



In vitro antioxidant, cytotoxic, apoptotic, and anti-migratory effects of methanolic and aqueous extracts of *Paronychia kurdica* on pancreatic cancer cells

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Paronychia kurdica'nın metanolik ve sulu ekstraktlarının pankreas kanser hücrelerinde in vitro antioksidan, sitotoksik, apoptotik ve anti-migrasyon etkileri

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Abstract: Pancreatic cancer is one of the most lethal malignancies due to rapid metastasis and treatment resistance, with high post-diagnosis metastasis risk and low five-year survival rates. This study investigated the phenolic and flavonoid content, antioxidant capacity, and biological effects of *Paronychia kurdica* extracts on pancreatic cancer cells. The methanolic (MeOH) extract exhibited higher total phenolic (26.39 mg GAE/g) and flavonoid (21.52 mg QE/g) contents than the aqueous extract and demonstrated moderate antioxidant effect according to DPPH and FRAP assays. LC-MS analysis identified chlorogenic acid, rutin, ferulic acid, and p-coumaric acid in both extracts, with higher rutin levels in the MeOH extract. Both extracts showed dose-dependent cytotoxicity on MIA PaCa-2 cells (IC₅₀: MeOH 411.4 µg/mL, aqueous 385.5 µg/mL). Scratch assays indicated that the MeOH extract inhibited cell migration by 50.56–70.14%. Apoptotic mechanisms were confirmed by qRT-PCR and Western blot analyses, with increased Bax and decreased Bcl-2 expression, particularly in MeOH extract-treated cells. Immunofluorescence revealed apoptotic morphological changes. These findings suggest that *P. kurdica* extracts possess antioxidant properties along with moderate cytotoxic and anti-migratory effects in pancreatic cancer cells, supporting their potential as a source of bioactive compounds for further pharmacological investigation.

Key words: *Paronychia kurdica*, phenolic compounds, antioxidant activity, pancreatic cancer, apoptosis

Özet: Pankreas kanseri, hızlı metastaz ve tedavi direnci nedeniyle en ölümcül malignitelerden biridir; tanı sonrası metastaz riski yüksek, beş yıllık sağkalım oranları ise düşüktür. Bu çalışmada, *Paronychia kurdica* ekstraktlarının fenolik ve flavonoid içerikleri, antioksidan kapasiteleri ve pankreas kanseri hücreleri üzerindeki biyolojik etkileri araştırılmıştır. Metanolik (MeOH) ekstrakt, sulu ekstrakta kıyasla daha yüksek toplam fenolik (26,39 mg GAE/g) ve flavonoid (21,52 mg QE/g) içerik göstermiş ve DPPH ile FRAP analizlerine göre orta düzeyde antioksidan etki sergilemiştir. LC-MS analizi, her iki ekstrakta da klorojenik asit, rutin, ferulik asit ve p-kumarik asidin bulunduğunu ortaya koymuş, MeOH ekstraktında rutin düzeyinin daha yüksek olduğu belirlenmiştir. Her iki ekstrakt da MIA PaCa-2 hücrelerinde doza bağlı sitotoksikite göstermiştir (IC₅₀: MeOH 411,4 µg/mL, sulu ekstrakt 385,5 µg/mL). Scratch analizleri, MeOH ekstraktının hücre göçünü %50,56–70,14 oranında inhibe ettiğini göstermiştir. Apoptotik mekanizmalar, qRT-PCR ve Western blot analizleri ile doğrulanmış; özellikle MeOH ekstraktı ile muamele edilen hücrelerde Bax ekspresyonunda artış ve Bcl-2 ekspresyonunda azalma gözlenmiştir. İmmünofloresan analizler apoptotik morfolojik değişiklikleri ortaya koymuştur. Bu bulgular, *P. kurdica* ekstraktlarının pankreas kanseri hücrelerinde antioksidan özelliklerin yanı sıra orta düzeyde sitotoksik ve anti-migratuvar etkiler gösterdiğini ve ileri farmakolojik araştırmalar için biyoaktif bileşik kaynağı olma potansiyeline sahip olduğunu desteklemektedir.

Anahtar Kelimeler: *Paronychia kurdica*, fenolik bileşikler, antioksidan aktivite, pankreas kanseri, apoptoz

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1. Introduction

Cancer remains a major global public health problem, posing a serious threat to human life due to its high morbidity and mortality rates worldwide (Manju et al., 2012; Ovais et al., 2016; Reddy and Grace, 2016). Cancer is caused by genetic abnormalities in DNA that impair normal cellular processes, including proliferation, differentiation, and cell death (Eröz et al., 2012). As a

result, cells proliferate uncontrolled, invading surrounding tissues and spreading to distant organs.

An essential part of preserving cellular homeostasis is apoptosis, an energy-dependent and genetically controlled process of cell death. Dysregulation of apoptotic pathways has been linked to pathological situations such as cancer, neurological disorders, and autoimmune diseases (Eröz et al., 2012). Pancreatic cancer is one of the deadliest cancers

due to its late detection, aggressive progression, and great resistance to standard therapy (WHO, 2025; GLOBOCAN, 2022).

Plant-derived natural compounds have attracted increasing attention as potential alternatives or complementary agents in cancer therapy. This is mainly due to their relatively low toxicity and broad spectrum of biological activities. It is estimated that approximately 50% of currently available pharmaceutical drugs are derived from natural sources, and many anticancer agents have originated from plant-based compounds (Talib and Mahasneh, 2010).

The Caryophyllaceae family is one of the five most species-rich plant families in the flora of Türkiye, represented by 559 species. This family exhibits a remarkably high endemism rate, with 230 endemic taxa accounting for approximately 41% of its total diversity (Davis et al., 1965–1985; Güner et al., 2012). While genera such as *Silene* L., *Dianthus* L., *Gypsophila* L., *Minuartia* L., and *Arenaria* L. comprise the majority of taxa within the family, several genera, including *Paronychia* Mill., are represented by a limited number of species (Davis et al., 1965–1985; Özhatay et al., 1999, 2009, 2011; Güner, 2000; Özhatay and Kültür, 2006; Güner et al., 2012; Ekim, 2014). The genus *Paronychia* Mill. comprises approximately 110 species worldwide, many of which are distributed in Türkiye (Kubitzki et al., 1993). Initially classified under the family *Illecebraceae* in Flora of Türkiye (Chaudhri, 1967), *Paronychia* was later transferred to the *Caryophyllaceae* family and placed within the subfamily *Paronychioideae* based on molecular phylogenetic evidence (Kubitzki et al., 1993).

In the present study, the potential anticancer effects of *Paronychia kurdica*, an endemic species of Türkiye, were investigated in the human pancreatic cancer cell line MIA PaCa-2. Methanolic (MeOH) and aqueous extracts obtained from the aerial parts of the plant were evaluated in terms of their total phenolic (TPC) and total flavonoid (TFC) contents, and their phytochemical profiles were characterized using LC–MS analysis. The extracts' antioxidant capabilities were evaluated utilizing DPPH and FRAP tests. Extracts were tested for their impact on cell survival, proliferation, morphology, migration, and apoptosis in MIA PaCa-2 cells. Furthermore, apoptotic mechanisms were elucidated by analyzing the expression of pro- and anti-apoptotic markers, including *Associated X protein (Bax)* and *B-cell lymphoma 2 (Bcl-2)* genes (Manju et al., 2012; Eröz et al., 2012; Talib and Mahasneh, 2010).

This comprehensive method seeks to reveal the potential anticancer capabilities of *P. kurdica* extracts, contribute to the development of natural product-based treatment options, and provide new preclinical data to the existing literature. Furthermore, comparing the biological effects of extracts obtained with different solvents emphasizes the relevance of solvent selection in phytotherapeutic research and provides a scientific foundation for future investigations (Manju et al., 2012; Smina et al., 2016).

2. Materials and Method

2.1. Plant material

Plant specimens of *P. kurdica* were gathered on July 27, 2023, from calcareous rock cracks west of the Gürgenboynu Plateau in the region of Almus district, Tokat

Province, Türkiye, at an elevation of 1770 m. The specimens were stored in the Herbarium of Ankara Yıldırım Beyazıt University with voucher number Koç 3705.

Prof. Dr. Murat Koç from Ankara Yıldırım Beyazıt University's Institute of Public Health, Department of Traditional, Complementary, and Integrative Medicine identified the plant material's taxonomy.

2.2. Preparation of plant extracts

The aerial parts of the plant were dried in accordance with herbarium procedures and ground into a fine powder prior to extraction. Ultrasound-assisted extraction (UAE) was carried out by mixing 10 g of powdered plant material with 100 mL of either absolute methanol or distilled water. The mixtures were subjected to ultrasonic treatment at a frequency of 60 kHz for 60 min at room temperature. Following extraction, the suspensions were filtered to remove plant residues. The methanolic extract was concentrated under decreased pressure using a rotary evaporator, while the aqueous extract was frozen at -80°C and then lyophilized at -50°C under 0.04 mbar pressure for 96 hrs. The dried extracts were weighed to determine extraction yields and kept at 4°C in the dark until further analysis.

2.3. Total Phenolic Content (TPC)

The total phenolic content (TPC) was determined using the Folin-Ciocalteu technique (Singleton and Rossi, 1965). Each extract was mixed with 30 μL of Folin-Ciocalteu reagent. 30 μL of a 5% sodium carbonate (Na_2CO_3) solution and 150 μL of distilled water should then be added. After the reaction was finished, the absorbance was measured at 725 nm. A calibration curve was created with gallic acid standards (7.8125, 15.625, 31.25, 62.5, 125 and 250 $\mu\text{g/mL}$). All assays were performed in triplicate ($n = 3$), and the results were expressed in milligrams of gallic acid equivalents per gram of extract (mg GAE/g).

2.4. Total flavonoid content (TFC)

Total flavonoid content (TFC) was determined using the aluminum chloride colorimetric method (Zhishen et al., 1999). 25 μL of each extract were mixed with 10 μL of 1 M sodium acetate, and 150 μL of distilled water. After adding 15 μL of 10% aluminum chloride (AlCl_3) solution, the mixture was incubated in the dark for 30 minutes. After incubation, absorbance was measured at 425 nm. A calibration curve was created with quercetin standards (7.8125, 15.625, 31.25, 62.5, 125 and 250 $\mu\text{g/mL}$), and the results were represented as milligrams of quercetin equivalents per gram of extract. All measurements were carried out three times.

2.5. Liquid Chromatography–Mass Spectrometry coupled with Quadrupole Time-of-Flight (LC–MS/QTOF) Analysis

The phenolic profile of the both MeOH and aqueous extracts were determined using an Agilent 1290 Infinity HPLC system with a Q-TOF mass spectrometer (Agilent Technologies, USA). The Agilent Poroshell C18 column (3.0 $\times$ 100 mm, 2.7 μm) was used for chromatography at 35°C . The mobile phase consisted of 0.1% formic acid in water (A) and acetonitrile (B) at a flow rate of 0.6 mL/min. The gradient elution was set as follows: 5% B (0 min), 20%

B (6 min), 70% B (18 min), 90% B (20-26 min), and re-equilibration to 5% B (32 min). Mass spectrometric detection was carried out in negative ESI mode over a m/z range of 30-1700, with a capillary voltage of 3000 V, a drying gas temperature of 325 °C, a nitrogen flow rate of 11 L/min, and a nebulizer pressure of 35 psi, with m/z 966.000725 as the reference ion. To identify and quantify eight phenolic compounds, retention durations and mass values were compared to authentic standards. Calibration curves were created for concentrations ranging from 1-100 µg/mL.

2.6. 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

The extracts' free radical scavenging activity was assessed using the DPPH assay (Braca et al., 2001). Different concentrations of the extracts (7.8125, 15.625, 31.25, 62.5, 125, 250 and 500 µg/mL) were prepared in methanol. Then, 100 µl of each sample solution was mixed with 100 µl of freshly prepared DPPH methanolic solution (0.1 mM). The reaction mixtures were incubated in the dark at room temperature for 30 min. After incubation, absorbance was measured at 517 nm using a spectrophotometer. Trolox was used as the reference antioxidant. Radical scavenging activity was calculated as percentage inhibition, and IC_{50} values were determined from concentration–response curves.

2.7. Ferric reducing antioxidant power (FRAP) assay

The reducing capacity of the extracts was determined using the Ferric Reducing Antioxidant Power (FRAP) assay, which evaluates the reduction of ferric (Fe^{3+}) ions to ferrous (Fe^{2+}) form. The FRAP working solution was freshly prepared by mixing acetate buffer, 2,4,6-tripyridyl-s-triazine (TPTZ) reagent, and ferric chloride solutions in appropriate proportions. For the analysis, 20 µL of extract sample was mixed with 150 µL of FRAP reagent, and the mixture was incubated at 37 °C in the dark for 30 min (Benzie and Strain, 1996). After incubation, the formation of the colored complex was monitored by measuring absorbance at 593 nm using a spectrophotometer. Antioxidant capacity was calculated using a Trolox calibration curve, and the results were expressed as Trolox equivalents.

2.8. In vitro anticancer activity

2.8.1. Cell culture

The American Type Culture Collection (ATCC) provided the MIA PaCa-2 human pancreatic cancer cell line. This cell line was derived from pancreatic tumor tissue of an adult male donor. The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM), which included %10 fetal bovine serum, 2 mM L-glutamine, and 1% penicillin/streptomycin (100 U/mL penicillin and 100 µg/mL streptomycin). Cultures were kept at 37°C in a humidified atmosphere with 5% CO_2 and used for studies when they achieved 80-90% confluency.

2.8.2. Preparation of plant extracts and cell treatment

To prepare stock solutions with a concentration of 10.000 µg/mL, methanolic and aqueous extracts of *P. kurdica* were first dissolved in DMSO. Treatment concentrations ranged from 0 to 1250 µg/mL, obtained through serial dilutions.

These prepared solutions were applied to MIA PaCa-2 cell cultures after they reached 70-80% confluence. After treatment, the cells were incubated for 24 hours in conventional culture conditions (37°C, 5% CO_2 , humidified environment).

2.8.3. MTT cell viability assay

The MTT assay was used to evaluate the effects of the extracts on MIA PaCa-2 cell viability (Mosmann, 1983). The cells were cultivated in 96-well plates (1×10^5 cells/well). The experiment was performed 24 hrs after the media was removed and the cells were washed with PBS. The extracts were dissolved in DMSO and the final DMSO concentration in the culture medium did not exceed 0.1% in any experimental condition. Then, MTT solution (0.5 mg/mL) was applied to each well, and the cells were incubated for 4 hrs at 37 °C. After incubation, the solution was removed, and DMSO was used to dissolve the formazan crystals in the well. A spectrophotometer was used to measure the optical density (OD) at 570 nm. The cell viability in the control group was considered to be 100%. The results were represented as percentages (%) based on the relative changes in OD_{570} values of the treatment groups compared to the control (Chang et al., 2018).

Cell viability (%) is calculated as follows: (Average OD_{570} of treated cells/Average OD_{570} of control cells) $\times$ 100.

2.8.4. Scratch-Wound healing assay

The impact of the extracts on MIA PaCa-2 cell migration capacity was evaluated using a wound-healing technique (Suarez-Arnedo et al., 2020; Liang et al., 2007). Cells were seeded onto 6-well plates at a density of 5×10^2 cells/well. Once the cells reached confluence, wounds were created using a sterile 200 µL pipette tip. The cells were then treated with IC_{50} doses of the plant's methanolic and aqueous extracts. Following treatment, the scratch area was quantified using ImageJ software and observed under an inverted microscope at 0th, 24th, 48th and 72nd hrs (Suarez-Arnedo et al., 2020). The scratch closure rate (%) was calculated:

The scratch closure rate (%) = [(Initial scratch-wound width – Final scratch-wound width) / (Initial scratch-wound width)] $\times$ 100.

2.8.5. Immunofluorescence staining

2.8.5.1. Phalloidin and 4',6-diamidino-2-phenylindole (DAPI) staining

Intracellular cytoskeletal organization of MIA PaCa-2 cells was evaluated by immunofluorescence staining. At a density of 5×10^2 cells per well, cells were seeded onto 6-well plates. The culture media was collected 24 hours after transfection, and the cells were fixed in 4% paraformaldehyde at room temperature for 10 min. Subsequently, cells were permeabilized with 0.1% Triton X-100 for 10 min. Actin filaments were stained using Alexa Fluor 594-Phalloidin (1:1000) for 60 min at room temperature in the dark. Nuclear counterstaining was performed with DAPI (1:1000). Fluorescence images were taken with a fluorescence microscope, and fluorescent signals were processed with Image J software (Handala et al. 2019).

2.9. Real-Time PCR (qRT-PCR)

To evaluate the apoptotic effects of *P. kurdica* MeOH and aqueous extracts on pancreatic cancer cells, the expression levels of *Bax* and *Bcl-2* were analyzed by qRT-PCR. MIA PaCa-2 cells were cultured in 6-well plates and treated with IC₅₀ concentrations of MeOH and aqueous extracts for 24 h. Untreated cells served as the control group. Total RNA was isolated using Tripure Isolation Reagent (Roche Life Sciences), and cDNA was synthesized from 1 µg of RNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific). qRT-PCR analysis was performed using a SYBR Green-based master mix according to the manufacturer's instructions. Gene expression analysis was performed using gene-specific primers on a Rotor-Gene Q system (Qiagen) (Table 1). *Gapdh* (*Glyceraldehyde-3-Phosphate Dehydrogenase*) was utilized as the reference gene, and relative gene expression levels were estimated using the 2^{-ΔΔCt} technique (Livak & Schmittgen, 2001). All experiments were conducted in triplicate (Freshney, 2000; Özdaş et al., 2022).

2.10. Western Blot analysis

The protein expression levels of *Bax* and *Bcl-2* were examined using Western blotting to determine the impact of *P. kurdica* MeOH and aqueous extracts on apoptosis-related signaling pathways. MIA PaCa-2 cells were cultivated in 6-well plates and exposed to IC₅₀ concentrations of MeOH and aqueous extracts for a duration of 24 hrs. The control group consisted of untreated cells. To produce total protein extracts, cells were collected after treatment, cleaned with cold PBS, and lysed in RIPA buffer supplemented with protease inhibitors (Özdaş et al., 2022; Özdaş, 2019). The Bradford test was used to quantify protein concentrations (Bradford, 1976). After being separated using 10% SDS-PAGE, 20 µg of protein was transferred to PVDF membranes.

After blocking the membranes with 5% non-fat milk in Tris-buffered saline containing 0.1% Tween-20 (TBST), primary antibodies anti-*Bax* antibody (ab53154), anti-*Bcl-2* antibody (ab59348) at 1:500 (Abcam, Cambridge, UK) were incubated overnight at 4 °C. Following washing, membranes were incubated for one hour at room temperature with secondary antibodies conjugated with

Table 1. Primers used for qRT-PCR analysis

Gene	Primer	Sequence (5'-3')
Bax	Forward	GTCCAATGTCCAGCCCATGA
	Reverse	AGCTGAGCGAGTGTCTCAAG
Bcl-2	Forward	GGTGAAGTGGGGGAGGATT
	Reverse	GGCAGGCATGTTGACTTCAC
Gapdh	Forward	ATCACCATCTTCCAGGAGCGAGA
	Reverse	GTCTTCTGGGTGGCAGTGATGG

Table 2. Total phenolic content (TPC), total flavonoid content (TFC), and extract yields of *P. kurdica* obtained using MeOH and aqueous extracts

Extraction Solvent	TPC (mg GAE/g)	TFC (mg QE/g)	Extract Yield (%)
MeOH	26.39 ± 0.01	21.53 ± 0.01	11.98
Aqueous	20.57 ± 0.01	12.66 ± 0.01	9.53

Abbreviations: TPC: Total Phenolic Content, TFC: Total Flavonoid Content, GAE: Gallic Acid Equivalent, QE: Quercetin Equivalent

HRP. Using an enhanced chemiluminescence (ECL) detection (Bio-Rad, USA) with the VersaDoc 4000MP imaging system, immunoreactive bands were visible (Özdaş et al., 2022). Using ImageLab software, band intensities were measured by densitometric analysis, normalized to β-actin, and reported as relative percentage changes in comparison to the control group (Taylor et al., 2013).

2.11. Statistical analysis

The data was processed with SPSS v24.0 (IBM Corp., Chicago, IL, USA) and presented as mean ± SD. Data normality and variance homogeneity were examined using the Kolmogorov-Smirnov and Levene tests, respectively (Levene, 1963; Mishra et al., 2019). One-way ANOVA was used to compare treated and control groups in MTT assay, scratch wound healing, qRT-PCR, and Western blot assays, followed by Tukey's post hoc test (Smalheiser, 2017). The IC₅₀ values were obtained with GraphPad Prism v8.4.3 (GraphPad Software, San Diego, CA, USA). A p-value < 0.05 was considered statistically significant.

3. Results

3.1. Phytochemical analysis

3.1.1. Extract yield, TPC, and TFC

The aerial parts of *P. kurdica* were extracted using an ultrasonic-assisted extraction method with methanol (MeOH) and water as solvents, and the extraction yields were determined. As presented in Table 2, the MeOH extract exhibited higher total phenolic content (TPC) (26.39 ± 0.01 mg GAE/g), total flavonoid content (TFC) (21.53 ± 0.01 mg QE/g), and extract yield (11.98%) compared to the aqueous extract, which showed TPC and TFC values of 20.57 ± 0.01 mg GAE/g and 12.66 ± 0.01 mg QE/g, respectively, with an extraction yield of 9.53%. The TPC values were determined according to the method of Singleton and Rossi (1965), while TFC values were determined using a modified method of Zhishen et al. (1999).

3.1.2. LC-MS/QTOF analysis

The phenolic composition of the methanolic (MeOH) and aqueous extracts of *P. kurdica* was investigated using Liquid Chromatography–Mass Spectrometry/Quadrupole Time-of-Flight (LC-MS/QTOF) analysis. Optimization studies were performed for phenolic standards, and chromatographic separation of compounds in the sample

was achieved. The analysis was carried out with a total runtime of 35 min. Calibration curves were constructed over a concentration range of 1–20 µg/mL (1, 2.5, 5, 10, 20 µg/mL), and the corresponding calibration equations and R² values (0.9870–0.9998) indicated high accuracy and reliability of the measurements. Both extracts were evaluated

for eight typical phenolic constituents, including catechin, chlorogenic acid, caffeic acid, syringic acid, p-coumaric acid, ferulic acid, rutin, and quercetin. Rutin was found to be the main constituent in both extracts, with 1.65 ± 0.20 $\mu\text{g}/\text{mg}$ in MeOH and 1.34 ± 0.20 $\mu\text{g}/\text{mg}$ in aqueous extract. Other phenolic compounds such chlorogenic acid, p-coumaric acid, and ferulic acid were detected at low quantities (< 0.5 $\mu\text{g}/\text{mg}$). These results are summarized in Table 3.

3.2. Antioxidant activity

3.2.1. DPPH radical scavenging activity and FRAP assay

The antioxidant activities of the methanolic (MeOH) and aqueous extracts of *P. kurdica* were evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay. In addition, the Ferric Reducing Antioxidant Power (FRAP) assay was performed to assess the reducing potential of the extracts. As presented in Table 4, the MeOH extract exhibited stronger antioxidant activity with a lower DPPH IC_{50} value (0.182 ± 0.020 mg/mL) and higher FRAP value (61.003 ± 0.063 $\text{mg TE}/\text{g}$) compared to the aqueous extract, which showed a DPPH IC_{50} value of 0.316 ± 0.015 mg/mL and a FRAP value of 45.337 ± 0.025 $\text{mg TE}/\text{g}$.

3.3. In vitro cell culture

3.3.1. Cytotoxic effects of *Paronychia kurdica* extracts on pancreatic cancer cells

A concentration-dependent and time-dependent decrease in cell viability was observed following treatment with both extracts. Significant decreases were observed at concentrations above 150 $\mu\text{g}/\text{mL}$ for both extracts (MeOH: 150 $\mu\text{g}/\text{mL}$, $P = 0.0103$; H₂O: 150 $\mu\text{g}/\text{mL}$, $P = 0.0127$), with higher concentrations exhibiting stronger effects ($P < 0.0001$) (Fig. 1a). Cell viability remained above 90% at concentrations below 250 $\mu\text{g}/\text{mL}$. The IC_{50} values were calculated as 411.4 ± 8.3 $\mu\text{g}/\text{mL}$ for the MeOH extract and 385.53 ± 7.89 $\mu\text{g}/\text{mL}$ for the aqueous extract (Fig. 1b). These IC_{50} concentrations were subsequently used for further experiments

3.3.2. Effects of *Paronychia kurdica* extracts on MIA PaCa-2 cell migration

The scratch-wound experiment was used to evaluate the effects of MeOH and aqueous *P. kurdica* extracts on MIA PaCa-2 cell migration. Cells were treated with IC_{50} concentrations of extracts for 24 hrs, and wound closure was monitored at 0, 24, 48, and 72 hrs using ImageJ

software (Fig. 2a). Both extracts significantly inhibited cell migration compared to control. At 24 hrs, wound closure decreased by $50.56 \pm 1.14\%$ (MeOH, $P = 0.0004$) and $5.34 \pm 1.30\%$ (H₂O, $P = 0.0155$). At 48 hrs, reductions were $60.18 \pm 1.07\%$ and $22.43 \pm 1.43\%$, and at 72 hrs, 70.14% and 26.92% , respectively (all $P < 0.0001$). MeOH extract consistently showed stronger inhibition than H₂O extract

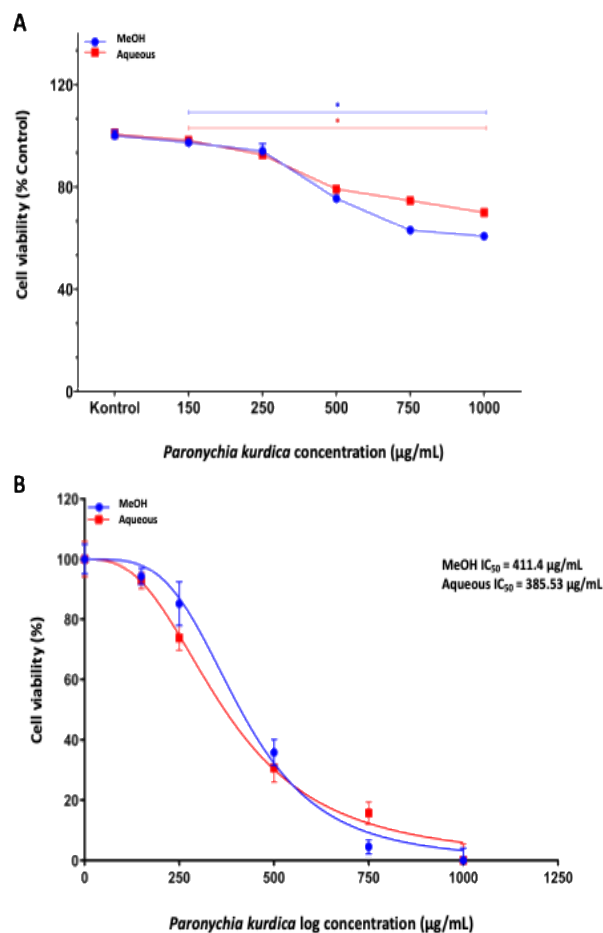


Figure 1. Effects of *Paronychia kurdica* Extracts on MIA PaCa-2 Cell Viability (MTT Assay); A) MIA PaCa-2 cell viability after 24 hrs of treatment with increasing MeOH concentrations and *P. kurdica* aqueous extracts. Untreated cells served as the control. B) IC_{50} values representing the half-maximal inhibitory concentration of *P. kurdica* extracts on cell viability. IC_{50} values were calculated using a nonlinear regression model. Data are expressed as mean $\pm$ SD ($n = 3$). Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test. * $P < 0.05$ indicates a significant difference compared to the control.

Table 3. Selected phenolic compounds of *P. kurdica* identified by LC-MS/QTOF

No.	Compound Name	Aqueous Extract ($\mu\text{g}/\text{mg}$)	MeOH Extract ($\mu\text{g}/\text{mg}$)	Retention Time (min)
1	Catechin	<1.00	<1.00	5.44
2	Chlorogenic acid	<1.00	<1.00	5.78
3	Caffeic acid	<1.00	<1.00	6.25
4	Syringic acid	<1.00	<1.00	6.92
5	p-Coumaric acid	<1.00	<1.00	8.86
6	Ferulic acid	<1.00	<1.00	10.40
7	Rutin	1.34 ± 0.20	1.65 ± 0.20	11.94
8	Quercetin	<1.00	<1.00	17.77

Note: Chlorogenic acid, p-coumaric acid, and ferulic acid were determined below the quantification limit (<0.5 $\mu\text{g}/\text{mg}$), whereas the other compounds were not detected in the extracts

Table 4. DPPH radical scavenging activity and FRAP values of *P. kurdica* extracts

Extract	DPPH IC ₅₀ (mg/mL)	FRAP (mg TE/g)
MeOH	0.182 ± 0.020	61.003 ± 0.063
Aqueous	0.316 ± 0.015	45.337 ± 0.025
Trolox (positive control)	0.0156 ± 0.001	–
Trolox (positive control)	0.0156 ± 0.001	–

Note: DPPH IC₅₀ represents the concentration required to scavenge 50% of DPPH radicals. FRAP values are expressed as Trolox equivalents (TE). There was a statistically significant difference between all extracts and the positive control (one-way ANOVA, post hoc Tukey, $p < 0.05$)

(24, 48, 72 hrs: 47.77%, 48.67%, 59.13%; $P \leq 0.0031$) (Fig. 2b). The inhibition of cell migration was both dose- and time-dependent.

3.3.3. Effects of *Paronychia kurdica* extracts on MIA PaCa-2 cell morphology

MIA PaCa-2 cells treated with IC₅₀ doses of MeOH or aqueous *P. kurdica* extracts for 24 hrs were examined by inverted phase-contrast and F-actin immunofluorescence microscopy (Bianchi et al., 2022). Untreated cells served as control (Fig. 3). Control cells exhibited spindle-shaped and

rounded morphologies with organized F-actin filaments. Extract-treated cells, especially with MeOH, showed cell shrinkage, microtubule reduction, nuclear condensation, and increased spherical cells in the supernatant—consistent with apoptotic morphology. H₂O extract induced similar but less pronounced effects (Fig. 3a,b).

3.4. Effect of *Paronychia kurdica* extracts on Bax and Bcl-2 gene expression in MIA PaCa-2 cells

The effect of *P. kurdica* extracts on apoptosis-related Bax and Bcl-2 mRNA levels in MIA PaCa-2 cells was evaluated using qRT-PCR (Pisani et al., 2020). Cells were treated with the IC₅₀ concentration of the extracts for 24 hrs. In untreated control cells, Bax and Bcl-2 mRNA levels were 1.38 ± 0.02 and 3.69 ± 0.02 , respectively. Treatment with MeOH and aqueous extracts increased Bax expression by $27.49 \pm 2.27\%$ and $9.48 \pm 1.87\%$, and decreased Bcl-2 expression by $18.85 \pm 1.31\%$ and $8.11 \pm 0.50\%$, respectively (all $P < 0.0001$). Compared to aqueous extract-treated cells, MeOH extract treatment led to a 16.44% increase in Bax and an 11.68% decrease in Bcl-2 expression ($P < 0.0001$) (Fig. 4).

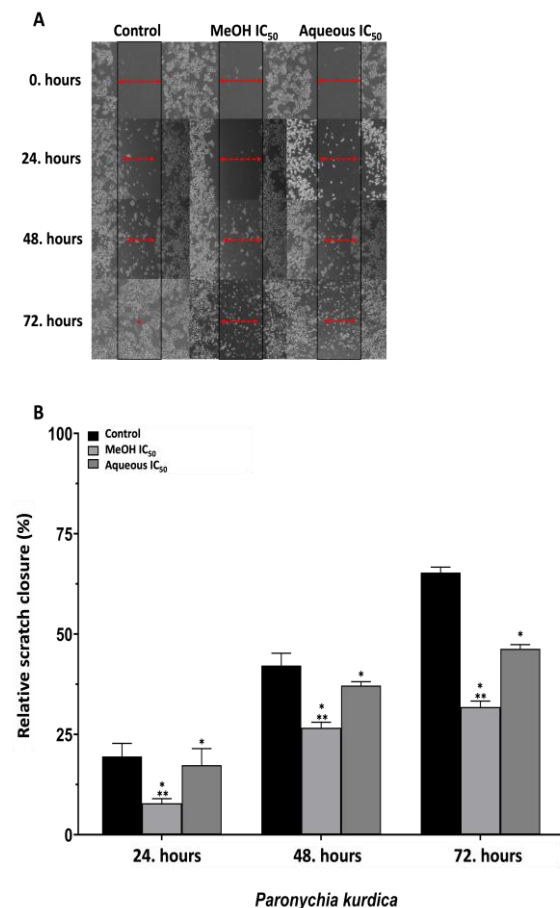


Figure 2. Effects of *Paronychia kurdica* Extracts on MIA PaCa-2 Cell Migration (Scratch Assay); MIA PaCa-2 cells were grown in 6-well plates, and the control group consisted of untreated cells. The scratch-wound test was used to measure cell migration when cells were exposed to IC₅₀ concentrations of MeOH or aqueous *P. kurdica* extracts during 0–72 hrs. (A) Representative microscopic images (10X) show changes in the wound area. Initial and final wound boundaries are marked in black and red, respectively (scale bar: 100 µm). (B) Wound closure was quantified using ImageJ. Data are presented as mean ± SD (n = 3). Statistical analysis was performed using one-way ANOVA followed by Tukey's test. 24, 48, and 72 hrs: * $P < 0.05$ vs. control; ** $P < 0.05$ vs. H₂O extract. **Abbreviations:** MeOH: Methanol; IC: Half-maximal inhibitory concentration; SD: Standard deviation.

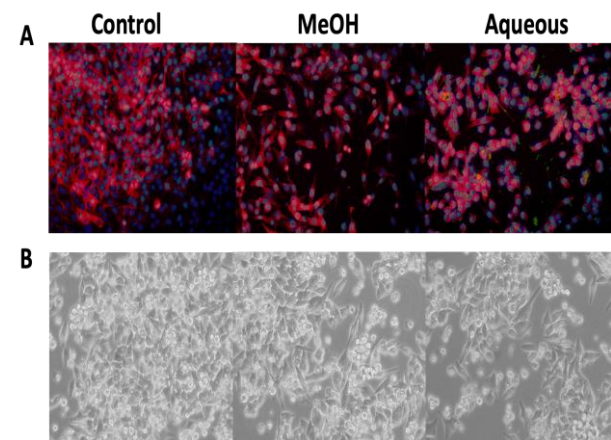


Figure 3. Effects of *Paronychia kurdica* Extracts on MIA PaCa-2 Cell Morphology; MIA PaCa-2 cells were treated for 24 hrs with IC₅₀ doses of MeOH or aqueous *P. kurdica* extracts or left untreated as control, and analyzed by inverted phase-contrast and F-actin/nuclear immunofluorescence staining. (A) Representative immunofluorescence images (20X) showing F-actin (Phalloidin-iFluor, red) and nuclei (DAPI, blue). Merged images highlight cytoskeletal and nuclear morphology (scale bar: 50 µm). (B) Representative inverted phase-contrast images (20X) showing overall cell morphology (scale bar: 50 µm).

Abbreviations: MeOH: Methanol; IC₅₀: Half-maximal inhibitory concentration; DAPI: 4',6-diamidino-2-phenylindole dihydrochloride.

3.5. Effect of *Paronychia kurdica* extracts on Bax and Bcl-2 protein expression in MIA PaCa-2 cells

The effect of *P. kurdica* extracts on apoptosis-related Bax and Bcl-2 protein levels in MIA PaCa-2 cells was assessed using Western blotting (Pisani et al., 2020). Cells were

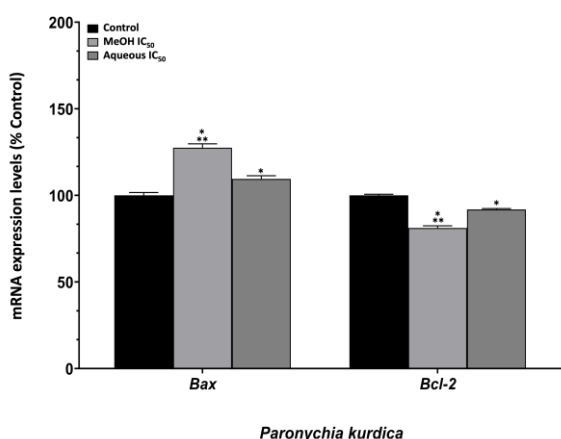


Figure 4. Effect of *Paronychia kurdica* Extracts on Bax- and Bcl-2 mRNA Expression Levels in MIA PaCa-2 Cells; mRNA expression levels of Bax and Bcl-2 in untreated and *P. kurdica*-treated MIA PaCa-2 cells were measured by qRT-PCR. Cells in the treatment groups were exposed to the IC₅₀ concentrations of MeOH and aqueous extracts of *P. kurdica* for 24 hrs. *Gapdh* was used for normalization. Relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method and presented relative to the untreated control group. Data are presented as mean $\pm$ SD (n = 3 independent experiments). Statistical significance was assessed using one-way ANOVA followed by Tukey's test. *P < 0.0001 vs. untreated control; **P < 0.0001 vs. aqueous extract.

Abbreviations: Bax: Bcl-2-associated X protein; Bcl-2: B-cell lymphoma 2; *Gapdh*: Glyceraldehyde-3-Phosphate Dehydrogenase; qRT-PCR, quantitative reverse transcription polymerase chain reaction; MeOH, methanol; IC₅₀, inhibitory concentration; SD, standard deviation.

treated with the IC₅₀ concentrations of the extracts for 24 hrs. Western blot analysis revealed differences in Bax and Bcl-2 protein levels between treated and untreated cells (Fig. 5a). Densitometric analysis of the protein bands showed that, compared to untreated control cells, treatment with MeOH and aqueous extracts increased Bax protein expression by $19.60 \pm 2.28\%$ and $6.37 \pm 1.90\%$ (P < 0.0001 and P = 0.0001, respectively), while Bcl-2 levels decreased by $10.29 \pm 1.14\%$ and $5.26 \pm 1.23\%$ (P < 0.0001 and P = 0.0002, respectively). Furthermore, compared to aqueous extract-treated cells, MeOH extract treatment led to a 12.43% increase in Bax and a 5.30% decrease in Bcl-2 expression (P = 0.0002 and P = 0.0008, respectively) (Fig. 5b).

4. Discussions

Pancreatic cancer is among the most aggressive malignancies due to its high mortality rate, early metastatic potential, and limited response to current therapeutic strategies, despite its relatively low incidence (Ferlay et al., 2024; Halbrook et al., 2023). The five-year survival rate of around 12% underscores the critical necessity for supplementary and supportive therapy approaches (Ma et al., 2024). In this context, medicinal plants high in phenolic and flavonoid chemicals are gaining popularity in anticancer research due to their multi-target biological properties.

In the present study, the phytochemical composition and biological activities of MeOH and aqueous extracts of *P. kurdica* were comprehensively evaluated for the first time. Although data on the *Paronychia* genus are limited, several species within the Caryophyllaceae family have been reported to be rich in phenolic compounds and to exhibit

protective effects against oxidative damage (Karimi et al., 2020).

The MeOH extract had considerably greater total phenolic (26.39 mg GAE/g) and flavonoid (21.52 mg QE/g) contents than the aqueous extract, which can be attributed to methanol's superior extraction efficiency for phenolic and flavonoid chemicals (Do et al., 2014). Similar solvent-dependent differences have also been reported in other Caryophyllaceae species (Karimi et al., 2020). Consistent with its phytochemical richness, the MeOH extract demonstrated superior antioxidant activity, as evidenced by a lower DPPH IC₅₀ value (0.182 mg/mL) and a higher FRAP value (61.00 mg TE/g) compared to the aqueous extract (IC₅₀ = 0.316 mg/mL; FRAP = 45.33 mg TE/g). Phenolic compounds are known to have antioxidant properties via free radical scavenging and metal ion reduction processes (Naveed et al., 2018). Although studies on the antioxidant potential of *Paronychia* species are scarce, comparable results have been reported for other Caryophyllaceae members. For instance, the MeOH extract of *Gypsophila arrostii* showed a DPPH IC₅₀ value of 0.224 mg/mL (Kahriman et al., 2019), supporting the notable antioxidant capacity of *P. kurdica*.

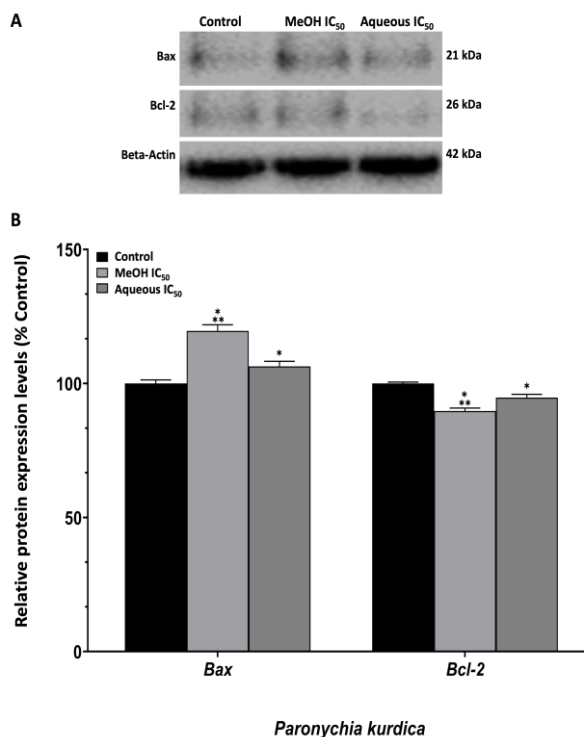


Figure 5. *Paronychia kurdica* Extracts Modulate Bax- and Bcl-2-Mediated Apoptosis in MIA PaCa-2 Cells; Bax and Bcl-2 protein levels in untreated and *P. kurdica*-treated MIA PaCa-2 cells were determined by Western blot. Cells were treated with IC₅₀ concentrations of MeOH and aqueous extracts for 24 hrs. A. Representative Western blot membranes (20 μ g/lane) showing Bax and Bcl-2 protein levels. B. Relative band intensities were measured densitometrically and normalized to β -actin. Band intensities are expressed as fold change relative to the control group.

Data are presented as mean $\pm$ SD (n = 3 independent experiments). Statistical significance was assessed using one-way ANOVA followed by Tukey's test. *P < 0.05 vs. control; **P < 0.05 vs. aqueous extract.

Abbreviations: Bax: Bcl-2-associated X protein; Bcl-2: B-cell lymphoma 2; MeOH, methanol; IC, inhibitory concentration; SD, standard deviation.

LC-MS/QTOF analysis, performed for the first time on *P. kurdica* extracts, demonstrated that rutin was quantitatively detected in both the MeOH and aqueous extracts. In contrast, chlorogenic acid, p-coumaric acid, and ferulic acid were detected below the quantification limit ($<0.5 \mu\text{g}/\text{mg}$), while catechin, caffeic acid, syringic acid, and quercetin were not detected in the extracts. Notably, rutin was detected at $1.65 \pm 0.20 \mu\text{g}/\text{mg}$ in the MeOH extract, whereas it was $1.34 \pm 0.20 \mu\text{g}/\text{mg}$ in the aqueous extract. The improved solubility of flavonoids in organic solvents further explains the higher TFC and enhanced biological activity of the MeOH extract (Zhishen et al., 1999; Do et al., 2014).

Similar solvent dependent phenolic patterns have been reported in other Caryophyllaceae species. In *Gypsophila aucherii*, methanol extract was richer in phenolic constituents and showed stronger antioxidant activity than water extract, consistent with the present findings (Altay, 2018). In conclusion, the MeOH extract of *P. kurdica* demonstrated a richer phenolic and flavonoid composition, stronger antioxidant capacity, and enhanced biological activity compared to the aqueous extract. Considering that multiple phenolic compounds were identified in the extracts, the observed biological effects are unlikely to be attributed to a single dominant compound alone. Instead, these effects may result from synergistic or additive interactions among the phytochemical constituents. These findings suggest that *P. kurdica* represents a promising natural source of antioxidants and a potential candidate for pharmacological applications. Nonetheless, more in vivo investigations are needed to corroborate these in vitro findings and determine this species' medicinal potential.

This study provides original in vitro evidence on the effects of *P. kurdica* extracts in the pancreatic cancer cell line MIA PaCa-2, contributing novel data to the limited literature on this genus. The results demonstrate that both MeOH and aqueous extracts reduce cell viability in a dose-dependent manner and exert anti-proliferative and pro-apoptotic effects, which may be associated with their antioxidant capacity (Mosmann, 1983; Wang et al., 2019). According to MTT assay results, the IC_{50} values of the MeOH and aqueous extracts were $411.4 \mu\text{g}/\text{mL}$ and $385.5 \mu\text{g}/\text{mL}$, respectively, indicating moderate cytotoxic activity. While a standard chemotherapeutic agent was not included as a positive control in this assay, the observed effects were interpreted in comparison with previously reported data under similar experimental conditions. The pronounced loss of cell viability observed at concentrations above $250 \mu\text{g}/\text{mL}$ suggests the activation of apoptotic mechanisms (Chang et al., 2018). Since the extracts represent complex phytochemical mixtures, conversion of IC_{50} values into molar units is not straightforward and may not accurately reflect the activity of individual constituents. Therefore, IC_{50} values are presented in mass concentration units ($\mu\text{g}/\text{mL}$). This approach is commonly used in phytochemical studies and allows for more reliable comparison with similar plant extract-based research.

Previous studies have reported that plant-derived extracts exhibit measurable cytotoxic and antiproliferative effects in pancreatic cancer models, including MIA PaCa-2 cells, with IC_{50} values generally within the range of several hundred $\mu\text{g}/\text{mL}$ depending on extract composition and experimental conditions (Gundogdu et al., 2020; Wang et al., 2019).

In addition, members of the Caryophyllaceae family have been shown to possess bioactive phytochemicals such as saponins and phenolic compounds, which are associated with anticancer activity. For instance, *Dianthus chinensis* extracts have been reported to induce apoptosis and inhibit cell proliferation in human cancer cell lines through mechanisms involving transcriptional regulation and oxidative stress pathways (Shin et al., 2013). Although selective toxicity could not be evaluated due to the absence of a normal cell line, this aspect should be addressed in future studies to determine the safety profile of the extracts. Collectively, these results indicate that *P. kurdica* extracts exhibit moderate cytotoxic and antiproliferative effects in pancreatic cancer cells. However, IC_{50} -based cytotoxicity data alone are insufficient, and detailed analyses of apoptotic pathways supported by in vivo studies are required.

Metastasis is a major cause of mortality in pancreatic cancer and is closely linked to the migratory and invasive capacities of cancer cells (Chen et al., 2021). The scratch (wound-healing) assay is widely accepted as a reliable in vitro method for assessing cell migration (Liang et al., 2007; Suarez-Arnedo et al., 2020). In the present study, both MeOH and aqueous extracts of *P. kurdica* significantly inhibited cell migration in MIA PaCa-2 cells. Notably, the MeOH extract suppressed migration by 50.56%, 60.18%, and 70.14% at 24, 48, and 72 hrs, respectively, whereas the aqueous extract exhibited a more limited inhibitory effect (5.34–26.92%). These results indicate a pronounced anti-migratory and potential anti-metastatic activity of the MeOH extract.

The observed inhibition of cell migration is consistent with previous findings reported for other Caryophyllaceae species. For example, ethanolic extracts of *Dianthus chinensis* reduced migration in A549 lung cancer cells via modulation of epithelial–mesenchymal transition (EMT) pathways (Zhang et al., 2016), while *Silene vulgaris* MeOH extract impaired cytoskeletal organization and reduced migration in MCF-7 cells (Ammar et al., 2020). Additionally, root extracts of *Gypsophila paniculata* were shown to inhibit cell adhesion and migration by targeting integrin and focal adhesion signaling (El-Deeb et al., 2012). The anti-migratory effects of *P. kurdica* extracts may be attributed to their phenolic and flavonoid constituents, which are known to regulate signaling pathways involved in cell motility. In particular, chlorogenic acid has been reported to inhibit the Wnt/ β -catenin pathway, suppress nuclear β -catenin translocation, and enhance E-cadherin expression, thereby limiting EMT and metastatic progression (Zeng et al., 2021). The higher abundance of lipophilic phenolic compounds in the MeOH extract likely underlies its stronger anti-migratory activity compared to the aqueous extract. In conclusion, this study demonstrates that *P. kurdica*, particularly its MeOH extract, significantly inhibits cell migration in pancreatic cancer cells. These findings are consistent with anti-migratory properties reported for other Caryophyllaceae species and highlight *P. kurdica* as a promising natural source with potential anti-metastatic activity warranting further mechanistic and in vivo investigations.

Apoptosis is a programmed cell death mechanism that maintains cellular homeostasis. It is characterized by morphological changes such as cell shrinkage, chromatin condensation, and cytoskeletal disruption. This process is

defined by morphological changes such as cell shrinkage, chromatin condensation, cytoskeletal disruption, and loss of cell adhesion (Mustafa et al., 2024). These structural alterations enable both qualitative and quantitative assessment of apoptosis through fluorescence staining techniques and microscopic analyses (Bianchi et al., 2022; Wang et al., 2019).

In the present study, the apoptotic effects of MeOH and aqueous extracts of *P. kurdica* on MIA PaCa-2 pancreatic cancer cells were evaluated using immunofluorescence staining methods. Cells in the control group had a spindle-shaped morphology consistent with previous research, strong adhesion to the culture surface, and well-organized microtubule networks reaching from the nucleus to the cell periphery (Sasaki et al., 2020). In contrast, cells treated with the MeOH extract particularly demonstrated pronounced cell shrinkage, reduced microtubule density, and accumulation of fluorescence signals around the nucleus, all of which are characteristic features of apoptosis. These effects may be attributed to the more lipophilic nature of the MeOH extract, which facilitates membrane permeability and enables more direct interaction with cytoskeletal structures (Sasidharan et al., 2011).

F-actin immunofluorescence analyses revealed that actin filaments in control cells were organized in a regular, radial pattern, whereas extract-treated cells exhibited marked actin disorganization, segmental losses, and fluorescence signal clustering. The more pronounced disruption observed in MeOH-treated cells further supports its stronger apoptotic potential compared to the aqueous extract (Predebon et al., 2019). In addition, DAPI staining revealed classical apoptotic nuclear features such as chromatin condensation, nuclear fragmentation, and cell shrinkage particularly in cells exposed to the MeOH extract (Kroemer et al., 2007). These findings indicate that cell death occurred predominantly through apoptosis.

To elucidate the molecular mechanisms underlying apoptosis induction, qRT-PCR analyses were performed. Treatment with MeOH and aqueous extracts resulted in increases in *Bax* gene expression of 27.49% and 9.48%, respectively, accompanied by decreases in *Bcl-2* expression of 18.85% and 8.11%. These results indicate that the MeOH extract, in particular, shifts the *Bax/Bcl-2* balance in favor of *Bax*, thereby effectively activating the intrinsic apoptotic pathway. Similar apoptotic gene regulation patterns have been reported for other plant extracts, including *Euphorbia billardieri* and *Eryngium planum*, as well as several members of the Caryophyllaceae family (Roshanravan et al., 2018; Kikowska et al., 2023).

These gene-level alterations were further confirmed at the protein level by Western blot analyses. *Bax* protein expression increased by 19.60% and 6.37% following treatment with MeOH and aqueous extracts, respectively, while *Bcl-2* protein levels decreased by 10.29% and 5.26%. Notably, the MeOH extract induced a more pronounced *Bax/Bcl-2* imbalance, thereby strongly promoting activation of the mitochondrial apoptotic pathway (Youle and Strasser, 2008). These protein-level changes were consistent with the apoptotic morphological features observed at the cellular level, including cytoplasmic shrinkage, chromatin condensation, and nuclear deformation (Elmore, 2007).

In conclusion, this study demonstrates that both MeOH and aqueous extracts of *P. kurdica* induce apoptosis in MIA PaCa-2 pancreatic cancer cells, with the MeOH extract exerting a markedly stronger effect. The MeOH extract, which is rich in phenolic and flavonoid compounds, was found to modulate the *Bax/Bcl-2* balance and activate the mitochondrial apoptotic pathway. It also inhibited cell migration. These findings suggest that *P. kurdica* may represent a potential source of bioactive compounds for further investigation in pancreatic cancer models. Nevertheless, further *in vivo* studies and detailed investigations into the pharmacokinetic and pharmacodynamic properties of the active constituents are required to support clinical translation.

5. Conclusions

In this study, the phytochemical composition and biological activities of *P. kurdica* extracts obtained using different solvents were systematically evaluated. The results demonstrated that the MeOH extract possessed significantly higher TPC and TFC contents compared to the aqueous extract. This enriched phytochemical profile was strongly associated with the superior antioxidant capacity of the MeOH extract, as evidenced by DPPH radical scavenging and FRAP reducing power assays. *In vitro* cell culture experiments further revealed that the MeOH extract of *P. kurdica* effectively inhibited cell proliferation, suppressed cell migration, and induced apoptosis in MIA PaCa-2 pancreatic cancer cells. Collectively, these findings suggest that *P. kurdica* may represent a biologically valuable natural source with antioxidant activity and moderate cytotoxic potential. This study also highlights the critical influence of solvent selection and extraction strategy on the phytochemical yield and biological efficacy of plant extracts. The observed relationship between polyphenolic content and biological activity provides a relevant scientific basis for the development of plant-based therapeutic and nutraceutical products. Nevertheless, the present findings are limited to *in vitro* models. Future studies should focus on *in vivo* validation, detailed elucidation of molecular mechanisms, and comprehensive toxicological assessments. Furthermore, isolation and structural characterization of the active constituents will be essential to fully define the pharmacological potential of *P. kurdica*.

6. Limitations of the Study

Despite the comprehensive experimental approach, certain limitations of the present study should be considered. The absence of a normal epithelial cell line limits the assessment of selective cytotoxicity and prevents a direct comparison between anticancer activity and potential toxicity toward non-cancerous cells. Therefore, the therapeutic selectivity and safety profile of the extract could not be fully determined under the current experimental conditions. In future studies, evaluating the extract at the reported IC_{50} concentrations in healthy cell models will be essential to clarify its selectivity index and potential biomedical applicability. In addition, no standard chemotherapeutic agent was included as a positive control, which restricts direct comparisons between the observed effects of the extract and currently used anticancer drugs. Comparative studies using conventional chemotherapeutics under identical experimental conditions are necessary to better define the relative efficacy and translational relevance of

the extract. Furthermore, the findings are limited to in vitro conditions and lack in vivo validation. Although apoptosis was assessed through Bax/Bcl-2 expression analysis, additional mechanistic investigations, including mitochondrial membrane potential and caspase-3 activation assays, are required to further elucidate the underlying pathways.

Conflict of Interest

Authors have declared no conflict of interest.

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Authors' Contributions

Salih MOLLAHALİLOĞLU: Conceptualization, Methodology, Investigation, Writing – original draft, Writing – review & editing. Derya ALTAY: Investigation, Formal analysis, Statistical analysis. Murat KOÇ: Plant collection and identification. Sibel ÖZDAŞ: Investigation, Formal analysis, Statistical analysis. İpek CANATAR: Investigation, Formal analysis, Statistical analysis.

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