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RESEARCH ARTICLE

Exploring the anticancer and apoptotic potential of *Verbena officinalis* L. extracts in combination with conventional chemotherapy for pancreatic cancer: A multifaceted *in vitro* study on PANC-1 cells

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Abstract

This study evaluated the anticancer and apoptotic potential of different *Verbena officinalis* (VO) extracts on human pancreatic adenocarcinoma PANC-1 cells compared to normal HUVEC cells. We assessed the antioxidant capacity through total phenolic content (TPC) as milligrams of gallic acid equivalents (GAE) per gram and DPPH radical scavenging assays. Our results showed that the aqueous VO extract exhibited a high TPC of 875.70 ± 0.75 mg GAE/g, while the methanol extract demonstrated the most effective DPPH scavenging with an IC_{50} 60.65 µg/mL. Cytotoxicity assays, both with VO alone and in combination with gemcitabine, revealed potent anticancer effects against PANC-1 cells (IC_{25}-IC_{50} value: 25-37.40 µg/mL) but no significant impact on normal HUVECs. Apoptosis analysis using flow cytometry indicated that VO with gemcitabine induced more significant apoptosis than either treatment alone. Furthermore, VO extracts modulated the expression of apoptotic genes, reducing anti-apoptotic *Bcl-2* levels while increasing pro-apoptotic *Bax* and *Caspase-3* in PANC-1 cells, as confirmed by qRT-PCR. This study supports the potential of VO as an anticancer agent, offering insights into the mechanisms underlying its traditional medicinal applications. Further research could establish VO as an effective complementary natural therapy for pancreatic cancer.

Keywords

anticancer activity; apoptosis; pancreatic cancer; *Verbena officinalis*

Introduction

Pancreatic cancer, a highly lethal malignancy, ranked as the seventh leading cause of cancer-related deaths worldwide in 2020, accounting for 2.6% of new cases and 4.7% of global cancer-related mortality (1). In the United States and Europe, it is the fourth most common cause of death from cancer (2). Despite being relatively less common compared to other cancer types (1), its incidence and mortality rates are projected to increase globally (3). Pancreatic cancer is characterized by uncontrolled proliferation and aberrant functioning of pancreatic cells (4). Unfortunately, despite extensive research, pancreatic cancer has a dismal prognosis, with a 5-year survival rate of under 8% (1-5). This poor prognosis is primarily due to late diagnosis, aggressive metastasis and limited response to existing therapies, such as FOLFIRINOX, which includes gemcitabine, capecitabine or folinic acid, fluorouracil, irinotecan and oxaliplatin (1-2). The increasing resistance to standard treatments underscores the urgent need for innovative therapeutic approaches against this disease (2).

Most cases of pancreatic cancer are diagnosed at advanced stages when therapy is less likely to succeed (6). The absence of effective screening techniques contributes to late-stage diagnoses, further compromising outcomes (1). These challenges emphasize the importance of exploring new treatment regimens and complementary therapies, including medicinal herbs (3). Recently, there has been a growing interest in investigating natural compounds and herbal extracts for cancer prevention and treatment (7-8). These natural product-based approaches offer a promising therapeutic strategy, particularly as supplemental therapies, given the limitations of existing treatment options (2). To identify novel agents from natural sources against pancreatic cancer, further research into new molecular targets and signaling pathways is imperative. In summary, natural compounds and herbal medicines have become significant research areas for developing innovative therapies for this deadly cancer.

Verbena officinalis L., commonly known as common vervain, is a perennial herb belonging to the Verbenaceae family. Indigenous to Europe, Western Asia and North Africa, it exhibits adaptability to diverse climates and has a rich history in traditional medicine systems across various cultures (9-10). *V. officinalis* can grow to a 25-100 cm height and produces delicate spikes bearing pale purple or pink flowers (10). Extensive research has identified a range of major bioactive constituents within VO extracts, including flavonoids, terpenoids, phenolic acids, phenylpropanoids and iridoids (11). LC-MS analysis has pinpointed compounds such as hedergonic acid and serjanic acid in VO extracts (10). The pharmacological potential of VO has been well-documented in preclinical studies, highlighting its antioxidant (12), anti-inflammatory (11-13-14), antimicrobial (15) and anticancer effects (16). Research has also demonstrated the growth-inhibitory effects of VO extracts on human choriocarcinoma cells and hepatoma cells (17). This plant extract has displayed the ability to suppress the growth of human choriocarcinoma JAR cells and ongoing investigations seek to elucidate its mechanism (18). These findings collectively underscore the potential of VO as a promising natural source for anticancer agents, potentially offering synergistic effects when used alongside conventional chemotherapy drugs. Further research is warranted to characterize the specific anticancer bioactive compounds found in *V. officinalis* and delve into their therapeutic potential and mechanisms of action, both as standalone treatments and in combination with conventional anticancer therapies (19).

Pancreatic cancer remains a formidable disease with limited treatment options. Preliminary studies have shown that *V. officinalis* L. possesses anticancer properties. However, there needs to be more research investigating the apoptotic and anticancer effects of *V. officinalis*, particularly in combination with conventional chemotherapy. This study takes a multifaceted approach, including cytotoxicity assays, gene expression analysis and morphological observations, to assess the potential of *V. officinalis* extracts as a novel therapy for pancreatic cancer. Human pancreatic adenocarcinoma PANC-1 cells serve as an *in vitro* model for this evaluation.

Materials and Methods

Preparation of V. officinalis extracts

Verbena officinalis was obtained from the Iranian Biological Resources Center Herbarium in Tehran, Iran (voucher No. IBRC P1000615). Aerial parts were washed with deionized water before oven-drying at 60 °C. Extracts of *V. officinalis* was prepared with different solvents, including methanol (MeOH), ethanol (EtOH) and water. For each extract, 20 g of the powdered material was combined with 50 mL of a boiling solvent-either 60% EtOH, MeOH, or water-in a flask and left to incubate for 72 hr. Extracts were filtered through Whatman No. 1 filter paper, solvents were removed by rotary evaporation (Rv10 digital, Germany) and the resulting solids were distilled twice. Final extracts were stored at 4 °C.

Total Phenolic Content (TPC) determination

The TPC assessment was conducted using a commercial kit (Naphenol TM, Navand Salamat Pishtaz Co, Urmia, Iran) according to the manufacturer's instructions. In brief, 8 µL of VO extracts at a concentration of 100 µg/mL were meticulously mixed with 24 µL of reagent-1, along with various standard concentrations ranging from 0 to 500 mg/mL (specifically 0, 50, 100, 150, 200, 250 and 500 mg/mL). After a 5 min incubation, 40 µL reagent-2 was added and the amalgamation was allowed to incubate for 45 min at room temperature while being kept in darkness. The absorbance was subsequently measured at 630 nm using a spectrophotometer (BioTek, Inc, USA). TPC was then quantified and expressed in milligrams of gallic acid equivalents (GAE) per gram extract.

DPPH radical-scavenging activity determination

The free radical scavenging ability was assessed using the DPPH (1,1-diphenyl-2-picrylhydrazyl) assay kit (Zantox, Birjand, Iran). The procedure was as follows: 31.25 to 500 µg/mL extract concentrations were added into a solution containing 0.15 mM DPPH in ethanol. This mixture was then vigorously shaken. After an incubation period of 30 min, the residual DPPH radicals were measured at 517 nm utilizing a microplate reader (Benchmark Plus, Bio-Rad). The scavenging activity represented inhibition concerning a control solution (Trolax). The blank utilized was 96% ethanol. The negative control consisted of 1.0 mM DPPH in 2.5 mL ethanol (20-21).

Cytotoxicity assay

Cell culture : The PANC-1 cell line (Human pancreatic cancer, IBRC C10071) and normal HUVECs (Human umbilical vein endothelial cells, IBRC C10102) cell lines were provided by the Iranian Biological Resource Center (IBRC) and the Pasteur Institute in Tehran, Iran. These cell lines were cultured in DMEM medium, supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. The incubation was conducted at 37 °C in a humidified atmosphere with 5% CO_2.

Cell viability assay : Human PANC-1 and normal endothelium cells were seeded in 96-well plates at a density of 5×10^4 cells per well. They were subjected to various treatments, including different concentrations (ranging from 3.125 µg/mL to 100 µg/mL) of VO extract, Gemcitabine (GEM) and a combination of Gemcitabine with *V. officinalis*.

These treatments were administered over three intervals (24, 48 and 72 hr). Cell viability was assessed using the 3-(4,5 -dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. In brief, MTT solution was added to each well at a 5 mg/mL concentration and incubated for 2 hr at 37 °C. Formazan crystals formed were dissolved by adding 100 µL of dimethyl sulfoxide (DMSO) and incubating under standard conditions for an additional 4 hr. The optical density of each well was then measured at 570 nm using an ELISA multi-well plate reader. The absorbance of each well was compared to control wells, which were not treated with any specific materials, to determine the IC_{50} values.

Apoptosis analysis : Evaluation of apoptosis induction was performed by the Annexin V-FITC/PI kit (eBioscience, San Diego, CA, USA). Briefly, the cells were treated with selected IC_{50} concentrations of GEM, VO extract and VO /GEM. 5 x 10^5 cells were seeded into 6-well plates. After 24 hr when cells became 80% confluent, the cells were treated with pre-calculated IC_{50} concentration. Both control and treated cells were incubated for 48 hr in an incubator (37 °C, 5% CO_2). The cells were harvested using 2.5% trypsin and then washed with PBS. Later, the cells were resuspended in 500 µL of 1X binding buffer. The cells were stained with 5 µL of Annexin V -FITC and 5 µL of propidium iodide staining solution for exactly 15 min at room temperature and protected from light. Finally, the cells were immediately analyzed via flow cytometry FACS Calibur (BD Biosciences) using BD and Flowjo software.

qRT-PCR : Total RNA was extracted from both untreated and treated cells (using IC_{50} concentrations) utilizing the TRIzol reagent (Invitrogen, San Diego, CA, USA), following a protocol outlined in previous studies (22-23). The purity and quantity of the extracted RNA were assessed by measuring the absorbance ratios at 260/280 and 260/230 nm. Complementary DNA (cDNA) synthesis was carried out using the PrimeScript™ RT reagent kit (Takara Bio, Japan) according to the manufacturer's instructions.

To assess the expression levels of *BAX*, *BCL2* and *CASP3* genes, quantitative real-time PCR (qRT-PCR) was conducted using the SYBR Green method. Glyceraldehyde-3 -phosphate dehydrogenase (*GAPDH*) was used as the internal control for normalizing the expression levels. Primer sequences used for the reactions are listed in Table 1. qRT-PCR was performed on the QIAGEN Rotor-Gene Q system (Qiagen, Germany), with each sample run in triplicate. Data were analyzed using the cycle threshold (Ct) values and were expressed as fold changes using the $2^{-\Delta\Delta CT}$ method (24).

Western Blotting : To evaluate the impact of VO extract, gemcitabine (GEM) and their combination on the expression of Bax, Caspase-3 and Bcl-2 proteins, cells were exposed to their respective IC_{50} concentrations for 48 hr. Afterward, the cells were lysed in RIPA buffer, followed by denaturation of the lysates. Proteins were resolved on a 12% SDS-PAGE gel and transferred to an Immobilon-P membrane (Millipore Co., Bedford, MA, USA) after equilibration in a transfer buffer [50 mM Tris, pH 9.0, 40 mM glycine, 0.375% SDS, 20% methanol].

The membranes were then blocked with 5% non-fat milk at room temperature for 30 min. For protein detection, primary antibodies against caspase-3 (ABclonal, A2156), Bax (Upstate, Lake Placid, NY, USA) and Bcl-2 (Upstate, Lake Placid, NY, USA) were diluted in antibody dilution buffer (Coolaber, SL1360) and incubated overnight at 4 °C with the membranes. Afterward, the membranes were treated with horseradish peroxidase-conjugated secondary antibodies at room temperature for 2 hr.

Unbound antibodies were washed off, leaving only the target proteins, which were detected by the binding of the secondary antibodies to the primary antibodies. GAPDH was used as a loading control to normalize protein levels. The quantification of protein bands was performed using densitometric analysis with ImageJ software (National Institutes of Health).

Table 1. Specific primer sequences are used for RT-qPCR

Genes Name		Sequence (5'to3')	Length	GC%	Tm °C
CASP3	Forward	TTCAGAGGGG ATCGTTGTAG	20	50	**60**
	Reverse	TCAATGCCAC AGTCCAGTTC	20	50	**60**
BAX	Forward	GGTTGTCGCC CTTTTCTAC	19	52.00	**58**
	Reverse	CGGAGGAAGT CCAATGTC	19	57.9	**60**
BCL2	Forward	GATGTGATGC CTCTGCGAAG	20	55.00	**62**
	Reverse	CATGCTGATG TCTCTGGAAT C	21	47.6	**62**
GAPDH	Forward	TGCCTCCTGC ACCACCAAC	19	63.16	**62**
	Reverse	CGGAGGGGCC ATCCACAG	18	72.22	**62**

Statistical analysis

The results were expressed as the mean ± standard deviation (SD) from 3 independent experiments. Statistical comparisons between groups were made using Student's *t*-test, with a *P*-value < 0.05 considered statistically significant. IC_{50} values were calculated by plotting data points in triplicate across a range of concentrations, followed by regression analysis. Graphs were generated using GraphPad Prism 8 software (GraphPad Software, Inc., La Jolla, CA, USA).

Results

Amount of total phenolic content in VO extracts

This study evaluated the TPC of VO extracts using 3 different solvents: aqueous, EtOH and MeOH. The TPC content exhibited variations among the different extraction methods. Specifically, the TPC content was highest in the aqueous extract (875.70 ± 0.75 mg GAE/g), followed by the EtOH extract (742.26 ± 0.66 mg GAE/g) and the MeOH extract had the lowest TPC content (547.26 ±

0.78 mg GAE/g).

DPPH radical-scavenging activity

The antioxidant potential of VO extracts, prepared using different solvents (aqueous, EtOH and MeOH), was assessed through a DPPH radical scavenging assay. The VO extracts were tested at 31.25 to 500 µg/mL concentrations. Among the 3 extracts, the MeOH extract of VO displayed the highest free radical scavenging activity, with the lowest IC_{50} value of 60.65 µg/mL. In comparison, the IC_{50} values for the EtOH-VO and AqVO were 94.27 and 215.00 µg/mL respectively (Fig. 1). The increased radical scavenging activity of the methanol extract may be attributed to the enhanced solubility and extraction efficacy of antioxidant compounds, such as flavonoids and phenolics when compared to aqueous and ethanol. The IC_{50} value of the Trolax standard was 161.100 µg/mL, which is more potent compared to the aqueous extracts tested.

Cytotoxicity effects analysis

The cytotoxic effects of various VO extracts were assessed using MTT assays on PANC-1 and HUVEC cells. Initially, the IC_{50} of VO extracts on PANC-1 cells was determined within the 50–100 µg/mL concentration range. Significantly, cytotoxic effects ($p > 0.01$) were observed after 24, 48 and 72 hr of exposure. The analysis demonstrated that all VO extracts inhibited the tested cells (Table 2). The EtOH extract showed the lowest IC_{50} values against the PANC-1 cell line after 24 hr (IC_{50} >100 µg/mL). Conversely, the aqueous extract displayed the highest anticancer potential against the PANC-1 cell line, with IC_{50} = 17.18 µg/mL and 13.31 µg/mL after 48 and 72 hr respectively. Treatment with increasing concentrations of AqVO extracts resulted in a dose and time-dependent inhibition of cell growth compared to the untreated controls.

Furthermore, exploration was conducted to ensure whether the cytotoxic impact of VO extract extended to normal cells by subjecting HUVECs to the plant treatment. Interestingly, cytotoxicity was only observed at higher concentrations, indicating that VO extracts had a more pronounced inhibitory effect on the growth of PANC-1 pancreatic cells. The IC_{50} for noncancerous HUVEC cells was as high as 100 µg/mL. The viability of untreated control cells corresponds to 100% because none of the extracts were cytotoxic to the normal cells, although the selectivity index (SI) was not measured.

The cell viability of PANC-1 cells exhibited a significant dose-dependent reduction when exposed to gemcitabine, with an IC_{50} value of 43.53 µg/mL after 48 hr. After establishing the dose-response relationships for cytotoxicity of the AqVO + GEM extract in PANC-1 cells, we examined the co-treatment with VO and gemcitabine. This co-treatment led to a dose-dependent decrease in cell viability, with an IC_{50} value of 37.40 µg/mL for AqVO and 25

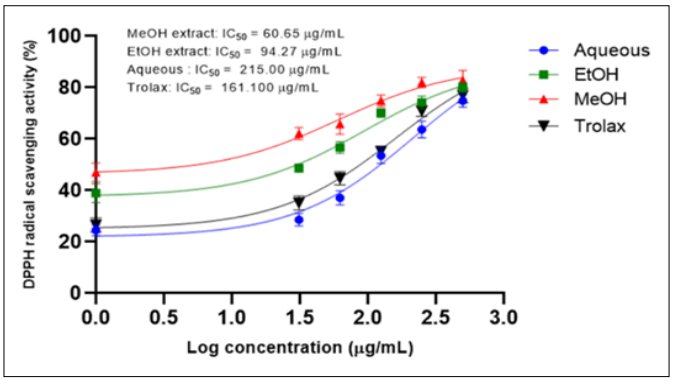

Fig. 1. DPPH radical scavenging activity (%) of various *V. officinalis* extracts and trolax

Table 2. Determined IC_{50} values (µg/mL) of the aqueous, EtOH, MeOH of *V. officinalis* extracts and Gemcitabine against PANC-1 cell lines

Extract	PANC-1			HUVEC
	IC_{50} (µg/mL)			
	24 hr	48 hr	72 hr	24 hr
Aqueous extract	24.45	17.83	13.31	> 100
EtOH extract	> 100	36.03	21.03	> 100
MeOH extract	31.63	36.94	67.03	> 100
Gemcitabine	> 100	43.53	22.59	> 100

Each value in the table is represented as mean ± SD. Ethanol: EtOH; methanol: MeOH; IC_{50}: The half maximal inhibitory concentration, PANC-1: Human pancreatic Adenocarcinoma

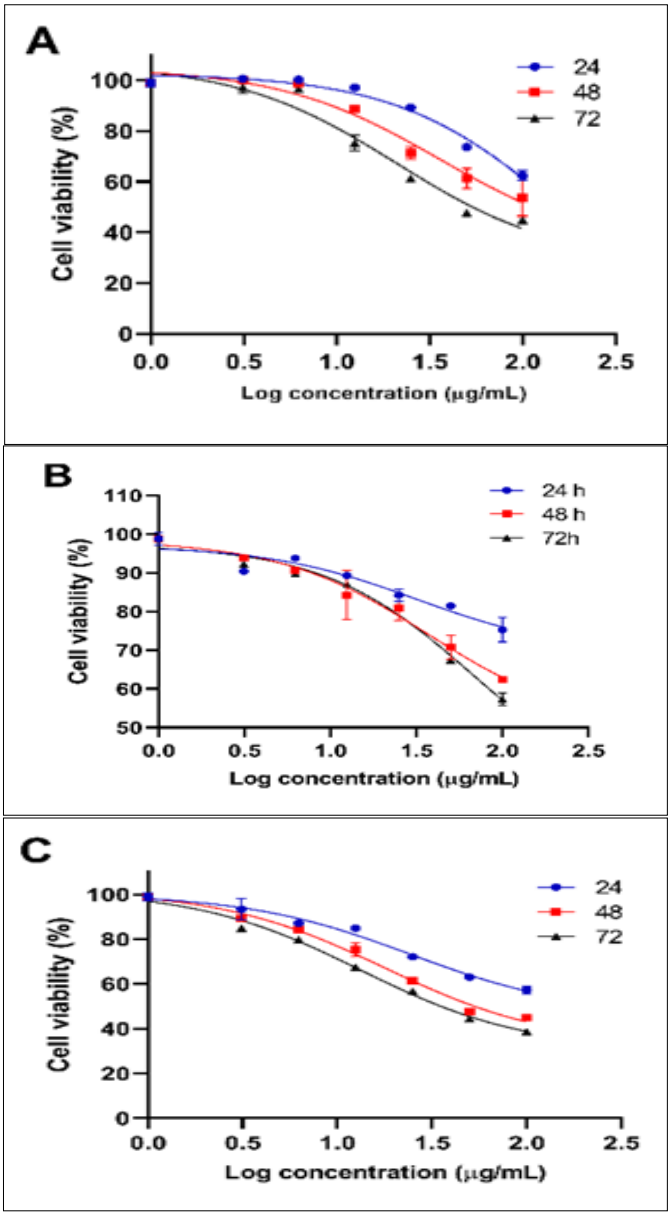

Fig. 2. *In vitro* cytotoxic activity of various extracts in PANC-1 cells was assessed by the MTT assay at different exposure times (24, 48 and 72 hr). **A)** Ethanolic extract, **B)** Methanol extract and **C)** Aqueous extract. All the values represent the mean ± SD

µg/mL for gemcitabine after 48 hr.

Observation of morphological changes and induction of PANC-1 cells apoptosis by combined V. officinalis and gemcitabine treatment

Inverted phase contrast microscopy was used to evaluate the impact of 48 hr AqVO and GEM treatment on the morphology of PANC-1 cells. In the control group, cells remained small and circular for over 6 days, showing continuous proliferation. In contrast, combining GEM and AqVO (AqVO + GEM) extract led to significantly more extensive and elongated cells with less densely packed neurites than the control group (Fig. 3B).

In Fig. 3A(a), it can be observed that the viability of untreated PANC-1 cells was 96.7%. Fig. 3A(b) shows that, compared to untreated PANC-1 cells, 13.1% of cells were early-apoptotic after treatment with 37.40 µg/mL AqVO extract. The percentage of apoptotic PANC-1 cells induced by GEM treatment alone was 15.7% (Fig. 3A(c)). However, when PANC-1 cells were treated with 25-37.40 g/mL AqVO + GEM, early-apoptosis was significantly increased to 58.2% (Fig. 3D) compared to treatment with the extract alone (Fig. 3A(b)). These findings indicate that both AqVo and the AqVO + GEM extracts can induce apoptosis in PANC-1 cells, with the combination therapy showing the highest apoptotic effect. Apoptotic percentages after AqVO extract, GEM and combination therapy are compared in Fig. 3C.

Determination of mRNA levels of BAX, BCL-2 and CASP3 expression

Fig. 4A-C presents the effect of AqVO on the gene expression of *BAX*, *BCL-2* and *CASP3* in PANC-1 cells following 48 hr of treatment with 50 µg/mL GEM, 37.40 µg/mL AqVO, or their combination (25-37.40 µg/mL). In Fig. 4A, the relative expression of the pro-apoptotic gene, *BAX*, is shown when cells were treated with AqVO and GEM and the combination of both (AqVO + GEM). AqVO and GEM, AqVO + GEM extracts increased *BAX* expression 2.1-fold (p = 0.0031), 1.94-fold (p = 0.0065) and 1.86-fold (p > 0.05) compared to the control group respectively (Fig. 4A). Fig. 4B illustrates the impact of AqVO extract on anti-apoptotic *Bcl-2* gene expression. *Bcl-2* expression significantly decreased with AqVO (0.72-fold, p = 0.0008), GEM (0.35-fold, p < 0.0001) and AqVO + GEM (0.05-fold, p < 0.0001) compared to untreated cells (Fig. 4B). As shown in Fig. 4C, *CASP3* mRNA levels were upregulated by 2.09-fold (p < 0.0001) in the AqVO + GEM extract. At the same time, they were downregulated by GEM (p = 0.0397).

Western Blot Analysis of VO Extract and Gemcitabine in Combination on PANC-1 Cells

To further investigate the molecular mechanisms involved in apoptosis and necrosis triggered by VO extract (37.40 mL), gemcitabine (GEM, 50 µg/mL) and their combination (VO + GEM, 25–37.40 µg/mL), Western blot analysis was performed. This allowed us to measure the expression of key mitochondrial apoptosis-related proteins, including Caspase-3, Bax and Bcl-2.

Results indicated that the combination of VO and GEM led to an increase in Caspase-3 and Bax protein levels, which confirms the apoptosis-promoting effects of *M. sativa* and GEM on PANC-1 cells. Additionally, the combination treatment significantly reduced Bcl-2 protein

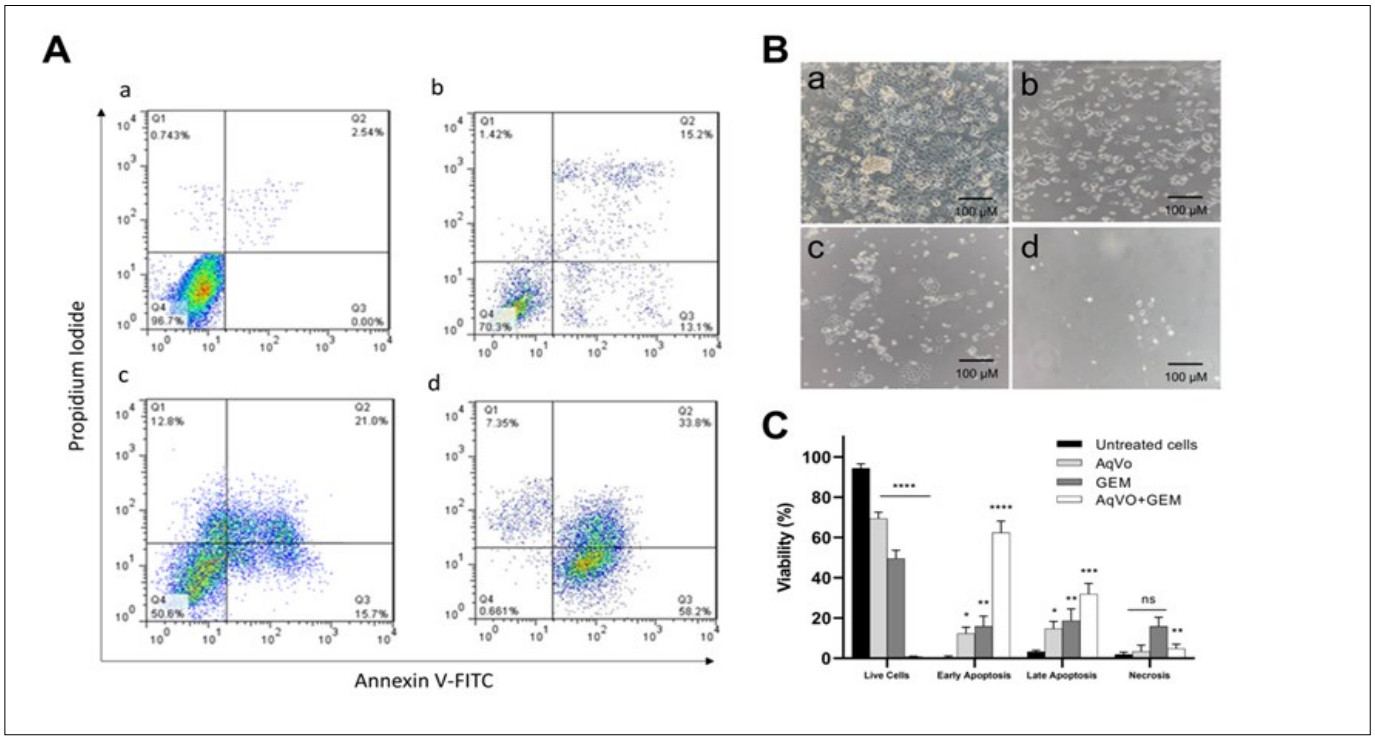

Fig. 3. A) Apoptosis induction in PANC-1 cells by *V. officinalis* and GEM measured by flow cytometry after 48 hr of treatment. **(a)** Untreated cells (control), **(b)** cells treated with AqVO at IC_{50} 37.40 µg/mL, **(c)** cells treated with GEM 50 µg/mL, **(d)** cells treated with a combination of AqVO + GEM 25-37.40 µg/mL; **B)** Representative photomicrographs of PANC-1 cells prior to treatment **a)** untreated cells of PANC-1 cell line, **b)** PANC-1 cells treated with GEM (50 µg/mL), **c)** PANC-1 cells treated with AqVO (43.13 µg/mL), **d)** PANC-1 cells treated with AqVO + GEM (25-37.40 µg/mL); **C)** Comparison of apoptosis induced by untreated cell, AqVO + GEM, AqVO and GEM. Q1, necrotic cells (Annexin-FITC−, PI+), Q2, late apoptotic cells (Annexin-FITC+, PI+), Q3, early apoptotic cells (Annexin-FITC+, PI-). Q4, healthy viable cells (Annexin-FITC-, PI-). * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001

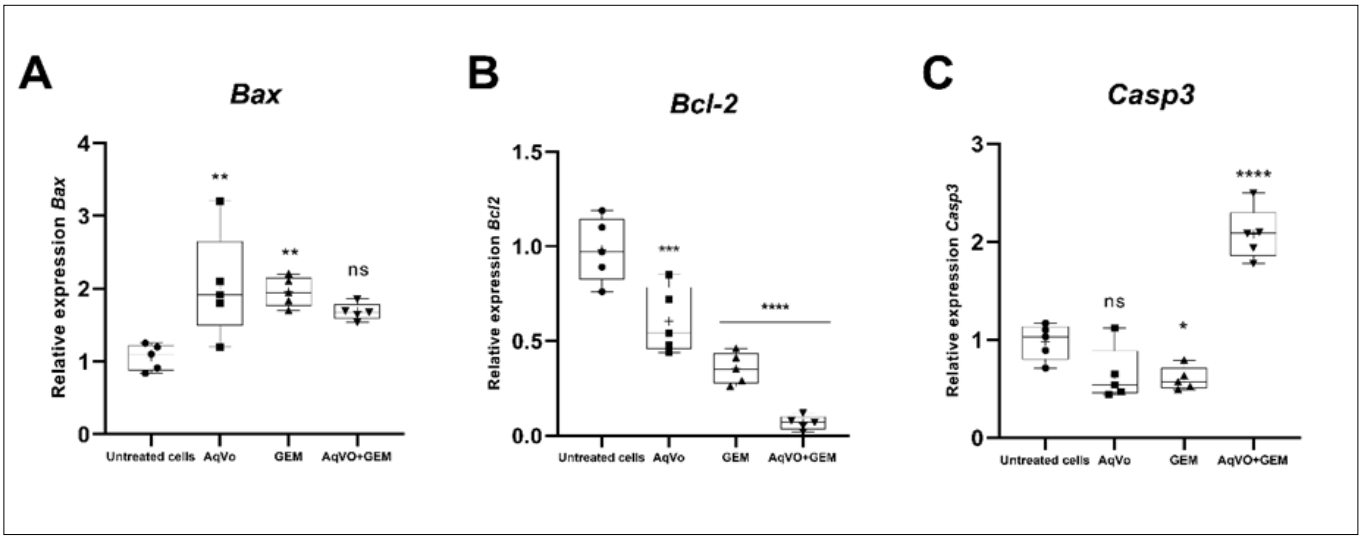

Fig. 4. Relative gene expression of *Bax*, *Bcl-2* and *CASP3* in different concentrations of AqVO, GEM and AqVO + GEM. **A)** *Bax*, **B)** *Bcl-2*, **C)** *CASP3*; Results of experiments are expressed as mean ± SD (n = 5). Statistical significance was assessed using Student's *t*-test. Compared with the control group, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$, and ns = not significant

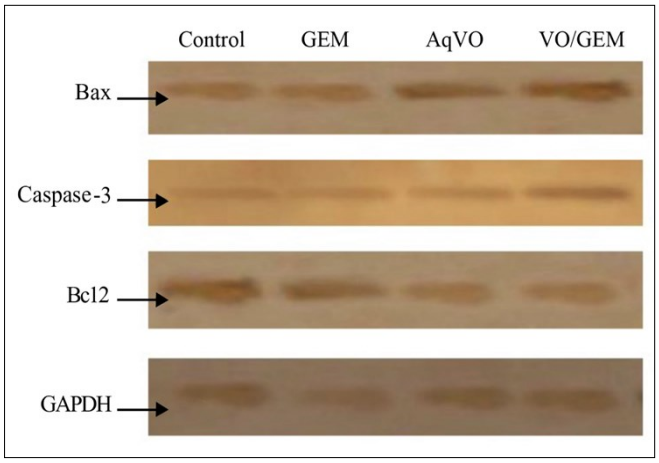

Fig. 5. Western blot bands of Caspase-3, Bax and Bcl-2 protein expression levels in AqVO Extract, GEM and AqVO + GEM for 48 hr treatment in PANC-1 cells. Each protein band was normalized to the intensity of GAPDH used

levels as is shown in Fig. 5 and GAPDH was used as a reference (Fig. 5).

Discussion

Pancreatic cancer has a poor prognosis, emphasizing the necessity for novel treatment approaches, such as natural compounds (6-25). In this study, we demonstrated the anticancer potential of *Verbena officinalis*, a medicinal plant with emerging evidence of therapeutic properties (26 -27). This research finding shows that the aqueous *V. officinalis* extract has a high TPC of 875.70 ± 0.75 mg GAE/g. Polyphenols may enhance antioxidant effects by donating hydrogens and forming stable radicals (28). Antioxidant activity neutralizes free radicals, including enzymes and chelating metals (21-29). DPPH scavenging activity assay validate the antioxidant potential using different *V. officinalis* extracts. The MeOH extract exhibited the highest free radical scavenging, while the aqueous extract showed the lowest despite having the highest TPC. This suggests that the specific phenolic compounds influence antioxidant effects more than total content. This findings contrast with (9) who found that the 50% ethanolic extract had the greatest DPPH scavenging (IC_{50} = 21.04 1.61 µg/ mL) compared to the 33.8 0.43 µg/mL found in the aqueous extract . In our results, the MeOH extract showed higher antioxidant activity than the ethanol extract (94.27 µg/mL), followed by the AqVO extract (215 µg/mL). However, its total phenolic content was lower than the AqVO extract. It is essential to emphasize that the detailed kinetic processes underlying radical scavenging by *V. officinalis* extracts remain a subject yet to be fully characterized. On the other hand, total phenolic and total flavonoid concentrations have been shown to correlate with the antioxidant activity of aqueous and hydroalcoholic extracts in three *in vitro* model systems, as reported (9). They also found that 4 components of the aqueous extract showed significant antioxidant activity. The antioxidant capabilities of these phenolic compounds may be responsible for the anticancer action of *V. officinalis* extracts. Although the antioxidant properties and compounds in extracts were characterized (9). While this research not only investigates the antioxidant and phenolic effects but also examines the anticancer effects of 3 different extract conditions on pancreatic cancer cell line.

In this study, we demonstrated that the aqueous extract of *V. officinalis* inhibits pancreatic cancer cell proliferation in a time- and dose-dependent manner, with an IC_{50} value of 37.40 µg/mL at 48 hr. These antiproliferative effects align with previous research showing that plant extracts rich in phenolic antioxidants suppress cancer cell growth (30-31). Our results corroborate past studies indicating pro-apoptotic effects of *V. officinalis* components, such as essential oils, flavonoids and polysaccharides, in cancer models (12-16). For example, *V. officinalis* essential oil may directly activate procaspase 3, leading to their pro-apoptotic action (16).

A study was primarily conducted in patients with Chronic Lymphocytic Leukemia (CLL), which differs from our pancreatic cancer cell line. It demonstrated that the *V. officinalis* extract significantly affects apoptosis. Our findings show that the *V. officinalis* extract indeed enhances apoptotic processes in pancreatic cancer cells (16).

Another study revealed that polysaccharides extracted from *V. officinalis* were optimized using a Box-

Benhnken design and found to have significant inhibitory effects on the invasion and metastasis of colorectal cancer cells (32).

While the precise apoptotic mechanisms require further elucidation, *V. officinalis* shows potential as a selective anticancer agent, given its cytotoxicity against pancreatic cancer cells but not normal cell lines. *V. officinalis* extract displayed anti-tumor effects in H22 tumor-bearing mice without impairing immune function. The extract showed anti-tumor activity with an inhibition rate of 38.78%, increasing the spleen index somewhat. It did not cause significant changes in delated-typed hypersensitivity and hemolysin levels compared to the model group, indicating no damage to immune function (18). This indicates that *V. officinalis* could be an ideal candidate for pancreatic cancer activity (IC_{50} < 100 µg/mL), as it showed no toxicity toward normal cells. However, more research is needed to understand its mechanisms of action fully.

The aqueous extract in our study demonstrated the highest anticancer potential against the PANC-1 cell line, with an IC_{50} of 17.83 µg/mL compared to other extracts. This result aligns with the long-standing use of plant products in treating various human disorders dating back to ancient times (33). Iridoid glycosides, secondary plant metabolites with considerable medicinal value, have been extensively studied for their beneficial roles in plants. These compounds, including numerous iridoid class phytochemicals, exhibit a wide array of bioactivities such as cardiovascular, anti-viral, anti-hepatotoxic, anti-inflammatory, anti-cancer, immunomodulatory, anti-spasmodic, hypolipidemic, choleretic, purgative and hypoglycemic properties (33). Several important components from the aqueous extracts of Verbena and lemon verbena, such as apigenin, mono- and di-glucuronides of luteolin, hastatoside, iridoids, verbenalin and various flavonoids identified (12).

It is conceivable that the enhanced presence of these bioactive compounds in the aqueous extract may contribute to its superior anticancer activity, potentially facilitating better bioavailability and interaction with the target cancer cells. However, it is suggested that further studies be undertaken to explore the specific mechanisms of action of these compounds within the aqueous extract. Such studies would be crucial to gaining a deeper understanding of the molecular pathways and interactions at play, allowing for a more comprehensive evaluation of the extract's therapeutic potential.

In summary, this study demonstrates the therapeutic potential of *Verbena officinalis*, particularly the aqueous extract, in inhibiting the growth of PANC-1. Notably, the extract induces apoptosis and alters the expression of pro- and anti-apoptotic genes. Additionally, combining the aqueous *V. officinalis* extract with the chemotherapy drug gemcitabine leads to enhanced inhibition of PANC-1 cell viability, highlighting the potential of this combination for pancreatic cancer treatment. These findings validate the anticancer effects of *V. officinalis* against pancreatic cancer cells and shed light on the mechanisms involved. Further research is warranted to confirm these results and thoroughly explore the therapeutic applications of *V. officinalis* for pancreatic cancer and potentially other malignancies. *In vivo* investigations will be critical to translate these *in vitro* findings into clinical applications for improving pancreatic cancer outcomes. Overall, this study establishes a foundation for developing *V. officinalis* as a novel and alternative therapeutic option, either alone or as an adjunct to standard chemotherapy, providing hope for more effective management of this aggressive disease.

Conclusion

This study elucidates the promising anticancer potential of *V. officinalis* L. extracts, specifically regarding its synergistic effects when administered alongside gemcitabine for the treatment of pancreatic adenocarcinoma (PANC-1 cells). The findings strongly suggest that *V. officinalis* can augment the anticancer efficacy of conventional chemotherapy, thereby facilitating the exploration of natural compounds as adjuncts to established treatment protocols. Notably, the aqueous extract of *V. officinalis* exhibited the highest phenolic content and the MeOH extract showed significant DPPH radical scavenging activity, which underscores its potent antioxidant properties. Moreover, the extract was demonstrated to inhibit PANC-1 cell viability and induce apoptosis, primarily through the modulation of apoptotic gene expression. Furthermore, investigating the broader implications of *V. officinalis* across various cancer types and in conjunction with other therapies may unveil innovative treatment strategies, thereby reinforcing its potential as an alternative or complementary therapeutic option in the field of oncology.

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Authors' contributions

Author contributions Conceptualization, SMS and MDA; Methodology, VC and ST; Data gathering, VC and ST; Validation, MDA; Formal Analysis, MDA and VC; Investigation, SMS, ST and VC; Writing - Review and Editing, MDA and SMS.

Compliance with ethical standards

Conflict of interest: The authors declare that they have no competing interests.

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Declaration of generative AI and AI-assisted technologies in the writing process

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