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Original research article

In vitro inhibition of migration and adhesion in cholangiocarcinoma cells by ovalitenin A through PI3K modulation

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ABSTRACT

Ovalitenin A, a chalcone extracted from the roots of *Millettia brandisiana* Kurz, has exhibited cytotoxic properties in various human cancer cells. However, the effects of this on cholangiocarcinoma (CCA), an aggressive malignancy of the bile duct epithelium, are still not well understood. This study investigated the anti-metastatic potential of ovalitenin A and its associated molecular mechanisms in CCA cells. Sulforhodamine B (SRB), wound-healing, and adhesion assays were used to test cell viability, migration, and adhesion, respectively. Western blotting was performed to quantify phosphorylated PI3K and VEGF proteins, and qRT-PCR was used to measure *MMP-9* and *TIMP-1* mRNA. Ovalitenin A suppressed the proliferation of CCA cells in a concentration-dependent manner. Following 24 hours of treatment, it markedly diminished cell migration and adhesion. Network pharmacology analysis found 32 common targets between ovalitenin A and CCA utilizing the SwissTargetPrediction and GeneCards databases. Moreover, protein–protein interaction analysis revealed a robust association among these targets, indicating the involvement of PI3K-related signaling pathways. PIK3CA and MMP-family proteins were prioritized as candidate targets based on their centrality within the network and their well-established roles in CCA progression, metastasis, and extracellular matrix remodeling. As predicted, treatment of ovalitenin A reduced the levels of phosphorylated PI3K and VEGF proteins and lowered the ratio of *MMP-9* to *TIMP-1* mRNA. These findings suggest that ovalitenin A may reduce CCA metastasis, possibly by altering PI3K signaling and its downstream molecular effectors.

Keywords: Ovalitenin A; *Millettia brandisiana*; bile duct cancer; metastasis, PI3K signaling

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1. Introduction

Cholangiocarcinoma (CCA) is a cancer that affects the epithelial lining of the bile duct. It is one of the most common types of liver cancer, causing about 13% of liver cancer-related deaths around the world.[1] Its occurrence is remarkably elevated in specific areas, notably Northeast Thailand, attributable to various risk factors, including chronic infections by the liver fluke *Opisthorchis viverrini*.[2] The prognosis for CCA remains poor, especially for people who are diagnosed at an advanced or unresectable stage. These people usually get gemcitabine-cisplatin chemotherapy, and their median overall survival (mOS) is about 12 months, compared to about 51 months for those eligible for surgical intervention.[3,4] This difference is mostly due to CCA cells spreading aggressively, which makes current treatments less effective and greatly increases the risk of death.[5]

Metastasis, a multifaceted and intricate process, necessitates a series of steps, including epithelial–mesenchymal transition (EMT), which endows cancer cells with augmented migratory and invasive abilities.[6] A crucial molecular regulator in this context is the activation of phosphatidylinositol 3-kinase (PI3K), especially its phosphorylated variant, which significantly influences cell survival, migration, and invasion through subsequent signaling cascades.[7,8] Furthermore, PI3K activation can elevate the expression of vascular endothelial growth factor (VEGF) and matrix metalloproteinases (MMPs), both of which are essential mediators of metastatic progression.[9] Specifically, VEGF contributes to metastasis by enhancing vascular permeability and promoting the development of an extra-vascular matrix that fosters endothelial proliferation.[10] Conversely, matrix metallo-proteinases (MMPs) facilitate invasion through the degradation of the extracellular matrix, a process meticulously controlled by tissue inhibitors of metalloproteinases (TIMPs).[11–13] The activity of MMPs can be mitigated by increased TIMP concentrations, thus impacting the fluctuating equilibrium between matrix degradation and tissue remodeling.[14]

Natural products remain a crucial resource in the search for anticancer drugs, offering a diverse array of structurally unique molecules with considerable biological activity. *Millettia* species are known for their production of various bioactive secondary metabolites, especially chalcones, which possess considerable pharmacological potential.[15] *Millettia brandisiana* Kurz is a significant source of these compounds, particularly ovalitenin A, a chalcone containing an α,β-unsaturated ketone moiety, which has shown greater cytotoxic effectiveness than structurally similar compounds such as lonchocarpine. Previous studies have shown that ovalitenin A displays cytotoxic effects against several human cancer cell lines, including those derived from prostate (DU145), cervical (HeLa), chronic myelogenous leukemia (K562), hepatoma (HepG2), leukemia (HL60), and cholangiocarcinoma (KKU-M156) cancers.[15] Although ovalitenin A's cytotoxic properties have been established, its anti-metastatic potential and the specific mechanisms by which it operates in cholangiocarcinoma (CCA) are not yet fully understood. Notably, chalcones such as ovalitenone from *Millettia erythrocalyx* inhibit migration, invasion, and EMT in human lung cancer cells, suggesting that Millettia chalcones, including ovalitenin A, may also possess anti-metastatic activity in addition to cytotoxicity, supporting their investigation in CCA.[16]

This investigation sought to assess the anti-metastatic effects of ovalitenin A within CCA cells, while also elucidating the associated molecular mechanisms. Network pharmacology analysis facilitated the identification of shared targets between ovalitenin A and CCA. The significance of the predicted signaling pathway was subsequently evaluated through experimental methods. These findings could potentially provide new perspectives on the capacity of ovalitenin A as a natural agent for the suppression of metastasis in CCA.

2. Materials and Methods

2.1 Materials

Trichloroacetic acid (T6399-100G) and sulforhodamine B (SRB) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Tris (K7023-22) was obtained from Vivantis Technologies (Selangor Darul Ehsan, Malaysia). Polyvinylidene fluoride (PVDF) membrane was sourced from Merck Millipore Corporation, while RIPA lysis buffer was acquired from VMR International. Antibodies targeting β-actin (sc-47778), phosphorylated PI3K (p-PI3K; sc-293115), VEGF (sc-7269) and horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (sc-2357) were obtained from Santa Cruz Biotechnology (Dallas, TX, USA). Primers for *MMP-9, TIMP-1*, and *β-Actin* were acquired from Bio Basic Canada Inc. (Ontario, Canada).

2.2 Cell culture and treatments

The human cholangiocarcinoma (CCA) cell line KKU-452 was developed at the Cholangiocarcinoma Research Institute, Khon Kaen University.[17] Cells were cultured in Ham's F12 complete medium supplemented with 10% fetal bovine serum (FBS), 25 mM HEPES (pH 7.3), sodium bicarbonate, penicillin (100 µg/mL), and gentamicin (80 µg/mL). Cultures were maintained at 37°C in a humidified environment with 5% CO_2.[17]

2.3 Preparation of ovalitenin A

Ovalitenin A was graciously supplied by Prof. Chavi Yenjai, Faculty of Science, Khon Kaen University. Briefly, ovalitenin A was isolated from 5 kg of air-dried *M. brandisiana* roots extracted with 20 L of EtOAc. The 200 g crude extract was fractionated by silica gel column chromatography, and ovalitenin A (56.0 mg) was obtained from subfraction F5.7 via preparative TLC. Its structure was confirmed by ^{1}H and ^{13}C NMR spectroscopy.[15]

2.4 Cell viability assay

Cell viability was evaluated using the sulforhodamine B (SRB) assay.[18] In this procedure, 7,500 cells were plated in each well of 96-well plates and allowed to incubate overnight. Following this, the cells were treated with ovalitenin A at concentrations ranging from 0 to 10 µg/mL for periods of 24, 48, and 72 hours. After the treatment, the cells were fixed with 10% trichloroacetic acid, stained with 0.4% SRB, and the bound dye was then solubilized using 10 mM Tris base. Absorbance was measured at 540 nm using a Sunrise™ microplate reader (Tecan Group, Ltd., Switzerland). Cell viability was reported as a percentage of the untreated control, and the half-maximal inhibitory concentration (IC_{50}) values were determined using GraphPad Prism 8.

2.5 Wound-healing assay

For migration studies, 300,000 cells per well were seeded in 24-well plates and incubated overnight.[19] A linear scratch was made using a sterile pipette tip, and, images were recorded at 0 hour (t = 0). Cells were subsequently exposed to ovalitenin A (0.31, 0.63, and 1.25 µg/mL) and incubated for 24 hours. The wound closure was re-photographed, and migration was assessed with Image-Pro Plus software by assessing the relative distance of cell separation between the wound edges.

2.6 Cell adhesion assay

To evaluate cell adhesion, 25,000 cells per well were seeded in fibronectin-coated 96-well plates and subjected to ovalitenin A treatment (0.00–1.25 µg/mL) for 45 minutes. Adherent cells were fixed with cold methanol, stained with 0.25% crystal violet, pictures were captured, and the dye was solubilized for measurement at 540 nm using a microplate reader (Tecan Group Ltd., Switzerland).

2.7 Network pharmacology

The canonical SMILES of ovalitenin A was submitted to the SwissTargetPrediction database (http://www.swisstargetprediction.ch) to identify potential targets. Targets associated with cholangiocarcinoma were retrieved from the GeneCards database (https://www.genecards.org) using the keyword "cholangiocarcinoma." Overlapping targets between ovalitenin A and CCA were identified using VENNY 2.1 (https://bioinfogp.cnb.csic.es/

tools/venny). A protein–protein interaction (PPI) network was then constructed using the STRING v11.0 database (https://string-db.org) under *Homo sapiens* conditions with a confidence threshold of 0.9. The resulting networks were visualized and analyzed using Cytoscape software, and hub genes were identified using the CytoHubba plugin based on the degree centrality.

2.8 Western blot assay

Cells (250,000 cells/well) were seeded in 6-well plates and incubated overnight.[18] On the subsequent day, cells were treated with ovalitenin A at concentrations of 0.31, 0.63, and 1.25 µg/mL for 24 hours. Post-treatment, cells were lysed in RIPA buffer containing 1× Halt™ Protease Inhibitor Cocktail (Thermo Fisher Scientific, USA). The lysates were subjected to centrifugation, and the supernatants were collected. Protein concentrations were measured by the Bradford assay (Bio-Rad Laboratories, USA). Equal amounts of protein (25 µg) were separated using 10% SDS-PAGE and transferred onto PVDF membranes. Following blocking, membranes were incubated with primary antibodies overnight at 4°C, subsequently exposed to HRP-conjugated secondary antibodies. Protein bands were visualized using the ChemiDoc™ MP imaging equipment, and densitometric analysis was conducted with Gel-Pro Analyzer 4. Protein expression was standardized against β-actin.

2.9 Quantitative real-time polymerase chain reaction (qRT-PCR)

Cells (250,000 cells/well) were plated in 6-well plates and allowed to incubate overnight. The following day, the cells were exposed to ovalitenin A (0-1.25 µg/mL) for a duration of 24 hours. Total RNA was then extracted utilizing TRIzol reagent, adhering to the manufacturer's protocol. Subsequently, the RNA was reverse-transcribed into cDNA employing iScript™ Reverse Transcription Supermix (Bio-Rad Laboratories, USA). Quantitative PCR was performed using SYBR™ Green Master Mix on a QuantStudio™ 6 Flex system (Thermo Fisher Scientific, USA). The cycling parameters were established as follows: 95°C for 30 seconds, succeeded by 40 cycles of 95°C for 15 seconds and 60°C for 30 seconds, culminating in a final melt curve analysis. Relative mRNA levels were determined using the $2^{-}\Delta\Delta Ct$ method, with β-actin serving as the internal reference.

2.10 Statistical analysis

All experiments were conducted independently and in triplicate. Data are presented as the mean ± standard error of the mean (SEM). Statistical analyses utilized one-way ANOVA accompanied by Tukey's post hoc test, with $p < 0.05$ being set as statistically significant.

3. Results

3.1 Effect of ovalitenin A on CCA cell viability

The cytotoxic effects of ovalitenin A on KKU-452 cholangiocarcinoma (CCA) cells were evaluated via the sulforhodamine B (SRB) assay. Ovalitenin A treatment significantly diminished cell viability in a concentration-dependent manner (Fig. 1). Furthermore, the calculated IC_{50} values for ovalitenin A decreased with prolonged exposure (Fig. 1). These findings suggest that ovalitenin A effectively inhibits the proliferation of CCA cells. Consequently, subsequent experiments utilized concentrations below the IC_{50} to assess the compound's impact on cell migration and adhesion, in addition to investigating its effects on associated intracellular mechanisms.

3.2 Effect of ovalitenin A on CCA cell migration

The effect of ovalitenin A on cell migration was assessed by the wound-healing assay. Treatment with ovalitenin A markedly reduced the migratory ability of KKU-452 cells in a concentration-dependent fashion. After 24 hours, the wound gap remained broader at high concentrations (0.31–1.25 µg/mL; Fig. 2), with significantly impaired motility of the treated CCA cells.

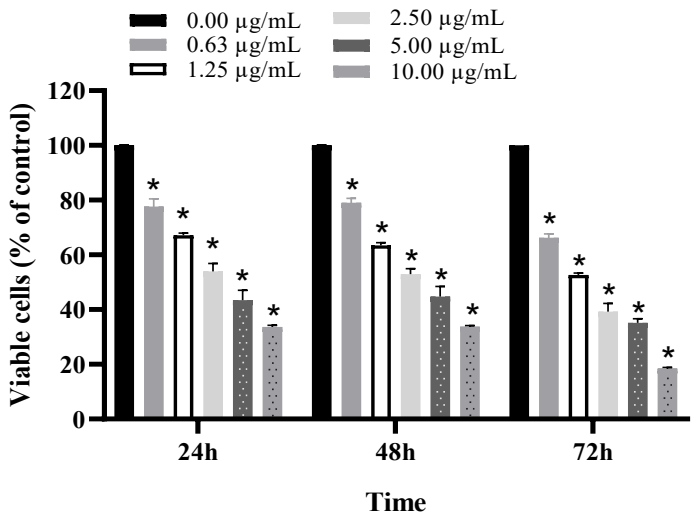
The data indicate that ovalitenin A significantly inhibits CCA cell migration.

3.3 Effect of ovalitenin A on CCA cell adhesion

The effect of ovalitenin A on cell adhesion was assessed by a short-term adhesion experiment. A 45-minute exposure to ovalitenin A significantly diminished the adhesive ability of KKU-452 cells in a concentration-dependent manner, with the percentage of adherent cells declining from 73.4% to 47.9% as the concentration increased from 0.31 to 1.25 µg/mL (Fig. 3). The findings indicate that ovalitenin A interferes with initial adhesive contacts critical for metastatic progression.

Table 1. Nucleotide sequences of primers used in qRT-PCR.

Genes	Forward primer	Reverse primer	PCR product
β-Actin	5'-GCACAGAGCCTCGCCTT-3'	5'-GTTGTCGACGACGAGCG-3'	92 bp
MMP-9	5'-ACTTGGTCCACCTGGTTCAA-3'	5'-GAAGATGCTGCTGTTCAGCG-3'	216 bp
TIMP-1	5'-GAGTGGGAACAGGGTGGACA-3'	5'-AGGCTCTGAAAAGGGCTTCCA-3'	150 bp



Time (h)	IC_{50} (µg/mL)
24	2.01 ± 0.40
48	1.86 ± 0.33
72	1.54 ± 0.50

Fig. 1. Effect of ovalitenin A on the viability of KKU-452 cells. Cells were pretreated with increasing concentrations of ovalitenin A (ranging from 0.63 to 10 µg/mL) for durations of 24, 48, and 72 hours and the IC_{50} values of ovalitenin A are presented in the insert table. The data are expressed as the mean ± SEM; $^{*}P < 0.05$ in comparison to the control group.

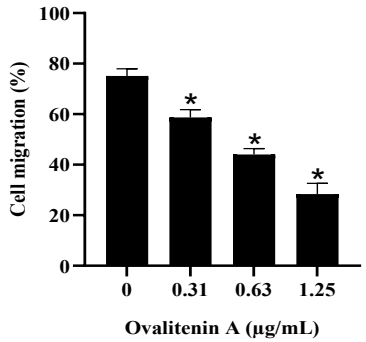


Fig. 2. Effect of ovalitenin A on the migratory behavior of KKU-452 cells. The data are expressed as the mean ± SEM; $^{*}P < 0.05$ in comparison to the control group.

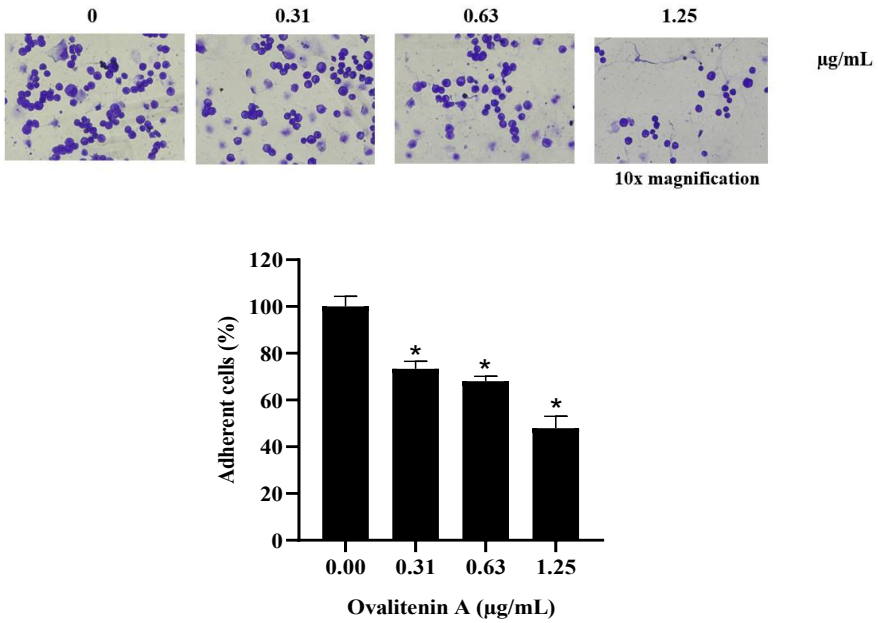


Fig. 3. Effect of ovalitenin A on the adherence of KKU-452 cells. The data are represented as the mean ± SEM; $^{*}P < 0.05$ in comparison to the control group.

3.4 Network pharmacology study of the targets of ovalitenin A

The SwissTargetPrediction database identified 101 possible protein targets for ovalitenin A, whereas the GeneCards database yielded 2,495 CCA-related targets. The convergence of these datasets revealed 32 common targets (Fig. 4A). A protein–protein interaction (PPI) network, derived from the STRING database, consisted of 32 nodes and 24 edges, indicating high-confidence interactions (Fig. 4B). The average node degree and clustering coefficient were 1.5 and 0.442, respectively, demonstrating functional interconnectivity among these targets.

3.5 Effect of ovalitenin A on the PI3K pathway

Western blotting revealed a significant reduction in phosphorylated PI3K (p-PI3K) expression, in a dose-dependent

manner, after 24 hours of ovalitenin A treatment (Fig. 5A). Moreover, time-course analysis revealed a progressive decline in p-PI3K levels at 6, 12, and 24 hours post-treatment with 1.25 µg/mL ovalitenin A (Fig. 5B). These findings suggest that ovalitenin A effectively suppresses the activation of the PI3K signaling pathway within CCA cells.

3.6 Effects of ovalitenin A on metastasis-related genes and proteins

RT-qPCR analysis showed that a 24-hour treatment with ovalitenin A significantly reduced *MMP-9* mRNA levels while increasing *TIMP-1* expression, leading to a lower *MMP-9*/*TIMP-1* ratio (Fig. 6A). In addition, Western blot analysis showed that ovalitenin A significantly decreased VEGF protein levels (Fig. 6B). Therefore, these results suggest that ovalitenin A might inhibit the spread of cancer in CCA cells by affecting the PI3K-dependent regulation of MMP-9, TIMP-1, and VEGF.

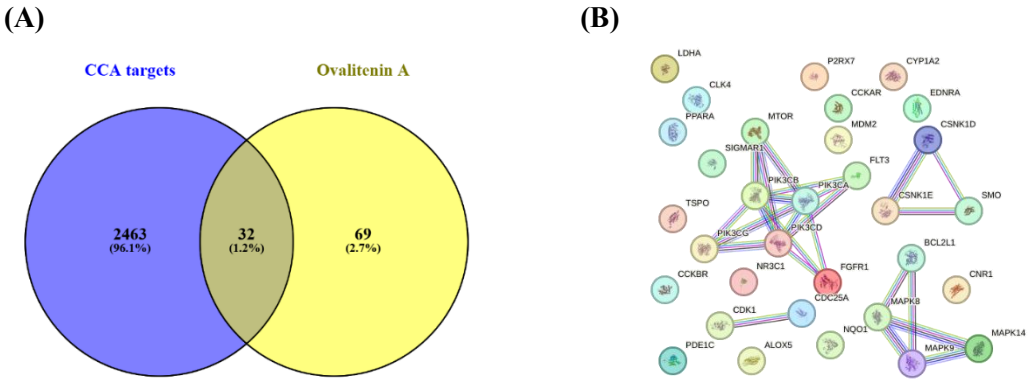


Fig. 4. (A) Venn diagram illustrating the intersection of ovalitenin A targets and CCA targets and (B) PPI network of 32 potential ovalitenin A targets against CCA obtained from the STRING database.

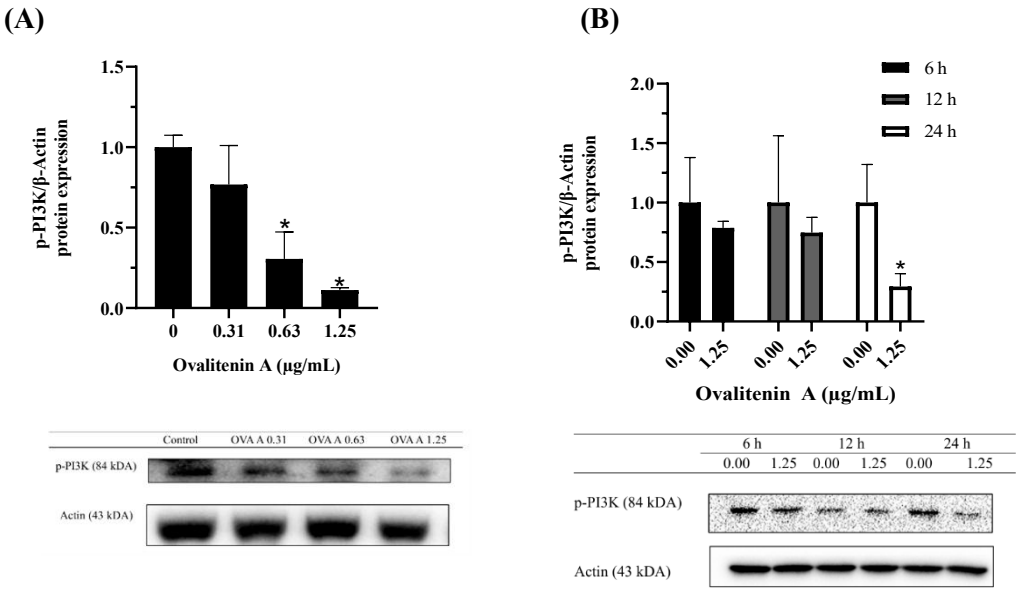


Fig. 5. Effects of ovalitenin A on p-PI3K protein expression in KKU-452 cells. Cells were treated with (A) ovalitenin A (0.31–1.25 µg/mL) for 24 hours, or with (B) 1.25 µg/mL ovalitenin A for 6, 12, and 24 hours. The data are expressed as the mean ± SEM; $^{*}P < 0.05$ in comparison to the control group.

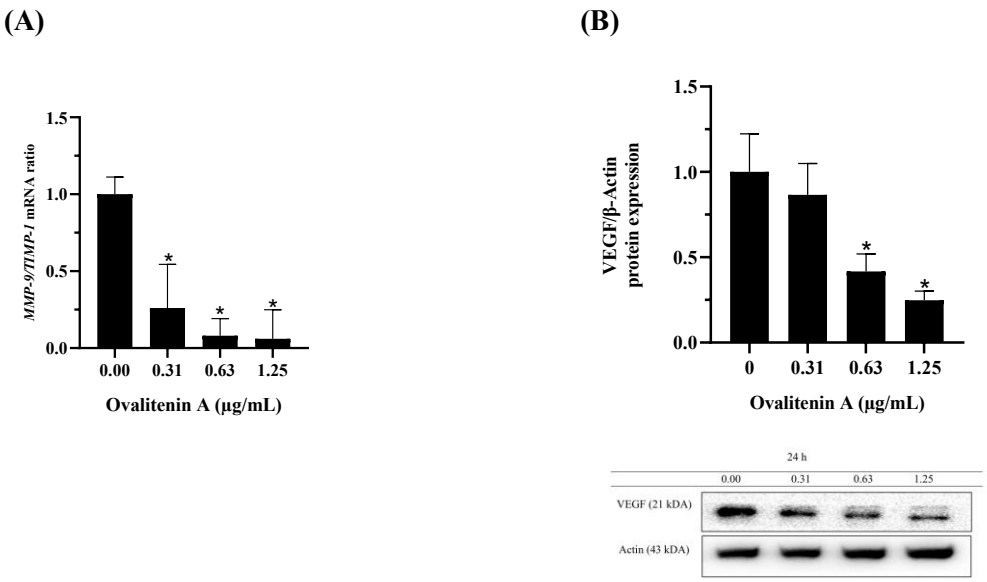


Fig. 6. (A) Effects of ovalitenin A on *MMP-9/TIMP-1* gene expression and (B) VEGF protein expression in KKU-452 cells. Cells were treated with ovalitenin A (0.31–1.25 µg/mL) for 24 hours. The data are presented as the mean ± SEM; $^{*}P < 0.05$ in comparison to the control group.

4. Discussion

CCA constitutes a significant public health concern owing to its aggressive nature and restricted treatment alternatives, especially in advanced or metastatic phases.[3,4] The inadequate results linked to existing chemotherapy regimens underscore the pressing necessity for innovative agents that can impede tumor progression and metastasis. Natural products have historically served as a prolific source of anticancer agents, with various bioactive metabolites from *Millettia* species exhibiting notable cytotoxic and anti-metastatic effects.[20,21] Among these, ovalitenin A, a chalcone extracted from *Millettia brandisiana* Kurz, has surfaced as a promising candidate owning to its robust cytotoxicity against diverse human cancer cell lines.[15]

In agreement with earlier studies, this research revealed that ovalitenin A substantially inhibited the proliferation, migration, and adhesion of CCA cells. The compound displayed a clear dose- and time-dependent cytotoxic effect, with IC_{50} values of 2.01, 1.86, and 1.54 µg/mL at 24, 48, and 72 hours, respectively. Comparable cytotoxicity was observed in KKU-213A cells (IC_{50} = 2.07 µg/mL, maximum cytotoxicity = 75.0%) and slightly reduced effects in non-malignant MMNK-1 cells (IC_{50} = 3.24 µg/mL, maximum cytotoxicity 51.1% at 10 µg/mL, unpublished data), suggesting a trend toward selective toxicity for CCA cells. These results are consistent with prior investigations and support the notion that ovalitenin A exerts anticancer effect on many types of CCA. Previous mechanistic studies have shown that ovalitenin A induces mitochondrial-mediated apoptosis through the modulation of COX-2, PARP-1, and Bcl-2/Bax expression, thereby validating its pro-apoptotic properties.[22]

Natural product-derived compounds frequently exhibit multiple biological actions, and ovalitenin A appears to follow this pattern. Our findings indicate that its anti-migratory and anti-adhesive activities are not solely attributable to cytotoxicity. At concentrations that reduced viability by approximately 25% and 32% (0.63 and 1.25 µg/mL), migration was inhibited by 47% and 60%, respectively, an effect notably

disproportionate to the degree of cytotoxicity and suggestive of targeted regulatory actions rather than nonspecific cellular stress. In line with this, adhesion was reduced by nearly 50% after only 45 minutes of 1.25 µg/mL treatment, underscoring its capacity to disrupt early steps of metastatic dissemination. To further minimize the influence of cytotoxicity, a lower concentration (0.31 µg/mL; ~10% viability reduction) was evaluated and still produced consistent inhibitory trends in migration and adhesion. These observations collectively support the view that ovalitenin A's anti-metastatic activities are not merely secondary to cell loss but involve specific functional modulation.

Network pharmacology is an approach that uses bioinformatic data to provide an overview of potential drug targets, based on the concept that compounds exert their effects through multiple targets and pathways rather than a single molecule.[23] In this study, we applied network pharmacology to predict the potential targets of ovalitenin A using integrated bioinformatic datasets. The analysis showed that the main protein–protein interaction (PPI) cluster was enriched with PI3K-related targets. Specifically, four of the seven proteins (PIK3CA, PIK3CB, PIK3CD, and PIK3CG) were directly involved in PI3K function. All four PI3K isoforms have been previously reported to be upregulated or mutated in various cancers, with PIK3CA being the most frequently reported; its activating mutations drive oncogenic signaling, while its inhibition has been shown to suppress cancer cell growth.[24] This suggests that ovalitenin A's effects in our experiments may be largely mediated through modulation of the PI3K pathway. Supporting this idea, Western blot analysis showed that ovalitenin A decreased the phosphorylated form of PI3K. This implies a reduction in PI3K signaling, which could contribute to the observed decrease in downstream processes related to cell migration and invasion. The PI3K pathway's hyperactivation has been extensively associated with the advancement of CCA, primarily through its role in promoting EMT, enhancing invasiveness, and extending the lifespan of neoplastic cells.[25] As a result, targeting this signaling pathway presents a plausible approach to achieving the observed anti-metastatic outcomes.

Ovalitenin A's ability to suppress PI3K signaling was associated with a decrease in VEGF expression. VEGF is important for tumor angiogenesis and metastasis because it increases blood vessel permeability and promotes the formation of an extracellular matrix, which supports the growth of endothelial cells.[10] A similar decrease in VEGF expression has been seen with cucurbitacin B in CCA cells, as well as with rice bran hydrolysates, which inhibit various pathways, including PI3K.[26,27] The consistent reduction of VEGF after PI3K inhibition highlights the importance of this pathway in regulating the potential for metastasis.

Furthermore, even at the lowest concentrations of ovalitenin A, where cytotoxicity is minimal, we observed robust suppression of PI3K and MMP-9/TIMP-1 expression, key drivers of cancer cell migration and metastasis. Ovalitenin A reduced the *MMP-9/TIMP-1* expression ratio, indicating an additional mechanism that enhances its anti-metastatic properties. MMP-9, a pivotal enzyme involved in extracellular matrix degradation, facilitates cancer cell invasion, while TIMP-1 functions as a natural inhibitor that preserves tissue integrity.[28–29] A lowered *MMP-9/TIMP-1* ratio indicates reduced matrix breakdown and invasive capacity.[30] These findings are consistent with previous research demonstrating that PI3K pathway suppression alters MMP/TIMP balance in multiple malignancies, including hepatocellular carcinoma.[31] A limitation of this study is the absence of protein-level validation for MMP-9 and TIMP-1. Future studies should include Western blot or ELISA analysis to confirm whether the observed transcriptional changes

translate into corresponding alterations at the protein level.

Notably, similar effects have been observed with ovalitenone, a related chalcone from *Millettia erythrocalyx*. In a recent study, ovalitenone inhibited the migration of lung cancer cells by suppressing the AKT/mTOR pathway and EMT, highlighting a comparable mechanism of anti-metastatic action.[16] These findings support the notion that chalcones from *Millettia* species, including ovalitenin A and ovalitenone, can effectively target key signaling pathways involved in tumor cell migration and invasion.

The findings of this investigation indicate that ovalitenin A suppresses the proliferation and metastasis of CCA cells, likely through the inhibition of PI3K signaling, which in turn diminishes VEGF and MMP-9/TIMP-1 expression. Ovalitenin A's ability to modulate key factors involved in tumor invasion underscores its potential as a lead compound for the development of anti-metastatic agents targeting CCA. Nevertheless, further research, particularly *in vivo* studies and analyses across diverse CCA models, is essential to confirm these mechanistic findings and assess the potential clinical uses of ovalitenin A.

5. Conclusion

This investigation reveals that ovalitenin A possesses significant anti-metastatic effects on cholangiocarcinoma cells. The substance demonstrated a concentration-dependent inhibition of cell proliferation, migration, and adhesion, concurrently down-regulating key molecular targets associated with metastasis. Ovalitenin A's action involved the suppression of PI3K phosphorylation and a reduction in VEGF expression, alongside a decrease in the MMP-9/TIMP-1 mRNA ratio, thereby suggesting a disruption of the PI3K-mediated signaling pathway. These observations imply that ovalitenin A may impede metastatic progression in CCA. While further validation in *in vivo* and clinical contexts is necessary, the present results underscore ovalitenin A as a potentially valuable candidate for the development of novel therapeutic agents aimed at CCA metastasis.

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Conflicts of Interest

The authors declare no conflict of interest in the manuscript.

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