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ANTI-BREAST CANCER ACTIVITY OF PTEROSPERMUM DIVERSIFOLIUM: APOPTOSIS INDUCTION AND SELECTIVE SYNERGY WITH LAPATINIB

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Summary

Pterospermum diversifolium Blume, a Vietnamese indigenous medicinal plant, along with other species in the same genus, has been preliminarily reported to possess anti-cancer properties. However, its breast cancer subtype-specific effects and underlying mechanisms remain largely unexplored. In this study, we evaluated the effects of *P. diversifolium* ethanol extract (PDE) on cell viability, proliferation, and apoptosis in two breast cancer cell lines, including MCF-7 (ER-positive) and SKBR3 (HER2-positive). PDE inhibited the viability of both MCF-7 and SKBR3 cells, with IC_{50} values of 38.09 µg/mL and 35.68 µg/mL, respectively. Moreover, PDE showed a remarkable degree of selective cytotoxicity, with a selectivity index (SI) ranging from 3.56 to 3.80 when compared to non-malignant HEK293 cells. Mechanistically, PDE suppressed the expression of the proliferation marker Ki67 at 40 µg/mL and induced apoptosis at concentrations as low as 20 µg/mL. Notably, PDE exhibited a synergistic cytotoxic effect with lapatinib against HER2-positive SKBR3 cells but showed no synergism with tamoxifen in ER-positive MCF-7 cells. These original findings warrant further investigation into the potential use of PDE as an adjuvant agent in breast cancer therapy.

Keywords: *Apoptosis; Breast cancer; Lapatinib; Pterospermum diversifolium; Synergism.*

1. Introduction

Breast cancer (BC) is the most prevalent cancer and the second leading cause of cancer-related death among women [1]. Due to tumor heterogeneity, treatment strategies depend largely on molecular markers. Hormone receptor-positive (HR+) BC, the most common subtype (approximately 70% of cases), is primarily treated with hormone therapy. Around 20% of BC cases overexpress human epidermal growth factor receptor 2 (HER2) and respond well to HER2-targeted therapies [2]. Conversely, 10-20% of tumors lack expression of hormone receptors and HER2, a subtype known as triple-negative breast cancer (TNBC), which initially responds to chemotherapy [2]. Although advances in chemotherapeutics and targeted therapies have significantly reduced mortality, drug resistance, whether intrinsic or acquired, remains a major obstacle, often leading to treatment failure, relapse, metastasis, and death [3]. This underscores an urgent need for adjunctive therapeutic options to improve treatment efficacy.

In recent decades, there has been an explosion of research focused on identifying potential medicinal plants for cancer treatment. Specifically in BC, at least seventy plant species have been reported to exhibit cytotoxic effects against cancer cells to varying degrees [4]. These plants contain bioactive compounds such as flavonoids, terpenoids, phenolic acids, and alkaloids, which have been shown to inhibit breast cancer cell proliferation through multiple mechanisms, including the activation of both intrinsic and extrinsic apoptotic pathways and the induction of cell cycle arrest. Notably, many medicinal plants can also act as adjuvant agents by enhancing the cytotoxic effects of conventional chemotherapies, which is especially relevant since most plant extracts show only moderate activity when used alone [5],[6].

In a preliminary screening of plant extracts for anti-BC activity, *Pterospermum diversifolium* Blume (also known in Vietnam as Long mang) exhibited promising cytotoxic effects. While other

species in the *Pterospermum* genus (e.g., *P. truncatolobatum*, *P. acerifolium*) have shown significant cytotoxicity against various cancer cell lines, including BC cells, the anti-cancer properties of *P. diversifolium* remain poorly understood [7]. Therefore, this study aims to investigate the anti-breast cancer potential of *P. diversifolium* extract, both as a single agent and in combination with standard chemotherapeutic drugs across different BC subtypes.

2. Materials and Methods

2.1. Chemicals and reagents

Culture media, trypsin/ethylene diamine tetraacetic acid (EDTA) solution, and supplements (penicillin/streptomycin/amphotericin B solution, fetal bovine serum (FBS)) were purchased from PAN-Biotech GmbH (Aidenbach, Germany). Doxorubicin, tamoxifen, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT), dimethyl sulfoxide (DMSO), and solvents for extraction were procured from Sigma-Aldrich (Saint Louis, Missouri, USA). Lapatinib was provided by Beyotime (Shanghai, China). Primary antibody against Ki67 and Alexa-488 conjugated secondary antibody against rabbit IgG were acquired from Abcam (Waltham, MA, USA). One-step TUNEL *in situ* apoptosis kit was bought from Elabscience (Houston, Texas, USA).

2.2. Sample collection and preparation of plant extract

Leaves of *Pterospermum diversifolium* were collected in Bac Giang province in April 2025 and taxonomically identified by MSc. Lai Viet Hung (Center of Medicinal Material Resources). A voucher specimen (NIMM-20134) was deposited at the Herbarium of Center of Medicinal Material Resources (NIMM), Vietnam. After collecting, the plant material was cleaned, air-dried, and coarsely ground. A total of 100 grams of the dried material was subjected to three-time reflux extraction with 70% ethanol (3×500 mL) for 3 h each. The combined extracts were filtered, and the solvent was completely evaporated under reduced pressure, followed by water-bath concentration to obtain the *P. diversifolium* ethanol extract (PDE), with an extraction yield of 4.69%.

For cell-based assays, PDE was dissolved in absolute DMSO to prepare a stock solution at a concentration of 100 mg/mL and stored at - 20°C until use. Prior to incubation with cells, the stock solution was diluted with culture medium to the desired concentrations. The final DMSO concentration in the culture medium did not exceed 0.2%. This concentration of DMSO was confirmed to have no effect on the viability of the cell lines used in this study.

2.3. Cell culture

MCF-7, SKBR3, and HEK293 cell lines were obtained from American Type Culture Collection (Rockville, MD, USA). MCF-7 and HEK293 cells were routinely cultured in high-glucose DMEM medium, while SKBR3 cells were cultured in RPMI-1640 medium. All culture media were supplemented with 10% fetal bovine albumin (FBS) and 1% antibiotic/antimycotic reagent. Cells were maintained in a 37°C in a humidified incubator with 5% CO_2.

2.4. Cell viability assessment and synergistic anti-cancer effect analysis

Cell viability was assessed using the MTT assay [8]. Briefly, cells were seeded in 96-well plates at a density of 10^4 cells/well and incubated overnight. Cells were then treated with PDE or reference compounds at increasing concentrations for 72 hours. Following treatment, 10 µL of MTT solution (final concentration: 0.5 mg/mL) was added and incubated for 2 hours. After removing the medium, formazan crystals were dissolved in 200 µL of DMSO, and absorbance (OD) was measured at 570 nm using a microplate reader. The inhibitory proportion of cell viability was calculated as follows:

$$I(\%) = (OD_{control} - OD_{sample}) \times 100 / OD_{control}.$$

The potential synergistic effects between PDE and tamoxifen or lapatinib were evaluated using the Chou-Talalay method [9]. IC_{50} values (the concentration required to inhibit 50% of cell viability) for each agent were first determined. Subsequently, cells were treated with tamoxifen and PDE (or lapatinib and PDE), alone or in combination, at concentrations equivalent to

~0.25×, 0.5×, 1.0×, 1.5×, and 2.0× their respective IC_{50} values. The agents were combined at a fixed 1:1 ratio based on their IC_{50} values.

2.5. Immunocytochemistry (ICC)

ICC was used to detect nuclear expression of Ki67 as previously described [10]. Cells were seeded into an 8-well glass slide chamber at a density of 4×10^4 cells/well. After overnight incubation, cells were treated with PDE or tamoxifen for 48 h. Then, cells were fixed with 4% paraformaldehyde (20 min, room temperature), permeabilized with 0.2% Triton X-100 (10 min, room temperature), and blocked with 3% bovine serum albumin (200 µL each solution/well) for 30 min at room temperature. A primary antibody against Ki67 (dilution ratio of 1:200) and an Alexa-488 conjugated secondary antibody (dilution ratio of 1:500) were sequentially incubated with cells for 16 h (4°C) and 90 min (room temperature), respectively. The slide was covered with a coverslip using a DAPI (4′,6-diamidino-2-phenylindole) containing mounting reagent (Abcam). Finally, images were acquired using a fluorescent microscope (ECLIPSE Ti2, Tokyo, Japan).

2.6. Tunel assay

Apoptotic cells were detected by the TUNEL assay according to the manufacturer's instructions. Cells were seeded into an 8-well glass slide chamber with a density of 4×10^4 cells/well. After overnight incubation, cells were treated with PDE or doxorubicin (DOX) for 72 h. Cells were then fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100. The labeling solution was freshly prepared immediately before use by mixing the TdT buffer, labeling reagent, and TdT enzyme provided in the manufacturer's kit. Cells were first incubated with the TdT buffer at 37°C for 20 min. Subsequently, the labeling solution was added, and the cells were incubated for an additional 60 min at 37°C. Images were acquired using a fluorescent microscope (ECLIPSE Ti2) and analyzed using ImageJ.

2.7. Caspase-3/7 activity assay

Apoptosis was also assessed by measuring caspase-3/7 activity using a quantitative assay kit from Promega (Caspase-Glo® 3/7 assay system) as we described previously [11]. Cells were seeded in a white 96-well plate. After overnight incubation, cells were treated with PDE or DOX. At the end of the treatment period, 30 µL of the caspase-3/7 reagent was added to each well containing 100 µL of medium, mixed gently, and incubated for 60 minutes. Finally, the luminescence of each well was measured using an appropriate plate reader (Varioskan LUX Multimode Microplate Reader).

2.8. Statistical analysis

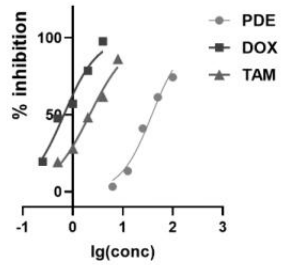
All statistical analyses were performed using GraphPad Prism 10.1. Data are presented as mean ± standard error (SE) from three independent experiments. Statistical differences between groups were assessed using one-way ANOVA followed by Tukey's post hoc test. A p-value < 0.05 was considered statistically significant. IC_{50} values were estimated by non-linear regression analysis. Synergism was confirmed by the Chou-Talalay analysis using COMPUSYN software.

3. Results

3.1. PDE decreases the viability of breast cancer cells compared to non-malignant cells

The anti-breast cancer activity of PDE was first assessed using the MTT assay. As shown in Fig. 1A and 1B, PDE reduced the viability of MCF-7 and SKBR3 cells in a dose-dependent manner, with IC_{50} values of 38.09 µg/mL and 35.68 µg/mL, respectively. Compared to conventional anti-breast cancer drugs, including doxorubicin (DOX), tamoxifen (TAM), and lapatinib (LAPA), PDE exhibited lower cytotoxic potency. In non-malignant HEK293 cells, PDE showed reduced toxicity, with an IC_{50} of 135.5 µg/mL. The selective index (SI) of PDE ranged from 3.56 to 3.80, indicating moderate selectivity for cancer cells. While this SI was lower than that of TAM or LAPA, it was higher than that of DOX (1.73-2.29).

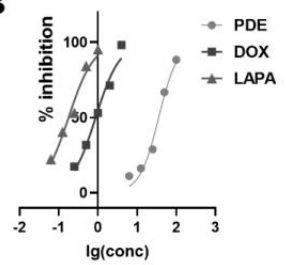
A



MCF-7

Agent	IC_{50} (μg/mL)	95% CI
PDE	**38.09 ± 6.58**	27.98 – 53.76
DOX	**0.6630 ± 0.1411**	0.4274 – 0.9806
TAM	**2.174 ± 0.3304**	1.620 – 2.915

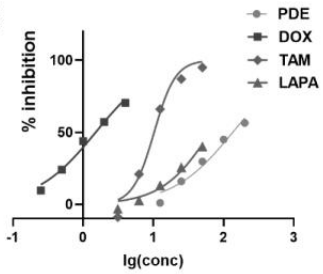
B



SKBR3

Agent	IC_{50} (μg/mL)	95% CI
PDE	**35.68 ± 5.143**	26.91 – 47.07
DOX	**0.8770 ± 0.1561**	0.6159 – 1.228
LAPA	**0.1780 ± 0.0335**	0.1212 – 0.2526

C



HEK293

Agent	IC_{50} (μg/mL)	95% CI
PDE	**135.5 ± 40.58**	92.41 – 251.5
DOX	**1.515 ± 0.220**	1.167 – 2.029
TAM	**10.29 ± 1.785**	7.502 – 14.50
LAPA	**> 50**	-

D

Agent	Selective index (SI)	
	MCF7/HEK293	SKBR3/HEK293
PDE	**3.56**	**3.80**
DOX	**2.29**	**1.73**
TAM	**4.73**	-
LAPA	-	**> 280**

Fig.1. Effects of PDE on cell viability. Cells were treated with PDE for 72 h and cell viability was examined by MTT assay. (A, B) Dose–response curves of PDE in MCF-7 and SKBR3 cells. (C) Dose–response in HEK293 cells. IC_{50} data were expressed as mean ± SE (n = 3).

3.2. PDE inhibits the proliferation of breast cancer cells

To assess the effect of PDE on cell proliferation, nuclear expression of Ki67 was analyzed using immunocytochemistry. As shown in Fig. 2, Ki67 (green) was localized exclusively in the nuclei, counterstained with DAPI (blue). In the control group, most cells were Ki67-positive, indicating active proliferation. In contrast, PDE-treated cells showed a marked reduction in Ki67 expression. Quantitative analysis using ImageJ revealed a significant decrease in total Ki67 fluorescence intensity in cells treated with 40 μg/mL of PDE. This reduction was similar to that observed in cells treated with 2 μM of tamoxifen (TAM), a well-established anti-proliferative agent in breast cancer therapy.

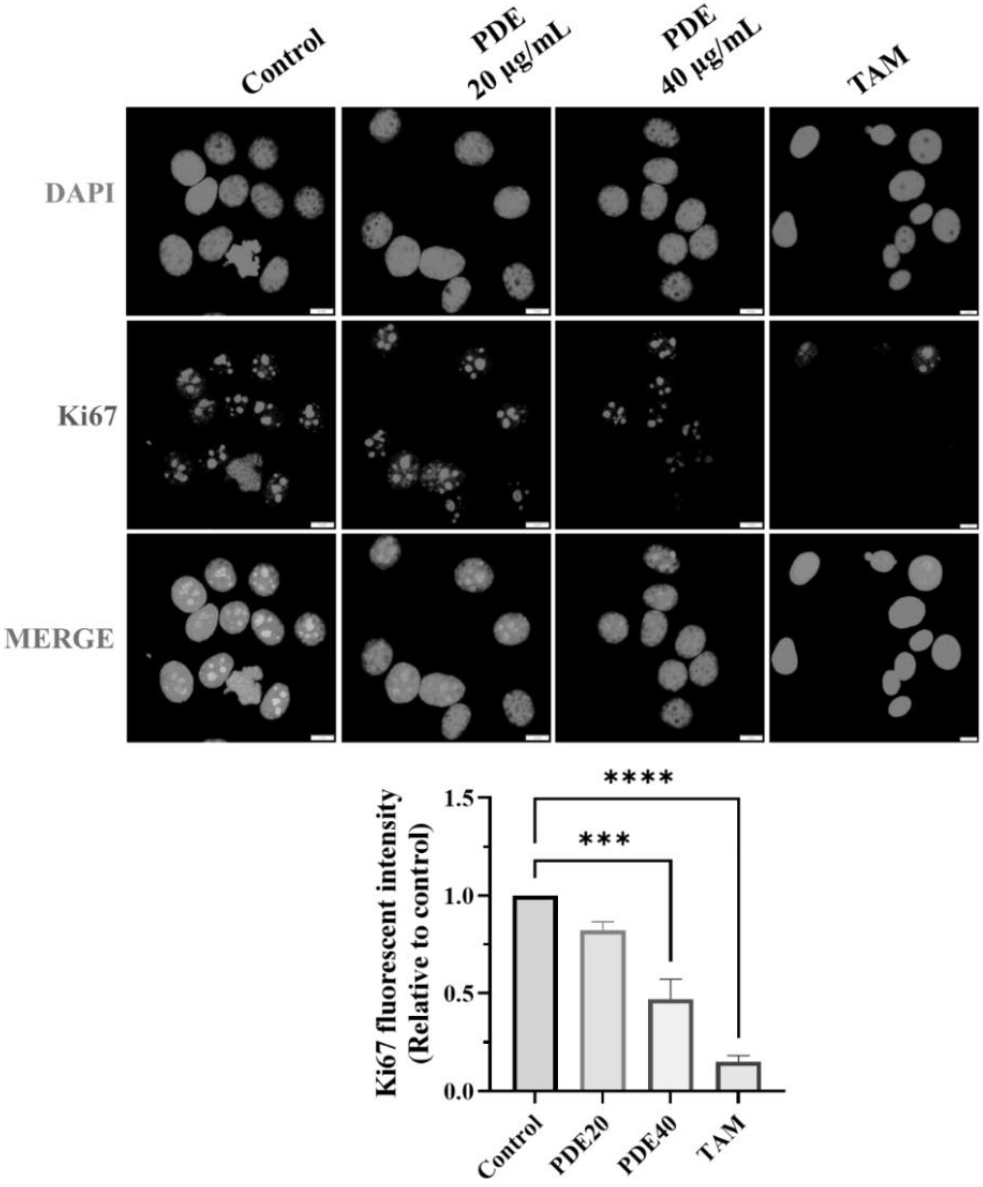


Fig. 2. PDE reduces nuclear Ki67 expression in MCF-7 breast cancer cells.
****p<0.001 and ****p<0.0001 compared to control (n=3); scale bar: 10 µm; 400X.*

3.3. PDE induces the apoptosis of breast cancer cells

Apoptosis in MCF-7 cells was assessed using the TUNEL assay. In this assay, apoptotic cells were identified by red fluorescence (Fig. 3A). While few TUNEL-positive cells were observed in the control group, the number of apoptotic cells increased markedly following treatment with PDE at 20 and 40 µg/mL. Notably, the effect of PDE at 40 µg/mL was comparable to that of doxorubicin (DOX) at 0.5 µg/mL. Likewise, PDE enhanced the activity of caspase-7, another marker of apoptosis, in a dose-dependent manner (Fig. 3B). These results suggest that PDE inhibits the growth of breast cancer cells, at least in part, through the induction of apoptosis.

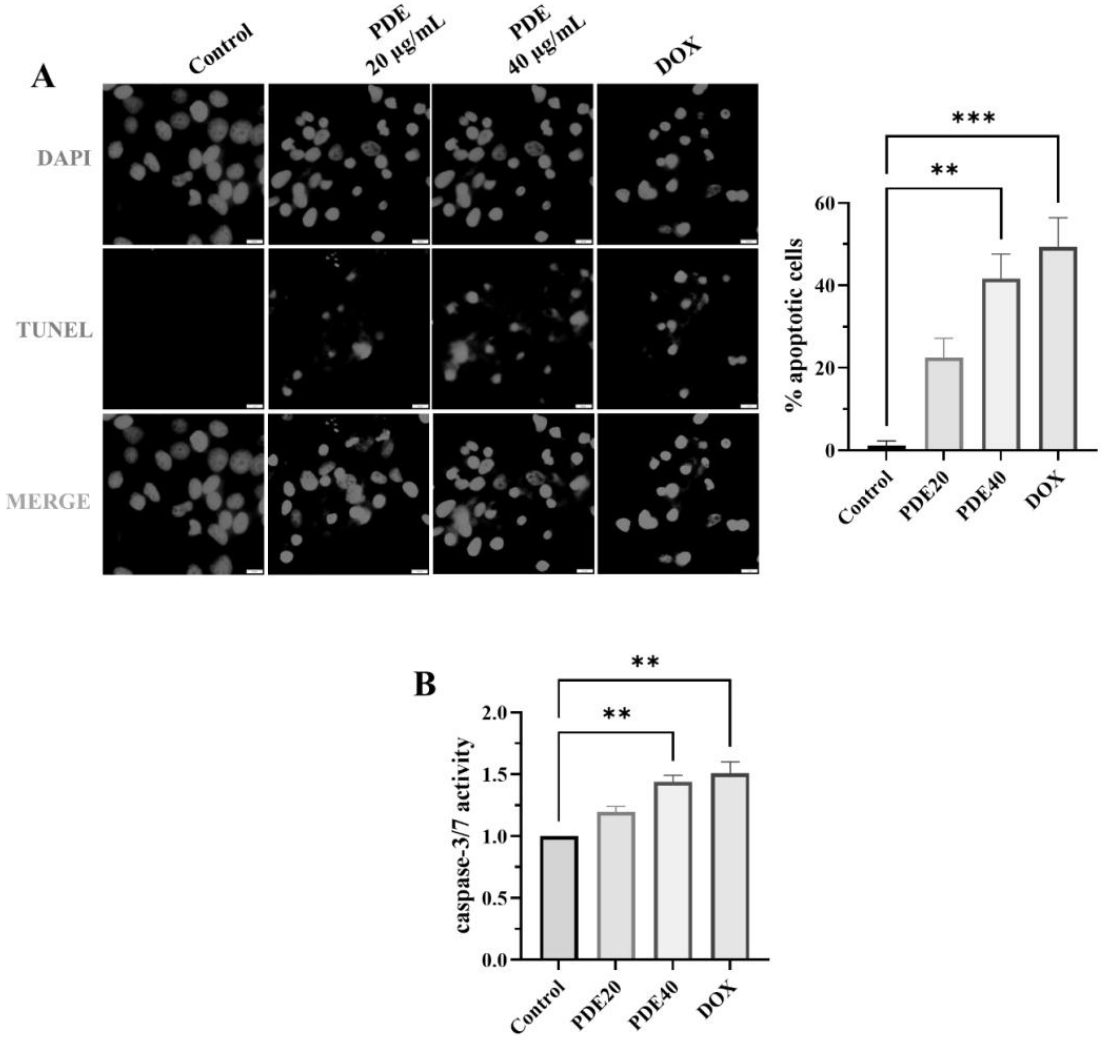


Fig. 3. Effects of PDE on apoptosis in MCF-7 breast cancer cells.
***p<0.01 and ***p<0.001 compared to control (n=3); scale bar: 10 µm; 100X.*

3.4. PDE exhibits a synergistic effect with lapatinib in SKBR3 breast cancer cells

We next investigated whether PDE could exert synergistic anti-cancer effects when combined with established therapeutic agents such as tamoxifen (TAM) and lapatinib (LAPA). Co-treatment with PDE and either TAM or LAPA resulted in greater inhibition of breast cancer cell viability compared to treatment with each agent alone (Fig. 4). However, PDE did not show significant synergy with TAM, an estrogen receptor (ER) antagonist, in MCF-7 cells (Fig. 4A). In contrast, a clear synergistic effect was observed between PDE and LAPA, a HER2 inhibitor, in SKBR3 cells, as confirmed by Chou–Talalay analysis (Fig. 4B). Consistent with this, the DRI plots indicated that combining PDE with LAPA reduced the doses required for each agent to achieve the same effect (Fig. 4C). These findings suggest that the pharmacodynamic interaction between PDE and standard breast cancer therapies is context-dependent and may vary according to molecular subtypes of breast cancer.

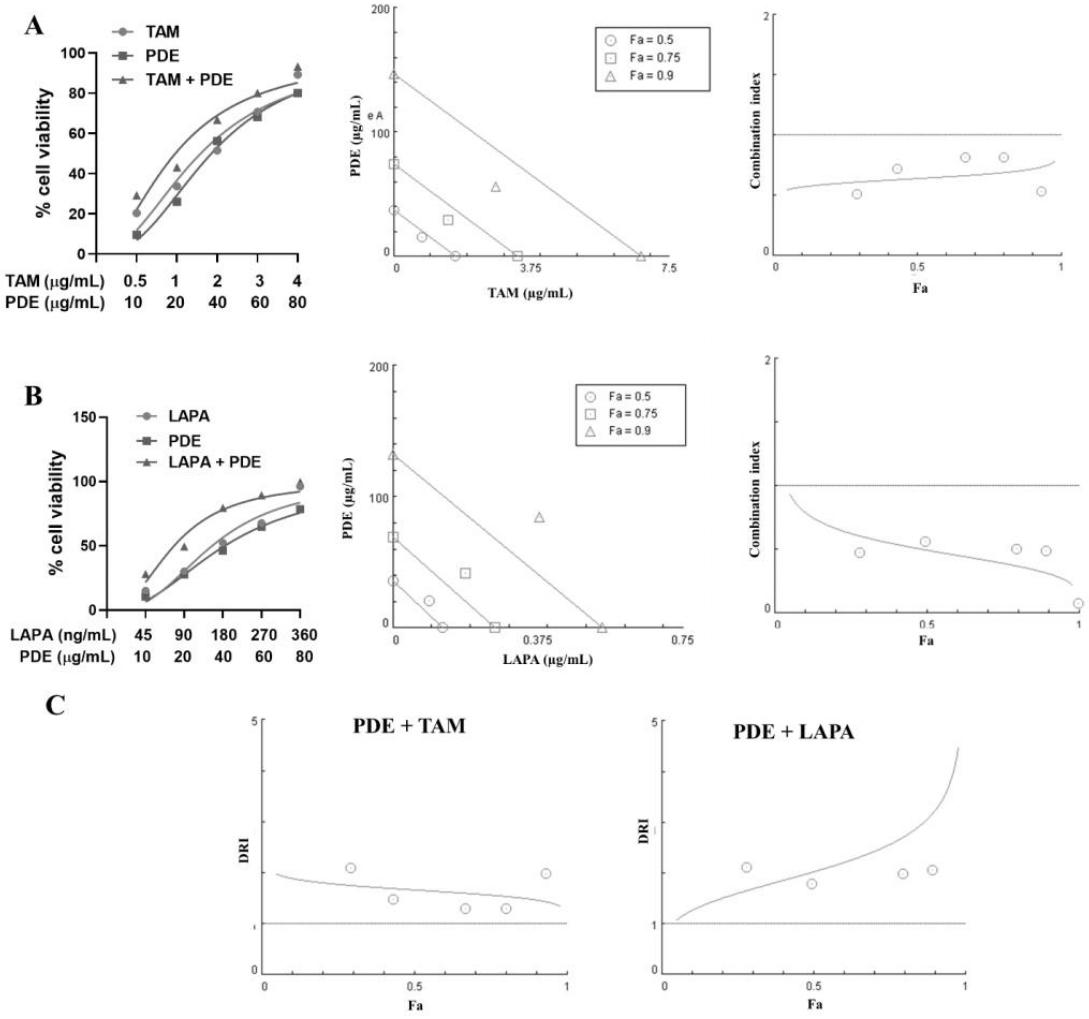


Fig. 4. Combined effects of PDE and LAPA or TAM on breast cancer cell viability.
(A) *MCF-7 cells were treated with PDE (10-80 μg/mL), TAM (0.5-4.0 μg/mL), or their combination at fixed ratio based on IC_{50} values. Cell viability was assessed after 72 h using the MTT assay. Isobologram/combination index (CI) analysis of PDE and TAM combination in MCF-7 cells based on the Chou-Talalay method.* ***(B)*** *SKBR3 cells were treated with PDE (10-80 μg/mL), LAPA (45-360 ng/mL), or their combination at fixed ratios. Cell viability was determined by MTT assay after 72 h. Isobologram/combination index analysis of PDE and LAPA in SKBR3 cells shows synergistic interaction.*
*(**C**) Dose reduction indexes for PDE-TAM and PDE-LAPA combinations.*

4. Discussion

In this study, three cell lines were selected, including two breast cancer cell lines (MCF-7 and SKBR3) and one non-malignant cell line (HEK293), in order to investigate the effects of PDE on breast cancer cells with distinct genotypic/phenotypic characteristics and to preliminarily evaluate its selectivity between cancerous and normal cells. MCF-7 is an estrogen receptor-positive (ER^+) breast cancer cell line that responds well to tamoxifen (TAM), whereas SKBR3 is a HER2-positive line representative of breast cancers treatable with HER2-targeted tyrosine kinase inhibitors such as lapatinib (LAPA). PDE exhibited comparable cytotoxic effects on both MCF-7 and SKBR3 cells, with IC_{50} values of 38.09 and 35.68 μg/mL, respectively. This suggests that PDE's anti-breast cancer activity may not primarily involve modulation of hormone receptor or growth factor receptor signaling, but rather alternative, receptor-independent mechanisms.

While data on the anticancer activity of *P. diversifolium* (PDE) remain limited, a recent study reported that silver nanoparticles synthesized using *P. heterophyllum* extract inhibited the growth of triple-negative MDA-MB-231 breast cancer cells, with an IC_{50} of 50.4 µg/mL [12]. However, the effects of this agent on ER^+ or $HER2^+$ breast cancer cells have not yet been evaluated, and its underlying mechanisms of action remain unexplored. Furthermore, the selectivity between cancerous and non-cancerous cells is also a critical factor in evaluating the therapeutic potential of a cytotoxic agent. Using HEK293 cells, a human embryonic kidney-derived cell line, as a non-malignant model, we found that PDE exhibited significantly lower cytotoxicity toward this cell line compared to breast cancer cell lines. Although the selectivity index (SI) of PDE was lower than that of target-specific agents such as TAM and LAPA, it was notably higher than that of the cytotoxic chemotherapeutic agent doxorubicin (DOX). However, it is important to note that HEK293 cells do not fully represent normal cells [13]. Therefore, the effects of PDE on primary non-malignant cells should be further investigated in future studies to more accurately estimate its safety profile.

The anticancer activity of an agent may result from modulation of the cell cycle or the induction of programmed cell death (apoptosis). To investigate the underlying mechanisms of PDE's anti-breast cancer effects, we evaluated its impact on each process in comparison with the antiproliferative control tamoxifen (TAM) and the apoptosis control doxorubicin (DOX). Ki67 is a nuclear proliferation marker expressed during interphase, and its nuclear localization reflects the proliferative status of cells [14]. As expected, PDE treatment at a higher concentration (40 µg/mL) significantly reduced Ki67 expression, indicating a potential effect on cell cycle regulation. In addition, TUNEL staining demonstrated that PDE strongly induced apoptosis. Notably, apoptotic activity was evident even at a lower concentration (20 µg/mL), suggesting that apoptosis induction may be the predominant mechanism underlying PDE's cytotoxic effect on breast cancer cells.

The therapeutic role of an anticancer agent, particularly a crude plant extract, is often best realized as an adjuvant to standard chemotherapeutic regimens [15]. In fact, several plant-derived compounds previously shown to possess cytotoxic activity against breast cancer cells were later found to antagonize the effects of tamoxifen (TAM), significantly limiting their clinical potential [16]. Therefore, evaluating whether PDE exhibits synergistic interactions with standard treatments such as TAM and lapatinib (LAPA) across different breast cancer subtypes is essential for better understanding its pharmacological profile. Interestingly, PDE demonstrated a synergistic effect only in combination with LAPA in HER2-positive SKBR3 cells, but not with TAM in ER-positive MCF-7 cells. These findings suggest that PDE may have greater potential as a supportive therapy for HER2-positive breast cancer than for hormone receptor-positive subtypes.

Given the reported anti-breast cancer activity of PDE, it is important to elucidate the relationship between its chemical constituents and biological effects. To date, the specific compounds present in PDE remain poorly characterized. Preliminary qualitative screening of *P. diversifolium* leaves indicated the presence of flavonoids, phenolics, tannins, and saponins [17]. Of particular interest are flavonoids, reported in other *Pterospermum* spp. (e.g., luteolin, kaempferol), which have been shown to induce apoptosis in breast cancer cells [18]. Similarly, triterpenoids such as taraxerol and betulonic acid, isolated from related *Pterospermum* taxa, have demonstrated marked cytotoxicity against cancer cell lines [19],[20]. Future work should therefore focus on comprehensive phytochemical profiling (qualitative and quantitative), isolation and structure elucidation of active constituents, and mechanistic studies to identify which compounds (or combinations thereof) mediate PDE's anti-breast cancer effects.

This is the first report on the role of PDE in the inhibition of breast cancer cells. While the inhibitory activity of PDE on the two cell lines, MCF-7 and SKBR3, is similar, there is a marked difference in its synergistic effects with agents that are specifically used to treat each breast cancer subtype. Since PDE exhibits synergy with LAPA but not with TAM, further investigations of the effects of PDE on SKBR3 cells are needed to elucidate the mechanism of action of PDE in this cell line. Another limitation

of the present study is the lack of evaluation of the influence of plant-related factors and extraction procedures on the anticancer activity of PDE. In fact, although most previous studies have investigated the biological activities of *P. diversifolium* leaf extracts, anticancer activity has also been reported in other parts of *Pterospermum* species, such as the bark, roots, and stems [18],[19]. Similarly, identification of the active constituents, as discussed above, would facilitate the optimization of the solvent system and extraction protocol in order to maximize the anticancer activity of PDE.

5. Conclusions

This study demonstrated that PDE possesses significant cytotoxic effects against both ER-positive and HER2-positive breast cancer cells. These effects appear to be mediated through the suppression of cell proliferation and the induction of apoptosis. When combined with targeted therapies, PDE showed a synergistic interaction with lapatinib (LAPA) in HER2-positive SKBR3 cells but not with tamoxifen (TAM) in ER-positive MCF-7 cells. Taken together, these findings suggest that PDE may serve as a promising therapeutic candidate, particularly in the treatment of HER2-positive breast cancer.

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