In vitro anticancer activity of tomentosin on human pancreatic cancer cells

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Research Article

Keywords: Tomentosin, pancreatic cancer, apoptosis, migration, invasion

Posted Date: April 4th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1508545/v1

Abstract

Tomentosin is a natural sesquiterpene lactone with anti-inflammatory, anti-fungal and neuroprotective effects. In recent years, anticancer activity of tomentosin has been emphasized. The aim of this study was to determine possible anticancer effect of tomentosin on pancreatic cancer cells. The cytotoxic effect of tomentosin on PANC-1 and MIA PaCa-2 human pancreatic cancer cells was determined by XTT analysis and doses of tomentosin that inhibit 50% cell viability (IC_{50}) were found for both cell lines. After treatment with tomentosin, apoptosis rate was determined using FITC Annexin V and migration, invasion and colony formation analyzes were performed. Reactive oxygen species (ROS) level and the change in mitochondrial membrane potential (MMP) were evaluated. The effect of tomentosin on expression levels of apoptosis-related genes was determined by qRT-PCR. The levels of Caspase-3 and Caspase-9 were analyzed by western blot method. The IC_{50} dose of tomentosin was found to be 31.11 μM in PANC-1 cells and 33.93 μM in MIA PaCa-2 cells for 48 hours. Treatment with tomentosin induced apoptosis and ROS production in pancreatic cancer cells. In addition, after tomentosin treatment, MMP decreased and migration, invasion and colony formation of pancreatic cancer cells were suppressed. According to qRT-PCR and western blot analyzes, while tomentosin affected expression level of apoptosis-related genes, an increase was observed in Caspase-3 and Caspase-9 protein levels. Considering all the data together, this study reveals that tomentosin has anticancer effects on pancreatic cancer cells, and therefore it predicts that tomentosin can be evaluated as an effective agent against pancreatic cancer.

Introduction

Pancreatic cancer is one of the deadliest malignancies with a death rate close to the number of cases. Despite the development of treatment methods in recent years, pancreatic cancer is one of the cancers with the lowest 5-year survival rate, only 10%. And, it is thought that pancreatic cancer will rank third in cancer-related deaths by 2025 [1–3]. Pancreatic cancer has a rather poor prognosis and is often not detected at an early stage due to the lack of symptoms until it has developed significantly. Accordingly, the feasibility of surgical resection in pancreatic cancer patients is limited. In addition, the recurrence rate after resection is quite high. Therefore, chemotherapy is an important part of pancreatic cancer treatment [4, 5]. Gemcitabine is used in the first-line treatment of pancreatic cancer. However, gemcitabine has a very short half-life and is rapidly converted to fluorodeoxyuridine which is inactive metabolite of gemcitabine. In addition, uptake of gemcitabine into cells is low. Because of these properties, gemcitabine should be given in high doses that can cause systemic toxicity. And also, resistance to gemcitabine is observed in some cases [6–8]. Therefore, there is a clear need to develop new treatment strategies for pancreatic cancer.

Natural products with various pharmacological properties can inhibit or suppress carcinogenesis process by various mechanisms such as regulation of reactive oxygen species (ROS) level, control of gene expression, inhibition of cell proliferation, induction of apoptosis and modulation of signaling pathways [9]. One of these natural products with anticancer activity is sesquiterpene lactones, which are natural terpenoids with a 15-carbon skeleton containing a lactone, generally found in genera from *Asteraceae*

family [10]. Tomentosin, a natural sesquiterpene lactone with various pharmacological properties such as anti-inflammatory and anti-fungal, as well as neuroprotective effects, is found in *Inula* sps belongs to *Asteraceae* family [11–14]. In recent years, it has been shown that tomentosin causes anticancer effects on cervical cancer [15], osteosarcoma [16], gastric cancer [17], multiple myeloma [18], Burkitt's lymphoma [19], leukemia [20] and hepatocellular carcinoma [21] by various mechanisms.

In this study, it was aimed to investigate anticancer activity of tomentosin in PANC-1 and MIA PaCa-2 human pancreatic cancer cells. For this, the cytotoxic and apoptotic effects of tomentosin in pancreatic cancer cells were determined, and its effects on migration, invasion and colony formation capacities of cells were evaluated. In addition, while the expression levels of important genes and proteins that play a role in apoptosis were determined at the molecular level, changes in the ROS level and mitochondrial membrane potential (MMP) were also investigated.

Materials And Methods

Cell culture

PANC-1 (CRL-1469™) and MIA PaCa-2 (CRL-1420™) human pancreatic cancer cell lines obtained from ATCC. Cells were grown in high glucose DMEM medium containing 10% fetal bovine serum, 1% penicillin-streptomycin, 1% non-essential amino acids and 1% sodium pyruvate at 37°C, 5% CO_2 and 95% humidity.

Cytotoxicity assay

The cytotoxic effect of tomentosin on pancreatic cancer cells was evaluated by XTT analysis. PANC-1 and MIA PaCa-2 cells were seeded at $2x10^3$ cell in 96-well plates. After 24 hours, varying concentrations of tomentosin (0-300 µM) were treated to cells for 24, 48 and 72 hours. At the end of the specified hours, XTT solution (Biological Industries, 20-300-1000) was added to wells and after approximately 4 hours, absorbance value of each well was read in a microplate reader (BioTek Epoch) at 450 nm wavelength and 630 nm reference range. % cell viability was calculated and IC_{50} doses of tomentosin in PANC-1 and MIA PaCa-2 cells were determined with GraphPad Prism 8.0.2 software.

Apoptosis assay

After tomentosin treatment, % apoptosis rate was determined by a flow cytometry using FITC Annexin V apoptosis kit (BioLegend, 640.922). Cell suspensions ($1x10^6$ cells/ml) were prepared from control and dose group cells with Dulbecco's PBS. And, 5 µl of FITC Annexin V and 5 µl of 7-ADD dye were added to 100 µl of cell suspensions. After incubation at room temperature for 15 minutes in the dark, 400 µl of Annexin V binding buffer was added to cell suspensions and % apoptotic cells were determined in a flow cytometer (BD FACSAriaTM III).

Wound healing assay

The effect of tomentosin on cell migration in pancreatic cancer cells was determined by wound healing assay. For this, an artificial wound was created with the help of a yellow pipette tip in control and dose group cells, which were seeded in 24-well plates ($1x10^5$ cells/well). Cell migration was evaluated by imaging under the microscope at 24, 48 and 72 hours. Wound areas were measured using ImageJ software and % rate of wound closure was determined.

Invasion assay

The effect of tomentosin on invasion of pancreatic cancer cells was determined using the 24-well Corning BioCoatMatrigel Invasion Chamber (Corning, 354480) Cell suspensions ($2.5x10^5$cells/ml) were prepared with serum-free medium from control and dose group cells. 500 µl of cell suspension was added to upper chamber and medium containing serum was added to lower chamber. After 22 hours at 37°C, non-invasive cells were removed from upper surface of membrane with a swab, while invasive cells that migrated to lower part of membrane were fixed with 100% methanol and stained with 0.1% crystal violet. After washing, three random fields were counted under a light microscope.

Colony assay

For the colony assay, control and dose group cells, which were seeded in 6-well plates ($2x10^3$), were cultured for 10 days and medium changed every 2 days. At the end of 10 days, washing was done with PBS and 100% methanol was added to wells for fixation. After 10 minutes, methanol was removed and 5% crystal violet was added to wells. Colonies were counted after washing.

Measurement of mitochondrial membrane potential (MMP)

The effect of tomentosin on MMP in pancreatic cancer cells was performed according to manufacturer's instructions (Abcam, ab113850). Briefly, control and dose group cells ($2x10^4$ cells/well) were washed with 1X dilution buffer in 96-well plates. Then, cells were stained with dilution buffer containing JC-1 dye. After washing with 1X dilution buffer, 100 µl of medium was added to wells. The fluorescence of JC-1 was measured at Ex/Em = 475/530 nm for green fluorescence and Ex/Em = 535/590 nm for red fluorescence in a fluorescent microplate reader (BioTek Epoch).

Intracellular reactive oxygen species (ROS) detection

After tomentosin treatment, intracellular ROS level was determined using ROS red dye, which reacts with cellular ROS to produce a red fluorescent signal (Abcam, ab186027). For this, 100 µl of ROS Red Working

Solution was added to control and dose group cells ($2x10^4$ cells/well) in 96-well plates and plates were incubated for 1 hour at 37°C. After incubation, 20 µL of Assay buffer was added to wells. After 15 minutes, the red fluorescence measured in a fluorescent microplate reader (Ex:520 nm/Em:605 nm) (BioTek Epoch).

RNA extraction and qRT-PCR analysis

qRT-PCR analysis was performed to determine the effect of tomentosin on expression levels of apoptosis-related genes in pancreatic cancer cells. Firstly, total RNA isolation was performed using RiboEx (GeneAll, 301-001) from control and dose group cells seeded in 6-well plates ($3x10^5$ cells/well). Then, cDNA synthesis was performed from total RNAs in accordance with the manufacturer's instructions (Bio-Rad, 170-8891). Primers for target genes and *ACTB* used in qPCR analysis were designed with IDT PrimerQuest (https://eu. idtdna. com/ Prime rquest/ Home/ Index). 5 µl of BrightGreen 2X qPCR MasterMix, 5 pMol forward primer, 5 pMol reverse primer and 2 µl cDNA were used in qRT-PCR. The PCR protocol, consisting of 10 minutes at 95˚C, 15 seconds at 95˚C and 60 seconds at 60˚C, was carried out in Bio-Rad CFX ConnectTM Real-Time System as 40 cycles. The analysis was performed with $2^{-\Delta\Delta CT}$ method using "RT2 Profiler ™ PCR Array Data Analysis" software.

Wetern blot analysis

The effect of tomentosin on Caspase-3 and Caspase-9 protein levels in pancreatic cancer cells was evaluated by western blot analysis. Protein isolation was performed using RIPA solution (Cell signaling, 9806) from control and dose group cells, which were seeded in 6-well plates ($5x10^5$ cells/well). 50 µg of protein was separated in 8-15% SDS-PAGE and transferred to PVDF membrane. After blocking with 5% non-fat milk, membrane was incubated with anti-Caspase-3 (StJohn's Laboratory, STJ92021, 1:500), anti-Caspase-9 (StJohn's Laboratory, STJ92027, 1:500) and anti-β-Actin (Bioss Antibodies, BS-0061R, 1:1000) primary antibodies at +4°C overnight. At the end of the incubation, washing was done with TBST and membrane was treated with secondary antibody (Jackson Immuno Research, 211-035-109) for 2 hours. After washing, chemiluminescence solution (Biovision, K820-500) was added to membrane for imaging and chemiluminescence was detected on Azure Biosystems™ c280. ImageJ software was used for analysis.

Statistical analysis

All experiments were performed in triplicate and data were presented as mean ± standard deviation. Comparison between control and dose groups was performed with GraphPad Prism version 8.0.2 using Student's t test. In all analyzes, $p < 0.05$ was considered statistically significant.

Results

Tomentosin inhibits cell proliferation and induces apoptosis in pancreatic cancer cells

The effect of tomentosin on cell proliferation in PANC-1 and MIA PaCa-2 cells was determined by cytotoxicity analysis. For this, cells were treated with 5 μM, 10 μM, 15 μM, 20 μM, 25 μM, 30 μM, 40 μM, 50 μM, 75 μM and 100 μM of tomentosin for 24, 48 and 72 hours. The effect of tomentosin on viability of pancreatic cancer cells is presented in Fig.1A. Accordingly, tomentosin inhibited proliferation of pancreatic cancer cells in a dose- and time-dependent manner. In addition, IC_{50} dose of tomentosin in PANC-1 and MIA PaCa-2 cells was found to be 31.11 μM and 33.93 μM for 48 hours, respectively. In further analysis, cells were treated with IC_{50} doses of tomentosin for 48 hours.

To evaluate the effect of tomentosin on apoptosis in pancreatic cancer cells, cells were treated with IC_{50} doses of tomentosin for 48 hours and apoptosis rates were determined with FITC Annexin V. Accordingly, apoptosis rates in PANC-1 cells were found to be 5.3% in control group and 15.2% in tomenstosin-treated group. In MIA PaCa-2 cells, apoptosis rate was 13.73% in tomenstosin-treated group, while it was 6.26% in control group. According to the apoptosis analysis results, tomentosin significantly increased apoptosis rate in PANC-1 and MIA PaCa-2 cells compared to control group (Fig. 1B) ($p < 0.05$).

Tomentosin inhibits migration, invasion and colony formation of pancreatic cancer cells

The effect of tomentosin on cell migration was evaluated by wound healing analysis in pancreatic cancer cells. After treatment of tomentosin at IC_{50} doses, migration of PANC-1 and MIA PaCa-2 cells was evaluated for 24, 48 and 72 hours. According to the wound healing analysis results, tomentosin did not cause a significant change on cell migration in both cell lines for 24 hours. However, it was determined that tomentosin significantly reduced migration of PANC-1 and MIAPAa2 cells for 48 and 72 hours when compared to control group (Fig. 2) ($p < 0.05$).

The effect of tomentosin on invasion of pancreatic cancer cells was evaluated with Matrigel invasion chamber. After 48 hours with IC_{50} doses of tomentosin, invasion of PANC-1 and MIA PaCa-2 cells was significantly reduced compared to control group (Fig. 3A) ($p < 0.05$). In addition, according to the colony analysis results, colony forming capacities of PANC-1 and MIAPACA cells were significantly suppressed after treatment with IC_{50} doses of tomentosin for 48 hours (Fig. 3B) ($p < 0.05$).

Tomentosin increases intracellular ROS level and decreases MMP in pancreatic cancer cells

After tomentosin treatment, intracellular ROS level and MMP were evaluated in pancreatic cancer cells. Accordingly, treatment of tomentosin at IC_{50} doses significantly increased intracellular ROS level and

significantly decreased MMP in pancreatic cancer cells (Fig. 4A-B) ($p < 0.05$).

Tomentosin effects expression level of genes and proteins associated with apoptosis in pancreatic cancer cells

The effect of tomentosin on apoptosis in pancreatic cancer cells was evaluated at the molecular level by qRT-PCR and western blot analysis. After tomentosin treatment, the expression levels of *BAX*, *BCL2*, *CASP3*, *CASP7*, *CASP8*, *CASP9*, *CYCS*, *PPARG*, *FAS*, *FADD*, *TNF* and *TNFR1* genes, which are associated with apoptosis, were determined by qRT-PCR analysis at mRNA level. Accordingly, in PANC-1 cells, tomentosin treatment at IC_{50} dose for 48 hours caused a significant increase in the expression level of *BAX*, *CASP3*, *CASP7*, *CASP8*, *CASP9*, *CYCS*, *FAS*, *FADD* and *TNF* genes. On the other hand, in MIA PaCa-2 cells, a significant increase was seen in the expression level of *BAX*, *CASP3*, *CASP7*, *CASP8*, *CASP9*, *CYCS*, *FAS*, *FADD*, *TNF* and *TNFR1* genes after tomentosin treatment (Fig. 5A) ($p < 0.05$). The effect of tomentosin on Caspase-3 and Caspase-9 protein levels was evaluated by western blot analysis. Accordingly, a significant increase in Caspase-3 and Caspase-9 protein levels was detected in tomentosin-treated PANC-1 and MIA PaCa-2 cells, compared to control group (Fig. 5B) ($p < 0.05$).

Discussion

In recent years, both *in vitro* and *in vivo* studies have shown that various natural products have chemotherapeutic activity in a wide variety of cancers, including pancreatic cancer [22–24]. In this respect, investigation of anticancer effects of various natural products constitutes an important area in cancer studies and the results are considered promising. In this study, we investigated anticancer effect of tomentosin, a sesquiterpene lactone, on PANC-1 and MIA PaCa-2 human pancreatic cancer cells.

According to our results, tomentosin inhibited proliferation of pancreatic cancer cells in a dose and time dependent manner. And, IC_{50} doses of tomentosin in PANC-1 and MIA PaCa-2 cells were 31.11 μM and 33.93 μM for 48 hours, respectively. The possible anticancer effect of tomentosin on pancreatic cancer cells was evaluated by apoptosis, wound healing, invasion and colony formation assays. Accordingly, treatment of tomentosin at IC_{50} doses induced apoptosis in both cell lines and suppressed migration, invasion and colony formation capacity of pancreatic cancer cells. These results are consistent with studies investigating the anticancer effect of tomentosin. In a study with human cervical cancer cells, it has been shown that tomentosin induces apoptosis and arrests cell cycle at G2/M [15]. Lee et al. (2019) stated that tomentosin induces apoptosis, arrests cell cycle in G2/M and suppresses migration, invasion and colony formation of MG-63 human osteosarcoma cells [16]. Similarly, it has been observed that tomentosin stimulated apoptosis and decreased invasion and migration in AGS gastric cancer cells [17] Yu et al. (2021) also showed that tomentosin induces apoptosis, arrests cell cycle in G2/M and inhibits colony formation in hepatocellular carcinoma cells [21].

In this study, we also demonstrated that tomentosin increases intracellular ROS level and decreases MMP in pancreatic cancer cells. Similarly, in cervical cancer, osteosarcoma, leukemia and gastric cancer, it has been reported that tomentosin induces ROS production and causes a decrease in MMP [15–17, 20]. Although it is known that abnormal ROS production increases cell proliferation and stimulates the transformation of normal cells into cancer cells, high ROS level in cancer cells arrests cell cycle and induces cell death [25, 26]. Pancreatic cancer cells may also become sensitive to oxidative stress-induced cell death with increased ROS levels [27]. Therefore, induction of ROS production in cancer cells is an important therapeutic strategy and numerous natural products, like some chemotherapeutics, exert antitumor effects on human cancer cells by increasing ROS level [28–30].

A successful anticancer agent should kill cancer cells without damaging normal cells too much. This is achieved by inducing apoptosis in cancer cells in a controlled manner. Therefore, a natural product thought to have anticancer activity is also expected to induce apoptosis [31]. Apoptosis occurs in cells by two main pathways, extrinsic pathway mediated by death receptors and intrinsic pathway mediated by mitochondria [32]. The extrinsic pathway is initiated by binding of death ligands such as Fas, TRAIL and TNF-α to their receptors. Then, with the activation of adapter proteins such as FADD and TRADD, caspase-8 or -10 initiator caspases are stimulated and cell death occurs via caspase cascade [33]. In intrinsic pathway of apoptosis, mitochondrial membrane permeability changes as a result of activation of BCL-2 family members Bax and Bak, and various proapoptotic molecules such as cytochrome c are released from the mitochondria to the cytoplasm. Binding of cytochrome c to Apaf-1 forms an apoptosome and apoptosome activates caspase-9. Then caspase-3 and caspase-7 are activated and cell death occurs [34]. Increased ROS production can stimulate intrinsic apoptosis pathway, as well as extrinsic apoptosis pathway [35]. And, occurrence of a decrease in MMP is an important step in intrinsic pathway [36]. In this study, the decrease in MMP as well as an increase in ROS production in pancreatic cancer cells after tomentosin treatment indicates that mitochondria-mediated intrinsic pathway may be induced.

On the other hand, apoptotic effect of tomentosin on pancreatic cancer cells was determined at the molecular level by evaluating expression levels of genes encoding proteins involved in the intrinsic and extrinsic pathways of apoptosis. In this context, *CASP8*, *PPARG*, *FAS*, *FADD*, *TNF* and *TNFR1* genes for extrinsic pathway and *BAX*, *BCL2*, *CASP3*, *CASP7*, *CASP9* and CYCS genes for intrinsic pathway were evaluated in gene expression analysis. In addition, Caspase-3 and Caspase-9 protein levels were also analyzed by western blot method. The gene expression analysis results show that tomentosin significantly increases expression of genes involved in both intrinsic and extrinsic pathways of apoptosis in pancreatic cancer cells. Western blot analysis results also reveal that protein levels of Caspase-3 and Caspase-9, which are important regulators of apoptosis, increase after tomentosin treatment. In the study performed with multiple myeloma cells in which antiproliferative and apoptotic effects of tomentosin were investigated by gene expression analysis at the molecular level, it has been shown that tomentosin affects the expression of genes involved in neoplastic processes such as growth, proliferation, migration, invasion and apoptosis. In the same study, it has been stated that tomentosin induced both intrinsic and extrinsic pathways of apoptosis in multiple myeloma cells [18]. In the study of the same researchers with

Burkitt lymphoma cells, it was determined that tomentosin stimulates both intrinsic and extrinsic pathways of apoptosis in these cells, and it was revealed that tomentosin regulates the expression of genes involved in proliferation and cell growth as well as immune system pathways [19]. When the results of ROS, MMP, gene expression and western blot analyzes are evaluated together in this study, it is thought that tomentosin can induce both intrinsic and extrinsic pathways of apoptosis in pancreatic cancer cells as in multiple myeloma and burkit lymphoma cells.

Conclusion

In conclusion, with this study, we showed for the first time that tomentosin causes anticancer effects in pancreatic cancer cells. And, we revealed that tomentosin stimulates apoptosis, reduces migration, invasion and colony formation abilities, causes a decrease in MMP and increases ROS production in pancreatic cancer cells. Our results reveal the *in vitro* effect of tomentosin on pancreatic cancer cells, and, we hope that this study will pave the way for future *in vivo* studies and contribute to development of new therapeutic strategies against pancreatic cancer.

Declarations

Author contributions

Ebru Güçlü: Conceptualization, Investigation, Methodology, Formal analysis, Writing - Original Draft, Writing - Review & Editing, Visualization. İlknur Çınar Ayan: Conceptualization, Investigation, Methodology, Formal analysis, Writing - Original Draft, Writing - Review & Editing, Visualization. Hatice Gül Dursun: Resources, Writing - Review & Editing, Supervision. Hasibe Vural: Resources, Writing - Review & Editing, Supervision.

Funding

The authors received no financial support for the research, authorship, and/or publication of this article.

Data availability

Enquiries about data availability should be directed to the authors.

Declarations

Conflıct of interest

The authors declare that they have no conflict of interest.

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Figures

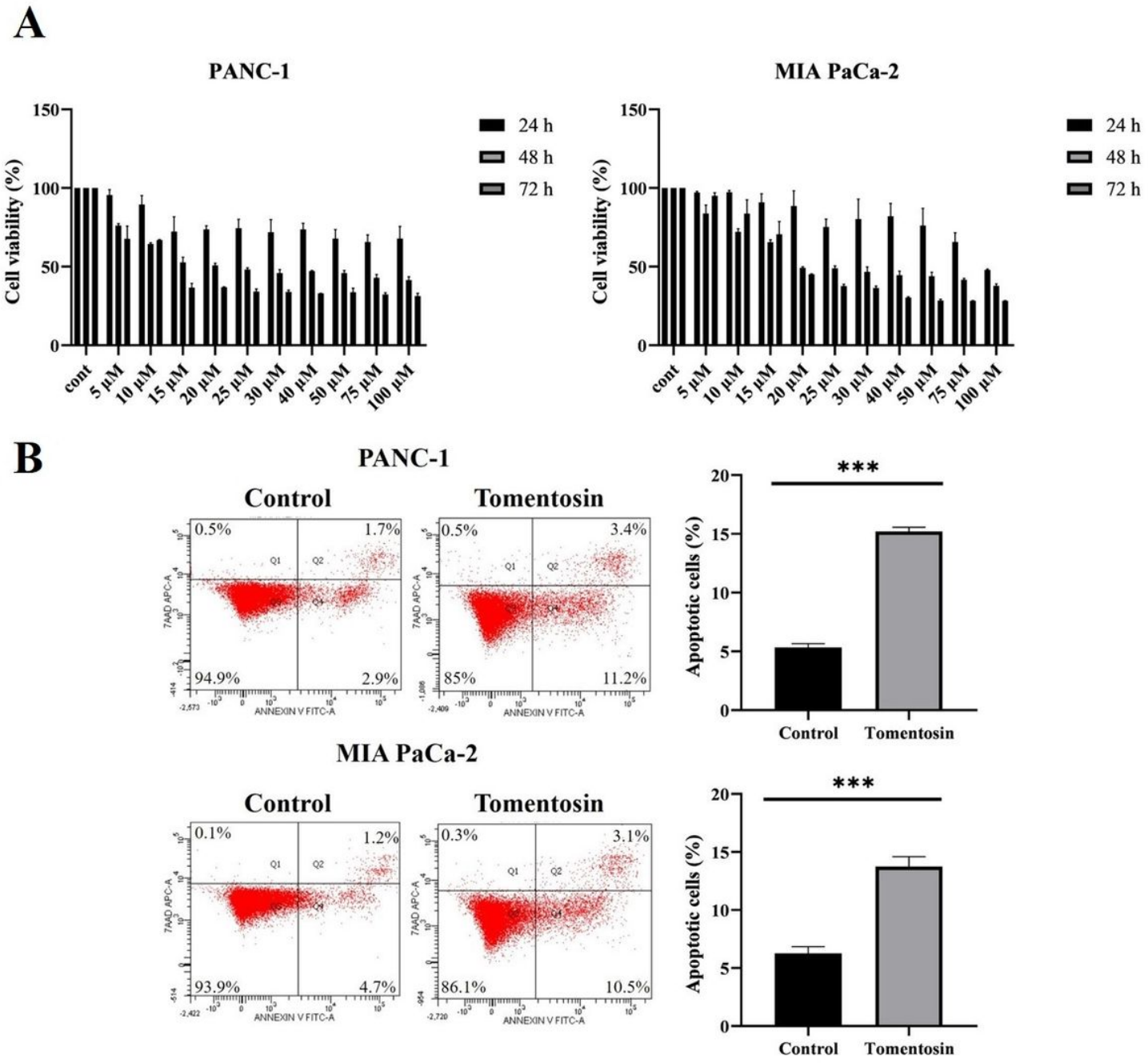

Figure 1

Effect of tomentosin on cell viability and apoptosis in PANC-1 and MIA PaCa-2 cells. **A** Cells were treated with different doses of tomentosin for 24, 48 and 72 hours and cell viability was determined by XTT analysis. The IC_{50} dose of tomentosin in pancreatic cancer cells was found using XTT data. **B** Cells were treated with IC_{50} dose of tomentosin for 48 hours and apoptosis rates were determined with FITC Annexin V. ****p < 0.001*

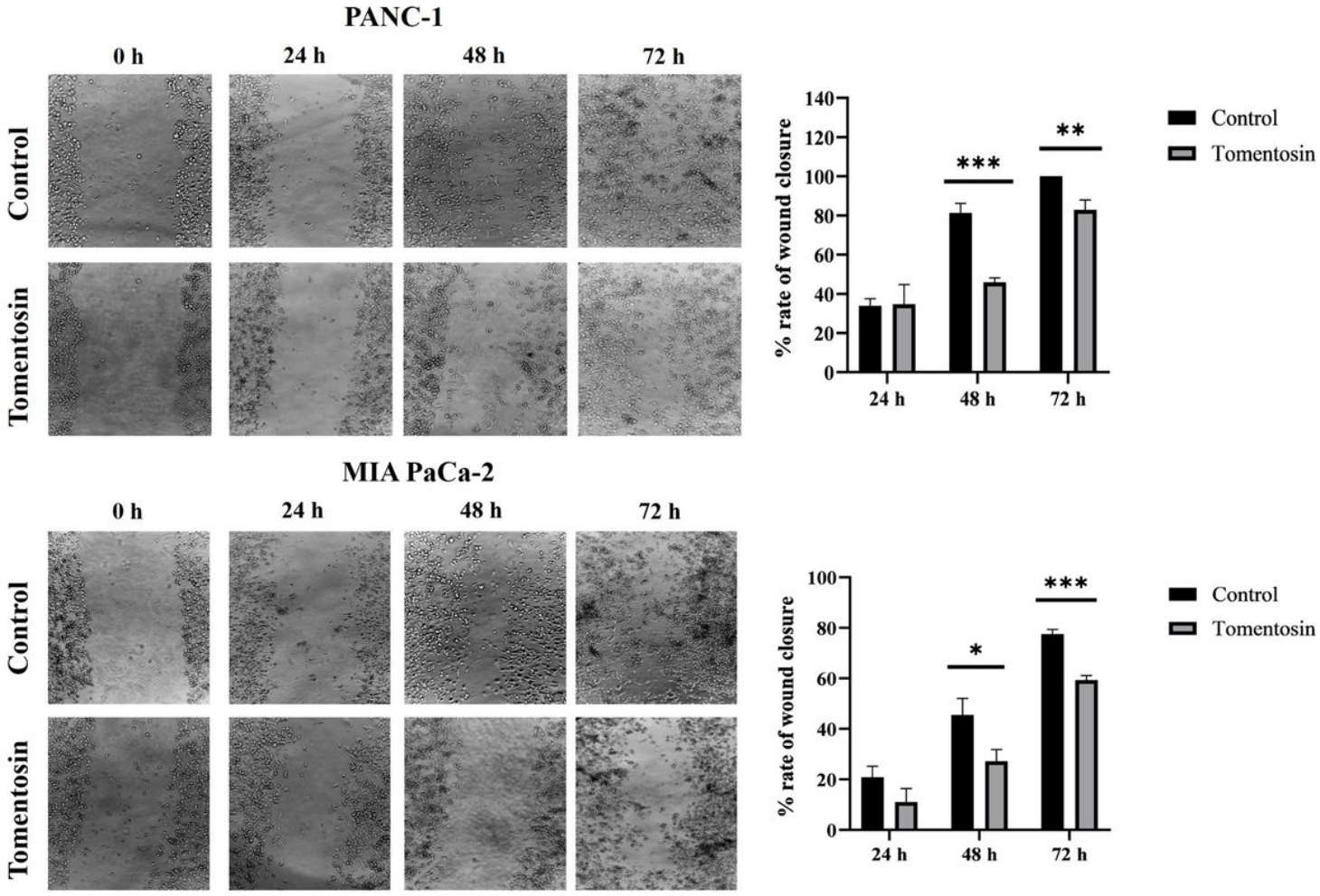

Figure 2

Effect of tomentosin on migration of PANC-1 and MIA PaCa-2 cells. Cells were treated with IC_{50} doses of tomentosin and cell migration was evaluated with wound healing assay. Wound areas were measured with ImageJ software and % rate of wound closures were calculated for 24, 48 and 72 hours. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

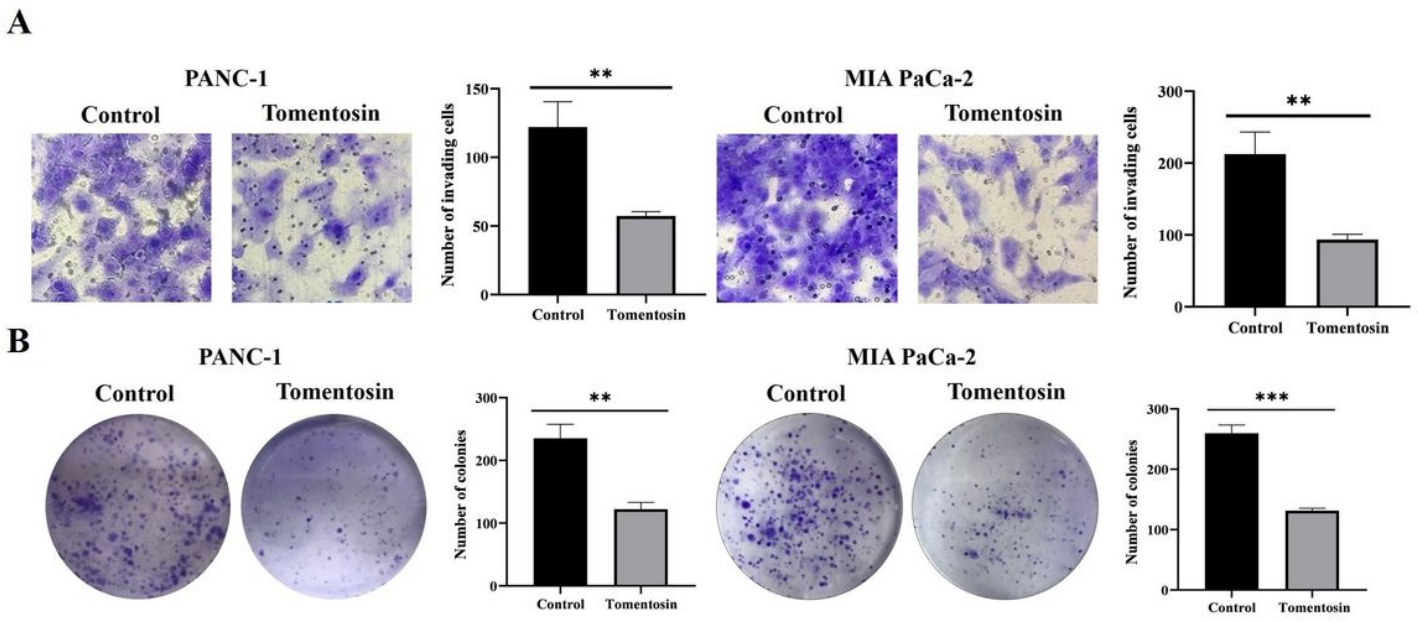

Figure 3

Effect of tomentosin on invasion and colony formation of PANC-1 and MIA PaCa-2 cells. **A** Cells were treated with tomentosin at IC_{50} doses for 48 hours and cell invasion was evaluated with Matrigel invasion chamber. Invaded cells were stained with crystal violet and counted. **B** Cells were treated with IC_{50} doses of tomentosin for 48 hours and cultured for 10 days. Colonies were stained with crystal violet and counted. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

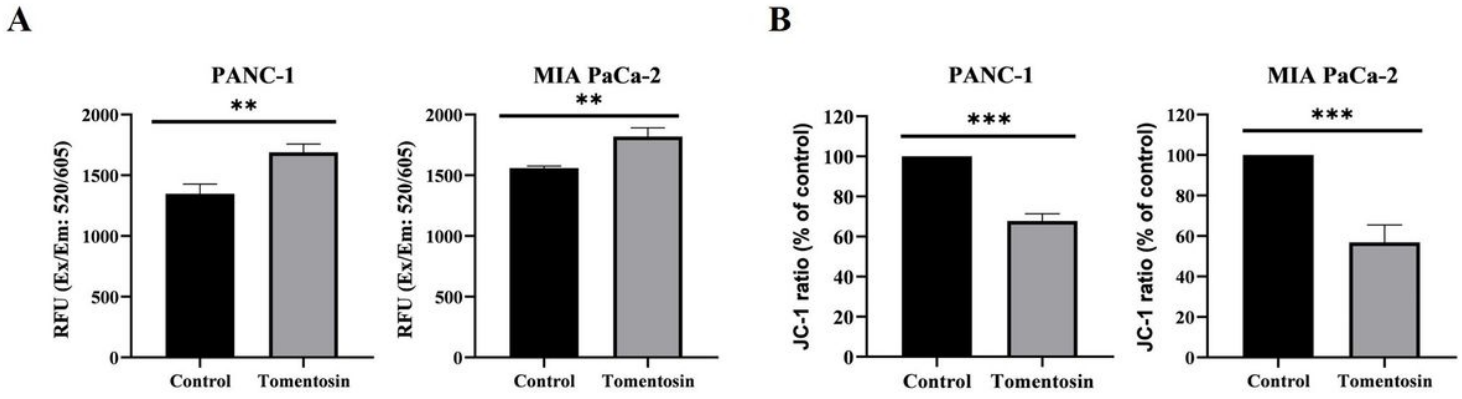

Figure 4

Effect of tomentosin on intracellular ROS level and MMP. **A** Cells were treated with IC_{50} doses of tomentosin for 48 hours and ROS level was evaluated by measuring the red fluorescence at Ex/Em = 520/605 nm. **B** The change in MMP was evaluated with JC-1 dye after treatment of tomentosin at IC_{50} doses for 48 hours. Readings were taken at Ex/Em = 535/590 nm for red fluorescence and at Ex/Em = 475/530 nm for green fluorescence. The change in MMP was determined by ratio of red fluorescence to green fluorescence and results are presented as % of control. **$p < 0.01$; ***$p < 0.001$.

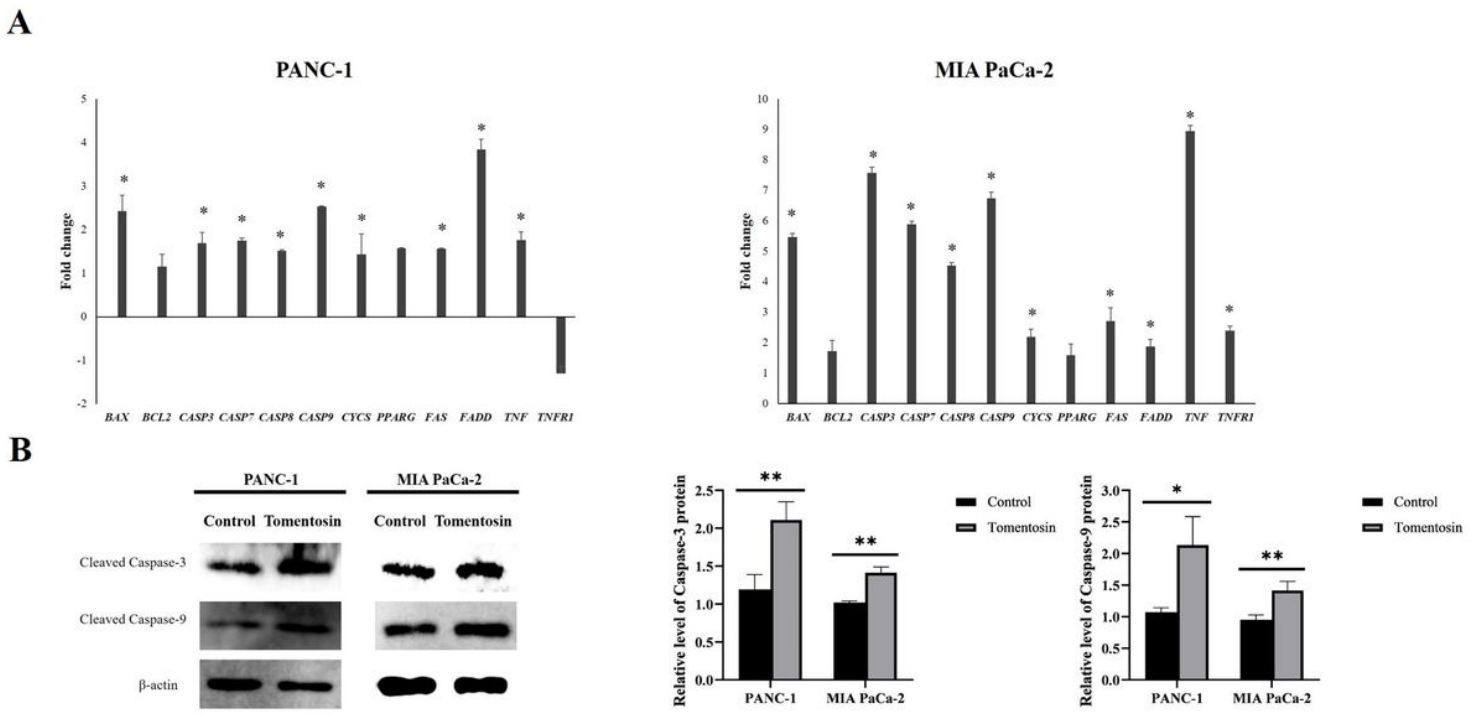

Figure 5

Effect of tomentosin on level of genes expression and proteins associated with apoptosis. **A** The effect of tomentosin on the expression levels of important genes associated with apoptosis was evaluated by qRT-PCR analysis. RNA was isolated from control and tomentosin-treated cells, and cDNA synthesis was performed. After qRT-PCR, fold changes were determined by $2^{-\Delta\Delta CT}$ method. *ACTB* was used as a reference gene. **B** The effect of tomentosin on Caspase-3 and Caspase-9 protein levels was evaluated by western blot analysis. Band thicknesses were calculated with Image J program. The results were presented as relative protein level by normalizing with β-actin. *p < 0.05; **p < 0.01; ***p < 0.001.