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IN VITRO SCREENING OF HERBAL MEDICINE FOR SYNERGISTIC EFFECT WITH TAMOXIFEN ON MCF-7 BREAST CANCER CELLS

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Summary

***In vitro* Screening of Herbal Medicine for Synergistic Effect with Tamoxifen on MCF-7 Breast Cancer Cells**

Breast cancer stands as the most prevalent form of malignancy and is the second leading cause of death among women. Despite tamoxifen (TAM) continuing to be a primary choice for the prevention and treatment of estrogen-receptor (ER) positive breast tumors, resistance to TAM poses a significant clinical hurdle in breast cancer therapy. Consequently, there has been a rising interest in discovering new agents, particularly those derived from plants, that can augment TAM sensitivity in breast cancer cells. This study aims to explore herbal medicines capable of enhancing the anticancer effects of TAM in ER-positive breast cancer cells, specifically MCF-7 cells, utilizing conventional bioassays such as MTT, colony formation assay, and immunocytochemistry. Among 25 samples tested, *Polygala chinensis* L. extract (PCE) showed strong synergism with TAM against MCF-7 cell survival, evidenced by the combination of Bliss analysis and Chou-Talalay analysis. Additionally, co-treatment of MCF-7 cells with PCE and TAM more significantly suppressed colony formation and the nuclear expression of proliferative marker Ki67 compared to TAM alone. These findings imply that PCE potentiates the anti-proliferative effect of TAM in MCF-7 breast cancer cells and may become a promising candidate in the treatment of breast cancer.

Keywords: *Polygala chinensis* L.; *Synergistic effect; Tamoxifen; Breast cancer.*

1. Introduction

Breast cancer stands as the most prevalent cancer type and is the second leading cause of cancer-related deaths among women. Given the genetic diversity of breast tumor cells, treatment strategies are tailored based on the expression of molecular markers. Among the various types of breast cancer cells, hormone receptor positive tumors represent the primary pathological variant, constituting 70% of all breast cancer cases. First-line adjuvant therapeutic approaches for this variant include anti-estrogen therapies, such as selective estrogen receptor modulators, selective estrogen receptor degraders, and aromatase inhibitors [1].

Despite the recent introduction of newer drug classes, tamoxifen serves as the gold standard hormonal therapy for ER-positive breast cancers, functioning as an antagonist to estrogen in breast tissue. It is also employed as adjuvant therapy in breast cancer to diminish the risk of recurrence [2]. Despite its established efficacy, approximately half of ER+ breast cancers do not respond to tamoxifen. Furthermore, a notable limitation is the diminishing effectiveness of the drug with prolonged usage, as many tumors eventually develop resistance to tamoxifen [3],[4]. Additionally, adverse side effects, such as uterine cancer and thromboembolic disease, have been linked to tamoxifen use. Concerns regarding the toxicity of chemotherapeutic drugs have led to an increased interest in herbal and natural products for cancer treatment [5]. The concurrent use of natural products alongside conventional anticancer drugs is believed to potentially enhance treatment efficacy through additive or synergistic effects. Moreover, this combination approach may hold the promise of mitigating chemotherapy's side effects. However, the purported advantages of natural products in this context lack scientific validation.

In the present study, we aimed to screen herbal medicine that might potentiate the anticancer effect of TAM in ER-positive breast cancer cells (MCF-7 cells) using conventional bioassays such as MTT, colony formation assay, and immunocytochemistry.

2. Materials And Methods

2.1. Cell culture and reagents

MCF-7 breast cancer cell line was a kind gift of Prof. Suresh Awale from the University of Toyama (Japan) and was routinely cultured in DMEM media supplemented with 10% FBS and 1% penicillin/streptomycin. Cells were

maintained in a humidified incubator at 37°C with 5% CO_2. All reagents for cell culture were purchased from Thermo Scientific (Waltham, MA, USA). Apparatuses for cell culture (tissue culture 96-well plates, 10-mm dishes, 35-mm dishes, and 8-well slides) were acquired from Corning Incorporated (Somerville, Massachusetts, USA).

2.2. Collection and preparation of plant extract

Twenty-five herbal plants were selected for screening synergistic effects with tamoxifen on MCF-7 breast cancer cells. Criteria for selecting medicinal plants: medicinal plants that have been documented or belong to a genus with species known to exhibit anti-tumor activity or the ability to inhibit the growth of cancer cells, particularly breast cancer cells.

Samples were collected at the Research Center of Medicinal Plants, Hanoi, in August 2022. Botanical identification was performed by MSc. Lai Viet Hung (Center of Medicinal Material Resources). Voucher specimens were deposited at the Herbarium of Medicinal Material Resources Centre (NIMM), Vietnam. The dried plant samples were coarsely ground and extracted thrice with 70% aqueous ethanol at a ratio of ethanol to samples of 10/1, 8/1, and 8/1, respectively, under reflux for 3 h at a temperature of 60-70°C. The extracts were then combined and filtered and the solvent was evaporated completely under reduced pressure to give total ethanol extracts.

2.3. Cell viability assay

Cell viability assay was carried out as described previously [6]. In brief, cells were seeded into a 96-well plate at the density of 10^4 cells/well. After overnight incubation, cells were treated with tamoxifen (T5648, Sigma-Aldrich, USA) with or without extracts at increasing concentrations for 48 h. Cells were then incubated with MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide, M-2128, Sigma-Aldrich, USA) solution at the final concentration of 0.5 mg/mL for 2 h. Subsequently, media were removed, and the resultant formazan crystals were dissolved in 200 µL dimethyl sulfoxide (Sigma-Aldrich, USA). Finally, the absorbance was measured at 570 nm using a microplate reader (SpectraMax iD3, Molecular Devices, USA). The percentage of viable cells was calculated as follows:

$$\% \text{ Cell viability} = \frac{Absorbance\ of\ the\ tested\ sample}{Absorbance\ of the\ control} \text{ x } 100$$

2.4. Evaluation of synergistic effects

For the evaluation of synergistic effects, Bliss analysis and Chou-Talalay analysis were employed. Bliss score (BC) was calculated as follows:

Bliss score (BC) = S_A x $S_B - S_{AB}$

Where S_A, S_B, and S_{AB} are the percentages of viable cells after treatment with tamoxifen (5 µM), medicinal plants (50 µg/mL), and the combination of tamoxifen and medicinal plants at the indicated concentrations, respectively. BC > 0 indicates synergism; BC = 0 indicates additivity; BC < 0 indicates antagonism [7].

For Chou-Talalay analysis, IC_{50} values (concentrations that reduce the cell viability by 50%) for tamoxifen and PCE were first estimated. Then, the effect of tamoxifen and PCE (alone or in combination) on the cell viability was examined at ~ 0.25×IC_{50}, 0.5×IC_{50}, 1.0×IC_{50}, 1.5×IC_{50}, and 2.0×IC_{50} for each agent (combination ratio was fixed at 1:1 based on IC_{50} values). Combination index (CI) and dose-reduction index (DRI) were obtained from CompuSyn software (USA). CI < 1 indicates synergy; CI = 1 indicates additivity; CI > 1 indicates antagonism [8].

2.5. Colony formation assay

Colony formation assay was performed as previously described [6]. Cells were seeded into 35-mm dishes at a low density (2×10^3 cells/dish). On the next day, cells were treated with tamoxifen and/or PCE for 24 h. After that, treatment media were replaced with fresh media to allow cells to grow. Media were then changed every 3 days. On the 14^{th} day, cells were fixed with 70% ice-cold ethanol, followed by staining with crystal violet (61135, Sigma-Aldrich, USA) solution (0.5%) for 30 min. Cells were properly washed with PBS and the images were acquired using a light microscope (Zeiss, Germany). The number of cell colonies (defined as clusters of at least 50 cells) was counted using Image J software.

2.6. Immunocytochemistry

Immunocytochemistry (ICC) was used to determine the nuclear level of Ki67, a proliferative marker [6]. Cells were seeded into an 8-well glass chamber slides at the density of 4×10^4 cells/well. After treatment with tamoxifen and PCE, cells were fixed with 4% paraformaldehyde for 20 min,

followed by permeabilization with 0.2% Triton X100 in PBS for 10 min and blocking with 3% bovine serum albumin for 45 min. Then, cells were sequentially incubated with a primary antibody against Ki67 (1:200) (ab15580, Abcam) for 16 h at 4ºC and an Alexa-488 conjugated secondary antibody (1:500) (ab150077, Abcam) for 90 min at room temperature. The slides were covered with a coverslip using a mounting reagent containing DAPI (ab104139, Abcam). The images were acquired using a fluorescent microscope (Nikon, Tokyo, Japan). The proportion of Ki67-positive cells was calculated based on Image J software.

2.7. Statistical analysis

Data acquired from at least three independent experiments are expressed as mean ± standard error (SE). Statistical analyses were performed using GraphPad Prism 8.3.0 (San Diego, CA, USA). In particular, IC_{50} values were estimated using a non-linear regression model. Statistical differences among groups were determined by one-way ANOVA in combination with post-hoc Turkey's test or t-test (if only 2 groups were presented). In addition, the Chou-Talalay method along with CompuSyn software were employed to specifically estimate the combination index (CI) and dose-reduction index (DRI).

3. Results

3.1. Bliss analysis of synergism between TAM and selected herbal medicines

Among 25 samples tested, only *Polygala chinensis* exerted synergistic effect with tamoxifen, evidenced by the Bliss score calculated more than 0 (Table 1). As such, we next evaluated the synergistic effect of the combination between tamoxifen and *P. chinensis* extract (PCE) on MCF-7 cells by using the Chou-Talalay method and proliferation assays, including colony formation and immunocytochemistry assays.

Table 1. Bliss score of tamoxifen combined with 25 selected herbal plants

No.	Samples	Part use	Bliss score (Mean)	95% CI
1	*Carica papaya* L.	Leaf	-0.117	(-0.163) – (-0.071)
2	*Paris polyphylla* Smith	Rhizome	-0.016	(-0.055) – 0.022
3	*Polygala chinensis* L.	Whole plant	**0.267**	**0.176 – 0.377**
4	*Polygala tenuiflora* Willd.	Whole plant	-0.159	(-0.200)– (-0.119)
5	*Cyperus rotundus* L.	Whole plant	-0.173	(-0.187)– (-0.161)
6	*Salvia miltiorrhiza* Bunge	Root	-0.018	(-0.023)– (-0.012)
7	*Morinda officinalis* F.C.How	Root	-0.108	(-0.136)– (-0.079)
8	*Corydalis balansae* Prain	Root	-0.118	(-0.137)– (-0.098)
9	*Phyllanthus amarus* Schum. and Thonn	Whole plant	-0.159	(-0.249)– (-0.069)
10	*Prunella vulgaris* L.	Aerial part	-0.147	(-0.181)– (-0.113)
11	*Atractylodes macrocephala* Koidz.	Rhizome	-0.117	(-0.161)– (-0.073)
12	*Acorus gramineus* L.	Rhizome	-0.183	(-0.262)– (-0.105)
13	*Morus alba* L.	Leaf and small branch	-0.187	(-0.250)– (-0.123)
14	*Dioscorea persimilis* Prain & Burk.	Rhizome	-0.183	(-0.263)– (-0.102)
15	*Mimosa pudica* L.	Root	-0.155	(-0.214)– (-0.096)
16	*Uncaria sp.*	Bark	-0.160	(-0.184)– (-0.137)
17	*Cymbopogon citratus* (DC.) Stapf	Whole plant	-0.161	(-0.238)– (-0.085)
18	*Chrysanthemum indicum* L.	Flower	-0.122	(-0.213)– (-0.032)
19	*Crinum latifolium* L.	Leaf	-0.032	(-0.196) – 0.132
20	*Croton tonkinensis* Gagnep.	Leaf and small branch	-0.021	(-0.058) – 0.099
21	*Clinacanthus nutans* (Burm.f.) Lindau.	Whole plant	-0.094	(-0.153) – 0.035
22	*Celastrus hindsii* Benth.	Whole plant	-0.072	(-0.314) – 0.170
23	*Artemisia annua* L.	Aerial part	-0.052	(-0.173) – 0.069
24	*Arctium lappa* L.	Leaf and root	-0.036	(-0.182) – 0.109
25	*Scutellaria barbata* Wall	Whole plant	-0.153	(-0.293)– (-0.013)

3.2. Suppressive effect of PCE on the viability of MCF-7 breast cancer cells

We next evaluated the effect of PCE on MCF-7 cell viability using MTT assay to estimate IC_{50} value. Our results showed that PCE decreased the MCF-7 cell survival in a concentration-dependent manner (Figs. 1A and 1B). The non-linear regression analysis suggested a sigmoidal dose-response model for PCE which was essentially similar to TAM. The IC_{50} of PCE was 28.43 µg/mL (95% CI: 27.20 – 29.73), demonstrating a relatively potent anti-breast cancer activity of PCE.

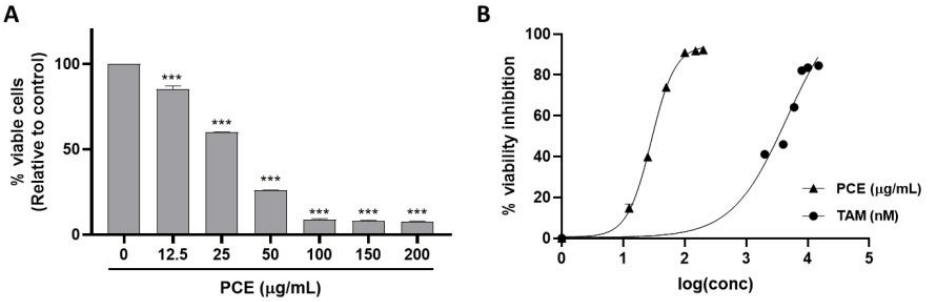

Fig. 1. Effect of PCE on the cell viability of MCF-7 breast cancer cells

(A) MCF-7 cells were treated with PCE at indicated concentrations for 48 h. Cell viability was examined by MTT assay. (B) The concentration-response curves of PCE and TAM were established based on a non-regression regression analysis by GraphPad software. *** $p < 0.001$ compared to the control cells; TAM: Tamoxifen; PCE: crude ethanol extract of *P. chinensis*.

3.3. Chou-Talalay analysis of synergism between TAM and PCE

Based on the estimated IC_{50} values of PCE and TAM (28.43 µg/mL and 4.56 µM, respectively), we designed a series of concentrations for PCE alone, TAM alone, and PCE-TAM combination (with fixed ratios of 1:1) ranging from approximately 0.25 to 2.0×IC_{50} value for each agent. As shown in Fig. 2A, the combination of PCE and TAM decreased MCF-7 cell survival more significantly than PCE or TAM alone at all the tested concentrations. Importantly, Chou-Talalay analysis clearly indicated a synergistic effect of PCE and TAM since the combination index (CI) < 1 was observed at almost effect level (Fig. 2B). It is also worth noting that the synergistic effect of PCE and TAM increased in a dose-dependent manner.

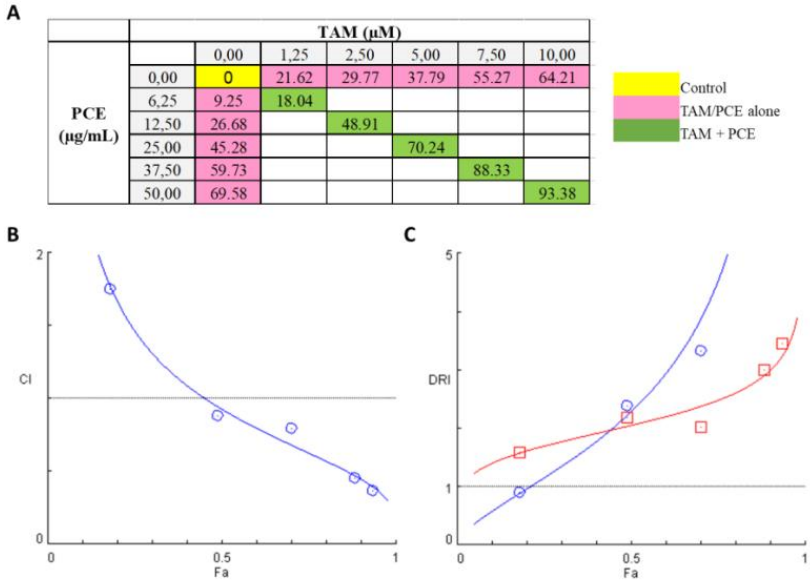

Fig. 2. The synergistic effects of TAM and PCE on the suppression of MCF-7 cell viability. (A) Effects of TAM and PCE on MCF-7 cell viability were examined at the concentrations ranging from ~ 0.25 to ~ 2.0 × IC_{50} value of each agent. The effect of TAM in combination with PCE was also evaluated at the fixed combination ratios of 1:1. The percentage of cell survival inhibition was shown. (B and C) Results obtained from Chou-Talalay analysis using CompuSyn software included the Fa-CI plot (B) and Fa-DRI plot (C). Fa: effect level; CI: combination index; DRI: dose-reduction index; TAM: Tamoxifen; PCE: crude ethanol extract of *P. chinensis*.

As a result, co-treatment of TAM and PCE led to a substantial reduction in the cancer-cell inhibitory concentration of each agent. In particular, dose-reduction index (DRI) for TAM and PCE at concentrations of approximately 0.25, 0.5, 1.0, 1.5, 2.0 × IC_{50} was 0.89, 2.38, 3.32, 8.31, 12.74 and 1.59, 2.18, 2.02, 2.99, 3.45, respectively (Fig. 2C). These findings imply that co-treatment with PCE may help reduce dose of TAM required for suppressing breast cancer cell growth.

3.3. PCE potentiates the suppressive effect of TAM on cell proliferation in MCF7 breast cancer cells

Having established that PCE enhances TAM's ability to suppress MCF-7 cell viability, we further validated this synergistic effect using additional methods, including the colony formation assay and ICC.

The colony formation assay evaluates the long-term effects of a treatment on a single cell's ability to proliferate into a large colony through multiple cell divisions. Given the high sensitivity of this assay, the lowest tested concentrations of TAM (1.25 μM) and PCE (6.25 μg/mL) were utilized. Notably, while TAM or PCE alone caused a slight reduction in colony numbers, their combined use significantly inhibited colony formation (Fig. 3A and 3B). These findings suggest that even brief exposure to the PCE/TAM combination can exert a prolonged suppressive effect on the proliferation of MCF-7 breast cancer cells.

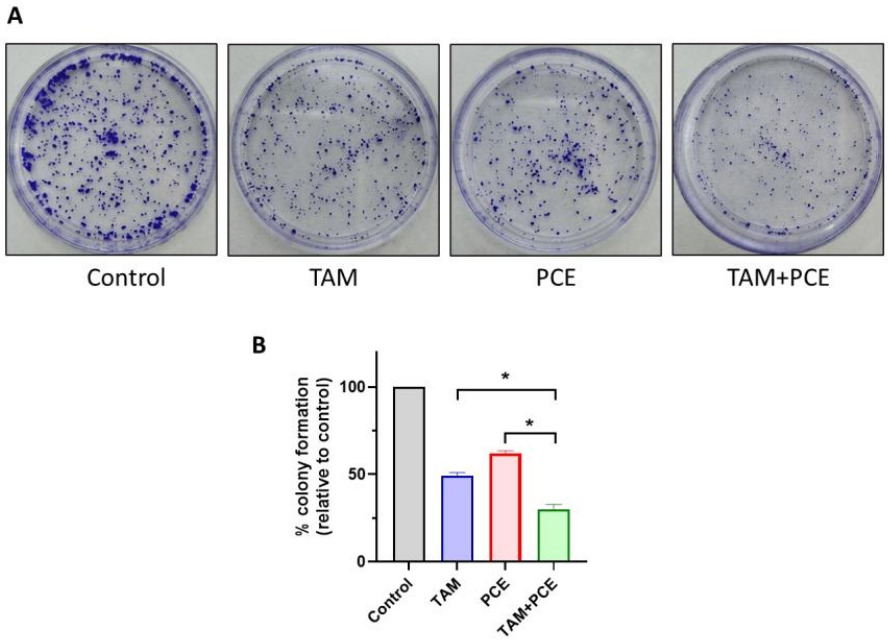

Fig. 3. The synergistic effect of TAM and PCE on the suppression of colony formation
Cells were treated with TAM (1.25 μM) and/or PCE (6.25 μg/mL) for 24 h, followed by further culturing in full DMEM media without TAM/PCE for 13 days. Cells were then stained with crystal violet. (A) Representative images from three independent experiments were shown. The relative number of colonies in each group was calculated based on Image J software. *$p < 0.05$ compared to the control cells; TAM: Tamoxifen; PCE: crude ethanol extract of *P. chinensis*.

Ki67 is a well-established marker of cell proliferation, primarily localized within cellular nuclei [9]. Its nuclear expression is widely utilized to assess the proliferative activity of cancer cells. In this study, immunocytochemistry (ICC) analysis revealed that approximately 70% of MCF-7 cells were Ki67-positive under control conditions. However, this proportion significantly decreased following treatment with TAM. Interestingly, cotreatment with PCE further reduced nuclear Ki67 expression by over 40% compared to TAM alone (Fig. 4A and 4B). Collectively, these findings indicate that the combination of PCE and TAM exerts a synergistic inhibitory effect on MCF-7 cell proliferation.

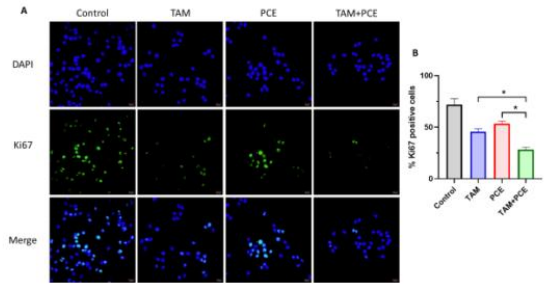

Fig. 4. The synergistic effect of TAM and PCE on the suppression of nuclear Ki67 expression
Cells were treated with TAM (5 μM) and/or PCE (25 μg/mL) for 24 h, followed by immunostaining with Alexa fluor 488 conjugated antibodies (green). Nuclei were counterstained with DAPI (blue). Scale bar: 20 μm. (A) Representative images for each group were shown. (B) Proportion of Ki67 positive cells was calculated from three independent experiments.
* $p < 0.05$ compared to the control cells; TAM: Tamoxifen; PCE: crude ethanol extract of *P. chinensis*.

4. Discussion

In recent decades, significant progress has been made in cancer treatment, leading to the cure of many cancer types. However, challenges such as adverse drug reactions and drug resistance persist in cancer therapy. Combination therapy, involving the simultaneous administration of conventional anti-cancer drugs with targeted agents or other supportive treatments, is recognized as one of the most effective strategies to address these challenges. It has the potential to enhance the anti-proliferative activity on cancer cells, broaden the spectrum of anti-cancer therapy to combat tumor cell heterogeneity, and prevent or delay the development of drug resistance. Additionally, in some cases, combining drugs can reduce the therapeutic dose of cytotoxic agents, thereby minimizing severe adverse reactions in patients [10]. Our study aimed to screen herbal plants with synergistic effects when combined with TAM, a key agent in the adjuvant treatment of breast cancer, by applying the Bliss analysis and Chou-Talalay analysis. These two methods are based on fundamentally opposing principles, effectively covering all possible drug interaction scenarios. Particularly, the Bliss analysis is based on the principle that the two drugs act through different mechanisms of action, targeting distinct pathways, whereas the Chou-Talalay analysis is based on the Loewe additivity model which assumes that the two drugs act through similar mechanisms, targeting the same pathway [11]. When the precise mechanisms of action for the drugs under study are unknown—or even when they are known but cannot entirely rule out unknown differences in their interactions—it becomes essential to combine both models. This dual approach provides a comprehensive framework for evaluating drug synergy [12]. Therefore, in the present study, we employed the Bliss analysis first to quickly determine whether there is a synergistic interaction between TAM and herbal plants, followed by the Chou-Talalay analysis to re-evaluate these interactions at various concentration combinations. This step verifies the synergistic effect and quantifies the doses that produce the highest level of synergy, contributing to further studies on dose reduction for optimal efficacy. This methodology ensures a higher level of accuracy and reliability compared to using a single model. Herein, we determined that *P. chinensis* extract (PCE) exhibits a significant synergistic interaction with TAM in MCF-7 breast cancer cells. Notably, the calculated Combination Index (CI) was as low as 0.368, strongly indicating synergy between PCE and TAM. Furthermore, the Chou-Talalay method enabled precise quantification of dose reduction. For instance, achieving a 90% reduction in viable cells required a 12-fold lower TAM dose when combined with PCE.

Ki-67 is a nuclear protein associated with cell proliferation. This protein is present during the active phases of the cell cycle (G1, S, G2, and M phases) but is absent in the quiescent G0 phase. The expression level of Ki-67, or the Ki-67 index, is used as a characteristic marker to assess the proliferative activity of cells [13]. In this study, the co-administration of PCE and TAM enhances the suppression of MCF-7 cell proliferation through a synergistic interaction. Furthermore, while the treatment with TAM and PCE modestly decreased the number of colonies, co-administration of the two agents resulted in dramatical inhibition of colony formation. It is noteworthy that unlike the MTT assay and the assessment of Ki-67 expression levels using immunocytochemistry, in which cell viability/proliferation is evaluated over a short period (approximately 48 hours), the colony formation assay allows for the assessment of drug effects over a prolonged duration (approximately two weeks). This further confirms the synergistic effect of tamoxifen and PCE in inhibiting MCF-7 cell proliferation.

P. chinensis has been traditionally used as a remedy for snake bites, coughs, and bronchitis [14]. Previous studies showed that *P. chinensis* exerts antioxidant, antidiabetic, antihyperlipidaemic, and hepatoprotective effects [14],[15],[16],[17]. However, modern studies exploring its potential role in cancer therapy are still limited. To date, only one study has examined the anticancer effect of this plant. That research demonstrated that *P. chinensis* effectively reduces tumor volume, decreases cancer cell viability, and prolongs the lifespan of mice in Dalton's lymphoma ascites tumor models [18]. In this study, we evaluated *P. chinensis* extract (PCE) for the first time as a potential synergistic partner to TAM. Our findings indicate that *P. chinensis* has the potential to enhance the therapeutic efficacy of TAM while reducing its required dosage and minimizing associated side effects, making it a promising candidate for breast cancer treatment optimization.

5. Conclusions

This study has demonstrated for the first time that the crude extract of *Polygala chinensis* possesses a synergistic effect with tamoxifen on the suppression of breast cancer cell growth. Future studies will be needed to unveil the possible role of *P. chinensis* in tamoxifen-resistant breast cancer, the mechanism underlying how *P. chinensis* enhances the tamoxifen sensitivity of breast cancer cells, and the chemical composition-biological activity relationship.

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