RESEARCH ARTICLE

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Betulinic Acid Extracted from Guava (*Psidium guajava* L.) Leaves Inhibits Human Cholangiocarcinoma Cell Metastasis by Activating E-cadherin and Inhibiting MMP-9 and VEGF

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Abstract

Background: Cholangiocarcinoma, a bile duct cancer, is highly metastatic. Betulinic acid (BA), a pentacyclic triterpene glycoside found in the leaves of guava (*Psidium guajava* L.), exhibits various biological activities, including inhibition of cancer progression in multiple cancer cell types. However, the mechanism of action of BA in human cholangiocarcinoma (HuCCA) invasion and metastasis remains unclear. **Objective:** This study investigated the effects of BA on HuCCA cell apoptosis, invasion, and metastasis. **Methods:** The effects of BA treatment on apoptosis, invasion, and metastasis were determined using immunofluorescence staining, transwell invasion assay, wound healing assays, and Western blot analysis, respectively. **Results:** The findings revealed that BA effectively reduced metastasis and invasion in HuCCA cells. Mechanistically, this effect was associated with an increase in E-cadherin expression and a corresponding downregulation of MMP-9 and VEGF. Furthermore, BA-induced apoptosis was correlated with an increase in caspase-3 activity. **Conclusion:** These findings suggest that BA extract from guava leaves not only induces apoptosis but also actively counteracts invasion and metastasis by upregulating *E*-cadherin and suppressing the expression of MMP-9 and VEGF. Therefore, BA from guava leaves shows significant promise as a therapeutic agent against the development and metastasis of HuCCA.

Keywords: Betulinic acid- guava leaves- cholangiocarcinoma- metastasis- E-cadherin- MMP-9- VEGF

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Introduction

Cholangiocarcinoma is a type of cancer that affects the bile ducts [1]. Its etiology remains unclear, although it has been associated with liver fluke infection and other factors [2]. Cholangiocarcinoma is highly metastatic, contributing to its status as an incurable cancer, largely attributed to late diagnosis leading to severe disease with high invasion and metastasis rates, ultimately increasing mortality. Metastasis involves a series of biological and pathological processes, including the movement of cancer cells, their intravasation into the blood or lymph circulation, and subsequent extravasation to distant organs [3, 4]. Its initial step involves the loss of cell adhesion, with E-cadherin playing a critical role as a cell adhesion molecule in epithelial tissues [5]. Thus, reduced E-cadherin expression, observed in various cancers, is correlated with disease progression and metastasis [6]. Metastasis also requires detachment from the basement membrane and extracellular matrix (ECM), facilitated by cancer cell-produced proteolytic enzymes [7, 8]. Matrix metalloproteinases (MMPs), zinc-dependent endopeptidases, are key contributors to ECM detachment, correlating with cancer cell invasion and metastasis [9]. In particular, MMP-9 is crucial for ECM detachment and plays a role in angiogenesis and invasion during metastasis [10, 11]. Cancer development relies on new blood vessels providing nutrients and oxygen [12], and vascular endothelial growth factor (VEGF) serves as a significant proangiogenic factor [13] contributing to angiogenesis and lymphangiogenesis during cancer development and metastasis [14]. *Psidium guajava* L. (order Myrtales; family Myrtaceae) is a medicinal plant native to Hawaii, Malaysia, the Philippines, Taiwan, and Thailand [15]. Various parts of the guava are used in traditional medicine [16], with its phytochemicals including flavonoids, tannins, and terpenoids [17-19]. Guava leaf extract also contains terpenoids, particularly betulinic acid (BA) [20, 21]. BA is a pentacyclic triterpene that can be isolated from a wide range of plant species. It is particularly abundant in the outer bark of the Betulaceae family, such as *Betula alba*, *Betula pendula* [22-24], and *P. guajava* [21, 24]. This naturally occurring compound exhibits a broad spectrum of biological activities, notably its

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antibacterial, anti-inflammatory, antiparasitic, antiviral, and anticancer properties [25, 26]. Furthermore, BA has been demonstrated to inhibit the proliferation of various types of cancer [27]. BA extracted from guava leaves inhibits human cholangiocarcinoma (HuCCA) cell growth [21]. However, the antimetastatic mechanisms of BA in HuCCA cells remain unknown. In this study, we examined the impact of BA extracted from guava leaves on HuCCA cell invasion and metastasis. In summary, BA increased E-cadherin expression while reducing MMP-9 and VEGF expression, resulting in anti-invasion and anti-metastasis effects in HuCCA cells.

Materials and Methods

Chemicals and reagents

BA was reported by Phonarknguen et al. to be extracted from guava leaves [21]. The molecular structure of BA is shown in Figure 1. The cell culture reagents were procured from Invitrogen-Gibco (Grand Island, NY, USA). Antibodies for caspase-3, E-cadherin, MMP-9, and VEGF were acquired from Santa Cruz Biotechnology (Santa Cruz, CA, USA), whereas β-actin was sourced from Cell Signaling Technology. Secondary antibodies were procured from Abcam and Thermo Fisher Scientific.

Cell lines

HuCCA cells (CL6) were obtained from Associate Professor Dr. Adisak Wongkajornslip (Department of Pharmacology, Faculty of Medicine, Siriraj Hospital, Mahidol University). Dulbecco's modified Eagle medium was used to cultivate CL6 cells, supplemented with 10% fetal bovine serum, 100 IU/mL penicillin, and 100 µg/mL streptomycin. They were kept in a humidified incubator with 5% CO_2 at 37°C.

Cell migration analysis

The migration capacity of CL6 cells was evaluated through a wound healing assay. The cells were cultured in 6-well plates at a density of 5×10^6 cells/well and incubated overnight at 37°C. After removing the culture medium, a scratch was made using a 200 µL pipette tip. The cells were treated with varying concentrations of BA (50-800 µg/mL) after being washed with PBS to remove floating cells and debris. The wound area was observed for 0, 24, and 48 h post-treatment using a phase contrast microscope (40× magnification; Nikon Instruments Inc.). Quantification of cell migration distances was performed using ImageJ software, and the migration percentage was calculated. Migration data are presented as the mean ± SD from independent triplicate measurements (n = 3) carried out for all samples.

Cell invasion test

A transwell invasion assay was conducted using modified chamber inserts with an 8 µm pore size and Matrigel coating (Corning, NY, USA) in a serum-free medium. The upper chamber was seeded with CL6 cells (5×10^4) in serum-free medium and treated with various BA doses (50-800 µg/mL), whereas the lower chamber was filled with serum and medium. Following a 4 h incubation in 5% CO_2 at 37°C, the chambers were thoroughly washed three times with PBS, the sample was fixed in ice-cold methanol for 15 min, and then stained with crystal violet for another 15 min. Uninvaded cells were removed from the upper chamber using a cotton swab. The study utilized a light microscope (40× magnification; Nikon Instruments Inc.) to photograph and count invasive CL6 cells on the lower filter surface. The number of invasive cells was quantified using ImageJ software. The invasive data are presented as the mean ± SD from five independent experiments (n = 5).

Immunofluorescence assay

CL6 cells were cultured on cover slips and placed in 6-well plates at a density of 1×10^5 cells/well before treatment with varying BA concentrations (50–800 µg/mL). Cells were fixed with 4% paraformaldehyde in cold PBS after the medium was removed and washed with PBS. They were then incubated for 15 minutes at 37°C. Permeation of CL6 cells was observed by 0.25% Triton X-100 for 10 min, then blocked for an hour using 2% bovine serum albumin. Subsequently, they were treated with primary antibodies (caspase-3, E-cadherin, MMP-9, and VEGF) at 37ºC overnight, followed by secondary antibody treatment. Descriptions of primary and secondary antibodies are presented in Table 1. Coverslips were

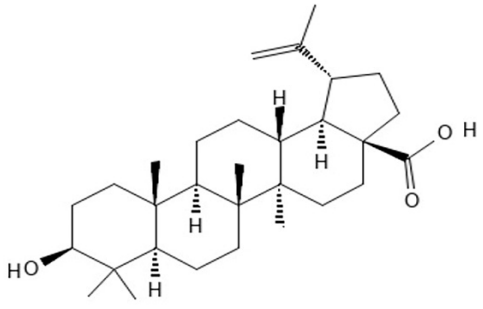

Figure 1. The Molecular Structure of Betulinic Acid (BA)

Table 1. Antibody Details for Immunofluorescence and Western Blotting

Antibodies	Dilution	Companies	Catalog number
Caspase-3	1/100	Santa cruz	sc-7148
E-cadherin	1/100	Santa cruz	sc-8426
MMP-9	1/100	Santa cruz	sc-13520
VEGF	1/100	Santa cruz	sc-7269
Donkey anti rabbit IgG	1/1,000	Thermo Fisher scientific	A-21206
Donkey anti mouse IgG	1/1,000	Abcam	Ab150105
β-actin	1/500	Cell signaling	4970s

placed on glass slides using antifade mounting medium containing 4',6-diamidino-2-phenylindole (DAPI; Vectashield, Vector Laboratories, Burlingame, CA, USA) before being viewed and imaged using a fluorescence microscope (400× magnification; Nikon Instruments Inc). Expression levels of active caspase-3, E-cadherin, MMP-9, and VEGF were quantified by measuring fluorescence intensity via ImageJ, with data normalized to the control group. Data are expressed as the mean ± SD, which sample independently analyzed in triplicate (n =3).

Western blot assay

CL6 cells were treated with BA doses ranging from 50 to 800 µg/mL after being seeded into 6-well plates at a density of 2×10^6 cells/well. Following treatment, whole cell lysates were prepared using ice-cold RIPA lysis buffer (Sigma-Aldrich) following the guidelines provided by the manufacturer. A BCA assay kit (Thermo Fisher Scientific) was used to measure the total protein concentrations. The JessTM Simple Western System (ProteinSimple, San Jose, CA, USA) was utilized for western blot analysis using an automated capillary-based size separation technique. Important proteins were found and measured using 12–230 kDa Jess separation modules (SM-W004; ProteinSample) following the directions given by the manufacturer. The primary and secondary antibodies used are listed in Table 1. Chemiluminescence detection was performed using peroxide/luminol-S (ProteinSample), and chemiluminescent images were analyzed using Compass Simple Western (version 5.0.1) software (Buil 0911, Protein Sample), which automatically determines the intensity of chemiluminescence. Relative densitometric analysis was performed following BA treatment. Data for positive cells and protein density are presented as mean ± SD, with all measurements independently performed in triplicate for each sample (n =3).

Statistical analysis

Data are shown as the means ± SD of three independent experiments. Statistical analyses were performed using GraphPad Prism version 8.0.1 (244), with one-way analysis of variance and the Sidak post hoc test employed to compare the treatment and control groups. Statistical significance was set at $p < 0.05$.

Results

BA induces caspase-3-mediated apoptosis

The study examined the impact of BA extracted from guava leaves on the apoptosis of HuCCA cells. Specifically, CL6 cells were treated with various BA concentrations, and apoptosis was assessed using immunofluorescence staining for caspase-3. We observed increased cytoplasmic localization in BA treatment compared to the untreated control (Figure 2A). Additionally, BA concentrations of 100–800 µg/mL significantly increased the caspase-3

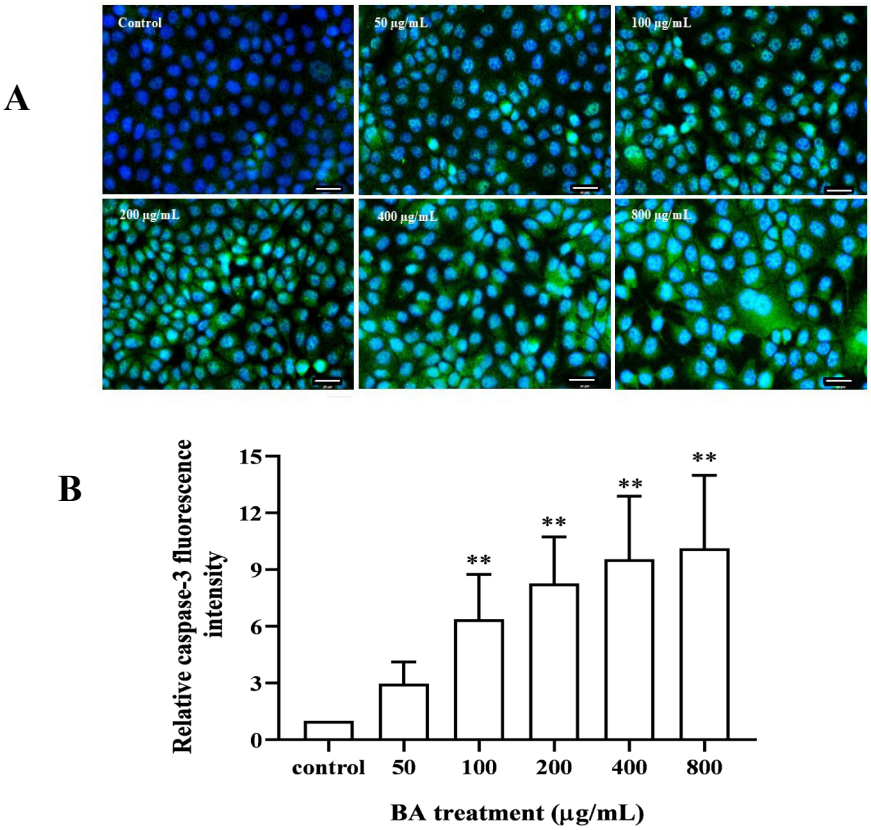

Figure 2. Quantitative Assessment of BA-Induced Apoptosis in CL6 Cells. (A) Representative immunofluorescence images showing DAPI-stained nuclei (blue) and active caspase-3 localized within the cytoplasm (green) in control and BA-treated cells. Scale bar = 20 µm. (B) Quantitative analysis of caspase-3 fluorescence intensity, expressed as mean ± SD, with each sample analyzed independently in triplicate (n = 3). ** indicates a statistically significant difference compared to controls ($p < 0.01$).

fluorescence intensity in a concentration-dependent manner relative to the untreated control (Figure 2B). Thus, BA-induced apoptosis in CL6 cells, with higher concentrations correlating with increased apoptotic cell proportions.

BA suppresses cell migration and invasion

The study examined the efficacy of BA in inhibiting CL6 cell migration and invasion. Migration analysis using a wound healing assay revealed that untreated control cells nearly filled the scratch area within 48 h, whereas BA-treated cells demonstrated impaired migratory capacity (Figure 3A). Moreover, BA concentrations of 100–800 µg/mL significantly inhibited CL6 cell migration at 24 and 48 h compared with untreated cells (Figure 3B). Transwell invasion assay revealed that exposure to BA (100–800 µg/mL) resulted in a significant, concentration-dependent reduction in the number of invading cells at 48 h compared to the control group (Figure 4A, 4B). Collectively, these findings demonstrate that BA exerts a dose-dependent inhibitory effect on the migratory and invasive abilities of CL6 cells.

BA Modulates E-cadherin, MMP-9, and VEGF Expression

To assess whether BA modulates metastasis-associated protein expression, CL6 cells were treated with various concentrations of BA (50–800 µg/mL) for 48 h. Protein expression levels were then evaluated by immunofluorescence and western blot analyses, with emphasis on key proteins associated with cell migration and invasion. BA treatment increased membrane localization of E-cadherin, as demonstrated by immunofluorescence staining compared with the control group (Figure 5A). BA (100–800 µg/mL) significantly increased the E-cadherin fluorescence intensity compared with untreated controls (Figure 5B). Western blot results showed that BA markedly upregulated E-cadherin expression (Figure 5C), with protein quantification indicating a significant increase at BA concentrations of 100–800 µg/mL compared with the control group (Figure 5D). Conversely, MMP-9 staining in the control group exhibited stronger cytoplasmic localization compared with the BA-treated group (Figure 6A). Treatment with BA (100–800 µg/mL) led to a significant reduction in theMMP-9 fluorescence intensity compared with the control group (Figure 6B). Western blot analysis confirmed that BA treatment effectively suppressed MMP-9 expression (Figure 6C), with quantification revealing significant decreases in protein levels at BA concentrations of 100–800 µg/mL compared with the control group (Figure 6D). VEGF staining by immunofluorescence was stronger in the cytoplasm of control cells than in BA-treated cells (Figure 7A). BA (100–800 µg/mL) significantly decreased the VEGF fluorescence intensity compared with untreated controls (Figure 7B). Western blot analysis revealed a marked decrease in VEGF expression following BA treatment (Figure 7C), and densitometric quantification demonstrated that VEGF protein levels were significantly reduced at concentrations of 100–800 µg/mL relative to control (Figure 7D).

Discussion

Cholangiocarcinoma, a major global health concern, is particularly prevalent in several Asian countries [28]. In Thailand's northeastern region, high incidences

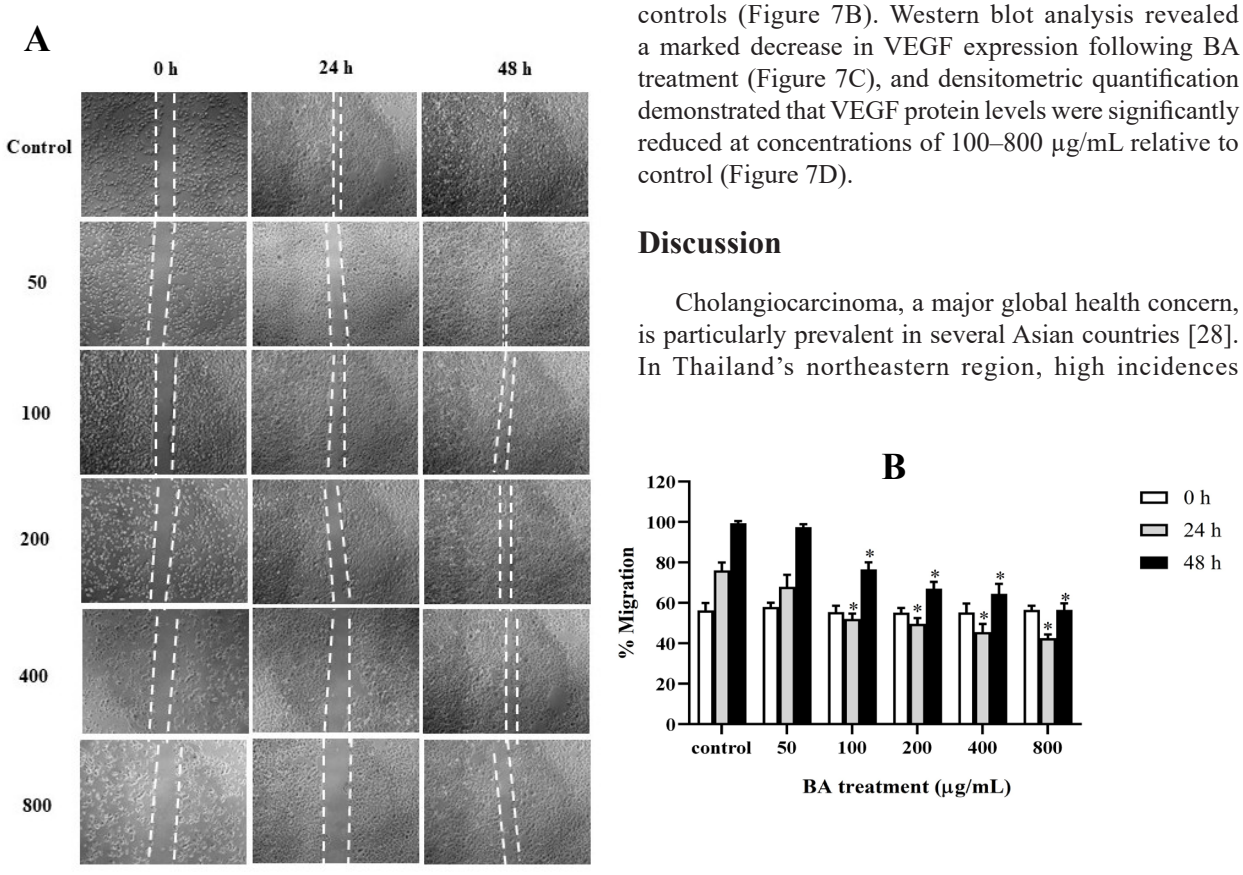

Figure 3. Wound-Healing Assays were Employed to assess the Effect of BA on CL6 Cell Migration. (A) The migration of the cells was viewed with a phase contrast microscope. Scale bar: 20 µm. (B) The percentage of cell migration was calculated relative to untreated control cells. Migration data are presented as the mean ± SD. All samples were analyzed using independent triplicate measurements (n = 3). Statistical significance was defined as $p < 0.05$ when compared to the control group (*).

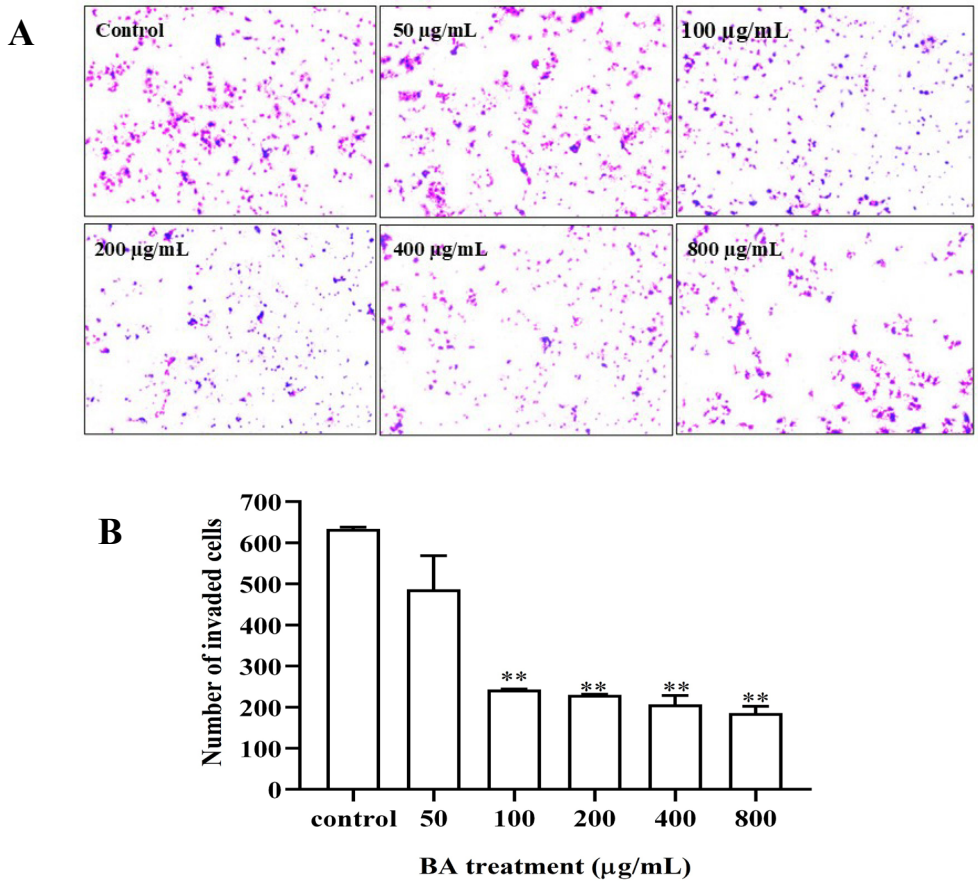

Figure 4. The Impact of BA on CL6 Cell Invasion was Evaluated Using Transwell Invasion Assays. (A) Cell invasion was observed after staining with crystal violet. Scale bar: 20 μm. (B) The number of CL6 cells that invaded was evaluated per field. Invasion data are presented as the mean ± SD from five independent experiments (n = 5). Statistical significance relative to the control group is denoted by an asterisk (**) at $p < 0.01$.

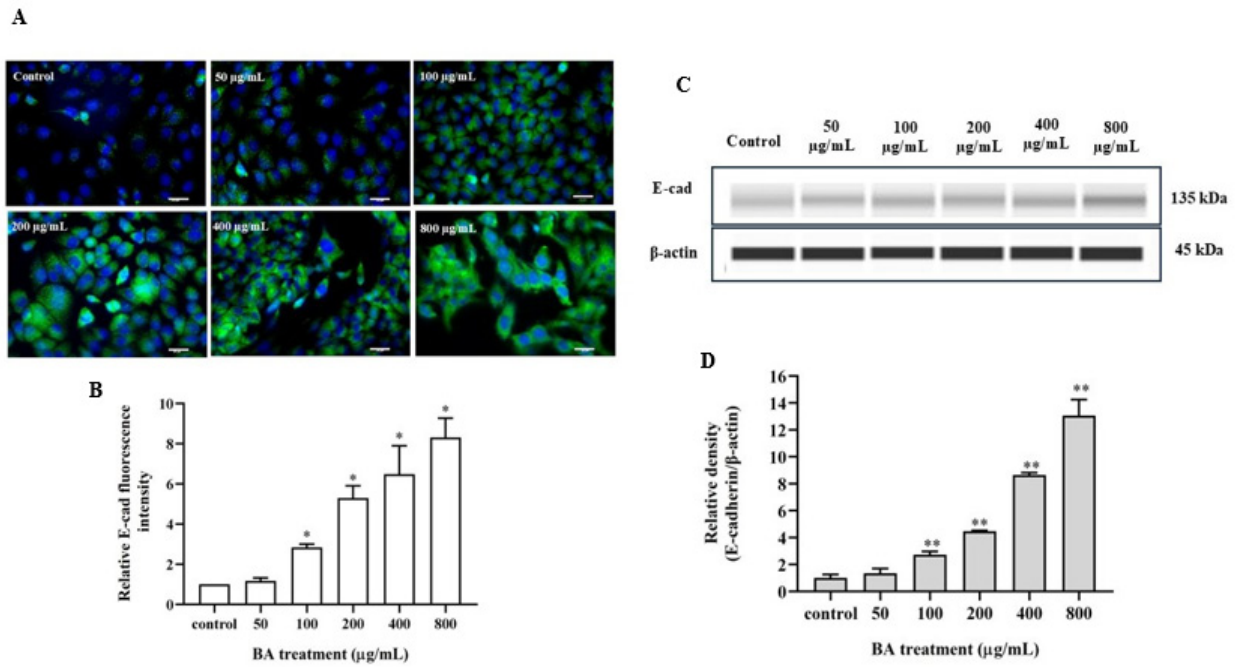

Figure 5. The Effect of BA on E-cadherin Protein Levels in CL6 Cells. (A) Representative immunofluorescence images of control and BA-treated cells; nuclei are counterstained with DAPI (blue), and active E-cadherin is localized at the membrane (green). Scale bar = 20 μm. (B) Quantitative analysis of E-cadherin fluorescence intensity. (C) Western blot analysis of E-cadherin expression levels. (D) Densitometric quantification of E-cadherin protein levels. Data are presented as mean ± SD from three independent experiments (n = 3). Statistical significance is indicated by * ($p < 0.05$) and ** ($p < 0.01$) relative to the control group.

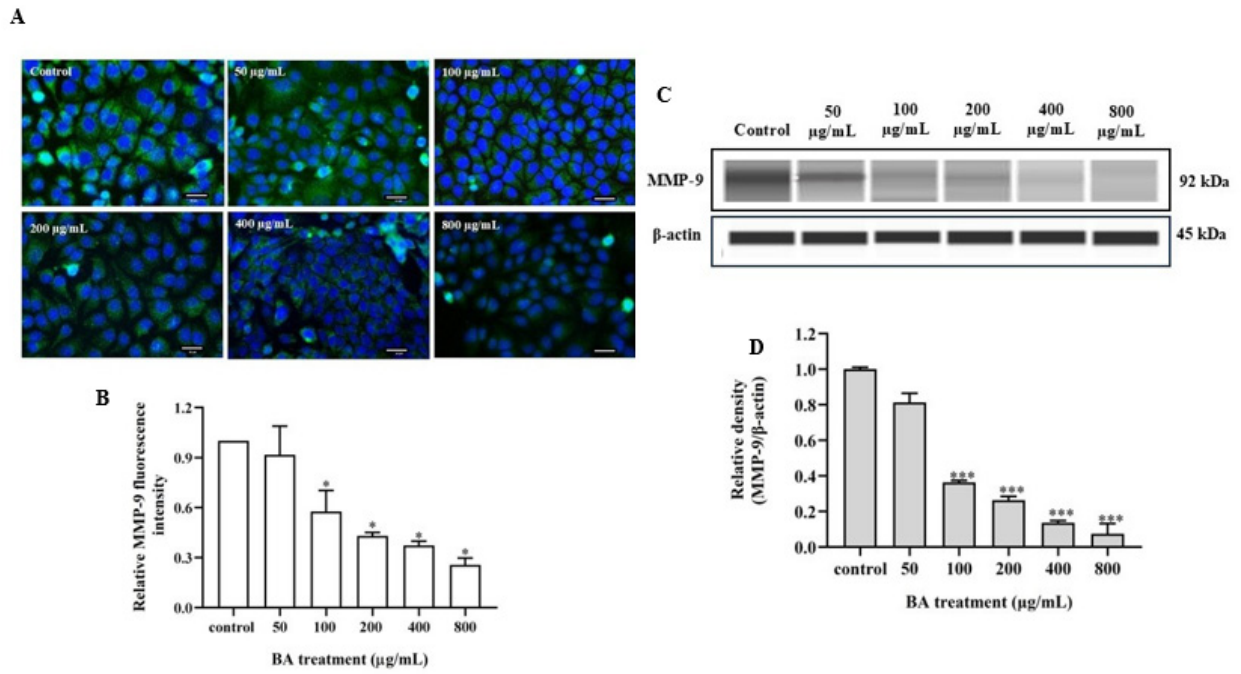

Figure 6. The Effect of BA on Protein Levels in CL6 Cells. (A) Representative immunofluorescence images showing DAPI-stained nuclei (blue) and active MMP-9 localized within the cytoplasm (green) in control and BA-treated cells. Scale bar = 20 µm. (B) Quantitative analysis of MMP-9 fluorescence intensity. (C) MMP-9 expression was determined by Western blot analysis. (D) Densitometric quantification of MMP-9 levels in response to BA treatment, expressed as mean ± SD, with each sample analyzed independently in triplicate (n = 3). Statistical significance is denoted by * ($p < 0.05$) and *** ($p < 0.001$), comparing the treatment groups to the control.

are linked to the consumption of raw freshwater fish, contaminated with liver fluke metacercaria cysts [2, 29]. Despite its increasing prevalence and mortality rates, current treatments for cholangiocarcinoma do not effectively extend patient survival. Thus, the development of new treatment options is imperative to increase cholangiocarcinoma treatment sensitivity, driving researchers' interest in discovering novel natural compounds with anticancer properties.

BA, as a natural pentacyclic triterpene glycoside

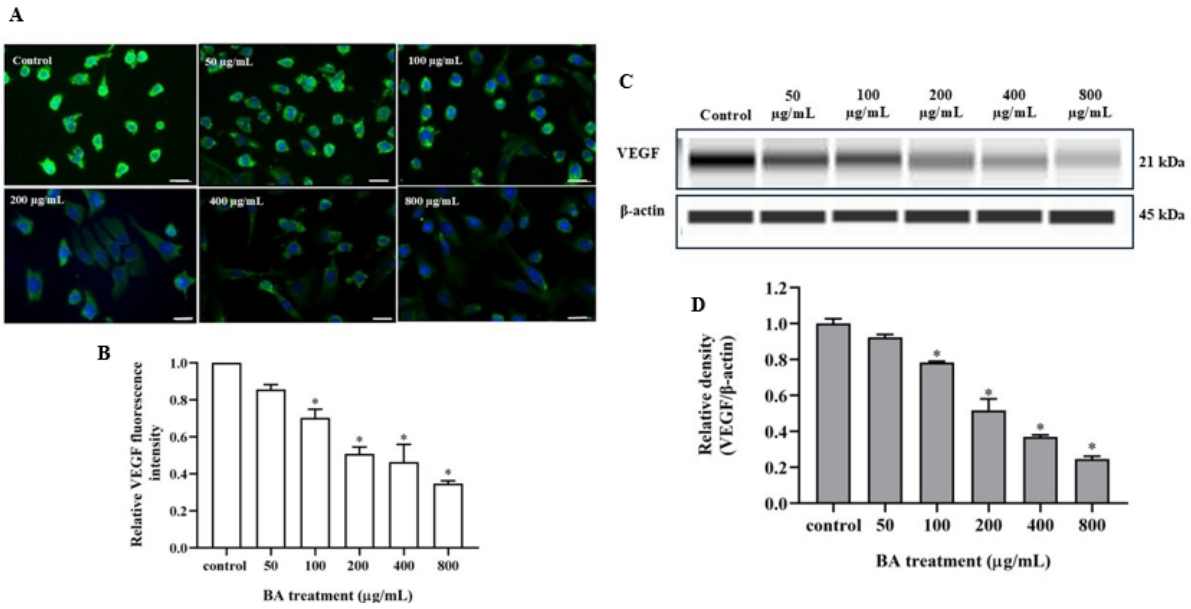

Figure 7. The Effect of BA on VEGF Protein Levels in CL6 Cells. (A) Representative immunofluorescence images showing DAPI-stained nuclei (blue) and active VEGF localized within the cytoplasm (green) in control and BA-treated cells. Scale bar = 20 µm. (B) Quantitative analysis of VEGF fluorescence intensity. (C) VEGF expression was determined by Western blot analysis. (D) Densitometric quantification of VEGF levels in response to BA treatment, expressed as mean ± SD, with each sample analyzed independently in triplicate (n = 3). Statistical significance is denoted by * ($p < 0.05$), comparing the treatment groups to the control.

extracted from several plant species [21-24], has been shown to prevent the growth of cancer [5]. Specifically, BA extracted from guava leaves is known to inhibit HuCCA cell growth without showing toxicity toward normal cells [21]. In the present study, we found that BA extracted from guava leaves effectively induced apoptosis in HuCCA cells while inhibiting their invasion and metastasis. Specifically, we observed that caspase-3 expression, associated with apoptosis, was elevated in BA-treated CL6 cells. caspase-3 plays a crucial role in the mitochondrial pathway of intrinsic apoptosis induction, a therapeutic target in cancer treatment. Thus, BA appears to induce apoptotic cell death through caspase-3 stimulation. These findings align with those of Lee et al. [30] regarding the ability of BA extracted from Cornus walteri to enhance caspase-3 expression. The results demonstrate BA's potential to induce apoptosis in HuCCA cells by utilizing the mitochondrial pathway.

Cancer cell migration and invasion are critical processes in metastasis across various cancers [31]. Therefore, we investigated the potential of BA to inhibit CL6 cell migration and invasion. Wound healing migration assay results revealed that BA treatment markedly reduced the migration efficiency of CL6 cells, resulting in a concentration-dependent decrease in the percentage of migrating cells. Additionally, transwell invasion assay results showed that BA effectively reduced CL6 cell invasion in a concentration-dependent manner. Taken together, the study reveals that BA effectively inhibits both the migration and invasion of CL6 cells. To elucidate the mechanisms underlying BA's antimetastatic effects on HuCCA cells, we examined metastasis-related proteins using immunofluorescence staining and western blot analysis. E-cadherin, a transmembrane protein that contributes to cell adhesion [32, 33], has been linked to both cancer invasion and metastasis [34-36]. Our study demonstrated that BA treatment significantly increased E-cadherin expression in CL6 cells, correlating with reduced migration and invasion effects. These findings suggest that BA inhibits CL6 migration and invasion, possibly by upregulating E-cadherin, thereby enhancing cell adhesion and tissue integrity. The results support earlier research indicating that diminished E-cadherin expression in cancer cells correlates with disease development [33]. This is because loss of E-cadherin compromises cell–cell adhesion, promoting increased cancer cell development, migration, invasion, and metastasis [37]. Conversely, elevated E-cadherin expression levels have been shown to reverse mesenchymal invasion to epithelial noninvasive phenotypes [38]. Several cancer cell types reportedly produce enzymes that can cleave E-cadherin, resulting in decreased cell adhesion and tissue integrity, which in turn supports cancer invasion and metastasis [39-41]. MMPs, proteolytic enzymes that degrade the ECM during metastasis, have been shown to regulate cancer growth and angiogenesis [42]. They also break down E-cadherin, producing extracellular fragments [43]. In particular, MMP-9 plays a crucial role in breaking down type IV collagen, one of the main parts of the basement membrane [44], and increased MMP-9 expression is a primary contributor to cancer invasion and metastasis [42]. Our results indicated that BA treatment decreased MMP-9 expression in CL6 cells, aligning with the findings of Wang et al. [45] and Zhang et al. [37], who found that chemically synthesized BA decreased MMP-9 expression levels. These findings suggest that BA effectively inhibits HuCCA cell invasion and metastasis by suppressing MMP-9 expression. VEGF is a cytokine that promotes angiogenesis in cancer, thereby enhancing cancer cell proliferation and metastasis [46-49]. VEGF expression has previously been linked to metastasis in several cancers, including colon, gastric, ovarian, and breast cancer, as well as osteosarcoma [45, 50]. Notably, Chen et al. [13] showed that inhibiting VEGF prevented nasopharyngeal cancer cell migration and invasion. In the present study, BA treatment effectively reduced VEGF expression in CL6 cells, resulting in decreased CL6 cell migration and invasion. Similarly, Chintharlapalli et al. [51] found that chemically synthesized BA decreased VEGF expression in prostate cancer cells. Gao et al. [52] highlighted the interplay among MMP-9, E-cadherin, and VEGF in cancer invasion and metastasis. Specifically, MMP-9-mediated E-cadherin degradation was associated with VEGF expression, which promotes angiogenesis. Our research discovered that BA treatment simultaneously decreased MMP-9 expression and increased E-cadherin expression, which helped maintain the integrity of the extracellular matrix (ECM) and basement membrane. Furthermore, BA therapy reduced VEGF expression, resulting in further inhibition of cancer invasion and metastasis. In summary, our findings confirm that BA inhibits HuCCA cell migration and invasion through a mechanistic pathway involving the upregulation of E-cadherin and the suppression of MMP-9 and VEGF. Future research must encompass the quantification of secreted VEGF levels through ELISA to attain a thorough comprehension of BA's impact on the tumor microenvironment, combined with in vivo validation to assess its viability as a therapeutic candidate for cholangiocarcinoma treatment.

Author Contribution Statement

Research design, K.A., R.P., and W.S.; methodology, K.A., R.P., and W.S.; investigation, K.A., R.P., and W.S.; data analysis, K.A., R.P., and W.S.; data curation, K.A., R.P., and W.S.; conclusion, K.A., R.P., and W.S.; writing original draft preparation, K.A.; project administration, K.A.; All authors have read and agreed to the published version of the manuscript.

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Conflict of Interest

The authors report no conflicts of interest.

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