



Pomegranate root extract possesses anti-metastatic potential by suppressing invasiveness and vasculogenic mimicry capability of cancer cells

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ABSTRACT

Cancer metastasis is a serious problem in cancer treatment. Metastasis is driven by cancer invasiveness and facilitated by angiogenesis of endothelial cells. Collective evidence revealed that highly invasive cancer cells possess vasculogenic mimicry (VM) capability by forming non-endothelial capillaries that mimic blood vessels. Moreover, VM also resists anti-angiogenic drugs. Thus, anti-invasion and *anti*-VM are necessary approaches required for anti-metastasis therapy. Pomegranate plants (*Punica granatum* L.) have been used in traditional medicines since ancient times. Different parts of the pomegranate tree possess the ability to inhibit cancer cell invasion and migration, but there is no report for pomegranate roots. This study aimed to explore anti-metastatic and *anti*-VM effects of pomegranate root extract (PR) in a human lung cancer cell line (A549) and a VM-forming human hepatocellular carcinoma cell line (SK-Hep-1). At less cytotoxic concentrations (lower than IC₂₀ values), PR dose-dependently reduced invasion, migration, and MMPs production in both A549 and SK-Hep-1 cell lines. PR also inhibited VM formation in SK-Hep-1 cells. Mechanistic studies revealed that, in A549 cells, PR inhibited hepatocyte growth factor (HGF)-induced activation of MET and its downstream AKT and ERK pathways. During VM formation of SK-Hep-1 cells, PR downregulated AKT and ERK signaling pathways without affecting their upstream activator, FAK phosphorylation. Phytochemical profiling of PR analyzed by LC-MS revealed tannins and ellagic acid derivatives were the major classes of natural products present in PR. This research reveals a novel health benefit of pomegranate roots in cancer metastasis therapy.

1. Introduction

Cancer represents a significant public health concern and is a leading cause of worldwide death. Non-small cell lung cancer (NSCLC) and hepatocellular carcinoma (HCC) are the two cancers with three highest cancer deaths in year 2022 [1]. Approximately 90 % of death from cancers result from metastasis [2]. Cancer metastasis is a multi-step process of cancer cell spreading from the original tumor to other parts of the body. The process starts from (i) invasion of cancer cells into the adjacent tissue of the tumor, (ii) transendothelial migration of cancer cells into the nearly blood or lymphatic vessels, (iii) cancer cells travelling in the circulatory system, (iv) penetration of cancer cells from bloodstream into tissue of target organs, and (v) colonization of cancer cells in the target organs [3]. Cancer invasion is the critical step of metastasis and involves cell migration, extracellular matrix (ECM)-degrading enzymes such as matrix metalloproteinase enzymes (MMPs), and cell-ECM interactions [4]. Thus, anti-invasive agents have received

attention in anti-metastatic therapy [5].

Tumor growth is typically supported by angiogenesis or the formation of new blood vessels mediated by endothelial cells and also acts as the route of metastasis [6]. On the other hand, highly invasive cancer cells can form tumor blood channels without endothelial cell involvement which is termed vasculogenic mimicry (VM) [7,8]. VM has been observed in multiple cancer types including hepatocellular carcinoma (HCC), lung cancer, gastric cancer, colorectal cancer, prostate cancer, melanoma, glioblastoma, and osteosarcoma [9–16]. Importantly, anti-angiogenesis drugs which target endothelial cells, cannot inhibit VM formation [17]. Moreover, anti-angiogenic therapy can also accelerate metastasis by eliciting VM formation [18]. Thus, it is important to search for *anti*-VM therapy. VM capability is associated with invasiveness of cancer cells because cell migration and ECM-remodeling are necessary for cancer cells to assemble into vessel-like structures, similar to that required for endothelial cells during angiogenesis [19]. Therefore, anti-invasive agents might also have a potential to inhibit VM

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formation.

Pomegranate (*Punica granatum* L.) is a fruit tree which has been used in various traditional medicines [20]. The plant parts may be divided into flowers, fruit (peel, seed, and juice), leaf, bark, stem, and root. All parts of pomegranate are used in pharmaceuticals worldwide: flower and fruit are used as a food supplement to treat diabetes, pericarp is used for treatment of diarrhea, metrorrhagia, and bellyaches, while bark and root possess anthelmintic properties [20]. Pomegranate has been reported to prevent cancer metastasis of various cancers including bladder, breast, colon, liver, lung, ovarian, prostate, renal, and skin cancer [21]. Pomegranate seed oil, juice polyphenols, and pericarp polyphenols inhibited proliferation, xenograft growth, and invasion in prostate cancer. In addition, pomegranate leaf extract induces apoptosis and inhibits migration and invasion in non-small cell lung carcinoma [22]. Although suppressive effects of pomegranate juice, peel, and seed on cancer metastasis have been demonstrated, there is no report on the effect of pomegranate root on cancer metastasis. In this study, we explored *in vitro* anti-invasion and *anti-VM* properties of pomegranate root extract (PR) in NSCLC and HCC cell lines, and also investigated the mechanisms of action.

2. Materials and methods

2.1. Plant material and extraction

Dried pomegranate roots and leaves were purchased from a local Thai medicine herb shop (Vejpong Pharmacy (HOCK ANN TUNG) Co., Ltd.) in Bangkok, Thailand. The pomegranate extracts were prepared using ethanol extraction. Briefly, the dried roots and leaves (50 g) were ground and extracted with absolute ethanol (150 ml) at room temperature for 24 h. The solution was filtered with Whatman filter paper No. 4 and then freeze-dried by using a lyophilizer. The dried roots and leaves yielded 22.30 mg of root extract (PR) and 23.97 mg of leaf extract (PL), respectively. The freeze-dried extracts were stored at -20°C and dissolved in dimethyl sulfoxide (DMSO) at 250 mg/ml for stock preparation. The final concentration of DMSO in all experiments was 0.2 % (v/v).

2.2. Phytochemical profiling analysis

Phytochemical constituent profile of crude extracts was analyzed by LC-MS technique carried out at Medicinal Plants Innovation Center of Mae Fah Luang University, using Agilent 1290 infinity LC instrument coupled to an Agilent 6540 series QTOF-MS equipped with an ESI source, a diode-array detector (Agilent Technologies, Santa Clara, CA, USA). The extracts were dissolved in methanol at 1 mg/ml, and filtered through 0.2 μm PTFE membrane filters before subjected to analysis. Sample separation was carried out on a reversed-phase column (Agilent Poroshell 120 EC-C18) that maintained at 35°C , with 200 $\mu\text{l}/\text{min}$ flow rate of gradient mobile phase consisting of 0.1 % formic acid in water (eluent A) and 0.1 % formic acid in acetonitrile (eluent B), as following conditions: 0–5 min, 5 % B; 1–10 min, 5–17 % B; 10–13 min, 17 % B; 13–20 min, 17–100 % B; 20–25 min, 100 % B; 25–27 min, 100–5 % B; 27–33 min, 5 % B. Peak identification was performed in both positive and negative modes. MassHunter workstation software (Qualitative Analysis, version B.08.00) was utilized for instrument control, data acquisition, and processing. Compound matching was performed with Personal Compound Database and Library (PCDL) of Medical Plants Innovation Center of Mae Fah Luang University. The compounds having PCDL scores higher than 70 and mass error less than ± 5 ppm were further subjected to *m/z* verification and MS/MS analysis.

2.3. Chemicals and antibodies

MTT (3-[4,5-Dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) was purchased from Sigma (St. Louis, MO, USA). MET-specific inhibitor

(SU11274), PI3K-specific inhibitor (LY294002; LY), and ERK-specific inhibitor (U012620) were obtained from Calbiochem (San Diego, CA, USA). Recombinant human hepatocyte growth factor (HGF) was purchased from Peprotech (Rocky Hill, NJ, USA). Matrigel® Basement Membrane Matrix (cat. no. 356234) was obtained from Corning (Corning, MA, USA). Primary antibody against MET (Cat. no. sc-10) was obtained from Santa Cruz Biotechnology (Dallas, TX, USA). Primary antibodies against Phospho-MET (Y1234/1235, Cat. no. 3126), AKT (Cat. no. 9272), phospho-AKT (S473, Cat. no. 9271), ERK (Cat. no. 9102), phospho-ERK (T202/Y204, Cat. no. 9101), FAK (Cat. no. 3285), Phospho-FAK (Y397, Cat. no. 8556), and horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA).

2.4. Cell culture

A549 (ATCC CCL-185) human lung cancer cell line and SK-Hep-1 (ATCC HTB-52) human hepatocellular carcinoma cell line were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). The cell lines were grown in RPMI 1640 (Gibco, Grand Island, NY, USA), supplemented with 10 % fetal bovine serum (JR Scientific, Woodland, CA, USA), and 1 % Antibiotic-Antimycotic solution (Gibco). All cultures were maintained at 37°C in a humidified atmosphere of 5 % CO_2 . Fibroblast-conditioned media were prepared according to previous report [23] and used as chemoattractant for invasion and migration assays.

2.5. Cell viability assay

Cell viability was determined after treatment according to previous report [24]. Cells were seeded into 96-well plates at a density of 1×10^4 cells/well and incubated for 24 h. After that, cells were incubated with or without test sample for 24 h. At the end of treatment, culture media in each well were replaced with media containing MTT and further incubated for 2 h. Then, the media in each well were replaced with DMSO to solubilize formazan product. Absorbance was measured by using a microplate reader, absorbance at 550 nm was subtracted with absorbance at 650 nm. The number of viable cells was determined from the absorbance and expressed as percent cell viability compared with control.

2.6. Cell invasion and migration assays

Invasion and migration capability of cells were evaluated by using Transwell chambers with 8 μm pore size (Corning) according to previous study [24]. For invasion assay, insert of Transwell chamber was pre-coated on upper surface with Matrigel (30 μg Matrigel protein/insert), while migration assay employed uncoated insert. Briefly, 200 μl of cell suspension (2×10^5 cells) in culture media containing test sample was seeded into the insert, and 600 μl of fibroblast-conditioned media containing the test sample was added into the lower chamber. Cells were allowed to invade or migrate for 24 h (for A549 cells) or 5 h (for SK-Hep-1 cells), and then the invaded/migrated cells attached on the lower surface of the insert were fixed with 25 % methanol and stained with 0.5 % crystal violet solution. The stained cells in each insert were extracted with an acid-methanol solution (0.1 N HCl). The absorbance was measured at 550 nm. Data were expressed as percent cell invasion or cell migration compared with control.

2.7. Gelatin zymography

MMP-2 and MMP-9 production by cancer cells was assessed by gelatin zymography as previously described [25]. Briefly, A549 cells (2×10^5 cells) or SK-Hep-1 cells (2×10^5 cells) in 24-well plate were cultivated in serum-free media with or without test sample for 24 h. After treatment, the conditioned media were collected and

electrophoresed in non-reducing gelatin-incorporated SDS-polyacrylamide gel. After electrophoresis, the gel was incubated in incubation buffer for 18 h at 37 °C, to allow gelatin digestion by MMPs. Then, the gel was stained with 0.3 % Coomassie blue dye for 1 h, followed by de-staining with a solution of 30 % methanol and 10 % acetic acid. Gelatinolytic activity of MMPs was visualized as clear band on a blue background. The gel was scanned and band intensity was determined using Image J software, and expressed as relative intensity compared with control.

2.8. Western blot analysis

For A549 cells, HGF which presence in the fibroblast-conditioned media was used to activate invasion-related cell signaling pathways. The cells were cultured in 6-well plates (5×10^5 cells/well) for overnight prior to a 24 h serum starvation. After that, the cells were incubated with or without test sample for 30 min, and then stimulated with 10 ng/ml HGF for 30 min. For SK-Hep-1 cells, VM-related signaling pathways were activated by attachment of the cells to extracellular matrix (Matrigel). Cell suspension with or without test sample was seeded into Matrigel-coated 6-well plate (200 µg Matrigel protein/well) at a density of 7×10^5 cells/well, and further incubated for 5 h. After treatment, whole cell proteins were extracted with radio-immunoprecipitation assay lysis buffer containing phosphatase and protease inhibitors cocktail. The extracted proteins were separated by 7.5 % SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane (Merck, Darmstadt, Germany). The membrane was incubated with primary antibodies specific to proteins of interest for overnight at 4 °C, followed by incubation with HRP-conjugated secondary antibodies for 40 min at room temperature. Band visualization was performed using enhanced chemiluminescence detection kit (GE Healthcare, Buckinghamshire, United Kingdom). Band intensity was quantified by using ImageQuant LAS 400 mini (GE Healthcare, Little Chalfont, U.K.).

2.9. Matrigel tube formation assay

Vasculogenic mimicry (VM) capability of SK-Hep-1 cells was evaluated by Matrigel tube formation assay, as previously described [25]. Briefly, 96-well plates were pre-coated with Matrigel (50 µl/well) and left solidified. The cells were seeded into the Matrigel pre-coated 96-well plates at a density of 1.8×10^4 cells/well, with or without test sample, and allowed to form tube-like structures for 5 h. Photographs were taken with a digital camera attached to an inverted phase-contrast microscope. Angiogenesis Analyzer for Image J was used to measure total length of the tube-like structures [26]. Data were expressed as relative tube length compared with control.

2.10. Statistical analysis

All results were presented as means $\pm$ S.E.M. The statistical significance of differences between control and treatment groups was determined by one-way analysis of variance (ANOVA) with Bonferroni multiple comparisons, and *p*-value less than 0.05 was considered to be significant. All data were statistical analyzed using GraphPad Prism 9.0 software (GraphPad, San Diego, CA, USA).

3. Results

3.1. Cytotoxicity and anti-invasion activity of pomegranate leaf and root extracts in A549 lung cancer cells

Initially, cytotoxicity of pomegranate leaf extract (PL) and pomegranate root extract (PR) were determined in A549 lung cancer cells to identify the inhibitory concentrations at 20 % and 50 % (IC₂₀ and IC₅₀) of the extracts. As shown in Fig. 1A, IC₂₀ and IC₅₀ values of PL were about 80 and 300 µg/ml, respectively, while IC₂₀ and IC₅₀ values of PR were about 90 and 520 µg/ml, respectively. The results indicated that PR was less cytotoxic than PL. Anti-metastatic potential assessment of a compound must be performed at non- or less-cytotoxic concentration. Therefore, anti-metastatic potential of PL and PR was assessed at IC₂₀ concentrations in subsequent studies. Since invasion is an early step of metastasis, we evaluated the anti-metastatic potential of the extracts towards A549 cell invasion. Interestingly, while PL showed a small effect on the cell invasion, PR markedly inhibited the invasion by 94 % reduction, compared to the control (Fig. 1B). Since the anti-invasion activity of PR was more potent than that of PL, we selected PR for further investigation.

3.2. Inhibition of A549 cell migration and MMP-2 production by pomegranate root extract

We further determined dose-dependent effect of PR over the non-cytotoxic concentration range (10–100 µg/ml) on A549 cell invasion. PR decreased invasion, in a dose-dependent manner, with 25–93 % inhibition being observed over the concentration range (Fig. 2A). Cell migration and MMPs production are necessary processes for cell invasion, so we further explored whether PR could inhibit the two processes. PR significantly decreased cell migration by 18–88 % inhibition over the concentration range of 10–100 µg/ml, in a dose-dependent fashion (Fig. 2B). Gelatin zymography showed that A549 cells actively produced MMP-2 enzyme and the production was suppressed by PR treatment in a dose-dependent manner with 38–98 % inhibition being observed over the concentration range (Fig. 2C). The inhibitory effects of PR on cell migration and MMP-2 production were correlated with the anti-invasion activity of PR, indicating that PR reduced invasion capability of A549

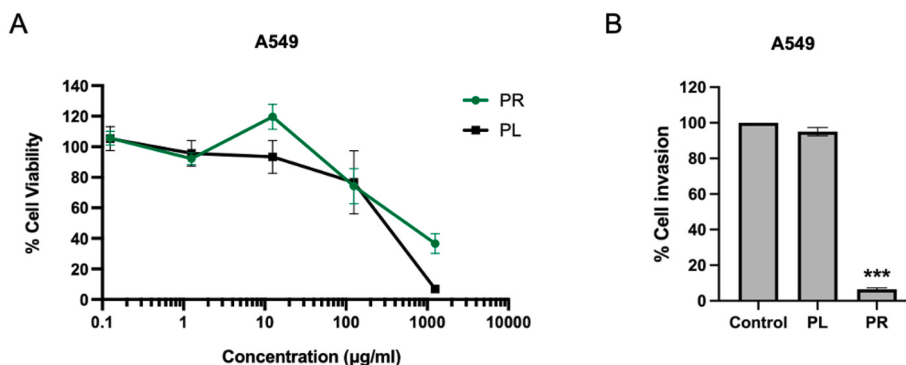


Fig. 1. Effects of pomegranate leaf extract (PL) and pomegranate root extract (PR) on A549 cell viability and invasion after 24 h treatment. (A) Cell viability was determined by MTT assay. (B) Anti-invasion activity was assessed by Transwell invasion assay. The cells were treated with PL and PR at IC₂₀ concentrations of 80 and 300 µg/ml, respectively, in Transwell chambers for 24 h. Data are presented as means $\pm$ S.E.M. from three independent experiments, ****p* < 0.001 versus control.

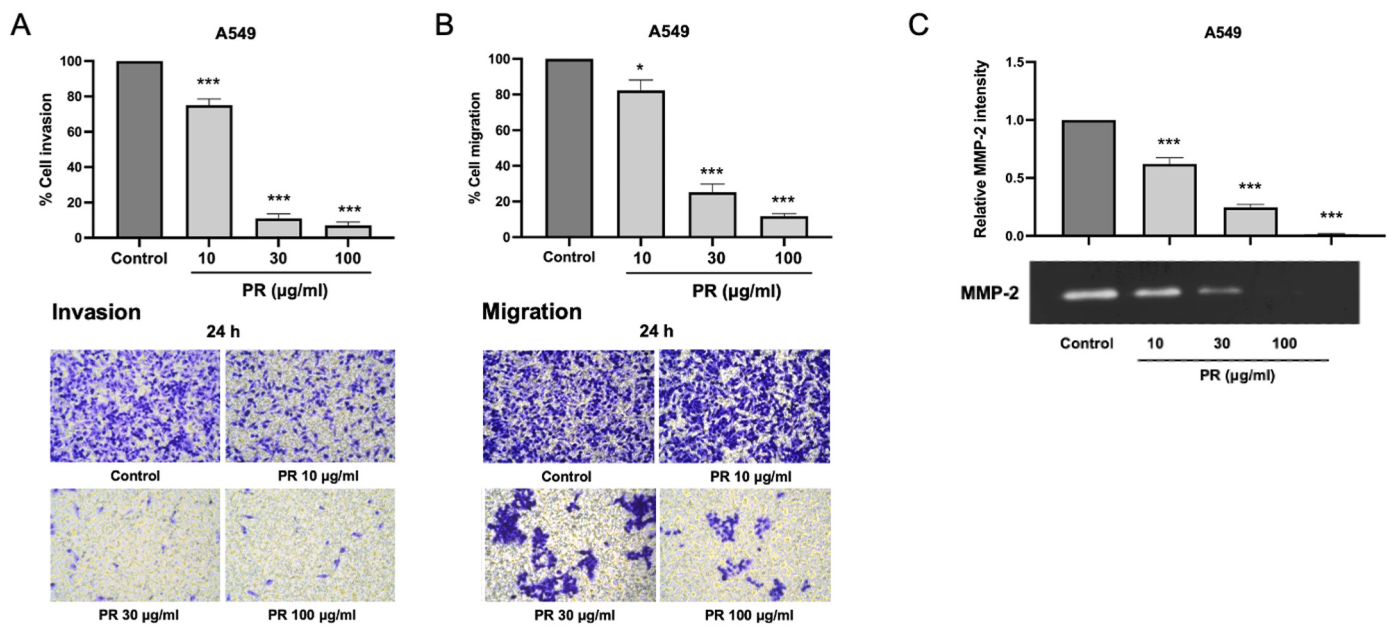


Fig. 2. Dose-dependent effects of pomegranate root extract on invasion, cell migration, and MMP-2 production in A549 cells. The cells were treated with PR at concentrations of 10–100 µg/ml for 24 h. (A and B) Invasion and migration of A549 cells determined by Transwell chamber assay. Representative photographs were taken at a magnification of $\times 40$ of crystal violet-stained invaded and migrated cells attached on Transwell filters. (C) MMP-2 production by A549 cells was detected by gelatin zymography. Activity of MMP-2 was displayed as clear bands on a blue background. Data are presented as means $\pm$ S.E.M. from three independent experiments, * $p < 0.05$ and *** $p < 0.001$ versus control.

cells by interfering with both principal processes of cell invasion.

3.3. Pomegranate root extract inhibits hepatocyte growth factor signaling pathway in A549 cells

Hepatocyte growth factor (HGF) is a protein secreted by fibroblasts

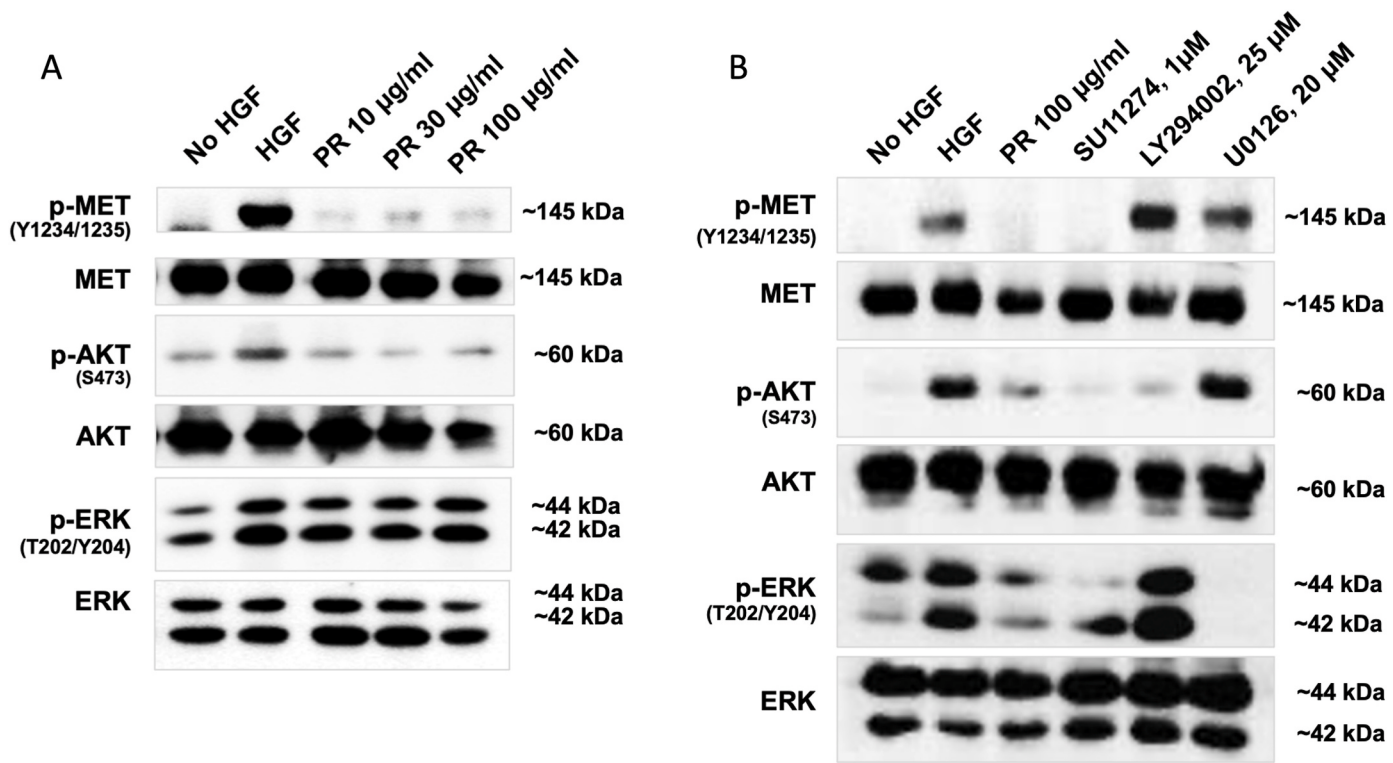


Fig. 3. Effect of pomegranate root extract on HGF signaling pathway in A549 cells. The cells were pretreated with PR or specific inhibitors for 30 min before a 30-min stimulation with HGF. Western blot analysis was used to detect phosphorylation of MET, AKT, and ERK proteins. (A) Dose-dependent effect of PR on HGF signaling activation. (B) Effect of MET-specific inhibitor (SU11274, 1 µM), PI3K/AKT-specific inhibitor (LY294002, 25 µM), and ERK-specific inhibitor (U0126, 20 µM) on HGF signaling activation.

and found to act as a tumor-promoting factor within tumor environment, by promoting invasion, migration, and survival of cancer cells [27]. Since HGF is present in the fibroblast-conditioned media used to induce invasion and migration of A549 cells in this study, we examined the effect of PR on activation of HGF signaling proteins including HGF receptor (MET) and its downstream components (AKT and ERK) after HGF stimulation. Our results showed PR treatment markedly abolished HGF-induced MET phosphorylation, as well as reducing AKT phosphorylation, in a concentration-dependent manner, while partial inhibition of ERK phosphorylation was also observed (Fig. 3A). The inhibition profile of PR (100 $\mu\text{g/ml}$) was similar to that of MET-specific inhibitor (SU11274, 1 μM), by decreasing levels of p-MET, p-AKT, and p-ERK (Fig. 3B). These results suggested that MET was a target of PR, and that the inhibition of HGF signaling pathway was a mechanism underlying the anti-invasion activity of PR in A549 lung cancer cells.

3.4. Pomegranate root extract reduce vasculogenic mimicry capability of SK-Hep-1 cells

Since vasculogenic mimicry (VM) capability is associated with cancer invasiveness as determined by cell migration capability and MMPs production [19], we further examined whether PR could inhibit VM formation. SK-Hep-1 is a highly invasive hepatocellular carcinoma cell line, and possesses VM capability, while A549 cells could not form VM. Thus, the effect of PR on VM formation was explored in SK-Hep-1 cells.

Firstly, cytotoxicity of PR in SK-Hep-1 cells was determined. In this cell line, IC_{20} and IC_{50} values of PR were about 210 and 650 $\mu\text{g/ml}$, respectively (Fig. 4A), which were greater than those with A549 cells,

suggesting that SK-Hep-1 cells showed more tolerance to PR than A549 cells.

Next, effects of PR on cell migration and MMPs production of SK-Hep-1 cells were assessed over the non-cytotoxic concentration range of 10–100 $\mu\text{g/ml}$, as used in the studies in A549 cells. Unlike A549 cells, SK-Hep-1 cells produced MMP-9 instead of MMP-2. After 5 h treatment, PR exhibited dose-dependent reduction of cell migration and MMP-9 production (Fig. 4B–C), similar to that observed in A549 cells. At the highest concentration (100 $\mu\text{g/ml}$), the inhibition of cell migration and MMP-9 production were 82 % and 50 %, respectively. These results confirmed that PR exerted anti-metastatic potential across different cancer types, at least, including lung and liver cancer.

Additionally, inhibitory effect of PR on VM capability of SK-Hep-1 cells was also observed. At a concentration of 100 $\mu\text{g/ml}$, formation of tube-like structures was significantly decreased by 31 % inhibition (Fig. 4D). Taken together, the results obtained in SK-Hep-1 cells suggested that PR could reduce VM capability of cancer cells by inhibiting ability of cancer cells to migrate and produce MMPs.

3.5. Inhibitory effect of pomegranate root extract on VM-related signaling pathways in SK-Hep-1 cells

The interaction with extracellular matrix components, such as laminin, is required for tube-like structure formation by VM-forming cancer cells [28]. Adhesion of cells to the extracellular matrix is mediated by integrins, which trigger phosphorylation of focal adhesion kinase (FAK) at Y397, leading to activation of FAK and its downstream signaling pathways. In this study, we used Matrigel adhesion to stimulate

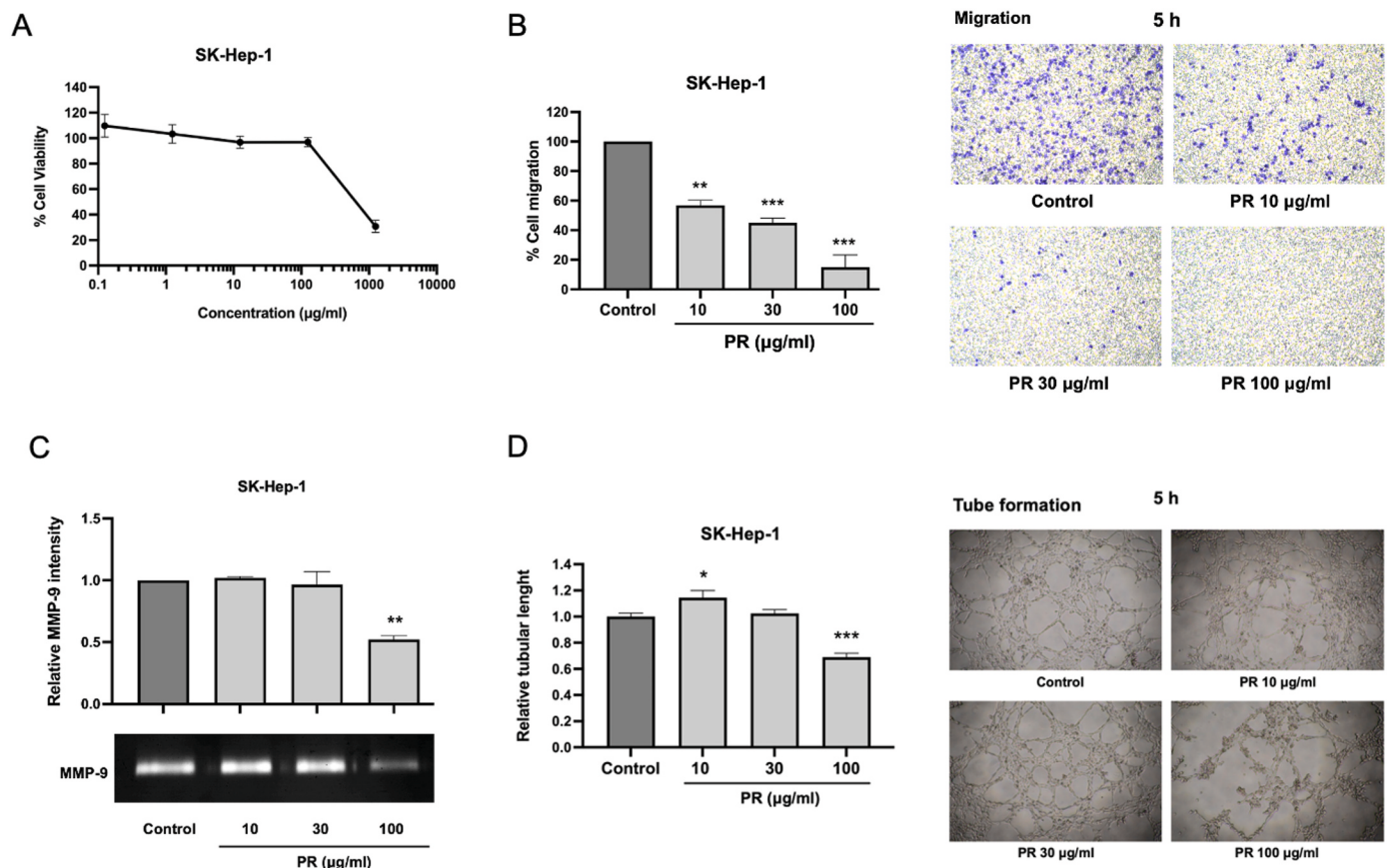


Fig. 4. Effects of pomegranate root extract on cell viability, cell migration, MMP-9 production, and VM capability of SK-Hep-1 cells. The cells were treated with PR at concentrations of 10–100 $\mu\text{g/ml}$. (A) Cell viability after 24 h treatment. (B) Cell migration after 5 h treatment. (C) MMP-9 production after 5 h treatment. Activity of MMP-9 was displayed as clear bands on a blue background. (D) VM formation after 5 h treatment. The cells were allowed to form tube-like structures in the presence or absence of PR for 5 h (original magnification of $\times 100$). Data are presented as means $\pm$ S.E.M. from three independent experiments, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ versus control.

VM-related signaling pathways in SK-Hep-1 cells to explore mechanism of VM inhibition by PR. As shown in Fig. 5, adhesion of untreated cells to Matrigel induced phosphorylation of FAK, AKT, and ERK, compared to untreated suspended cells. PR treatment dose-dependently decreased AKT and ERK phosphorylation, but AKT showed greater inhibition than ERK, while FAK phosphorylation was not affected (Fig. 5). Taken together, the results indicated that FAK was an upstream of AKT activation during VM formation in SK-Hep-1 cells. However, the AKT inhibition by PR did not result from FAK inhibition, suggesting that PR suppresses VM capability of SK-Hep-1 cells by affecting other molecules upstream of AKT.

3.6. Phytochemical profiling of pomegranate leaf and root extracts

We further investigated the potential anti-metastatic compounds present in PR, by exploring the differential chemical composition of PL and PR. Phytochemical constituents of PL and PR were analyzed by LC-MS technique. A total of 8 positive mode compounds and 49 negative mode compounds were identified (Supplementary Table S1). Fifteen compounds were present only in PR, including ellagic acid derivatives, flavonoids, steroid, cyclic polyol, phenolic acid, tannins, coumarin, xanthone, and lignan (Table 1). While 19 compounds were found in both PL and PR, including fatty acids, tannins, phenolic acids, flavonoids, ellagic acid derivatives, coumarin, steroid, triterpenoid and fatty acid (Table 2). The results indicated that tannins and ellagic acid derivatives were the major classes of natural products present in PR. However, future investigation is required to identify the active compounds responsible for anti-invasion and anti-VM activities of PR.

4. Discussion

Numerous research has shown that several parts of the pomegranate tree (juice, fruit, peel, seed, and leaf) exerted anti-metastatic potential in

multiple types of cancer, but there is no report on the anti-metastatic effect of pomegranate root [21]. Our study is the first to report the anti-metastatic potential of pomegranate root extract (PR) by decreasing invasiveness and VM capability of cancer cells and investigating the mechanisms of actions.

While pomegranate leaf extract (PL) did not inhibit invasion of A549 cells in our experimental study, a previous study by Li et al. showing that PL at a concentration close to the IC₂₀ value (25 µg/ml) could inhibit migration and invasion of H1299 lung cancer cells [22]. The discrepancy between our study and the previous study might be caused by the differences in both cell lines used (A549 in our study vs H1299 in Li et al.'s study) and extraction methods. Li et al. extracted fresh pomegranate leaves with 40 % ethanol for 2 h, while our study extracted dried leaves with absolute ethanol for 24 h. It might be possible that some anti-invasive substances presence in pomegranate leaves may be lost when the leaves were dried. Another explanation is the difference in polarity of extraction solvents, namely the 40 % ethanol used by Li et al. can lead to higher yields of water-soluble phenolic compounds, such as punicalagin, compared to absolute ethanol used in our study.

Until now, there is no standard anti-VM drug for use as a positive control in VM study. Nevertheless, we previously demonstrated that curcumin, a pharmacological active ingredient of turmeric, could suppress VM formation of SK-Hep-1 cells. Curcumin (30 µM or 11.05 µg/ml) could decrease VM formation by 92 % inhibition [25]. In this study, PR at a concentration of 100 µg/ml was able to decrease VM formation by 31 % reduction (Fig. 4D). Compared to curcumin, the relative anti-VM potency of PR crude extract (100 µg/ml) is around one-third of pure compound curcumin (11.05 µg/ml).

HGF is a pro-metastatic growth factor produced by fibroblasts and it potentiates invasiveness of carcinoma cells through activation of MET signaling [27]. HGF/MET signaling is overactivated in a wide range of cancers and is a therapeutic target for preventing metastasis [29]. In our experiment, we demonstrated that PR treatment could inhibit HGF-induced activation of MET/AKT/ERK signaling pathway in A549 cells. These results revealed a promising property of pomegranate as a new source of natural compounds targeting HGF/MET signaling.

Although the downregulation of AKT and ERK activation is the target shared between anti-invasion activity (in A549) and anti-VM activity (in SK-Hep-1) of PR, but upstream activators of these two signaling molecules are different. PR treatment disrupted HGF-induced MET auto-phosphorylation in A549 cells leading to abolish the downstream AKT and ERK phosphorylation. In contrast, the upstream of AKT and ERK signaling activation in SK-Hep-1 cells during VM formation was FAK (Y397) phosphorylation which triggered by binding of integrins to ECM components in Matrigel. However, VM suppression by PR did not correlate with FAK phosphorylation status but correlated with reduction of AKT and ERK phosphorylation, in a dose-dependent fashion (Fig. 5). These results suggested that inhibition of AKT activation by PR occurred at downstream of FAK activation in FAK/PI3K/AKT signaling. The possible target of PR should be a non-FAK protein and is upstream of AKT phosphorylation in VM regulation.

In addition to the integrin/FAK signaling, a model of VM signaling proposed by Paulis et al. indicates that a receptor tyrosine kinase EphA2 is upstream and can activate PI3K/AKT signaling in VM-forming cancer cells [30]. During VM formation, EphA2 is co-localized with VE-cadherin at the cell membrane and then directly activates PI3K which subsequently induces AKT phosphorylation. It is possible that the active compounds in PR might inhibit EphA2 function in VE-cadherin/EphA2/PI3K/AKT signaling.

VE-cadherin can also influence VM formation via mechanism mediated by EphA2-independent manner. Recently, Delgado-Bellido et al. demonstrated that, in uveal melanoma cell line with VM capability, the active FAK regulated VM formation by phosphorylating VE-cadherin at Y658 residue, allowing re-localization and association of phospho-VE-cadherin with p120-catenin/Kaiso and β-catenin/TCF4 complexes in the nucleus, leading to upregulation of VM-promoting genes [31,32].

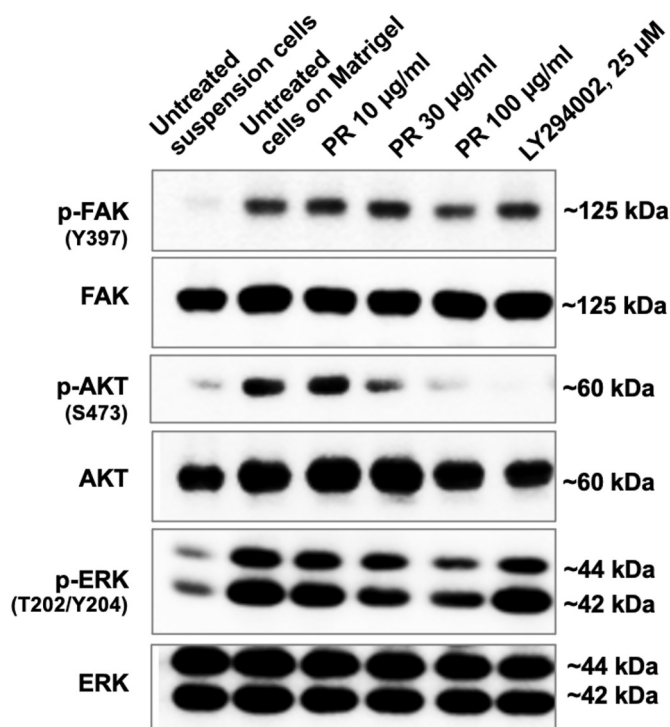


Fig. 5. Effect of pomegranate root extract on VM-related signaling pathways in SK-Hep-1 cells. Cell suspensions were treated with PR or specific inhibitors for 30 min before seeding into Matrigel-coated 6-well plates and further incubated for 5 h. Phosphorylation of FAK, AKT, and ERK proteins were detected by western blot analysis.

Table 1
List of compounds present only in PR, as identified by LC-MS.

No	RT (min)	Mass	<i>m/z</i> (Expected)	Chemical Formula	Identification	Compound Classification
Positive mode						
1	1.771	320.0529	343.043	C ₁₅ H ₁₂ O ₈	ethyl brevifolincarboxylate	Ellagic acid derivative
2	11.364	436.1362	437.1438	C ₂₁ H ₂₄ O ₁₀	phloridzin	Flavonoid
3	21.225	268.0385	286.0723	C ₁₅ H ₈ O ₅	coumestrol	Flavonoid
4	24.455	384.3403	402.3744	C ₂₇ H ₄₄ O	5-cholest-7-en-3-one	Steroid
Negative mode						
1	1.686	192.063	191.0558	C ₇ H ₁₂ O ₆	quinic acid	Cyclic polyol
2	4.914	198.053	243.0509	C ₉ H ₁₀ O ₅	syringic acid	Phenolic acid
3	5.414	934.071	933.0639	C ₄₁ H ₂₆ O ₂₆	2-O-galloyl-4,6(S,S) gallagoyl-d-glucose	Tannin
4	8.231	276.0267	275.0196	C ₁₃ H ₈ O ₇	3,4,8,9,10-pentahydroxy-dibenzo[b,d]pyran-6-one	Coumarin
5	8.642	474.043	533.0565	C ₂₁ H ₁₄ O ₁₃	valoneic acid	Tannin
6	8.877	918.0749	917.0681	C ₄₁ H ₂₆ O ₂₅	mallotusin	Tannin
7	9.2	802.0843	801.0775	C ₃₄ H ₂₆ O ₂₃	punigluconin	Tannin
8	10.257	422.0852	481.0986	C ₁₉ H ₁₈ O ₁₁	mangiferin	Xanthone
9	17.097	328.0217	327.0144	C ₁₆ H ₈ O ₈	ellagic acid, 3'-O-methyl-3, 4-methylene	Ellagic acid derivative
10	17.273	344.0523	343.0453	C ₁₇ H ₁₂ O ₈	ellagic acid, 3,3', 4'-tri-O-methyl	Ellagic acid derivative
11	24.554	362.1717	361.1646	C ₂₀ H ₂₆ O ₆	secoisolaricresinol	Lignan

Table 2
List of compounds present in both PL and PR, as identified by LC-MS.

No	RT (min)	Mass	<i>m/z</i> (Expected)	Chemical Formula	Identification	Compound Classification
Positive mode						
1	8.312	278.2252	301.214	C ₁₈ H ₃₀ O ₂	Punicic acid	Fatty acid
Negative mode						
1	1.831	482.0691	481.0617	C ₂₀ H ₁₈ O ₁₄	2,3-(S)-hexahydroxydiphenoyl-d-glucose	Tannin
2	1.86	782.0602	781.0526	C ₃₄ H ₂₂ O ₂₂	punicalin	Tannin
3	3.327	170.0212	169.014	C ₇ H ₆ O ₅	gallic acid	Phenolic acid
4	5	168.0418	167.0343	C ₈ H ₆ O ₄	vanillic acid	Phenolic acid
5	8.199	1084.068	1083.061	C ₄₈ H ₂₈ O ₃₀	punicalagin	Tannin
6	8.258	484.0853	483.0781	C ₂₀ H ₂₀ O ₁₄	gallic acid 3-O-β-D-(6'-O-galloyl)-glucopyranoside	Tannin
7	8.434	154.0265	153.0187	C ₇ H ₆ O ₄	protocatechuic acid	Phenolic acid
8	10.665	634.0816	633.0742	C ₂₇ H ₂₂ O ₁₈	punicacortein B	Tannin
9	11.252	450.1155	449.1077	C ₂₁ H ₂₂ O ₁₁	hovetrichoside C	Flavonoid
10	11.487	636.0939	635.087	C ₂₇ H ₂₄ O ₁₈	1,4,6-tri-O-galloyl-β-d-glucose	Tannin
11	12.074	248.0318	247.0247	C ₁₂ H ₈ O ₆	brevifolin	Ellagic acid derivative
12	13.953	448.0632	447.0561	C ₂₀ H ₁₆ O ₁₂	eschweilenol C	Ellagic acid derivative
13	14.775	302.0058	300.9986	C ₁₄ H ₆ O ₈	ellagic acid	Ellagic acid derivative
14	16.89	430.0882	475.0862	C ₂₁ H ₁₈ O ₁₀	luteolin-3'-O-β-D-xyloside	Flavonoid
15	19.239	192.0778	251.0915	C ₁₁ H ₁₂ O ₃	5-methylmellein	Coumarin
16	20.032	428.3288	487.3428	C ₂₈ H ₄₄ O ₃	ergosterol peroxide	Steroid
17	20.032	488.3499	487.3428	C ₃₀ H ₄₈ O ₅	asiatic acid	Triterpenoid
18	20.678	256.2392	315.2531	C ₁₆ H ₃₂ O ₂	palmitic acid	Fatty acid

However, there is lacking direct evidence to show that the elevated nuclear β-catenin/TCF4 complex can lead to AKT activation. Therefore, there is a low possibility for PR to suppress AKT activation through inhibiting VE-cadherin/β-catenin signaling.

Nevertheless, the VE-cadherin (Y658) phosphorylation is another downstream event of FAK activation and being upstream of AKT phosphorylation in VM regulation. Moreover, it is unclear whether VE-cadherin phosphorylation is necessary for EphA2/VE-cadherin colocalization during VM formation. Therefore, in addition to EphA2, the expression or phosphorylation of VE-cadherin might be a possible target modulated by the active compounds in PR to suppress VM formation through VE-cadherin/EphA2/PI3K/AKT signaling. However, it remains possible that the active compounds in PR might directly inhibit AKT and ERK. Therefore, identification of the anti-invasive and *anti-VM* compounds in PR and their targets should be performed in the future studies.

To our knowledge, this is the first time to report the phytochemical profile of pomegranate roots. Several ellagic acid derivatives and tannins were found in PR; however, none of them have been demonstrated to inhibit cancer invasion. While the flavonoids (coumestrol and phloridzin) and a xanthone (mangiferin), which only present in PR, have been demonstrated to possess anti-invasion activity. Coumestrol has been reported to inhibit invasion, migration, and MMP-9 expression in SKMEL-5 skin cancer cells, through suppression of PI3K/AKT signaling

[33]. Additionally, coumestrol also inhibited AKT phosphorylation in PC3 and LNCaP prostate cancer cell lines, resulting in reduction of cell migration capability [34]. Phloridzin displayed its anti-invasion and anti-migration activities in KYSE450 and KYSE30 esophageal cancer cell lines [35]. Interestingly, mangiferin has been shown to attenuate cancer invasion and migration in multiple cancer cell types, including lung cancer cells [36], breast cancer cells [37], pancreatic cancer cells [38], and osteosarcoma cells [39]. Moreover, mangiferin could suppress PI3K/AKT and ERK activation stimulated by phorbol myristate acetate (PMA) in astrogloma cell lines, resulting in reduction of invasion capability and MMP-9 production of the PMA-stimulated cells [40]. However, the effect of these compounds on VM formation requires further investigation. Taken together, pomegranate root is enriched in bioactive compounds with anti-invasion activity that might correlate with *anti-VM* potential. This research highlights the potential of pomegranate roots as a source for drug discovery in anti-metastatic therapy.

5. Conclusion

In summary, phytochemicals present in ethanolic extract of pomegranate roots possessed anti-metastatic potential by decreasing invasiveness of lung cancer cells and HCC cells, as well as reducing VM capability of aggressive HCC cells. Mechanistic studies revealed that the

inhibition of AKT and ERK signaling pathways was a mechanism shared by the anti-invasive and *anti*-VM activities of PR. These findings may lead to the development of effective anti-metastatic drugs from pomegranate for improving quality of life in cancer patients.

CRedit authorship contribution statement

Khajeelak Chiablaem: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Kriengsak Lirdprapamongkol:** Writing – original draft, Project administration, Conceptualization. **Jisnusun Svasti:** Writing – review & editing, Supervision, Funding acquisition.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bbrep.2025.102290>.

Data availability

Data will be made available on request.

References

- [1] F. Bray, M. Laversanne, H. Sung, J. Ferlay, R.L. Siegel, I. Soerjomataram, A. Jemal, Global cancer statistics 2022: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries, *CA Cancer J. Clin.* 74 (3) (2024) 229–263.
- [2] C.L. Chaffer, R.A. Weinberg, A perspective on cancer cell metastasis, *Science* 331 (6024) (2011) 1559–1564.
- [3] F. van Zijl, G. Krupitza, W. Mikulits, Initial steps of metastasis: cell invasion and endothelial transmigration, *Mutat. Res.* 728 (1–2) (2011) 23–34.
- [4] P. Friedl, K. Wolf, Tumour-cell invasion and migration: diversity and escape mechanisms, *Nat. Rev. Cancer* 3 (5) (2003) 362–374.
- [5] A. Gandalovicova, D. Rosel, M. Fernandes, P. Vesely, P. Heneberg, V. Cermak, L. Petruzella, S. Kumar, V. Sanz-Moreno, J. Brabek, Migrastatics-Anti-metastatic and anti-invasion drugs: promises and challenges, *Trends Cancer* 3 (6) (2017) 391–406.
- [6] N. Nishida, H. Yano, T. Nishida, T. Kamura, M. Kojiro, Angiogenesis in cancer, *Vasc. Health Risk Manag.* 2 (3) (2006) 213–219.
- [7] S.L. Wechman, L. Emdad, D. Sarkar, S.K. Das, P.B. Fisher, Vascular mimicry: triggers, molecular interactions and in vivo models, *Adv. Cancer Res.* 148 (2020) 27–67.
- [8] A.J. Maniotis, R. Folberg, A. Hess, E.A. Seftor, L.M. Gardner, J. Pe'er, J.M. Trent, P. S. Meltzer, M.J. Hendrix, Vascular channel formation by human melanoma cells in vivo and in vitro: vasculogenic mimicry, *Am. J. Pathol.* 155 (3) (1999) 739–752.
- [9] C. Jue, W. Zhifeng, Z. Zhisheng, C. Lin, Q. Yayun, J. Feng, G. Hao, I. Shintaro, T. Hisamitsu, G. Shiyu, L. Yanqing, Vasculogenic mimicry in hepatocellular carcinoma contributes to portal vein invasion, *Oncotarget* 7 (47) (2016) 77987–77997.
- [10] S.C. Williamson, R.L. Metcalf, F. Trapani, S. Mohan, J. Antonello, B. Abbott, H. S. Leong, C.P. Chester, N. Simms, R. Polanski, D. Nonaka, L. Priest, A. Fusi, F. Carlsson, A. Carlsson, M.J. Hendrix, R.E. Seftor, E.A. Seftor, D.G. Rothwell, A. Hughes, J. Hicks, C. Miller, P. Kuhn, G. Brady, K.L. Simpson, F.H. Blackhall, C. Dive, Vasculogenic mimicry in small cell lung cancer, *Nat. Commun.* 7 (2016) 13322.
- [11] J. Lv, B. Sun, H. Sun, Y. Zhang, J. Sun, X. Zhao, Q. Gu, X. Dong, N. Che, Significance of vasculogenic mimicry formation in gastric carcinoma, *Oncol. Res. Treat.* 40 (1–2) (2017) 35–41.
- [12] W. Li, S. Zong, Q. Shi, H. Li, J. Xu, F. Hou, Hypoxia-induced vasculogenic mimicry formation in human colorectal cancer cells: involvement of HIF-1 α , Claudin-4, and E-cadherin and vimentin, *Sci. Rep.* 6 (2016) 37534.
- [13] H. Wang, H. Lin, J. Pan, C. Mo, F. Zhang, B. Huang, Z. Wang, X. Chen, J. Zhuang, D. Wang, S. Qiu, Vasculogenic mimicry in prostate cancer: the roles of EphA2 and PI3K, *J. Cancer* 7 (9) (2016) 1114–1124.
- [14] O.K. Provan, V.O. Oria, T.T. Tran, J.I. Caulfield, C.R. Zito, A. Aguirre-Ducler, K. A. Schalper, H.M. Kluger, L.B. Jilaveanu, Vascular mimicry as a facilitator of melanoma brain metastasis, *Cell. Mol. Life Sci.* 81 (1) (2024) 188.
- [15] K. Maddison, N.A. Bowden, M.C. Graves, P.A. Tooney, Characteristics of vasculogenic mimicry and tumour to endothelial transdifferentiation in human glioblastoma: a systematic review, *BMC Cancer* 23 (1) (2023) 185.
- [16] L. Yan, R. Li, D. Li, Y. Zhu, Z. Lv, B. Wang, Development of a novel vasculogenic mimicry-associated gene signature for the prognostic assessment of osteosarcoma patients, *Clin. Transl. Oncol.* 25 (12) (2023) 3501–3518.
- [17] D.W. van der Schaft, R.E. Seftor, E.A. Seftor, A.R. Hess, L.M. Gruman, D. A. Kirschmann, Y. Yokoyama, A.W. Griffioen, M.J. Hendrix, Effects of angiogenesis inhibitors on vascular network formation by human endothelial and melanoma cells, *J. Natl. Cancer Inst.* 96 (19) (2004) 1473–1477.
- [18] Y. Xu, Q. Li, X.Y. Li, Q.Y. Yang, W.W. Xu, G.L. Liu, Short-term anti-vascular endothelial growth factor treatment elicits vasculogenic mimicry formation of tumors to accelerate metastasis, *J. Exp. Clin. Cancer Res.* 31 (1) (2012) 16.
- [19] K. Lirdprapamongkol, K. Chiablaem, M. Sila-Asna, R. Surarit, A. Bunyaratvej, J. Svasti, Exploring stemness gene expression and vasculogenic mimicry capacity in well- and poorly-differentiated hepatocellular carcinoma cell lines, *Biochem. Biophys. Res. Commun.* 422 (3) (2012) 429–435.
- [20] R. Wang, Y. Ding, R. Liu, L. Xiang, L. Du, Pomegranate: constituents, bioactivities and pharmacokinetics, *Fruit, Veg. Cereal Sci. Biotechnol.* 4 (2010) 77–87. Global Science Books.
- [21] N. Ahmadiankia, Molecular targets of pomegranate (*Punica granatum*) in preventing cancer metastasis, *Iran J. Basic Med. Sci.* 22 (9) (2019) 977–988.
- [22] Y. Li, F. Yang, W. Zheng, M. Hu, J. Wang, S. Ma, Y. Deng, Y. Luo, T. Ye, W. Yin, *Punica granatum* (pomegranate) leaves extract induces apoptosis through mitochondrial intrinsic pathway and inhibits migration and invasion in non-small cell lung cancer in vitro, *Biomed. Pharmacother.* 80 (2016) 227–235.
- [23] K. Lirdprapamongkol, C. Mahidol, S. Thongnest, H. Prawat, S. Ruchirawat, C. Srisomsap, R. Surarit, P. Punyari, J. Svasti, Anti-metastatic effects of aqueous extract of *Helixanthera parasitica*, *J. Ethnopharmacol.* 86 (2–3) (2003) 253–256.
- [24] P. Jantaree, K. Lirdprapamongkol, W. Kaewsri, C. Thongsornkleeb, K. Choowongkomon, K. Atjanasuppat, S. Ruchirawat, J. Svasti, Homodimers of vanillin and apocynin decrease the metastatic potential of human cancer cells by inhibiting the FAK/PI3K/Akt signaling pathway, *J. Agric. Food Chem.* 65 (11) (2017) 2299–2306.
- [25] K. Chiablaem, K. Lirdprapamongkol, S. Keeratchamroen, R. Surarit, J. Svasti, Curcumin suppresses vasculogenic mimicry capacity of hepatocellular carcinoma cells through STAT3 and PI3K/AKT inhibition, *Anticancer Res.* 34 (4) (2014) 1857–1864.
- [26] G. Carpentier, S. Berndt, S. Ferratge, W. Rasband, M. Cuendet, G. Uzan, P. Albanese, Angiogenesis analyzer for ImageJ - a comparative morphometric analysis of "Endothelial Tube Formation Assay" and "Fibrin Bead Assay", *Sci. Rep.* 10 (1) (2020) 11568.
- [27] B.Y. Owusu, R. Galemno, J. Janetka, L. Klampfer, Hepatocyte growth factor, a key tumor-promoting factor in the tumor microenvironment, *Cancers (Basel)* 9 (4) (2017).
- [28] C. Martini, M. DeNichilo, D.P. King, M.P. Cockshell, B. Ebert, B. Dale, L.M. Ebert, A. Woods, C.S. Bonder, CD36 promotes vasculogenic mimicry in melanoma by mediating adhesion to the extracellular matrix, *BMC Cancer* 21 (1) (2021) 765.
- [29] C.D. Mohan, M.K. Shanmugam, S.G.S. Gowda, A. Chinnathambi, K.S. Rangappa, G. Sethi, c-MET pathway in human malignancies and its targeting by natural compounds for cancer therapy, *Phytomedicine* 128 (2024) 155379.
- [30] Y.W. Paulis, P.M. Soetekouw, H.M. Verheul, V.C. Tjan-Heijnen, A.W. Griffioen, Signalling pathways in vasculogenic mimicry, *Biochim. Biophys. Acta* 1806 (1) (2010) 18–28.
- [31] D. Delgado-Bellido, A. Chacon-Barrado, J. Olmedo-Pelayo, C. Jordan Perez, P. Gilabert-Prieto, J. Diaz-Martin, A. Garcia-Diaz, F.J. Oliver, E. de Alava, Chromosomal 3p loss and 8q gain drive vasculogenic mimicry via HIF-2 α and VE-cadherin activation in uveal melanoma, *Cell Death Differ.* 32 (8) (2025) 1473–1483.
- [32] D. Delgado-Bellido, E. Zamudio-Martinez, M. Fernandez-Cortes, A.B. Herrera-Campos, J. Olmedo-Pelayo, C.J. Perez, J. Exposito, E. de Alava, A. Amaral, F. O. Valle, A.G. Diaz, F.J. Oliver, VE-Cadherin modulates beta-catenin/TCF-4 to enhance vasculogenic mimicry, *Cell Death Dis.* 14 (2) (2023) 135.
- [33] W. Kuang, W. Hu, H. Ren, Y. Shao, B. Liu, Plant derived coumestrol phytochemical targets human skin carcinoma cells by inducing mitochondrial-mediated apoptosis, cell cycle arrest, inhibition of cell migration and invasion and modulation of m-TOR/PI3K/AKT signalling pathway, *Saudi J. Biol. Sci.* 28 (5) (2021) 2739–2746.
- [34] W. Lim, M. Jeong, F.W. Bazer, G. Song, Coumestrol inhibits proliferation and migration of prostate cancer cells by regulating AKT, ERK1/2, and JNK MAPK cell signaling cascades, *J. Cell. Physiol.* 232 (4) (2017) 862–871.

- [35] Z. Jia, Y. Xie, H. Wu, Z. Wang, A. Li, Z. Li, Z. Yang, Z. Zhang, Z. Xing, X. Zhang, Phlorizin from sweet tea inhibits the progress of esophageal cancer by antagonizing the JAK2/STAT3 signaling pathway, *Oncol. Rep.* 46 (1) (2021).
- [36] Y.S. Lin, K.L. Tsai, J.N. Chen, C.S. Wu, Mangiferin inhibits lipopolysaccharide-induced epithelial-mesenchymal transition (EMT) and enhances the expression of tumor suppressor gene PER1 in non-small cell lung cancer cells, *Environ. Toxicol.* 35 (10) (2020) 1070–1081.
- [37] Q. Deng, Y.X. Tian, J. Liang, Mangiferin inhibits cell migration and invasion through Rac1/WAVE2 signalling in breast cancer, *Cytotechnology* 70 (2) (2018) 593–601.
- [38] L. Yu, M. Chen, R. Zhang, Z. Jin, Inhibition of cancer cell growth in gemcitabine-resistant pancreatic carcinoma by mangiferin phytochemical involves induction of autophagy, endogenous ROS production, cell cycle disruption, mitochondrial mediated apoptosis and suppression of cancer cell migration and invasion, *J. BUON* 24 (4) (2019) 1581–1586.
- [39] J. Wen, Y. Qin, C. Li, X. Dai, T. Wu, W. Yin, Mangiferin suppresses human metastatic osteosarcoma cell growth by down-regulating the expression of metalloproteinases-1/2 and parathyroid hormone receptor 1, *AMB Express* 10 (1) (2020) 13.
- [40] J.S. Jung, K. Jung, D.H. Kim, H.S. Kim, Selective inhibition of MMP-9 gene expression by mangiferin in PMA-Stimulated human astrogloma cells: involvement of PI3K/Akt and MAPK signaling pathways, *Pharmacol. Res.* 66 (1) (2012) 95–103.