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CONTENTS VOL. 30(5), 2025
(Pages 305 - 384)

Journal of Medicinal Materials, 2025, Vol. 30, No. 5 (375 - 384)

STANDARDIZED EXTRACTS OF *PARIS POLYPHYLLA* VAR. *CHINENSIS* SMITH RHIZOMES AND *PIPER LONGUM* L. FRUITS SUPPRESS BREAST CANCER CELL MIGRATION

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Summary

This study aimed to investigate the effects of two standardized extracts, *Paris polyphylla* var. *chinensis* rhizomes and *Piper longum* fruits, on breast cancer cell migration, a critical characteristic of cancer cell metastasis. Cytotoxicity of the standardized extracts of *P. polyphylla* var. *chinensis* rhizomes (PPCR-SE) and *P. longum* fruits (PLF-SE) was evaluated on MCF-7 and MDA-MB-231 breast cancer cell lines by MTT assay to determine appropriate concentrations for the migration assay. Subsequently, a cellular scratch assay was performed to evaluate wound closure rates after treatment with various extract concentrations. The results indicated that at concentrations of 1.25-5.0 µg/mL, PPCR-SE significantly inhibited MCF-7 cell migration with wound closure rates of 58.55-22.73%. PLF-SE, at concentrations of 2.5 and 5.0 µg/mL, inhibited MCF-7 cell migration with wound closure rates of 43.84% and 48.72%, respectively. Similar results were observed for MDA-MB-231 cells, with wound closure rates of 45.69% and 35.95% at 1.25 and 2.5 µg/mL of PPCR-SE, respectively, and ranging from 56.30-44.92% at 1.25-5.0 µg/mL of PLF-SE. The results suggested that both *P. polyphylla* var. *chinensis* rhizomes and *P. longum* fruits hold the potential to inhibit breast cancer migration. Further research is warranted to elucidate the precise molecular mechanisms underlying their effects on breast cancer cell migration.

Keywords: *Paris polyphylla* var. *chinensis* rhizomes; *Piper longum fruits; Standardized extracts; Migration; Breast cancer cells; Scratch assay.*

1. Introduction

Breast cancer is a dangerous and highly fatal disease in women. According to the GLOBOCAN 2022 report, there were 2.3 million new cases and 666,103 deaths due to breast cancer. In Vietnam, the incidence of breast cancer is clearly on the rise, with 11,067 cases and 4,671 deaths recorded in 2012, and subsequently increased to 24,563 new cases and 10,008 deaths in 2022 [1],[2]. Among these, luminal A breast cancer accounts for the largest proportion of patients (33.2%) [3]. This molecular subtype is distinguished by the positive expression of estrogen receptor (ER) and/or progesterone receptor (PR), and the absence of human epidermal growth factor receptor 2 (HER2) overexpression. A favorable prognosis and high survival rates are generally observed in patients diagnosed with Luminal A, primarily attributed to the less aggressive and invasive characteristics of breast cancer [3]. In contrast, while triple-negative breast cancer (TNBC) presents with the lowest prevalence (13.1%), it is characterized by highly aggressive and invasive features, typically manifesting as high-grade tumors and advanced stages at diagnosis. TNBC is frequently associated with early recurrence and a significantly poorer patient prognosis, primarily due to the absence of ER, PR, and HER2 expression (ER-, PR-, HER2), which severely restricts available therapeutic interventions [3]. Current breast cancer treatment methods include surgery, radiation therapy, and chemotherapy. However, these methods all have limitations, including causing pain and adverse side effects that impact patient's health. Therefore, many alternative cancer treatments and therapies have been employed, including medicinal plant extracts. Numerous studies have shown that medicinal plant extracts containing bioactive compounds such as flavonoids, alkaloids, terpenoids, and polyphenols possess anti-breast cancer activity [4],[5],[6],[7].

Paris polyphylla var. *chinensis* Smith is a perennial herbaceous plant classified under the genus *Paris* within the Trilliaceae family. Its primary geographical distribution encompasses China, Vietnam, and Myanmar. Historically, this plant has been extensively utilized in traditional medicine for a variety of therapeutic purposes,

including the treatment of venomous snake and insect bites, epidemic encephalitis, pyogenic dermatitis, tuberculous meningitis, asthma, diphtheria, mumps, and hemorrhoids [8]. A broad spectrum of pharmacological properties of *P. polyphylla* has been reported in research such as anti-cancer, anti-inflammatory, antipyretic, analgesic, sedative, antimicrobial, and hemostatic effects [9],[10],[11]. Among these diverse bioactivities, its anti-cancer potential stands out as particularly noteworthy and has gained significant research interest. Previous studies focusing on breast cancer cell lines have consistently demonstrated the capacity of *P. polyphylla* rhizomes to inhibit cellular proliferation. For instance, extracts derived from the leaves of *P. polyphylla* var. *chinensis* exhibit cytotoxic effects across multiple cell lines, including A549 (human lung carcinoma), HepG2 (human hepatocellular carcinoma), A431 (epidermoid carcinoma cell), HBE (human bronchial epithelial cell), and MCF-7 (human breast carcinoma) [9]. Notably, both the *n*-butanol fraction and chloroform extracts demonstrated high levels of toxicity against the MCF-7 cell line (IC_{50} value of 11.8 and 12.6 μg/mL, respectively) [9]. Another study indicated that polyphyllin D, a major active component isolated from *P. polyphylla*, effectively reduced the viability of MCF-7 and MDA-MB-231 breast cancer cells, exhibiting IC_{50} values of 5 μM and 2.5 μM, respectively, after 48 hours of treatment [12]. Its mechanism of action involves the induction of apoptosis, characterized by DNA fragmentation, cell cycle arrest, phosphatidylserine externalization, and the loss of cell membrane integrity. Mechanistically, polyphyllin D exerts its effects on the mitochondrial membrane, leading to a diminished expression of Bcl-2 and an augmented expression of Bax, alongside the activation of caspase-9. *In vivo* studies further corroborate these findings, with polyphyllin D administration (2.73 mg/kg, i.v.) over 10 days significantly reducing tumor growth by 50% in both weight and size in MCF-7 cell-bearing mice [12]. Similarly, paris saponin II, an isolate from *P. polyphylla* var. *chinensis*, has demonstrated potent cytotoxic activity against MCF-7 breast cancer cells [13]. Paris saponin II notably elevates the expression levels of proteins associated with cell proliferation, G2/M phase progression, and apoptosis, such as p53, p21, p27, and Bax [13]. Concurrently, it significantly inhibits the expression of cyclin D1, a key cell cycle regulatory protein.

Piper longum L., commonly known as long pepper, is a member of the Piperaceae family widely distributed across tropical and subtropical regions globally. Chemical investigations of this plant have revealed the presence of a diverse array of compounds, including significant quantities of alkaloids, terpenoids, sterols, and essential oils, which have been isolated from its fruits, roots, leaves, and seeds [14]. In traditional medicine, *P. longum* has been historically employed for treating various respiratory ailments, such as sinusitis, tuberculosis, general respiratory infections, and childhood asthma. Regarding its anticancer properties, extracts of *P. longum* have also been investigated for their ability to inhibit metastasis and invasion in Glioblastoma multiforme, prostate cancer, and lung metastasis induced by B16F10 melanoma cells in C57BL/6 mice [15],[16],[17]. Recent studies have reported the anti-breast cancer properties of *P. longum fruits.* Specifically, two key compounds isolated from *P. longum*, piperlongumine and piperine**,** have been demonstrated to be effective against breast cancer cells. Cytotoxic effects of piperlongumine have been expressed on breast cancer cells as MCF-7 (IC_{50} approximately 25 μM) and MDA-MB-231 (IC_{50} approximately 23 μM) after 24 hours of treatment [18]. Piperlongumine also effectively inhibits the proliferation and migration of MCF-7 cells [19]. Piperine, on the other hand, has been shown to suppress the growth and migration of TNBC cells. This effect is mediated through a mechanism involving the downregulation of G1-binding proteins (e.g., cyclin D3, CDK4, E2F-1) and G2-binding proteins (e.g., cyclin B, CDK1, Cdc25C), alongside an increased expression of p21 Waf1/Cip1 in the MDA-MB-468 cell line [20].

While previous studies have highlighted the antiproliferative and cytotoxic effects of *P. polyphylla* var. *chinensis* rhizomes and *P. longum* on breast cancer cells, studies specifically addressing their influence on breast cancer cell metastasis remain limited. Therefore, this study aims to conduct a preliminary investigation into the effects of standardized extracts of *P. polyphylla* var. *chinensis* rhizomes and *P. longum*

fruits on one of the critical processes in metastasis including the migration of breast cancer cell lines, luminal A MCF-7 and TNBC MDA-MB-231.

2. Materials and methods

2.1. Plant materials and standardized extracts preparation

Rhizomes of *Paris polyphylla* var. *chinensis* Smith were collected in November 2017 in Lao Cai, Vietnam. Fruits of *Piper longum* L. were collected in September 2020 in Dak Song, Dak Nong, Vietnam. The plants were identified by Assoc. Prof. Pham Thanh Huyen and MSc. Nguyen Van Hieu in the National Institute of Medicinal Materials. The samples were cleaned and dried at 55°C until reaching a constant weight. The samples were then stored in vacuum-sealed bags at room temperature.

The standardized extracts of *Paris polyphylla* var. *chinensis* rhizomes (PPCR-SE) [21] and *Piper longum* L. fruits (PLF-SE) [22] were prepared following the previously reported procedure.

2.2. Cell culture

Human breast cancer cell lines (MCF-7 and MDA-MB-231 cells) were cultured in high glucose Dulbecco's Modified Eagle Medium (DMEM) with 10% (*v/v*) fetal bovine serum (FBS), 1% (*v/v*) penicillin-streptomycin in a 5% CO_2 atmosphere in an incubator (Esco Lifesciences, Singapore) at 37°C. Cells were removed from the monolayer using 0.25% trypsin in phosphate-buffered saline, resuspended in medium, and seeded at a suitable density in the plate. Chemicals were purchased from Sigma-Aldrich, US.

2.3. Cell viability assay

The breast cancer cells were seeded at a density of 1×10^4 cells/well in a 96-well plate (SPL Life Sciences, Korea) and incubated under 5% CO_2 at 37°C for 24 h to form a cell monolayer. After 24 h, the cells were treated with PPCR-SE or PLF-SE at different concentrations and incubated for 24 h. After that, the cells were exposed to 0.5 mg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent (Sigma-Aldrich, US) and incubated for 3 h at 37°C in a 5% CO_2 atmosphere. Supernatants were removed, and 100 µL of DMSO (Merck, Germany) was added. The plates were then incubated for 15 minutes to solubilize the formazan crystals, and absorbance was measured at 570 nm using a plate reader (BioTek, USA). Doxorubicin (DOXO) (Sigma-Aldrich, US), a standard chemotherapeutic drug, was used as a positive control. Dimethyl sulfoxide (DMSO) (Merck) at an appropriate final concentration was used as a control. The viability of cells treated with and without the test samples was expressed as cell viability percentage (%) by comparing the mean absorbance of cells from different groups using the following formula: Cell viability (%) = [(Mean absorbance treated cells - Mean absorbance blank)/(Mean absorbance untreated cells - Mean absorbance blank)] × 100 [23].

2.4. Scratch wound assay

The breast cancer cells were seeded at a density of 2×10^5 cells/well in a 6-well plate and incubated in a 5% CO_2 atmosphere at 37°C for 24 h to grow a confluent monolayer. The monolayers were scratched off using a sterile 1000 µL pipette tip to make a straight scratch, and PBS was added to quickly remove debris and floating cells. After that, the cells were treated with non-cytotoxic concentrations of PPCR-SE or PLF-SE in DMEM supplemented with 1% FBS and incubated for 24 h at 37°C. The cells were observed and photographed at two-time points (0 and 24 h) to determine the wound closure under a microscope (Nikon Eclipse Ts2R-FL) at 100× magnification. The areas of the scratches were measured using ImageJ. DOXO was used as a positive control. DMSO at an appropriate final concentration was used as a control. Wound closure rate was calculated using the formula: Wound closure (%) = [(Scratch area at time 0 h - Scratch area at time 24 h)/Scratch area at time 0 h] × 100 [24].

2.5. Statistical analysis

All experiments were repeated at least three times (Mean ± Standard deviation). The IC_{50} value was calculated using GraphPad Prism software version 8.0.2 (US). One-way ANOVA with Tukey's test was used to determine the difference between the experimental groups ($p < 0.05$ was considered statistically significant).

3. Results

3.1. Cytotoxicity of PPCR-SE and PLF-SE in breast cancer cell lines

The cytotoxicity of different PPCR-SE and PLF-SE concentrations on MCF-7 and MDA-MB-231 breast cancer cell lines was evaluated

using the MTT assay over a 24-hour period. As depicted in Fig. 1A-C, the PPCR-SE and PLF-SE demonstrated a potent inhibitory effect on MCF-7 cells. PPCR-SE, PLF-SE, and DOXO caused concentration-dependent inhibition of cell proliferation towards MCF-7. PPCR-SE (12.5-100 µg/mL), PLF-SE (25-100 µg/mL), and DOXO (2.5-20 µg/mL) significantly reduced MCF-7 cell viability compared to the untreated control with IC_{50} values of 27.62, 57.20, and 4.84 µg/mL, respectively, indicating effective cytotoxicity against MCF-7 cells.

PPCR-SE (5-80 µg/mL) and DOXO (5-20 µg/mL) also notably reduced MDA-MB-231 TNBC cell viability compared to the untreated control with IC_{50} values of 24.75 and 17.01 µg/mL, respectively (Fig. 1D, 1F). Meanwhile, PLF-SE (1.25-100 µg/mL) did not significantly affect the viability of MDA-MB-231 cells, with the percentage of viable cells not significantly different from the untreated control, so the IC_{50} value was not determined (Fig. 1E). Thus, the PLF-SE displayed a lower degree of cytotoxicity compared to PPCR-SE on both MCF-7 and MDA-MB-231 cells.

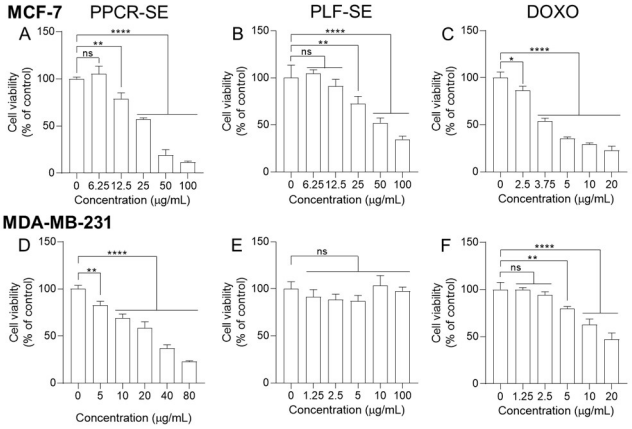

Fig. 1. Cytotoxic activity of PPCR-SE and PLF-SE in MCF-7 and MDA-MB-231 cells after 24 hours of treatment. PPCR-SE, PLF-SE, and DOXO are standardized extracts of *P. polyphylla* var. *chinensis* and *P. longum*, and doxorubicin, respectively. $^{ns}p > 0.05$, $^{**}p < 0.01$, and $^{****}p < 0.0001$ compared to the untreated control (0 µg/mL) by Tukey's test.

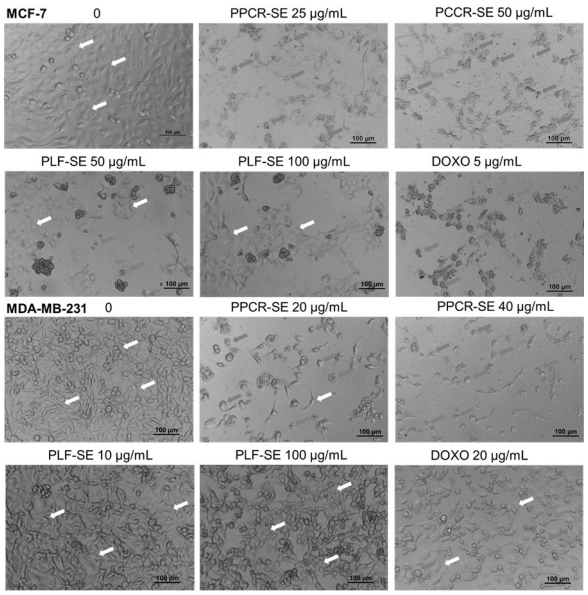

Fig. 2. Representative images illustrating the morphology of MCF-7 and MDA-MB-231 cells after 24 h of exposure to PPCR-SE and PLF-SE, in comparison to the untreated control (0 µg/mL). White arrow indicates living, adherent cells; red arrow shows cells that became rounded and shrunken; yellow arrow points dead cells. Scale bar = 100 µm.

As shown in Fig. 2, the untreated MCF-7 and MDA-MB-231 cells maintained their original morphology and appeared to have close contact with each other after 24 h. In contrast, MCF-7 and MDA-MB-231 cells lost their original shape, became sparser, and many suspension cells (dead cells) appeared after 24 hours of PPCR-SE treatment at 25-50 μg/mL and 20-40 μg/mL, respectively. MCF-7 cells did not have a polygonal or trigonal shape, while MDA-MB-231 cells lost their elongated spindle shape. For PLF-SE treatment, MCF-7 cells lost their original shape, became sparse, and many dead cells appeared after 24 hours at 50 and 100 μg/mL, respectively. MCF-7 cells did not have a polygonal or trigonal shape, while the morphology of MDA-MB-231 cells treated with PLF-SE 10 and 100 μg/mL appeared similar to that of the control. For DOXO treatment, most of the MCF-7 cells lost their original shape and showed many death cells after 24 h of DOXO treatment at 5 μg/mL concentration, while MDA-MB-231 cells became rounder and lost their long spindle shape at 20 μg/mL DOXO.

3.2. PPCR-SE and PLF-SE suppress breast cancer cell migration

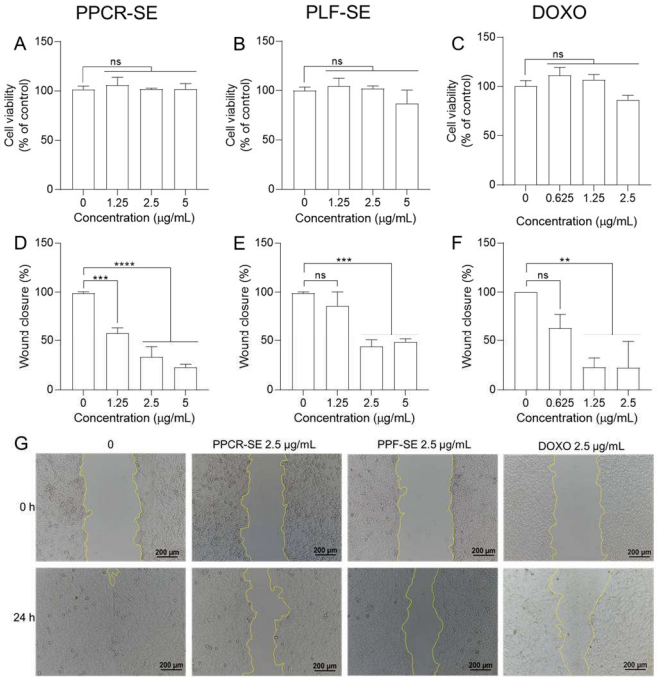

Fig. 3. Migration inhibitory activity of PPCR-SE and PLF-SE in MCF-7 cells after 24 hours of treatment. PPCR-SE, PLF-SE, and DOXO are standardized extracts of *P. polyphylla* var. *chinensis* and *P. longum*, and doxorubicin, respectively. A–C: cell viability (%), D–F: migration inhibitory activity, G is representative cell images of inhibition of MCF-7 cell migration by PPCR-SE and PLF-SE at 2.5 μg/mL concentration after 24 h, scale bar = 200 μm. $^{ns}p > 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$ compared to the untreated control (0 μg/mL) by Tukey's test.

To comprehensively assess the anti-migratory efficacy of the two standardized extracts, effective concentrations were selected to ensure non-cytotoxic conditions, defined as maintaining a cell survival rate exceeding 80% with no significant difference compared to the untreated control [25]. Consequently, based on preliminary cytotoxicity assays, concentrations of 1.25, 2.5, and 5 μg/mL were chosen for the scratch wound assay conducted on MCF-7 cells for both standardized extracts (Fig. 3A-C).

As shown in Fig. 3D-E, the migration inhibitory effect of both extracts was demonstrated; notably, PPCR-SE showed more pronounced migration inhibition than PLF-SE. Specifically, at the concentration of 1.25 μg/mL, PPCR-SE significantly inhibited cell migration. In contrast, MCF-7 cells treated with PLF-SE at this concentration showed no statistically significant difference compared to the untreated control group. Meanwhile, PPCR-SE and PLF-SE (2.5 and 5 μg/mL) significantly decreased cell motility and

migration of MCF-7 cells. The wound closure rates of MCF-7 cells treated with PPCR-SE at concentrations of 1.25, 2.5, and 5 µg/mL were 58.55%, 33.29%, and 22.73%, respectively, compared to the untreated control of 99.10% after 24 h (Fig. 3D). The wound closure rates of MCF-7 cells treated with PLF-SE at concentrations of 1.25, 2.5, and 5 µg/mL were 86.32%, 43.84%, and 48.72%, respectively, compared to the untreated control of 99.24% after 24 h (Fig. 3E). These results showed that PPCR-SE and PLF-SE at a concentration of 2.5 µg/mL inhibited the migration of MCF-7 cells by more than 50%. DOXO at concentrations of 1.25 and 2.5 µg/mL also inhibited more than 50% of the migration of MCF-7 cells with wound closure percentages of 23% and 22.48%, respectively, remarkably lower than the untreated control of 100% after 24 h (Fig. 3F).

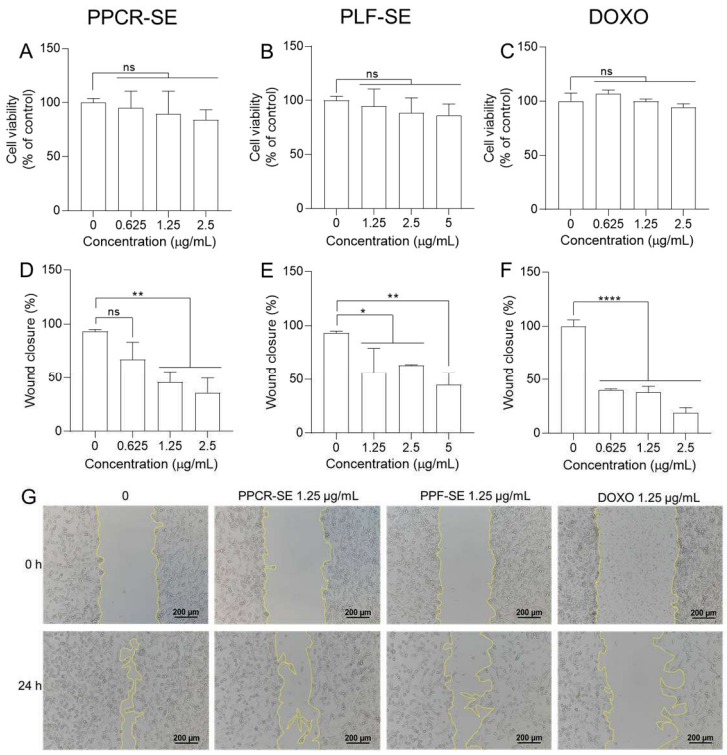

Fig. 4. Migration inhibitory activity of PPCR-SE and PLF-SE in MDA-MB-231 cells after 24 hours of treatment. PPCR-SE, PLF-SE, and DOXO are standardized extracts of *P. polyphylla* var. *chinensis* and *P. longum*, and doxorubicin, respectively. A-C are cell viability (%), D-F are migration inhibitory activity, G is representative cell images of inhibition of MDA-MB-231 cell migration by PPCR-SE and PLF-SE at 1.25 µg/mL concentration after 24 h, scale bar = 200 µm. $^{ns}p > 0.05$, $^{**}p < 0.01$, and $^{****}p < 0.0001$ compared to the untreated control (0 µg/mL) by Tukey's test.

Similarly, the effect of PPCR-SE and PLF-SE on MDA-MB-231 TNBC cell migration was also examined using the scratch assay at non-cytotoxic concentrations (Fig. 4A-C). As shown in Fig. 4D-E, the migration inhibitory effect of both extracts was demonstrated; notably, PPCR-SE also showed more pronounced migration inhibition than PLF-SE. Specifically, at the concentration of 1.25 µg/mL, both PPCR-SE and PLF-SE significantly inhibited cell migration by approximately 50% after 24 h, compared to the untreated control (93.27%). When the concentration was increased to 2.5 µg/mL, the migration inhibitory effect of PPCR-SE was observed to be higher than that of PLF-SE, with wound closure rates of 35.95% and 63.15% after 24 h, respectively, compared to the untreated control. Results also showed that 2.5 µg/mL PPCR-SE and 5 µg/mL PLF-SE inhibited the migration of MDA-MB-231 TNBC cells by more than 50%. DOXO at concentrations of 0.625-2.5 µg/mL also inhibited more than 50% of the migration of MDA-MB-231 cells with wound closure percentages of 40.26 – 19.00%, remarkably lower than the untreated control after 24 h (Fig. 4F).

Table 1. Comparison of migration inhibition (%) in the scratch assay and the IC_{50} values (MTT) for PPCR-SE and PLF-SE in MCF-7 and MDA-MB-231 cells

Cell line	Conc. (µg/mL)	PPCR-SE		PLF-SE		DOXO	
		Migration inhibition (%)	IC_{50} (µg/mL)	Migration inhibition (%)	IC_{50} (µg/mL)	Migration inhibition (%)	IC_{50} (µg/mL)
MCF-7	0.625	nd	27.62	nd	57.20	36.58	4.84
	1.25	41.82		13.79		77.00	
	2.5	67.31		56.59		77.52	
	5	77.97		51.67		nd	
MDA-MB-231	0.625	35.22	24.75	nd	nd	62.18	17.01
	1.25	58.23		46.85		64.07	
	2.5	68.67		39.51		84.31	
	5	nd		59.06		nd	

nd: not determined

The abbreviation nd denotes not determined. PPCR-SE, PLF-SE, and DOXO are standardized extracts of *P. polyphylla* var. *chinensis* and *P. longum*, and doxorubicin, respectively

Overall, PPCR-SE demonstrated higher cytotoxic activity and greater migration inhibition compared to PLF-SE on both breast cancer cell lines (Table 1). At the same concentrations of 1.25, 2.5, and 5 µg/ml, PPCR-SE demonstrated significantly higher migration inhibition than PLF-SE in the MCF-7 cell line. The inhibition rate for PPCR-SE progressively increased from 41.82% to 77.79%, while the rates for PLF-SE were considerably lower (13.79% - 51.67%). This trend was also observed in MDA-MB-231 cells. The results of the IC_{50} and migration inhibition on the two breast cancer cell lines by the positive control, DOXO, were also recorded and are presented in Table 1.

4. Discussion

In recent years, research into anti-cancer drugs has consistently garnered significant interest. Addressing the limitations of current cancer therapies like surgery, chemotherapy, and radiation, medicinal plants are increasingly viewed as an effective supportive treatment method for cancer. They contain numerous compounds capable of inducing cytotoxicity and inhibiting tumor proliferation and metastasis. *P. polyphylla* var. *chinensis* and *P. longum* were two species belonging to the *Paris* and *Piper* genus, respectively. These two genera are known for possessing compounds with anti-cancer effects, such as steroidal saponins and alkaloids, by activating the apoptosis pathway and showing cytotoxic and antiproliferative effects on cancer cells. Despite numerous studies on the anti-breast cancer effects of *P. longum*, similar to *P. polyphylla* var. *chinensis*, there is still a lack of research on the anti-metastatic capabilities of these two species in breast cancer. Therefore, this study was conducted to provide an initial assessment of the impact of standardized extracts of *P. polyphylla* var. *chinensis* rhizomes (PPCR-SE) and *P. longum* fruits (PLF-SE) on breast cancer metastasis, focusing on migration.

The study demonstrated the cytotoxic potential of both standardized extracts, PPCR-SE and PLF-SE, on the breast cancer cell lines MCF-7 and MDA-MB-231. Consequently, to assess the impact of PPCR-SE and PLF-SE on breast cancer cells without confounding cytotoxic effects, the concentrations selected for further evaluation were kept at non-cytotoxic levels for both cancer and normal cells. Based on the MTT assay results, concentrations of 1.25, 2.5, and 5 µg/mL were chosen for investigating the migration inhibitory properties of PPCR-SE and PLF-SE.

Metastasis is a multi-stage process involving cell proliferation and invasion, migration into blood and lymphatic vessels, cell adhesion, extravasation, and tumor development. Among these stages, cell migration plays a critical role in enabling cancer cells to spread from the primary tumor to various organs throughout the body. In this study, a scratch assay was used to initially evaluate the ability of PPCR-SE and PLF-SE to inhibit *in vitro* cell migration. Previous research indicates that DOXO (0.01-0.25 µg/mL) is capable of inhibiting the migration of osteosarcoma cells (OSCORT) after 24 hours, with the percentage of non-migrating cells reaching over 75% [26]. Furthermore, DOXO (2.5-5 nM) has also been shown to inhibit the migration of HT1080 fibrosarcoma cells by approximately 70% in a 3D model [27]. In another study, DOXO (3 µM) was shown to inhibit the migration of MDA-MB-231

breast cancer cells by nearly 70%, and this effect was more pronounced when combined with rhoifolin [28]. Research on the synergistic migratory inhibitory effects of DOXO with other compounds has also been demonstrated [29, 30]. At the molecular level, DOXO reduces the expression of integrin β1, a component of the extracellular matrix (ECM) that plays a crucial role in cell migration [27]. Therefore, DOXO will be used as a positive control for the scratch assay. The results showed that both PPCR-SE and PLF-SE effectively inhibited the migration of MCF-7 and MDA-MB-231 breast cancer cells in the scratch assay. Notably, at a low concentration of 2.5 μg/mL, PPCR-SE and PLF-SE were observed to inhibit migration in both MCF-7 and MDA-MB-231 cells. Previous studies supported these observations regarding migration inhibition. It has been shown that polyphyllin VI, isolated from *P. polyphylla* Sm., has demonstrated the ability to inhibit metastasis in 4T1 and MDA-MB-231 breast cancer cells [31]. Specifically, at a concentration of 3 μM, polyphyllin VI inhibited the migration and invasion capabilities of 4T1 cells by nearly 50%. Furthermore, MDA-MB-231 cells treated with 3 μM polyphyllin VI also exhibited over 40% inhibition of migration. Besides, it has also demonstrated that ethanol extract from *P. polyphylla* can inhibit the migration of J82 cancer cells at concentrations of 0.8 and 1.6 μg/mL [32]. Saponins isolated from *P. polyphylla*, such as saponin II, polyphyllin I, and polyphyllin II, have also been demonstrated to play a role in inhibiting cancer cell migration. Specifically, *P. polyphylla* saponin II inhibits migration and invasion by promoting autophagy in B16 and B16F10 melanoma cells and by suppressing epithelial-mesenchymal transition (EMT) through the inhibition of signaling pathway proteins [33]. Polyphyllin I has been shown to inhibit EMT by targeting the TGFβ1/Smad2/3 signaling pathway at concentrations of 3 mg/kg and 6 mg/kg [34]. Polyphyllin II is involved in regulating the expression of EMT-related genes by increasing E-cadherin expression and decreasing the expression of N-cadherin, Snail family transcriptional repressor 2, Twist family bHLH transcription factor 1, and matrix metallopeptidase (MMP)-2 and MMP-9 genes [35]. Piperlongumine, an active compound in *P. longum*, has been reported to inhibit the migration of various cancer cell types, including MCF-7 breast cancer cells in a dose-dependent manner (10-40 μM) and glioblastoma cells (LN229 and U87 at 1, 2, 5 μM) by promoting reactive oxygen species (ROS) accumulation, which subsequently upregulates p38/JNK signaling and inhibits NF-κB nuclear translocation [15],[19]. Furthermore, piperine, found in *P. longum*, has also been shown to reduce breast cancer cell migration in 4T1 murine models at concentrations of 70-140 μmol/L by downregulating MMP-9 and MMP-13 expression [36]. MMPs are crucial proteases that play a crucial role in degrading the ECM, a process essential for metastasis into the bloodstream. Research on HT-1080 cells indicated that piperine's inhibition of MMP-9 expression is mediated through the suppression of NF-κB expression [37]. NF-κB is a key promoter of tumor formation, inducing morphological changes and cell migration by upregulating snail expression and inhibiting E-cadherin expression, both of which are critical regulators of tumor invasion and EMT. Therefore, inhibiting NF-κB expression contributes to preventing tumor invasion.

Overall, the cytotoxicity and migration inhibition results for both PPCR-SE and PLF-SE indicate that they have antiproliferative and antimigratory effects on both MCF-7 and MDA-MB-231 breast cancer cell lines. The MCF-7 cell line is a representative of ER+ (luminal A subtype) breast cancer, characterized by an epithelial phenotype and low metastatic potential. In contrast, MDA-MB-231 is a model for triple-negative breast cancer, exhibiting a mesenchymal phenotype and high metastatic potential. These findings suggest that both PPCR-SE and PLF-SE may be effective against both subtypes of breast cancer. Furthermore, the results also show that PPCR-SE appears to be more potent against both cell lines than PLF-SE. The anticancer effects of *P. longum* L. are primarily attributed to piperine and piperlongumine, focusing on mechanisms such as reduced expression of MMP-9 and MMP-13, upregulation of p38/JNK signaling, inhibition of NF-κB nuclear translocation, and ROS accumulation [15],[19],[36]. Meanwhile, *P. polyphylla* is known for its potent anticancer capabilities. *P. polyphylla* is a species characterized by its substantial content of steroidal saponins. A significant proportion of these saponin compounds is implicated in the inhibition of cancer cell metastasis, migration, and invasion, operating through diverse cellular pathways. Steroidal saponins exhibit potent suppressive effects on tumor metastasis and invasion, primarily by inhibiting angiogenesis, targeting the signaling molecules and regulatory

proteins involved in the epithelial-mesenchymal transition (EMT) pathway and matrix metalloproteinases (MMPs). Comprehensive analyses of saponins derived from *P. polyphylla*, specifically polyphyllin I, polyphyllin II, and polyphyllin E, have demonstrated their capacity to impede the expression and enzymatic activity of metalloproteinases MMP-2 and MMP-9 [38]. Polyphyllin I has been shown to downregulate the mRNA and protein expression of VEGF, which plays a crucial role in angiogenesis, a factor that promotes cancer metastasis and invasion [39]. Furthermore, this compound exerts an inhibitory influence on the Wnt/β-catenin and TGFβ1/Smad2/3 signaling pathways, thereby effectuating EMT inactivation [34, 40]. Concomitantly, polyphyllin II, isolated from *P. polyphylla*, has been documented to curtail cell migration by diminishing cofilin activity and downregulating MMP2 and MMP9 expression, specifically targeting the Akt/NF-kB signaling axis [41]. An analogous mechanistic action has been observed with polyphyllin E [42]. Moreover, a notable reduction in the levels of N-cadherin, Snail family transcriptional repressor 2, Twist family bHLH transcription factor 1, MMP2, MMP9, and other EMT-associated proteins has been observed following exposure to Polyphyllin II [35].

5. Conclusion

This study indicated that standardized extracts from *Paris polyphylla* var. *chinensis* rhizomes and *Piper longum* fruits exhibited cytotoxic activity against MCF-7 and MDA-MB-231 breast cancer cells. Additionally, these extracts effectively inhibit cell migration in both cell lines, suggesting their potential as a herbal candidates for alternative breast cancer therapies. However, further research is necessary to thoroughly evaluate the safety of these extracts on normal cells and to elucidate the underlying molecular mechanisms involved in this process.

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