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Comparative evaluation of the anti-proliferative effects of alkaloid-rich extract of jujube seed and paclitaxel on MDA-MB-231 breast cancer cell line

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ABSTRACT

Introduction: Cancer is the second cause of death in the world, and among all types of cancer, triple-negative breast cancer (TNBC) has one of the worst prognoses. Repeated use of chemotherapy drugs is associated with drug resistance. This study aims to investigate the effect of jujube alkaloid-rich extract on drug sensitivity and its anti-tumor effects on paclitaxel-resistant MDA-MB-231 cells in vitro.

Materials and methods: The MDA-MB-231 breast cancer cell line and its paclitaxel-resistant variant were used. The XTT method was employed to measure cell viability, while Annexin-PI assays were used to detect apoptosis. Various concentrations of the jujube extract alone and in combination with paclitaxel assessed synergistic effects in 2D and 3D cell culture models.

Results: The presence of alkaloids in the extract obtained from the jujube seed was confirmed by the dragendorff test and its amount was 0.395 g in 5.0 g of jujube seed powder. Paclitaxel and jujube extract exhibited dose-dependent cytotoxic effects on paclitaxel-resistant MDA-MB-231 cells. The combination of jujube extract and paclitaxel significantly decreased IC_{50} from 541.3 μg/mL to 124.3 μg/mL, and the IC_{20} decreased from 92.1 μg/mL to 23.9 μg/mL when combined with paclitaxel in 2D culture model. The combination's toxicity was reduced in the 3D culture model. Apoptosis rates were 0.75 % in the control group, 13.89 % in the alkaloid-rich extract group, 2.59 % in the paclitaxel group, and 42.90 % in the combination group.

Conclusion: Combining paclitaxel with alkaloid-rich jujube seed extract enhances cytotoxicity and apoptosis in paclitaxel-resistant MDA-MB-231 breast cancer cells in both 2D and 3D culture models. This suggests that such combinations might be a viable strategy to overcome drug resistance in TNBC treatments.

1. Introduction

Cancer remains one of the most severe diseases worldwide, with more than 10 million new cases reported each year. The primary causes of cancer development are mutations in oncogenes that result in a dominant gain of function and the malfunctioning of tumor suppressor genes [1,2]. Breast cancer is the most frequently diagnosed malignancy among women globally and is the leading cause of death from malignant

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tumors. The incidence of breast cancer continues to rise in all parts of the world, with more than one million new cases globally each year, making up around 25 % of all malignancies in females worldwide [3,4]. Breast cancer exhibits considerable heterogeneity and can be categorized based on numerous clinical and pathological characteristics. This classification aids in predicting outcomes and tailoring treatments to those who are most likely to benefit. Presently, the status of estrogen receptors (ER), progesterone receptors (PR), and human epidermal growth factor receptor-2 (HER2) are commonly used as predictive markers to guide the selection of appropriate adjuvant therapies [5]. Triple-negative breast cancer (TNBC) encompasses a heterogeneous group of fundamentally different diseases with different histologic, genomic, and immunologic profiles, which are aggregated under this term because of their lack of ER, PR, and HER2 expression [6]. The MDA-MB-231 cell lines exemplify the mesenchymal subtype of triple-negative breast cancer [7].

Chemotherapy is a crucial component in the treatment of breast cancer, using chemotherapeutic drugs to destroy cancer cells and prevent their spread. It is particularly useful for treating advanced and aggressive forms of breast cancer, as well as reducing the risk of recurrence. The effectiveness of chemotherapy in shrinking tumors and improving survival rates makes it a vital option in both preoperative and postoperative cancer care [8]. Paclitaxel (PTX) has exhibited efficacy as an anticancer drug against a range of cancers, such as lung, breast, ovarian, leukopenia, and liver cancer [9]. PTX is a crucial frontline chemotherapy agent for treating breast cancer, particularly in advanced metastatic cases. PTX functions as a microtubule stabilizer, specifically targeting cells in the G2/M phase of the cell cycle. Its cytotoxic effects are concentration and time-dependent, leading to the inhibition of cell division and ultimately inducing cell death [10]. The development of resistance to PTX significantly disrupts clinical treatment and has detrimental effects on patient prognosis, commonly emerging with prolonged exposure to PTX, posing challenges in managing patients effectively [11]. Resistance to chemotherapy diminishes its efficacy in cancer treatment. Tumors can either be intrinsically resistant to drugs or acquire resistance during treatment. Acquired resistance poses a significant challenge, as tumors develop resistance not only to initially prescribed drugs but may also become cross-resistant to other medications with distinct mechanisms of action [12]. To enhance the efficacy of PTX, investigations are exploring the use of herbal drugs in combination with PTX. Herbal drugs, known for their diverse bioactive compounds, can serve as chemo-sensitizers to reverse drug resistance and augment the therapeutic effects of PTX [13]. Plants play a crucial role in cancer treatment as a source of natural compounds with therapeutic potential. Over the past few decades, a significant proportion of anticancer drugs have been derived from plant compounds, highlighting the importance of exploring and harnessing the bioactive components of plants in the fight against cancer [14].

Jujube (*Ziziphus Jujuba* Mill.) belongs to the genus *Ziziphus* (family: Rhamnaceae) and is largely cultivated in the subtropical and tropical regions, particularly in Australia, southern and eastern Asia, and Europe [15]. Jujube fruit possesses various beneficial properties, including antioxidant, anti-inflammatory, hepato-protective, blood pressure and lipid reduction, and anticancer properties. Recent phytochemical and pharmacological studies have shown that alkaloids are one of the major biologically active components of the Jujube fruit. Alkaloids with diverse structural variations, serve as nitrogenous organic compounds, contributing significantly to the protection of plants against both biological and abiotic stresses [16]. Mahanimbine, a compound rich in alkaloids found in curry leaves, has demonstrated potent anticancer effects by modulating P-glycoprotein (P-gp) activity. Additionally, when combined with the chemotherapeutic drug gefitinib, mahanimbine synergistically increased its intracellular accumulation in lung cancer cells, suggesting its potential to overcome chemoresistance [17].

Combination therapy involving *Piper nigrum*, rich in alkaloids, demonstrates potent anticancer effects, as evidenced by the sensitization of paclitaxel-resistant cervical cancer cells to paclitaxel. Piperine, a major component, enhances cell apoptosis and decreases Mcl-1 protein expression when combined with paclitaxel, showcasing the potential of *P. nigrum* alkaloids, particularly dimeric amide alkaloids, in cancer treatment [18]. This research aimed to assess the efficacy of jujube seed extract, known for its alkaloid content, on drug (paclitaxel) sensitivity and its antitumor effects on paclitaxel-resistant MDA-MB-231 cells in vitro and provide insights into the potential of jujube seed extract as a novel therapeutic option for breast cancer treatment.

2. Materials and Methods

2.1. Materials

The MDA-MB 231 human breast cancer cell line was supplied by the National Cell Bank of Iran (Pasteur Institute of Iran, Tehran, Iran). Polycaprolactone (PCL) (80,000 Mn, sigma-aldrich™, USA, Cat# 440744), Roswell Park Memorial Institute (RPMI) medium and Paclitaxel were purchased from Sigma Aldrich™ (USA). Trypsin-Ethylene diamine tetra acetic Acid (EDTA) and Dulbecco's Modified Eagle's medium (DMEM), Penicillin-Streptomycin, and fetal bovine serum (FBS) were purchased from GIBCO™ (USA). DNase I, RNase free was purchased from Millipore™ (USA). Phosphate buffered saline (PBS), ethanol 96 %, Chloroform ($CHCl_3$), Hydrochloric (HCl), Sulfuric acid (H_2SO_4), Ethyl acetate ($C_4H_8O_2$), Dimethyl sulfoxide (DMSO), and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Merck™ (Germany). Annexin V Apoptosis Detection Kit FITC was purchased from eBioscience™ (China).

2.2. Plant collection and alkaloid-rich extraction

Jujube fruit specimens were collected from Birjand City in late September in their optimal stage of formation in the South Khorasan province of Iran and transported to the Faculty of Pharmacy in Sari. A voucher specimen was deposited at the Herbarium of the Faculty of Pharmacy, Mazandaran University of Medical Science, Iran (herbarium number: MAZUM-A1018). The fruits were dried in an oven at 55 °C to prevent decay. Subsequently, the seeds were isolated, and powdered using an electric mill to increase the surface area for subsequent processing. The powdered seed material was extracted with 96 %, ethanol under reflux for 4-h. The resulting extract was then filtered, and the solvent was removed by evaporation using a rotary evaporator at 40 °C under reduced pressure. The remaining residue was dissolved in water and acidified (pH = 2) with H_2SO_4, followed by partitioning with chloroform to remove lipophilic, acidic, and neutral components. After alkalizing the acidic solution (pH = 10) with dropwise ammonia (NH_3), it was extracted with ethyl acetate and washed with distilled water to achieve a neutral solution (pH = 7). The ethyl acetate extract was further concentrated using a rotary evaporator to obtain the final alkaloid-rich extract, which was subsequently powdered using a freeze dryer [19].

2.3. Preparation of plant extracts for qualitative determination of alkaloids

The 96 % ethanol extract of powdered seed of jujube (20 g) was prepared by maceration method (6 h on the shaker), then concentrated by rotary evaporator. The 96 % ethanol extract (1 g) from the previous step was mixed with 2N HCl (3 ml) and distilled water (20 ml). This mixture was heated in a water bath (bain-marie) for 5 min. After filtering through cotton, sodium chloride (0.5 g) was added to the solution. Subsequently, ammonia (6 ml) and chloroform (30 ml) were added, and the solution was decanted. The chloroform layer was evaporated to dryness in an oven at 55 °C. The residue was dissolved in 2N HCl (10 ml) and distributed into test tubes: I. Mayer's test: A white precipitate indicates alkaloids. II. Dragendorff's test: A red-brown

precipitate indicates alkaloids. III. Wagner's test: A red-brown precipitate indicates alkaloids [20,21].

2.4. Determination of total alkaloids by titrimetric methods

The powdered plant material (5 g) was mixed with *n*-butanol (20 ml) and vigorously stirred. The resulting mixture was transferred into a reagent bottle and kept overnight at room temperature. Afterward, it underwent centrifugation at 6000 rpm (4025 g-force) for 10 min, and the supernatant was adjusted to 50 ml with *n*-butanol. The supernatant was used to determine the total alkaloid content through titrimetric methods. Specifically, the supernatant (10 ml) was poured into a 100 mL separating funnel, followed by the addition of 0.1N HCl (10 ml), which was thoroughly mixed for 2–3 min to enhance alkaloid solubility. This resulted in the separation of alkaloids neutralized with 0.1N HCl in the lower layer and *n*-butanol in the upper layer. The HCl (10 ml) portion was then transferred into a beaker, where methyl red (2–3 drops) was added, causing the solution to turn slightly reddish. The contents of the beaker were titrated against 0.1N NaOH until the color changed from red to pale yellow, indicating neutralization. The titrant volume was calculated using the formula below and this procedure was repeated three times for accuracy [22].

$$V2 = \frac{N1.V1}{N2}$$

The total alkaloid amount was then determined by considering the following equivalent:

0.1N HCl (1 ml) ≡ 0.0162 g alkaloid

2.5. Cell culture and generation of MDA-MB-231 PTX resistant cell lines

Cells were cultivated in DMEM enhanced with 10 % (v/v) heat-inactivated fetal bovine serum and 1 % (v/v) penicillin-streptomycin. They were maintained at 37 °C and 5 % CO_2. Paclitaxel powder was dissolved in 1 % DMSO to create a 10 mM stock solution (853 μg/ml), which was aliquoted and stored at −20 °C. After long-term exposure of MDA-MB-231 cells to 1 nM (0.853 ng/ml) PTX, the concentration was gradually increased over 2 weeks, ultimately reaching 100 nM (39.85 ng/ml). This process resulted in the creation of a paclitaxel-resistant subline known as MDA-MB-231 PTXR [23].

2.6. Cryopreservation of MDA-MB-231 cells

The process begins by harvesting cells during the logarithmic growth phase, detaching them from the culture flask using EDTA and 0.25 % trypsin. The cells are then washed with RPMI medium and counted with a hemocytometer to assess viability and concentration. Once counted, 3×10^6 cells are transferred into each conical tube and centrifuged at 1500 rpm (252 g-force) for 5 min. After centrifugation, the supernatant is discarded, and the cell pellet is resuspended. For each conical tube containing 3×10^6 cells, 1 mL of freezing medium is added to cryovials. The vials are then cooled at a controlled rate (typically °C per minute) using a freezer container (Thermo Scientific™ Mr. Frosty™, USA) to −20 °C for 1 h, and subsequently transferred to a −70 °C freezer for 24 h. Finally, the cryovials are stored in liquid nitrogen tanks for long-term preservation. All cryopreserved samples are labeled with key information, including cell type, freezing date, and passage number. This approach ensures high post-thaw cell viability and minimizes damage, enabling reliable recovery for future research applications [24].

2.7. Thawing procedure for cryopreserved cells

The procedure begins by preparing sterile equipment and culture medium in a cell culture hood. Cryovials containing the frozen cells are removed from the liquid nitrogen tank and immediately placed into a 37 °C water bath. The vial is closely monitored, and once ice crystals have melted, the contents are quickly transferred into a sterile conical tube containing RPMI medium supplemented with 10 % fetal bovine serum (FBS). The tube is then centrifuged at 1500 rpm (252 g-force) for 5 min to separate the cells from residual DMSO. After centrifugation, the supernatant, which contains the DMSO, is discarded carefully to avoid disturbing the cell pellet. Cell viability is assessed using trypan blue exclusion or automated counting methods, and the cells are seeded into a T25 flask (Thermo Scientific™, USA) with 10 % FBS. After 24 h of incubation, the medium is replaced to remove any residual DMSO, and the cells are examined for proper morphology using an inverted microscope [24].

2.8. Cytotoxicity evaluation of paclitaxel on cancer cell and MDA-MB-231 PTXR cell line in 2D culture

The MDA-MB-231 cell lines were treated with varying concentrations of paclitaxel (ranging from 10 to 100 μg/ml). The 50 % inhibitory concentration (IC_{50}) was obtained by the 2, 3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) colorimetric assay. By adding 3×10^4 MDA-MB-231 cancer cells into each well of a 96-well plate, 0.1 ml of medium containing varying concentrations of paclitaxel was added. After 48 h incubation period at 37 °C, 5 % CO_2, XTT reagent was added to each well, and the absorbance was measured at 590 nm and 620 nm using a multifunctional ELISA reader, with all experiments repeated three times to ensure accuracy [25].

2.9. Cytotoxicity evaluation of alkaloid-rich extract on cancer cell and MDA-MB-231 PTXR cell line in 2D culture

XTT assay aimed to assess the cytotoxic effects of various extract concentrations (ranging from 0 to 1000 μg/ml) alongside a 7.1 nM dose of paclitaxel, which serves as the IC_{50} dose. After adding 3×10^4 cells into each well of a 96-well plate and allowed to adhere for 24 h, different concentrations of the extract and the IC_{50} dose of paclitaxel were then added in triplicate, with DMSO serving as the negative control. After a 48 h incubation period at 37 °C with 5 % CO_2, the XTT reagent was applied to assess cell viability. Absorbance was measured using an ELISA reader at 450 and 620 nm, with all experiments repeated three times to ensure accuracy [25].

2.10. Drug interaction analysis in MDA-MB-231 cells

To assess drug interactions in MDA-MB-231 cells, the CompuSyn software, based on the modified Chou-Talalay Combination Index (CI) method, was utilized. This software calculates the doses required, both individually and in combination, to induce varying levels of cytotoxicity. For each cytotoxic level, the software generates a Combination Index (CI) to evaluate drug interactions.

In this study, paclitaxel at a concentration of 7.1 nM (IC_{50}) was combined with different concentrations of an alkaloid-rich extract. The CI was used to define the type of interaction: a $CI < 1$ indicates synergy, $CI = 1$ suggests an additive effect, and $CI > 1$ implies antagonism.

Key plots generated from this analysis include:

I. **Dose-Effect Curve**: Illustrates the dose-response relationship for both the drug and the extract, showing effects on the vertical axis and doses on the horizontal axis.
II. **Fa-CI Plot**: Depicts synergy below the line where $CI = 1$ and antagonism above it.
III. **Median-Effect Plot**: Shows the relationship between Log (D), where D is the dose, and Log (Fa/Fu), the ratio of affected to unaffected fractions. A positive slope suggests stimulation, while a negative slope indicates inhibition.

IV. **Dose-Normalized Isobologram**: Visualizes interactions, with points below the diagonal representing synergy, above the line indicating antagonism, and on the line showing additive effects [26].

2.11. Cytotoxicity evaluation of alkaloid-rich jujube extract and paclitaxel in a 3D culture model

The fabrication of the nano-scaffold was achieved using an electrospinning device (Fanavaran Nano Meghyas Co, Iran) with precise parameters: a flow rate of 1 ml/h, a needle distance of 13 cm, and a total volume of 10 ml. The resulting structure was analyzed using scanning electron microscopy (SEM), which provided detailed images of the fiber arrangement and the diameter of the Electrospun Polycaprolactone (PCL) nano-scaffolds.

To enhance cell attachment and proliferation, the PCL nanofibers underwent O_2 plasma treatment. This process, conducted for 5 min at 0.4 mbars and 30 W in a microwave plasma chamber, improved the nanofibers' surface hydrophilicity, aiding in better cell interaction. The treated nanofibers were then sterilized with 70 % ethanol to eliminate any microbial contamination.

Post-sterilization, the nanofibers were rinsed three times with phosphate-buffered saline (PBS) to remove any residual ethanol, followed by freeze-drying to maintain their structural integrity. This combination of SEM analysis, plasma treatment, sterilization, and freeze-drying produced high-quality PCL nano-scaffolds that were suitable for 3D cell culture applications [27,28].

2.12. Cytotoxicity evaluation of alkaloid-rich jujube extract and paclitaxel on paclitaxel-resistant MDA-MB-231 cells in a PCL polymeric nano-scaffold

Following the determination of IC_{50} values for both the alkaloid-rich extract and paclitaxel in previous stages, cells were treated with concentrations ranging from 0 to 1000 μg/ml of the alkaloid-rich extract and 7.1 nM of paclitaxel under conditions closely mimicking the human body environment. After adding $3x10^4$ cells into each well of a 96-well plate and allowed to adhere to the polymeric scaffold for 24 h, various concentrations of the extract and paclitaxel were added in triplicate to the cells. An equal volume of PBS (buffer) was added in triplicate to the wells as a negative control. After 48 h of incubation at 37 °C with 5 % CO_2, the cells were exposed to XTT reagent to assess cell viability. Absorbance was measured using an ELISA reader at 450 and 620 nm. Notably, this experiment was repeated three times to ensure accuracy [29].

2.13. Flow cytometric analysis of cellular apoptosis

MDA-MB-231 cells were seeded at a density of 3×10^5 cells/mL and were treated with 100 μg/ml of alkaloid extract and 7.1 nM of paclitaxel for 24 h. Following the treatment period, cells were washed twice with cold phosphate-buffered saline (PBS) and detached using 1x trypsin-EDTA. The detached cells were then transferred to tubes containing 1 mL of medium and centrifuged at 1400 rpm (220 g-force) for 5 min. The supernatant was decanted, and 100 μL of binding buffer was added to each tube, followed by gentle pipetting. Except for the negative control group, where no dye was added, 10 μL of FITC-Annexin V and 10 μL of propidium iodide (PI) were added to each tube. The tubes were incubated in the dark at room temperature for 30 min. After incubation, an additional 400 μL of binding buffer was added, and the samples were analyzed using a FACSCalibur™ flow cytometer with FlowJo™ software [30].

2.14. Data analysis and statistics

The findings are reported as mean ± standard deviation (SD). All statistical analyses are done using the GraphPad Prism version 6 software and SPSS version 16. The normality of data distribution is assessed using the Kolmogorov-Smirnov test. Given the quantitative description of the study variables, data analysis involves paired t-tests and non-parametric Wilcoxon tests. In all tests, P value < 0.05 is considered significant.

3. Results

3.1. Qualitative determination of alkaloids

After adding a drop of various indicators to the test tubes, adding Dragendorff reagent to the tube containing the jujube's seed extract formed a distinct reddish-brown precipitate, indicating the presence of alkaloids (Table 1). The root bark and leaf extracts of jujube were also tested, but all three tests conducted for these extracts did not confirm the presence of alkaloids.

3.2. Quantification of total alkaloids

To quantify the total alkaloid content, a titration method was employed using an acidic solution (V1) containing the extract of jujube seed powder with NaOH solution (V2). The neutralization point was identified when the indicator in the titrated solution turned yellow. Using equations for the calculation of titrant volume and alkaloid amount, the average volume of NaOH required after three titrations was 24.43 ml. This resulted in an alkaloid content of 0.395 g in 5 g of jujube seed powder.

3.3. Cytotoxicity evaluation of paclitaxel on cancer cell and MDA-MB-231 PTXR cell line

XTT assay was used to evaluate the cytotoxicity of paclitaxel. As shown in Fig. 1, various concentrations of paclitaxel ranging from 0 to 100 nM were tested on the cells for 48 h. Paclitaxel showed dose-dependent cytotoxic effects on the MDA-MB-231 cells, with an IC_{50} of 7.1 nM. In contrast, MDA-MB-231 PTXR cells showed high survival rates, with 95 % viability at 7.1 nM and 83 % viability even at 100 nM of Paclitaxel.

3.4. Cytotoxicity evaluation of alkaloid-rich extract and its drug interaction analysis with paclitaxel on MDA-MB-231 PTXR cell line

The cytotoxic effects of the alkaloid-rich extract on MDA-MB-231 PTXR cells were evaluated using the XTT assay. As ill ustrated in Fig. 2 (blue line), various extract concentrations, ranging from 0 to 1000 μg/mL, were tested over a 48-h period. The results showed a dose-dependent cytotoxicity, with an IC_{20} (the concentration at which 80 % of cells survive) of 92.1 μg/mL and an IC_{50} (the concentration at which 50 % of cells survive) of 541.3 μg/mL.

Next, the combination of the alkaloid-rich extract with paclitaxel (at its IC50 concentration of 1.7 nM) was analyzed to assess any drug interaction. After 48 h (green line in Fig. 2), the IC_{50} of the extract was reduced from 541.3 μg/mL to 124.3 μg/mL, and the IC_{20} decreased from 92.1 μg/mL to 23.9 μg/mL. This significant reduction in IC values suggests a synergistic effect between the alkaloid-rich extract and paclitaxel in MDA-MB-231 PTXR cells.

The synergistic effect of this interaction was confirmed using

Table 1
Results of alkaloid presence in jujube's seed extract.

Reagent	Precipitate color	Confirmation of alkaloid presence
Mayer	–	–
Dragendorff	Reddish-brown	+
Wagner	–	–

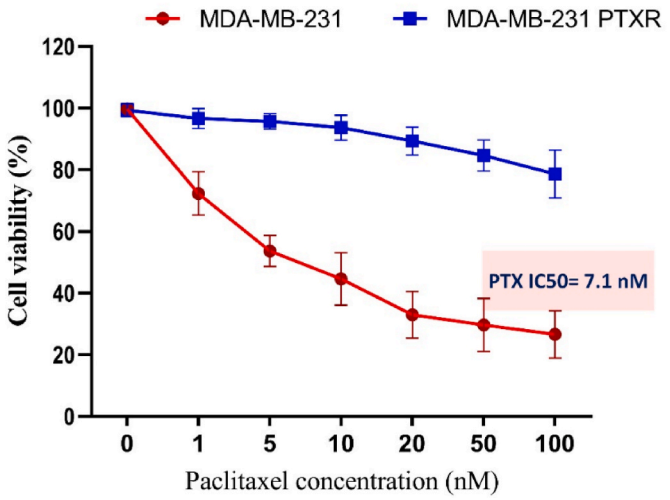

Fig. 1. Cytotoxicity evaluation of different concentrations of paclitaxel on cancer cell and MDA-MB-231 PTXR cell line.

CompuSyn software, which reported Combination Index (CI) values less than one for all concentrations, indicating synergism (Table 2). Notably, the concentration of 100 μg/mL showed the highest synergy (CI = 0.13375).

Detailed analysis using CompuSyn software provided various plots (Fig. 3):

1. **Dose-effect curve (Fig. 3A):** The combined treatment exhibited stronger dose-dependent effects compared to the extract alone, with green points (combination of Paclitaxel and extract) positioned lower than the blue line (extract alone), indicating higher cytotoxicity.
2. **Combination index plot (Fig. 3B):** All Fa-CI points were below line 1, indicating synergistic effects across all concentrations.
3. **Median-effect plot (Fig. 3C):** The log-transformed results showed positive slope lines, indicating increased response with higher doses. The intersection with the X-axis indicated the combined IC_{50} of 124.3 μg/mL.
4. **Dose-normalized isobologram (Fig. 3D):** This plot showed that all combined doses (colored points within the triangle) had synergistic effects with CI values less than one.

3.5. Cytotoxicity evaluation of alkaloid-rich extract on cancer cells and its drug interaction

3.5.1. Analysis with paclitaxel on MDA-MB-231 PTXR Cell line in a PCL polymeric nano-scaffold

To evaluate the cytotoxicity of an alkaloid-rich extract on MDA-MB-231 PTXR breast cancer cells, the effects of various concentrations of the extract on cell viability were assessed using the XTT assay. As shown in Fig. 4 (blue line), after 48 h of exposure to the extract at concentrations ranging from 0 to 1000 μg/mL, the extract demonstrated dose-dependent cytotoxic effects on MDA-MB-231 PTXR cells. The IC_{20} was calculated to be 382.3 μg/mL, while the IC_{50} was determined to be over 1000 μg/mL. Also as illustrated in Fig. 4 (green line), when the resistant cells were exposed to varying concentrations of the extract ranging from 10 to 750 μg/mL combined with 7.1 nM paclitaxel for 48 h, IC_{20} significantly decreased from 382.3 μg/mL to 65 μg/mL and IC_{50} also, significantly decreased from over 1000 μg/mL to 407.8 μg/mL.

3.5.2. Apoptosis induced in MDA-MB-231 PTXR cell lines

To quantify the apoptotic effects of paclitaxel, alkaloid extract alone, and in combination with each other on the MDA-MB-231 PTXR cell line, flow cytometry was used. PI and Annexin V-FITC staining helped identify viable, apoptotic, and necrotic cells. Untreated cell lines served as controls. After 24 h, the cells were treated with 100 μg/mL of alkaloid extract, 7.1 nM paclitaxel, and combinations of 100 μg/mL of alkaloid extract with 7.1 nM PTX. Fig. 5 illustrates the apoptotic cells in different treatment groups compared to the control group. As shown, the apoptosis rate increased in all treatment groups compared to the control group, with the highest rate observed in the group treated with both paclitaxel and the alkaloid extract. Specifically, the apoptosis rates were 0.75 % in the control group, 13.89 % in the alkaloid extract group, 2.59 % in the paclitaxel group, and 42.90 % in the combination group.

Table 2
Combination index of different concentrations of alkaloid-rich extract in combination with IC_{50} concentration (7.1 nM) of paclitaxel.

Alkaloid Concentration (μg/mL)	CI	Effect
10	33966/0	Synergism
50	21340/0	Synergism
100	13375/0	Synergism
250	14297/0	Synergism
500	16675/0	Synergism
1000	20473/0	Synergism

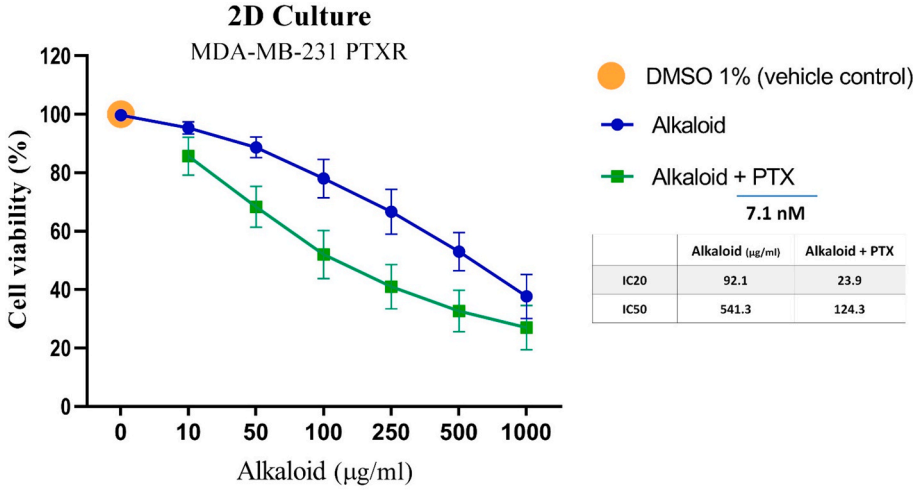

Fig. 2. Cytotoxicity evaluation of alkaloid-rich extract and in combination with paclitaxel on MDA-MB-231 PTXR cell line.

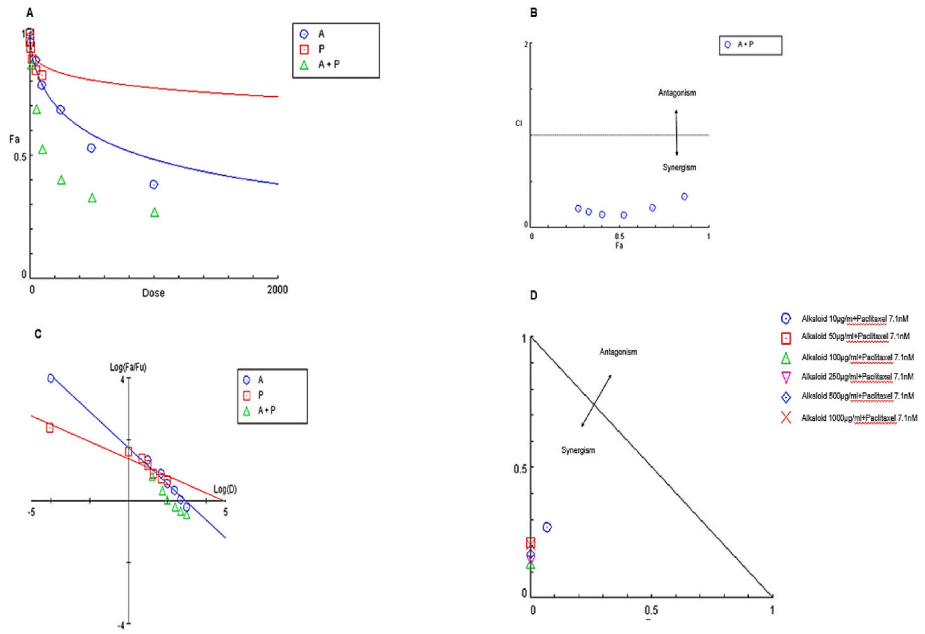

Fig. 3. Plot analysis of alkaloid-rich extract and paclitaxel on MDA-MB-231 PTXR cells using CompuSyn software
A) Dose-effect curve B) Combination index plot C) Median-effect plot D) Dose-normalized isobologram.

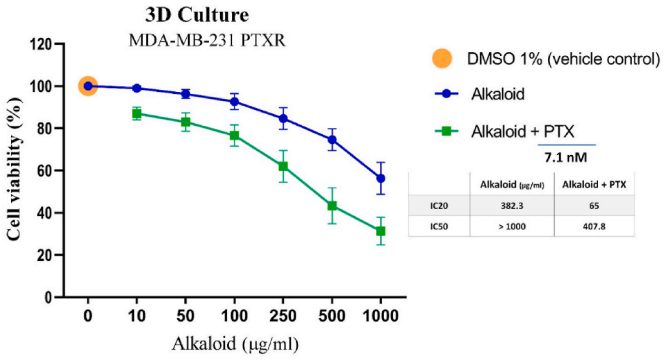

Fig. 4. Cytotoxicity evaluation of alkaloid-rich extract and alkaloid in combination with paclitaxel on MDA-MB-231 PTXR cells in a 3D Culture Model.

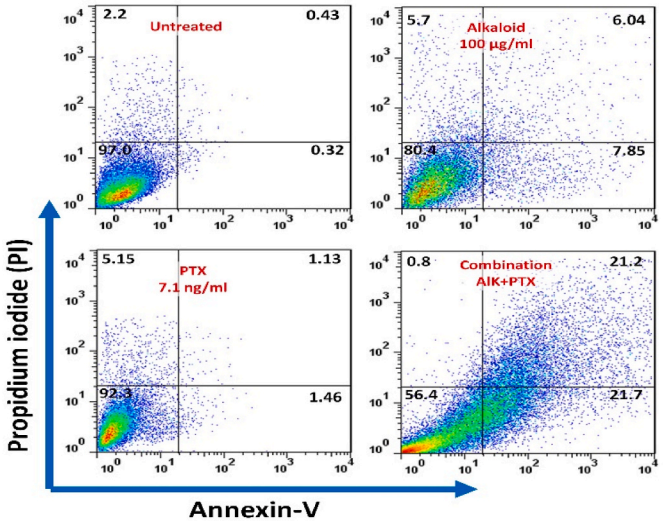

Fig. 5. Flow cytometry analysis of apoptosis in MDA-MB-231 PTXR cell lines.

4. Discussion

Breast cancer is a significant public health concern worldwide and is the most common cancer among women. It is predicted that by 2040, the global burden of breast cancer will rise to over 3 million new cases and 1 million deaths annually [31]. TNBC is the most aggressive form of breast cancer, characterized by high mortality rates and a significant likelihood of metastasizing to distant parts of the body beyond the breast [32].

Cryopreservation is a crucial method used to maintain the viability and functionality of MDA-MB-231 cells, over extended periods. This technique involves cooling the cells to sub-zero temperatures to halt their biological activity and preserve their integrity. Proper cryopreservation practices ensure cell line authenticity and support reproducibility in biomedical research, particularly for studies on breast cancer. The thawing process is critical for the efficient recovery of cryopreserved cells and must be performed rapidly to minimize damage caused by dimethyl sulfoxide (DMSO). Unlike the gradual freezing process, rapid thawing ensures optimal cell viability [24].

One of the chemotherapy drugs, paclitaxel, is commonly used in the treatment of various cancers, including breast cancer. The development of resistance to this drug, particularly in TNBC, hinders its therapeutic success. Alkaloids present a promising strategy for sensitizing drug-resistant cancer cells, enhancing the effectiveness of conventional treatments, and potentially overcoming chemoresistance [18,33]. Although further studies are needed to confirm the exact mechanism, jujube seed extract may combat paclitaxel resistance by targeting molecular pathways such as DNA binding, microtubule polymerization, enzyme inhibition, and epigenetic modulation. Similar to alkaloids from other plants, jujube seed alkaloids may disrupt cancer cell replication, induce apoptosis, and interfere with the molecular processes that allow cancer cells to survive chemotherapy. This preliminary finding suggests that jujube seed extract holds potential for overcoming drug resistance, particularly through its interaction with cellular proteins and nucleic acids [34]. Assessing cytotoxicity of a naturally occurring furoquinoline alkaloid and four acridone alkaloids towards multi-factorial drug-resistant cancer cells using the resazurin reduction method exhibit both the furoquinoline and acridone alkaloids cytotoxic effects with IC_{50} values below 138 μM in all tested cancer cell lines. The IC_{50} values for furoquinoline ranged from 41.56 μM in HepG2 cells to 90.66 μM in

HCT116 (p53−/−) cells. For 1-hydroxy-4-methoxy-10-methylacridone, IC_{50} values ranged from 6.78 μM in HCT116 (p53−/−) cells to 106.47 μM in MDA-MB-231 pcDNA cells. Norwogonin's IC_{50} values ranged from 5.72 μM in U87MG.ΔEGFR cells to 137.62 μM in CCRF-CEM cells. These alkaloids are potential natural cytotoxic products [35]. Cyclopeptide alkaloids, especially from the jujube plant, show strong cytotoxic effects on the MRC-5 lung cancer cell line. Different concentrations of 10 alkaloids derived from the *Rhamnaceae* plant family on the lung cancer cell line using the MTT assay, show IC_{50} values below 10 μg/mL, suggesting that these compounds have cytotoxic effects on the mentioned cell line [36]. The observed cytotoxic effects are consistent with the current study, where the natural extract exhibited dose-dependent toxicity against MDA-MB-231 PTXR cells, leading to a notable decrease in IC_{50} values. Tetrandrine (TET), a bis-benzylisoquinoline alkaloid, effectively reverses drug resistance mediated by P-Glycoprotein. Without tetrandrine, KBv200-resistant cells show significantly higher resistance to paclitaxel and doxorubicin, with IC_{50} values approximately 20–25 times greater than sensitive cells. Co-treatment with TET markedly increases sensitivity to paclitaxel and doxorubicin by about 10-fold. Additionally, TET enhances the cytotoxicity of doxorubicin and vincristine, effectively restoring sensitivity in KBv200 cells resistant to doxorubicin [37]. TET also reverses paclitaxel resistance in human ovarian cancer cells by inducing apoptosis and cell cycle arrest via the β-Catenin pathway. TET demonstrates the ability to sensitize SKOV3/PTXR cells to paclitaxel, significantly inhibiting their proliferation and enhancing the anticancer effects of PTX when used in combination [38]. These findings are in line with the present study in which the alkaloid-rich extract, in combination with paclitaxel, exhibited significantly stronger cytotoxic effects on MDA-MB-231 PTXR cells, leading to a significant decrease in IC_{50}.

In mammals, tissues and cells are not only connected but also to structures known as the extracellular matrix (ECM). Cells grow in an organized three-dimensional space, and their behavior is influenced by factors such as the nature and extent of their interactions with neighboring cells and the ECM [39]. In the present study, a 3D culture model was used to better mimic the in vivo environment, thereby aiding in a deeper understanding of cell-cell interactions in studies. The results of the present study from the 3D culture model demonstrate greater resistance to these compounds and require higher doses of the extract to exhibit toxicity and differ from those of the 2D culture in terms of IC_{50} and IC_{20} concentrations of the extract. Intracellular mediators contributing to doxorubicin and cisplatin resistance show greater resistance to chemotherapy in three-dimensional multicellular environments compared to two-dimensional cultures across three different endometrial cancer cell lines [40]. Cytotoxicity assay on breast cancer cell lines such as BT-474, MCF-7, T-47D, and MDA-MB-23 in both 2D plates and 96-well plates for 3D models treated with paclitaxel, doxorubicin, and 5-fluorouracil at concentrations ranging from 0.1 to 10x the areas under the curve (AUC) obtained in clinical pharmacokinetic studies shows different resistances. For 5-fluorouracil, there was no significant difference between the two cell culture models. However, T-47D, BT-474, and BT-549 cells exhibited greater resistance to paclitaxel and doxorubicin in 3D culture compared to 2D culture. The study attributed the increased resistance in the 3D model to hypoxic conditions, cell quiescence, or decreased expression of pro-apoptotic molecules like caspase-3, and the 3D model better mimics in vivo conditions, showcasing tumor dormancy and anti-apoptotic characteristics [41]. These findings are in line with the present study.

Piper nigrum (family: *Piperaceae*) reportedly contains nine amide alkaloids. Among them, seven compounds sensitized paclitaxel-resistant cervical cancer cells HeLa/PTX to paclitaxel. The combination therapy of the alkaloid extract of *P. nigrum* with paclitaxel, increased cellular apoptosis, with the involvement of reduced phospho-Akt and MCL-1 levels. Piperine (50 μM) combined with paclitaxel (200 nM) decreased MCL-1 protein expression by 35.9 ± 9.5 % ($p < 0.05$). Additionally, combined treatments of six dimeric amide alkaloids with paclitaxel all reduced MCL-1 protein expression in the range of 23.5 ± 9.7 % to 41.7 ± 7.2 % ($p < 0.05$). It was demonstrated that plant dimeric amide alkaloids have a significant sensitization effect on cancer cells to paclitaxel [18]. Noscapine, a benzylisoquinoline alkaloid, binds to a different site on tubulin compared to paclitaxel and induces mitotic arrest in paclitaxel-resistant ovarian cancer cells, leading to apoptosis [42]. These findings align with a present study where the apoptosis rate increased in all treatment groups compared to the control group.

Withania somnifera known as Ashwagandha (fam: *Solanaceae*) has demonstrated notable effects on apoptosis, particularly in cancer cells, and its ability to induce apoptosis through various mechanisms makes it a promising candidate in cancer therapy. In the murine breast cancer cells (EMT6), the combination of Ashwagandha extract and cisplatin significantly increased Caspase-3 activity by 2.98 times compared to the control group, demonstrating enhanced apoptotic activity. Similarly, in the cisplatin-resistant cell line, Ashwagandha extract alone increased Caspase-3 activity by 1.39 times compared to the control, while the combination treatment further elevated it by 2.31 times compared to the control, indicating substantial improvement over cisplatin treatment alone ($p < 0.001$). These findings underscore Ashwagandha's potential to sensitize cancer cells to chemotherapy-induced apoptosis, thereby enhancing treatment efficacy [43]. *Spica prunellae* (family: *Lamiaceae*) extract enhances the sensitivity of 5-fluorouracil-resistant human colorectal cancer cells (HCT-8/5-FU) to fluorouracil by increasing apoptosis rates and suppressing colony formation at low cytotoxic concentrations [44]. The apoptosis-inducing effects of plant extracts, as observed in various studies, including those on Ashwagandha and *S. prunellae*, are consistent with findings in the current study on jujube's seed alkaloid-rich extract.

5. Conclusion

The study demonstrated that the combination of paclitaxel and alkaloid-rich jujube seed extract exhibits higher cytotoxicity and induces greater apoptosis in paclitaxel-resistant MDA-MB-231 breast cancer cells compared to the individual treatments alone. This synergistic effect was observed in both 2D and 3D cell culture models, and it can be hypothesized that the alkaloids present in the extract might modulate cellular signaling pathways or mechanisms involved in drug resistance, such as drug efflux pumps or the inhibition of apoptosis. The findings suggest that jujube alkaloid-rich extract can potentially enhance the efficacy of paclitaxel chemotherapy, offering a promising approach to overcoming drug resistance in triple-negative breast cancer treatment. Further research is warranted to understand the mechanisms fully, how to deliver this extract to humans and explore this combination therapy's clinical applications.

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Data availability

All the data are available in the main text. All the data generated in this study can be obtained from the corresponding authors upon reasonable request.

Ethics approval

All experimental procedures adhered to the guidelines approved by the Research Ethics Committee of Mazandaran University of Medical

Sciences, Iran (IR.MAZUMS.REC.1401.14319).

CRediT authorship contribution statement

Emran Habibi: Writing – review & editing, Methodology, Funding acquisition, Conceptualization. **Amin Sepehrara:** Investigation, Data curation. **Hesamoddin Arabnozari:** Writing – original draft, Formal analysis. **Fariborz Sharifianjazi:** Data curation, Formal analysis. **Seyed Ehsan Enderami:** Investigation, Conceptualization. **Satyajit D. Sarker:** Writing – review & editing. **Hadi Hassannia:** Investigation, Conceptualization. **Lutfun Nahar:** Writing – review & editing, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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