

The cytotoxic effect of Berberine Chloride and its impact on the expression of regulatory miR-let-7a, a tumor suppressor, in the human ovarian cancer cell line A2780

Mahsa Shahrabi Farahani^a, Parichehr Hanachi^a, Shahradd Mohebbali^b, Roshanak Zarrin Ghalami^a

^a Department of Biotechnology, Faculty of Biological Sciences, Alzahra University, Tehran, Iran.

^b Department of Biology, Faculty of Science, Islamic Azad University Science and Research Branch, Tehran, Iran.

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Abstract

Ovarian cancer poses significant challenges as one of the leading causes of death among women with gynecological malignancies. This study investigates berberine chloride's efficacy in ovarian cancer treatment and its impact on miR-let7a expression. A2780 ovarian cancer cells were treated with various concentrations of berberine chloride, and cell viability was assessed by MTT assay. Additionally, miR-let7a expression levels were quantified by RT-PCR. Berberine chloride exhibited significant lethality against ovarian cancer cells, with the highest efficacy at 200 μmol ($p < 0.001$). Lower concentrations (12.5 μmol and 25 μmol) significantly increased miR-let7a expression ($p < 0.001$). Flow cytometry was employed to identify the optimal dose for further analysis. The findings demonstrate that berberine chloride is a potential therapeutic agent for ovarian cancer, effectively inducing cell death and upregulating miR-let7a expression.

Keywords: Berberine Chloride; Cytotoxic; Ovarian Cancer; MiR-let7-a.

1. Introduction

Ovarian cancer, the seventh most common cancer among women worldwide, is often diagnosed at advanced stages, contributing to high mortality rates. Risk factors include age, reproductive history, lifestyle, and metabolic disorders such as obesity, which is associated with increased incidence and poorer survival outcomes in ovarian cancer. Obesity alters adipose tissue function, leading to dysregulated adipokine levels, chronic inflammation, and altered signaling pathways that promote tumorigenesis [1, 2]. Understanding these

mechanisms is critical for developing effective therapeutic strategies.

MicroRNAs (miRNAs) are small non-coding RNAs that regulate gene expression and are often dysregulated in cancer, impacting hallmarks such as proliferative signaling and metastasis. [3, 4]. In ovarian cancer, specific miRNAs show diagnostic and prognostic potential, surpassing traditional markers like CA-125 [5, 6]. The let-7 miRNA family, particularly let-7a, is a tumor suppressor in various cancers, including ovarian cancer. Reduced let-7a expression is associated with

* Corresponding Author:

Parichehr Hanachi, Department of Biotechnology, Faculty of Biological Sciences, Alzahra University, Tehran, Iran. E-mail: p.hanachi@alzahra.ac.ir.

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aggressive disease and poor prognosis, while its overexpression enhances sensitivity to chemotherapy and radiation [7, 8]. However, in specific contexts, let-7a may promote cancer cell migration and chemoresistance, highlighting its complex role [9, 10].

Current research is exploring the mechanisms behind drug resistance in many types of cancer during chemotherapy. Scientists are investigating the potential of using active compounds derived from plants, either through extraction or synthesis, that can effectively eliminate cancer cells. The aim is to enhance the effectiveness of initial treatment methods [11–13].

Berberine chloride, an isoquinoline alkaloid derived from plants such as barberry and goldenseal, has emerged as a promising anticancer agent [14]. It inhibits cancer cell proliferation in a dose-dependent manner, induces cell cycle arrest at the G2/M phase, and promotes apoptosis by targeting pathways such as NF- κ B, AKT/mTOR, and Wnt/ β -catenin [15, 16]. Berberine also modulates miRNA expression, including let-7a, potentially influencing its tumor-suppressive or oncogenic roles [14, 17]. Its ability to suppress inflammation and induce programmed cell death, including necroptosis, makes it a promising candidate for overcoming chemoresistance in ovarian cancer [18, 19]. In this study, A2780 cells were treated with specific doses of berberine chloride to assess cell viability using MTT and trypan blue assays, and the IC₅₀ was subsequently determined. Additionally, apoptosis, necrosis, and cell cycle factors were assessed using flow cytometry with Annexin-V/FTIC kit at selected doses. Following these treatments, cells were treated at selected doses and subjected to RT-PCR for quantitative gene expression analysis.

2. Materials and Methods

2.1.1. Cell Culture and Chemical Reagents

Human A2780 ovarian cancer cells were obtained from the Gene Research Center (Tehran, Iran) and cultured in RPMI-1640 medium (Bioidea, Iran) supplemented with 10% fetal bovine serum (FBS; Gibco, USA), 1% non-essential amino acids (Gibco, USA), 1% L-glutamine (Bioidea, Iran), and 1% penicillin/streptomycin (P/S; Bioidea, Iran). Cells were maintained at 37°C in a humidified atmosphere with 5% CO₂. Berberine

chloride was purchased from Molekula (UK) and dissolved in DMSO to prepare a stock solution, diluted to working concentrations in culture medium.

2.1.2. Cell Proliferation Assay

2.1.2.1. Trypan Blue Cell Viability Assay

A2780 cells were seeded at 7×10^3 cells/well in 48-well plates and treated in triplicate with seven concentrations of berberine chloride (0, 6.25, 12.5, 25, 50, 100, and 200 μ M) for 24 and 48 hours at 37°C in 5% CO₂. After treatment, cells were detached using 0.25% EDTA/trypsin (Bioidea, Iran) and stained with trypan blue (Merck, Germany). Viable and non-viable cells were counted using a Neubauer chamber under an inverted microscope [9, 20].

2.1.2.2. MTT Cell Viability Assay

A2780 cells were seeded at 7×10^3 cells/well in 96-well plates and treated in quintuplicate with berberine chloride concentrations (0, 6.25, 12.5, 25, 50, 100, and 200 μ M) for 24 and 48 hours at 37°C in 5% CO₂. After treatment, 50 μ L of MTT solution (0.5 mg/mL; Sigma-Aldrich, USA) was added to each well and incubated for 2 hours at 37°C. The formazan crystals were dissolved in 50 μ L DMSO (ACS, USA), and absorbance was measured at 570 nm using an ELISA reader (BioTek Epoch, USA) [21–23].

2.1.2.3. IC₅₀ Calculation

Using dose-response curves, the half-maximal inhibitory concentration (IC₅₀) was calculated from MTT assay data. Cell viability percentages were normalized to the untreated control (0 μ M). IC₅₀ values were determined by analyzing the dose-response relationship across tested concentrations of 0, 6.25, 12.5, 25, 50, 100, and 200 μ M at 24 and 48 hours. The IC₅₀ was estimated using nonlinear regression analysis GraphPad Prism 10.3.1 version, based on the two concentrations where cell viability was closest to 50%, allowing for a precise interpolation between these points. The resulting dose-response curves were plotted to visualize the effect of berberine chloride concentration on cell viability, with IC₅₀ values annotated on the graphs, which are shown in Figure 5 [24].

2.1.3. Cell Cycle and Apoptosis Analysis

The selected concentration of 50 μmol berberine chloride was applied to A2780 cells for 24 hours in a flask after cell seeding. Subsequently, the treated cells and a control sample were sent for flow cytometry testing using an Annexin-V/FITC kit to assess the treatment's effects on the cell cycle, apoptosis, and necrosis. Gating was established using forward scatter (FSC) and side scatter (SSC) to exclude debris and doublets. Annexin-V/FITC and PI fluorescence were analyzed to distinguish early apoptosis (Annexin-V⁺ /PI⁻), late apoptosis (Annexin-V⁺ /PI⁺), and necrosis (Annexin-V⁻ /PI⁺). Cell cycle distribution was assessed by fixing cells in 70% ethanol, staining with PI, and analyzing DNA content. Data were processed using FlowJo software (v10.8) to quantify cell populations in G0/G1, S, and G2/M phases [25].

2.1.4. Gene Expression Analysis

A2780 cells were treated with berberine chloride at 12.5, 25, and 50 μM for 24 and 48 hours. Total RNA was extracted using the Trizol Genex Kit (Iran), and RNA quantity and quality were assessed using a Nano Drop spectrophotometer (Thermo Fisher, USA). cDNA was synthesized from 5 μg RNA using specific stem-loop primers for miR-let-7a and RNU6 (internal control) with a cDNA synthesis kit (Solis BioDyne, Estonia). Quantitative real-time PCR (qRT-PCR) was performed in triplicate using SYBR Green Master Mix (Ampliqon, Denmark) on an ABI StepOnePlus system (Applied Biosystems, USA). The thermal cycling conditions were: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 58–60°C for 1 min. Relative expression of miR-let-

7a was calculated using the $2^{-\Delta\Delta\text{Ct}}$ method, normalized to RNU6 [15, 26, 27]. Primer sequences, product sizes, and annealing temperatures are provided in Table 1.

2.1.5. Statistical Analysis

All obtained results were analyzed using SPSS version 27 software with a significance level set at $p < 0.001$ and $p < 0.05$ compared to the control group. The data were entered into SPSS version 27 for replication, and the mean and standard deviation relative to the control sample were calculated. A one-way analysis of variance (ANOVA) was performed to assess the overall significance of differences between means. A Post-hoc Tukey test investigated significant differences between various group levels. Additionally, the test of means was calculated at a significance level of $p < 0.001$ and $p < 0.05$. For gene expression, fold changes were analyzed using the $2^{-\Delta\Delta\text{Ct}}$ [28].

3. Results and discussion

3.1. Trypan blue cell viability

Berberine chloride induces cytotoxicity by promoting apoptosis in A2780 cells. As shown in Figure 1, different concentrations of 6.25-200 μmol berberine chloride at 24 and 48 hours increased cellular apoptosis compared to the control. However, after 48 hours, due to cellular resistance, the inhibition of cell proliferation decreased compared to 24 hours. However, the concentrations of 200 and 100 were shown to be the most effective in inducing apoptosis in A2780 cancer cells. At a concentration of 25 μmol , there was a rapid and significant rise in cellular apoptosis after 24 hours.

Table 1. Primer Sequences for RT-PCR

Gene	Primer Type	Sequence (5'-3')	Annealing Temp (°C)	Product Size (bp)
RNU6	Stem-loop	GTCGTATCCAGTGCAGGGTCCGAGGTATTTCGC ACTGGATACGACAAATATGG	60	70
RNU6	Forward	GCAAGGATGACACGCAAATTC	60	-
RNU6	Reverse (Universal)	GTGCAGGGTCCGAGGT	60	-
miR-let-7a-2-5p	Stem-loop	GTCGTATCCAGTGCAGGGTCCGAGGTATTTCGC ACTGGATACGACAACTAT	58	65
miR-let-7a-2-5p	Forward	CACGCTTGAGGTAGTAGGTTGT	58	-
miR-let-7a-2-5p	Reverse (Universal)	GTGCAGGGTCCGAGGT	58	-

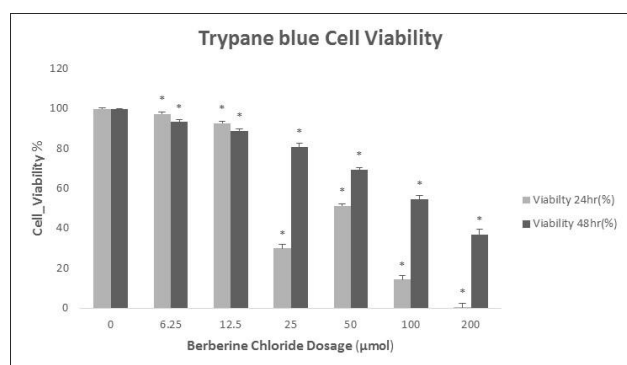


Figure 1. Trypan Blue Viability test. The graph illustrates the percentage of cell viability of A2780 cells using the trypan blue method at 24 and 48 hours, with a significance level of $p < 0.001$ indicating a significant difference compared to the control group.

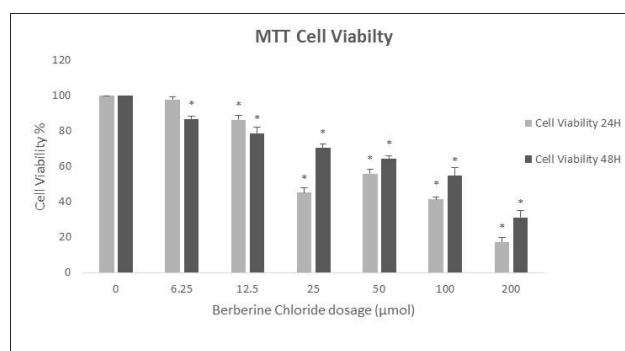


Figure 2. MTT assay Viability test. The graph depicts the percentage of cell viability of A2780 cells using the MTT assay at 24 and 48 hours, with a significance level of $p < 0.001$ indicating a significant difference compared to the control group.

3.2. MTT assay cell viability

Based on the MTT assay, it was found that berberine chloride reduced the viability of A2780 cells after 24 and 48 hours. As demonstrated in [Figure 2](#), the cytotoxic impact was amplified at higher concentrations [29].

Significantly, after 48 hours, there were observable alterations in cell structure and increased resistance, as shown in [Figures 3](#) and [4](#). The results of this test were consistent with the Trypan Blue test. [Figures 3A](#) and [4A](#) show microscopic images of the control samples at 24 and 48 hours post-culture, respectively.

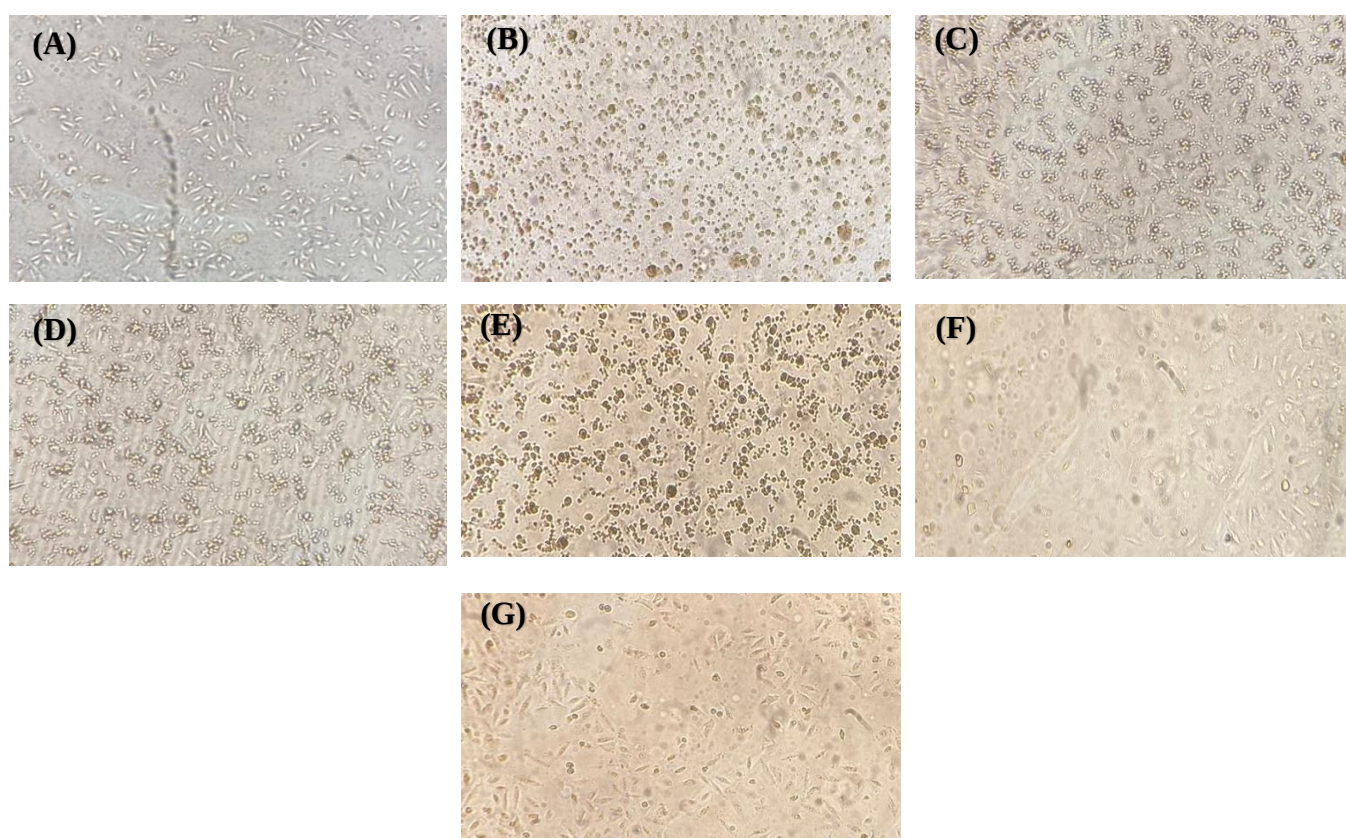


Figure 3. Photos taken from the cell under the CKX31/OLYMPUS inverted microscope after 24 hours of treatment. A: A2780 cell control sample. B: Cells treated with 200 μM concentration of berberine chloride. C: Cells treated with 100 μM. D: Cells treated with 50 μM. E: Cells treated with 25 μM. F: Cells treated with 12.5 μM. G: Cells treated with 6.25 μM. Black arrows show live cells.

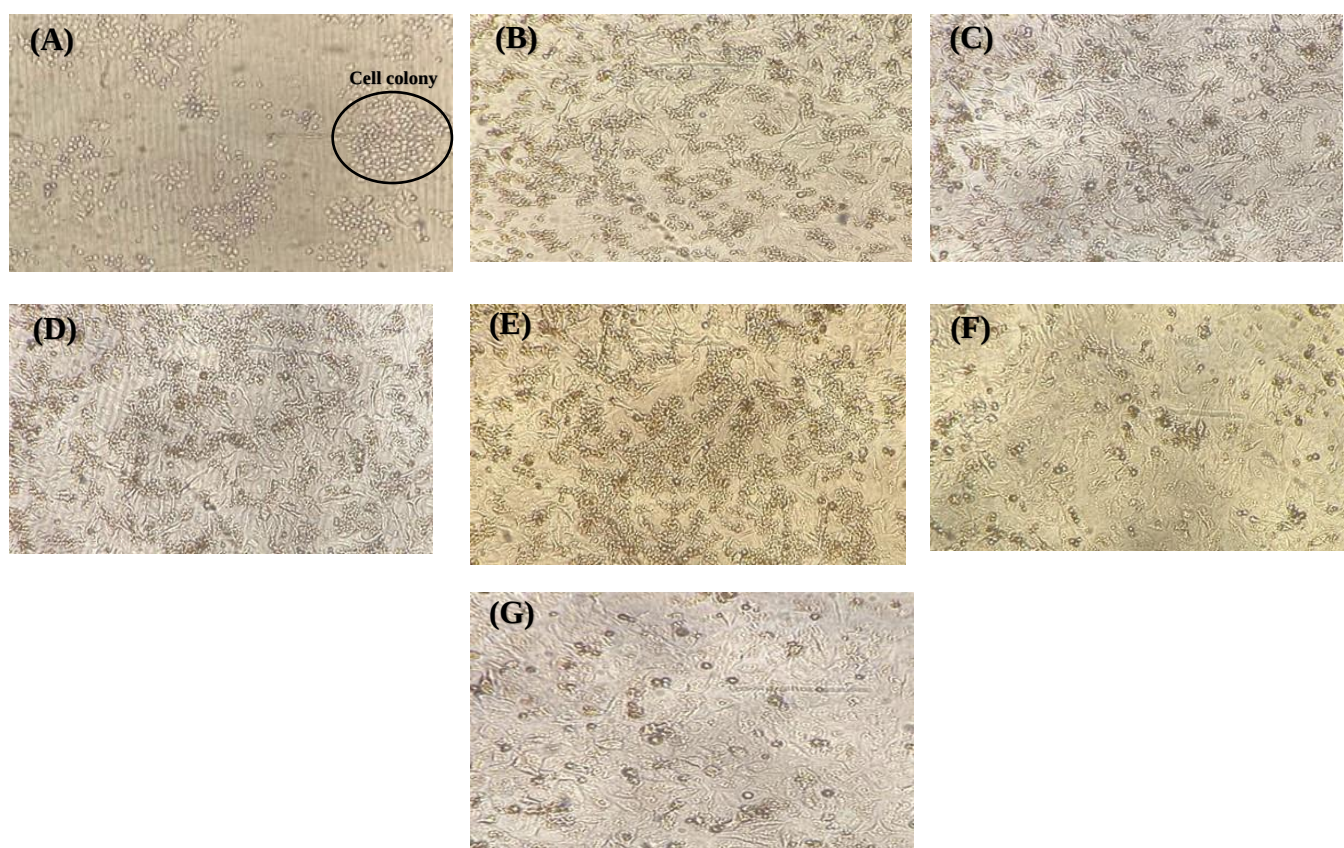


Figure 4. Photos taken from the cell under the CKX31/OLYMPUS inverted microscope after 48 hours of treatment show the morphology changes. A: A2780 cell control sample. B: Cells treated with 200 μM concentration of berberine chloride. C: Cells treated with 100 μM . D: Cells treated with 50 μM . E: Cells treated with 25 μM . F: Cells treated with 12.5 μM . G: Cells treated with 6.25 μM which showed the least morphological change. Black arrows show cell morphology changes.

Figures 3B and **4B** display cells treated with 200 μM berberine, while **Figures 3c** and **4c** show cells exposed to 100 μM berberine. Similarly, **Figures 3d** and **4d** illustrate cells treated with 50 μM berberine. **Figures 3E** and **4E** depict treatment effects with 25 μM berberine, and **Figures 3F** and **4F** show cells exposed to 12.5 μM berberine. Finally, **Figures 3G** and **4G** present cells treated with 6.25 μM berberine.

All images were captured at 24 and 48 hours post-treatment, respectively, providing insights into berberine's time- and concentration-dependent effects on cell morphology. Additionally, the IC_{50} was obtained at a concentration of 50 micromoles after 24 hours, which is shown in **Figure 5**.

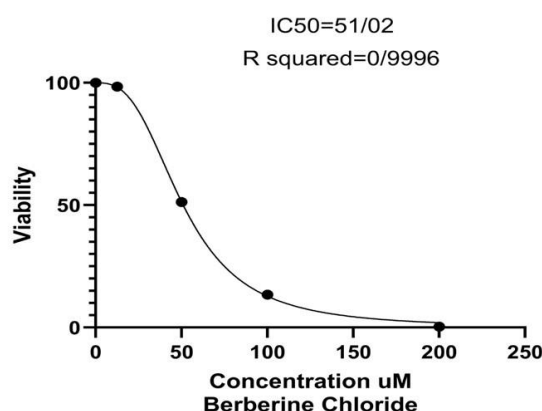


Figure 5. IC_{50} of Berberine chloride. The IC_{50} of berberine chloride on A2780 cells at 50 μmol for 24 hours was determined. This concentration was obtained based on the results of the MTT assay.

3.3. Cell Proliferation and Apoptosis by Flow Cytometry Annexin-V/FITC

Flow cytometry analysis was conducted using the Annexin V-FITC/PI kit to assess apoptosis, necrosis, and cell cycle dynamics in A2780 cells treated with 50 μ M berberine chloride for 24 hours, compared to an untreated control. The Annexin V/PI staining distinguishes cell populations based on membrane integrity and phosphatidylserine exposure: Q1 (Annexin V-/PI+) represents necrotic cells, Q2 (Annexin V+/PI+) indicates late apoptotic cells, Q3 (Annexin V+/PI-) corresponds to early apoptotic cells, and Q4 (Annexin V-/PI-) represents viable cells. In the treated group, 58.8% of cells were in Q1, indicating a high level of necrosis, compared to only 0.263% in the control group. Late apoptosis (Q2) was observed in 0.169% of treated cells, significantly lower than the control (0.431%). Early apoptosis (Q3) increased to 0.205% in treated cells, compared to 0.21% in the control, suggesting a slight induction of early apoptosis. The percentage of viable cells (Q4) decreased markedly to 42.9% in the treated group, compared to 99.1% in the control, indicating substantial cell death ($p < 0.001$) (Figure 6).

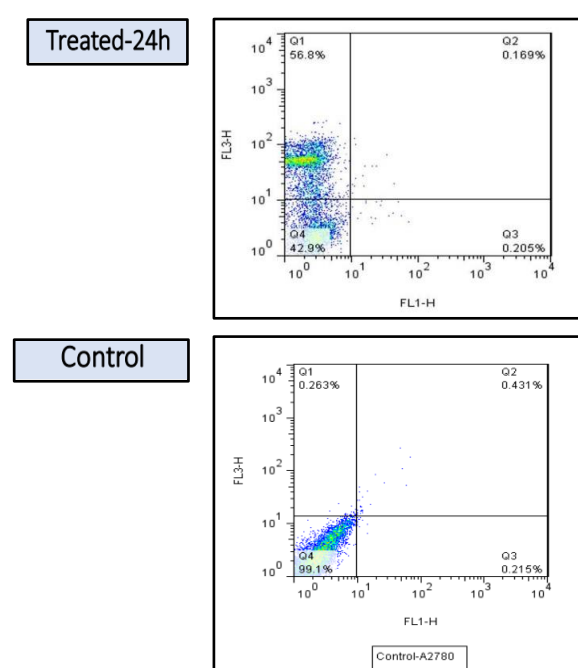


Figure 6. Flow Cytometry Results for Apoptosis and Necrosis. The results of the Annexin V-FITC/PI assay on A2780 cells treated with 50 μ M berberine chloride for 24 hours, compared to the control, show the distribution of cells across quadrants: Q1 (necrosis), Q2 (late apoptosis), Q3 (early apoptosis), and Q4 (viable cells) ($p < 0.001$ compared to control)

Cell cycle analysis further revealed significant alterations in the treated cells compared to the control (Figures 7 and 8 and Table 2). As summarized in Table 2, the berberine chloride-treated group showed an increase in the G0/G1 population and a marked increase in the sub-G1 population compared with the control group. Figure 7 shows the cell cycle distribution of the control A2780 cells at 24 hours, with the x-axis representing DNA content and the y-axis indicating the number of cells. Three distinct peaks were observed, corresponding to the G1, S, and G2/M phases. In the control, the G1, S, and G2/M phases accounted for a typical distribution of a healthy cell cycle. However, in the treated group (Figure 8), a prominent sub-G1 peak was detected, representing 21.06% of cells with reduced DNA content, indicating apoptosis. The S phase showed increased proliferative activity with a fold change of 11.34 compared to the control, suggesting more cells undergoing DNA replication.

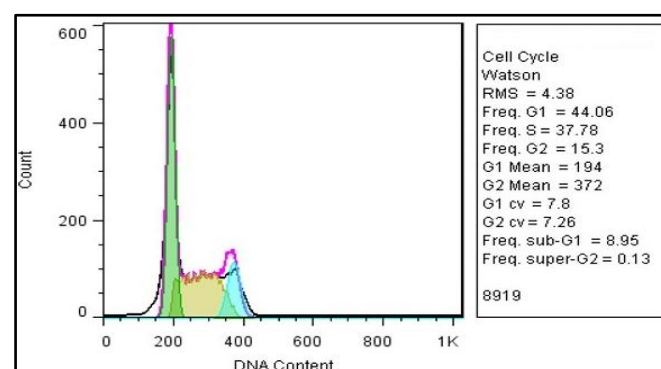


Figure 7. Flow Cytometry Cell Cycle Result (Control). The cell cycle distribution of untreated A2780 cells at 24 hours, with the x-axis indicating DNA content and the y-axis showing the number of cells, displays typical G1, S, and G2/M peaks.

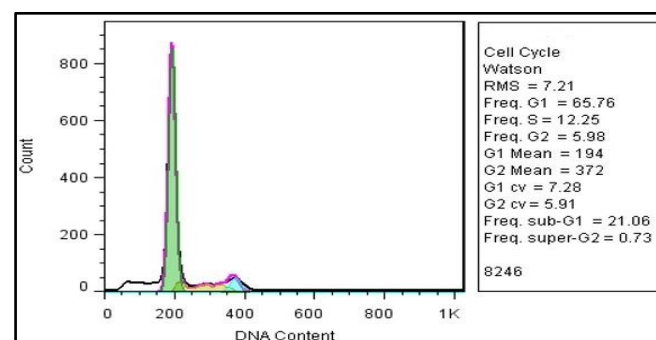


Figure 8. Flow Cytometry Cell Cycle Result (Treated). The cell cycle distribution of A2780 cells treated with 50 μ M berberine chloride for 24 hours shows a prominent sub-G1 peak (21.06%), indicating apoptosis, alongside altered G1, S, and G2/M phases ($p < 0.001$ compared to control).

Table 2. Cell cycle distribution of A2780 cells after 24 h treatment with Berberine chloride.

Group	G0/G1 (%)	S (%)	G2/M (%)	Sub-G1 (%)	Watson RMS
Control	44.06	37.78	15.30	8.95	4.38
Berberine chloride (50 μ M, 24 h)	65.76	12.25	5.98	21.06	7.21

The G1 and G2/M phases exhibited fold changes of 7.8 and 5.91, respectively, compared to the control. Notably, the proportion of cells in the G2 phase decreased to 5.98% in the treated group, compared to 98.5% in a typical healthy cell cycle. This indicates that treatment disrupted cell division, with many cells undergoing apoptosis before reaching mitosis ($p < 0.001$). The presence of the sub-G1 peak further confirms that berberine chloride induces apoptosis in A2780 cells, potentially signifying a therapeutic effect.

3.4. Gene Expression by RT-PCR

The relative expression of the miR-let-7a gene in A2780 cells was analyzed using quantitative real-time PCR (qRT-PCR) following treatment with berberine chloride at concentrations of 12.5, 25, and 50 μ M for 24 and 48 hours, compared to an untreated control (Figure 9). At 24 hours, a reduction in miR-let-7a expression was observed at 25 μ M and 50 μ M compared to the control, potentially reflecting an initial suppression of this microRNA in response to berberine chloride treatment. In contrast, at 48 hours, a significant upregulation of miR-let-7a expression was detected at 25 μ M and 50 μ M, with a p -value < 0.001 , indicating a time-dependent regulatory effect. At the lower concentration of 12.5 μ M, a modest increase in expression was noted at both 24 and 48 hours compared to the control, though this was less pronounced than at higher concentrations at 48 hours. The observed shift from downregulation at 24 hours to upregulation at 48 hours at 25 μ M and 50 μ M may suggest a delayed response of miR-let-7a to berberine chloride, possibly due to a time-dependent induction of cellular stress or activation of regulatory pathways, which warrants further investigation. These findings highlight the concentration- and time-dependent modulation of miR-let-7a expression by berberine chloride in A2780 cells.

This study demonstrates that berberine chloride exerts cytotoxic effects on A2780 ovarian cancer cells in a dose- and time-dependent manner, as evidenced by trypan blue and MTT assays. The IC_{50} of berberine chloride was determined to be 50 μ M at 24 hours, consistent across both assays, indicating its potential as a therapeutic agent for ovarian cancer. This finding aligns with previous studies, such as Liu *et al.* (2019), which reported that berberine induces cell death in ovarian cancer cells (OVCAR3) in a dose-dependent manner [27]. However, our results show a higher IC_{50} compared to Agnarelli *et al.* (2018), who reported reduced viability in MIA PaCa-2 and HDF cells at lower concentrations (2–50 μ M) after 48 hours [20]. This discrepancy may be attributed to differences in cell line sensitivity or experimental conditions, such as treatment duration and cell culture environment.

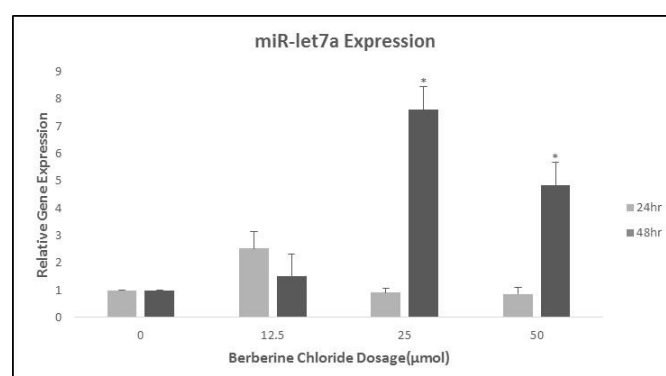


Figure 9. Gene Expression. The graph illustrates significant changes in the relative expression of the miR-let-7a gene in A2780 cells treated with 12.5, 25, and 50 μ M berberine chloride at 24 and 48 hours. Significant increases compared to the control sample were observed at 25 and 50 μ M at 48 hours ($p < 0.001$).

Flow cytometry analysis revealed a significant induction of necrosis in A2780 cells treated with 50 μ M berberine chloride at 24 hours, with 58.8% of cells in the Q1 region (necrotic) compared to 0.263% in the control, alongside a reduction in viable cells (Q4) from 99.1% in the control

to 42.9% in the treated group ($p < 0.001$). Early and late apoptosis (Q3 and Q2) showed minimal changes, suggesting that necrosis is the predominant mode of cell death at this concentration and time point. This observation is consistent with Liu et al. (2019), who reported that berberine induces both necrosis and apoptosis in OVCAR3 cells [27]. The high necrosis rate may reflect a rapid cytotoxic response to berberine chloride, potentially due to severe cellular stress or membrane disruption, which warrants further mechanistic studies.

Cell cycle analysis further supports the cytotoxic effects of berberine chloride, showing a prominent sub-G1 peak (21.06%) in treated A2780 cells, indicative of apoptosis. The S phase exhibited increased proliferative activity (fold change of 11.34 compared to control). In contrast, the G2/M phase was significantly reduced (5.98% compared to 98.5% in a typical healthy cell cycle), suggesting that berberine chloride disrupts cell division, with many cells undergoing apoptosis before reaching mitosis ($p < 0.001$). These findings are in line with Agnarelli et al. (2018), who reported an increase in sub-G1 cells and G2/M arrest in HDF and U343 cells treated with 50 μM berberine for 48 hours [20]. However, Zhang et al. (2020) observed G0/G1 arrest in HCT116 and SW480 cells treated with berberine, highlighting cell line-specific responses to berberine's effects on cell cycle regulation [26].

The expression of the tumor suppressor microRNA miR-let-7a was modulated by berberine chloride in a dose- and time-dependent manner. At 12.5 μM , a modest increase in miR-let-7a expression was observed at 24 and 48 hours compared to the control, suggesting a stimulatory effect at lower concentrations. However, at 25 and 50 μM , miR-let-7a expression decreased at 24 hours but significantly increased at 48 hours ($p < 0.001$), indicating a time-dependent regulatory response. This biphasic effect may reflect an initial stress response at higher concentrations, followed by a compensatory upregulation of miR-let-7a, which inhibits cancer cell proliferation and promotes apoptosis. Li et al. (2021) similarly reported that berberine upregulates miR-145, another tumor suppressor miRNA, in cancer cells [30]. The delayed upregulation of miR-let-7a at 48 hours in A2780 cells suggests that berberine chloride may

enhance tumor-suppressive pathways over time, potentially contributing to its anti-cancer effects.

Microscopic analysis confirmed the cytotoxic effects, with A2780 cells treated at 50 μM showing moderate apoptotic changes after 48 hours, while higher concentrations (200 μM) induced severe necrosis, supporting the flow cytometry findings. These observations align with the dose-dependent reduction in cell viability observed in trypan blue and MTT assays, reinforcing the therapeutic potential of berberine chloride in targeting ovarian cancer cells.

In summary, this study highlights the dose- and time-dependent cytotoxic effects of berberine chloride on A2780 cells, with an IC_{50} of 50 μM at 24 hours, predominantly inducing necrosis alongside apoptosis, as evidenced by flow cytometry and cell cycle analysis. The modulation of miR-let-7a expression further suggests a role for berberine chloride in regulating tumor-suppressive pathways. These findings provide a foundation for further mechanistic studies to explore berberine chloride's potential as a therapeutic agent in ovarian cancer treatment, particularly its effects on necrosis and miRNA regulation.

4. Conclusion

In conclusion, based on the results obtained from this study, berberine chloride acts as an effective alkaloid in regulating the positive expression of the tumour suppressor gene miR-let-7a in the A2780 human ovarian cancer cell line. Treatment at 24 hours likely enhanced cell lethality, potentially through mechanisms involving cell necroptosis, but further studies are required to confirm these effects. Extending treatment to 48 hours was associated with changes in cell morphology and increased cellular resistance to berberine chloride, indicating that treatment duration may affect response and could be considered in future experimental designs. The increased miR-let-7a expression during treatment resistance warrants further investigation into its potential role in cellular processes such as drug resistance or metastasis. These findings contribute to our understanding of berberine chloride's effects on cellular signalling in ovarian cancer cells and highlight areas for additional research to explore its mechanisms and potential applications.

Conflict of interest

The authors declare that they have no conflict of interest.

Data availability

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

Authors Contributions

All authors contributed to the study conception and design, data acquisition, analysis and interpretation of the results, and preparation and revision of the manuscript. All authors read and approved the final version of the manuscript.

Authors Orcid numbers:

Mahsa Shahrabi Farahani: 0009-0009-7818-3218

Parichehr Hanachi: 0000-0002-6450-203X

Shahrdad Mohebbali: 0000-0001-7240-9436

Roshanak Zarrin Ghalami: 0000-0003-0104-2395

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Using artificial intelligence chatbots

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