

Ethyl acetate extract from *Inula helenium* L. inhibits the proliferation of pancreatic cancer cells by regulating the STAT3/AKT pathway

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Abstract. Sesquiterpene lactones are bioactive compounds that have been identified as responsible for the anticancer activity of the medicinal herb, *Inula helenium* L. (IHL). However, the mechanisms of action involved in the anti-pancreatic cancer activity of IHL have yet to be elucidated. The present study used an optimized extraction strategy to obtain sesquiterpene lactones from IHL (the resulting product termed ethyl acetate extract of IHL; EEIHL), and examined the potential mechanisms involved in the anti-pancreatic cancer activity of EEIHL. Ethanol and ethyl acetate were used to extract sesquiterpene lactones from IHL to give the final product EEIHL. Cell Counting Kit-8, colony formation and Annexin V/propidium iodide assays were used to detect the anti-proliferative activity of EEIHL. Cell migration was determined with a wound healing assay. mRNA and protein expression levels were analyzed by reverse transcription-quantitative polymerase chain reaction and western blot analyses, respectively. It was identified that low concentrations of EEIHL caused CFPAC-1 cell cycle arrest in the G₀/G₁ phase, whereas high concentrations of EEIHL induced mitochondria-dependent apoptosis. In addition, EEIHL could inhibit the phosphorylation of the signal transducer and activator of transcription (STAT)3/AKT pathway, potentially resulting in impeded cell mobility. In conclusion, EEIHL could activate mitochondrial-dependent apoptosis and inhibit cell migration through the STAT3/AKT pathway in CFPAC-1 cells.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive malignancies and may become the second leading cause of cancer-associated mortality by the year 2030 (1). The majority of PDAC patients are diagnosed at advanced and aggressive stage, when surgery is not an option (2). Therefore, systemic chemotherapy is crucial for PDAC therapy at advanced stages (3). Although great efforts have been dedicated into developing efficacious compounds against PDAC in preclinical and clinical studies, the 5-year median survival rate remains at ~5% and has not changed significantly during the past 40 years (4). Thus, novel therapeutic compounds are urgently required to combat this aggressive malignancy.

Inula helenium L. (IHL) is a traditional Chinese medicinal herb that has been used to treat intestinal tuberculosis associated-enterorrhagia, bronchitis and chronic enterogastitis, and has recently been identified to possess anticancer activity (5-7). Sesquiterpene lactones are a family of compounds that being identified responsible for the anti-cancer activity of IHL (8-10). However, existing methods have proved to be insufficient to extract sesquiterpene lactones from IHL. Ineffective components in the IHL crude extract would undermine the anti-cancer activity (11-13). In addition, the anti-proliferative activity of IHL against PDAC cells has yet to be reported.

The present study optimized the extraction process of IHL. The dried rhizome and root of IHL was first extracted with 95% ethanol, and yielded extracts were partitioned by ethyl acetate to give the final product, ethyl acetate extract of IHL (EEIHL). It was identified that EEIHL induced CFPAC-1 cell cycle arrest in the G₀/G₁ phase, and induced mitochondria-dependent apoptosis. In addition, EEIHL demonstrated potent anti-migration activity compared with isoalantolactone, possibly through downregulating the phosphorylation of signal transducer and activator of transcription (STAT)3 and AKT, thus indicating that EEIHL may possess anti-proliferative activity against PDAC cells.

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Key words: *Inula helenium* L., pancreatic cancer, cell cycle, apoptosis, signal transducer and activator of transcription 3/AKT pathway

Materials and methods

Extraction and characterization of EEIHL. *Inula helenium* L. was obtained from the Bozhou traditional Chinese medicine market (Anhui, China). The dried rhizome and root of *Inula helenium* L. (10.0 kg) were crushed into coarse powder. The powder was soaked with 95% ethanol for 48 h, and extracted with 20-fold 95% ethanol at room temperature by percolation. The combined extract was concentrated in a vacuum to give a crude extract. The crude extract was suspended in 1.5 l warm water and partitioned with ethyl acetate (3x1.5 l). Subsequent to evaporation in reduced pressure, 370.16 g EEIHL was yielded (yield, 3.7%). To verify that EEIHL contained bioactive compounds, a vanillin-sulfuric acid colorimetry assay was applied to establish sesquiterpene lactones in EEIHL (14).

High performance liquid chromatography (HPLC) chromatogram of EEIHL. EEIHL was run through an Acquity UPLC system with a BEH C18 column (25°C, 2.1x100 mm, 1.7 μ m; Waters Corporation, Milford, MA, USA). CH₃CN and H₂O were used as the mobile phase, and a gradient program was applied as follows: 0–2.5 min, 30% of CH₃CN; 2.5–4 min, 30–45% of CH₃CN; 4–7 min, 45% of CH₃CN; 7–8 min, 45–60% of CH₃CN; 8–9 min, 60% of CH₃CN; 9–10 min, 60–85% of CH₃CN; 10–12 min, 85% of CH₃CN; 12–13 min, 85–30% of CH₃CN; and maintained at 30% of CH₃CN for the next 3 min. The sample solution was 5 mg of EEIHL dissolved in 1.0 ml MeOH, and the injection volume was 1 μ l with the flow rate of 0.3 ml/min. The detection wavelength was set at 210 nm.

Proton nuclear magnetic resonance (¹H-NMR) analysis of EEIHL. The presence of alantolactone, isoalantolactone and alloalantolactone was verified by ¹H-NMR. A total of 10 mg of sample was dissolved in CDCl₃, and then detected by NMR. Tetramethylsilane was used as internal standard. The ¹H-NMR was performed with a Bruker Ultra Shield Plus 600 MHz spectrometer (Bruker Corporation, Billerica, MA, USA).

Materials and cell culture. RPMI-1640 medium and fetal bovine serum (FBS) were purchased from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The Cycletest Plus DNA Reagent kit was purchased from BD Biosciences (Franklin Lakes, NJ, USA). DAPI was purchased from Beyotime Biotechnology (Shanghai, China). The Annexin V-FITC Apoptosis kit was purchased from BestBio Company (Shanghai, China). A Mitochondrial Membrane Potential Assay kit was purchased from Signalway Antibody LLC (College Park, MD, USA).

The primary antibodies against cyclin-dependent kinase (CDK)2 (cat. no. ab32147), phosphorylated (p)-CDK2 (cat. no. ab76146), CDK6 (cat. no. ab124821), cyclin D1 (cat. no. ab134175), p-Rb (cat. no. ab184796), cyclin E1 (cat. no. ab135380), Bcl-2 (cat. no. ab32124), Bax (cat. no. ab32503), Bim (cat. no. ab32158), Mcl-1 (cat. no. ab32087), poly(ADP-ribose) polymerase (PARP; cat. no. ab191217), caspase-3 (cat. no. ab13847), X-linked inhibitor of apoptosis protein (XIAP; cat. no. ab21278), AKT (cat. no. ab8805), p-AKT (cat. no. ab38449), signal transducer and activator of transcription 3 (STAT3; cat. no. ab68153), p-STAT3 (cat. no. ab76315)

and β -actin (cat. no. ab8226) were purchased from Abcam (Cambridge, MA, USA). Primary antibodies were diluted at 1:1,000.

CFPAC-1 human PDAC cells were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured with RPMI-1640 medium containing 10% FBS and 1% penicillin/streptomycin at 37°C in a 5% CO₂ humidified atmosphere. EEIHL was dissolved in DMSO at 100 mg/ml.

Cell viability assay. The rate of cell proliferation was measured by a Cell Counting Kit-8 (CCK-8) assay (BestBio Company). Cells were cultured in 96-well plates at a concentration of 7x10³/well. Cells were cultured for 24 h and treated with the indicated concentrations of EEIHL. At 48 h of treatment, the supernatant was removed, 100 μ l of CCK-8 solution was added to each well and the plates were incubated for a further 2 h at 37°C. Cell viability was quantified by a Multiskan Spectrum spectrophotometer (Thermo Fisher Scientific, Inc.) by the optical density (OD) at 450 nm. Cell viability was calculated as [(OD₄₅₀ of treated cells/OD₄₅₀ of control cells) x100%]. Three independent experiments were performed.

Colony formation assay. CFPAC-1 cells were seeded into 6-well plates at a density of 1x10³/well and incubated for 24 h. The cells were then treated with the indicated concentrations of EEIHL. Following 24 h of treatment, the supernatant was removed and cells were cultured for a further two weeks. Then the cells were fixed with 4% paraformaldehyde for 15 min and stained with Giemsa solution for 15 min at room temperature. Visible colonies were imaged with a ChemiDoc XPS system (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Cell cycle analysis. Exponentially growing cells were seeded in 6-well plates (2x10⁵/well) and cultured overnight in a 5% CO₂ atmosphere at 37°C. Following treatment with EEIHL, CFPAC-1 cells were harvested and washed twice with cold PBS and then fixed in 70% cold ethanol at 4°C overnight. Cells were stained with the Cycletest Plus DNA Reagent kit (BD Biosciences), according to the manufacturer's protocol. Cell cycle distribution was analyzed using a flow cytometer (BD Biosciences). Data were collected by BD FACSDiva software version 8.0.1 (BD Biosciences) and analyzed by ModFit LT for Windows version 4.1.7 (Verity Software House, Topsham, ME, USA). Three independent experiments were performed.

Apoptosis assay. Exponentially growing cells were seeded in 6-well plates (2x10⁵/well) and cultured overnight in a 5% CO₂ atmosphere at 37°C. Following treatment with EEIHL for 48 h, the cells were harvested and washed with PBS. Then the cells were stained with the Annexin V-FITC Apoptosis kit, according to the manufacturer's protocol, and analyzed with a flow cytometer (BD Biosciences). Three independent experiments were performed.

DAPI staining. CFPAC-1 cells (8x10⁴ cells/well) were cultured in 24-well plates. Following exposure to EEIHL, cells were fixed with 4% paraformaldehyde for 20 min, and stained with DAPI for 15 min at room temperature. Following washing with

PBS, cells were observed under a fluorescence microscope (Ti-E; Nikon Corporation, Tokyo, Japan).

Detection of mitochondrial membrane potential. Mitochondrial membrane potential was visualized by the 5,5',6,6'-tetrachloro-1,1',3,3' tetraethyl-imidacarbocyanine iodide (JC-1) stain from the Mitochondrial Membrane Potential Assay kit. Cells were seeded into 6-well plates at a density of 2×10^5 /well, and cultured for 24 h. Following treatment, the cells were collected, washed with PBS, and incubated with JC-1 for 15 min at 37°C. Subsequent to washing, cells were immediately analyzed using a flow cytometer (BD Biosciences). Three independent experiments were performed.

Caspase-3 activity assay. CFPAC-1 cells (2×10^5 cells/well, 6-well plate) were incubated with EEIHL for 48 h. Cells were washed with PBS and lysed in cell lysis buffer included in the Caspase-3 assay kit. Caspase-3 activity in cell lysates were determined colorimetrically using a BioVision colorimetric caspase assay kit (BioVision, Inc., Milpitas, CA, USA). Chromophore conjugated peptides, including DEVD-p-nitroanilide (pNA) and VEID-pNA, were substrates for caspase-3, releasing pNA on caspase activity, which was quantified according to the manufacturer's protocol.

Western blot analysis. Following treatment with different concentrations of EEIHL, total proteins (40 μ g) were extracted using radioimmunoprecipitation assay lysing buffer (RIPA; cat. no. P0013B; Beyotime Institute of Biotechnology, Shanghai, China) and subjected to 12% SDS-PAGE prior to being transferred onto PVDF membranes (Bio-Rad Laboratories, Inc.). The membranes were blocked with 5% non-fat milk at room temperature for 1 h, and then incubated with specific primary antibodies overnight at 4°C. Subsequent to washing in TBST, membranes were incubated with the secondary antibodies (dilution 1:5,000; HRP Goat Anti-Mouse cat. no. A21010 and HRP Goat Anti-Rabbit cat. no. A21020; Abbkine, Wuhan, Hubei, China) at room temperature for a further 1 h. The protein bands were visualized using an ECL system WBKLS0050 (EMD Millipore, Billerica, MA, USA) and analyzed using Bio-Rad Laboratories Quantity One software version 4.6.2 (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Wound healing assay. Exponentially growing cells were seeded in 6-well plates (2×10^5 /well) and cultured overnight in a 5% CO₂ atmosphere at 37°C. Following a 24-h incubation, a 1-ml pipette tip was used to gently scratch the monolayer at the center of the well. Detached cells were washed away with PBS, and the remaining cells were cultured with serum-free medium and the indicated doses of treatment. Cells were incubated for 24 h prior to the capture of images (DS-Ri2; Nikon Corporation).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from cells with TRIzol (Life Technologies; Thermo Fisher Scientific, Inc.), precipitated with isopropyl alcohol, and rinsed with 70% ethanol. Single-strand cDNA was prepared from the purified RNA using PrimeScript RT Master Mix (cat. no. RR036A; Takara Biotechnology Co., Ltd., Dalian, Liaoning, China), followed by



Inula helenium L.

Extracted by
ethanol and
ethyl acetate

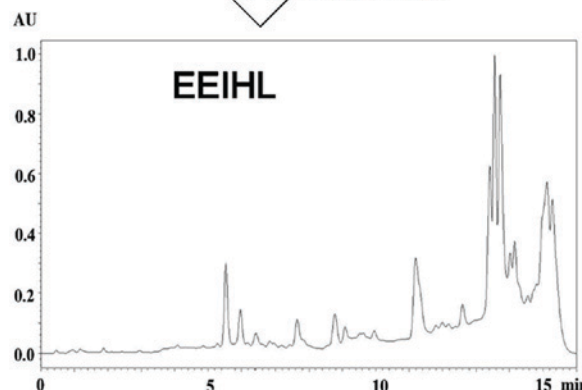


Figure 1. Image of *Inula helenium* L. Dried rhizomes and roots of *Inula helenium* L. were extracted with ethanol and partitioned with ethyl acetate. The resulting EEIHL product was analyzed by high performance liquid chromatography. EEIHL, ethyl acetate extract of *Inula helenium* L.

qPCR with SYBR-Green (Qiagen GmbH, Hilden, Germany) in the 7900HT Fast Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The thermocycling parameters were 94°C for 30 sec followed by 40 cycles of 58°C for 30 sec and 72°C for 30 sec, with one final cycle of 72°C for 90 sec. Three independent experiments were performed. The relative expression of target genes against the reference gene was obtained from $2^{-\Delta\Delta C_q}$ values (15).

The primers used were: E-Cadherin, forward, 5'-TTCTGCTGCTCTTGCTGTTT-3', reverse, 5'-TGGCTCAAGTCAAGTCCTG-3'; Snail, forward, 5'-GAAAGGCCTTCAACTGCAA-3', reverse, 5'-TGACATCTGAGTGGGTCTGG-3'; GAPDH, forward, 5'-GAGTCAACGGATTGTGGTCGT-3', reverse, 5'-TTGATTTTGGAGGGATCTCG-3'.

Statistical analysis. The results are expressed as the mean \pm standard deviation of at least three independent experiments. A two-sided Student's t test or one-way analysis of variance followed by a post hoc Fisher's least significant difference test was used to analyze the differences among groups. Graphs were prepared using SigmaPlot software version 12.0 (Systat Software, Inc., San Jose, CA, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Characterization of EEIHL. To extract sesquiterpene lactones from crude IHL roots efficiently, ethanol and ethyl acetate were

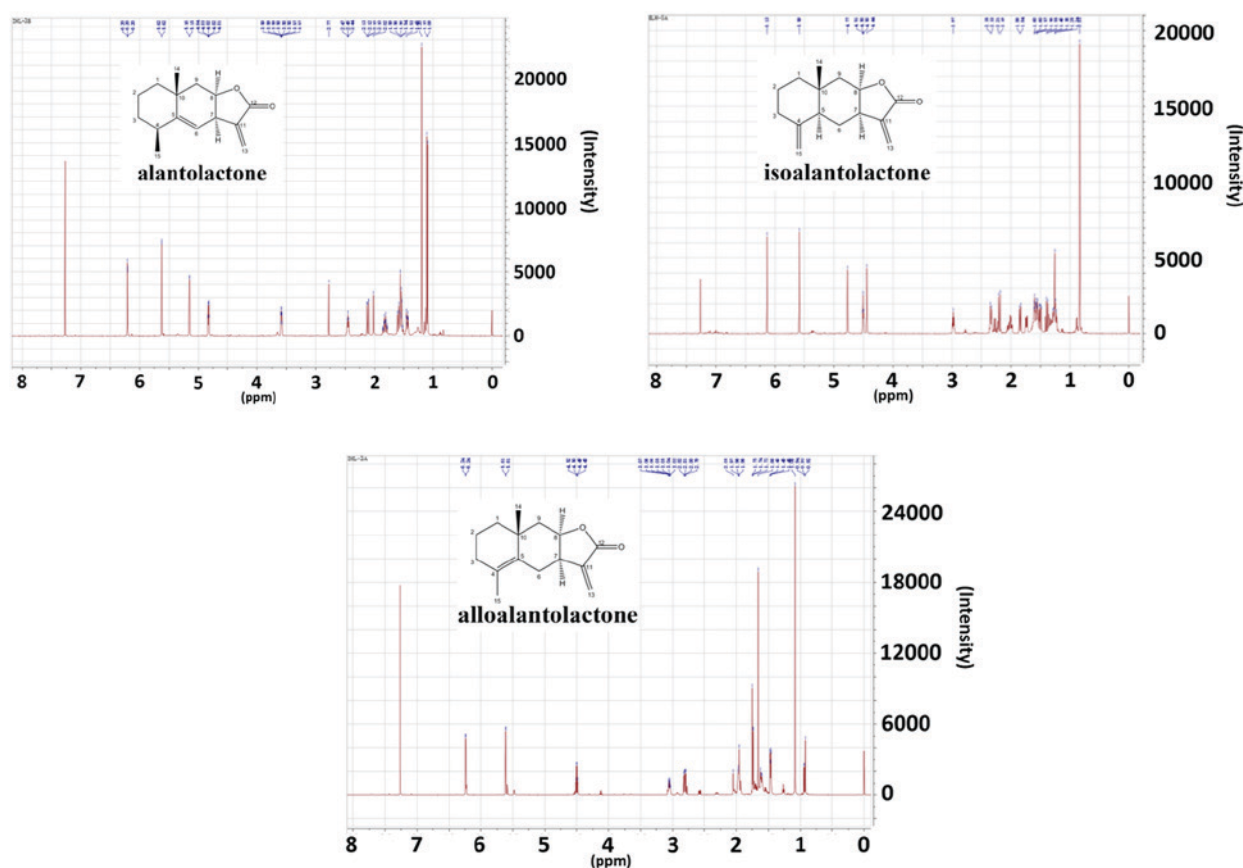


Figure 2. ¹H-NMR of alantolactone, isovalantolactone and alloalantolactone. A total of 10 mg sample was dissolved in CDCl₃ and detected with ¹H-NMR. The δ scale is relative to tetramethylsilane, an internal standard, at $\delta=0$. ¹H-NMR, proton nuclear magnetic resonance spectra.

used to partition IHL and produce the final product, EEIHL. A vanillin-sulfuric acid colorimetry assay was then applied to establish sesquiterpene lactones in EEIHL. The color of the mixture was purple or purple-red, indicating the existence of sesquiterpene lactones in EEIHL, as previously described (16). These three compounds were further confirmed by analysis with HPLC (Fig. 1). In addition, ¹H-NMR spectra were used to identify the presence and structure of alantolactone, isovalantolactone and alloalantolactone (Fig. 2).

EEIHL inhibited the proliferation of CFPAC-1 cells. The anti-proliferative activity of EEIHL against CFPAC-1 PDAC cells was determined by CCK-8 assay and colony formation assay. As shown in Fig. 3A, EEIHL and isovalantolactone dose-dependently inhibited the proliferation of CFPAC-1 cells at 48 h treatment. The inhibition rate was >90% following treatment with 6 μ g/ml of EEIHL for 48 h, and the half maximal inhibitory concentration (IC₅₀) was 4.3 and 2.8 μ g/ml for EEIHL and isovalantolactone, respectively. In addition, a relatively low concentration of EEIHL inhibited the formation of CFPAC-1 colonies (Fig. 3B). When cells were treated with 2 μ g/ml of EEIHL for 24 h and cultured for a further 2 weeks, the number of CFPAC-1 colonies was diminished. At concentrations of 1 or 2 μ g/ml, EEIHL demonstrated anti-proliferative activity after 24 h treatment. Therefore, EEIHL potentially inhibited the proliferation and colony formation of CFPAC-1 cells.

Low concentrations of EEIHL caused CFPAC-1 cell-cycle arrest at the G₀/G₁ phase. Following treatment with EEIHL for 24 h, CFPAC-1 cells were stained with propidium iodide (PI) followed by cell cycle analysis. As shown in Fig. 4A and B, 2 μ g/ml of EEIHL treatment resulted in a 15% increase in the proportion of cells in the G₀/G₁ phase. The protein expression of total CDK2 and p-CDK2 gradually decreased following treatment with EEIHL (Fig. 4C), whereas the protein level of cyclin D1 or cyclin E1 remained unchanged. Notably, pRb was downregulated following EEIHL treatment, indicating that EEIHL may downregulate CDK2 by affecting pRb.

High concentrations of EEIHL caused mitochondrial-dependent apoptosis in CFPAC-1 cells. As the EEIHL concentration was increased, CFPAC-1 cells displayed an increasing extent of mitochondrial-dependent apoptosis. As shown in Fig. 5A and B, 4 and 6 μ g/ml of EEIHL treatment induced 27 and 48% apoptosis, respectively, while untreated cells displayed an apoptosis rate of 6%. Meanwhile, 4 μ g/ml of EEIHL resulted in shrunken nuclei and intensified DAPI fluorescence (Fig. 5C).

To explore the role of the mitochondria in EEIHL-induced apoptosis, the JC-1 stain was applied to determine the mitochondrial membrane potential (Fig. 6). Following treatment with increasing concentrations of EEIHL, depolarized mitochondrial membrane potential gradually increased to 10.8% (Fig. 6A and B). Proteins determining mitochondrial fate were

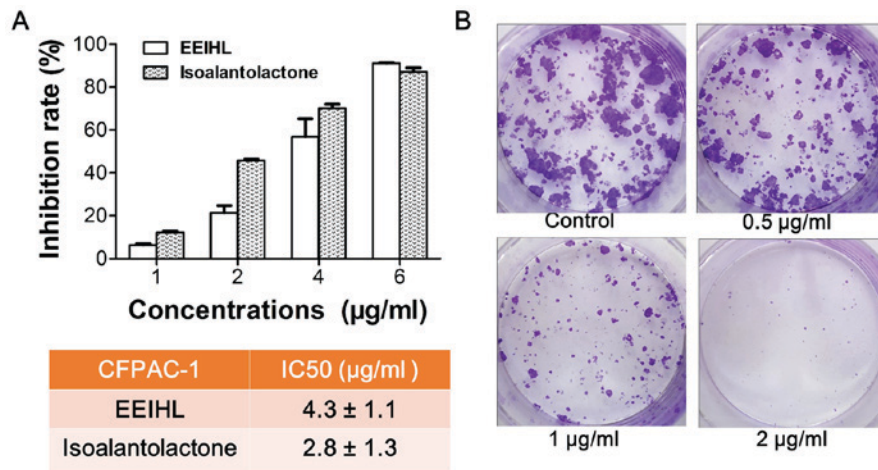


Figure 3. Anti-proliferative activity of EEIHL against CFPAC-1 pancreatic ductal adenocarcinoma cells. (A) A 48-h treatment of EEIHL dose-dependently inhibited the proliferation of CFPAC-1 cells. (B) CFPAC-1 cells were seeded into 6-well plates at a density of 1,000 cells/well and incubated for 24 h, exposed to EEIHL for 48 h, and cultured for a further 2 weeks. Cells were then fixed and stained with Giemsa solution prior to image capture. EEIHL, ethyl acetate extract of *Inula helenium* L.

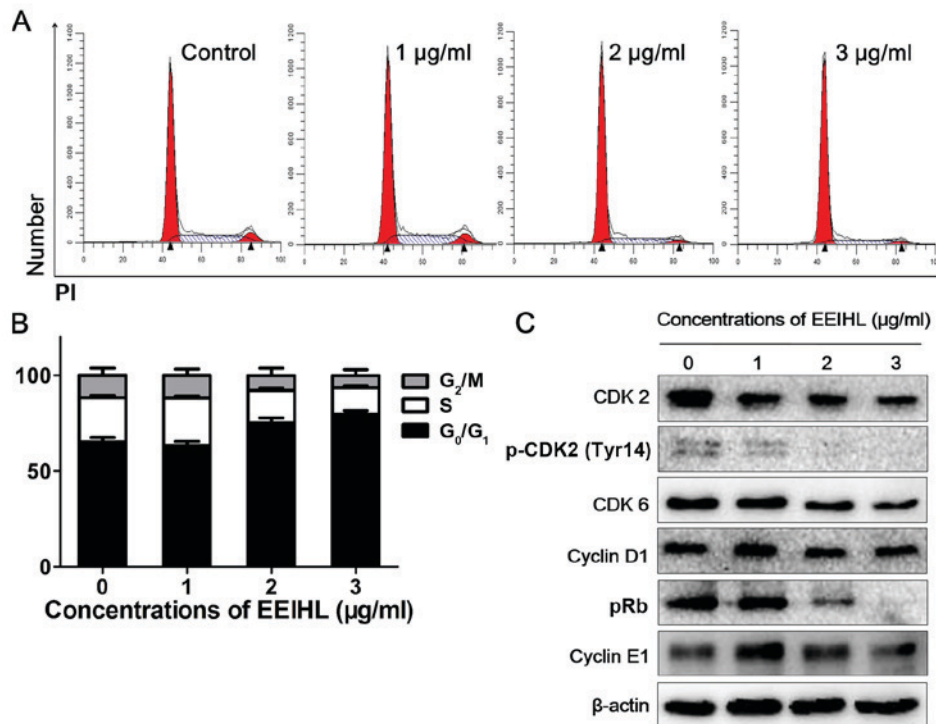


Figure 4. EEIHL induced G₀/G₁ cell cycle arrest in CFPAC-1 cells. (A) CFPAC-1 cells were seeded into 6-well plates (2x10⁵/well) and cultured for 24 h. Cells were then treated with the indicated concentrations of EEIHL for 24 h, followed by PI staining and flow cytometric analysis. (B) Quantitative analysis of cell cycle distribution. (C) Western blot analysis was used to analyze protein expression in CFPAC-1 cells following EEIHL exposure for 24 h. EEIHL, ethyl acetate extract of *Inula helenium* L.; PI, propidium iodide; CDK, cyclin-dependent kinase; p-, phosphorylated.

then analyzed by western blot (Fig. 6D). In accord with the depolarized mitochondrial membrane potential, pro-apoptotic protein Bim was evidently elevated, whereas anti-apoptotic proteins Bcl-2 and Mcl-1 were downregulated, suggesting the crucial role of the mitochondria in EEIHL induced apoptosis. Furthermore, as indicated by the appearance of cleaved PARP and decreased levels of XIAP, EEIHL induced caspase-dependent apoptosis; this was verified by determining the caspase activity following EEIHL treatment (Fig. 6C). In addition, EEIHL induced the decreased expression of p-AKT

and p-STAT3, suggesting alternative pathways involved in the anti-proliferation effect of EEIHL (Fig. 6D).

EEIHL inhibited CFPAC-1 cell migration. Cell migration was measured using a wound healing assay. When treated with 2, 4 or 6 µg/ml of EEIHL, the migration ability of CFPAC-1 cells was gradually attenuated (Fig. 7A and B). In comparison with isoalantolactone, 4 µg/ml of EEIHL was significantly more potent in impeding CFPAC-1 cell migration. Snail is a transcriptional factor that promotes the

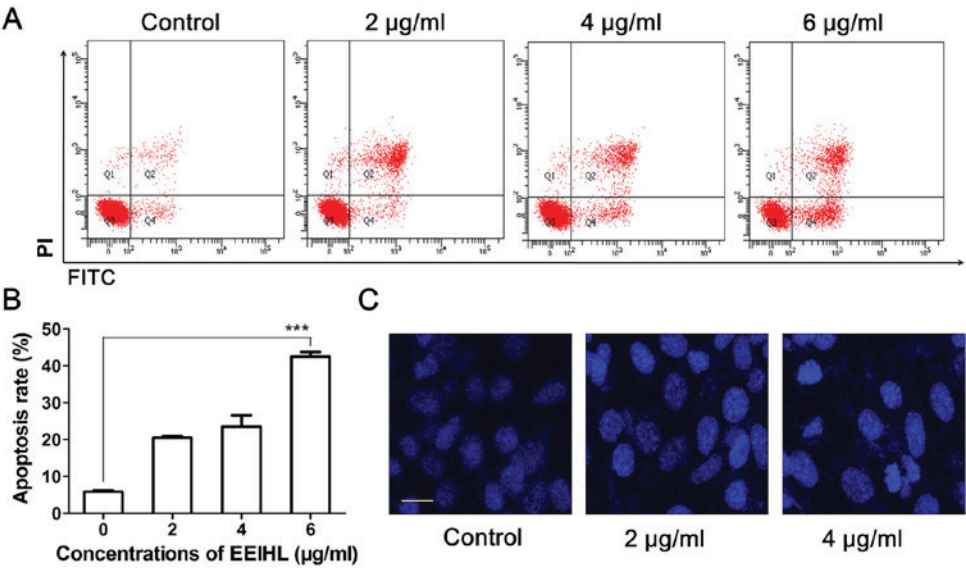


Figure 5. EEIHL induced apoptosis in CFPAC-1 cells. (A) CFPAC-1 cells were seeded into 6-well plates (2×10^5 /well) and cultured for 24 h. Cells were then treated with indicated concentrations of EEIHL for 48 h, followed by Annexin-V/PI staining and flow cytometric analysis. (B) Quantitative analysis of apoptotic cells following EEIHL exposure. (C) CFPAC-1 cells (8×10^4 cells/well) were cultured in 24-well plates. Following exposure to EEIHL, cells were fixed with 4% paraformaldehyde for 20 min, and stained with DAPI for 15 min. Scale bar, 20 µm. ***P<0.001. EEIHL, ethyl acetate extract of *Inula helenium* L.; PI, propidium iodide; FITC, fluorescein isothiocyanate.

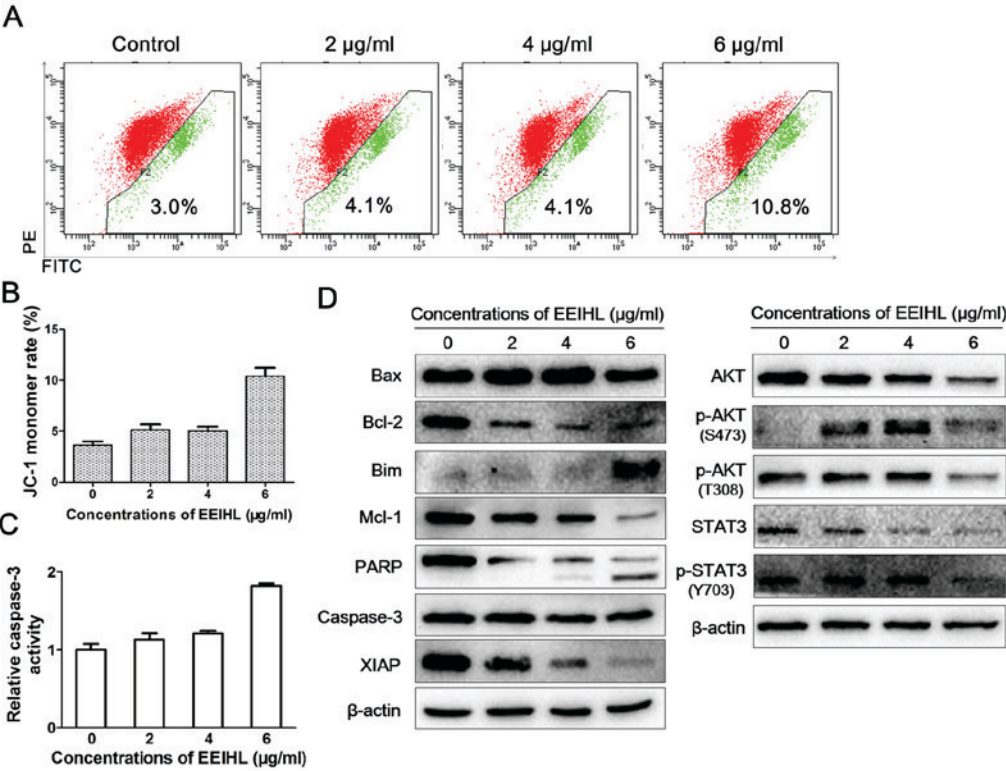


Figure 6. EEIHL promoted mitochondrion-dependent apoptosis. (A) CFPAC-1 cells were seeded into 6-well plates at a density of 2×10^5 /well, and cultured for 24 h. Following treatment with EEIHL for 24 h, the JC-1 stain was used to observe the depolarized mitochondria membrane potential by flow cytometry. (B) Quantitative analysis of depolarized mitochondria membrane potential. (C) CFPAC-1 cells were seeded into 6-well plates at the density of 2×10^5 /well, and cultured for 24 h. Following treatment with EEIHL for 48 h, caspase-3 activity in cell lysates were determined with a colorimetric assay. (D) Following treatment with indicated concentrations of EEIHL for 48 h, cells were lysed and proteins were extracted for western blot assay. EEIHL, ethyl acetate extract of *Inula helenium* L.; JC-1, 5,5',6,6'-tetrachloro-1,1',3,3' tetraethyl-imidacarbocyanine iodide; Bax, Bcl-2-like protein 4; Bim, Bcl-2-like protein 11; Mcl-1, Bcl-2-like protein 3; PARP, poly(ADP-ribose) polymerase; XIAP, X-linked inhibitor of apoptosis protein; STAT, signal transducer and activator of transcription; p-, phosphorylated.

repression of E-cadherin, which serves a key role in cell adhesion; the dysregulated transcription of Snail and E-cadherin is associated with the epithelial-mesenchymal transition (EMT) and tumor metastasis. As shown in Fig. 7C, the mRNA level

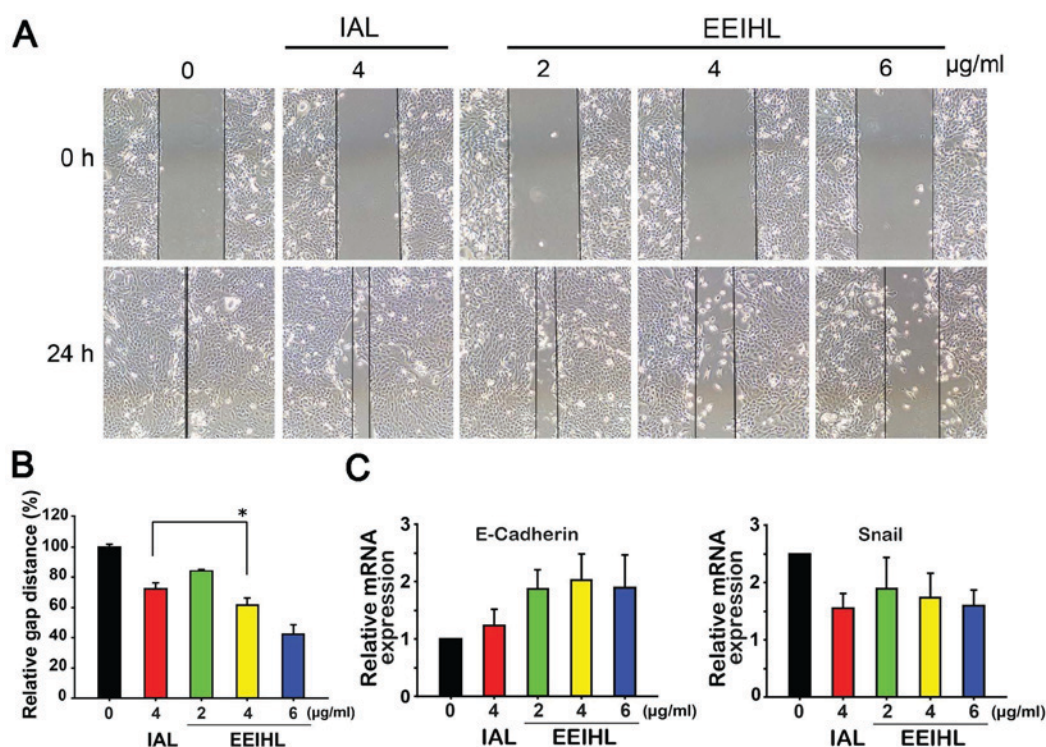


Figure 7. EEIHL inhibited CFPAC-1 cell migration through regulating epithelial-mesenchymal-transition-associated mRNA. (A) Cells were incubated for 24 h and were scratched by pipette tip across the center of the well. Following the removal of the detached cells, the remaining cells were allowed to grow for another 24 h before images were captured. (B) Quantitative analysis of wound lengths based on three independent experiments. (C) Following treatment with the indicated compounds for 24 h, total RNA was extracted followed by reverse transcription-quantitative polymerase chain reaction. * $P < 0.05$. EEIHL, ethyl acetate extract of *Inula helenium* L.; IAL, isoalantolactone.

of E-cadherin increased following EEIHL treatment to a greater extent than treatment with isoalantolactone, indicating the inhibitory effect of EEIHL on cell mobility. Meanwhile, the mRNA level of Snail was significantly downregulated, suggesting that EEIHL may also inhibit the Snail-induced regulation of EMT.

Discussion

Inula helenium L. is a traditional medicinal herb that may possess potent anti-cancer activity (5-7). As previously reported, sesquiterpene lactones are a class of chemical compounds responsible for the anti-cancer activity of IHL (17). Among those sesquiterpene lactones, alantolactone and isoalantolactone are the most extensively studied compounds obtained from the ethanol extracts of IHL (18,19). However, there remain other sesquiterpene lactones that may also exhibit anti-cancer activity but have yet to be intensively studied (20). For instance, isocostunolide was identified as inducing caspase-dependent apoptosis in melanoma cells, and its IC_{50} value was comparable to alantolactone or isoalantolactone (21). The anti-PDAC activity and potential mechanisms of sesquiterpene lactones are not fully understood. The present study optimized the separation strategy by extracting IHL with 95% ethanol and partitioning with ethyl acetate, aiming to maximize the content of the sesquiterpene lactones that exhibit anti-cancer activity.

Sesquiterpene lactones extracted from IHL have been reported to inhibit proliferation through inducing apoptosis, causing G_1 phase arrest or activating reactive oxygen

species (ROS) in several types of cancer, including breast cancer, leukemia and lung squamous cell carcinoma (22-24). To the best of our knowledge, there have been few studies regarding the effect on pancreatic cancer, apart from Khan *et al* (25) who identified that isoalantolactone could induce ROS-mediated apoptosis in PANC-1 cells. In the present study, EEIHL exhibited anti-proliferative activity against CFPAC-1 cells in a dose-dependent manner, without affecting the ROS level (data not shown) (26). Comparing the inhibitory effect of EEIHL with isoalantolactone, the 48 h IC_{50} value of EEIHL on CFPAC-1 cells was $4.3 \mu\text{g/ml}$, a similar level to isoalantolactone ($2.8 \mu\text{g/ml}$). However, the inhibitory effect of impurities could not be excluded in this preliminary study. Future studies, should isolate the mixture of alantolactone, isoalantolactone and alloalantolactone, and further quantify the ratio of these three major components with antitumor activity, with the aim to determine the exact mechanisms of action of EEIHL-induced apoptosis. Additionally, one cell line is insufficient to represent the phenotype of metastatic pancreatic cancer. Accordingly, the authors of the present study are working on using additional pancreatic cell lines, including BxPC-3 and Capan-1, and HPDE6-C7 immortal human pancreatic duct epithelial cells, to demonstrate the specific antitumor effect of EEIHL on pancreatic cancer cells.

Low concentrations of EEIHL induced CFPAC-1 cell cycle arrest at the G_0/G_1 phase rather than in S phase as in a previous study (27), indicating a distinct mechanism of action in CFPAC-1 cells. CFPAC-1 cells harbor allelic deletion in the SMAD4 gene, inducing the inactivation of

SMAD4. Loss of SMAD4 causes alterations to multiple kinase pathways, including the p38 and AKT pathways, and increases chemoresistance *in vitro* (28,29). The results of the present study indicated that high concentrations of EEIHL can downregulate the phosphorylation of AKT and STAT3, affecting mitochondrial apoptotic proteins, including Bim, Mcl-1 and Bcl-2. AKT is directly phosphorylated by PDK1 at T308, and forms an activation loop between SIN1, mTORC2 and p-AKT (T308), leading to the phosphorylation of AKT S473 by mTORC2 (30,31). In addition, it has been reported that STAT/AKT pathway inhibition could cause apoptosis in prostate cancer (32). In the present study, it was identified that p-AKT (T308) and p-STAT3 (Y703) decreased following exposure to 6 $\mu\text{g/ml}$ EEIHL, indicating STAT3/AKT inhibition following EEIHL treatment. To fully clarify the STAT3/AKT-associated mechanisms of EEIHL-induced apoptosis, it will be necessary to transfect cells with a hyper-phosphorylated STAT3 plasmid and knockdown AKT in a future study.

The present study compared the anti-migration activity of EEIHL with isoalantolactone by using a scratch wound healing assay. At a dose of 4 $\mu\text{g/ml}$, a 24-h treatment with EEIHL demonstrated an anti-migration effect comparable to isoalantolactone. Additionally, EEIHL treatment significantly increased the mRNA level of E-cadherin, decreased the transcription of Snail, and downregulated the phosphorylation of STAT3 and AKT. Saitoh *et al* (33) identified that STAT3 acted as a mediator that could enhance the induction of Snail at the mRNA level. It was previously identified that the STAT3/AKT pathway mediated EMT and its downstream target, Snail, in hepatocellular carcinoma (34,35). The function of STAT3/AKT/Snail in regulating EMT and thus, cell migration, has been established (36). Therefore, it was assumed that EEIHL could interfere with the phosphorylation of STAT3/AKT proteins, and downregulate the transcription of Snail, reducing the mobility of CFPAC-1 cells.

In conclusion, EEIHL produced anti-cancer activity in a concentration-dependent manner. Low concentrations of EEIHL induced G₀/G₁ phase arrest in CFPAC-1 cells, whereas high concentrations of EEIHL induced apoptosis, possibly via the downregulation of the phosphorylation of STAT3 and AKT. In addition, the inhibition of the STAT3/AKT pathway may affect downstream mRNA transcription, including of Snail and E-cadherin, leading to a reduced cell migration ability.

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