

Tetrastigma hemsleyanum (Sanyeqing) root extracts evoke S phase arrest while inhibiting the migration and invasion of human pancreatic cancer PANC-1 cells

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Research Article

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Abstract

Background: Ethyl acetate extracts from *Tetrastigma hemsleyanum* (Sanyeqing) (EFT), a member of the Vitaceae plant family, have been shown to exhibit efficacy against a variety of cancers. In this light, our current study seeks to examine the mechanism of efficacy between EFT extracts and human pancreatic cancer PANC-1 cells.

Methods: The chemical components of EFT were analyzed by gas chromatography–mass spectrometry. The cytotoxicity of EFT on PANC-1 cells was measured using an MTT assay. In order to investigate EFT induction of cell cycle arrest, changes in cell-cycle distribution were monitored by flow cytometry. Wound healing and transwell assays were employed to investigate whether migration and invasion of PANC-1 cells were inhibited by EFT. Relative protein expression was detected using Western blot.

Results: GC-MS analysis of the chemical composition of EFT revealed that the majority of constituents were organic acids and their corresponding esters. EFT exhibits measurable cytotoxicity and inhibition of PANC-1 invasion. Growth inhibition was primarily attributed to downregulation of CDK2 which induces cell cycle arrest in the S-phase. Inhibition of metastasis is achieved through downregulation of mesenchymal-associated genes/activators, including ZEB1, N-cadherin, Vimentin, and Fibronectin. Meanwhile, the expression of E-cadherin was significantly increased by EFT treatment. Furthermore, downregulation of MMP-2 and MMP-9 were observed.

Conclusion: Treatment of PANC-1 with EFT demonstrated measurable cytotoxic effects. Furthermore, EFT evoked S phase arrest while inhibiting the migration and invasion of PANC-1 cells. Additionally, EFT inhibited the epithelial to mesenchymal transition and MMPs expression in PANC-1 cells. This study serves to confirm the strong therapeutic potential of EFT while identifying the mechanisms of action.

1. Background

It is estimated that fatalities arising from pancreatic cancer will be second only to lung cancer by 2020. Overall 5-year survival rates for pancreatic cancer have remained unchanged for decades and patient prognosis remains poor despite efforts to better understand the tumor microenvironment [1–3]. Therefore, novel treatment strategies for pancreatic cancer are in dire need. Current chemotherapeutic regimens used in treating pancreatic cancer suffer from debilitating side effects which limit tolerable doses. Furthermore, many therapies exacerbate the mutation of cancers to metastatic and drug resistant phenotypes. Yet, the majority of modern cancer therapeutics are derived from plants [4]. Concerning anti-tumor effects, previous studies have reported that various natural products can demonstrate anticancer efficacy through inhibition of cell growth. Mechanisms of action range from inhibition of the cell cycle, apoptosis, migration inhibition and regulated expression of oncogenes [4–5].

Tetrastigma hemsleyanum Diels et. Gilg (Sanyeqing) is a rare, endangered medicinal plant native to China and traditionally used in folk medicine to treat a variety of cancers. Recent studies have found that extracts from Sanyeqing exhibited strong cytotoxicity toward a variety of cancers, including leukemia and

carcinomas of the lung, liver, stomach, and intestine [6–9]. Therefore, Sanyeqing shows strong therapeutic potential as an anticancer agent. Yet, exactly how Sanyeqing induces efficacy is not well understood. Therefore, the present study serves to investigate the possible mechanisms of ethyl acetate extracts from *Tetragium hemsleyanum* (EFT) cytotoxicity against PANC-1 such as cell cycle arrest and inhibition of metastasis.

2. Materials and methods

2.1 Antibodies and reagents

PANC-1 cells and BxPC-3 cells were purchased from the Stem Cell Bank, Chinese Academy of Sciences. Rabbit polyclonal antibodies against E-cadherin (1:1000, ab40772), CDK2 (1:1000, ab32147), Vimentin (1:1000, ab108333), Fibronectin (1:1000, ab2413), MMP-2 (1:1000, ab32124), MMP-9 (1:1000, ab76003) along with N-cadherin (1:1000, ab98952) were obtained from Abcam, England. Mouse monoclonal antibody against β -actin (1:1000, Mab1445), Goat anti-rabbit (1:1000, GAR0072) and Goat anti-mouse (1:1000, GAM0072) fluorescent marker conjugated secondary antibodies were obtained from MultiSciences Biotech, China. Rabbit polyclonal antibodies against ZEB1 (1:1000, #3396) were obtained from Cell Signaling Technology, USA. The BCA protein assay kit (P0011), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) cell proliferation and cytotoxicity assay kit (C0009) were obtained from Beyotime Biotech, China. Dulbecco's modified Eagle medium (DMEM) was obtained from HyClone, USA. RPMI-1640 was obtained from Gibco, USA. Fetal bovine serum was obtained from Sijiqing Biotech, China. Polyvinylidene fluoride (PVDF) membrane was obtained from Millipore Life Science, USA.

2.2 Preparation of extracts

Tetragium hemsleyanum roots were purchased from Pan'an in the Zhejiang Province of China, and authenticated by Dr. Shuili Zhang (College of Pharmaceutical Science, Zhejiang Chinese Medical University, Hangzhou, China). *Tetragium hemsleyanum* root was air dried for four days and dried in a hot air oven at 42 °C. Then the dried plant was ground to powder form. Two kilograms of the powdered *Tetragium hemsleyanum* root (particle size < 400 μ m) was refluxed in 75% ethanol three times, 2 h for each reflux. Three extractions were then combined and filtered. Subsequently, ethanol was removed using a rotary vacuum evaporator at 60°C under reduced pressure. The ethanol extract was suspended in water. Then the solution was sequentially partitioned with different solvents, including petroleum ether, diethyl ether, ethyl acetate and n-butanol. Ethyl acetate extracts from *Tetragium hemsleyanum* (EFT) was concentrated using a rotary evaporator. A stock solution of EFT was dissolved in dimethyl sulfoxide (DMSO), stored at 4 °C, and diluted to the desired concentration with DMEM before each experiment. The final concentration of DMSO was 0.1% (v/v) then filtered through a 0.22- μ m micro-filtrate membrane. The negative control was treated with medium containing 0.1% DMSO. The same DMSO concentration was used for each drug treatment.

2.3 Gas chromatography–mass spectrometry (GC–MS) analysis

GC–MS analysis (Thermo Fisher Scientific, USA) was performed to analyze EFT composition with HP-55% phenyl methyl siloxane capillary column (30 m * 0.25 mm * 0.25 µm). The carrier gas was helium at a flow rate of 1.0 ml/min with a split ratio of 50:1 (v/v). The temperature increased at a rate of 8°C /min from 30°C (hold 5 min) to 120 °C, then at a rate of 20°C min from 120 °C to 240°C (kept 10 min). The injector temperature was maintained at 230°C.

2.4 Cell culture and cell proliferation assay

The BxPC-3 and PANC-1 human pancreatic cancer cell lines were cultured in RPMI-1640 culture medium and DMEM culture medium, respectively, containing 10% fetal bovine serum (FBS) in a humidified incubator with 5% CO₂ at 37°C. Cells were plated in 96-well plates (at a density of 5×10³ cells/well) (corning, USA), incubated overnight, and treated with different fractions of *Tetrastigma hemsleyanum* at the concentrations of 0, 25, 50, 100, 200 and 400 µg/ml for 24h (or treated with different EFT at the concentrations of 0, 25, 50, 100, 200 and 400 µg/ml for 12, 24, 48, and 72 h). After treatment, cells were washed twice with phosphate-buffered saline (PBS) and incubated with 10 µl MTT (5mg/ml) and 80 µl DMEM at 37°C for 4 h. Then the supernatant was replaced by 150 µl DMSO to dissolve formazan. The absorbance was measured at 570 nm by a Microplate reader. The viability of PANC-1 cells was compared with relation to the control group (0.1% DMSO). Each assay was performed in triplicate. The percentage of cell growth inhibition was calculated as follows:

Cell viability inhibitory rate (%) = [A570 (control) – A570 (EFT)]/A570 (control) ×100%

2.5 Flow cytometric analysis of cell cycle

PANC-1 cells cultured in 6-well plates at a density of 5×10⁵ cells/ml were treated with EFT (50, 100, and 200 µg/ml) or 0.1% DMSO for 24 h. After treatment, cells were collected by centrifugation at 1000 g for 5 min. The pellet was washed with twice with PBS, fixed by 1ml of 70% cold ethanol and kept at – 20°C overnight. The ethanol was removed by centrifugation at 1000g for 5 min. Then cells were resuspended in PBS. Finally, cells were incubated in 50 µg/ml PI/RNase Staining Buffer in the dark at 37°C for 30 min and immediately analyzed by a flow cytometry (FC 500, Beckman, USA). MultiCycle software was used to analyze cell cycle data. Three independent experiments were performed in this analysis.

2.6 Wound healing assay

PANC-1 cells were seeded in six-well plates. When the cells formed a confluent monolayer, wounds were produced by scratching the middle of the well with a 200-µl pipette tip. The culture medium of PANC-1 cells was supplemented with EFT (50, 100, and 200 µg/ml) or 0.1% DMSO for 24 h. The initial wound photomicrographs were taken for comparison and to allow migration into blank areas for 24 h in PANC-1 cells. The percentage of wound closure was measured by light microscopy (Olympus, Tokyo, Japan). Experiments were conducted in triplicate. The migration distance was calculated as follows:

Migration distance = Edge distance at 0h - Edge distance at 24h

2.7 Migration and invasion assays

In order to assess cell migration *in vitro*, PANC-1 cells (1×10^4 in 500 μ L DMEM supplemented with 1% FBS) were seeded in the upper chamber a transwell migration chamber (8 μ m; Millipore Corp.). The lower chamber was filled with 750 μ L DMEM supplemented with 20% FBS used as the chemoattractant and indicated concentrations of EFT (50, 100 and 200 μ g/ml, 0.1% DMSO set as the vehicle). After 24 hours, cells in the lower chamber were fixed by methanol, stained with 0.2% DAPI for 15 min, 0.1% crystal violet for 30 min. After three washes with PBS, the samples were photographed in five independent, 100 X magnification fields for each well using a microscope (Olympus, Tokyo, Japan). Crystal violet was then dissolved with 33% acetic acid, and the absorbance was read at 570 nm. The cell invasion assay was performed similarly with matrigel-coated (BD Biosciences, USA) transwells. Experiments were repeated in triplicate for each experiment.

2.8 Western blotting

PANC-1 cells were seeded in 3.5 cm petri dishes (1×10^6 cells/ml). Then, PANC-1 cells were treated with 50, 100 μ g/ml EFT for 24 h and 200 μ g/ml EFT for 12, 24, 48 h (0.1% DMSO as a control). The total protein of treated cells was extracted with radio-immunoprecipitation assay lysis buffer (1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), and 1% sodium deoxycholate). Supernatants were collected and protein concentrations determined using a BCA protein assay kit. The lysates were boiled for 5 min at 100°C. Equal amounts of total protein were separated by 8–10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to PVDF, blocked with 5% nonfat milk in TBS-Tween buffer (pH 7.5, 0.12 M Tris-base, 1.5 M NaCl, 0.1% Tween-20) for 2 h at room temperature, incubated with the corresponding primary antibodies separately overnight at 4°C and then incubated with fluorescent marker-conjugated secondary antibodies (goat-antirabbit or goat-antimouse) at room temperature in the dark for 2 h. Detection was performed using an ECL system. Protein quantification was normalized to β -actin expression. ImageJ software was used to quantify band intensities.

2.9 Statistical analysis

The data are presented as the mean $\pm$ standard deviation from at least three independent experiments. Differences were analyzed using one-way analysis of variance (ANOVA) (three or more groups) or independent t-test (two groups). Statistical comparisons were performed with the SPSS 17.0 software (univariate analysis of variance). $P < 0.05$ was considered statistically significant.

3. Results

3.1 Chemical analysis of EFT composition by GC–MS

EFT composition was analyzed by GC–MS and different components were identified by comparing their retention times (RIs) with literature values and their mass spectrum with the data library (Fig. 1). As shown in Table 1, a total 36 components were identified, representing 93.20% of the total compounds. The major constituents were succinic acid (10.56%), benzoic acid, 2-borono-4,5-dimethoxy-, 1-methyl ester (8.65%), palmitic acid (12.02%), linoleic acid (14.37%), and 9,12,15-all-cis-Octadecatrienoic acid (9.46%).

Table 1
Chemical constituents of EFT analyzed by GC–MS.

Peak	Rt(min)	Molecular formula	Molecular weight	Compound	Area (%)
1	8.53	C ₉ H ₁₆ O	140	3,3,5-Trimethylcyclohexanone	0.74
2	8.99	C ₃ H ₆ O ₃	234	Lactic acid	1.42
3	13.07	C ₄ H ₁₀ O ₃	250	Diethylene glycol	0.25
4	13.88	C ₃ H ₈ O ₃	308	Glycerol	0.89
5	14.63	C ₄ H ₆ O ₄	262	Succinic acid	10.56
6	15.14	C ₃ H ₆ O ₄	322	2,3-dihydroxy-Propanoic acid	1.04
7	15.5	C ₁₁ H ₂₀ O ₃	200	5-Keto-2,2-dimethylheptanoic acid, ethyl(ester)	1.20
8	15.69	C ₄ H ₁₀ O ₃	322	1,2,4-Butanetriol	0.26
9	18.77	C ₇ H ₆ O ₃	282	Salicylic acid	3.12
10	24.48	C ₈ H ₁₂ O ₇	436	1,3-Dioxolane-4,5-dicarboxylicacid1,3-Dioxolane-4,5-dicarboxylicacid, 2-ethoxy-2-methyl-, (4R,5R)-	2.35
11	24.68	C ₁₂ H ₁₀ O ₅	438	Armillarisin A	3.28
12	24.82	C ₆ H ₁₀ O ₇	554	D-Glucuronic acid	3.31
13	24.9	C ₆ H ₁₂ O ₆	540	Dextrose	3.81
14	25.68	C ₁₁ H ₂₁ BO ₆	404	2-(1,3-Dioxolan-2-on-4-yl)-1-ethylboronic acid pinacol cyclic ester	0.24
15	26.48	C ₉ H ₈ O ₃	308	3-Phenylpyruvic acid	4.52
16	26.57	C ₃ H ₄ O ₄	320	Malonic acid	0.81
17	27.22	C ₁₈ H ₃₆ O ₂	284	Hexadecanoic acid, ethyl ester	0.24
18	27.77	C ₁₀ H ₁₃ BO ₆	420	Benzoic acid, 2-borono-4,5-dimethoxy-, 1-methyl ester	8.65
19	28.13	C ₁₆ H ₃₂ O ₂	328	Palmitic acid	12.02
20	28.85	C ₁₇ H ₃₆	240	Tetradecane, 2,6,10-trimethyl	0.45
21	29.57	C ₁₇ H ₃₄ O ₂	342	Methyl hexadecanoate	0.72

Peak	Rt(min)	Molecular formula	Molecular weight	Compound	Area (%)
22	29.81	C ₂₀ H ₃₆ O ₂	308	9,12-Octadecadienoic acid, ethyl ester	0.44
23	29.9	C ₂₁ H ₃₆ O ₄	352	9,12,15-Octadecatrienoic acid, 2,3-dihydroxypropyl ester	0.18
24	30.65	C ₁₈ H ₃₂ O ₂	352	Linoleic acid	14.37
25	30.74	C ₁₈ H ₃₀ O ₂	350	9,12,15-all-cis-Octadecatrienoic acid	9.46
26	31.02	C ₁₈ H ₃₆ O ₂	356	Stearic acid	1.28
27	33.38	C ₂₃ H ₄₆ O ₂ S	382	11-Eicosenoic acid, trimethylsilyl ester	0.30
28	33.72	C ₂₀ H ₄₀ O ₂	384	Eicosanoic acid	0.20
29	35.11	C ₁₆ H ₂₂ O ₄	278	1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester	0.34
30	35.75	C ₁₉ H ₃₈ O ₄	474	Hexadecanoic acid,(2S)-2,3-dihydroxypropyl ester	0.44
31	36.23	C ₂₂ H ₄₄ O ₂	412	Docosanoic acid	0.30
32	37.78	C ₂₁ H ₄₀ O ₄	500	9-Octadecenoic acid (Z)-, ester with 1,2,3-propanetriol	0.83
33	42.08	C ₁₉ H ₂₈ N ₄ O ₅	392	4-Azido-2-nitrobutyric acid, 2,6-di-t-butyl-4-methoxyphenyl ester	0.35
34	43.34	C ₂₈ H ₄₈ O	400	Campesterol	0.70
35	43.65	C ₂₉ H ₄₈ O	484	Stigmasterol	0.35
36	44.27	C ₂₉ H ₅₀ O	486	beta-Sitosterol	3.78

3.2 Cell proliferation cytotoxicity assay

To determine the efficacy of EFT against PANC-1 cells and BxPC-3 cells, an MTT assay was performed after incubating cells with the control (DMSO only), 25, 50, 100, 200, 400 µg/ml of EFT for 12, 24, 48 and 72 h. Cell viability and inhibitory rates were determined as seen from Fig. 2A and 2B. At very low doses (25 µg/ml), EFT showed no significant difference with the control cells in PANC-1 and BxPC-3 cells. The cell proliferation was inhibited in PANC-1 and BxPC-3 cells following treatment with 50 µg/ml EFT compared to the control ($P < 0.05$). The inhibitory effect was also found to increase in a time-dependent manner, with 72 h incubations demonstrating greater inhibition than earlier time points in PANC-1 and BxPC-3 cells ($P < 0.05$). The reduction in cell viability became constant at 48–72 h, the 50% inhibitory concentration of EFT (IC₅₀) at 72 h were 76.4 µg/ml and 175.9 µg/ml respectively in PANC-1 and BxPC-3 cells determined using SPSS 19.0 software (Fig. 2A and 2B).

3.3 Effects of EFT on cell cycle distribution in PANC-1 cells

To determine whether EFT induces cell cycle arrest, the distribution of cells in various phases of the cell cycle was determined by flow cytometry. It was found that treatment of PANC-1 cells with EFT at 50, 100 and 200 µg/ml for 24 h induced cell cycle arrest (Fig. 3A). EFT treatment resulted in S-phase arrest as indicated by an increase in the proportion of cells in the S-phase and a subsequent reduction of cells in the G0/G1 and G2/M cell populations ($P < 0.01$, $P < 0.05$) (Fig. 3B). These results demonstrated cell cycle arrest in the S phase under treatment of EFT. Subsequently, the levels of CDK2 were determined by western blot analysis. Results showed decreased expression of CDK2 compared with the control. (Fig. 3C and 3D). A course experiment (0, 12, 24, 48 h) with the maximum dose of 200µg/ml also showed a remarkable reduction in CDK2 expression ($P < 0.01$). (Fig. 3E and 3F).

3.4 EFT inhibited cell migration and invasion

To analyze how the migration and proliferation of PANC-1 cells occurred, a wound healing assay was employed. After a pipette tip was dragged across a surface of confluent cells, the migration distance could be examined. As shown in Fig. 4A, the migration distance was significantly decreased by EFT treatment (Fig. 4B, $P < 0.01$). In conjunction, transwell assays were conducted to examine whether migration could be inhibited by EFT. As shown in Fig. 4C and 4E, fewer cells were found to infiltrate the membranes with or without matrigel under the treatment of EFT, compared with untreated groups ($P < 0.05$) (Fig. 4D and 4F).

3.5 EFT inhibited mesenchymal properties and MMPs in PANC-1 cells

Considering that the mesenchymal transition is closely related to migration and invasion of cancer cells, key molecular markers of Epithelial-Mesenchymal Transition (EMT) were observed before and after EFT treatment. Cells were incubated with EFT at concentrations of 0, 50, 100, and 200µg/ml before analysis. It was shown that EFT significantly decreased the expression of mesenchymal-associated genes/activators, including ZEB1, N-cadherin, Vimentin and Fibronectin. Meanwhile, the expression of E-cadherin was significantly increased by EFT treatment (Fig. 5A and 5B, $P < 0.05$). Two key enzymes of Matrix metalloproteinases (MMPs) family (MMP-2 and MMP-9) were also evaluated after EFT treatment. Figure 5C and 5D illustrate a reduced expression of MMP-2 and MMP-9 following EFT incubation in a dose-dependent manner ($P < 0.05$). This result, however, reflects a change in the total expression of MMPs, further study should be involved to determine the changes of active MMPs. Yet, these results suggest that EFT is capable of inducing a pronounced inhibitory effect on the mesenchymal transition.

4. Discussion

Pancreatic cancer is a leading cause of morbidity and mortality in both developed and developing countries. Characterized by its aggressive biological nature and strong resistance against

chemotherapeutic treatment or radiation therapy, the 5-year survival rate of pancreatic cancer has shown little improvement in the past decade at < 5% survival. Because chemotherapy and radiation therapy produce detrimental side effects, an urgent need for treatment strategies has arisen for pancreatic cancer [1, 3, 10]. Recently, traditional Chinese medicines have garnered increasing attention due to their potential as anti-tumor agents. The majority of traditional Chinese medicines are derived from plant species, which have been used against cancer for centuries [4, 11, 12]. Yet, mechanisms underlying their efficacy remain unclear. Current research suggests that the mechanism of action occurs through cell cycle arrest, induction of apoptosis and inhibition of migration [7, 12–14]. Previous studies have shown that an ethyl acetate fraction of *Tetragium hemsleyanum* (EFT) can cause cell cycle arrest and induce apoptosis in human hepatoma HepG2 cells [6]. In this study, the underlying antitumor mechanisms were profiled in PANC-1 human pancreatic cancer cells treated with EFT.

Cell cycle control describes the regulatory process of cell growth that is dictated by coordinated interactions of various cyclins and their respective cyclin-dependent kinases (CDKs). Cyclins and CDKs together form various checkpoints that can either usher or inhibit progression of a cell through the cell cycle. If the key steps at each regulatory phase of the cell cycle are completed, cyclin/CDK interactions allow progression into the next phase of the cycle [15]. However, prolonged arrest at a particular checkpoint can occur due to genetic instability or mutation; both key contributors to cancer development [15–17]. Not surprisingly, a variety of anticancer agents arrest the cell cycle at particular checkpoints to induce apoptosis in cancer cells [13, 16]. As seen from Figure.3, S phase arrest was induced by EFT in a dose-dependent manner through downregulation of CDK2. This result indicates that the mechanism of EFT cancer therapy is, in part, due to the downregulation of CDK2 that induces cell cycle arrest and subsequent apoptosis. Yet, the mechanism of EFT is not solely limited to apoptosis. EFT has also shown potential to prevent migration and invasion of PANC-1 cells in a dose-dependent manner in Figure.4. Since pancreatic cancer is characterized by excessive migration and invasion, methods to specifically inhibit or reverse these malignant features are crucial in both basic and preclinical research [17–19]. Largely, EFT's anti-metastatic properties can largely be related to the epithelial to mesenchymal transition (EMT) and matrix metalloproteinases (MMPs).

EMT is a biological process where cells lose epithelial characteristics and obtain a mesenchymal phenotype, which improves cell motility by decreasing cell-cell adhesion [17]. Many studies point to the activation EMT as the critical mechanism for the acquisition of malignant phenotypes in epithelial cancer cells [19–22]. EMT is based on the down-regulation of epithelial markers such as E-cadherin while mesenchymal markers, such as ZEB1, Vimentin, N-cadherin and Fibronectin, are up-regulated. Therefore, EMT or mesenchymal properties are an ideal target for cancer therapy [23].

MMPs are a family of zinc-dependent endopeptidases essential for extracellular matrix degradation [24]. Among all MMPs, MMP-2 and MMP-9 are key to basement membrane type IV collagen degradation during cancer progression. Particularly MMP-2 and MMP-9 are well known for promoting tumor migration and invasion [25, 26]. Aside from direct regulation of basement membrane degradation, cross talk between MMPs and EMT can regulate the invasion or migration of cancer cells. It has been demonstrated

that overexpression of MMP-2 or MMP-9 led to induction of EMT in breast cancer [27]. Yet, a variety of EMT processes strengthen MMP expression in return, suggesting an intricate positive feedback loop between MMPs and EMT that synergistically contributes to migration and invasion in malignant tumors.

Therefore, strategies inhibiting both EMT and MMPs are an efficient approach to cancer therapy. As seen from Figure.5, downregulation of mesenchymal markers such as ZEB1, Vimentin, N-cadherin and Fibronectin and upregulation of epithelial markers such as E-cadherin demonstrate the reversal of the metastatic EMT phenotype induced by EFT. Furthermore, downregulation of MMP-2 and MMP-9 were also identified after EFT treatment. These results suggest that EFT interrupts the positive feedback loop between EMT and MMPs by downregulating key cytokines known for EMT while also downregulating MMPs. These results further contribute to the anti-invasion properties found of EFT against PANC-1 human pancreatic cancer cells.

In summary, pancreatic cancer remains one of the most lethal cancers with 5% of 5-year survival rate. Its poor prognosis is mainly attributed to the nature of the cancer which is both drug resistant and highly metastatic. For treatment of pancreatic cancer, EFT has been identified as a potent therapeutic. As seen from Table.1, GC – MS analysis has revealed that the chemical constituents of EFT are primarily organic acids and its esters, mostly palmitic acid (12.02%). Several studies have demonstrated that fatty acids possess anticancer activity [28–31]. Therefore, the high palmitic acid composition may be the chemical basis for EFT anticancer activity.

While EFT has shown potent efficacy against PANC-1 cells *in vitro*, its mechanism of efficacy is poorly understood. Studies performed herein demonstrated that EFT demonstrates significant cytotoxicity against PANC-1 through a cytotoxicity assay. Furthermore, EFT was found to inhibit PANC-1 migration through a wound healing assay. It was determined that EFT inhibits progression of PANC-1 through a collection of key properties. Firstly, EFT halts PANC-1 growth by inducing S-phase cell cycle arrest through downregulation of CDK2. Secondly, EFT tackles the issue of PANC-1 invasion and metastasis through a two-pronged approach. By increasing expression of cell-cell adhesion molecules, EFT substantially reverses the EMT, a key contributor to metastasis. Furthermore, EFT acts to downregulate MMP-2 and MMP-9 which are primarily known for inducing basement membrane degradation. By strengthening the basement membrane and promoting cell-cell adhesion, EFT interrupts the positive feedback loop between EMT and MMPs that are known contributors to the metastasis of PANC-1 cells. Based on the results of this study, the mechanisms by which EFT affects PANC-1 cells are summarized in Figure. 6.

5. Conclusion

This study confirmed the anti-cancer properties of EFT on human pancreatic cancer PANC-1 cells. EFT exhibits efficacy through multiple avenues; such as inhibition of cell growth, inhibition cell invasion, cell cycle arrest, inhibition of mesenchymal properties and MMPs. In conclusion, these results affirm that *Tetragium hemsleyanum* serves as a valuable therapeutic for the treatment of pancreatic cancer.

Declarations

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Availability of data and materials

The data will be accessible by contacting the corresponding author of this study.

Authors' contributions

Yifan Sun, Haiyan Qin, Chunchun Zhang, and Ting Zhang carried out GC-MS analysis of the chemical composition of EFT, participated in the cytotoxicity and cell cycle analysis, and drafted the manuscript. Yifan Sun and Haiyan Qin carried out the cell migration and invasion and Western blot analysis. Yifan Sun participated in the cell cycle analysis. Ting Zhang participated in the design of the study and performed the statistical analysis. Jian Xu and Ting Zhang participated in the grammar mistakes correcting and statistical analysis. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Ethics approval and consent to participate

This information is not relevant since our study does not involve neither animals nor humans.

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Figures

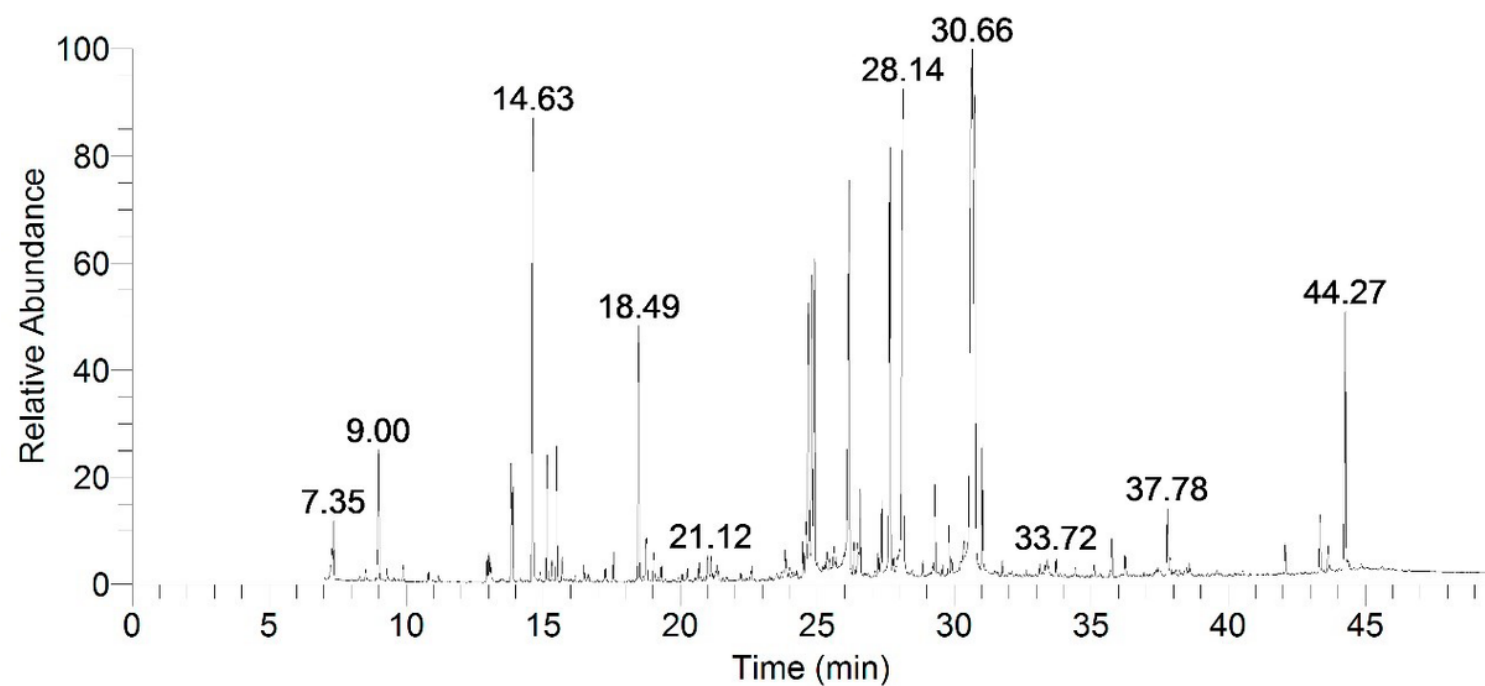


Figure 1

GC-MS analysis of EFT composition.

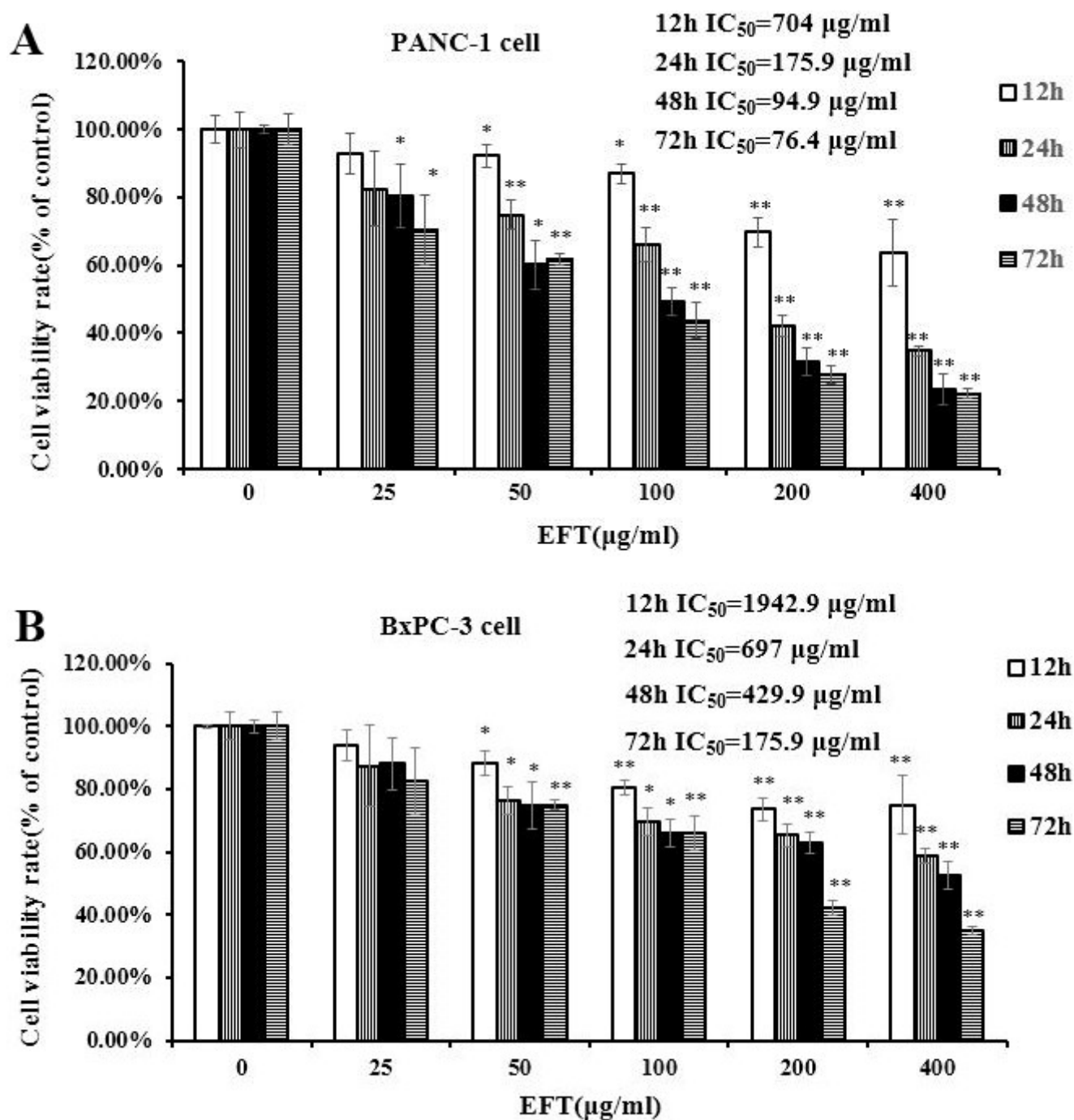


Figure 2

EFT inhibits cell proliferation in BxPC-3 and PANC-1 cells. PANC-1 cell (A) and BxPC-3 cell (B) viability inhibitory rate determined by MTT after incubation for 12h, 24h 48h, and 72h. Data represent the mean $\pm$ SD of three replicates and three independent experiments. * P <0.05, ** P <0.01, compared with control (0.1% DMSO only).

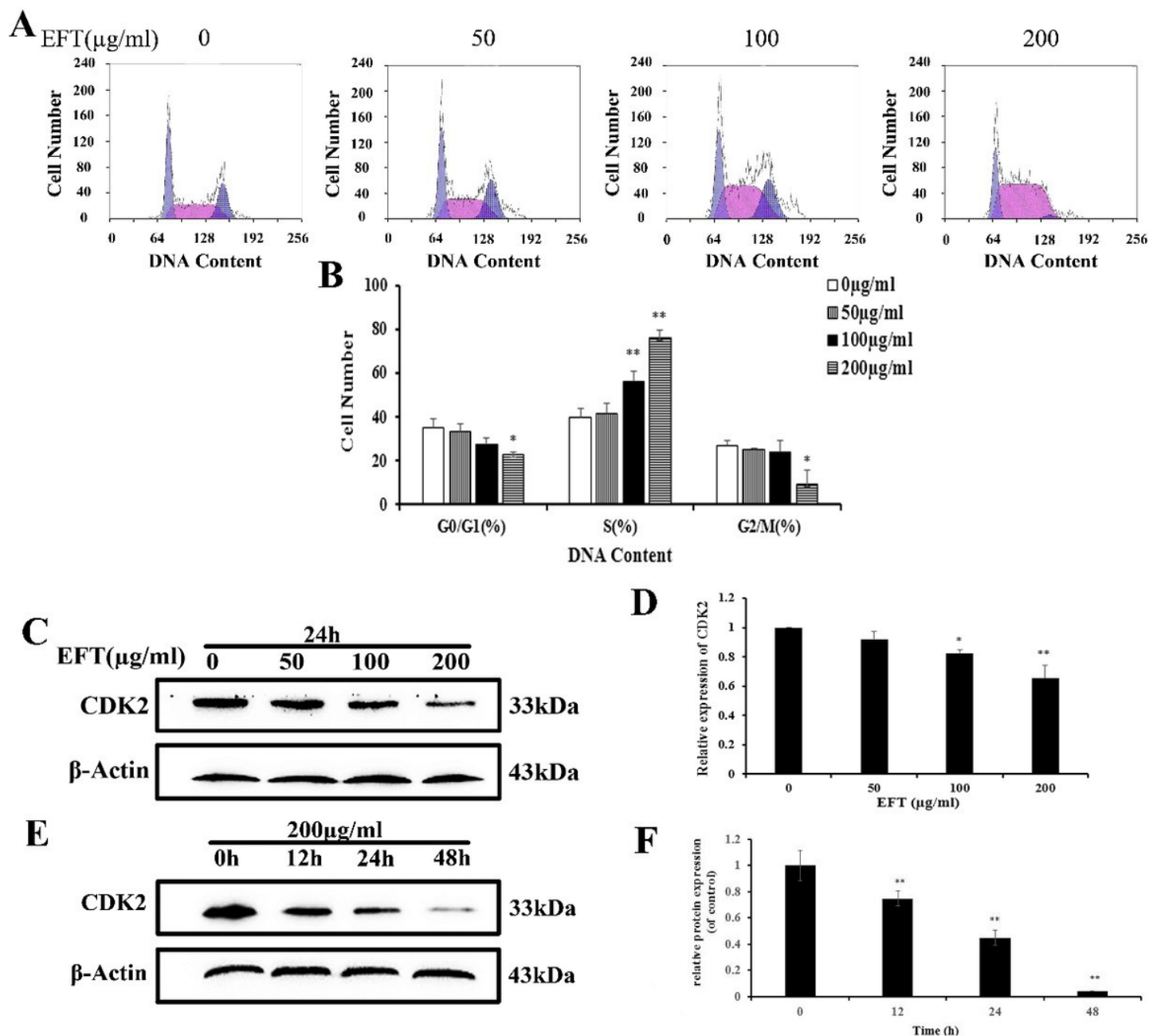


Figure 3

Effects of EFT on the cell-cycle in PANC-1 cells. (A) Cells were exposed to different concentrations of EFT for 24 hours (0.1% DMSO as a control). After treatment, cells were harvested and subject to flow cytometry to assess the cell cycle distribution. At least 10,000 cells were analyzed per sample. (B) The distribution of cells in each phase of the cell cycle after treatment with 50, 100 and 200 $\mu\text{g/ml}$ EFT (0.1% DMSO as a control). All tests were performed in triplicate and presented as mean $\pm$ standard deviation. (C and E) PANC-1 cells were treated with 50, 100 $\mu\text{g/ml}$ EFT for 24 h and 200 $\mu\text{g/ml}$ EFT for 12, 24, 48 h (0.1% DMSO as a control). The total protein of treated cells was determined. Western blot analysis of the effect of EFT on protein levels of CDK2 in PANC-1 cells. (D and F) Quantification of CDK2 ratios. * $P < 0.05$, ** $P < 0.01$, compared with control (0.1% DMSO only).

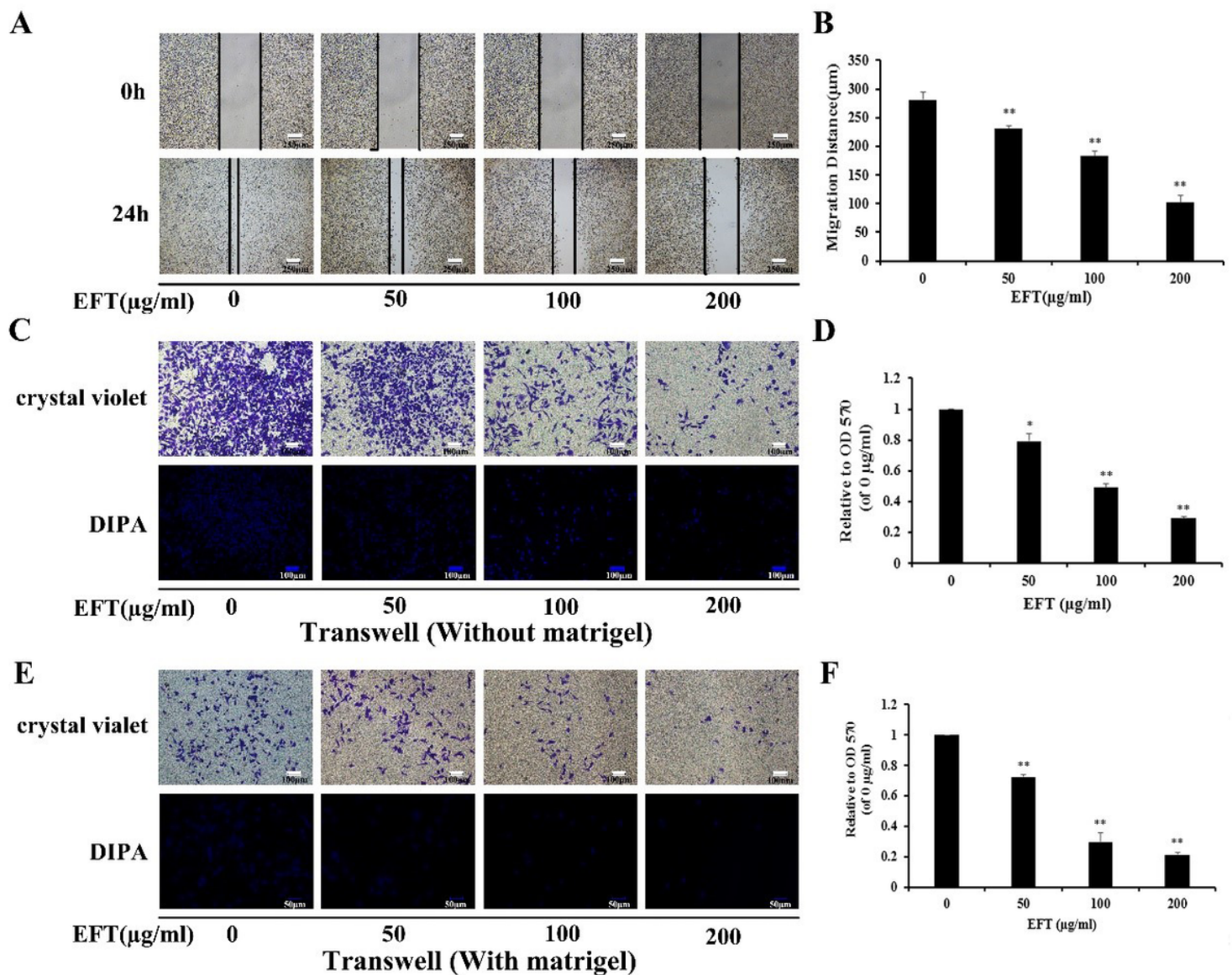


Figure 4

Effects of EFT on migration and invasion in PANC-1 cells. (A) The combined effects of migration and proliferation of EFT-treated PANC-1 cells were measured by a wound healing assay (magnification, $\times 40$). (B) The migration distance was significantly decreased by EFT treatment. (C) Cells were treated with indicated concentrations of EFT for 24 hours. The representative images of migrating cells at the bottom of the membrane stained with crystal violet and DAPI were visualized (magnification, $\times 100$). (E) Cells were incubated with indicated concentrations of EFT for 24 hours. Photographs illustrate cell invasion through the polycarbonate membrane stained by DAPI and crystal violet (magnification, $\times 100$). (D and F) The stained cells were eluted by 33% glacial acetic acid for 20 minutes. The elution was measured at 570 nm to obtain the OD 570 values, which are normalized to that of the control. * $P < 0.05$, ** $P < 0.01$, compared with control (0.1% DMSO only).

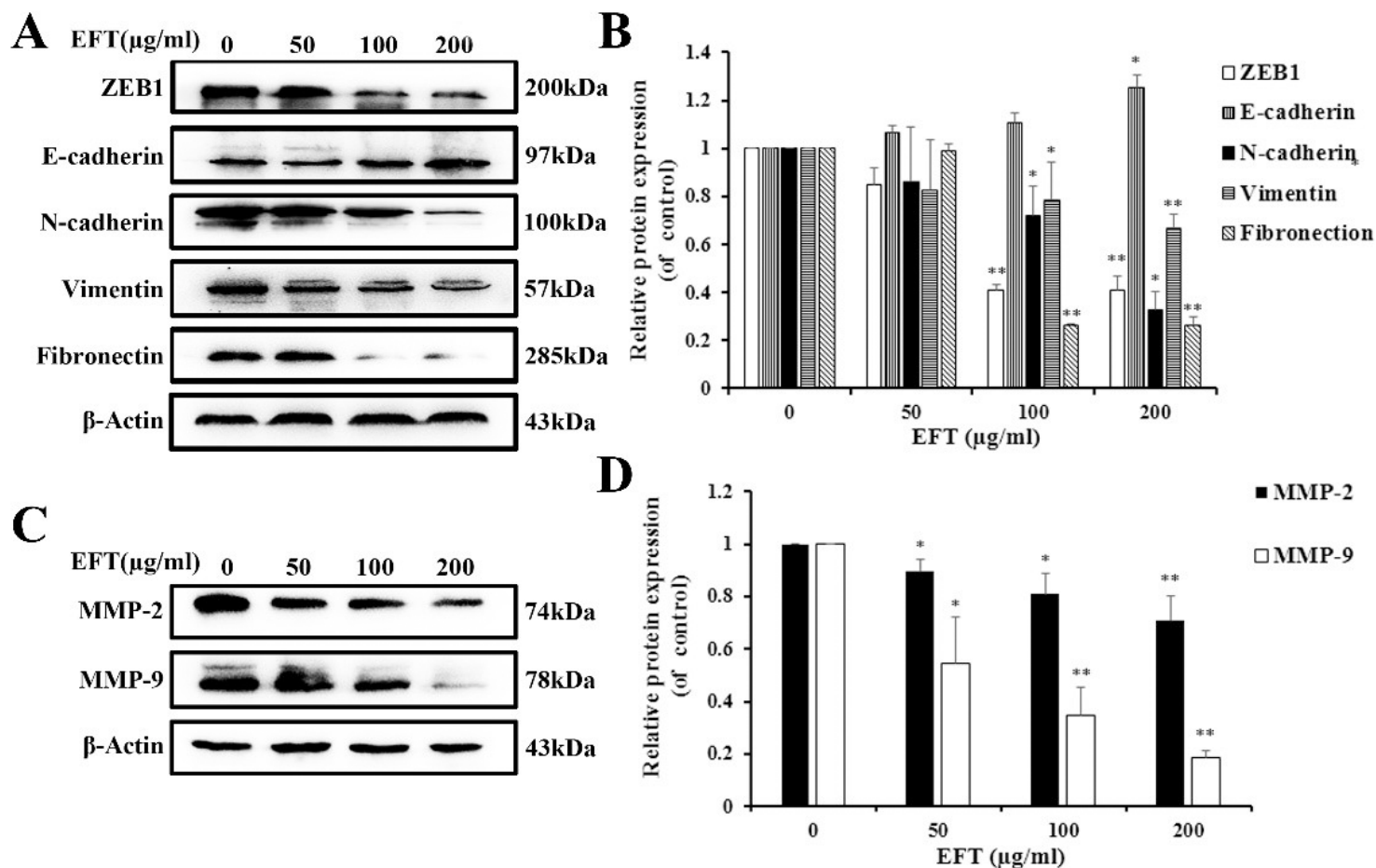


Figure 5

EFT inhibited mesenchymal properties and MMPs in PANC-1 cells (A and B) Total protein expression ratio was quantified against β -actin. (C and D) All tests were performed in triplicate and presented as mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$, compared with control (0.1% DMSO only).

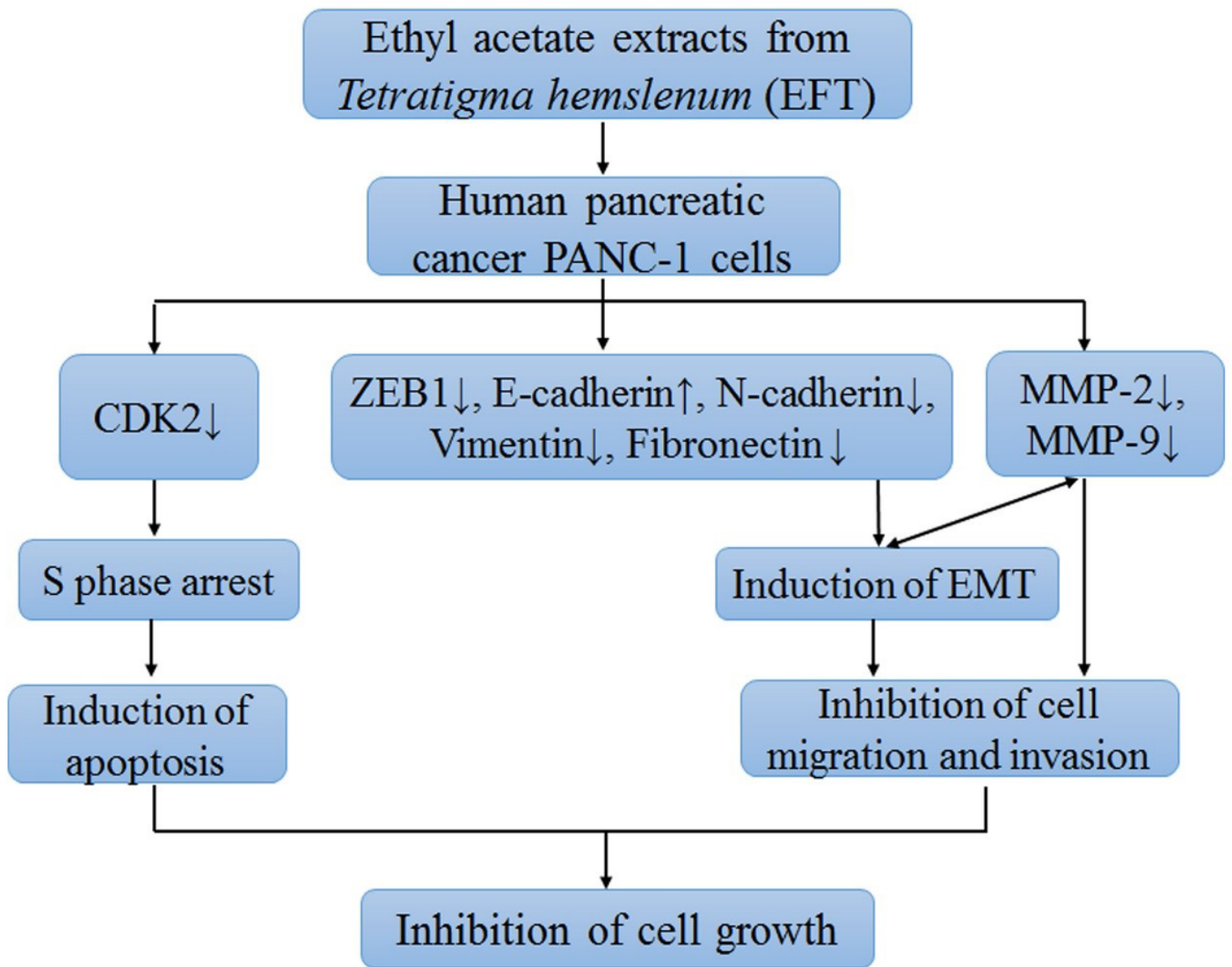


Figure 6

The proposed mechanism of EFT-induced S phase cell cycle arrest, inhibition of migration and invasion of PANC-1 cells.