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Inhibitory Effects of *Cannabis sativa* Seed Extract on the Pancreatic Cancer Cell Line PANC-1

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ABSTRACT

Background: Pancreatic ductal adenocarcinoma (PDAC) remains a highly lethal malignancy, underscoring the urgent need for novel therapeutic agents. *Cannabis* has recently gained attention as a promising plant-derived source of bioactive compounds; however, most studies investigating the anti-tumor properties of *Cannabis sativa* (*C. sativa*) have focused on leaf extracts, leaving its seeds largely unexplored. In this study, we investigated the anti-tumor potential of an ethanolic extract of *C. sativa* seeds against pancreatic cancer cells.
Methods: An ethanolic extract of *C. sativa* seeds was prepared and analyzed by gas chromatography mass spectrometry. The cytotoxicity of the extract on PANC-1 (pancreatic cancer) and Human Foreskin Fibroblast (HFF) normal cells was assessed using the MTT assay (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide). Colony formation and wound-healing assays evaluated the extract's impact on PANC-1 colony formation and migration. Flow cytometry analysis assessed the cell cycle phase of PANC-1 cells after treatment.
Results: The extract selectively suppressed PANC-1 proliferation without affecting HFF cell viability. It induced G1-phase arrest and increased the sub-G1 population in PANC-1 cells, inhibited colony formation, shifted colony composition toward a paraclone-dominant phenotype, and reduced migratory capacity.
Conclusion: Our *in vitro* findings demonstrate that *C. sativa* seed extract selectively inhibits PANC-1 cell proliferation and reduces cell migration and clonogenicity while sparing normal cells, suggesting its potential as a plant-derived candidate for PDAC therapy.

1. Introduction

Among all cancer types, pancreatic cancer is the 12th most common cancer worldwide, and due to its poor prognosis, it is the sixth leading cause of cancer-related deaths. Pancreatic ductal adenocarcinoma (PDAC), which accounts for more than 90% of diagnosed pancreatic cancer cases, is the most common type of pancreatic cancer and has a 5-year survival rate of less than 5% (Bray et al., 2024). Current data show that new PDAC cases are increasing by 1% annually (Siegel et al., 2023). PDAC is a particularly aggressive cancer owing to the following factors: rapid growth, high metastatic potential, poor prognosis, and resistance to current treatment options (Le Large et al., 2017). Due to the limited effectiveness of current PDAC treatments, with an average survival rate of only 5-6 months, there is a pressing need to develop new

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therapies (Bengtsson et al., 2020). Treatment options for PDAC vary depending on the disease progression and include radiation, chemotherapy, and surgical interventions. The surgical approach is an effective treatment for patients with localized tumors in the early stages. However, only a small percentage of patients are diagnosed at an early stage with localized tumors. Consequently, most patients present in advanced stages with metastatic tumors, where treatment options are more limited, and chemotherapy is typically used as the first-line standard treatment for these individuals. However, chemotherapy is associated with significant adverse systemic effects. Despite significant advancements in current treatment methods for pancreatic cancer, the impact of these therapies on patient survival remains minimal, and the survival rate has shown little improvement (Kolbeinsson et al., 2023). Accordingly, research into alternative treatments, including those derived from plant-based sources, is ongoing (Vasques Sr et al., 2024).

Cannabis (*Cannabis sativa*) is an annual plant that belongs to the Cannabaceae family (Kuddus et al., 2013; Hourfane et al., 2023). Recently, *cannabis* has received a lot of attention as a promising plant-based medication for treating a variety of illnesses (Shin et al., 2024). Recent studies have demonstrated that bioactive compounds isolated from this plant possess a wide spectrum of pharmacological activities, notably antimicrobial and antioxidant effects (Shin et al., 2024). To date, pharmacological investigations on *C. sativa* have primarily focused on its antimicrobial and analgesic properties, as well as its antidiabetic potential (Vasques Sr et al., 2024), anticancer activity (Erhabor et al., 2024), and anti-inflammatory and antidepressant effects (Shin et al., 2024). In addition, evidence suggests that *C. sativa* may exert protective effects against oxidative stress and may also have therapeutic potential in the management of neuropathic pain (Erhabor et al., 2024). A study investigating the cytotoxic effects of aqueous *cannabis* extracts on cell growth and proliferation demonstrated that these extracts inhibited the growth of MCF-7 human breast cancer cells and DU145 human prostate cells, suggesting their potential anticancer activity (Erhabor et al., 2024; Shin et al., 2024). Additionally, the extract inhibited angiogenesis, nitric oxide production, and oxidative stress in the studied cells (Erhabor et al., 2024). Many studies have shown the anticancer effects of extracts from different types of *cannabis* against various tumors (Hosami et al., 2021; Rožanc et al., 2021; Drozd et al., 2022; Emhemmed et al., 2022; Blal et al., 2023; Cherkasova et al., 2024). *Cannabis* can affect the tumor microenvironment through initiating anti-tumorigenic cell signaling, regulating apoptosis and angiogenesis (Velasco et al., 2016; Erhabor et al., 2024), and adjusting the release of different immunogenic and inflammatory cytokines (Yekhtin et al., 2022). In a recent study, the anti-proliferative and pro-apoptotic effects of two main *C. sativa* cannabinoids in a combination of Delta-9-tetrahydrocannabinol (THC) and cannabidiol (CBD) were shown on pancreatic cancer xenograft mouse models (Le et al., 2024). In another study, researchers used a new method to isolate a class of cannabinoids from crude *Cannabis sativa* leaf extract, yielding cannabinoid glycosides. The cannabinoid glycosides had improved anti-cancer activities, especially against pancreatic cancer cell lines MiaPaca-2 and Panc-1 (Nalli et al., 2024).

Because hemp seeds contain phenolic compounds, researchers have extensively studied the antioxidant, antibacterial, and cytotoxic activities of hemp seed extracts (Metouekel et al., 2024). The presence of bioactive compounds such as polyphenols, flavonoids, and cannabinoids in *Cannabis sativa* seeds has been associated with anti-inflammatory, antioxidant, and anticancer properties. Recently, silver nanoparticles biosynthesized with hemp seed extract have been developed and investigated in a lung cancer cell line. The results showed promising outcomes in terms of their potential effectiveness (Yontar & Çevik, 2024). While these findings suggest the effectiveness of *Cannabis sativa* seed extract silver nanoparticles on lung cancer, to date, the raw extract obtained from hemp seeds has not been directly used as a phytochemical agent against cancer cells. Given the aggressive nature of pancreatic cancer and the need for alternative treatment approaches, investigating the anticancer properties of *Cannabis sativa* seed extract against pancreatic cancer cells is of interest.

Previous studies on the anti-cancer properties of *Cannabis sativa* have mainly focused on its leaf and flower, with special attention to the two major secondary metabolites, THC and CBD. In this study, we explore the anti-cancer properties of *C. sativa* seeds. Seeds from *Cannabis sativa* have significant nutritional and medicinal benefits with a distinct chemical composition compared to the leaves and flowers (Leonard et al., 2020). Unlike marijuana, they contain low Tetrahydrocannabinol (THC levels (<0.1-1%), which ensure minimal psychoactive effects (Citti et al., 2019). They are used as a traditional snack in Iran, along with nuts, especially during winter. Here, we investigate the effect of an ethanolic *cannabis* seed extract on pancreatic cancer cells PANC-1. We seek to know whether *C. sativa* seed extract shows any inhibitory effect on the growth, migration, and colony formation capacities of PANC-1 cells. Since the PANC-1 cell line has shown a higher resistance to chemotherapy drugs in comparison to other pancreatic cancer cell lines, MIA-PaCa-2 and BxPC-3, it was chosen for this study.

2. Materials and Methods

2.1 Preparation of hemp seed extract

To prepare the local ecotype of *C. sativa* seed extract, 130 g of partially ground (fine <2 mm and coarse >2 mm particle sizes) dry seeds were subjected to maceration in 80% ethanol (v/v, 600 mL) for 48 h at 4°C in the dark, with continuous shaking and stirring. The mixture was subjected to an electric mixer at 60 RPM for 48 hr. The mixture was then filtered through cellulose filter paper, and the solvent was removed using a rotary vacuum evaporator at 40-45°C. The suspension was filtered through a Whatman #1 filter paper (pore size of 30 μm). To eliminate residual solvent, the extract was placed under a laminar flow hood at room

temperature for 3 days. A total of 3 g of dried extract was obtained and stored in the dark at 4°C.

2.2 Gas Chromatography–Mass Spectrometry (GC-MS) analysis

The GC-MS analysis was carried out using an Agilent Technologies GC-MS apparatus (GC 7890B; MS 5977A, USA), equipped with an auto-injector, under the following conditions: A Thermo Electron Focus gas chromatograph integrated with a Polaris Q ion trap mass spectrometer, employing an HP-5MS capillary column (50 m x 0.25 mm; 0.25 μm), was utilized for the analysis. The gas chromatograph oven temperature program was initiated at 50°C and increased at a rate of 2°C/min until it reached 290°C. Helium served as the carrier gas (2mL/min), and the injector and ion source temperatures were maintained at 280°C and 290°C, respectively, for one minute. The detector was operated in electron impact mode (70 eV), and the m/z range of 35-550 a.m.u. was used for detection. The NIST Mass Spectral Database was utilized to identify the matching compounds after all chromatogram peaks were examined using the Xcalibur ® Software. To calculate the retention indices (RI), a standard alkane solution for GC (C8-C20 in n-hexane) was used. The relative percentage of each component was computed using the GC peak areas without correction factors.

2.3 Cell culture

Human pancreatic cancer cells (PANC-1) and human foreskin fibroblasts (HFF) were obtained from the Iranian Biological Resource Center (IBRC, Tehran, Iran). Both cell lines were maintained in high-glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) for PANC-1 and 15% (v/v) FBS for HFF, in strict accordance with the suppliers' recommended protocols. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2.

2.4 MTT assay

The cytotoxic effects of the *C. sativa* seed extract on the viability and proliferation of PANC-1 cells were evaluated using the MTT assay. PANC-1 cells were seeded into 96-well plates at a density of 3×10^3 cells per well. After 24 h of incubation, the culture medium was removed, and the cells were treated with various concentrations of *C. sativa* seed extract for 72 h. After discarding the culture medium, 100 µL of dimethyl sulfoxide (DMSO > 99%) was added to each well to fully dissolve the insoluble formazan crystals. Absorbance was subsequently recorded at 570 nm using a microplate reader. All experimental conditions were conducted in three independent replicates. Appropriate vehicle-treated groups served as solvent controls, and cell viability values were normalized directly to the no-treatment control group. The half-maximal inhibitory concentration (IC50) values were derived via non-linear regression fitting using GraphPad Prism software (specifically utilizing the variable slope/least squares fit model).

2.5 Crystal violet staining

To evaluate the clonogenic potential of PANC-1 cells, a colony formation assay was performed. After 72 h of treatment with the extract, the culture medium and cell debris were removed, and the cells were gently washed with phosphate-buffered saline (PBS). Cells were fixed with 10% paraformaldehyde at room temperature for 30 min. Following fixation and washing, 0.02% (w/v) crystal violet solution was added to each well and incubated at room temperature for 5 min. Excess dye was removed, and the plates were washed with PBS. The colonies were visualized, classified, and enumerated manually using an inverted light microscope. Untreated cells and cells treated with the vehicle solvent alone served as negative and vehicle controls, respectively. All experiments were conducted in three independent biological replicates ($n = 3$).

2.6 Colony-Forming assay

PANC-1 cells were treated with *C. sativa* seed extract for 48 h. Cells were then trypsinized. After carefully pipetting into single cells, both groups (treated and control) were seeded in 6-well plates (200 cells/well). After two weeks, the colonies were fixed with formaldehyde 0.2% (v/v) for 30 min. The plates were stained with 0.05% (w/v) crystal violet for 30 min at RT. The stained colonies were counted and examined to determine the type of colony.

2.7 Cell migration

PANC-1 cells were seeded into 6-well plates at a density of 3.5×10^5 cells/well in DMEM supplemented with 10% FBS. After 24 h of incubation, cells were treated with various concentrations of the extract or vehicle control for 48 h until reaching approximately 90% confluence. To inhibit cell proliferation and ensure that wound closure was driven solely by cell migration, cells were pre-treated with mitomycin C (5 μg/mL) for 8 h at 37°C. A linear scratch wound was then introduced across the center of the confluent cell monolayer using a sterile 200 μL pipette tip. The culture medium was discarded, and the detached cells and cellular debris were gently washed twice with PBS. Fresh serum-deprived medium was added, and wound areas were photographed at 0, 24, 48, and 72 h post-scratching using an inverted light microscope at 40 × magnification. The scratched surface area was quantified using ImageJ software (version 1.8.0, National Institutes of Health, USA). The normalized percentage of wound closure (migration rate) was calculated using the following equation:

$$\text{Normalized percentage of migration (\%)} = \left(\frac{\text{Area of scratch at 0h} - \text{Area of scratch at imaging t}}{\text{Area0h}}\right) \times 100$$

2.8 Cell cycle analysis

PANC-1 cells were plated in 60 mm dishes (2×10^5 cell/dish). After 24 h, the cells were treated with *C. sativa* seed extract at 1.3 mg/mL for 48 h at 37°C. The cells were

detached from the plates and fixed with cold 70% ethanol at 4°C overnight. Subsequently, the cells were washed twice with PBS. Next, the cells were simultaneously treated with RNase A (50 µl of 100 µg/mL) and Propidium Iodide (PI) (200 µl of 50 µg/mL). The cells were then incubated in the dark at 37°C for 1 h. Cells were analyzed using a flow cytometer at a wavelength of 488 nm, and the results were examined using FlowJo (version 10.5.3).

2.9 Statistical analysis

Statistical analysis of the cell viability assay, colony formation assay, and migration assay was performed using either t-test or one-way ANOVA, followed by Dunnett's post hoc test. For colony types and cell cycle assays, the chi-square test was used. Stars were representative of the following *p-value* levels: * ($P \leq 0.05$), ** ($P < 0.01$), *** ($P < 0.001$), and **** ($P < 0.0001$).

3. Results and Discussion

3.1 Solubility and Selective Cytotoxicity of C. sativa Seed Extract against PANC-1 Cells

To determine the optimal vehicle for biological assays, the solubility of the *C. sativa* seed extract was evaluated in deionized water, PBS, and DMSO. The extract exhibited the highest solubility in DMSO (70 mg/mL), followed by deionized water (26.5 mg/mL), while remaining insoluble in PBS. Subsequently, the cytotoxic profile of the extract dissolved in DMSO was investigated across a wide concentration range (2.8×10^{-8} to 6.3 mg/mL) against PANC-1 pancreatic cancer cells after 72 h of exposure. The cellular response demonstrated a clear triphasic pattern. At low concentrations (2.8×10^{-8} to 3.5×10^{-6} mg/mL), no significant change in viability was observed, indicating a biologically inactive phase. Intermediate concentrations (3.9×10^{-5} to 5.2×10^{-2} mg/mL) induced a decline in cell viability. However, at these intermediate ranges, vehicle-associated toxicity (DMSO) was also observed, which partially masked the extract-specific cytotoxic efficacy. Conversely, at the highest concentrations (0.57 to 6.3 mg/mL), a distinct protective phenomenon was observed. While control cells exposed to equivalent concentrations of DMSO exhibited severe toxicity, those treated with the extract showed significantly higher viability ($P < 0.001$). This suggests that bioactive components within the extract may act to counteract solvent-induced stress (Supplementary Figure S1). To isolate the specific therapeutic efficacy of the extract without solvent interference, further quantitative and qualitative assays were conducted using the water-soluble fraction (0 to 2.4 mg/mL). Crystal violet staining qualitatively confirmed a dose-dependent reduction in PANC-1 cell density, showing severe monolayer disruption at elevated concentrations (Figure 1). Quantitatively, cell viability declined progressively, establishing a significant inhibitory effect starting at 0.51 mg/mL ($P < 0.05$) and reaching maximum suppression at 1.86 and 2.13 mg/mL ($P < 0.01$) (Figure 1B). Based on this dose-response profile, the half-maximal inhibitory concentration (IC50) of the water-soluble extract against PANC-1 cells was determined to be 1.3 mg/mL (Supplementary Figure S2). Our results align with previous literature demonstrating that *C. sativa* extracts exert anti-proliferative and cytotoxic effects across various cancer models, including MCF-7, HT-29, and PC-3 (Pino et al., 2023), MDA-MB-231 and HNSCC (Blal et al., 2023), HCC1806 (Li et al., 2022), U-87 (Nigro et al., 2020), A549 (Hosami et al., 2021), Caco-2 (Rožanc et al., 2021), and AsPC-1 (Emhemmed et al., 2022). The observed biphasic response, characterized by minor, non-significant growth stimulation at very low doses (0.12 mg/mL) followed by potent cytotoxicity at higher concentrations, reflects a classic hormetic effect often observed with polyphenol-rich botanical extracts in oncology models.

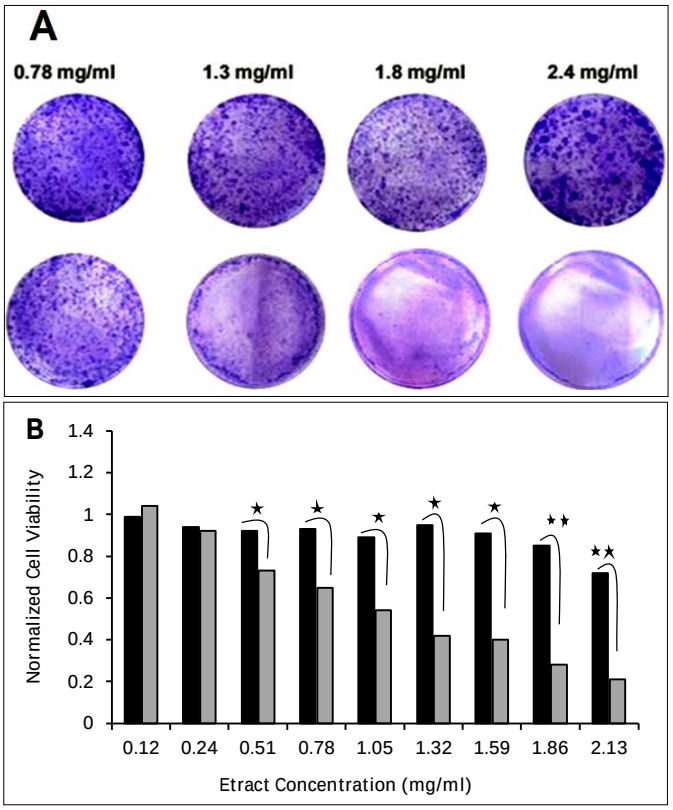


Figure 1. Selective cytotoxicity of *C. sativa* seed extract against PANC-1 and HFF cells. (A) Dose-dependent inhibition of PANC-1 cell proliferation is shown by representative images of crystal violet staining after 72 h of treatment with indicated concentrations (0.78, 1.32, 1.86, and 2.13 mg/mL). (B) Cell viability of normal HFF cells treated with water-dissolved extract (vehicle: deionized water) for 72 h. Data are expressed as mean ± SD (n = 3). Statistical significance was determined by one-way ANOVA followed by Dunnett's test

3.2 Effect of C. sativa Seed Extract on HFF Cell Viability

The effect of water-soluble *C. sativa* seed extract on the viability of normal HFF cells was evaluated after 72 h of treatment in the concentration range of 0.12 to 2.13 mg/mL. At lower concentrations (0.12 to 1.32 mg/mL), the extract did not show cytotoxicity and instead caused a moderate but significant increase in cell viability compared with the

control group. A significant effect was observed at 1.05 mg/mL ($P < 0.05$), suggesting possible growth-promoting or protective activity. At intermediate concentrations (1.59 to 1.86 mg/mL), this stimulatory effect was maintained, and cell viability remained significantly higher than that of the control group ($P < 0.01$ and $P < 0.001$). However, at the highest concentration tested (2.13 mg/mL), a sharp decrease in cell viability was observed ($P < 0.001$) (Supplementary Figure S3), indicating a toxic effect at this dose. These findings show that the extract is not cytotoxic at low to moderate concentrations, but its toxic effect becomes evident at higher doses. This pattern is important because, although the extract showed a strong concentration-dependent cytotoxic effect on PANC-1 cancer cells, its effect on normal HFF cells was much less severe and was only observed at the highest concentration. Our results are also consistent with previous studies reporting the selective toxicity of hemp seed extract on cancer cells, while normal cells were affected only at high doses.

3.3 Colony Formation Assay and Clonogenic Potential

The long-term effects of the water-soluble *C. sativa* seed extract on the proliferative capacity and stemness-related features of PANC-1 cells were evaluated using a colony formation assay (Figure 2). As evidenced by the results, the extract caused a significant reduction in the colony formation ability of PANC-1 cells.

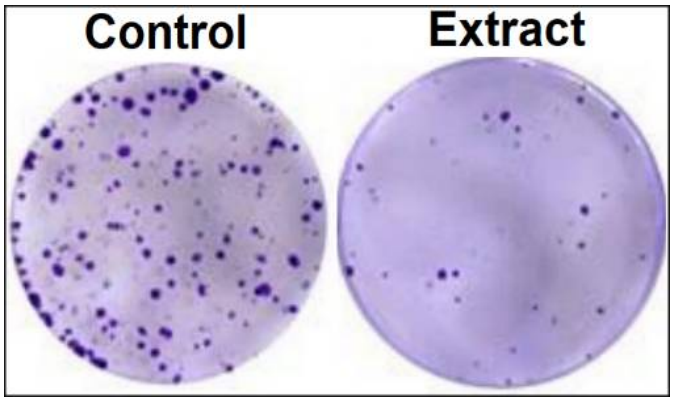


Figure 2. Effect of the water-soluble seed extract of *C. sativa* on colony formation in PANC-1 cells. Deionized water was used as the control. Representative crystal violet-stained images illustrating the clonogenic capacity of PANC-1 cells after treatment with 1 mg/mL *C. sativa* seed extract for 48 h

The colonies were categorized into three distinct morphological types: holoclones (large, dense, and highly proliferative), meroclones (intermediate), and paraclones (small, diffuse, and terminal). Our findings revealed a marked decrease in holoclones, accompanied by a significant shift in population distribution toward paraclones (Figure 3a and 3b). Previous studies have shown that holoclones are the primary tumorigenic population in vivo and have greater self-renewal and tumor-growth capacity than meroclones and paraclones (Tan et al., 2011; Kalirai et al., 2011). Therefore, the extract's ability to suppress holoclone formation and promote a shift toward the less aggressive paraclone phenotype suggests its potential in limiting the clonogenicity properties of PANC-1 cancer cells. These observations are consistent with prior research by Nigro et al. (2020), which reported that a lignanamide-rich fraction of *C. sativa* seed extract effectively decreased the colony formation capacity of U-87 (glioblastoma) cells. In the present study, the whole water-soluble extract of *C. sativa* not only reduced the total number of colonies but also qualitatively altered the clonal composition, further supporting its role as a potential anti-clonogenic agent in pancreatic cancer.

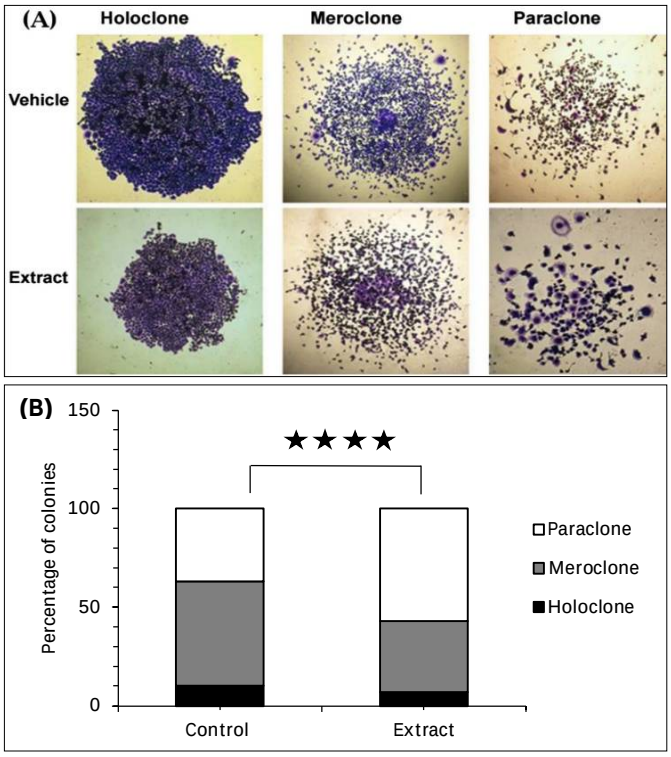


Figure 3. Effect of *C. sativa* seed extract on PANC-1 colony morphology and subtype distribution. (A) Representative phase-contrast light microscopy images illustrating the morphology of distinct colony subtypes (holoclones, meroclones, and paraclones) formed by PANC-1 cells following 48 h of treatment with 1 mg/mL water-soluble *C. sativa* seed extract or vehicle control (deionized water). (B) Quantitative percentage distribution of holoclones, meroclones, and paraclones after 48 h of exposure to 1 mg/mL extract compared with the control. Data represent the mean ± SD of three independent experiments (n = 3)

3.4 Comparative Cytotoxicity: PANC-1 vs. HFF Cells

To evaluate the selective index of the extract, its effects were simultaneously tested on HFF (human fibroblast) cells as a normal cell model. Interestingly, the HFF cells exhibited a biphasic response to the extract. At lower concentrations (0.12-1.32 mg/mL), the extract appeared to promote HFF viability, potentially due to the presence of antioxidant compounds such as lignanamides and phenolic derivatives, which are known to exert protective effects on normal cells (Metouekel et al., 2024). A stable differential window was observed between 1.59 and 1.86 mg/mL, where the extract

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maintained high viability in HFF cells while significantly inhibiting PANC-1 cells (Supplementary Figure S3 and Figure 1B). However, a critical cytotoxic threshold was reached at 2.13 mg/mL, where viability in HFF cells also began to decline. This selective cytotoxicity at moderate doses highlights the therapeutic potential of the *C. sativa* extract, as it inhibited the PANC-1 cells while leaving HFF cells viable at the same concentrations.

3.5 The effect of C. sativa seed extract on the migration capacity of PANC-1 cells

To investigate the effect of *C. sativa* seed extract on the migratory capacity of PANC-1 cells, a wound-healing assay was performed. As shown in Figure 4, the quantitative analysis demonstrated that the extract significantly inhibited cell migration in a time-dependent manner. After 24 h, migration was markedly reduced in the treated group compared with the vehicle control ($P < 0.05$). This inhibitory effect became more pronounced at 48 h, at which time untreated PANC-1 cells exhibited approximately 96% wound closure, whereas extract-treated cells showed only 68% closure ($P < 0.001$). These findings, derived from three independent experiments, indicate that *C. sativa* seed extract effectively suppresses the migratory behavior of PANC-1 cells. Cell migration is a critical event in cancer invasion and metastasis. Vaccani et al. (2005) reported that pure cannabidiol has a strong inhibitory effect on the migration of U87-MG glioma cells, and Solinas et al. (2012) similarly showed that this compound suppresses endothelial cell migration. Given that *C. sativa* seeds contain CBD, the inhibitory effect observed here might be attributable, at least in part, to this bioactive constituent. Consistently, the present results align with those of Nigro et al. (2020), who reported that a lignanamide-rich fraction of *cannabis* seed extract reduced migration in U-87 cells compared with the control group.

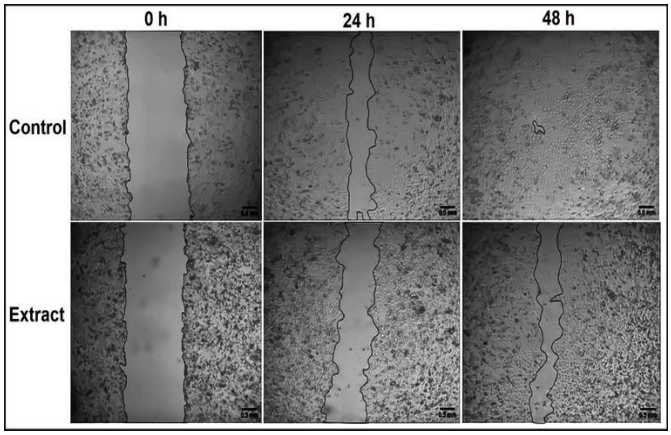


Figure 4. Inhibitory effect of *Cannabis sativa* seed extract on PANC-1 cell migration. Representative phase-contrast micrographs illustrating the progressive closure of scratch wounds in PANC-1 cells at 0, 24, and 48 h post-wounding. Cells were treated with either 1.5 mg/mL water-soluble *C. sativa* seed extract or deionized water as the vehicle control. To exclude the contribution of cell proliferation to gap closure, monolayer cultures were pre-treated with mitomycin C before scratch induction. Scale bars = 0.5 mm

3.6 Cell cycle distribution and induction of apoptosis

To elucidate the mechanism underlying the anti-proliferative activity of the *C. sativa* seed extract, the cell cycle distribution of PANC-1 cells was analyzed via flow cytometry. As illustrated in Figure 5, treatment with 1.3 mg/mL of the extract for 48 h induced a statistically significant redistribution of cells across the cell cycle phases ($P < 0.01$). Quantitative analysis revealed that the extract triggered a robust G0/G1-phase arrest, where the percentage of cells in the G1 phase increased from 46.0% in the vehicle-treated control to 56.5% in the extract-treated group, an absolute increase of 10.5%. Concomitantly, a marked decline was observed in the S-phase population, dropping from 24.6% to 13.1%, indicating a substantial blockade at the G1/S transition and the subsequent inhibition of DNA replication. Additionally, the G2/M population showed a slight decrease from 19.0% to 16.9%, further reflecting the cell cycle arrest at the G1/S interface. Furthermore, a notable elevation in the sub-G1 fraction, a hallmark of DNA fragmentation and apoptotic cell death, was observed, rising from 10.4% to 13.5%.

Together, these quantitative shifts confirm that the anti-proliferative impact of the *C. sativa* seed extract on PANC-1 cells is driven by a combination of G1-phase blockade and the induction of programmed cell death. While previous studies have reported the pro-apoptotic effects of *cannabis* leaf and flower extracts in various cancer models, including A549 lung cells (Hosami et al., 2021), ASPC-1 pancreatic cells (Emhemmed et al., 2022), and PC-3 prostate cells (Pino et al., 2023), our findings highlight the therapeutic potential specifically of the seed extract. *C. sativa* is known to produce a diverse array of secondary metabolites with documented antioxidant, anti-inflammatory, and anticancer properties (Nigro et al., 2020; Koltai & Shalev, 2022). Notably, most existing oncology research has focused on the leaves of the plant (Hussein et al., 2014; Yu et al., 2024); to the best of our knowledge, this is the first study to demonstrate the inhibitory effects of *C. sativa* seeds specifically on pancreatic cancer cells. Although the current data confirm the induction of G1 arrest and apoptosis, further investigations are required to elucidate the precise molecular pathways and specific metabolites responsible for these biological effects.

The observed changes in cell-cycle distribution suggest that the extract exerted cytotoxic and cell-cycle-modulatory effects on the treated cells. The increase in the sub-G1 population from 10.4% in the control group to 13.5% following extract treatment suggests an elevation in apoptotic cell death. In addition, the marked accumulation of cells in the G1 phase, accompanied by a reduction in the S-phase population, suggests that the extract may inhibit cell-cycle progression at the G1/S checkpoint. The decreased proportion of cells in the S and G2/M phases may further reflect reduced DNA synthesis and impaired cell proliferation. Overall, these findings indicate that the extract may suppress cellular growth through the induction of apoptosis and G1-phase cell-cycle arrest. However, additional assays, such as Annexin V/PI staining and the

evaluation of apoptosis-related proteins, are required to confirm the underlying mechanism.

3.7 Chemical characterization of C. sativa seed extract by GC–MS

GC–MS analysis was performed to profile the chemical constituents of the *Cannabis sativa* seed extract (Table 1). A total of 18 compounds belonging to diverse chemical classes were identified, collectively representing 75.24% of the total chromatographic area, with the remainder corresponding to unidentified minor peaks. The extract was predominantly characterized by fatty acids and fatty acid esters, which constituted 49.81% of the identified profile. The major constituents within this fraction were cis-9, cis-12-octadecadienoic acid (22.75%), hexadecanoic acid (15.61%), and cis-vaccenic acid (8.43%), followed by 9,12-octadecadienoic acid ethyl ester (1.45%), linolenic acid (1.16%), and octadecanoic acid (0.39%). In addition to free fatty acids, Table 1 classifies terpenes as accounting for 0.98% of the identified compounds, represented by phytol (0.98%). Other lipid-related derivatives, including 2-mono-palmitin (1.86%) and diisooctyl phthalate (1.08%), constituted 2.95% of the profile. The extract also contained the antioxidant tocopherol isomer, γ-tocopherol (1.77%), and a minor glucosinolate component, desulphosinigrin (0.08%). Furthermore, three phytocannabinoids were detected, contributing 7.96% of the identified profile: cannabidiol (CBD; 3.83%), Delta 9-tetrahydrocannabinol (Delta 9-THC; 2.86%), and cannabinol (CBN; 1.26%). The phytosterol fraction comprised γ-sitosterol (3.86%), campesterol (1.94%), and stigmasterol (0.92%), representing a total sterol content of 6.72%. The complex pharmacological activity of the whole *C. sativa* seed extract might be mediated by the collective and potentially synergistic actions of these diverse phytochemicals, aligning with the concept of the entourage effect (Hanuš & Hod, 2020). Although the current study evaluated the efficacy of the whole ethanolic extract rather than isolated compounds, the documented bioactivities of the main identified constituents support the observed antiproliferative and apoptotic effects in PANC-1 cells. However, because formal synergy analyses (such as combination index modeling) were not conducted with isolated compounds in the present study, this synergistic interaction remains a viable hypothesis that warrants further systematic and orthogonal investigation. Specifically, hexadecanoic acid has been shown to induce cell cycle arrest at the G1 phase and trigger apoptosis in various cancer cell lines, potentially through DNA topoisomerase I inhibition or the upregulation of intracellular reactive oxygen species (ROS) (Harada et al., 2002; Bharath et al., 2021). Similarly, octadecanoic acid has been reported to exert cytotoxic and antitumor effects by promoting membrane damage, necrosis, and apoptosis in gastrointestinal and hepatocellular carcinoma models (Yu et al., 2024).

The phytosterol fraction, particularly γ-sitosterol (3.86%), may also play a key role in the bioactivity of the extract. Phytosterols are well known for their chemopreventive properties, with studies indicating that γ-sitosterol can suppress cancer cell proliferation, downregulate *c-myc* expression, and initiate apoptotic cascades (Sundarraj et al., 2012; Endrini et al., 2014). The presence of the bioactive phytocannabinoids CBD and Delta-9-THC, although in lower percentages compared to typical flower or inflorescence extracts (Jin et al., 2021), further enriches the chemical complexity of the seed extract. While most research on *Cannabis* mediated oncology targets high cannabinoid aerial tissues, our findings demonstrate that the seed extract provides a unique, lipid-rich chemical profile containing a balanced mixture of unsaturated fatty acids, phytosterols, and moderate cannabinoid levels. These results suggest that the antiproliferative, antimigratory, and cell cycle-arresting effects observed in PANC-1 cells are likely the result of a multi-component synergy. Consequently, the whole *C. sativa* seed extract warrants further investigation via bioactivity-guided fractionation to fully elucidate the specific molecular mechanisms and synergistic interactions underlying its therapeutic potential.

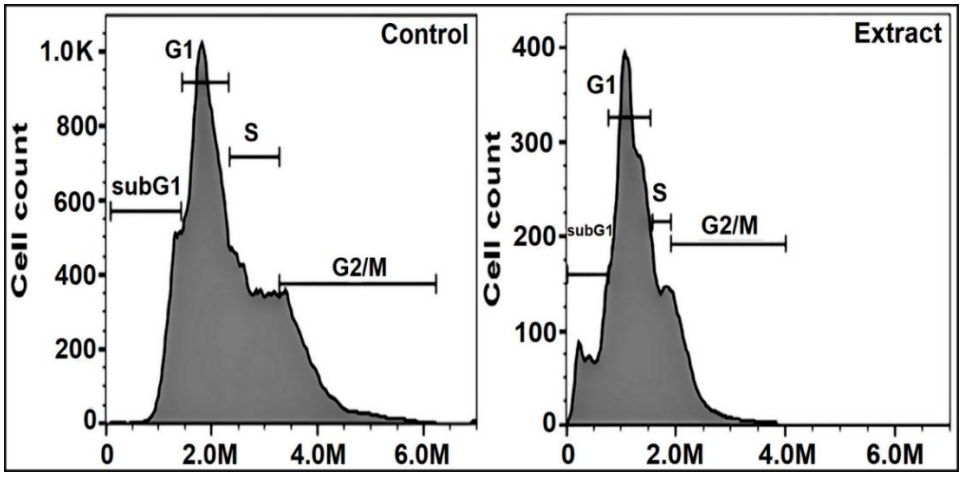


Figure 5. Effect of the water-soluble seed extract of *C. sativa* on cell cycle distribution in PANC-1 cells, as assessed by flow cytometry. Cells were treated with 1.3 mg/mL of the extract for 48 h. Deionized water was used as the vehicle control. Representative flow cytometry histograms showing the percentage of cells in different phases of the cell cycle in control and extract-treated cells

Table 1. Identification of the components of the *C. sativa* seed extract using GC-MS

Compound	Retention Time (minutes)	%	Chemical Family
Desulphosinigrin	55.14	0.08	Glucosinolate
Hexadecanoic acid	72	15.61	Long-chain fatty acids
Phytol	78.44	0.98	Terpene
cis-9,cis-12-Octadecadienoic acid	80.19	22.75	Unsaturated fatty acid
cis-Vaccenic acid	80.43	8.43	Monounsaturated fatty acids
9,12-Octadecadienoic acid, ethyl ester	80.75	1.45	Fatty acid esters
Octadecanoic acid	81.17	0.39	Saturated Fatty Acid
Cannabidiol	91.72	3.83	Phytocannabinoid
2-mono-Palmitin	95.26	1.86	Monoglyceride
Delta 9-Tetrahydrocannabinol (Dronabinol)	95.72	2.86	Phytocannabinoid
Diisooctyl phthalate	95.92	1.08	Fatty acid esters
Cannabinol	98.12	1.26	Phytocannabinoid
β-Mono-linolein	101.9	5.01	Monoglyceride
Linolenic acid	102.16	1.16	Polyunsaturated fatty acid
γ-Tocopherol	113.85	1.77	Tocopherol
γ-Sitosterol	116.94	3.86	Phytosterol
Campesterol	119.39	1.94	Phytosterol
Stigmasterol	120.22	0.92	Phytosterol

4. Conclusion

To the best of our knowledge, the present study is the first to demonstrate that an ethanolic extract of *Cannabis sativa* seeds possesses selective anticancer properties against the human pancreatic cancer cell line PANC-1, while exhibiting negligible cytotoxicity toward normal human foreskin fibroblast (HFF) cells. Our findings demonstrate that the extract significantly suppresses the proliferative and clonogenic potential of PANC-1 cells, notably shifting colony morphology toward less aggressive paraclones. The observed anti-tumor activity is mechanistically driven by the induction of G1-phase cell cycle arrest, accompanied by a modest increase in the sub-G1 fraction, suggestive of moderate apoptosis, as evidenced by flow cytometric analysis. Furthermore, the extract significantly attenuated the migratory capacity of PANC-1 cells. Chemical profiling via GC–MS suggests that these biological effects might be attributed to the collective actions of 18 identified compounds, dominated by bioactive fatty acids (e.g., hexadecanoic and octadecanoic acids), phytosterols (e.g., γ-sitosterol), and phytocannabinoids. Collectively, these results highlight the therapeutic potential of *C. sativa* seed extract as a novel, multi-component candidate for pancreatic cancer intervention. However, the in vitro nature of the study, the absence of in vivo animal validation, and the relatively high IC50 of the crude extract must be considered critical limitations when interpreting these findings in a physiological context. Further research is warranted to elucidate the specific molecular signaling pathways involved in cell cycle arrest and migration inhibition, as well as to evaluate the bioavailability, toxicity profiles, and efficacy in relevant in vivo models.

Authors' Contributions

Fatemeh Salah: Data curation; Formal analysis; Investigation; Methodology; Resources; Software; Visualization; Writing-original draft; Writing-review & editing. **Forouzan Ghasemian Roudsari:** Conceptualization; Data curation; Funding acquisition; Methodology; Project administration; Resources; Software; Supervision; Validation; Visualization; Writing-review & editing; Final English review & editing. **Mohammad Masoudi:** Conceptualization; Data curation; Funding acquisition; Methodology; Project administration; Resources; Software; Supervision; Validation; Visualization; Writing-review & editing; Final English review & editing. **Sadrollah Ramezani:** Data curation; Investigation; Methodology; Resources; Writing-review & editing; Final English review & editing. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare no conflicts of interest.

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Ethical Considerations

All ethical principles are considered in this article. This study was approved by the Ethics Committee of the University of Zanjan (Code: IR.ZNU.REC.1402.012).

Using Artificial Intelligence

The authors used Artificial Intelligence (AI) tools solely for proofreading and enhancing the readability of this manuscript. We explicitly declare that no generative artificial intelligence was used to create the content, data, or arguments presented in this study.

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