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Original Article

Anticancer activity of *Trigonella Foenumgraecum* (fenugreek) seed extract by inducing apoptosis in pancreatic cancer cell

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Abstract: Background/Aim: Pancreatic cancer exhibits resistance to currently available drugs in the pharmaceutical industry. The development of new drugs is crucial, and research on plant substances with biological activities against cancer is actively underway. This study explored the potential use of fenugreek seed extract (FSE) in pancreatic cancer treatment as the anticancer activity of FSE is still poorly understood. Materials and Methods: The anticancer activity of FSE on pancreatic cancer cells was evaluated using cell viability and apoptosis assays. The migration rate of cancer cells was quantified using wound healing and Transwell migration assays. Western blotting was utilized to assess relevant signaling pathways, and LC-MS/MS was employed to detect the active compounds in FSE. Results: FSE inhibited the proliferation of pancreatic cancer cell lines (Panc-1, Miapaca-2, SNU-213, and Aspc-1) in a time and dose-dependent manner without greatly affecting normal cells (293T). The inhibition of cancer cell proliferation was attributed to the activation of cleaved caspase-3 and Bax, a pro-apoptotic marker. The anticancer effects and inhibition of cell migration were mediated by the MAPK, Akt, MMP-9, and vimentin signaling pathways through inactivation of the phosphorylated proteins related to cell growth, differentiation, and migration. LC-MS/MS analysis detected various active compounds capable of inducing apoptosis in pancreatic cancer cells. Conclusion: We demonstrated that FSE has anticancer properties by inducing apoptosis and preventing metastasis in pancreatic cancer cells without affecting normal cells.

Keywords: Anticancer activity, apoptosis induction, cell migration, fenugreek seed extract (FSE), LC-MS/MS analysis

Introduction

Cancer is the leading cause of death worldwide with further increases expected due to modern lifestyles. The American Cancer Society, reported an estimated 62,210 new cases of pancreatic cancer in the United States in 2022, resulting in 49,830 deaths. Pancreatic cancer has the lowest 5-year survival rate at only 11% among all cancers [1]. This poor prognosis is attributed to the lack of reliable early detection markers and delayed treatment initiation, often resulting in cancer spread to other organs [2]. Current treatment options for pancreatic cancer include surgery and chemotherapy, but the success rate remains low due to high recurrence rates. The exploration of medicinal plants for cancer treatment has gained attention due to of their bioactive substances [3].

Trigonella foenum-graecum, commonly known as fenugreek, belongs to the *Fabaceae* family and is native to southeastern Europe and western Asia and it is now cultivated worldwide [4]. Fenugreek seeds, with both bitter and sweet tastes, are primarily used as spices and flavoring agents in cooking and pickling [5]. In addition to their culinary uses, fenugreek seeds have a history of medicinal use for treating diabetes, high cholesterol levels, and wound inflammation [6]. Research on fenugreek seeds has revealed their pharmacological activities including anti-inflammatory, antibacterial, antioxidant, chemotherapeutic, and antidiabetic

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properties [7]. Most of the medicinal effects of fenugreek seeds are attributed to their chemical composition, which includes proteins, fatty acids, essential oils, fibers, and steroid saponins [8].

While some studies have examined the impact of fenugreek seed extract on cancer cells, limited research has been done on its effects on apoptosis in pancreatic cancer. Out study aims to explore the inhibitory effects of fenugreek seed extract (FSE) on pancreatic cancer, as well as the signaling pathways that trigger apoptosis and inhibit metastasis.

Materials and methods

Plant material and extract preparation

T. foenum-graecum (fenugreek) seed extract (FSE) was sampled from Bangladesh and obtained from the Korea Research Institute of Bioscience and Biotechnology International Biological Material Research Center. The extract was prepared using 100% methanol.

Cell culture

Human pancreatic cancer cell lines, PANC-1, MiaPaca-2, AsPC-1, and SNU-213 were purchased from the Korean Cell Line Bank (KCLB, Seoul, Republic of Korea). The 293T kidney epithelial cell line, provided by the KCLB, was used as a non-cancerous control. PANC-1, MiaPaca-2, and 293T cells were maintained in DMEM (Gibco-BRL, Gaithersburg, MD, USA) supplemented with 10% heat inactivated fetal bovine serum (FBS, Gibco-BRL) and 1% penicillin-streptomycin (Pen-Strep, Gibco-BRL). AsPC-1 and SNU-213 cells were maintained in RPMI 1640 medium (Gibco-BRL) supplemented with 10% FBS and 1% Pen-Strep. All cells were incubated at 37°C, in a 5% CO_2 atmosphere.

Reagents and antibodies

EZ-Cytox cell viability assay kits were obtained from Daeil Lab Service, (Seoul, Republic of Korea). The Annexin V-FITC/PI Apoptosis Detection kit was procured from BD Pharmingen (San Diego, CA, USA). The M-PER Mammalian Protein Extraction Reagent for cell lysis was purchased from Thermo Fisher Scientific (USA). Primary antibodies such as phosphorylated Akt, Akt, phosphorylated ERK1/2, ERK1/2, phosphorylated p38, p38, cleaved caspase-3, caspase-3, Bax, MMP-9, vimentin and GAPDH were sourced from Cell Signaling Technology (Danvers, MA, USA). Secondary antibodies were purchased from Merck (Darmstadt, Germany). The BS ECL Plus Kit was obtained from Biosesang (Gyeonggi-do, Republic of Korea).

Cell viability assay

Cell viability was assessed using the WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium) solution. 293T, PANC-1, MiaPaca-2, AsPC-1, and SNU-213 cells were seeded in 12 well plates. After a 24 h incubation period, cells were treated with FSE in DMEM or RPMI at concentrations of 10, 15, 20, and 25 μg/mL for 72 h at 37°C. Post-treatment, cells were incubated with 5% WST-1 reagent for 30 min at 37°C in a 5% CO_2 incubator. Absorbance was measured at 450 nm using a Multiskan GO Spectrophotometer.

FITC-annexin V apoptosis assay

Apoptosis was detected using the FITC Annexin V Apoptosis Detection Kit from BD Pharmingen (San Diego, CA, USA). 293T, PANC-1, MiaPaca-2, AsPC-1, and SNU-213 cells were seeded in 6-well plates and treated with 20 μg/mL of FSE for 72 h. After treatment, cells were trypsinized, harvested, and washed with cold PBS. Cells were then resuspended in 500 μL of binding buffer containing Annexin V-FITC staining solution and incubated for 15 min at 37°C in the dark. Apoptotic cells were detected using an LSRFortessa flow cytometer (BD Biosciences).

Cell wound healing assay

Briefly, pancreatic cancer cell lines were seeded in 6-well plates and allowed to grow until they reached 90% confluence. The medium was then replaced with serum-free medium, and the cells were cultured for an additional 24 h. A 200 μl pipette tip was used to scratch the cell monolayer, and the cells were washed twice with PBS to remove any floating cells. The cells were imaged using a microscope at 0 and 12 h, and the wound area was measured using ImageJ software. The wound healing rate was calculated as follows: Wound healing rate = 1 - (wound area (12 hr)/wound area (0 hr)).

Transwell migration assay

A 24-well plate Boyden chamber was used to assess cancer cell migration. Briefly, the upper

well was pre-coated with 10 μg/mL of fibronectin (Sigma-Aldrich, St.Louis, MO, USA). The upper chamber contained cells suspended in a serum-free medium treated with FSE, while the lower chamber contained RPMI. The cells were incubated for 6 h at 37°C. After incubation, the cells were fixed with 4% paraformaldehyde (Biosesang, Seongnam, Korea) and stained with 0.2% crystal violet solution. The stained cells were imaged using a microscope, and the number of migrating cells was counted.

Western blot analysis

The pancreatic cancer cell lines were treated with 20 μg/mL of FSE for 12, 24, and 48 h in a 60 mm dish. Following treatment, the cells were lysed using M-PER cell lysis buffer containing 2 mM sodium vanadate, 30 mM sodium pyrophosphate, 100 mM sodium fluoride, 0.1 M PMSF, and protein inhibitors. The lysates were then centrifuged, and the protein concentration was quantified using the Bradford assay. Subsequently, the cell lysates were mixed with SDS-PAGE sample loading buffer and heated at 99°C for 5 min. The proteins were separated using 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes. The membrane was then blocked using 5% skim milk in TBST and incubated with primary antibodies including p-AKT, AKT, p-ERK1/2, ERK1/2, p-p38, p38, cleaved caspase-3, caspase-3, Bax, MMP-9, vimentin, and GAPDH overnight at 4°C. After washing with TBST, the membrane was incubated with secondary antibodies diluted in TBST (1:4000) for 1 h at room temperature. The bands were detected using a BS ECL kit per the manufacturer's instructions.

Identification of active compounds by LC-MS/MS

To identify the active compounds in FSE, LC-MS/MS analysis was performed. Briefly, the methanolic extract was separated using an Eclipse Plus C18 RRHD (1.8 μm, 2.1 × 50 mm) column. The mobile phase for solvent A was water containing 0.1% formic acid, and for solvent B was ACN containing 0.1% formic acid. The flow rate for the analysis was set at 0.2 mL/min. Data analysis was performed using Compound Discovery 3.3TM. The unknown metabolites were detected using the online databases Chemspider and mzCloud (ddMS2).

Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA) (GraphPad Prism 5.0 Software). Statistical significance was set at $P<0.05$.

Results

FSE suppresses pancreatic cancer cell proliferation rate

To investigate the proliferation rate of pancreatic cancer cell lines, they were exposed to varying concentrations of FSE (10, 15, 20, and 25 μg/mL) for durations of 24, 48, and 72 h. Cell viability was determined using the WST-1 assay, revealing a dose-and time-dependent inhibition of pancreatic cancer cell proliferation by FSE (**Figure 1A-E**). Specifically, 20 μg/mL of FSE decreased cell proliferation by approximately 70% in SNU-213 and AsPC-1 cells, and by 50% in Panc-1 and MiaPaca-2 cells after 72 h of treatment compared with the control group. In control 293T cells, treatment with 15 μg/mL of FSE exhibited no toxicity, whereas 20 μg/mL of FSE showed mild toxicity after 72 h. These findings suggest that FSE significantly hinders the proliferation of pancreatic cancer cell lines.

FSE induces apoptosis in pancreatic cancer cells

Apoptosis, a regulated cell death process, plays a crucial role in preventing inflammation [8]. To investigate how FSE triggers apoptosis in pancreatic cancer cells, flow cytometry was performed using annexin-V/PI double staining. Pancreatic cancer cells and 293T cells were treated with 20 μg/mL of FSE for 72 h (**Figure 2A** and **2B**). The results showed that SNU-213 cells exhibited an 88% increase in apoptosis rate, AsPC-1 cells showed a 75% increase, PANC-1 cells displayed a 51% increase, and MiaPaca-2 cells exhibited a 36% increase when treated with 20 μg/mL of FSE, compared to the control. Interestingly, the apoptotic rate of 293T cells was significantly lower. These findings suggest that FSE-induced cell death in pancreatic cancer cells is primarily mediated through apoptosis.

FSE activates apoptosis-related markers in pancreatic cancer cells

Activation of apoptosis-related markers was assessed by Western blot analysis. Caspase-3

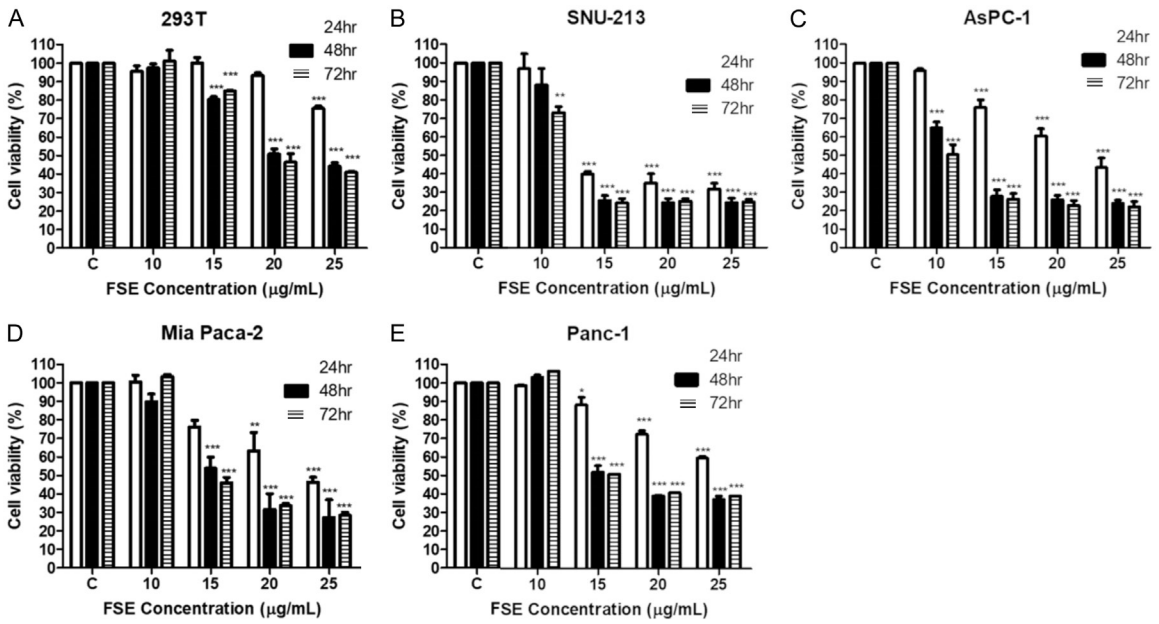

Figure 1. Cytotoxic effect of fenugreek seed extract (FSE) on human pancreatic cancer cell lines and human embryonic kidney cells at different concentrations and times. Cell viability was measured using WST-1 Assay. A. Cell viability of 293T human embryonic kidney cells after 24, 48, and 72 h of FSE treatment. B-E. Cell viability of human pancreatic cancer cell lines, SNU-213, AsPC-1, Mia Paca-2, and Panc-1 cell. Data are presented as mean ± SD from three independent experiments ((*P<0.05, **P<0.01, ***P<0.001), one-way ANOVA with Tukey's post-hoc test).

activation was confirmed 48 h after FSE treatment of pancreatic cancer cells (**Figure 3A** and **3B**). Expression of cleaved caspase-3 increased in a time-dependent manner however, there was no significant change in total caspase-3 expression. Expression of Bax, a pro-apoptotic marker, also increased in a time-dependent manner after FSE treatment (**Figure 3A** and **3B**). These results show that the proliferation of pancreatic cancer cells is inhibited by apoptosis through activation of caspase-3 and Bax.

FSE induces apoptosis via the MAPK and Akt signaling pathways

To identify the signaling pathways involved in inducing apoptotic responses and inhibiting cell migration in pancreatic cancer cells, the protein phosphorylation levels of ERK, P38, and Akt were determined by Western blot analysis (**Figure 4A** and **4B**). Following FSE treatment, phosphorylation of ERK, P38, and Akt was significantly altered. The phosphorylation levels of ERK1/2, p38, and Akt decreased in a time-dependent manner after FSE treatment. These findings demonstrate that FSE induces apoptosis and inhibits pancreatic cancer cell migration via the MAPK and Akt signaling pathways.

FSE inhibits cell migration of pancreatic cancer cells

The anti-migratory effect of FSE on pancreatic cancer cells was assessed using cellular wound healing and Transwell migration assays. After treatment with 20 μg/mL FSE for 12 h, the migration ability of MiaPaca-2 cells was inhibited by 70%, Panc-1 cells by 82%, AsPC-1 cells by 84%, and SNU-213 cells by 67% compared to control (**Figure 5A** and **5B**). FSE treatment significantly inhibited cancer cell migration in the Transwell assay (**Figure 5C** and **5D**). Additionally, expression of MMP-9 and vimentin, which are correlated with cell migration, was suppressed by FSE treatment (**Figure 5E** and **5F**). MMP-9 and vimentin expression decreased over time in all cancer cell lines tested. These results demonstrate that FSE has antimetastatic ability in pancreatic cancer cells.

LC-MS/MS analysis of methanolic extract of fenugreek seed

We aimed to detect the compounds present in the methanol extract of fenugreek seeds using LC-MS/MS analysis. LC-MS/MS spectra indicated the presence of numerous compounds

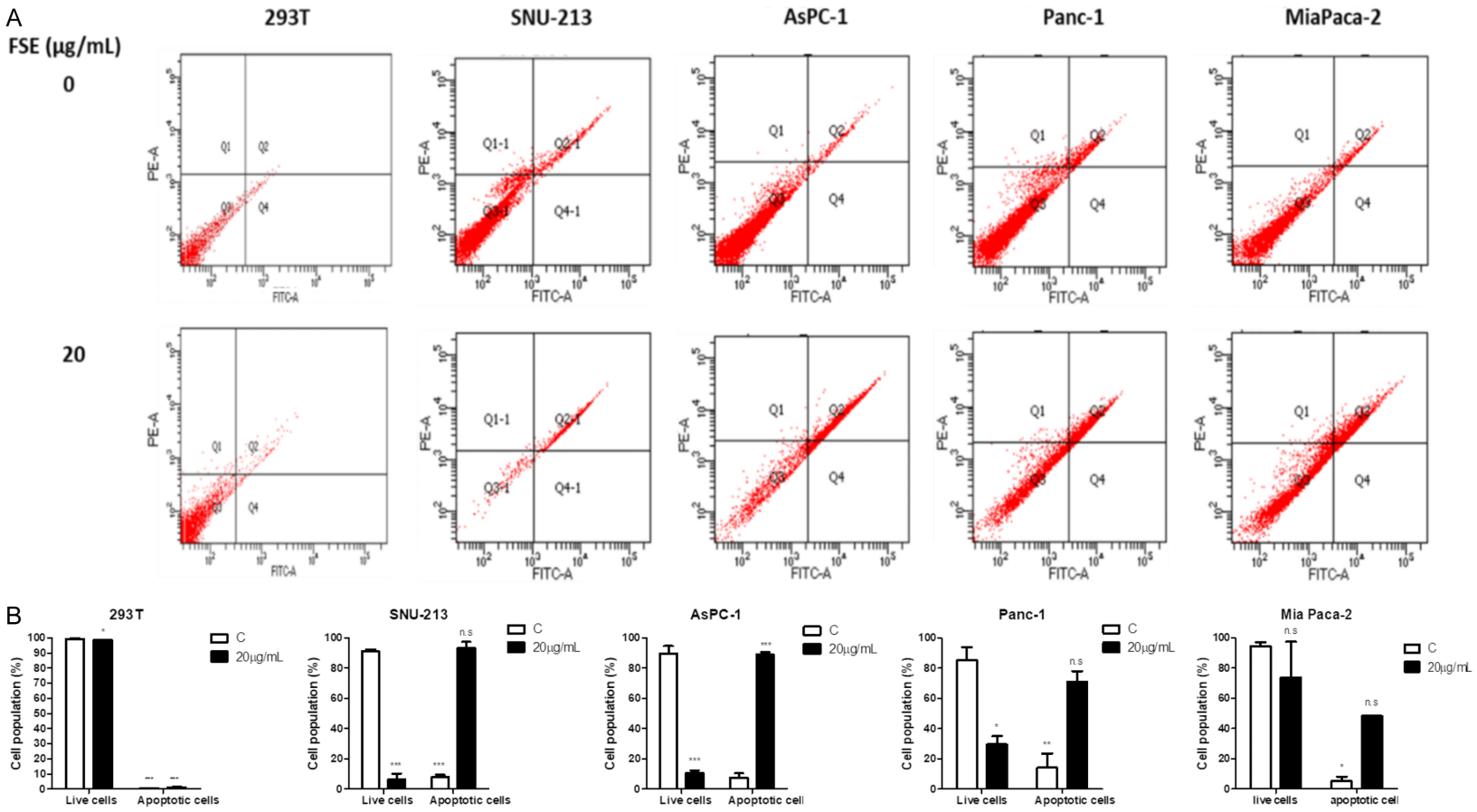

Figure 2. Fenugreek seed extract (FSE) induces apoptosis in pancreatic cancer cell lines. A. Flow cytometry analysis using Annexin V and PI staining in SNU-213, AsPC-1, MiaPaca-2, and Panc-1 cells after treatment with 0 and 20 μg/mL of FSE for 72 h. B. Quantitative analysis of the percentage of total apoptotic cells. Data are presented as mean ± SD from three independent experiments ((*P<0.05, **P<0.01, ***P<0.001), one-way ANOVA with Tukey's post-hoc test).

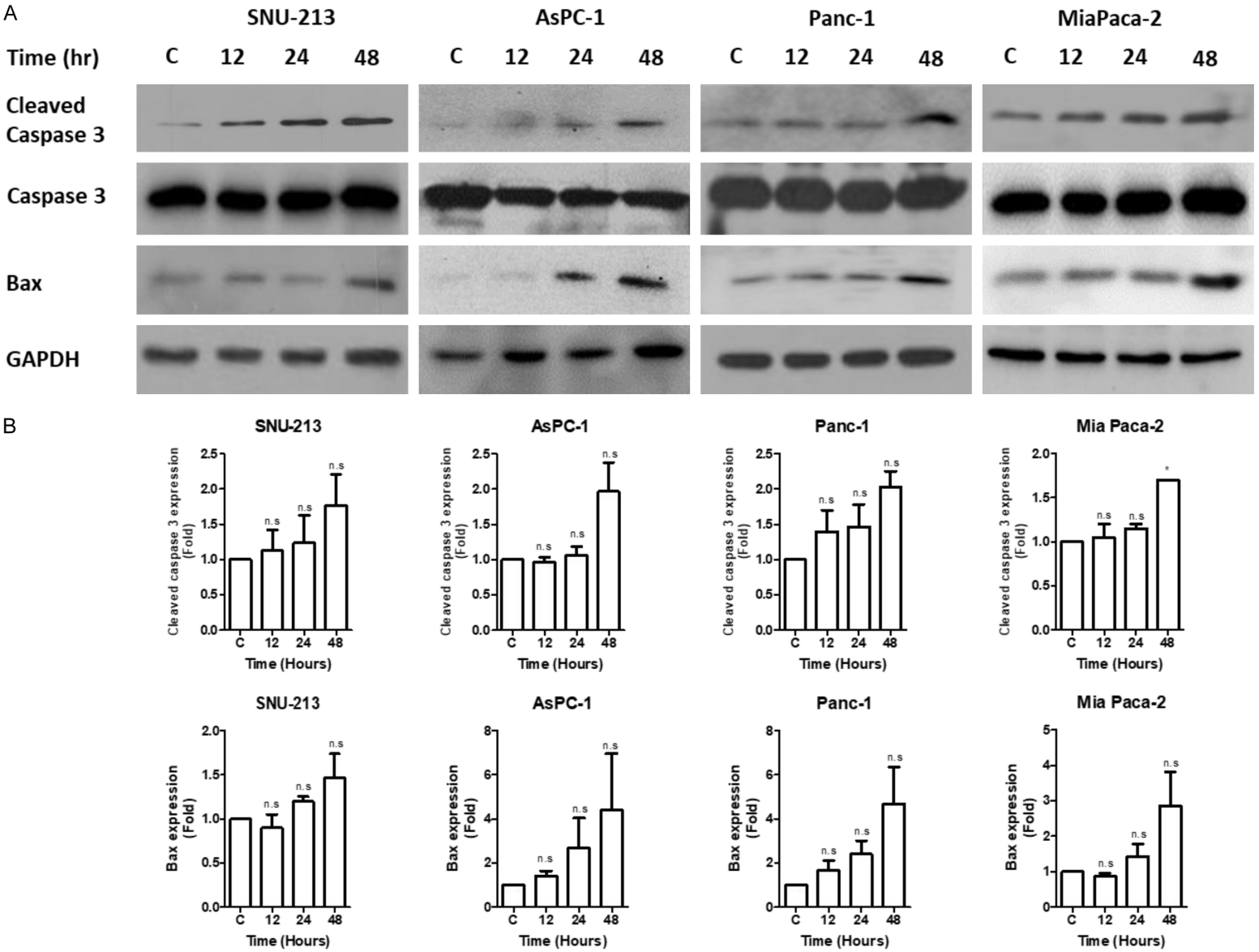

Figure 3. Fenugreek seed extract (FSE) activates apoptosis-related markers in pancreatic cancer cells. Western blot analysis of cleaved caspase-3, caspase-3, and Bax following treatment with 20 μg/mL of FSE for 12, 24, and 48 h. GAPDH was used as the loading control. Data are presented as mean ± SD from three independent experiments ((*P<0.05, **P<0.01, ***P<0.001), one-way ANOVA with Tukey's post-hoc test).

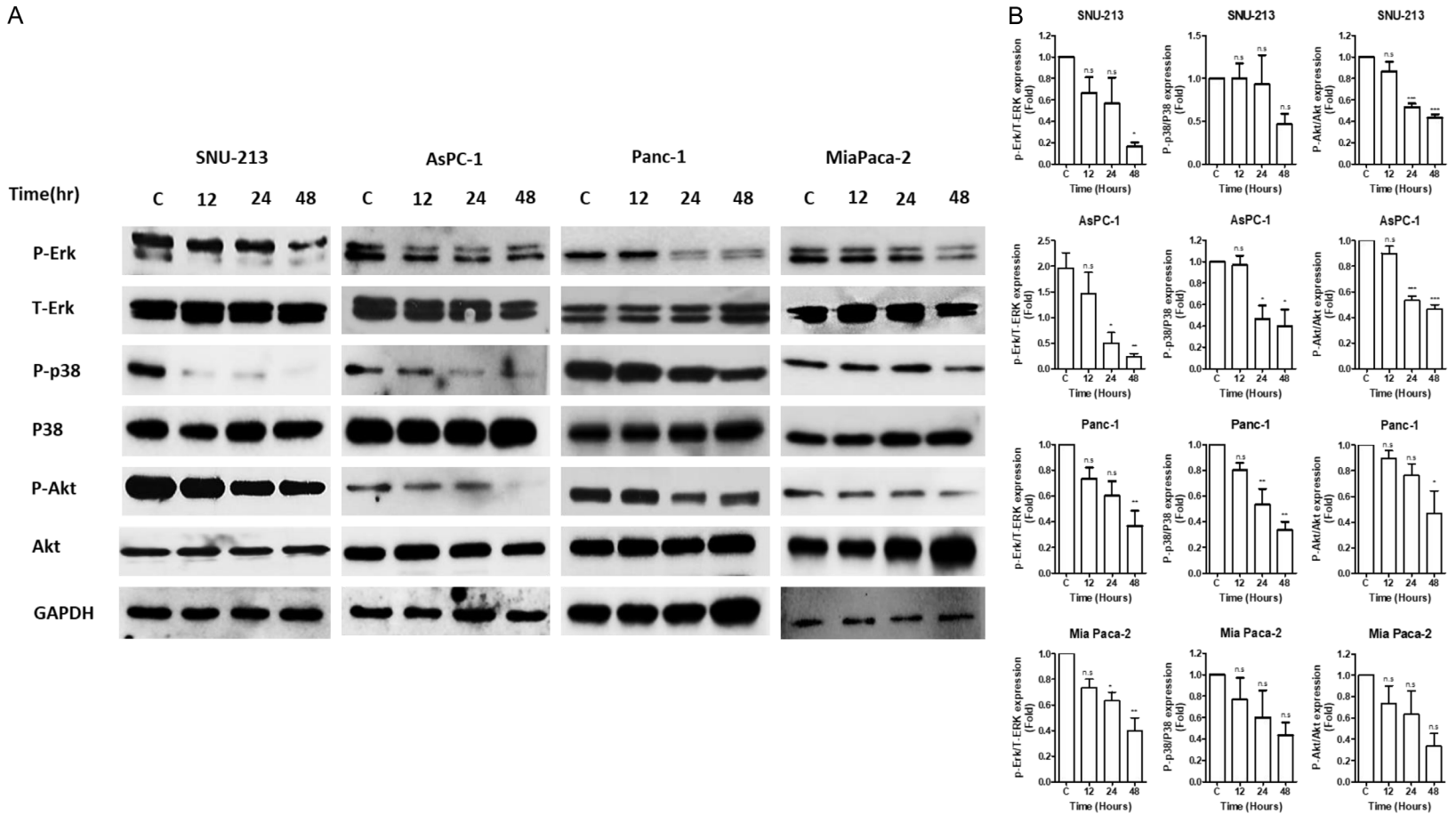

Figure 4. Fenugreek seed extract (FSE) induces apoptosis and inhibits cell migration in pancreatic cancer cell lines mediated by MAPK and Akt signaling pathways. Pancreatic cancer cell lines were treated with 20 μg/mL of FSE for 0, 12, 24, and 48 h. The protein levels of phospho-AKT and AKT, phospho-ERK1/2, ERK1/2, phospho-p38, and p38 were analyzed by western blotting. GAPDH was used as the loading control. Data are presented as mean ± SD from three independent experiments ((*P<0.05, **P<0.01, ***P<0.001), one-way ANOVA with Tukey's post-hoc test).

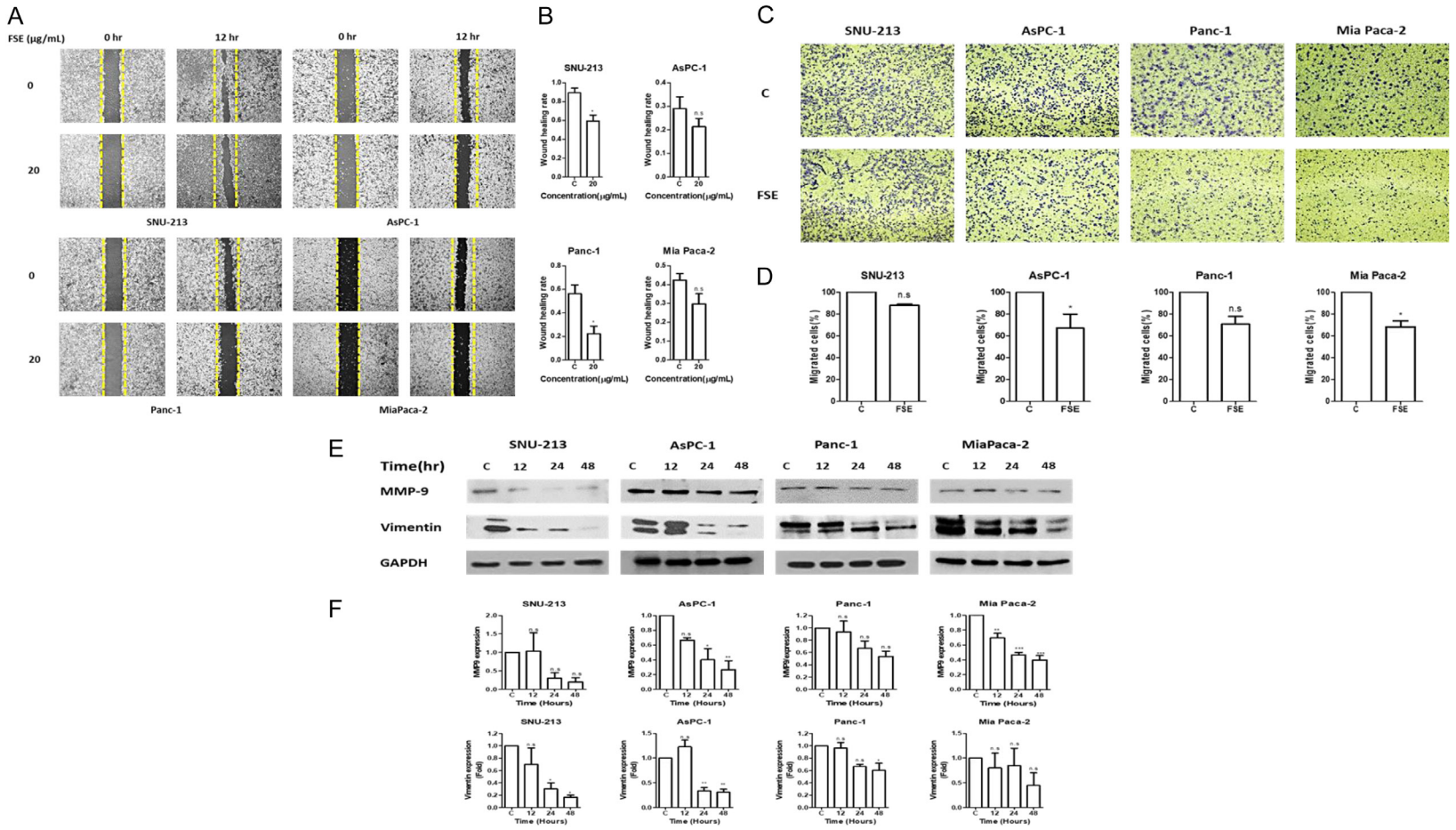

Figure 5. Fenugreek seed extract (FSE) inhibits cell migration of pancreatic cancer cell lines. A, B. Wound healing assay was performed by creating a wound in the cell monolayer and treating it with 0 and 20 μg/mL of FSE for 12 h. Photomicrographs were obtained at 0 and 12 h of treatment using an inverted microscope. The wound area was quantified using Image J Software. C, D. Cell migration ability in pancreatic cancer cells after treatment with 20 μg/mL of FSE determined using the Transwell migration assay. Magnification: ×100. E, F. Expression of migration-related proteins including MMP-9, and vimentin determined by western blotting. Data are presented as mean ± SD from three independent experiments ((*P<0.05, **P<0.01, ***P<0.001), one-way ANOVA with Tukey's post-hoc test).

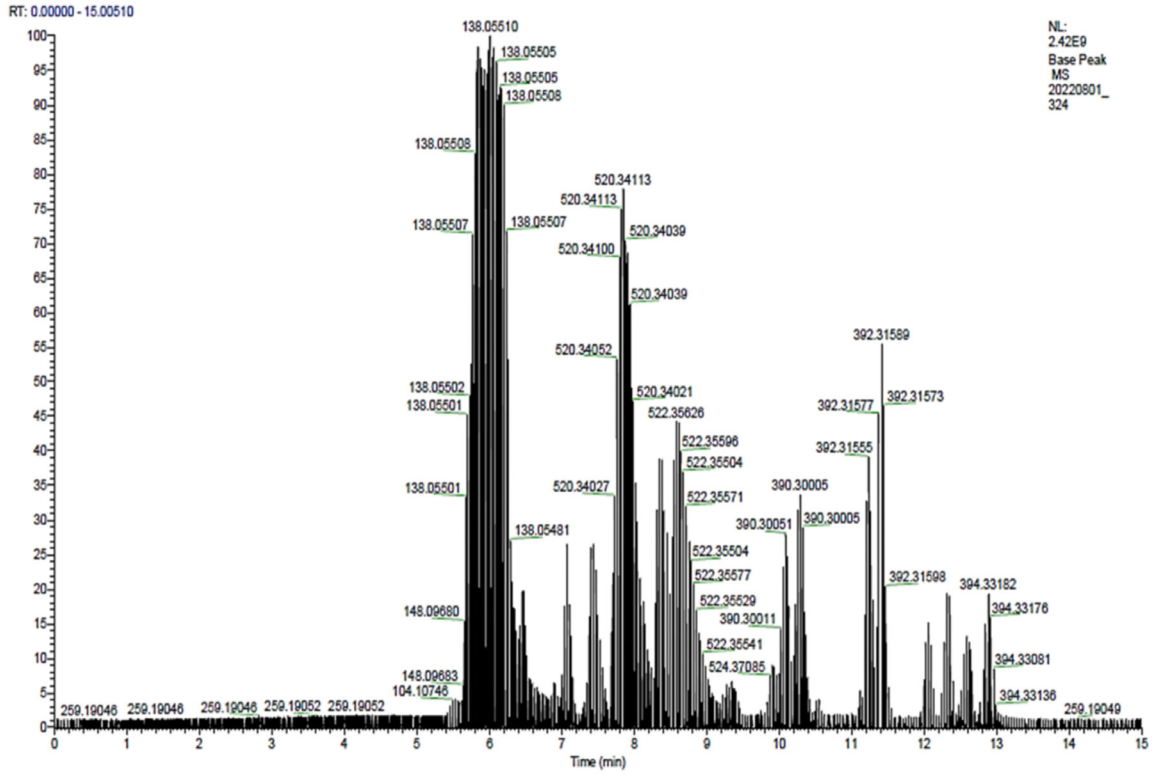

Figure 6. LC-MS/MS chromatogram of fenugreek seed extract (FSE). Data analysis was performed using Compound Discover 3.3™.

Table 1. Compounds identified by LC-MS/MS of fenugreek seed extract (FSE)

No.	Name	Formula	Calc. MW	RT [min]
1	Anthranilic acid	$C_7H_7NO_2$	137.04782	6.011
2	Choline	$C_5H_{13}NO$	103.10011	5.771
3	Betaine	$C_5H_{11}NO_2$	117.07931	5.839
4	Linoleoyl ethanolamide	$C_{20}H_{37}NO_2$	323.28277	9.332
5	α Eleostearic acid	$C_{18}H_{30}O_2$	278.22496	7.942
6	Melezitose	$C_{18}H_{32}O_{16}$	504.16883	5.659
7	Oleoyl ethanolamide	$C_{20}H_{39}NO_2$	325.29815	10.531
8	Picolinic acid	$C_6H_5NO_2$	123.03234	6.216
9	Stearamide	$C_{18}H_{37}NO$	283.28771	12.735
10	2,4-dihydroxyheptadec-16-en-1-yl-acetate	$C_{19}H_{36}O_4$	328.26113	7.31

(**Figure 6**). Ten active compounds, including anthranilic acid and betaine, were detected relatively reliably (**Table 1**). Among these compounds, anthranilic acid, choline, betaine, α-eleostearic acid, melezitose, and oleoyl ethanolamide are known to have anti-cancer and anti-inflammatory effects [9-15]. Most active compounds detected by LC MS/MS analysis have not been previously reported in FSE, highlighting the need for further research on FSE.

Discussion

According to the European Union cancer forecast for 2022, pancreatic cancer is expected to surpass breast cancer to become the third leading cause of cancer-related deaths. This is due to the lack of curative treatments and the fact that pancreatic cancer patients typically do not exhibit symptoms until the cancer has metastasized, making early detection and screen-

ing challenging [1]. Plant extracts and plant-derived natural products have been used to treat various diseases, including cancer [16]. Currently, the success rate of pancreatic cancer treatment is limited, prompting active research into new therapeutic options. In this context, compounds present in fenugreek seeds could be valuable for developing cancer treatments [7]. Previous studies have demonstrated that FSE induces apoptosis in other types of cancers, such as hepatocellular carcinoma [3], breast cancer [17-20], colon adenocarcinoma cell lines [21-23], and liver cancer [5]. However, to the best of our knowledge, there are no detailed studies on the effects of FSE on pancreatic cancer cells. In this study, we found that FSE inhibits pancreatic cancer cell proliferation and induces apoptosis by inhibiting the MAPK and Akt signaling pathways.

Our results showed that FSE decreased the cell viability of pancreatic cancer cell lines PANC-1, MiaPaca-2, AsPC-1, and SNU-213 in a concentration- and time-dependent manner (**Figure 1A-E**). Additionally, the same FSE concentration did not significantly affect the survival rate of 293T cells, indicating low toxicity to normal cells, and increasing the potential for developing a natural treatment.

Apoptosis is programmed cell death characterized by morphological changes in the cell, including membrane blebbing and the externalization of phosphatidylserine [24]. Apoptotic cell death in FSE-treated cells was detected using flow cytometry after Annexin V/PI staining. In this study, the cleavage of caspase-3 and expression of Bax were upregulated in a time-dependent manner (**Figure 3**). Bai et al. showed that S. sarmentosum Bunge extract induced apoptosis in pancreatic cancer cells by upregulating the expression of cleaved caspase-3 and Bax [25]. Therefore, FSE inhibits cell proliferation and induces apoptosis through the activation of caspase-3 and Bax proteins.

We analyzed cell migration signaling pathways in FSE-treated cells. Among these pathways, the MAPKs and AKT signaling pathways are known to regulate cell processes such as proliferation, apoptosis, and migration [26]. Mammalian cells have three MAPK signal transduction pathways, which are extracellular-regulated kinases (ERK), p38 MAPK, and c-Jun N-terminal kinase (JNK), which contribute to various cellular responses [27]. p38 MAPK, activated by environmental or genotoxic stress, plays critical roles in apoptosis, proliferation, and differentiation [28, 29]. Our results demonstrated that FSE treatment downregulated phosphorylation levels of ERK1/2 and p38, potentially contributing to the inhibition of cancer cell proliferation and apoptosis (**Figure 4**). The Akt signaling pathway is associated with cancer cell proliferation and migration, and its activation promotes these processes [30, 31]. In this study, FSE treatment inhibited Akt phosphorylation in pancreatic cancer cells, thereby suppressing their migration. Wound healing and Transwell migration assays showed that inhibition of AKT, ERK, and p38 MAPK phosphorylation is correlated with reduced cancer cell migration.

Metastasis complicates pancreatic cancer treatment. Epithelial-to-mesenchymal transition (EMT) facilitates metastasis by enabling cells to acquire mesenchymal characteristics necessary for migration [32]. Therefore, inhibiting cancer cell metastasis is crucial for improving pancreatic cancer prognosis. In this study, treatment with FSE significantly inhibited cell migration by 67%-84% in SNU-213, MiaPaca-2, Panc-1, and AsPC-1 cell lines. FSE also suppressed the expression of migration-related proteins, including MMP-9 and vimentin. These findings underscore the potential of FSE to inhibit pancreatic cancer cell migration and prevent metastasis.

Khalil et al. detected 14 bioactive compounds using GC-MS analysis with primary compounds identified as squalene and naringenin [3]. In this study, an additional 10 active compounds were identified through LC-MS/MS analysis: anthranilic acid, choline, betaine, linoleoyl ethanolamide, α-eleostearic acid, melezitose, oleoyl ethanolamide, picolinic acid, stearamide, and 2,4-dihydroxyheptadec-16-en-1-yl acetate. The anticancer or anti-inflammatory activities of these compounds have been established; anthranilic acid for instance, exhibits anticancer effects against pancreatic cancer, while betaine induces cell death in prostate cancer [9, 10]. Linoleoyl ethanolamide demonstrates anti-inflammatory activities in dermatitis by inhibiting the NF-κB signaling pathway [11]. Additionally, α-eleostearic acid inhibits cell proliferation and migration of tongue squamous cell carcinoma CAL-27 cells and induces anti-in-

flammatory effects in bowel disease by activating peroxisome proliferator-activated receptor-gamma (PPARγ) [12, 13]. Melezitose and oleoyl ethanolamide have been shown to inhibit lung cancer cell proliferation and induce apoptosis [14, 15]. Therefore, identifying the phytochemicals in fenugreek seeds, is crucial to assessing their potential for cancer treatment, and developing novel therapies.

Methanolic FSE effectively inhibits pancreatic cancer cell proliferation by inducing apoptosis through activation of cleaved caspase-3 and Bax. FSE also demonstrates potential in preventing metastasis by suppressing expression of MMP-9 and vimentin and regulating cancer cell migration. Furthermore, FSE treatment results in activation of the MAPK and Akt signaling pathways. LC-MS/MS analysis identified numerous active compounds in FSE, some of which are likely contributors to its anti-cancer effects against pancreatic cancer cells. Therefore, continued investigation into fenugreek seeds holds promise for advancing alternative approaches to treating pancreatic cancer.

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Disclosure of conflict of interest

None.

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