



Peanut hairy root culture extract suppresses migration and invasion of highly metastatic cholangiocarcinoma cells

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ABSTRACT

Cholangiocarcinoma (CCA) is a highly aggressive malignancy with poor prognosis, partly because of late-stage diagnosis, therapeutic resistance, and metastatic progression. This study investigated the anticancer and anti-migratory, and anti-invasive effects of peanut hairy root culture crude extract (PCE), a source of prenylated stilbenoids including trans-arachidin-1 and trans-arachidin-3. The effects of PCE on cell viability were initially screened in KKU-213B, A549, MCF-7, and KB cancer cells. PCE reduced cell viability in a concentration-dependent manner, with KKU-213B cells showing the greatest sensitivity among the tested cell lines. The effects of PCE on cell viability were subsequently evaluated in a panel of CCA cell lines comprising KKU-213A, KKU-213B, KKU-055, and the highly metastatic KKU-213AL5 subline. KKU-213AL5 cells exhibited the greatest sensitivity and were therefore selected for further investigation. At non-cytotoxic concentrations, PCE significantly suppressed KKU-213AL5 cell migration and invasion, as determined by wound-healing and Matrigel-coated Transwell assays, respectively. Gelatin zymography showed that PCE did not significantly alter MMP-2 or MMP-9 activity. Collectively, these findings demonstrate that PCE inhibits the cell viability, migration, and invasion of highly metastatic CCA cells, supporting further investigation of its active constituents and underlying molecular mechanism.

Keywords: cholangiocarcinoma; peanut hairy root culture extract; prenylated stilbenoids; cell migration; cell invasion

1. Introduction

Cholangiocarcinoma (CCA), or bile duct cancer, is a highly aggressive malignancy characterized by early invasion, resistance to treatment, and poor clinical outcomes. CCA is an important health challenge worldwide, with substantial geographic variation in incidence and risk factors. In Thailand, chronic infection with *Opisthorchis viverrini* is an established risk factor in endemic areas; however, the etiology of CCA is multifactorial and is not limited to liver fluke infection.^{1,2} The disease is frequently diagnosed at an advanced stage, when curative surgical resection is no longer feasible. Moreover, the effectiveness of systemic therapy remains limited, particularly in patients with aggressive or metastatic disease. Therefore, the identification of novel bioactive agents capable of suppressing CCA cell growth and invasive behavior remains an important area of investigation.

Stilbenoids are a group of plant-derived polyphenolic compounds with diverse biological activities, including antioxidant, anti-inflammatory, and anticancer effects.³ Representative stilbenoids include trans-resveratrol, trans-arachidin-1, trans-arachidin-3, pterostilbene, and various oligostilbenes. Among these compounds, trans-resveratrol has been extensively investigated for its ability to induce apoptosis, inhibit cancer cell migration and invasion, and enhance cellular responses to chemotherapeutic agents.⁴⁻⁶ However, its potential clinical application is limited by rapid phase II metabolism and low systemic bioavailability.⁷ Prenylated resveratrol derivatives, particularly trans-arachidin-1 and trans-arachidin-3, exhibit greater lipophilicity and more favorable glucuronidation profiles than non-prenylated resveratrol, suggesting that they may possess improved biological and pharmacological properties.^{7,8}

Peanut hairy root culture, generated through *Agrobacterium rhizogenes*-mediated transformation provides a sustainable biotechnological platform for production of high-value stilbenoids. Peanut hairy root

culture extract (PCE) is rich in prenylated stilbenoids, particularly trans-arachidin-1 and trans-arachidin-3. Previous studies have demonstrated that peanut hairy root-derived stilbenoid possess antioxidant, anti-inflammatory, DNA-protective, and anti-proliferative activities.⁹⁻¹² More recently, PCE was shown to modulate the anticancer activity of gemcitabine in a cell line-dependent manner and to suppress tumor growth in a CCA xenograft model.¹³ In addition, stilbenoids such as resveratrol and related derivatives have been reported to inhibit cancer cell migration and invasion by modulating signaling pathways and molecules associated with metastatic progression.⁴⁻⁶ These findings led us to hypothesize that the prenylated stilbenoid-rich PCE might also suppress the migratory and invasive behavior of CCA cells. However, its effects on highly metastatic CCA cells remain insufficiently characterized.

Therefore, the present study investigated the effects of PCE on cell viability, migration, and invasion in CCA cell models. The effects of PCE on cell viability were initially screened across different cancer cell lines and subsequently evaluated in a panel of CCA cell lines. Based on the cytotoxic screening, a CCA cell line was selected for further evaluation of the effects of non-cytotoxic PCE concentrations on cell migration, invasion, and the gelatinolytic activities of matrix metalloproteinase-2 and matrix metalloproteinase-9.

2. Materials and Methods

2.1 Cell lines and culture conditions

The human cancer cell lines used in this study included A549 lung adenocarcinoma cells, MCF-7 breast adenocarcinoma cells, and KB oral epidermoid carcinoma cells, which were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). The CCA cell panel comprised KKKU-055, a poorly differentiated adenocarcinoma cell line; KKKU-213B, a moderately to poorly differentiated adenocarcinoma cell line;

KKU-213A, a well-differentiated adenocarcinoma cell line; and KKU-213AL5, a highly metastatic CCA subline. All CCA cell lines were obtained from the Japanese Collection of Research Bioresources (JCRB Cell Bank; Osaka, Japan).

All cell lines were cultured in Dulbecco's modified Eagle medium (DMEM; Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco, Thermo Fisher Scientific) and 1% (v/v) penicillin–streptomycin solution (Gibco, Thermo Fisher Scientific), providing final concentrations of 100 U/mL penicillin and 100 µg/mL streptomycin. The cells were maintained at 37°C in a humidified incubator containing 5% CO₂.

2.2 Preparation and characterization of PCE

Peanut hairy root cultures were elicited with paraquat (PQ; Sigma-Aldrich, St. Louis, MO, USA), methyl jasmonate (MeJA; Cayman Chemical Michigan, USA) and methyl-β-cyclodextrin (CD; Sigma-Aldrich, St. Louis, MO, USA) for 72 hours according to a previously established elicitation protocol.¹⁴ After elicitation, the culture medium was collected and extracted with ethyl acetate. The organic phase was evaporated to dryness at 40°C using a rotary evaporator. A single production batch of PCE was used throughout the study to minimize batch-to-batch variation. The chemical profile of PCE was characterized by high-performance liquid chromatography (HPLC) with detection at 340 nm. The major prenylated stilbenoids, trans-arachidin-1 and trans-arachidin-3, were identified and quantified according to previously established methods.^{9,16} HPLC analysis was performed in three independent analytical runs to confirm the reproducibility of the chemical profile. The dried extract was stored at –20°C until use according to previously established storage conditions for maintaining

the chemical stability and biological activity of the major stilbenoids.¹⁷

2.3 Cell viability assay

Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT) assay. Briefly, cells were seeded in 96-well plates at a density of 1.5×10^4 cells/well and incubated for 24 hours. The cells were then treated with PCE at concentrations ranging from 0 to 800 µg/mL for 48 hours. Cells treated with the corresponding concentration of dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) were used as the vehicle control. Following treatment, the culture medium was replaced with fresh medium containing 0.5 mg/mL MTT (Bio Basic Inc., Markham, ON, Canada). After incubation, the resulting formazan crystals were dissolved in DMSO, and the absorbance was measured at 540 nm using a microplate reader (Labsystems, Helsinki, Finland). Cell viability was expressed as a percentage relative to the vehicle control. The half-maximal inhibitory concentration (IC₅₀) values were calculated using GraphPad Prism version 9.0.

2.4 Wound healing assay

Cell migration was evaluated using a scratch wound-healing assay. Cells were seeded in 6-well plates at a density of 3×10^5 cells/well and cultured for 24 hours to form a confluent monolayer. A linear scratch was created across the cell monolayer, after which the wells were washed with phosphate-buffered saline (PBS; AMRESCO, Solon, OH, USA) to remove detached cells. The cells were then treated with PCE at concentrations of 0, 25, 50, and 100 µg/mL. Images of the scratched areas were captured at 0, 6, 12, 18, and 24 hour using an inverted microscope (Leica Microsystems, Wetzlar, Germany) at 10× magnification. Wound closure was quantified by measuring changes in the wound area or wound distance relative to the value at 0 hour.

2.5 Transwell invasion assay

Cell invasion was evaluated using a Matrigel-coated Transwell assay. Briefly, Transwell inserts with an 8.0- μ m pore size (Corning, Corning, NY, USA) were coated with Matrigel basement membrane matrix (Corning; Cat. No. 356234) at a concentration of 0.4 mg/mL. Cells were treated with PCE at the indicated concentrations for 24 hours, harvested by trypsinization, and resuspended in serum-free DMEM. A total of 2.0×10^4 cells were seeded into each upper chamber, while the lower chambers were filled with 500 μ L of DMEM supplemented with 10% (v/v) FBS as a chemoattractant. After incubation for 16 hours, non-invading cells remaining on the upper surface of the membrane were removed. Cells that had invaded through the Matrigel and reached the lower surface of the membrane were fixed with 4% (v/v) paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA; Cat. No. 441244) and stained with 0.5% (w/v) crystal violet (PanReac AppliChem, Barcelona, Spain). The invading cells were counted in five randomly selected microscopic fields per insert at 100 $\times$ magnification. Cell invasion was expressed as a percentage relative to the untreated control.

A sequential screening strategy was used to evaluate the effects of PCE on cell viability. In the initial screening, KKU-213B was included as a representative CCA cell line together with A549, MCF-7, and KB cells to compare the response to PCE among cancer cells of different tissue origins. The screening was subsequently expanded to a panel of CCA cell lines, including KKU-213A, KKU-213B, KKU-055, and KKU-213AL5, to compare their relative sensitivity to PCE and to select a CCA cell model for subsequent migration and invasion assays.

Previous concentration–response analyses demonstrated that the 48-hour IC_{50} values of PCE were 449.4 ± 29.4 , 643.2 ± 24.2 , 498.5 ± 25.7 , and 403.7 ± 14.8 μ g/mL in KKU-213A, KKU-213B, KKU-055, and KKU-213AL5 cells, respectively.¹² In the present

study, 200 μ g/mL PCE for 48 hours was selected as a confirmatory screening condition because this concentration was below the previously determined IC_{50} values for all four CCA cell lines and allowed comparison of their relative responses without extensive cytotoxicity.

2.6 Gelatin zymography

Cells were seeded at a density of 2×10^5 cells per 60-mm culture dish. When the cells reached approximately 70–80% confluence, the culture medium was removed, and the cells were washed twice with PBS. The cells were then treated with the indicated concentrations of PCE in serum-free medium for 24 hours. The conditioned media were collected and centrifuged at 4,000 rpm for 30 min to remove cells and cellular debris. The resulting supernatants were concentrated 10-fold using centrifugal filter units (Merck Millipore, Burlington, MA, USA). The gelatinolytic activities of MMP-2 and MMP-9 in the conditioned media were subsequently evaluated by gelatin zymography.

Briefly, 20 μ L of each sample was separated on 7.5% (v/v) polyacrylamide gels (AMRESCO, Solon, OH, USA) copolymerized with 0.1% (w/v) gelatin (AMRESCO). Electrophoresis was performed at 200 V for 1 hour. The gels were then washed twice with renaturing buffer containing 2.5% (v/v) Triton X-100 (Loba Chemie Pvt. Ltd., Mumbai, India) at room temperature to remove sodium dodecyl sulfate (AMRESCO). Subsequently, the gels were incubated in developing buffer containing 50 mM Tris-HCl, pH 7.5, 10 mM $CaCl_2$, 1 mM $ZnCl_2$ (Ajax Finechem, Seven Hills, NSW, Australia), and 1% (v/v) Triton X-100 at 37°C for 16–18 hours. After incubation, the gels were stained with Coomassie Brilliant Blue (Fluka, Buchs, Switzerland) and destained until clear gelatinolytic bands appeared against the stained background. Bands corresponding to MMP-2 and MMP-9 were quantified by densitometric analysis.

2.7 Statistical analysis

All quantitative data are presented as the mean $\pm$ standard deviation (SD) from at least three independent experiments. Statistical differences between groups were analyzed using Student's *t*-test. Comparisons among multiple groups were performed using one-way analysis of variance (ANOVA) followed by Dunnett's or Sidak's multiple comparisons tests, as appropriate. All statistical analyses were performed using GraphPad Prism software version 9.0 (GraphPad Software, San Diego, CA, USA). A *p*-value of less than 0.05 was considered statistically significant. Statistical significance is indicated as follows: **p* < 0.05, ***p* < 0.01, and ****p* < 0.001.

3. Results

3.1 Preparation and characterization of peanut hairy root crude extract

PCE was prepared from a single production batch and characterized by HPLC. The chromatographic profile consistently showed trans-arachidin-1 and trans-arachidin-3 as the major prenylated stilbenoids across three independent analytical runs. Their contents were 301.8 and 96.9 $\mu\text{mol/g}$ extract, respectively (Fig. 1).

3.2 Effects of PCE on cell viability of cancer cell lines

The effect of PCE on cell viability was initially evaluated in KKU-213B, A549, MCF-7, and KB cancer cells (Fig. 2A). PCE reduced cell viability in a concentration-dependent manner in all tested cell lines. KKU-213B cells were the most sensitive to PCE, whereas the viability of A549, MCF-7, and KB cells remained above 60% at the highest concentration tested, 800 $\mu\text{g/mL}$. In contrast, the viability of KKU-213B cells decreased to approximately 5% following treatment with 800 $\mu\text{g/mL}$ PCE.

Previous concentration–response analyses demonstrated that the 48-hour IC_{50} values of PCE were 449.4 ± 29.4 , 643.2 ± 24.2 , 498.5 ± 25.7 , and 403.7 ± 14.8 $\mu\text{g/mL}$ in KKU-213A, KKU-213B, KKU-055, and KKU-213AL5 cells, respectively.¹² KKU-213AL5

therefore exhibited the lowest IC_{50} value among the four CCA cell lines. In the present study, the relative responses of these cell lines were further evaluated using 200 $\mu\text{g/mL}$ PCE for 48 hours as a confirmatory screening condition. Consistent with the previous concentration–response data, KKU-213AL5 cells showed the greatest reduction in cell viability under this condition (Fig. 2B). Based on the previously reported IC_{50} values and the present confirmatory screening result, KKU-213AL5 cells were selected for subsequent migration and invasion experiments.

3.3 Determination of non-cytotoxic PCE concentrations

To determine suitable concentrations for the migration and invasion assays, the viability of KKU-213AL5 cells was evaluated following treatment with 0, 25, 50, 100, or 200 $\mu\text{g/mL}$ PCE for 48 hours. Treatment with 200 $\mu\text{g/mL}$ PCE significantly reduced cell viability, whereas concentrations of 25, 50, and 100 $\mu\text{g/mL}$ did not cause a significant reduction compared with the control group (Fig. 3). Therefore, PCE concentrations of 25–100 $\mu\text{g/mL}$ were selected for subsequent migration and invasion assays.

3.4 Inhibitory effect of PCE on KKU-213AL5 cell migration

The effect of PCE on the migration of KKU-213AL5 cells was evaluated using a wound-healing assay (Fig. 4). Cells were treated with non-cytotoxic concentrations of PCE and monitored at 0, 6, 12, 18, and 24 hour. In the untreated control group, the wound area progressively narrowed and was nearly closed by 24 hour. In contrast, PCE-treated cells exhibited a persistent wound gap, indicating reduced migratory ability (Fig. 4A). This inhibitory effect was more pronounced at 50 and 100 $\mu\text{g/mL}$. Quantitative analysis showed that PCE significantly delayed wound closure in a concentration- and time-dependent manner (Fig. 4B). Because the selected concentrations did not significantly reduce cell viability

under the conditions of the MTT assay, the delayed wound closure was unlikely to result from overt cytotoxicity. However, cell proliferation was not independently controlled

in the wound-healing assay; therefore, a contribution from altered proliferation cannot be excluded.

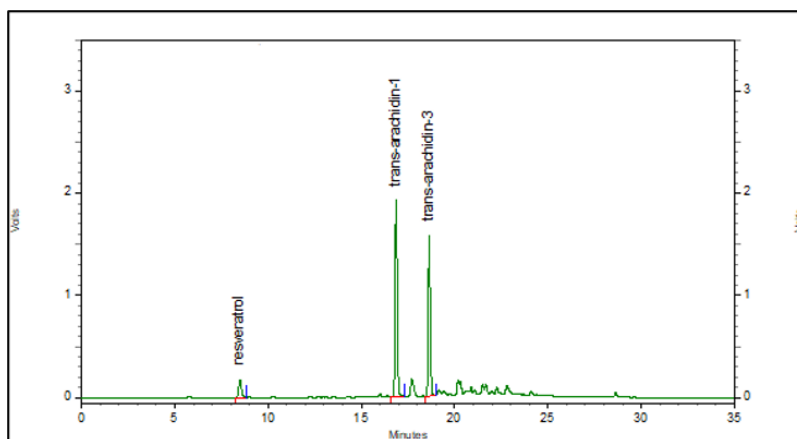


Fig 1. Representative HPLC chromatogram of PCE showing the major prenylated stilbenoids trans-arachidin-1 and trans-arachidin-3. Peanut hairy root cultures were elicited with PQ, MeJA and CD for 72 hours.

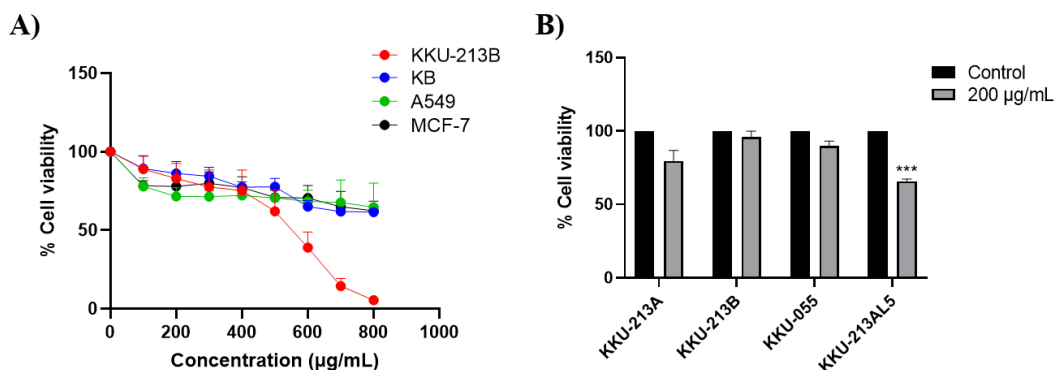


Fig 2. Effects of PCE on the viability of different cancer cell lines. (A) Cell viability of KKKU-213B, A549, MCF-7, and KB cells following treatment with PCE at concentrations of 0–800 µg/mL for 48 hours. (B) Cell viability of KKKU-213A, KKKU-213B, KKKU-055, and KKKU-213AL5 cholangiocarcinoma cells following treatment with 200 µg/mL PCE for 48 hours. Data are presented as the mean $\pm$ SD of at least three independent experiments. *** p < 0.001 vs. control group.

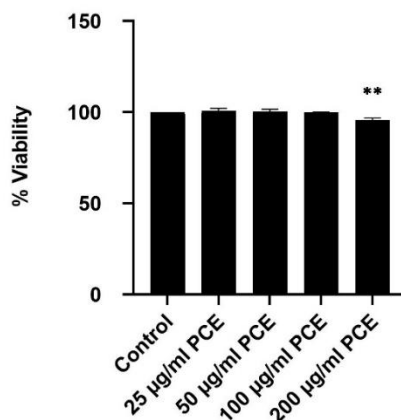


Fig 3. Effect of PCE on the viability of KKKU-213AL5 cells. Cells were treated with various PCE at concentrations of 0–200 µg/mL for 48 hours, and cell viability was determined using the MTT assay. Data are presented as mean $\pm$ SD of at least three independent experiments. ** $p < 0.01$ vs. control group.

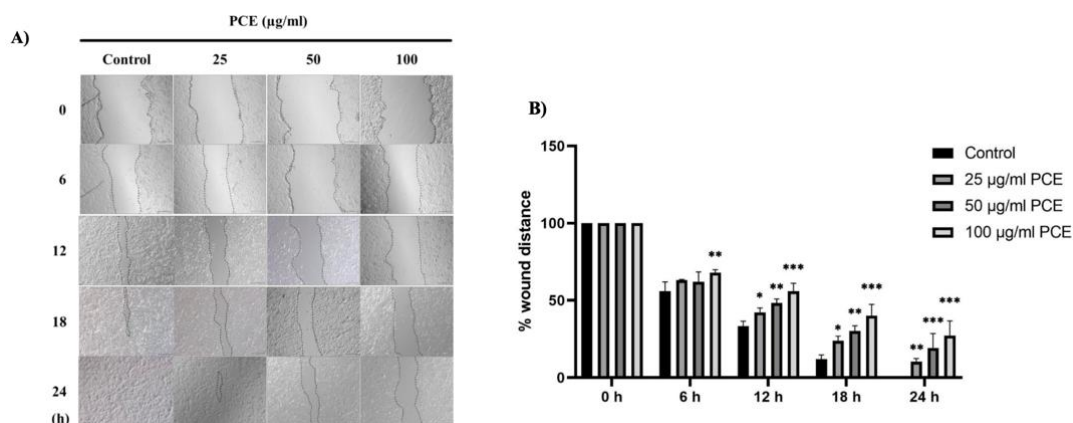


Fig 4. Effect of PCE on wound closure in KKKU-213AL5 cells. (A) Representative images of the wound-healing assay at 0, 6, 12, 18, and 24 hour after treatment with non-cytotoxic concentrations of PCE. (B) Quantitative analysis of the remaining wound distance expressed as a percentage relative to the wound distance at 0 hour. All data are presented as mean $\pm$ SD of at least three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. the corresponding control group at each time point.

3.5 Inhibitory effect of PCE on KKKU-213AL5 cell invasion

The effect of PCE on KKKU-213AL5 cell invasion was evaluated using a Matrigel-coated Transwell assay. KKKU-213AL5 cells were treated with non-cytotoxic concentrations of PCE for 24 hours and subsequently subjected to the invasion assay. Representative images showed a reduction in the number of invading cells in PCE-treated groups

compared with the untreated group (Fig. 5A). Quantitative analysis demonstrated that PCE significantly inhibited KKKU-213AL5 cell invasion in a concentration-dependent manner. The percentages of invading cells were $85.3 \pm 2.9\%$, $47.4 \pm 5.6\%$, and $10.8 \pm 3.1\%$ following treatment with 25, 50, and 100 µg/mL PCE, respectively (Fig. 5B). These results indicate that PCE suppresses the invasive ability of KKKU-213AL5 cells.

3.6 Effect of PCE on MMP-2 and MMP-9 activities in KKU-213AL5 cells

The effects of PCE on the gelatinolytic activities of MMP-2 and MMP-9 were evaluated by gelatin zymography. Conditioned media from KKU-213AL5 cells treated with non-cytotoxic concentrations of PCE in serum-free medium were collected and analyzed. Gelatinolytic bands corresponding to MMP-2 and MMP-9 were detected at approximately 72 and 92 kDa, respectively (Fig. 6A). Densitometric analysis showed that PCE treatment did not significantly alter the activities of either MMP-2 or MMP-9 compared with the untreated control group (Fig. 6B and C). These findings indicate that the inhibitory effect of PCE on KKU-213AL5 cell invasion was not accompanied by changes in MMP-2 or MMP-9 gelatinolytic activity.

4. Discussion

The present study demonstrated that PCE reduced the viability of several cancer cell lines and suppressed the migration and invasion of highly metastatic KKU-213AL5 CCA cells at non-cytotoxic concentrations. Among the tested CCA cell lines, KKU-213AL5 cells exhibited the greatest reduction in viability following PCE treatment. In addition, the inhibitory effect of PCE on cell invasion was not accompanied by significant changes in the gelatinolytic activities of MMP-2 or MMP-9. These findings extend current knowledge of the anticancer properties of peanut-derived stilbenoids by demonstrating their effects on the migratory and invasive behavior of aggressive CCA cells.

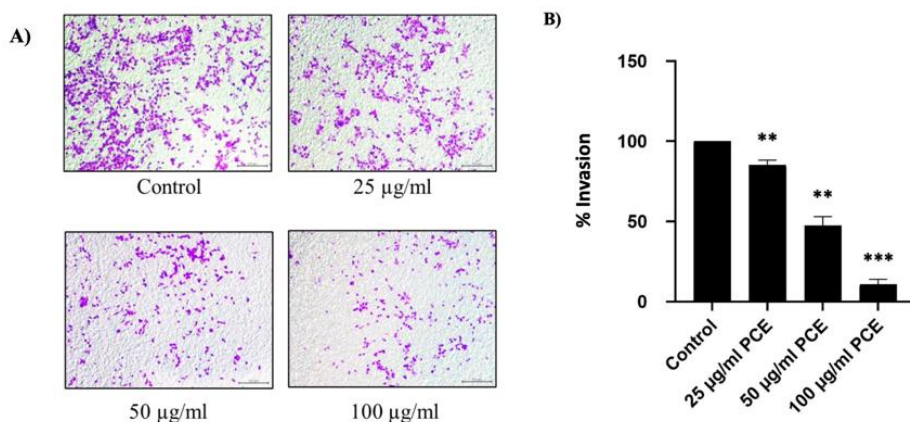


Fig 5. Effect of PCE on KKU-213AL5 cell invasion. (A) Representative images of invading cells stained with crystal violet after 16 hours of incubation in a Matrigel-coated Transwell chamber. (B) Quantitative analysis of cell invasion expressed as a percentage relative to the control group. Data are presented as the mean $\pm$ SD of at least three independent experiments. ** $p < 0.01$, *** $p < 0.001$ vs. control group.

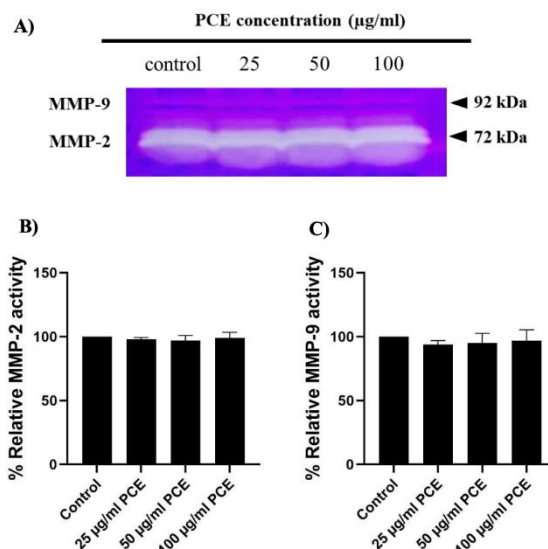


Fig 6. Effect of PCE on MMP-2 and MMP-9 activities in KKU-213AL5 cells. (A) Representative gelatin zymogram showing MMP-2 and MMP-9 activities in conditioned media collected after 24 hours of PCE treatment. (B and C) Densitometric analysis of MMP-2 and MMP-9 activities, respectively, expressed relative to the control group. Data are presented as the mean $\pm$ SD of at least three independent experiments.

The response to PCE varied among the tested cancer cell lines. CCA cells showed a greater reduction in viability, whereas A549 lung and MCF-7 breast cancer cells were relatively less responsive. However, because only one cell line from each non-CCA cancer type was examined, this finding indicates a cell line-dependent response rather than CCA-selective toxicity. Previous studies have similarly reported cell line-dependent responses to resveratrol, including relatively modest effects in MCF-7 and A549 cells.¹⁸ Differences in cellular signaling and antioxidant or xenobiotic-defense capacity may contribute to such differential sensitivity.^{19,20} Nevertheless, these factors were not examined in the present study, and the molecular basis of the response to PCE remains to be clarified.

Notably, KKU-213AL5 cells showed the greatest response to PCE among the tested CCA cell lines. KKU-213AL5 is a highly metastatic subline derived from KKU-213A through repeated *in vivo* selection and

exhibits greater migratory, invasive, and metastatic capacities than its parental cell line.²¹ The lower 48-hour IC_{50} previously reported for KKU-213AL5, together with the confirmatory screening in the present study, suggests that highly aggressive CCA phenotypes may be more responsive to PCE. This differential response may reflect cell line-specific differences in signaling pathways, stress-response mechanisms, membrane composition, or expression of molecular targets; however, these possibilities were not directly examined in the present study. Trans-arachidin-1 and trans-arachidin-3 have affinity for cannabinoid receptors,⁷ and altered cannabinoid receptor 2 expression has been reported in cancer.²² Nevertheless, the expression and functional involvement of cannabinoid receptors in KKU-213AL5 cells remain unknown. Further studies comparing molecular profiles and target expression among CCA cell lines are required to clarify the basis of their differential sensitivity to PCE.

Cell migration and invasion are essential steps in cancer progression and metastatic dissemination. In the present study, non-cytotoxic concentrations of PCE significantly delayed wound closure and reduced Matrigel invasion in KKKU-213AL5 cells. Although the selected PCE concentrations did not markedly reduce cell viability, wound closure is influenced by both cell migration and proliferation. Since proliferation was not directly controlled or independently quantified during the wound-healing assay, the contribution of altered proliferation to the delayed wound closure cannot be excluded. Therefore, further studies using proliferation-controlled migration assays are required to confirm the direct anti-migratory effect of PCE. Nevertheless, the marked reduction in Matrigel invasion supports an inhibitory effect of PCE on the invasive behavior of KKKU-213AL5 cells. These findings are consistent with previous studies showing that stilbenoids, including resveratrol and related compounds, can suppress cancer cell migration and invasion in several malignancies.^{23,24} Reported mechanisms include modulation of PI3K/Akt and MAPK signaling and downregulation of factors associated with angiogenesis, hypoxic responses, and invasive behavior.^{25,26} Other stilbenoids, such as pinosylvin, have also been reported to inhibit cancer cell migration and invasion through regulation of cyclooxygenase-2 and matrix metalloproteinases.²⁷

MMP-2 and MMP-9 are gelatinases that contribute to extracellular matrix degradation and cancer cell invasion. However, gelatin zymography showed that PCE did not significantly alter the activities of either enzyme in conditioned media from KKKU-213AL5 cells. Thus, the suppression of cell invasion observed in this study was not accompanied by changes in MMP-2 or MMP-9 gelatinolytic activity. Other mechanisms may therefore contribute to the anti-invasive effect of PCE; however, these possibilities were not investigated in the present study and should be regarded as proposed mechanisms.

Previous studies have shown that resveratrol and related stilbenoids can inhibit cancer cell invasion by modulating epithelial-mesenchymal transition-associated pathways and the urokinase-type plasminogen activator system.²⁸⁻³⁰ For example, resveratrol has been reported to suppress TGF- β 1-induced epithelial-mesenchymal transition and invasion in lung cancer cells and to reduce cancer cell invasion through downregulation of uPA/uPAR signaling.²⁸⁻²⁹ Pterostilbene, a resveratrol analogue, has also been shown to inhibit migration and invasion through regulation of uPA and cytoskeletal signaling.³⁰ Whether PCE affects these pathways in KKKU-213AL5 cells remains unknown and requires direct experimental evaluation.

Several limitations should be acknowledged. First, the migration and invasion experiments were performed only in KKKU-213AL5 cells. Although this cell line was selected based on its greater response to PCE and its highly metastatic phenotype, the findings may not be generalizable to other CCA cell lines because of the biological heterogeneity of CCA. Confirmation in additional CCA cell models is therefore required. Second, the present study used *in vitro* assays and did not directly assess metastatic dissemination *in vivo*. Third, the wound-healing assay does not fully distinguish cell migration from cell proliferation, and possible effects of PCE on proliferation may have contributed to the delayed wound closure. Fourth, PCE is a complex extract containing multiple bioactive constituents; therefore, the relative contributions of trans-arachidin-1, trans-arachidin-3, and other components remain to be determined. Finally, although MMP-2 and MMP-9 activities were evaluated, other signaling pathways and invasion-related proteins were not examined.

5. Conclusion

In conclusion, PCE suppressed the viability, migration, and invasion of highly metastatic cholangiocarcinoma cells. The

inhibitory effect on cell invasion was not accompanied by significant changes in MMP-2 or MMP-9 gelatinolytic activity, suggesting that other molecular mechanisms may be involved. These findings provide a basis for further investigation of PCE and its prenylated stilbenoid constituents in CCA models.

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Conflicts of Interest

The authors declare no conflict of interest.

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