin (**PN4**) and phyllanthin (**PN5**) exerted weak cytotoxic activity against MCF-7 cells with nearly the same IC_{50} values (74.2 ± 1.5 and 73.4 ± 2.1 μM, respectively). However, more potent cytotoxicity was observed against MCF-7^{ADR} cells after treatment with **PN4** and

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PN5 (with IC_{50}'s values of 58.7 ± 1.2 and 29.5 ± 0.9 μM, respectively). Therefore, we further studied the influence of **PN4** and **PN5** on the efficacy profile of DOX in breast cancer-resistant cells MCF-7^{ADR}, where **PN5** was found to be 2-fold more potent than **PN4**.

3.3. Effect of Hypophyllanthin (PN4) and Phyllanthin (PN5) on the Cytotoxic Profile of DOX in MCF-7^{ADR}. To investigate the influence of **PN4** and **PN5** on the cytotoxic profile of DOX, we evaluated the dose−response curve of DOX alone relative to its effect when combined with **PN4** and **PN5** in MCF-7^{ADR} cells (Table 1 and Figure 3). DOX alone exerted gradient cytotoxicity with increasing concentration with an IC_{50} value of 17.0 ± 1.9 μM. However, an equitoxic combination of **PN4** or **PN5** with DOX markedly improved the cytotoxic profile of DOX, as indicated by the decrease in its IC_{50} value to 9.5 ± 0.6 and 1.1 ± 0.03 μM, respectively. The determined combination index values for DOX with **PN4** or **PN5** were 0.65 and 0.091, respectively. This observation indicates a potent synergistic interaction, especially with **PN5** in MCF-7^{ADR} (Table 1).

Table 1. Comparative Cytotoxicity of DOX alone and in Combination with Hypophyllanthin (PN4) and Phyllanthin (PN5) against MCF-7^{ADR}[a]

	MCF-7^{ADR}		
treatments	IC_{50} (μM)	*R*-value (%)	CI-value
DOX (alone)	17.0 ± 1.9	1.2 ± 0.05	
DOX + **PN4**	9.5 ± 0.6	5.4 ± 0.4	0.652 [synergism]
DOX + **PN5**	1.1 ± 0.03	6.2 ± 0.31	0.091 [synergism]

[a]The value of the IC_{50}'s of DOX is determined alone and from the equitoxic ratios of DOX/**PN4** (1:10) and DOX/**PN5** (1:5). Data are presented as mean ± SD; n = 3.

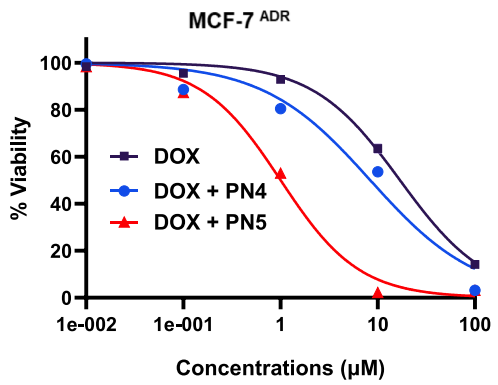

Figure 3. Chemomodulatory effect of **PN4** and **PN5** on the cytotoxicity of DOX in the MCF-7^{ADR} cell line. Cells were treated with serial dilution of DOX alone or in combination with **PN4** and **PN5** in equitoxic combination (1:10) and (1:5), respectively, for 72 h. Cell viability was evaluated by the SRB assay, and data are expressed as mean ± SD (n = 3).

3.4. Effect of Hypophyllanthin and Phyllanthin alone or in Combination with DOX on the Cell Cycle in MCF-7^{ADR} Cells. DNA flow cytometry was used to assess the effect of hypophyllanthin and phyllanthin alone or in combination with DOX on the cell cycle distribution of the MCF-7^{ADR} cell line (Figure 4). **PN4** showed no significant change in the cell population of the G_0/G_1-phase (the non-proliferating cell fraction) and the S phase compared to the control cells, but **PN4** significantly decreased the cell population in the G_2/M phase from 22.19 ± 1.2 to 18.37 ± 0.95% concomitantly with doubling cell population in the pre-G phase from 1.46 ± 0.01 to 2.91 ± 0.03%.

Unlikely, phyllanthin (**PN5**) treatment showed significant changes in all cell phases compared to control cells. **PN5** caused a meaningful increase in the G_o/G_1 phase from 54.13 ± 1.3 to 64.40 ± 1.6% that resulted in cell death manifested by a significant increase in the pre-G phase from 1.46 ± 0.01 to 7.32 ± 0.2% compared to control cells. Reciprocally, **PN5** caused a substantial decrease in the S phase from 23.69 ± 1.7 to 16.67 ± 0.9% and in the G_2/M phase from 22.19 ± 1.2 to 18.92 ± 0.77%.

Compared to control cells, treatment with DOX resulted in a significant cell cycle arrest at the G_2/M phase from 21.8 ± 1.6 to 75.87 ± 2.14%, indicating the difficulty of repairing the intracellular damaged DNA. Moreover, DOX treatment resulted in a significant cell death manifested by a marked increase in the Pre-G phase from 1.46 ± 0.01 to 30.17 ± 1.8% compared with control cells.

The combination of DOX with **PN4** caused a very potent antiproliferative effect compared to DOX treatment alone as manifested by the increase in the cell population in the G_0/G_1 phase from 6.34 ± 0.2 to 36.13 ± 2.53% and in the S phase population from 17.79 ± 1.06 to 32.88 ± 1.8%, while this combination caused a significant decrease in the Pre-G phase cell population from 30.17 ± 1.8 to 18.37 ± 0.56% compared to DOX treatment alone.

Unlikely, the combination of **PN5** with DOX significantly induced more cell death when compared to DOX treatment alone; cells in the pre-G phase were increased from 30.17 ± 1.8 to 55.75 ± 2.78%. In addition, the combination of **PN5** with DOX also showed antiproliferative effect and caused a significant increase in the G_0/G_1 phase from 6.34 ± 0.12 to 47.21 ± 0.66% and in the S phase from 17.79 ± 1.06 to 22.44 ± 1.2% when compared to DOX alone.

3.5. Modes of Cell Death Involved in the Synergistic Effect of Combined Treatment. To discern if hypophyllanthin (**PN4**) and phyllanthin (**PN5**) induced their cytotoxic activity via apoptosis or necrosis, MCF-7^{ADR} cells were treated with the IC_{50} values of **PN4** and **PN5** alone and in combination with DOX for 48 h, and then the cells were assessed using PI and Annexin-V/FITC staining coupled with flow cytometry. In MCF-7^{ADR} cells, treatment with **PN4** and **PN5** showed no significant apoptotic or necrotic cell death compared to control cells (Figure 5A,B).

DOX is a chemotherapeutic drug that can damage DNA and alter biological processes, such as apoptosis and necrosis. Herein, DOX alone caused a significant increase in total apoptosis (early and late) from 1.1 ± 0.17 to 24.2 ± 1.9% and necrosis from 0.62 ± 0.06 to 59.9 ± 1.13% compared to control untreated MCF-7^{ADR} cells (Figure 5B).

Compared to DOX alone, the combination of DOX with **PN4** and **PN5** resulted in more total cell death. DOX combined with **PN4** induced further significant necrosis (79.91 ± 0.15 vs 60.4 ± 1.13% of DOX alone), while the combination of DOX with **PN5** induced further significant apoptosis (35.4 ± 1.35 vs 24.7 ± 1.9% of DOX alone). These data provided more about different modes of cell death elicited by **PN4** and **PN5** alone and as an adjuvant to DOX against MCF-7^{ADR} cells.

3.6. Reversal of ADR Resistance by Hypophyllanthin and Phyllanthin in Combination with DOX Is Linked to Autophagy Inhibition. Currently, the autophagy role in

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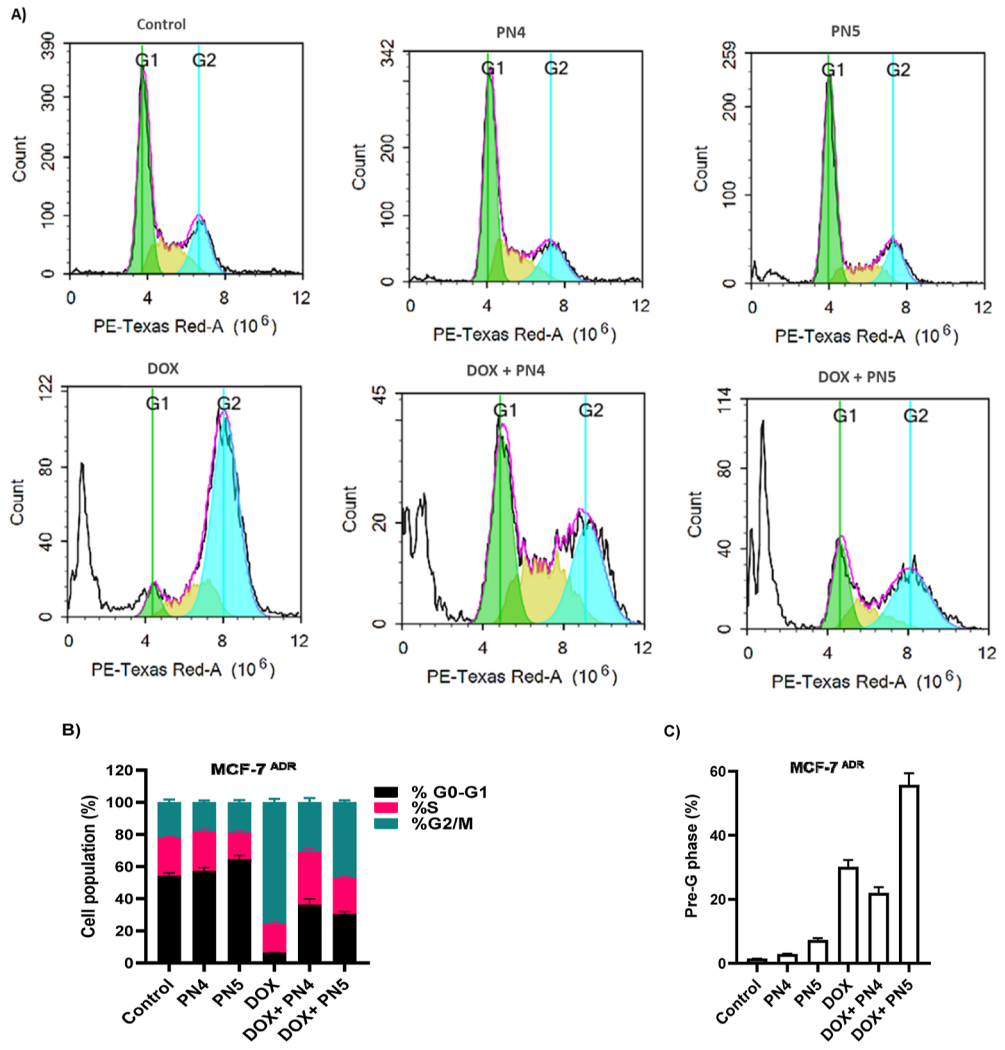

Figure 4. (A) MCF-7^{ADR} cells were exposed to DOX, (hypophyllanthin) **PN4**, (phyllanthin) **PN5**, and their combinations for 48 h, and cell cycle distribution was determined using DNA cytometry analysis. (B) Cell phase percentages were plotted as the percent of total events. (C) Pre sub-G phase was plotted as the percentage of total events. Data are presented as mean ± SD; n = 3.

cancer resistance to DOX and the involved mechanisms have become an area of interest to many investigators. Hence, to verify whether the reversal of ADR resistance by hypophyllanthin (**PN4**) or phyllanthin (**PN5**) in combination with DOX is related to autophagy, MCF-7^{ADR} cells were separately treated with **PN4** and **PN5** alone and in combination with DOX for 48 h, and autophagy was assessed using acridine orange dye coupled with flow cytometry (Figure 6A).

The results showed that DOX treatment alone significantly induced autophagic signal by nearly 100% (2-fold increase) compared to untreated control cells, further indicating that autophagy is associated with DOX resistance. However, the treatment of hypophyllanthin and phyllanthin combined with DOX markedly attenuated the autophagic induction of DOX by 37 and 40%, respectively (Figure 6B), suggesting that **PN4** and **PN5** combinations may reverse DOX autophagy-related resistance in MCF-7^{ADR}. Next, we further detected the autophagy-associated protein LC3II/I by western blotting. As shown in Figure 6C, DOX markedly enhanced the expression level of LC3II/I compared to that of control cells, while **PN4** or **PN5** combinations inhibited its expression compared to DOX alone.

On the other hand, treatment with **PN4** alone significantly induced the autophagic signal higher than control cells by 54%, while treatment with **PN5** inhibited autophagy by 15% compared with control cells (Figure 6B), indicating opposing effects of each compound alone in autophagy. Indeed, the results showed that compared with the control, **PN4** alone enhanced the expression of autophagy-associated protein LC3II/I, while a slight reduction in LC3II/I expression was observed after treatment with **PN5** (Figure 6C).

3.7. Hypophyllanthin and Phyllanthin Overcome Resistance of DOX in MC F-7^{ADR} through Down-regulating SIRT1/Akt. Research suggested a critical role of the SIRT1/Akt axis in the progression of breast cancer. SIRT1 was found to maintain the genomic integrity that regulates DOX resistance in breast cancer by activating the Akt pathway. To examine whether the inhibitory effect of hypophyllanthin (**PN4**) and phyllanthin (**PN5**) on MCF-7^{ADR} cells was associated with SIRT1 and Akt, MCF-7^{ADR} cells were untreated or treated with DOX or in combination with **PN4** or **PN5** for 48 h, and expressions of SIRT1 and P-Akt were assessed by western blot (Figure 7).

Cells treated with DOX alone exhibited a decrease in the levels of SIRT1 and P-Akt, whereas treatment with **PN4** and

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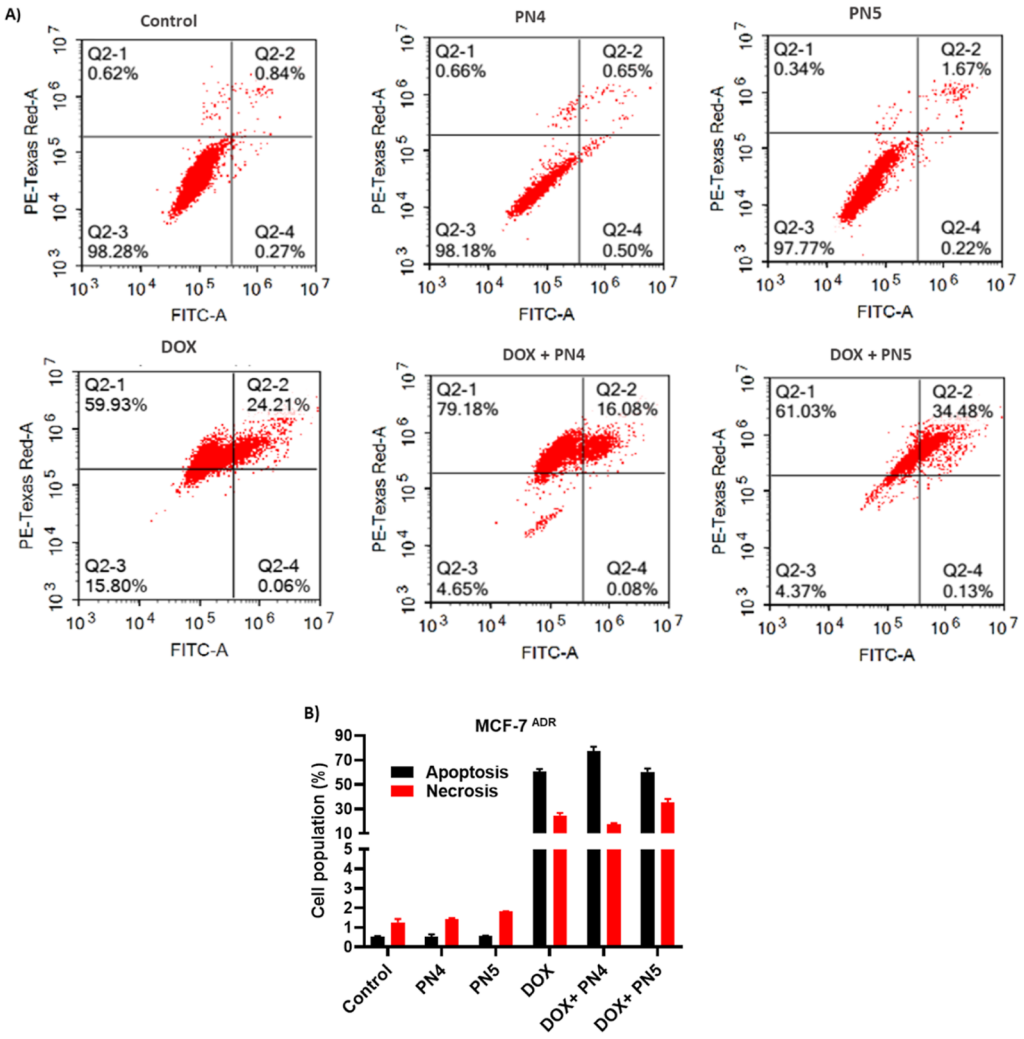

Figure 5. (A) Apoptosis/necrosis assessment in MCF-7^{ADR} cells after treatment with DOX, (hypophyllanthin) **PN4**, (phyllanthin) **PN5**, and their combinations for 48 h. Cells were stained with PI/Annexin V-FITC. (B) Different cell populations were plotted as the percentage of total events. Data are presented as mean ± SD; n = 3.

PN5 was more potent than DOX alone (compared with untreated control cells) in decreasing the levels of SIRT1 and P-Akt. Also, treatment with DOX in combination with **PN4** or **PN5** significantly reduced the expression of SIRT1 and AKT compared to DOX alone. Hence, we hypothesize that **PN4** and **PN5** can ameliorate DOX resistance in a SIRT1-dependent manner via decreased SIRT1 and P-Akt expressions in MCF-7^{ADR} cells.

3.8. Hypophyllanthin and Phyllanthin Inhibited the Migration Ability of MCF-7^{ADR} Cells and Downregulated the Expression of EMT Markers. Herein, we evaluated the effect of hypophyllanthin (**PN4**) and phyllanthin (**PN5**) on cell migration of MCF-7^{ADR} via the cell scratch assay. Yet, cells were treated with **PN4**, **PN5**, and DOX either alone or in combination, and wound closure was assessed daily until the closure of control untreated cells (96 h).

The results of the cell scratch test suggested that the migratory potential of MCF-7^{ADR} cells was not significantly affected after treatment with DOX alone compared to untreated cells, while both **PN4** and **PN5** showed a notable decrease in cell migration of MCF-7^{ADR} cells compared to untreated cells, indicating the potential antimigratory effect of both compounds.

Interestingly, the combination treatment of DOX with **PN4** and **PN5** achieved a remarkably more potent migration-inhibitory effect than DOX (Figure 8A,B), giving more evidence about their potential effect in inhibiting MCF-7^{ADR} migration and enhancing the DOX effect in controlling the migration of these resistant cells.

Recent research suggests the involvement of EMT in the sensitivity of breast cancer cells to DOX treatment. Since MCF-7^{ADR} cells exhibited EMT-like phenotype and enhanced metastatic potential, thus we tested the expressions of EMT-related proteins, specifically mesenchymal cell markers (N-cadherin and β-catenin) by western blot analysis after treatment with **PN4** and **PN5** and/or DOX (Figure 8C). We observed that the protein expression level of N-cadherin decreases after treatment with DOX, which indicates that DOX administration to MCF-7^{ADR} cells might suppress EMT. Additionally, it was observed that the protein level of N-cadherin and β-catenin decreased significantly after treatment with predetermined IC_{50} of **PN4** and **PN5** compared with the untreated or DOX-treated cells. This observation indicates that exposure of MCF-7^{ADR} cells to **PN4** or **PN5** may further suppress EMT. Also, in the cells treated with a combination of DOX with **PN4** or **PN5**, the expression of N-cadherin and β-

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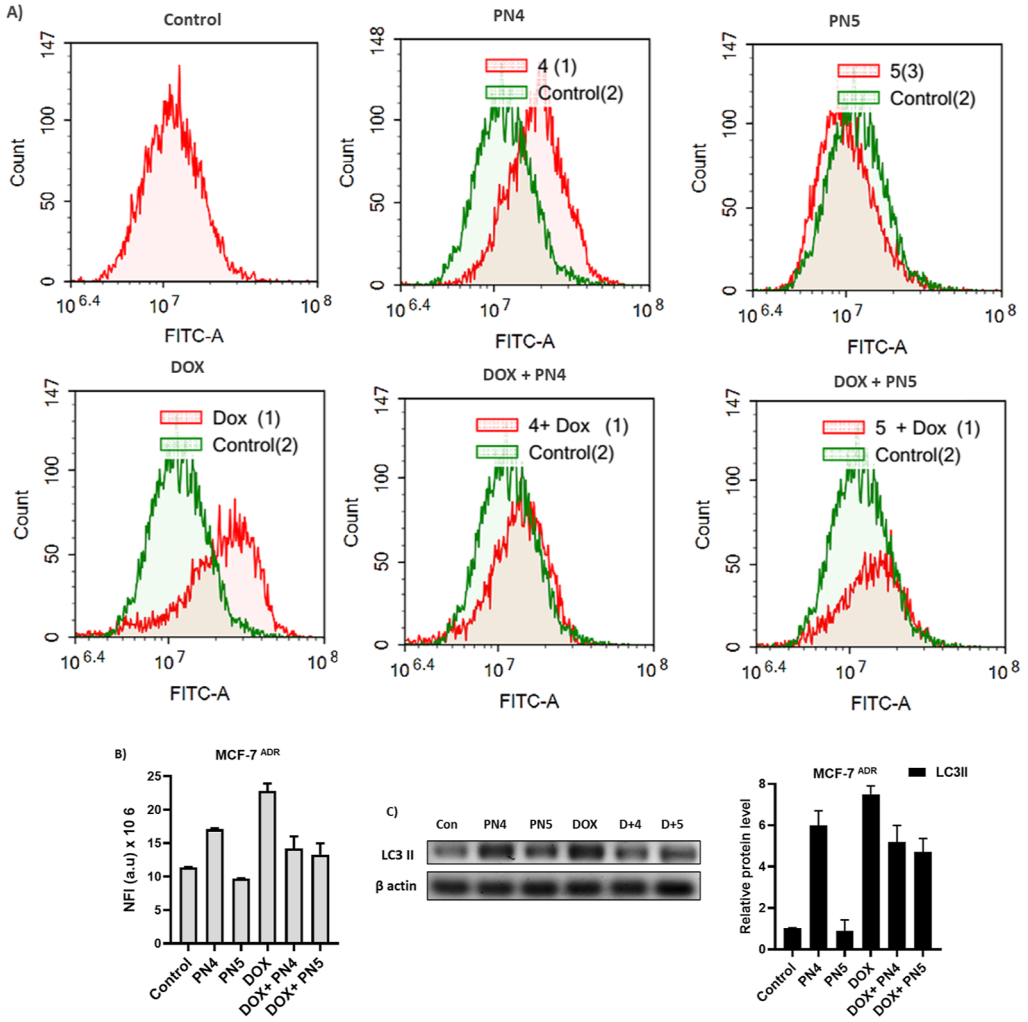

Figure 6. (A) Autophagic cell death evaluation in MCF-7^{ADR} cells after exposure to DOX, hypophyllanthin (**PN4**), and phyllanthin (**PN5**) alone or in combination with DOX for 48 h. The cells were stained with acridine orang dye. (B) NFI was plotted in comparison with the basal fluorescence of untreated MCF-7^{ADR} cells. (C) Western blot to detect the expression of the autophagy-related protein (LC3II). Data are displayed in triplicate.

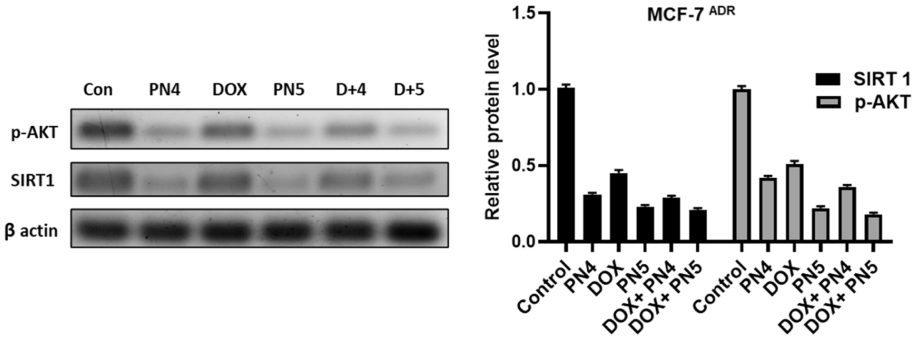

Figure 7. Hypophyllanthin (**PN4**) and phyllanthin (**PN5**) enhanced the effect of DOX by reducing the SIRT1/Akt pathway in MCF-7^{ADR} . The cells were treated with **PN4** and **PN5** alone and in combination with DOX for 48 h; then, cells were collected to detect the protein expressions of SIRT1 and P-Akt by western blot analysis. Data are presented as mean ± SD.

catenin proteins was downregulated compared with DOX alone. These results indicate that **PN4** and **PN5** can enhance the MCF-7^{ADR} cells' sensitivity to DOX by EMT inhibition.

4. DISCUSSION

Despite the remarkably improved prognosis of breast cancer patients diagnosed at early stage and received standard chemotherapy after surgical resection, the 5 year survival rates of breast cancer patients remain high, mainly due to drug resistance.[35] Moreover, for patients with advanced and metastatic cancers who have no opportunities to operate, chemotherapy is considered the most efficient approach to alleviate symptoms and improve survival rate. Yet, a subset of patients presents poor survival outcomes owing to drug resistance.[36] Therefore, overcoming resistance is still a big challenge in breast cancer chemotherapy.[37]

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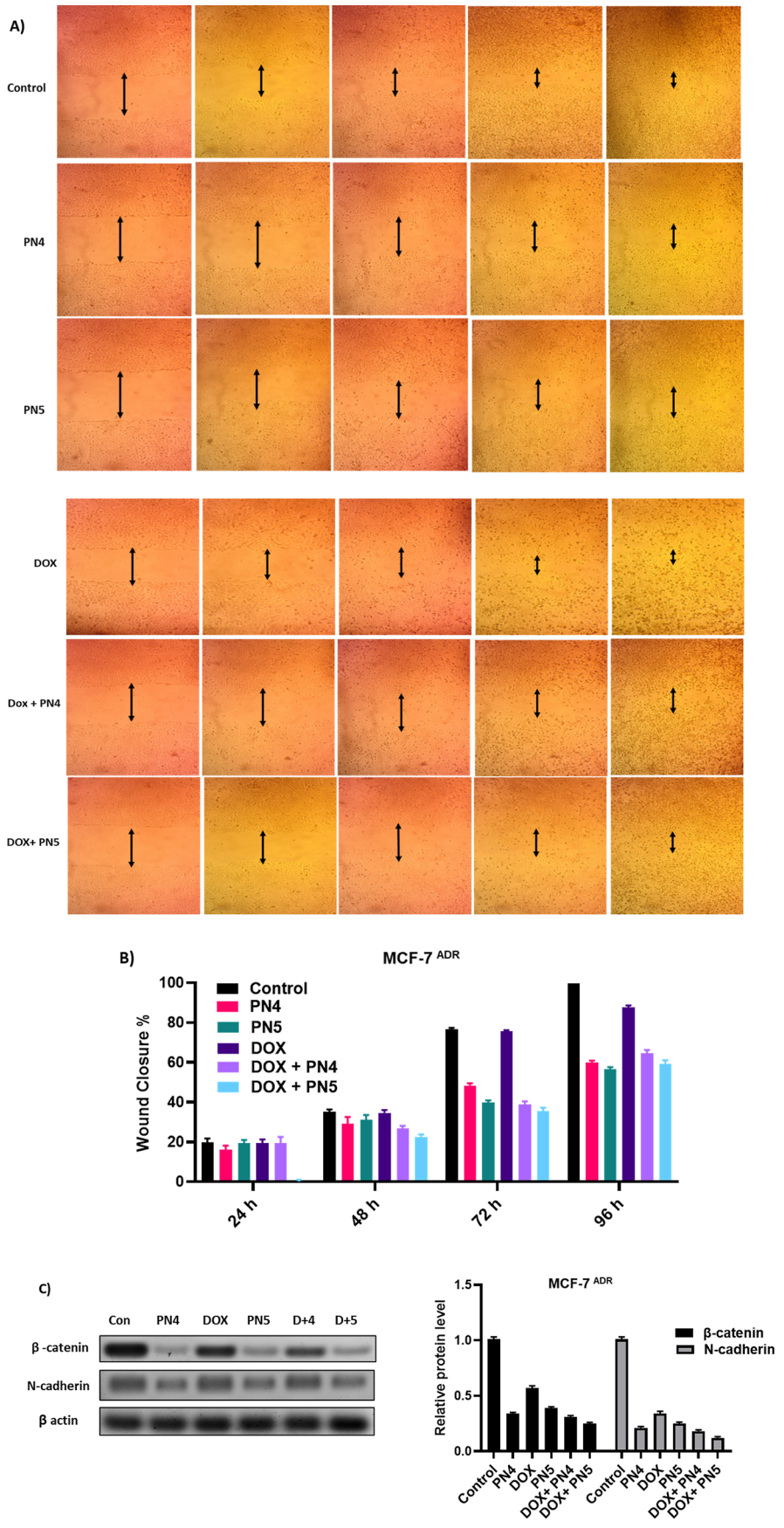

Figure 8. Hypophyllanthin (**PN4**) and phyllanthin (**PN5**) alone and in combination with DOX inhibit cell migration of MCF-7^{ADR} cells. (A) The migration distances were measured after different treatments daily for 96 h. (B) Data were blotted as wound closure % at each time interval. (C)

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Figure 8. continued

The protein level of EMT-related proteins (β-catenin and N-cadherin) was determined by western blot analysis in MCF-7^{ADR} cells. Data are presented as triplicates.

DOX is a commonly used chemotherapeutic drug in breast cancer which is affected by the development of acquired drug resistance and EMT upon prolonged use, resulting in chemotherapy failure.[38] Accordingly, we are investigating several chemomodulators to work as a sensitizing agent to DOX particularly in resistant breast cancer cells.[39,40]

Herein, we showed for the first time that two lignans, hypophyllanthin and phyllanthin, separated from *P. niruri*, significantly inhibited the growth and migration capacity of DOX-resistant breast cancer cells (MCF7^{ADR} cells) when combined with DOX.

Surprisingly, the killing effect of compounds isolated from *P. niruri* (**PN2**−**PN5**) was more potent in MCF-7^{ADR} cells compared to the naive MCF-7 cells. Moreover, MCF-7^{ADR} cells became more susceptible to DOX in the presence of hypophyllanthin and phyllanthin, indicating a sensitization effect of both compounds in resistant breast cancer cells. Accordingly, we further investigated the potential underlying mechanisms of this sensitization in MCF-7^{ADR} cells.

Phyllanthus species have been studied in recent decades for cancer treatment. However, few studies were concerned with *P. niruri* constituents in various plant parts and their biological activities, especially in cancer.[41] In general, studies investigating the anticancer properties of *Phyllanthus* species on breast cancer were scanty. Earlier in 2014, a study examined the in vitro and in vivo anticancer potential of hypophyllanthin and phyllanthin isolated from *P. amarus* against breast cancer.[42] Another study showed that four *Phyllanthus* species inhibit metastasis of human breast cancer cells via the suppression of ERK1/2.[43] Recently in 2021, a study showed the effective combination of *P. niruri* extract with *Curcuma longa* extract for treating metastatic breast cancer stem cells.[18] Herein, this is the first study investigating the potential anticancer activity of compounds derived from *Phyllanthus* species against resistant breast cancer cells.

Several studies establish that the development, metastasis, and recurrence of tumors are closely correlated with the cell cycle.[44,45] In addition, one of the strategies for improving the sensitivity of cancer cells to chemotherapeutics is to combine them with cell cycle regulators.[46] Hypophyllanthin alone caused a significant decrease in the G_2/M phase, while phyllanthin alone caused a significant increase in the G_0/G_1 phase. This indicates the antiproliferative potential of hypophyllanthin and phyllanthin, and they can hinder the progression of the cell cycle in MCF-7^{ADR} cells. Our study revealed a prominent G_2/M phase arrest for MCF-7^{ADR} cells after DOX treatment, consistent with several previous studies.[47,48] This cell cycle arrest-induced stress ultimately induces apoptosis in breast cancer.[40] On the contrary, DOX-treated MCF-7^{ADR} cells after co-treatment with hypophyllanthin and phyllanthin were markedly accumulated in the G_0/G_1 phase, suggesting that hypophyllanthin and phyllanthin distinctly modulated cell cycle arrest induced by DOX and revealed the potential of hypophyllanthin and phyllanthin as co-chemotherapeutic agents. Moreover, causing the G_0/G_1 phase arrest of MCF-7^{ADR} after co-treatment indicated the potent antiproliferative effects of both combinations. Phyllanthin exerted a more potent antiproliferative activity than hypophyllanthin that was accompanied by more cell death and more cells undergoing DNA fragmentation represented by the increase in pre-G population, compared with DOX alone. On the other hand, co-treatment with hypophyllanthin showed less cell death represented by a decrease in pre-G population.

In conclusion, the combination with hypophyllanthin was more cytostatic than cytotoxic. These results encouraged us to continue biological investigations on such combinations to understand the targeted mechanism of combination treatments by assessing apoptotic and autophagy-programmed cell death.

Among its other cellular effects, DOX induces ER stress and dysregulate autophagy, and on top of inducing apoptosis, DOX might induce necrotic cell death.[49,50] Consistent with these previous studies, our data revealed that DOX effectively induced cell apoptosis as well as necrosis in MCF7^{ADR} cells. Studies that investigated apoptosis induction of *Phyllanthus nuri* on cancer cells were scanty. Our study revealed that each of hypophyllanthin and phyllanthin alone did not induce significant apoptosis in MCF-7^{ADR}. However, the combination of hypophyllanthin with DOX shifted cell death more toward necrosis, while phyllanthin combination with DOX showed an exceptional synergistic effect in terms of apoptosis, suggesting that phyllanthin cytotoxicity is mainly via apoptosis induction. Apoptosis is the preferred mechanism of cancer cell death in response to chemotherapy because necrosis induces inflammatory collateral effects which are not desirable.[51] Therefore, apoptosis is considered an ideal target in cancer therapy, and consequently, phyllanthin combination with limited necrosis is more promising in resistant breast cancer cells.

Despite the complex and controversial role of autophagy in cancer, either as a pro-survival or pro-death, studies demonstrated autophagy as a new reversal strategy in multidrug resistance cancer therapy. Recently, autophagy-mediated drug resistance in tumor cells has been widely investigated,[6] with high autophagy levels noted in resistant breast cancer cells. DOX simultaneously triggers differently regulated cell death pathways, including induction of autophagy, at low doses.[39] In line with these studies, we found that DOX treatment activates autophagy in MCF7^{ADR} cells, confirmed with the high expression of LC3II (autophagy golden marker), and providing stronger evidence that the autophagy is associated herein with DOX resistance.[52,53] More and more preclinical data are concerned with reversing DOX resistance through modulating autophagy as one of the promising therapeutic strategies.[54] In this context, autophagy acts as a salvage mechanism or apoptosis-escape mechanism advocative for drug resistance. Various *Phyllanthus* species inhibited autophagy in several cancers like gastric cancer,[55] ovarian cancer,[56] and HCC.[57] Herein, we reported for the first time the effect of hypophyllanthin and phyllanthin on autophagy in resistant breast cancer cells. In this study, we showed that hypophyllanthin and phyllanthin combined with DOX inhibited autophagy by inhibiting the expression of LC3II, thereby reversing drug resistance. However, treatment with hypophyllanthin and phyllanthin alone showed opposing mechanisms regarding autophagy. Hypophyllanthin activated autophagy, while phyllanthin inhibited autophagy, confirming the different modes of cell death induced by the two

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compounds and explaining in part the observed potent sensitizing effect of phyllanthin over hypophyllanthin.

To date, more and more strategies to overcome resistance are concerned with the roles of SIRT1 in breast cancer progression and the development of treatment resistance.[9] SIRT1, a member of the mammalian sirtuin protein family, is significantly involved in several biological processes, including DNA repair, gene silencing, cell survival, metabolism, and aging.[58] Previous studies showed discrepancies in the expression of SIRT1 within different tumors, suggesting that SIRT1 can function either as a promoter or a suppressor in chemotherapy resistance. In breast cancer, SIRT1 expression was upregulated in drug-resistant cancer cell lines through increasing expression level of multidrug-resistant protein 1, suggesting SIRT1's promotional role in chemoresistance. Previous research revealed the crosslink between SIRT1 and Akt in the progression of breast cancer,[59] suggesting that SIRT1 directly activates Akt. In breast cancer, overactivation of Akt can cause uncontrolled cell proliferation and prevent programmed cell apoptosis. Consequently, disrupting the interrelation between SIRT1 and Akt would lead to inhibiting proliferation and invasive ability of MCF-7^{ADR} cells. In the current study and as expected, DOX modulates the expression of SIRT1 in MCF-7^{ADR} cells, which might be influencing DOX resistance. Currently, non-specific SIRT1 inhibitors like tenovin-6 and cambinol suppress tumor growth and can be utilized in different therapeutic purposes.[60] Herein, we found that hypophyllanthin and phyllanthin treatment alone or in combination with DOX are significantly downregulating the expression of SIRT1 and its downstream activity marker, p-Akt protein, in MCF7^{ADR} cells, providing a new insight into the potential use of hypophyllanthin and phyllanthin in the treatment of resistant breast cancer phenotype. In addition, SIRT1 signaling is reported to be associated with EMT transcriptional factors in cancer, which in turn enhances cancer cell migration, invasion, and metastasis.[61,62] EMT is a biological transformation of epithelial cells to mesenchymal cells through which cellular polarity and connections are lost, which leads to stronger migratory and invasive cancer cell properties. EMT not only promotes metastasis of cancer cells but also enhances the development of DOX resistance.[63] EMT transcriptional factors are overexpressed in MCF-7^{ADR} cells, and such resistant tumors carry more mesenchymal properties and are 3 times less likely to respond to chemotherapy than non-resistant cells.

Hence, the identification of novel agents which can inhibit EMT is of ultimate interest in improving the response of resistant tumors. Resistance to DOX treatment was also reported to be mediated by EMT in cancers via complex mechanisms, among which the β-catenin signaling pathway is the most frequently reported pathway.[64,65] In the present study, β-catenin was highly expressed in MCF7^{ADR} cells, confirming its essential role in DOX resistance of breast cancer.[66] Interestingly, it was indicated for the first time that hypophyllanthin and phyllanthin decreased the migration capacity of DOX-resistant breast cancer cells (MCF-7^{ADR}) and suppressed the expression of EMT markers (N-cadherin) and EMT inducer (β-catenin) together with synergizing the activity of DOX. These data suggested that hypophyllanthin and phyllanthin had an inhibitory effect on the EMT phenotype of MCF7^{ADR} cells.

5. CONCLUSIONS

Hypophyllanthin and phyllanthin isolated from *P. niruri* showed a moderate antiproliferative/cytotoxic property against resistant breast cancer cells. Yet, both agents significantly synergized DOX-induced anticancer properties against resistant breast cancer cells. This enhanced activity was explained by further promoting DOX-induced apoptosis and suppressing the apoptosis-escape autophagy feature of the resistant breast cancer cells. On the molecular level, both compounds (hypophyllanthin and phyllanthin) interfere with the SIRT1/Akt pathway and suppress N-cadherin/β-catenin axis, confirming the observed antiproliferative, cytotoxic, and anti-invasive effects of hypophyllanthin and phyllanthin.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c02953.

ESI-MS, ^{1}H, and ^{13}C NMR data for compounds **PN1−5** (PDF)

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M.R.M., A.E.H, A.M.A.-A, and E.A.-S. contributed to conceptualization; O.E.A.S., R.M.A., S.H.S., M.K., and A.E.H. contributed to methodology; O.E.A.-S., R.M.A., S.O.M., A.M.E.-D., and A.E.H. contributed to investigation; all authors contributed to writing—original draft preparation; all authors contributed to writing—review and editing; M.R.M. and A.M.A.-A contributed to supervision; and M.R.M., A.M.A.-A, A.E.H, S.H.S., M.K., and E.A.-S. contributed to project administration. All authors have read and approved the final manuscript.

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Notes

The authors declare no competing financial interest.

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