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RESEARCH ARTICLE

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Pomegranate extract inhibits migration and invasion of oral cancer cells by downregulating matrix metalloproteinase-2/9 and epithelial-mesenchymal transition

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Abstract

Discovering drug candidates for the modulation of metastasis is of great importance in inhibiting oral cancer malignancy. Although most pomegranate extract applications aim at the antiproliferation of cancer cells, its antimetastatic effects remain unclear, especially for oral cancer cells. The aim of this study is to evaluate the change of two main metastasis characters, migration and invasion of oral cancer cells. Further, we want to explore the molecular mechanisms of action of pomegranate extract (POMx) at low cytotoxic concentration. We found that POMx ranged from 0 to 50 μg/mL showing low cytotoxicity to oral cancer cells. In the case of oral cancer HSC-3 and Ca9-22 cells, POMx inhibits wound healing migration, transwell migration, and matrix gel invasion. Mechanistically, POMx downregulates matrix metalloproteinase (MMP)-2 and MMP-9

Sheng-Yao Peng and Chien-Chou Hsiao contributed equally to this work.

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activities and expressions as well as epithelial-mesenchymal transition (EMT) signaling. POMx upregulates extracellular signal-regulated kinases 1/2 (ERK1/2), but not c-Jun N-terminal kinase (JNK) and p38 expression. Addition of ERK1/2 inhibitor (PD98059) significantly recovered the POMx-suppressed transwell migration and MMP-2/−9 activities in HSC-3 cells. Taken together, these findings suggest to further test low cytotoxic concentrations of POMx as a potential antimetastatic therapy against oral cancer cells.

KEYWORDS

MAPK, migration assay, MMP, oral cancer therapy, POMx

1 | INTRODUCTION

Oral cancer is one of the top 10 prevailing cancers worldwide, showing high morbidity and mortality.[1] Poor prognosis in oral cancer is mainly attributed to its tendency for highly local invasion[2] and recurrence rate.[3] Metastasis consists of several processes, including cancer cell proliferation, angiogenesis, migration, invasion, and microenvironment interaction.[4] Accordingly, metastasis becomes an important factor for oral carcinogenesis.[5] To modulate metastasis is of great importance for the therapy of oral cancer malignancy.[6] Recently, a number of natural products were reviewed to have inhibitory potential against cancer invasion and metastasis.[7,8]

Pomegranate (*Punica granatum* L.) is a fruit crop that has abundant polyphenols.[9,10] POMx, a commercial dietary ingredient made from pomegranate extract was" generally recognized as being safe" (GRAS) by the United States Food and Drug Administration (FDA), and was sold before standardizing its polyphenolic ellagitannin content.[11] POMx was chosen as the material for pomegranate extract to investigate several biological and clinical effects.[12–16]

Most studies for pomegranate extracts focused on apoptosis and antiproliferation effects against cancer cells.[14,17,18] Generally, antitumor studies using pomegranate extracts were at high cytotoxic concentrations. For example, POMx was treated with higher than IC_{50} concentrations to inhibit proliferation and trigger apoptosis against cancer cells or prostate (PC3),[14] lung (A549),[19] breast (MCF7),[17] leukemia (K562),[18] and fibrosarcoma (WEHI-164).[20]

Natural products may show concentration effects when applied as anticancer drugs. For example, berberine at a low concentration (50 μM) effectively suppresses cell migration and invasion of nasopharyngeal cancer HONE1 cells than that of high concentration (300 μM).[21] The migration inhibitory effect of high concentrations of berberine can be explained by mitotic arrest and apoptosis effects induced in HONE1 cells.[21]

Although most of pomegranate extract studies focused on antiproliferation against cancer cells, its antimetastatic effects remain unclear, especially for oral cancer cells. To avoid the indirect effect of apoptosis on inhibitory migration, we chose a low cytotoxic concentration to evaluate whether the commercial dietary ingredient made from pomegranate extract, POMx, is sufficient to inhibit the two main metastasis characteristics, migration and invasion of oral cancer cells. If this could be shown, we further want to explore the underlying molecular mechanisms for the inhibition of metastasis.

2 | MATERIALS AND METHODS

2.1 | Cell culture and reagents

Oral cancer (SCC9, Ca9-22, and HSC-3) and normal oral (HGF-1) cell lines were obtained from commercial sources. Oral cancer (OC-2) cells were a gift from Dr. Wan-Chi Tsai (Kaohsiung Medical University, Taiwan).[22] All cells were cultured with DMEM/F-12 (Dulbecco's Modified Eagle Medium [DMEM]/Nutrient Mixture F-12) (Gibco, Grand Island, New York) at 3:2 or 4:1 (HGF-1) mixture containing 10% fetal bovine serum (Gibco) and commonly used antibiotics.

Pomegranate extract powder was purchased from POMx (POM Wonderful, LLC, Los Angeles, California), which is a commercial aqueous extract of polyphenols isolated from pomegranates (*Punica granatum* L.) and contains 95% glycone ellagitannins and 3.4% ellagic acid.[16,23] POMx was dissolved in dimethyl sulfoxide (DMSO) for experiments. PD98059, the mitogen-activated protein kinase (MAPK) inhibitor for extracellular signal-regulated kinases 1/2 (ERK1/2), was purchased from Abmole BioScience (Houston, Texas). Other chemicals were obtained from Sigma-Aldrich (St. Louis, Missouri).

2.2 | Cell viability

Cell viability was determined by ATP assay (PerkinElner Life Sciences, Boston, Massachusetts)[24] or trypan blue staining.

2.3 | Wound healing assay

Cellular 2D migration ability was evaluated by wound healing assay as described.[25] A pipette tip was used to scrape confluent cell monolayers, washed with 1× PBS, and generated the gap for migration assay. At incubation time intervals, the wound gaps for POMx-treated with oral cancer cells were photographed and analyzed using the free software "TScratch" (https://www.cse-lab.ethz.ch/software/).

2.4 | 3D migration and invasion assay

Cellular 3D migration was evaluated by transwell chambers (8 μm pore size; Greiner Bio-One ThinCert CellCoat 24 well Culture Inserts; Vilvoorde, Belgium). Cells were cultured in the upper chamber under serum-free medium and incubated with 10% FBS-containing medium with vehicle or POMx for 18 hours (HSC-3 cells) or 21 hours (Ca9-22 cells) in the bottom chamber. After wiping the nontranswell cells in the upper compartment, the transwell cells in the bottom chamber were fixed with 4% paraformaldehyde for 5 minutes and stained with Giemsa solution overnight. Finally, the transwell cells were counted by the image J software.

Cellular 3D invasion was evaluated by transwell chambers topped with 100 μL 0.5% Matrigel (BD Matrigel Basement Membrane Matrix, BD Biosciences, Bedford, Massachusetts). Other steps were the same as described for the transwell assay above.

2.5 | Zymography

Gelatin zymography was performed as previously described with some modification.[26] In brief, cells were seeded and incubated in 10% FBS medium overnight. After washing with 1X PBS, cells were incubated in serum-free medium containing POMx for 48 hours to harvest the conditioned medium. Conditioned medium was mixed with 5X nonreducing dye (156.25 mM Tris-HCl [pH 6.8], 0.625% SDS, 25% glycerol, and 0.2% bromophenol blue) in 4:1 ratio and loaded for electrophoresis in 7.5% SDS polyacrylamide/0.2% gelatin gel at 90 V for 4 hours. Subsequently, the SDS in PAGE gel was removed by twice washing off the gel with washing buffer (2.5% Triton X-100) and one washing of the gel with development buffer (50 mM Tris-base, 50 mM NaCl, 13.25 mM $CaCl_2$, and 0.05% Brij35) at room temperature. Finally, gel was stained by 0.125% Coomassie blue for 1 hour and washed with destaining solution (50% methanol and 10% acetic acid). Gelatinase activity for matrix metalloproteinase (MMP)-2 and MMP-9 was reflected by the size of the clear zone in the gel and analyzed by the Image J software.

2.6 | Quantitative RT-PCR (qRT-PCR)

Total RNA was extracted by Trizol reagent (Invitrogen, Carlsbad, California) and reverse-transcribed by OmniScript RT kit (Qiagen, Valencia, California) as described.[27] cDNA was reacted with iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, California) for preforming Real-Time RT-PCR using a MyiQ real-time machine (Bio-Rad). Epithelial-mesenchymal transition (EMT) signaling such as *focal adhesion kinase* (*FAK*), *E-cadherin* (*E-cad*), *Slug*, twist-related protein 1 (*Twist*), *vimentin* (*Vim*), *N-cadherin* (*N-cad*), and *GAPDH* genes were studied by a touch-down PCR program (50 cycles).[28] The primer and PCR amplicon information[29–32] is provided in Table 1. The relative mRNA expression (fold activation) was calculated using the $2^{-\Delta\Delta Ct}$ method[33] in the reference of housekeeping gene, *GAPDH*. ADH-1 (Glpbio.com; Montclair, California), a N-cadherin inhibitor, was used to treat cells as an EMT downregulation control.

2.7 | Western blotting

Total protein (45 μg) was run on a 10% SDS-PAGE and transferred to a PVDF membrane. Blocking was performed with 5% nonfat milk overnight. Primary antibodies such as ERK1/2, c-Jun N-terminal kinase 1/2 (JNK 1/2), p38, p-ERK1/2, p-JNK1/2, and p-p38 were purchased from the MAPKs Family Antibody Sampler Kit and Phospho-MAPK Family Antibody Sampler Kit (Cell Signaling Technology, Inc., Danvers, Massachusetts). Primary antibodies for MMP-2 and MMP-9 were purchased from iREAL Biotechnology Comp. (Hsinchu, Taiwan). Primary antibodies for EMT signaling such as E-cadherin, Slug, and vimentin were purchased from the EMT Antibody Sampler Kit (Cell Signaling Technology) and N-cadherin was purchased from BD Biosciences (Bedford, Massachusetts). GAPDH antibody (GeneTex International Corp.; Hsinchu, Taiwan) served as internal control. After processing with secondary antibodies, signals were generated by enhanced chemiluminescence (Biotools Inc., Jupiter, Florida). The band intensity was measured by the Image J software.

2.8 | Statistical analysis

A significant difference for multiple comparisons was performed by ANOVA with Tukey HSD test (JMP13; SAS Institute, Cary, North Carolina). Treatments without the same letter labeling differ significantly.

TABLE 1 Primer information for EMT-associated genes[aa]

Genes	Forward primers (5′ → 3′)	Reverse primers (5′ → 3′)	Length
E-cad	CGACCCAACCCAAGAATCTATC[30]	GCAGCAGAATCAGAATTAGCAAA	251 bp
Slug	AGAGGAAAGACTACAGTCCAAGCTT	TCCCCCGTGTGAGTTCTAATG	303 bp
Twist	GGACTCCAAGATGGCAAGCT	CTCTGGAAACAATGACATCTAGGTC	166 bp
FAK	CCAGAGGAGTGGAAATATGAATTGAG	GTTTTGGCCTTGACAGAATCCAG	308 bp
Vim	AGCATGTCCAAATCGATGTGG[29]	CGTTCCAGGGACTCATTGGTTC	271 bp
N-cad	ATCCTGCTTATCCTTGTGCTGAT	CCCTCATTAATGAAGTCCCCA	317 bp
GAPDH	CCTCAACTACATGGTTTACATGTTCC[31]	CAAATGAGCCCCAGCCTTCT[32]	220 bp

[a]Primers without reference were designed in this study.

3 | RESULTS

3.1 | Cell viability of POMx-treated oral cancer cells

In a ATP content-based viability assay (Figure 1), POMx treatment under 25 and 50 μg/mL shows >80% cell viability in four oral cancer cells (HSC-3, Ca9-22, SCC9, and OC-2) after 24 hours. This result suggests that POMx is little cytotoxic to oral cancer cells at these concentrations, which are suitable for the investigation of regulatory effects on cell migration by POMx in the following experiments.

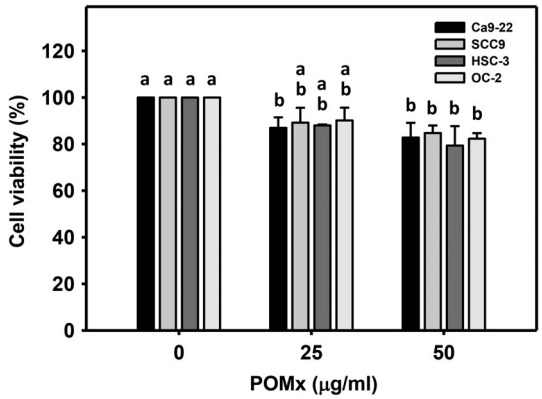

FIGURE 1 Effect of POMx on cell viability in oral cancer cells. Oral cancer cells (Ca9-22, SCC9, HSC-3, and OC-2) were treated with (0 [control with DMSO only], 25, and 50 μg/mL) of POMx for 24 hours ATP content assay. Multiple comparisons between different treatments and cells were analyzed. Treatments showing different labels (a-b) differ significantly: $P < .05$ to .0001. Data, mean ± SD (n = 3)

3.2 | POMx inhibits wound healing migration in oral cancer cells rather than oral normal cells

To identify the 2D migration effect of POMx in oral cancer HSC-3 and Ca9-22 cells and normal oral HGF-1 cells, the wound healing assay for POMx-treated cells were performed (Figure 2A-C). As shown in Figure 2D,E, the cell-free area (%) of the control decreased over time for oral cancer HSC-3 and Ca9-22 cells. At 9, 12 or 16 hours treatments of POMx, its cell-free areas (%) are higher than those of the control in a dose-responsive manner. In contrast, the cell-free areas (%) between control and POMx treatments in normal oral HGF-1 cells are similar in the 48 and 72 hours observations (Figure 2F). A long-term observation (up to 72 hours) for HGF-1 cell migration experiments was suggested by another study.[34] Accordingly, POMx inhibits the 2D migration in oral cancer cells rather than in normal oral cells.

3.3 | POMx inhibits transwell-based migration and invasion in oral cancer cells

To identify the 3D migration effect of POMx in oral cancer cells, the transwell and Matrigel invasion assays for POMx-treated HSC-3 and

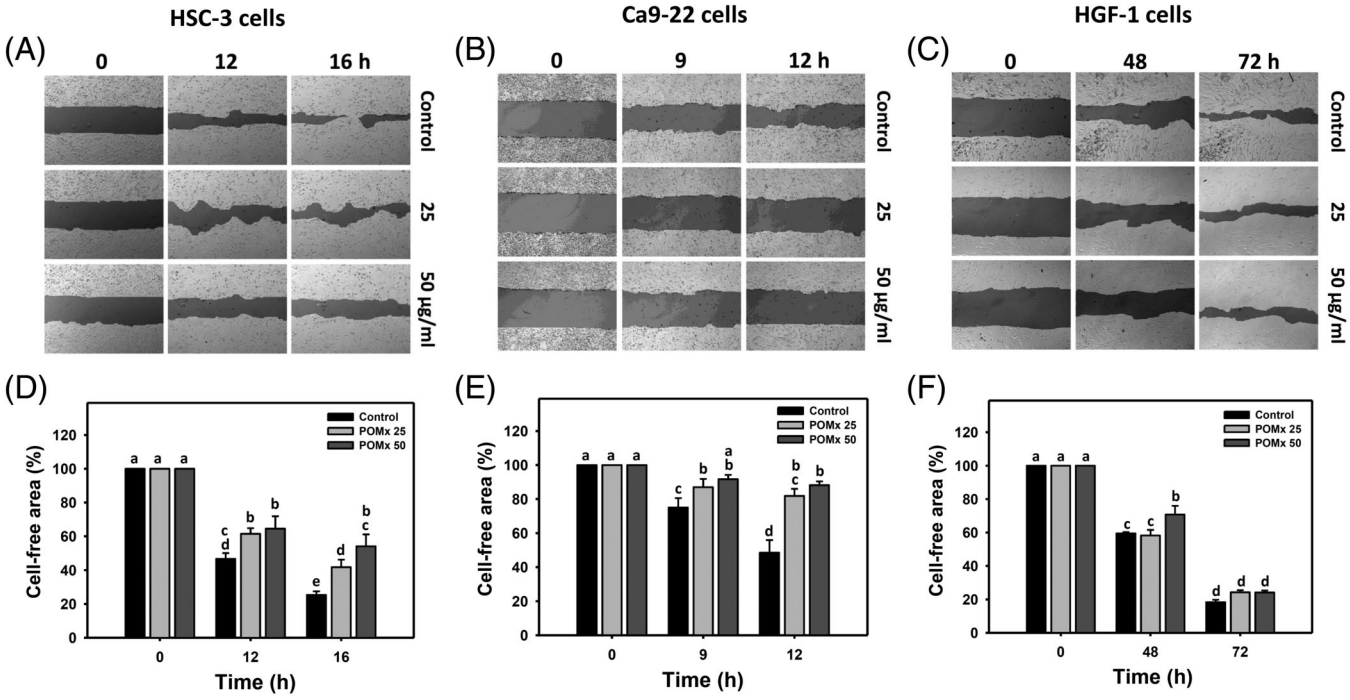

FIGURE 2 POMx inhibits wound healing migration in oral cancer and normal oral cells. (A-C) Images in wound healing assay. Oral cancer HSC-3 and Ca9-22 cells and normal oral HGF-1 cells were treated with POMx (0 (control with DMSO only), 25 (POMx 25), and 50 (POMx 50) μg/mL) for 0/12/16 hours, 0/9/12 hours, or 0/48/72 hours, respectively. (D-F) Statistics for (A-C). Multiple comparisons between different treatments were analyzed. Treatments showing different labels (a-e) differ significantly: $P < .05$ to .0001. Data, mean ± SD (n = 3)

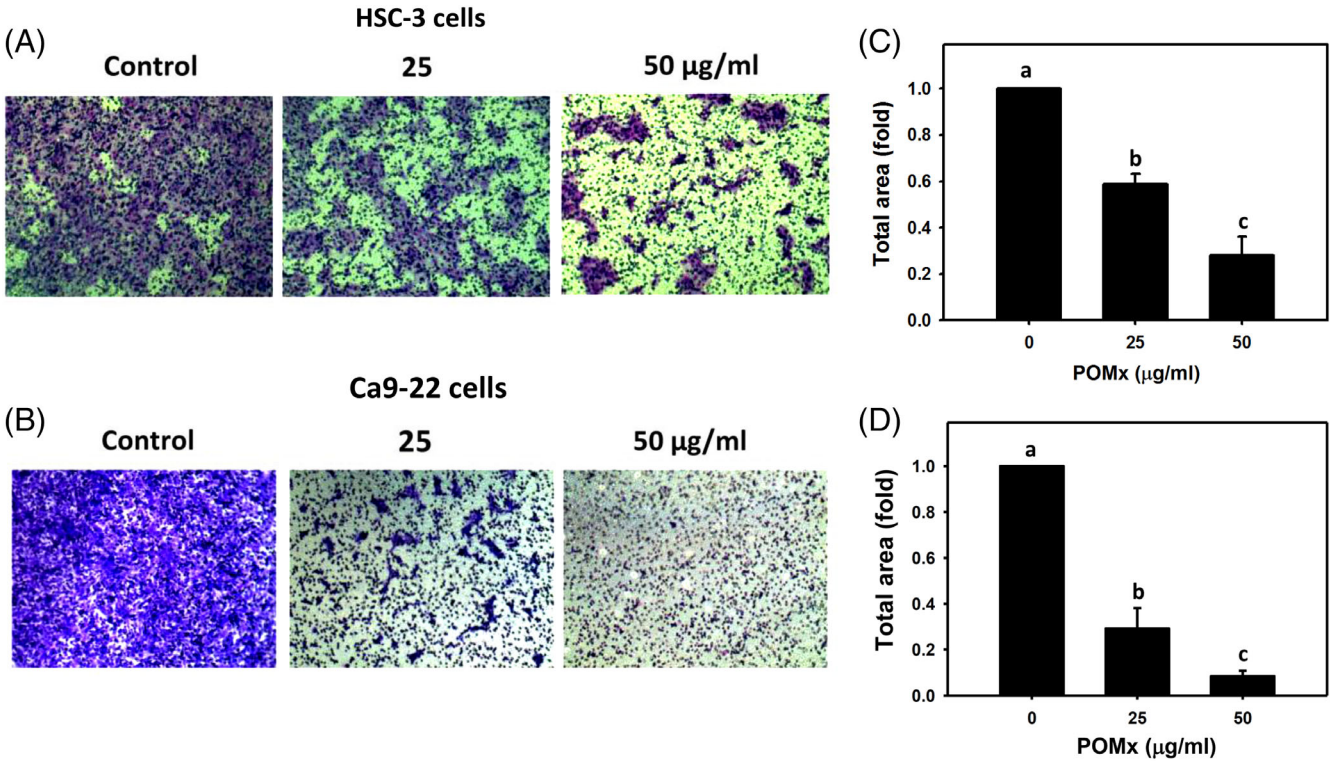

FIGURE 3 POMx inhibits transwell migration in oral cancer cells. (A, B) Images in transwell assay. HSC-3 and Ca9-22 cells were treated with POMx (0 (control with DMSO only), 25, and 50 μg/mL) for 18 or 21 hours, respectively. (C, D) Statistics for A and B. Total area indicates the amounts of stained cells. Multiple comparisons between different treatments were analyzed. Treatments showing different labels (a-c) differ significantly: $P < .001$ to .0001. Data, mean ± SD (n = 3) [Color figure can be viewed at wileyonlinelibrary.com]

Ca9-22 cells were performed (Figures 3A,B and 4A,B, respectively). As shown in Figures 3C,D and 4C,D, POMx dose-responsively inhibited the transwell-based migration and Matrigel invasion abilities in both HSC-3 and Ca9-22 cells. Accordingly, POMx has an inhibitory effect on 3D migration of oral cancer cells.

3.4 | POMx reduces the activities and expressions of MMP-2/-9 in oral cancer cells

MMP-2/-9 proteins are known to enhance cell migration and invasion.[35] To examine the effect on MMP-2/-9 activity of POMx treatments in oral cancer cells, a zymography assay was performed. To avoid the contamination of culture serum-containing MMP-2/-9 activators, zymography is commonly performed using serum-free condition medium. As shown in Figure 5A, trypan blue viability assay shows that POMx does not show significant differences on cell viability of oral cancer HSC-3 and Ca9-22 cells. Accordingly, the possibility that serum-free incubation may affect its cell viability and subsequently inhibits migration was excluded.

Figure 5B,C shows zymography patterns for POMx-treated oral cancer cells. In Figure 5D,E, the intensities of MMP-2/-9 bands, resulting from zymographic separation, are proportional to MMP-2/-9 enzyme activities, and dose-responsively reduced at 48 hours of POMx treatment for oral cancer HSC-3 and Ca9-22 cells. Moreover, MMP-2/9 protein expressions of oral cancer cells at 24 hours POMx treatment are lower than those at 12 hours. At 24 hours POMx treatment, Ca9-22 cells show lower MMP-2 levels than HSC-3 cells. Therefore, POMx reduces MMP-2/-9 activities and expressions in oral cancer cells.

3.5 | POMx regulates EMT-associated signaling in oral cancer cells

MMPs are known to regulate cell migration through the extracellular matrix (ECM).[36] To determine whether EMT signaling, including E-cadherin, Slug, Twist, FAK, vimentin, and N-cadherin, is dysregulated in POMx-treated oral cancer cells, qRT-PCR was performed (Figure 6A). For 12 hours treatment, the mRNA expression levels of *FAK* and EMT transcription factor (*Slug*) were decreased in oral cancer HSC-3 cells compared to that of control. After 24 hours treatment, the mRNA levels of mesenchymal markers (*vimentin* and *N-cadherin*) in addition to *FAK* and EMT transcription factors (*Slug* and *Twist*) were decreased in HSC-3 cells compared to that of control. Accordingly, *FAK*, EMT transcription factors, and mesenchymal markers were downregulated by POMx at 12 hours and/or 24 hours. In contrast, the epithelial marker *E-cadherin* was upregulated at 12 hours and maintained at basal levels at 24 hours in POMx-treated HSC-3 cells.

For protein expressions after 12 hours POMx treatment, the EMT signaling such as Slug, vimentin, and N-cadherin were decreased in HSC-3 and Ca9-22 cells (Figure 6B) and maintained at basal levels for

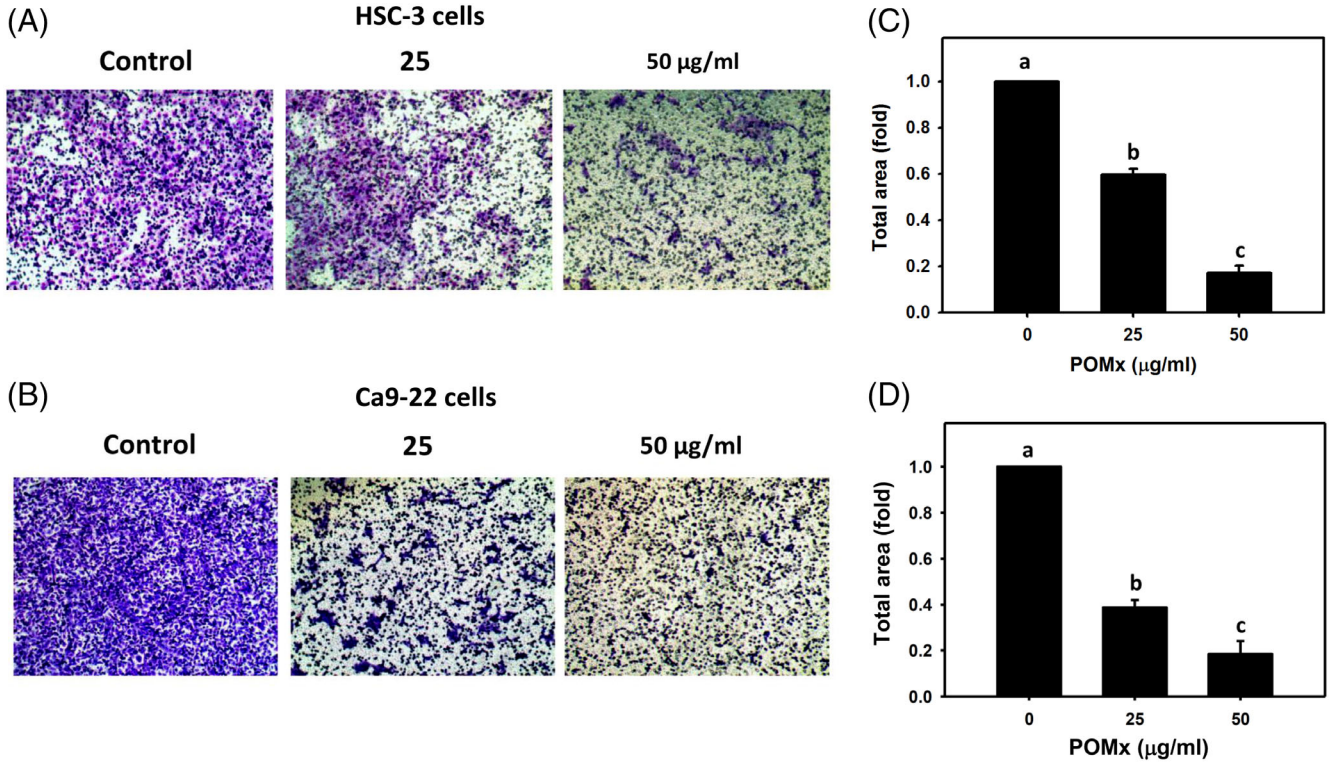

FIGURE 4 POMx inhibits invasion in oral cancer cells. (A, B) Images of invasion assay. HSC-3 and Ca9-22 cells were treated with POMx (0 (control with DMSO only), 25, and 50 μg/mL) for 18 or 21 hours, respectively. (C and D) Statistics for A and B. Total area indicates the amounts of stained cells. Multiple comparisons between different treatments were analyzed. Treatments showing different labels (a-c) differ significantly: $P < .0001$. Data, mean ± SD (n = 3) [Color figure can be viewed at wileyonlinelibrary.com]

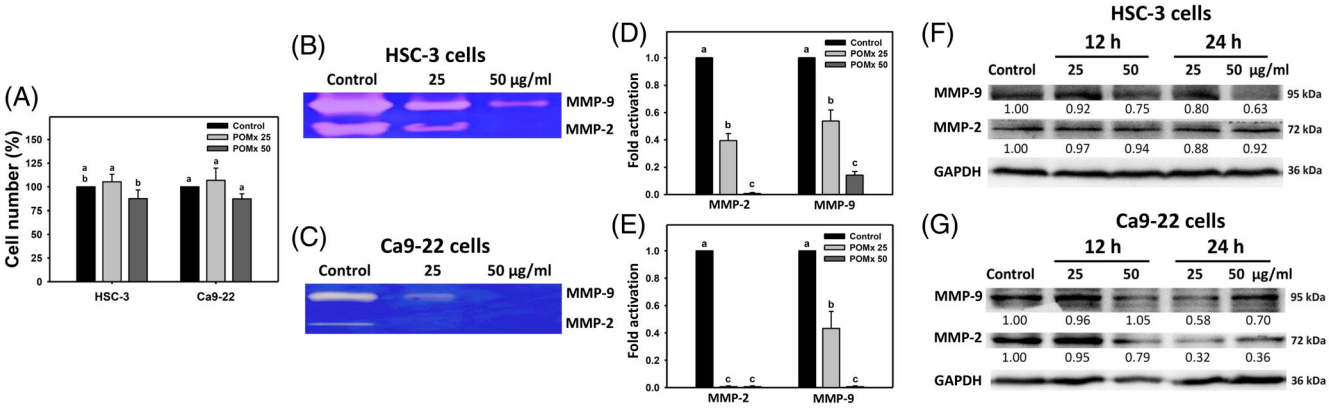

FIGURE 5 POMx inhibits the MMP-2 and MMP-9 activities and expressions in oral cancer cells. (A-C) Cell viability and zymography for oral cancer cells. HSC-3 and Ca9-22 cells were treated with POMx (0 (control with DMSO only), 25 (POMx 25), and 50 (POMx 50) μg/mL) for 48 hours under serum-free medium incubation. Cell viability was determined by trypan blue staining. Conditional medium was harvested for zymography assay. (D, E) Statistics for B and C. Multiple comparisons of the same MMP between different treatments were analyzed. Treatments showing different labels (a-c) significantly differ: $P < .005$ to .0001. Data, mean ± SD (n = 3). (F, G) Western blotting analysis for MMP-2 and MMP-9 expressions. HSC-3 and Ca9-22 cells were treated with POMx (0 (control with DMSO only), 25, and 50 μg/mL) for 12 and 24 hours [Color figure can be viewed at wileyonlinelibrary.com]

E-cadherin. For protein expressions after 24 hours POMx treatment, the levels of EMT signaling such as Slug, vimentin, and N-cadherin were lower than those of E-cadherin in in HSC-3 and Ca9-22 cells (Figure 6B). We conclude that POMx at relatively low concentrations downregulates EMT-associated signaling in oral cancer cells.

3.6 | POMx activates ERK1/2 among MAPK signaling in HSC-3 cells

MAPK was reported to regulate EMT signaling.[37] To evaluate the role of MAPK in EMT changes as shown in Figure 6, the protein

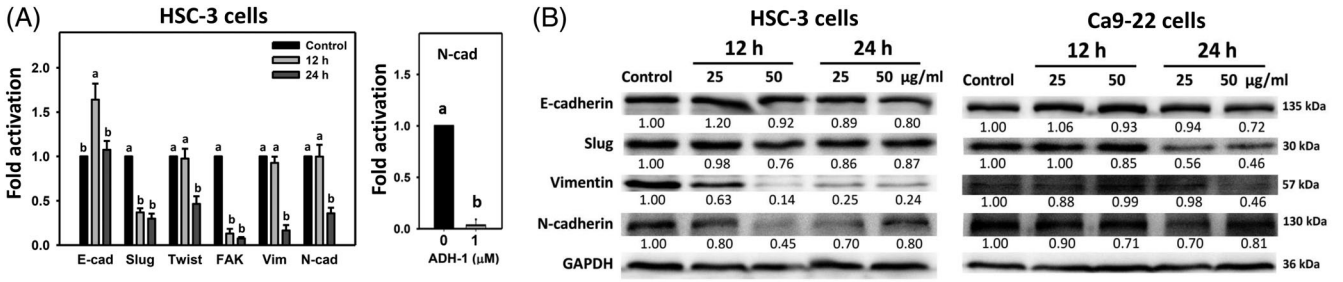

FIGURE 6 POMx regulates mRNA and protein expressions of EMT-associated genes in oral cancer cells. A, Quantitative RT-PCR for EMT gene expressions. HSC-3 cells were treated with POMx (0 (control with DMSO only), and 50 μg/mL) for 12 or 24 hours and then used qRT-PCR assays to observe mRNA expressions of EMT-associated gene. ADH-1 (1 μM, 24 hours), the N-cadherin inhibitor, was used as the control. Different treatments of the same gene were compared each other. Treatments showing different labels (a-b) differ significantly: $P < .05$ to .0001. Data, mean ± SD (n = 3). B, Western blotting for EMT protein expressions for POMx-treated HSC-3 and Ca9-22 cells. Cells were treated with POMx (0 (control with DMSO only), 25, and 50 μg/mL) for 12 and 24 hours. The intensity ratio for each EMT expression was adjusted to its matched GAPDH intensity

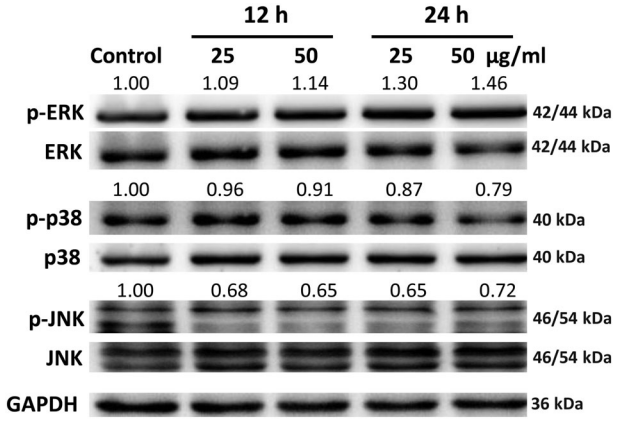

FIGURE 7 POMx regulates protein expression of MAPK in oral cancer cells. HSC-3 cells were treated with POMx (0 (control with DMSO only), 25, and 50 μg/mL) for 12 or 24 hours. Western blotting to MAPK signaling was performed. GAPDH is internal control. Band intensities were calculated by Image J freeware. Ratio is calculated by pMAPK/MAPK

expressions of three MAPK members such as ERK, p38, and JNK were examined (Figure 7). POMx at relatively low concentrations (25 and 50 μg/mL) induces phosphorylation of ERK1/2 at 12 and 24 hours but inhibits phosphorylation for both p38 and JNK in HSC-3 cells. These results suggest that ERK signaling is activated by POMx in oral cancer cells.

3.7 | ERK mediates POMx-inhibited 3D migration and MMP-2/-9 activities in oral cancer cells

To further validate the role of ERK signaling in the regulation of 3D migration (Figure 8A) and MMP-2/-9 (Figure 8C) in POMx-treated oral cancer cells, a specific ERK1/2 inhibitor PD98059 was applied. PD98059 significantly recovered the POMx-inhibited transwell ability on HSC-3 cells (Figure 8B). Furthermore, PD98059 substantially recovered the POMx-downregulated MMP-2/-9 activities (Figure 8D). The results suggest that POMx inhibits cell migration and MMP-2/-9 activation through ERK activation.

4 | DISCUSSION

The tested concentrations of POMx show low cellular cytotoxicity upon oral cancer cells in the present study. However, POMx causes significant inhibition of oral cancer cell migration (Figures 2 and 3) and invasion (Figure 4), MMP-2/-9 activities and expressions (Figure 5) as well as EMT-related gene mRNA and protein expressions (Figure 6). Such migratory inhibition affects oral cancer cells (Figure 2C,D) significantly more than oral normal cells (Figure 2F). The detailed mechanisms for both the POMx-mediated migration and invasion suppression are discussed in the following.

4.1 | Migration studies commonly use low cytotoxic concentration of drugs

When natural products affect cancer cells at high concentrations, this can be explained by their potency to induce apoptosis.[21] However, low cytotoxic concentrations of compounds are commonly chosen for migration studies.[38–40] As for POMx, at low cytotoxic concentrations it also shows wound healing inhibition in breast cancer stem cells.[16] Consistent with the above, our ATP assay (Figure 1) shows more than 80% cell viability for oral cancer cells, implying that anti-migration/invasion effects of POMx are not caused by general cytotoxicity.

4.2 | MMP-2/-9 inactivation is associated with POMx-inhibitory migration of oral cancer cells

MMP-2/-9 is required for cell migration and invasion, and has important functions in the metastasis (migration and invasion) of cancer

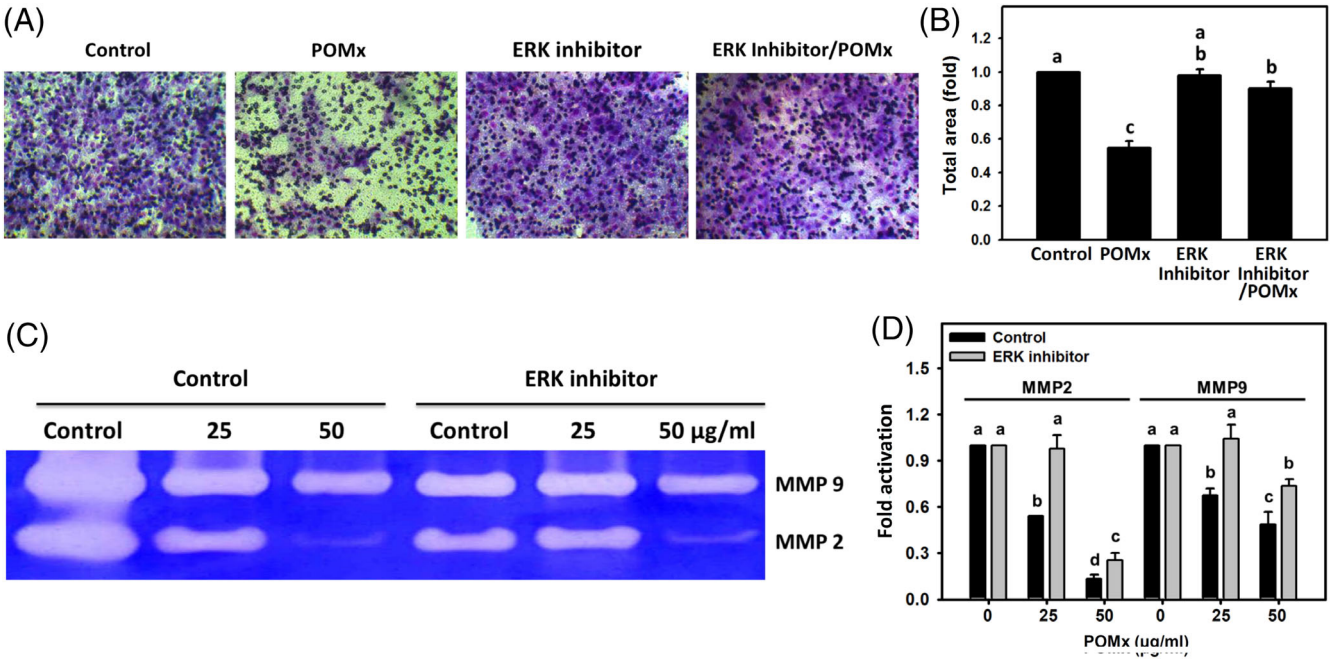

FIGURE 8 ERK affects transwell inhibition and MMP-2/MMP-9 activity of POMx-treated HSC-3 cells. A, Transwell assay. HSC-3 cells were pretreated with or without ERK inhibitor (20 μM, 1 hour) and posttreated with POMx (0 (control with DMSO only) and 50 μg/mL) for 18 hours followed by analysis of transwell assay. B, Statistics of transwell change in A. C, Zymography. HSC-3 cells were pretreated with or without ERK inhibitor (20 μM, 1 hour) and posttreated with POMx (0 [DMSO only], 25, and 50 μg/mL) for 48 hours. Conditional medium was harvested for zymography assay. D, Statistics for C. Treatments showing different labels (a-d) differ significantly: $P < .05$ to .0001. Data, mean ± SD (n = 3) [Color figure can be viewed at wileyonlinelibrary.com]

cells.[35] Inhibition of MMP-2/-9 by siRNA suppresses migration in retinoblastoma cells.[35] β-mangostin downregulates MMP-2/-9 protein expression and this way inhibits liver cancer cell invasion.[41] Similarly, we found that POMx suppresses the MMP-2/-9 activities and expressions based on zymography and western blotting in oral cancer HSC-3 and Ca9-22 cells (Figure 5). Accordingly, POMx-inhibitory migration on oral cancer cells may be mediated by inactivation of MMP-2/-9. It also demonstrates that POMx is a potential antimetastasis agent against oral cancer cells.

4.3 | EMT is involved in POMx-inhibitory migration of oral cancer cells

In general, epithelial cells are adhesive and highly expressing epithelial markers such as E-cadherin while mesenchymal cells are migrational and highly expressing mesenchymal markers such as N-cadherin.[42] FAK upregulates EMT in adenomyosis[43] and TGF β-mediated EMT in hepatocytes.[44] In EMT, epithelial markers are downregulated but FAK, EMT transcription factors (Slug and Twist), and mesenchymal markers are upregulated. In contrast, EMT inhibition shows the upregulation of epithelial markers and the downregulation of FAK, EMT transcription factors, and mesenchymal markers. Breast cancer stem cells show inhibitory migration change to low cytotoxic concentration of POMx, that is, POMx inhibits wound healing migration ability, downregulate EMT signaling gene *Twist* mRNA expression, and inhibit mammosphere formation.[16] Consistently, we demonstrated that the mRNA levels of FAK, EMT transcription factors (Slug and Twist), and mesenchymal markers (vimentin and N-cadherin) were downregulated, whereas the mRNA levels of the epithelial marker (E-cadherin) were upregulated in HSC-3 cells, indicating that the EMT process was suppressed by POMx in oral cancer cells which inhibit their migration and invasion. Moreover, we found that EMT-associated signaling proteins in oral cancer HSC-3 and Ca9-2 cells were downregulated by POMx (Figure 6B).

4.4 | POMx shows differentially regulation to MAPK family

The MAPK protein family (ERKs, JNKs and p38) regulates diverse cellular function such as proliferation, migration, invasion, and apoptosis.[45] However, these complex regulations are sometimes of reversed function and show variable effects among different cell types.[46,47]

Some studies for pomegranate extracts were focusing on MAPK expression and apoptosis without considering its migration effect.[13,14,19] At high cytotoxic concentration of POMx or other pomegranate extracts, different members of the MAPK family may be inhibited. POMx at 100 μg/mL (>IC_{50} 78 μg/mL) inhibited the phosphorylation of ERK1/2 in prostate cancer PC3 cells. However, no other member of the MAPK family was examined.[14] Lab-made pomegranate extracts ranging from 50 to 150 μg/mL cause growth inhibition of lung cancer A549 cells with a viability ranging between 67%, 60%, and 53% as indicated by the trypan blue assay in a 72 hours treatment.[19] Under cell viabilities of 67% to 53%, this lab-made

pomegranate extract inhibits phosphorylation of the MAPK family such as ERK1/2, JNK and p38 in lung cancer A549 cells.[19] However, these studies[14,19] focus only on the apoptosis and tumor inhibition effects at high cytotoxic concentrations of pomegranate extracts without investigating its migration effect, which is effective only at lower concentrations as the present study shows.

For comparison, at low cytotoxic concentration (10 μg/mL; with a cell viability = 80%) of POMx induces JNK phosphorylation in prostate cancer LAPC4 cells at 24 hours.[13] However, this study focuses only on the synergistic apoptosis effect for the combined treatment of POMx with IGFBP-3 without investigating the migration effect of POMx. In the present study, we found that at low cytotoxic concentrations (25 and 50 μg/mL; cell viability = 86% and 80%, respectively) of POMx induces ERK phosphorylation but inhibits JNK and p38 phosphorylation in oral cancer HSC-3 cells at 24 hours. Accordingly, different concentrations of POMx or different cancer cell types show different responses to regulate the expression among different members of the MAPK family.

4.5 | Role of ERK phosphorylation in POMx-induced migration and invasion of oral cancer cells

A number of studies have demonstrated that MAPKs regulate the MMP-2/-9 activation, leading to regulatory cell migration and invasion.[48,49] Our results show that the effects of POMx are mediated by activating ERK1/2 signaling, but not by the JNK and p38 signaling (Figure 7). Moreover, ERK1/2 inhibitor recovers the POMx-induced inhibition of transwell migration and MMP-2/-9 activation (Figure 8). We conclude accordingly that POMx inhibits the MMP-2/-9 activation, cell migration, and invasion through activating ERK1/2 signaling in oral cancer cells.

5 | CONCLUSION

The present study demonstrated that low cytotoxic concentrations of POMx inhibited cellular migration and invasion by suppressing MMP-2/-9 activities through activating the ERK1/2 and EMT signaling pathways. We suggest that POMx provides an anticancer effect by inhibiting the migration and invasion potential of oral cancer cells. Therefore, low cytotoxic concentrations of POMx provide a potent antimetastatic agent against oral cancer cells suggesting for further detailed investigation.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest among them.

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