RESEARCH ARTICLE

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# *Medicago sativa* Extracts Enhance the Anticancer Efficacy of GEM in PANC-1 Cells through Apoptosis Induction and *BAX/BCL-2/CASP3* Expression Modulation

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## Abstract

**Introduction:** Pancreatic cancer (PC) has a poor prognosis and limited response to therapies. Combinatorial approaches, such as natural product-based therapies, can enhance anticancer efficacy while minimizing side effects. This study evaluated M. sativa's anticancer properties and its potential as adjunctive therapy with Gemcitabine (GEM) to sensitize PANC-1 cells to chemotherapy. **Methods:** The antioxidant activity (AA) and total phenolic content (TPC) of M. sativa extracts (Methanol, Ethyl acetate, and water) were assessed using the DPPH radical scavenging assay. Cytotoxic effects on PANC1 and HUVEC cells were also evaluated by utilizing the MTT assay. Then, apoptosis detection was performed by Annexin V/PI-flow cytometry (FC). Besides, the DNA fragmentation analysis was conducted utilizing agarose gel electrophoresis (AGE). *Bcl-2*, *Bax*, and *CASP3* expression levels in PANC-1 cells using western blot analysis and qRT-PCR. **Results:** Herein, DPPH $IC_{50}$ values for M. sativa extracts (water, MeOH, EtOH) were 76.21, 110.32, and 65.39 µg/ml, respectively. The water extract of M. sativa exhibited the highest TPC (4612.15 ± 119.4 mgGAE/g). The cytotoxicity $IC_{50}$ values for EtOH M. sativa extract, GEM, and combined GEM with EtOH M. sativa on PANC1 cells were 68.74, 43.53, and 41.22 µg/ml M. sativa + 25 µg/ml GEM, respectively, with no toxicity observed in HUVEC cells. FC analysis revealed that Combining GEM and EtOH M. sativa yielded the highest apoptosis rate (25.6%). Expression changes in *Bcl-2*, *Bax*, and *CASP3*, as well as morphological alterations and DNA fragmentation, indicated apoptotic cell death. **Conclusion:** Our findings suggested that combining M.sativa EtOH extracts with GEM may represent a promising strategy for treating PC.

**Keywords:** Medicago sativa- PC- GEM- Apoptosis- Combination therapy

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## Introduction

Pancreatic cancer (PC) is the seventh most prevalent source of death from tumour globally and ranks 12th out of all malignancies [1]. It continues to be a major global burden because of its extreme aggression and low survival rate [2]. Unfortunately, only around 20% of PC patients are surgical candidates [3]. The pyrimidine antimetabolites GEM and 5-fluorouracil were the first effective therapies for PC. Multiagent regimens based on these antimetabolite substrates expanded the therapeutic insights decades later. Despite the emergence of novel chemotherapy approaches in recent decades, medication resistance remains a significant barrier [4]. To enhance the PC patients' overall survival rates, a large body of research has investigated the combination of gemcitabine (GEM) with different agents, e.g., natural products (NPs) [5-8]. NPs are vital in developing innovative treatments, accounting for over 60% of all recognized pharmaceuticals [9]. In vitro and animal model studies have suggested that compounds with diverse structures from numerous natural sources can exert both cancer chemo-preventive and chemotherapeutic effects [10]. The use of herbal medications as an alternative or supplemental cancer treatment option has been widely accepted [10, 11]. Consequently, several new cytotoxic chemicals are discovered in plants yearly, offering new avenues for cancer treatment [12, 13]. Clinical investigations suggest that using plant-derived chemicals in standard anti-tumor regimens may enhance the effectiveness of cancer treatment [14]. The considerable advances achieved in this research area have resulted in the therapeutic use of many secondary plant compounds, whereas others are being investigated in clinical trials serving as anticancer medicines [12].

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Secondary metabolites are common compounds that naturally arise in microbes and plants, and their structure and biological properties are determined by the metabolic routes through which they are created [15, 16]. Secondary structural metabolites include alkaloids, terpenoids, organosulfur compounds, and polyphenols [17]. Polyphenols, which are the most frequent natural secondary metabolites, include stilbenes, phenolic acids, and flavonoids, with flavonoids accounting for around two-thirds of all polyphenolic substances [18]. Their chemo-preventive properties, e.g., anticarcinogenic, antioxidant, anti-inflammatory, and antimutagenic effects can be attributed to phenolic bioactivities. They are also involved in cellular mechanisms, e.g., apoptosis (aka programmed cell death), cell cycle arrest, regulating the metabolism of carcinogens, ontogenesis expression, inhibition of DNA binding, as well as cell migration, adhesion, proliferation, or differentiation, and blockage of signaling pathways [19]. Therefore, these non-toxic "NPs" derived from natural resources may be effective in combination with traditional chemotherapeutic medicines for treating human malignancies with fewer side effects and greater efficacy.

Alfalfa (Medicago sativa [M. sativa]), commonly referred to as lucerne, a perennial flowering plant species in pea family (Fabaceae or Leguminosae), which has long been used in China, India, Iran, Iraq, South Africa, Turkey, and the US as a traditional herbal medicine [20]. It has caught researchers' attention thanks to its high nutritional value, which includes high concentrations of amino acids, folate, proteins, vitamins, minerals (e.g., calcium and iron), and phytochemicals (e.g., L-canavanine, coumestrol, isoflavones, carotene, and chlorophyll) [21-24]. Phytoestrogens (e.g., lignans, coumestans, and isoflavones,) found in M. sativa have been associated with improved human health, including osteoporosis prevention, cardiovascular disease, menopausal symptoms, and cancer chemoprevention along with treatment [25]. M. sativa has demonstrated beneficial effects on cytotoxicity and apoptosis in mammary cancer and human erythroleukemia due to its flavonoid contents [26]. Additionally, M. sativa has medicinal benefits, including preventing diabetes, anemia, endometriosis, gastric ulcers, and poor bone density [27, 28].

Apoptosis is essential for preserving tissue homeostasis during development. Defective apoptosis can promote cancer and uncontrolled cell growth. Human cancers often exploit this mechanism by evading cell death, which promotes tumor expansion [29]. Most tumors' inability to initiate apoptosis in response to adequate stimuli represents one of the greatest unresolved difficulties in oncology [29]. Apoptosis can be induced by activating two distinct signaling pathways. The chemical-induced mitochondria-related apoptotic pathway is controlled by important apoptotic proteins (e.g., cytochrome c, *Bax*, and Bcl-2) and subsequently by the activation of poly(ADP-ribose) polymerase (PARP) and caspase-3 [30, 31]. Because natural compounds may affect apoptotic pathways that are typically inhibited in human malignancies, they may provide new opportunities for cancer therapy development. Natural compounds have garnered significant interest since they safely and efficaciously overcome tumor cells' apoptosis resistance, given the success rate and issues of currently available synthetic medicines for PC [32].

Consequently, this research sought to assess the unique anticancer and antiapoptotic properties of Medicago sativa, as well as its effectiveness as a GEM chemosensitizer to apoptosis in PC.

## Materials and Methods

*Chemicals and Reagents*

RPMI1640 medium, Pen-Strep, and trypsin-EDTA were obtained from Thermo Fisher Scientific Inc. (Grand Island, NY, USA), whereas fetal bovine serum (FBS) was obtained from Cytiva (Kremplstraße, Pasching, Austria). The Naphenol™ total phenol assay kit (Navand Salamat Pishtaz Co, Urmia, Iran) was utilized to determine the TPC. Also, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was acquired from Invitrogen (Eugene, Oregon, USA). The Annexin V-FITC/PI Apoptosis Detection Kit (BioLegend, USA) was used for apoptosis detection. GEM hydrochloride was acquired from Sigma-Aldrich Corporation (Saint Louis, Missouri, USA).

*Cell Lines and Culture Condition*

The PANC-1 (i.e., Human pancreatic adenocarcinoma, IBRC 10156) and HUVEC CRL-1730 (i.e., Human Umbilical Vein Endothelial Cells) lines as normal cells were acquired from the cell bank of the Iranian Biological Resource Center (IBRCTM, Tehran, Iran) and Royan Stem Cell Bank (Tehran, Iran), respectively. They were then cultured in a mixture media of RPMI1640, 10% FBS, and 1% Pen-Strep (100 units penicillin and 100 µg streptomycin per ml). Afterward, they were incubated at 37°C, 95% relative humidity, and 5% $CO_2$.

*Isolation of plant extract*

The taxonomic identity of the Medicago sativa (M. sativa) was authenticated by the Iranian Plant Protection Research Institute (IRIPP), Tehran, Iran, and a voucher specimen was deposited in the herbarium with the accession number IRIPP: 3460. The aerial parts of the plant were first cleaned by rinsing with deionized water to remove any contaminants, and then oven-dried at 60°C to preserve the plant material. The dried aerial parts were subsequently ground into a fine powder using a mixer to increase the surface area for extraction. Extractions of M. sativa were carried out using different solvents, including methanol (MeOH), ethyl acetate (EtOAc), and water. For each extraction, 20 grams of the dried powder were placed in a flask, and 50 mL of the respective solvent (boiled water, 60% ethanol, or MeOH) was added. The mixtures were then incubated for 72 hours to allow for maximum extraction of the bioactive compounds. Following incubation, the extracts were filtered through Whatman filter paper (No. 1) to remove any impurities. The resulting extracts were stored frozen at −20°C to preserve their chemical composition and biological activity for later use in the study.

*Total Phenol Capacity Determination*

TPC is determined by the total phenol assay kit (Naphenol™, Navand Salamat Pishtaz Co, Urmia, Iran) according to the manufacturer's manual. In brief, 8µl of M.sativa extracts (100 µg/ml) and various concentrations of the standard solution (0, 50, 100, 150, 200, 250, 500 mg/ml) were mixed with 24µl of Reagent-1. This was followed by adding 40 µl of Reagent-2 to the resultant mixture after 5 min and incubating at average thermal condition indoors for 45 min in darkness. The mixture absorbance was calculated at 630 nm and recorded as mg gallic acid equivalents (GAE) per gram of extract using a UV-spectrophotometer (BioTek, Inc, USA). Each experiment was conducted in triplicates, whose results were indicated as mean±SD.

*Determining Antioxidant Properties*

The extracts' free-radical-scavenging capacity was evaluated using the DPPH (1,1-diphenyl-2-picrylhydroxyl) radical scavenging assay kit (Zantox, Birjand, Iran) according to the manufacturer's manual. In short, 0.15 mM DPPH ethanol solution was mixed with extract solutions at varying concentrations, followed by the vigorous shaking of the reaction mixture. After 30 min, a BioRad Benchmark Plus Microplate Reader (Hercules, California, USA) was used to quantify the remaining DPPH radicals at 517 nm. The extracts' AA was reported as percentage inhibition (I%) relative to a reference solution (e.g., Trolox). Each experiment was conducted in triplicates, whose results were recorded as mean±SD. Ninety six percent ethanol was used as the blank solution, while the negative control comprised a mixture of 2.5 ml of 96% ethanol and 1.0 mM DPPH solution.

The percentage of DPPH radical scavenging activity can be calculated using the following formula: DPPH radical scavenging % = [(A0 – A1)/A0] × 100 Where A0 is the absorbance of the DPPH solution and A1 is the absorbance of the sample.

*Cell Viability*

At a confluence of 80%, the cells were passaged in trypsin/EDTA, each day; cells were examined for morphology and contaminants. They were then seeded in 96-well plates at $5\times10^4$ cells/wells and thereafter incubated in standard conditions for 24 h. Next, they were treated three times with varying concentrations of M. sativa (0-150µg/ml), GEM (0-1,000 µg/ml), and a combination of M. sativa with GEM for 24 and 48h and 72h. The HUVEC cell line was also treated with various concentrations of M. sativa (0-150µg/ml) for 24, 48h, and 72. Cell viability was evaluated by means of the MTT assay. In brief, the MTT solution was thereafter added per well at 5 mg/ml and then incubated for 2 h at 37°C. Formazan crystals formed were solubilized by adding 100µl DMSO and incubated in standard condition for four hours. The optical density (OD) was then calculated (read) by an ELISA multi-well plate reader at 570 nm. The absorbance of each well compared to control wells (wells not treated with any unique material) was examined and analyzed to determine $IC_{50}$.

Cell viability (%) = (treated cell OD - blank OD)/ (vehicle control OD - blank OD) × 100 [33].

*Morphological alterations assay*

The PANC-1 cells were seeded at $5\times10^5$ cells/wells in 6-well plates and incubated thereafter for 24 h in standard conditions. They were then treated with $IC_{50}$ of M. sativa and a combination of M. sativa with GEM for 10-30 h. In the end, an inverted phase-contrast microscope was utilized to monitor any observable morphological changes.

*Apoptosis Detection Flowcytometry*

The evaluation of apoptotic cells induced by M. sativa, GEM, and their combination on PANC-1 was conducted by Flowcytometry using BioLegend's Annexin V-FITC Apoptosis Detection Kit (USA) based on the manufacturer's manual. The cells ($5\times10^5$) were then seeded in 6-well plates, after 24h which cells became 80% confluent, they were treated with a pre-calculated $IC_{50}$ concentration. This was followed by incubating both treated and untreated cells at 37°C and 5% $CO_2$ for 48 h. Next, they were harvested using 2.5% trypsin and thereafter risened with phosphate-buffered saline (PBS). They were then resuspended in 500µl1X binding buffer, followed by staining with PI staining solution (5 µL) and Annexin V-FITC (5 µL) for exactly 15 minutes at room temperature in darkness. Finally, they were immediately analyzed via a BD Biosciences' FACSCalibur flow cytometer by utilizing BD and FlowJo software.

*Gene expression assay*

Invitrogen TRIzol reagent (Carlsbad, California, USA) was employed to extract the total RNA from the cells that were untreated and treated with $IC_{50}$ concentration. Also, a NanoDrop Technologies ND-1000 Spectrophotometer was employed to calculate RNA concentrations at 260/280 nm by UV spectrophotometry. Agarose gel electrophoresis (AGE) was adopted as a measure of nucleic acid purity. The reverse transcription of 1µg RNA contained in each sample was performed using the Thermo Scientific RevertAid RT Reverse Transcription Kit (Cat No. K1691).

A Rotor-Gene Q MDx PCR cycler (Qiagen GmbH) was used to evaluate *Bax*, *Bcl-2*, and *CASP3* expressions. Based on the manufacturer's instructions, the *Bax*, *Bcl-2*, and *CASP3* genes were amplified by cDNA fragments as templates by employing 2X Real-Time PCR Master Mix(Biofact™, Korea, Cat No. DQ383-40H). Here is how the experimental protocol was performed: i) Thermocycling conditions included initial activation at a temperature of 95˚C for 15 min, and subsequently, 35 cycles at 94˚C for 35 sec and 60˚C for 40 sec; and ii) melting curve analysis. Table 1 lists qPCR's primer sequences. The *GAPDH* gene is employed as an endogenous normalizing gene. Gene expression levels were measured according to the Ct values by utilizing the $2^{-\Delta\Delta Ct}$ formula.

*Western Blotting*

To investigate the effects of M. sativa, GEM, and a combination of the two on Caspase 3, Bcl-2, and *Bax*, protein expression, the cells underwent treatment with

Table 1. Specific Primer Sequences for qRT-PCR

| Name | | Sequence (5'to3') | Length | GC% | Tm° C |
|---|---|---|---|---|---|
| *CASP3* | Forward | TTCAGAGGGGATCGTTGTAG | 20 | 50.00 | 56.36 |
| | Reverse | TCAATGCCACAGTCCAGTTC | 20 | 50.00 | 58.10 |
| *BAX* | Forward | CCACCCTGGTCTTGGATCCAGCCC | 24 | 66.67 | 68.71 |
| | Reverse | CCTGTGCACCAAGGTGCCGGAACT | 24 | 62.50 | 69.46 |
| *BCL2* | Forward | TTGTGGCCTTCTTTGAGTTCGGTG | 24 | 50.00 | 63.77 |
| | Reverse | GGTGCCGGTTCAGGTACTCAGTCA | 24 | 58.33 | 66.18 |
| *GAPDH* | Forward | TGCCTCCTGCACCACCAAC | 19 | 63.16 | 62.79 |
| | Reverse | CGGAGGGGCCATCCACAG | 18 | 72.22 | 62.18 |

the indicated $IC_{50}$ concentrations of M. sativa, GEM, and their combination for 48 h. Following treatment, cell lysis was performed in RIPA buffer and denatured. The protein samples were first equilibrated in a transfer buffer that contained 50 mM Tris, 40 mM glycine, 20% methanol, and 0.375% SDS, with pH 9.0. Afterward, they were separated on a 12% SDS-PAGE gel and transferred thereafter to an Immobilon-P Transfer Membrane (Millipore Co., Bedford, Massachusetts, USA).

The transferred membrane blocking was done for 30 min at room temperature with 5% non-fat milk. Once diluted in primary antibody dilution buffer (Coolaber, SL1360), primary antibodies were placed in an incubator overnight at 4°C with the nitrocellulose membranes. These included anti-caspase-3 (ABclonal, A2156), anti-*Bax* (Upstate, Lake Placid, NY, USA), and anti-Bcl-2 (Upstate Biotechnologies, Lake Placid, NY, USA). Next, the membranes were put into the incubator with HRP-conjugated secondary antibodies corresponding to them for two hours at room temperature. These antibodies were then added to the membrane upon washing to remove any unbound primary antibodies. The target proteins can be discovered because secondary antibodies can recognize and bind to primary antibodies. Protein levels were standardized by comparing them to the densitometry of *GAPDH*, which served as an internal reference, by employing the ImageJ software by the National Institutes of Health for quantified analysis of the band.

*DNA Fragmentation Assay*

The biochemical apoptotic hallmark is typically thought to be the DNA cleavage into oligonucleosomal fragments, which can be identified as a DNA ladder on AGE. The DNA fragmentation study was performed by selecting the IC50 dose of M. sativa, GEM, and a combination of the two on PANC-1. This was followed by washing the cells (5x105 cells per sample) with PBS and lysing them using a cell lysis buffer [150mM NaCl, 10mM EDTA, 0.5% SDS, 10mM Tris-HCl (pH: 7.5), 500 mg/l proteinase K]. Thereafter, the mixture was incubated overnight at 50°C. This was followed by isolating the lysate with phenol, isopropyl alcohol, and chloroform, and subsequently, precipitating the DNA with ethanol and 3M sodium acetate (pH: 5.2). RNase A (20 g/ml) was then added to the Tris-EDTA buffer solution (10mM Tris, 1mM EDTA, pH: 8.0), and the DNA pellet was put into the incubator thereafter for 30 min at 37°C. The concentration of DNA at 260/280 nm was determined by a NanoDrop ND-1000 UV-VIS spectrophotometer. DNA electrophoresis (20 μg) was done on a 1%w/v EtBr-stained AG. The bands of DNA fragmentation were photographed and analyzed under UV light .

*Statistical Analysis*

The t-test was adopted to compare all experimental data, and the results were demonstrated as mean±SD of the three abovementioned experiments ($P<0.05$ was taken to be statistically significant). The IC50 values were computed by regression analysis after triplicate data points were plotted over a concentration range. The graphs were created by employing GraphPad Prism 8.0 (GraphPad, La Jolla, California, USA).

## Results

*TPC in M. sativa extracts*

In this research, M. sativa extraction was assessed for TPC. Three different solvents (Water, EtOAc, and MeOH) were utilized throughout the extraction TCP process. The TPC content of the water extraction was the highest (4612.15±119.4 mgGAE/g), followed by the extraction of EtOH (3623.21 ± 152.39mg mgGAE/g) and MeOH (2523.18 ± 135.88 mgGAE/g), respectively.

*DPPH radical-scavenging activity*

Herein, the M.sativa extract was utilized to evaluate the antioxidant effects of DPPH radical scavenging assays. As a stable free radical, DPPH decolorizes after being exposed to antioxidants [34]. M.sativa has proven to have anti-inflammatory and antioxidant effects [35]. The scavenging effect of each solvent (water, EtOH, and MeOH) extract on DPPH was evaluated. Among extracts studied, EtOH exhibited powerful antioxidant abilities compared to other extracts with 65.39 ±0.015 $IC_{50}$. The $IC_{50}$ values of M.sativa water and MeOH extract were 76.21±0.016 and 110.32±0.017 μg/ml, respectively (Figure 1). The results of this study are consistent with previous research that has shown the antioxidant activity of M. sativa extracts. The DPPH radical scavenging assay is a reliable method for evaluating the antioxidant activity of plant extracts, and the results obtained in this study suggest that the EtOH extract of M. sativa has potent antioxidant properties. The antioxidant activity of plant extracts is often attributed to the presence of phenolic compounds, which are known

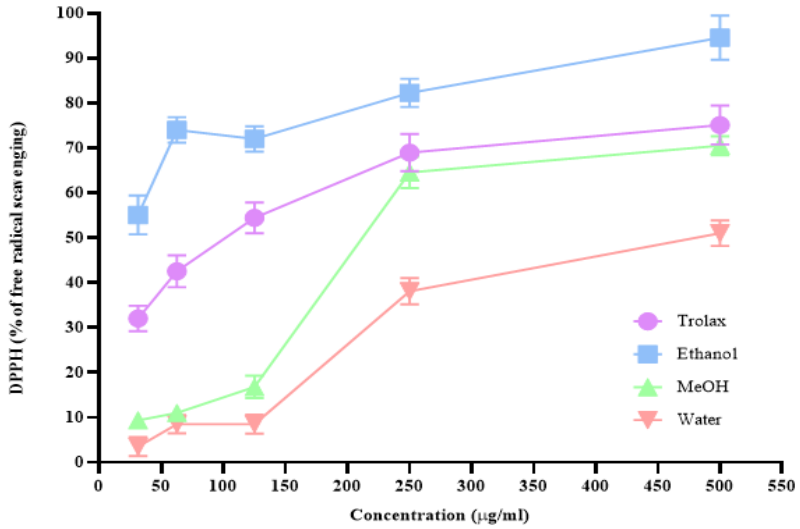


Figure 1. The Antioxidant Properties by DPPH Radical Scavenging of Different Extracts (MeOH, EtOAc, and water) of M.sativa and Trolox

to have radical scavenging properties. The total phenolic content of the extracts was not determined in this study, but it is likely that the EtOH extract had a higher content of phenolic compounds, which contributed to its higher antioxidant activity. In conclusion, the results of this study demonstrate the antioxidant effects of M. sativa extract using the DPPH radical scavenging assay. The EtOH extract exhibited the highest antioxidant activity, suggesting that it may be a useful natural antioxidant for various applications. Further studies are needed to fully characterize the antioxidant properties of M. sativa extracts and to explore their potential uses.

*In vitro anticancer activities*

*Cytotoxic effects of M. sativa extracts on PANC-1 and HUVEC cell lines*

The cytotoxic effects of various extracts (EtOH, MeOH, and H2O) of M. sativa on PANC-1 and HUVEC cells at concentrations of 0-150 µg/mL were examined by the MTT colorimetric assay. The cell viability percentage was also calculated following 24, 48, and 72 h.

Treating PANC-1 with various M. sativa extract concentrations indicated the dose-dependent cytotoxicity of the M. sativa extracts. The amount of inhibitory concentration ($IC_{50}$) was 68.74± 121.878, 98.66± 141.378, and 83.12± 102.12 µg/mL for EtOH, MeOH,

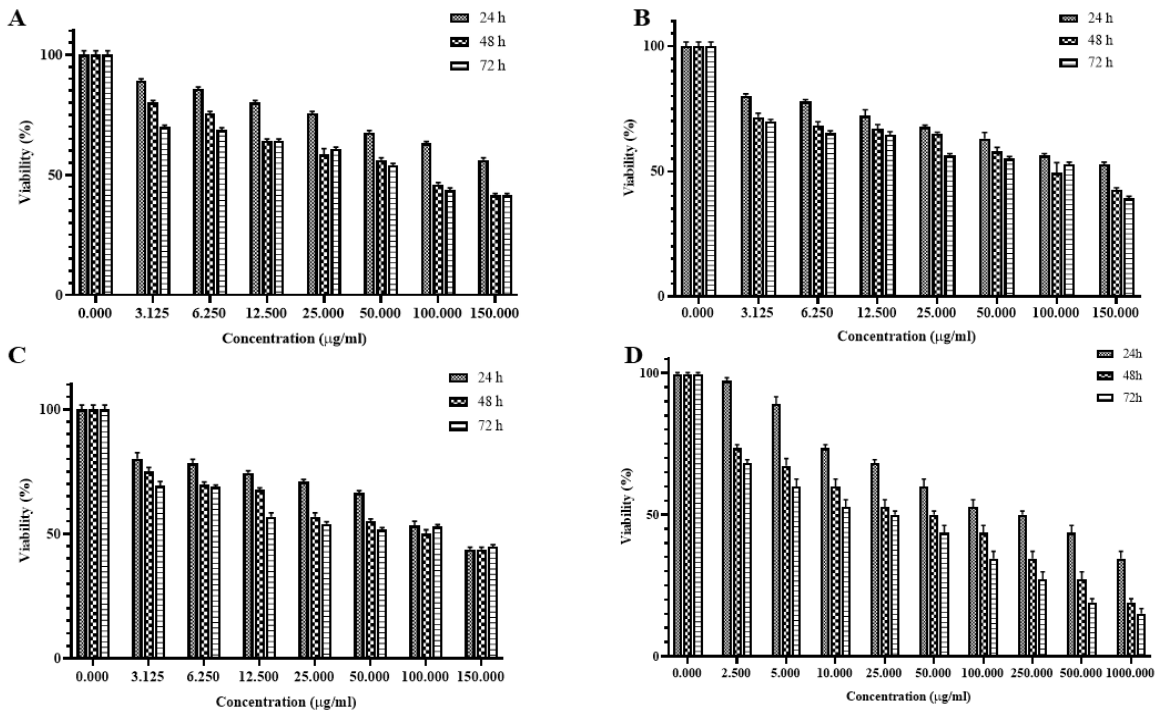


Figure 2. Cytotoxicity Evaluation of Different Extracts and GEM at 24, 48, and 72h on PANC-1 Cells. (A) EtOH extract; (B) MeOH extract; (C) water extract of M. sativa and (D) GEM.

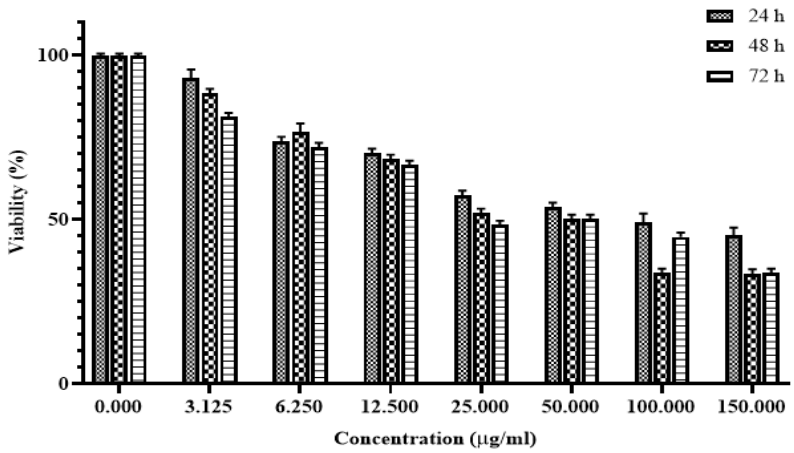


Figure 3. M.sativa + GEM after 48h (GEM = 25 µg/mL) on PANC-1 Cancer Cell Line (The Results were Expressed as Survival Percentages in Comparison with Control Samples.)

and water extract, after 48h, respectively (Figure 2 A, B, C). Furthermore, it was tested whether the M.sativa extract's cytotoxic impact was extended to normal cells; extract treatment on HUVEC was used for this purpose. Interestingly, cytotoxicity was only observed at higher dosages on normal cells and showed that M. sativa extracts more inhibited the PANC-1 growth. The $IC_{50}$ of the noncancerous HUVEC cell was up to 150 µg/ml(1,345µg/ml).

To investigate the $IC_{50}$ of GEM by MTT assay of PANC-1 cells that were treated with varying GEM concentrations (e.g., 2.5, 5, 10, 25, 50, 100, 250, 500, and 1,000 µg/ml). After a 48-hour incubation, the $IC_{50}$ of GEM was seen at 43.53 µg/mL concentration, and the cell viabilities were 49.954 ± 1.286, as shown in Figure 2D.

*Cytotoxic effects of EtOH M. sativa extract combined with GEM on PANC-1*

The cytotoxic effects of the combination EtOH M. sativa (0-150 µg/ml) and $IC_{25}$ of GEM (25µg/ml) were evaluated and showed enhanced cytotoxic effects on PANC-1 cells (Figure 3). By administering various dosages of EtOH M. sativa extract to the cells, we were able to detect $IC_{50}$ 41.22 µg/ml M. sativa + 25 µg/ml GEM. The effect of GEM (25 µg/ml) combined with EtOH extract of M.sativa (41.22 µg/ml) further reduced cell viability in PANC-1 after 48h against the use of each alone (Figure 3).

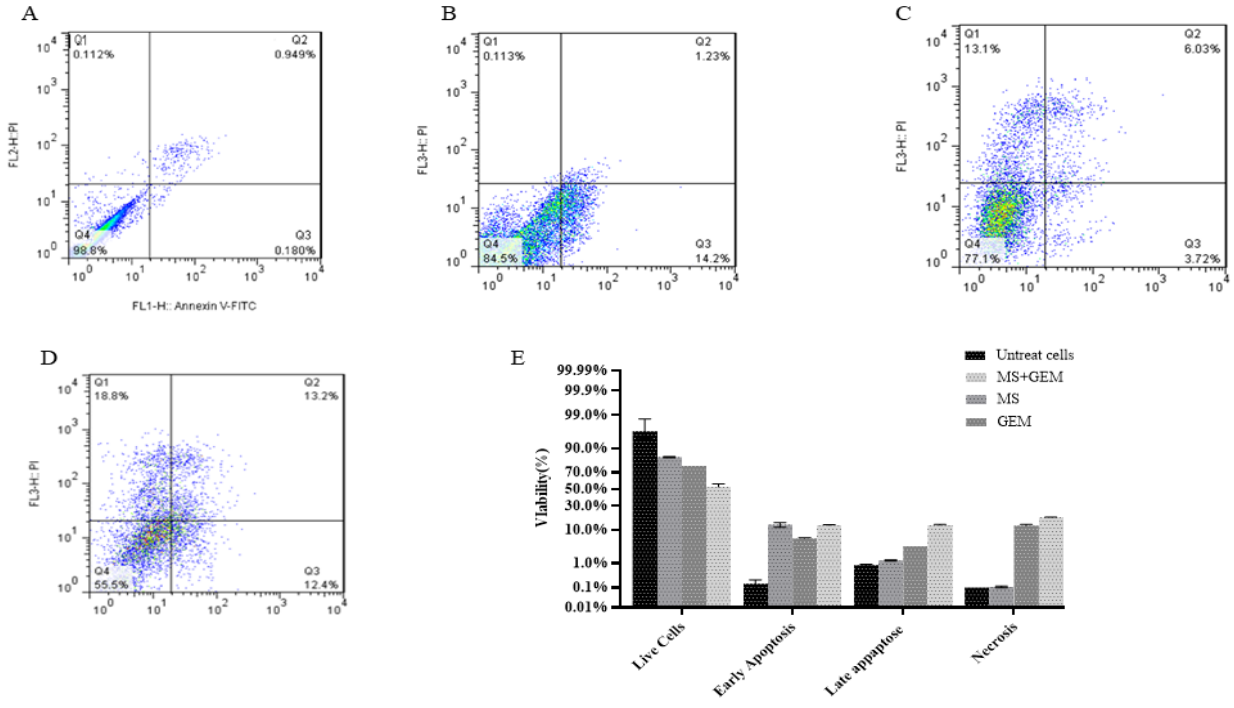


Figure 4. Histograms of the FCA of Apoptotic Cell Death upon Annexin V-FITC/PI Staining in PANC-1 Cells. (A) untreated cells; (B, C, D) IC50 concentration of EtOH M. sativa extract, GEM, and their combination in 48 h, respectively; (E) The apoptosis rate (late and early) comparison in the treated and untreated cells with IC50 concentration of EtOH M. sativa extract, GEM, and their combination.

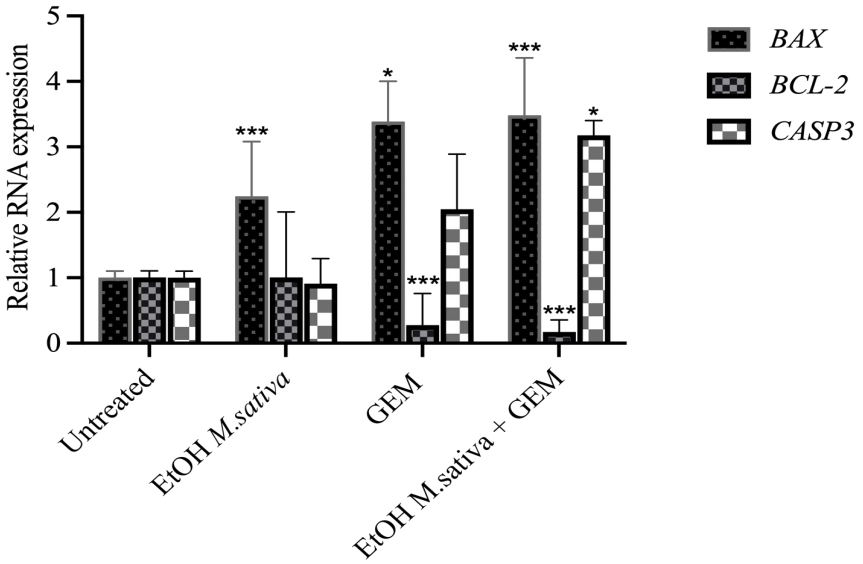


Figure 5. Relative Gene Expression of *BAX, BCL2*, and *CASP3* in Different Concentrations of EtOH M.sativa, GEM, and EtOH M.sativa Combined with GEM. (A) *BAX* (B) *BCL2* (C) *CASP3*; Experiments' results are reported as mean±SD (where n=3). [p<0.05*, p<0.01**, and p<0.001*** were taken as statistically significant compared to the untreated group using Student's t-test.]

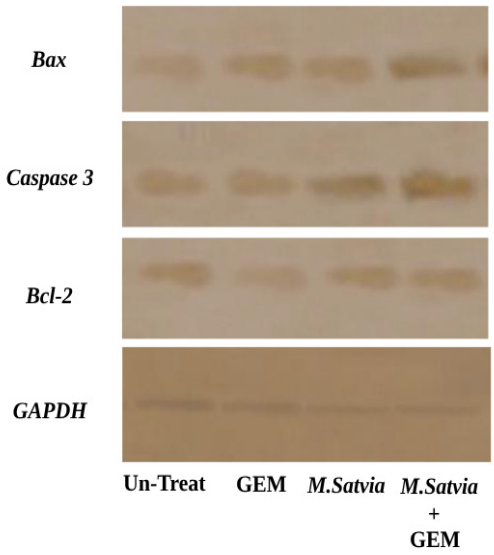


Figure 6. Western Blot Bands of *Caspase-3 Bcl-2,* and *Bax* Expression Levels in M.sativa, GEM, and M.sativa+GEM for 48h treatment in PANC-1. Each protein band was normalized to the intensity of GAPDH used.

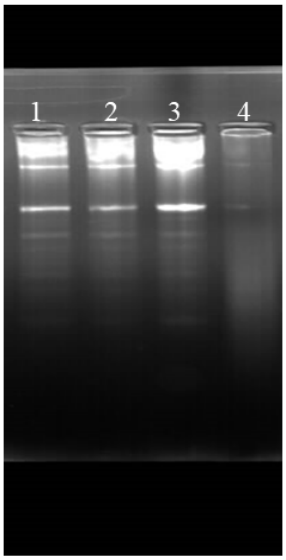


Figure 7. Electrophoresis of DNA fragmentation induced by EtOH M. sativa, GEM, and EtOH M. sativa combined with GEM for 48h, Lane1: EtOH M. sativa, Lane 2: GEM, Lane 3 EtOH M. sativa + GEM, Lane 4: Naked DNA.

*EtOH M. Satvia extract induces cell apoptosis*

To determine if the reduced cell viability was partially caused by apoptotic induction, the EtOH M. sativa extract, GEM, and their combination with an $IC_{50}$ concentration of 68.74µg/mL, 43.53µg/ml, and 41.22+25 µg/mL were utilized for Annexin V/PI expression research on PANC-1. Figure 4 A, B, C, and D depict the results obtained from this research on PANC-1, indicating the cell percentage undergoing apoptosis/necrosis and if viable cells are present. The untreated PANC-1 had a greater number of living cells (98.8%). The EtOH M. sativa extract-treated cells exhibited apoptosis in 15.43% and necrosis in 0.113%, and GEM-treated cells showed apoptosis in 9.75% and necrosis in 13.1%, respectively (Figure 4C). The apoptosis of 25.6% in PANC-1 cells treated with EtOH M. sativa extract combined with GEM was observed and dramatically higher than in the GEM, and EtOH M. sativa-treated cells (Figure 4D).

*qRT-PCR analysis*

To examine the role molecular EtOH M. sativa extract may play in anti-cancer effects and gain a better understanding of the partial cell death mechanism by apoptosis, the *Bcl-2*, *Bax*, and *CASP3* expression were

examined. In particular, their expression was evaluated after 48 hours of treatment with the IC50 dose of EtOH M.sativa, GEM, and EtOH combined with GEM. Our analysis revealed that treatment with EtOH M.sativa, GEM, and EtOH M. sativa combined with GEM dramatically increased the mRNA expression of *Bax* in PANC-1 (2.245, 3.387, and 3.482 fold, respectively, $p<0.05$, $p=0.04$, $p<0.05$) (Figure 5A). In contrast, mRNA expression of *Bcl-2* significantly decreased in response to GEM and EtOH M. sativa combined with GEM treatment (0.274 and 0.169-fold, respectively, $p<0.001$) (Figure 5B). Additionally, EtOH M. sativa combined with GEM treatment significantly increased mRNA expression of *CASP3* in PANC-1 (3.17-fold, respectively, $p=0.036$). Our findings indicated that the regulation of genes related to apoptosis in PANC-1 by EtOH M.sativa combined with GEM treatment was more profound than that observed with M. sativa and GEM treatments. Overall, our results provide important insights into the potential anti-cancer effects of EtOH M. sativa extract and the molecular mechanisms underlying its action.

*Western Blot Analysis of M. sativa Combined with GEM on PANC-1*

To clarify the molecular mechanism underlying necrosis and apoptosis induced by M. sativa (68.74µg/ml), GEM (43.53 µg/ml), and M. sativa+GEM (41.22 µg/ml and 25µg/ml), we conducted Western blotting to assess the levels of proteins related to mitochondrial apoptosis, including *Bax*, *Caspase-3, Bcl-2*, and *GAPDH*.

The data revealed that M. sativa+GEM increased *Bax* and *Caspase-3* levels, confirming the apoptosis-inducing effects of M. sativa+GEM on PANC-1 cells. Also, M. sativa+ GEM decreased Bcl2 protein levels (Figure 6).

*DNA fragmentation*

Apoptosis is characterized by DNA degradation into a unique fragment pattern. AGE was used to identify these fragments as a DNA ladder. Apoptosis-associated DNA fragmentation is distinguished by DNA cleavage on a regular basis, unlike necrosis-associated random fragmentation. After treating PANC-1 with the IC50 of EtOH M. sativa, GEM, and EtOH M. sativa combined with GEM for 48h, the genomic DNA from cells underwent AGE. In an EtBr-stained AG, a clear DNA fragment ladder was discovered (Figure 7, lanes 2-4). The fragmentation pattern was observed in cells treated with EtOH M. sativa, GEM, and EtOH M. sativa combined with GEM concentrations for 48h (Figure 7). On the contrary, the fragmentation pattern was not observable in the untreated control cells.

## Discussion

Secondary metabolites from plants have a unique potential for discovering novel anticancer medicines with diverse structural and chemical profiles [36, 37]. Phenolics are the most abundant secondary metabolites found in plants, and they are used as antioxidants as well as in herbal medicine [38]. Some research has been done on the secondary metabolites produced by M.sativa, particularly phenolic compounds with AA, and researchers have emphasized M.sativa's potential medicinal and pharmacological properties [39, 40]. NPs are gaining popularity in cancer treatment because they have fewer side effects, are less costly, and are more easily accessible than popular medications. A lecture through a published research review by Bouyahya et al, [41] showed that phenolic compounds are great sources of naturally occurring anticancer agents, offering various therapeutic and preventive options for the treatment of numerous cancer types. These chemicals can be employed solely or in conjunction with currently available anticancer medicines. However, additional research involving human subjects and other pharmacokinetic characteristics must be carried out to guarantee the safety of the medications above before being prescribed. Furthermore, clinical studies could be used to track the development process of a standardized dosage or extract.

The antioxidant properties of phenolic compounds are widely recognized, which are important in combating oxidative stress-related chronic diseases, e.g., Cardiovascular diseases, Alzheimer's disease, cancer, and diabetes [42, 43]. Based on our data, we observed that the water extraction method yielded the highest TPC content (4612.15 mgGAE/g) in comparison to EtOAc (3623.21mg mgGAE/g) and MeOH (2523.18 mgGAE/g) extractions. Our results indicate that the recovery of phenolic compounds is affected by the solvent type, polarity, and solubility in the extraction solvent. In Akkol et al.'s study [44], TPC was determined in the sage extracts (Salvia virgata and Salvia halophila), varying in the range 2830-21230 mg/100g of extract, depending on the utilized extraction solvent. Moreover, solvent polarity contributes to enhancing the solubility of phenolic compounds. The hydroxyl group-containing TPC in M.sativa significantly contributes to its antioxidant capacity by releasing hydrogen and producing persistent radical intermediates [45].

Therefore, it is crucial to carefully consider extraction method factors when evaluating the antioxidant activities of plant extracts. Our results on the DPPH• scavenging test are consistent with previous studies. Kudan and Anupam reported that M. sativa extracts exhibit high AA, which can be ascribed to numerous phenolic components, e.g., quercetin and kaempferol [46]. Similarly, Rana et al. demonstrated that extracts from M. sativa roots possess high AA [47]. Overall, our findings suggest that M.sativa extract is a promising source of natural antioxidants with potential benefits for health.

This research examined the impact of M.sativa treatment on PANC-1 cells at various doses and time points. Our results indicate that the reduction in PANC-1 cell viability was time-/dose-dependent, with a best IC50 value of 68.74 µg/mL for the EtOH extract from M.sativa following 48 hours of treatment. Previous research has also reported that M.sativa treatment reduces cell viability in several cancer cells, including leukemia, cervix, and breast cancer, in a dose-dependent manner [48]. Notably, M.sativa exhibits low cytotoxicity against normal cells, making it a potential candidate for safe cancer therapy. To explore the cytotoxic effect mechanism of M.sativa

on PANC-1 cells, we conducted microscopic, flow cytometry, and molecular evaluation. Our results revealed that M.sativa-treated PANC-1 cells exhibited rounder morphology compared to untreated cells, indicating apoptotic cell death. The morphological analysis further demonstrated that M.sativa induces apoptosis in PANC-1 cells, as demonstrated by nuclear condensation, fragmentation, and the production of apoptotic bodies. Also, flow cytometric analysis revealed a significant increase in early apoptosis (14.2%) and late apoptosis (1.23%) in cells treated with an IC50 concentration of EtOAc M.sativa extract compared to untreated cells.

Our findings align with earlier research regarding the apoptotic effects of Medicago sativa. Gatouillat et al. demonstrated that the alfalfa leaf extracts' cytotoxic effects were evaluated on a number of multidrug-resistant (MDR) and sensitive tumor cell lines, including the P388 mouse leukemia cell line as well as its doxorubicin-resistant counterpart (i.e., P388/DOX). According to a study by Gatouillat et al., apoptosis induction caused by PARP cleavage and caspase-3 activation was the mechanism by which alfalfa leaf extracts inhibited cell growth. According to their research, alfalfa leaf extract may be useful for cancer treatment and chemoprevention [26]. Additionally, Samani et al. investigated the anticancer effect of twenty medicinal herbs from Chaharmahal and Bakhtiari Province in Iran on PC cell lines. The plants were extracted using 70% ethanol, and The MTT assay was used to evaluate their anti-proliferative activity on DU145, HDF, and PC-3 cell lines [49]. According to Samani et al.'s results, Achillea wilhelmsii and Euphorbia szovitsii Fisch. & C.A.Mey. demonstrated the strongest anti-proliferative activity on PC-3. Besides, M. sativa, Urtica dioica, and Euphorbia szovitsii Fisch. & C.A.Mey. had the lowest $IC_{50}$ values on DU-145 and a strong anti-proliferative activity on PC cells. These plants could provide efficient sources of NPs for developing novel anti-PC drugs [49].

Apoptosis is a desirable cell death process in many cancer therapy regimens, and various genes, including *Bcl-2*, *Bax*, and *CASP3*, regulate it. In the current study, we used qRT-PCR and western blot use to explore the impact of M. sativa on the protein and mRNA expression of anti- and pro-apoptotic genes *Bcl-2*, *Bax*, and *CASP3* in PANC1 cells. Our qRT-PCR findings suggested that *Bcl-2* expression was high in untreated PANC-1 cells and decreased significantly in response to M.sativa treatment in a time-dependent manner. Additionally, our qRT-PCR results revealed low expression of *Bax* in untreated PANC-1 cells, which significantly increased in response to M.sativa treatment in a time-dependent manner. Both M.sativa and M.sativa+GEM in PANC-1 cells resulted in an activated intrinsic pathway of apoptosis through reducing anti- apoptotic protein (i.e., Bcl-2) and increasing pro-apoptotic proteins (i.e., *Caspase-3* and *Bax*). Programmed cell death is inhibited by the anti-apoptotic gene *Bcl-2*, whereas cell death is promoted by the pro-apoptotic gene *Bax*. An imbalance between *Bcl-2* and *Bax* expression can lead to chemoresistance, making cancer cells less susceptible to chemotherapy-induced apoptosis [50]. Thus, chemo-preventive drugs that can decrease *Bcl-2* expression and increase *Bax* expression may enhance sensitivity to anticancer treatments by promoting apoptosis in cancer cells.

Recent years have seen a significant interest in using NPs to enhance the chemotherapeutic effects of GEM, particularly in the field of PC. Previous research has shown that NPs can improve GEM's therapeutic benefits in cancer treatment. Many standard chemotherapies are known to cause significant toxicity, highlighting the need for innovative combination approaches that can induce apoptosis without causing harmful side effects [51-54].

This research aimed to investigate the potential of combining GEM, a chemotherapeutic drug, with M. sativa at lower doses (25 - 41.22 µg/ml). We found that treating PANC-1 cells with GEM alone resulted in a time-/dose-dependent reduction of cell viability, with an $IC_{50}$ of 43.53 µg/ml at 48 hours of treatment. Our findings indicated that M. sativa enhances the growth inhibitory effects of Gem at sub-lethal doses, with the combination leading to a more pronounced decrease in cell viability than either compound alone. This suggests that M. sativa improves the efficacy of GEM against cancer cells, particularly at lower dosages, thus reducing damage to normal cells and improving overall survival. Combining M.sativa with conventional treatment strategies, such as GEM, has the potential to minimize adverse effects associated with these treatments.

According to scientific articles, cancer is the second most prevalent cause of mortality immediately after CVD [55, 56]. However, conventional cancer treatments have limitations due to their poor selectivity and adverse side effects. The term "cancer chemoprevention" refers to the role of external agents in suppressing cancer development; numerous plants have been identified to contribute to the chemoprevention process through various mechanisms [57]. In recent times, NPs have acquired significant attention from researchers as potential chemo-preventive agents owing to their widespread availability, low toxicity, and cost-effective production [58]. Plant-derived NPs could be used to develop innovative and improved cancer treatments that can effectively target multiple hallmarks of cancer [59]. NPs are diverse in their chemical structure and have fairly low toxicity, making them a promising option for further exploration [59].

The trend of presenting studies using plant extracts in combination with cancer therapies is increasing, Cancan Zhou et al. argued that resveratrol can enhance PC cells' GEM sensitivity by inhibiting lipid synthesis through the downregulation of SREBP1, a key lipid synthesis regulator [60].

Originating from Chinese herbs, emodin (1,3,8-trihydroxy-6-methylanthraquinone [61-63]) is an anthraquinone derivative found in the rhizomes and roots of various plants (e.g., Polygonum cuspidatum, Rheum palmatum, Cassia obtusifolia, Polygonum multiflorum, and Aloe vera) and various fungal species (e.g., Aspergillus wentii and Aspergillus ochraceus). This herb has been proven to both sensitize tumor cells to radiotherapy and chemotherapy as well as to block pathways that result in treatment resistance. In vitro, studies have shown that emodin can reverse resistance to GEM in PC cell lines by downregulating MDR-1 (P-gp),

Bcl-2, and NF-κB expression, upregulating cytochrome-C, *Bax*, and caspase-3 and caspase-9 expression levels, and inducing cell apoptosis. Notably, emodin has shown the capability of inducing apoptosis in GEM-resistant PC cell lines [64]. In addition, in vitro/in vivo studies have suggested that emodin can downregulate NF-κB and XIAP and enhance apoptosis in mice with human PC cells [53]. A recent study also surveyed the anticancer properties of C5E, an herbal mixture extract, in PC cells (i.e., PANC-1) with or without GEM treatment. As the co-treatment with C5E and GEM, these findings suggest that the combined treatment of GEM and C5E may synergistically affect PANC-1 cells [65].

In conclusion, our results demonstrate that M. sativa extracts have high total phenolic content and AA. The combination of EtOH M. sativa with GEM leads to a sharp decrease in cell viability and induction of apoptosis in PANC-1 cells. Besides, this combined treatment led to reduced *Bcl-2* expression and increased *Bax* and *CASP3* expression, confirming apoptotic cell death. Importantly, M. sativa extracts were not toxic to HUVEC normal cells.

The data obtained from this study is crucial for determining the appropriate dosage for future pre-clinical studies. additional research must be conducted to investigate the impacts of combining GEM and M. Satvia extract in PC xenograft models. These results may lay the foundation for the development and production of herbal medicines and pave the way for performing clinical trials on patients with PC in the future. Additionally, future studies should investigate the efficacy of M. Satvia extract and its derivatives as a stand-alone treatment or when combined with other chemotherapy medicines for PC treatment.

## Author Contribution Statement

Vahid Chaleshi, Hamid Asadzadeh-Aghdaei: Conceptualization, Supervision, Reviewing, and Editing, designed the work plan, Project administration, Funding acquisition; Nazanin Jamshidi, Vahid Chaleshi, and Farnaz RoshanFarzad: Methodology, Examination, Authorship, Preparation of the Original Draft; Maha Chehresaz and Negar Jamshidi: Data curation, Validation, Formal analysis.

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*Ethical statement*

This research was approved by The Board of the Gastroenterology and Liver Diseases Research Center, Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences, Tehran, Iran.

*Competing Interest Statement*

There is no conflict of interest disclosed by the authors.

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