

The cytotoxic activity of *Ziziphus Jujube* on cervical cancer cells: *In Vitro* studyR. Hoshyar^{1,2}, S. Jamali¹, M. Fereidouni³ and M. R. Abedini^{1,4}*¹ Cellular & Molecular Research Center, Birjand University of Medical Sciences, Birjand, Iran² Department of Biochemistry, Faculty of Medicine, Birjand University of Medical Sciences, Birjand, Iran³ Department of Immunology, Faculty of Medicine, Birjand University of Medical Sciences, Birjand, Iran⁴ Department of Pharmacology, Faculty of Medicine, Birjand University of Medical Sciences, Birjand, Iran**Corresponding author:** Dr. M. R. Abedini, Department of Pharmacology, Faculty of Medicine, Birjand University of Medical Sciences, Birjand, Iran, P.O. Box: 9717853577. Email: mohrabe@yahoo.com, mohrabe@bums.ac.ir**Abstract**

Recently, there are tendency to use natural products such as *Ziziphus Jujube* (*Jujube*) as therapeutic agents for cancer. Understanding the molecular mechanisms of anti-cancer effects of *Jujube* may improve the current therapeutic strategies against cervical cancer. Our MTT data showed a significant dose- and time-dependent inhibition of OV-2008 cell proliferation following *Jujube* administration. Moreover, qRT-PCR analyses significantly revealed the suppression of cyclin D1 and the enhancement of P53, P21 and P27 expression in treated cells. These results suggest that the herb exerts a cytotoxic effect on cervical cancer cells through alternation of the expression of the genes that are involved in regulation of cell cycle.

Key words: Cervical cancer, Cyclin D1, P21; P27, P53, *Ziziphus Jujube*.**Introduction**

Cervical cancer is one of the most common cancers among women worldwide (1). Recently, due to anxiety concerning the side effects of chemical drugs, there has been a trend toward using medicinal plants in the preatment of cancer (2-4). *Jujube* is a herbal product widely used as a remedy for different disorders (5). The fruit has been used as an antioxidant and anticancer agent including proliferation inhibition and apoptosis induction in cancer cells (6-8). There is a growing body of evidence that cancer occurs as a result of cell cycle abnormality. Several key regulators including cyclin D1, P27, P21 and P53 modulate cell cycle directly and enable cells to move through their normal cycle (9). Although *Jujube* inhibited the growth of cancer cells, the molecular pathways involved in anti-proliferative activity are not clear. In the current study, we evaluated the cytotoxic effects of an aqueous extract from Iranian *Jujube* on the expression of several genes involved in cell cycles, including cyclin D1, P27, P21 and P53 on cervical cancer cells.

Materials and Methods***Jujube* extract preparation**

The preparation of *Jujube* extraction performed according to our previous study (8).

Cell culture and Cell viability assay

OV2008 cell line (cervical cancer cells) was kindly provided by Doctor Benjamin K. Tsang's laboratory (Department of Obstetrics, Gynecology and Cellular and Molecular Medicine, University of Ottawa, Canada). The cells were cultured in RPMI-1640 which supplemented with 10 % FBS serum, 100 units/mL penicillin and 100 mg/mL streptomycin. The cell line was grown

at 37 °C in humidified atmosphere containing 5 % CO₂ (10,11). The cells were seeded in 96-well plates (5 x 10³ cells/ well) and allowed to attach overnight. Then the cells were treated with different concentrations of *Jujube* (0–2 mg/ml) at various time intervals (0–72 h). The in vitro cytotoxic effect of *Jujube* extract was evaluated by MTT assay. For analysis of the cytotoxic efficiency, the IC₅₀ value of *Jujube* was calculated using the dose- and time-dependent curves by linear interpolation (12).

RNA extraction and qRT-PCR endpoint analysis

Total RNA was extracted by using RNeasy Mini Kit (Qiagene-USA) and cDNA synthesized using Thermo-scientific Kit (Thermo Scientific, USA). qRT-PCR for genes was carried out by designed primers. P21: 5'-GGCAGACCAGCATGACAGATT-3' (forward) and 5'-GCGGATTAGGGCTTCCTCTT-3' (reverse), P53: 5'-ACCACCATCCACTACAATA-3' (forward) And 5'-ACAAACACGCACCTCAAAGC-3' (reverse), P27: 5'-AAGAAGCCTGGCCTCAGAAG-3' (forward) and 5'-TCCATTCCATGAAGTCAGCGAT-3' (reverse), cyclinD1: 5'-CGGTGTCCTACTTCAAATGTGTG-3' (forward) and 5'-AAGACCTCCTCCTCGCACT-3' (reverse), β-actin: 5'-TGGCACCCAGCACAATGAA-3' (forward) 5'-CTAAGTCATAGTCCGCCTAG-3' (reverse). Gene amplification was performed in the ABI Step One™ Real-Time PCR System (Applied Biosystems, Foster City, CA) with 40 cycles of denaturation at 95°C for 30s, annealing and extension at 60°C for 30s and data collection 80°C for 20s. β-actin gene was used to normalize the relative expression for interested genes calculated by 2^{ΔΔCT} method and SYBR Green kit according to our previous reports (13).

Statistical analysis

Results are expressed as the means ± SEM of at least three independent experiments (n=3). Data were

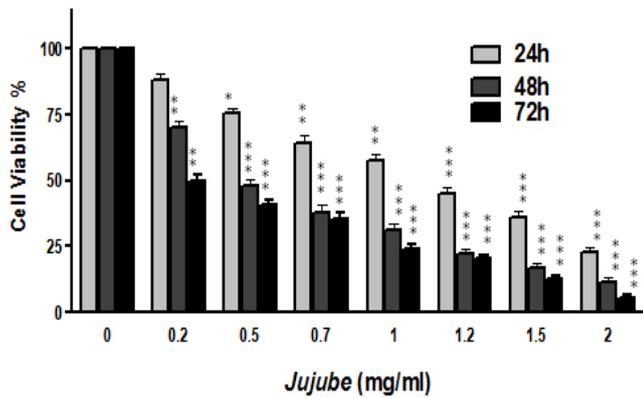


Figure 1. The OV-2008 cells were treated with different concentrations of *Jujube* for 0-72 h. Data are expressed as mean \pm SEM (n = 3). Values are statistically significant at * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$ vs. respective control group (One-way ANOVA followed by Tukey's post hoc test).

analyzed using one-way ANOVA and with Tukey's post hoc test to assess differences between experimental groups. Statistical significances were inferred at $P \leq 0.05$, (PRISM 5.0; Graph- Pad Software Inc.).

Results

As shown in Figure 1, *Jujube* induced a significant decrease in cancer cell proliferation in a dose- and time-dependent manner. Analyses of the cell survival showed that the IC₅₀ values of *Jujube* on the OV2008 cells at 24, 48, and 72 h treatments were 1.2 ± 0.03 , 0.5 ± 0.05 and 0.2 ± 0.02 mg/ml, respectively.

After 1.2 mg/ml (IC₅₀ at 24h) treatment with *Jujube* (0- 24h incubation), cells were collected, total RNA from each sample were isolated and then real-time PCR was used for the gene expression. This medicinal herb dramatically decreased the mRNA level of cyclin D1 in a time-dependent manner (Figure 2A). As shown in Figure 2B, P53, P27 and P21 mRNA levels were sharply elevated at 6, 12, and 24h incubation times when compared with the control group.

Discussion

Medicinal plants constitute an adjunct therapeutic strategy for cancer in many countries (14). *Jujube* exerts an inhibitory effect on the proliferation of various cancers (15). We have already shown that treatment of a tumour-bearing rat with *Jujube* extract significantly increased the serum antioxidant capacity and reduced anaemia, neutrophilia and early-stage leukaemia in cancerous rats (8). *Jujube* (1.2 mg/ml; 0-72 h) caused morphological changes in treated OV2008 cells including cell shrinkage, blebbing, piknotic nuclei, condensation and deformation of the cell membrane in a time dependent manner. In addition, the extract induced a strong decrease in cancer cell proliferation in a dose- and time-dependent manner (Figure 1). It has also been shown that treatment with *Jujube* fruits facilitated cell death among a variety of cancer cells (7, 15). More than 20 types of flavonoids as antioxidants have been isolated from the jujube fruit (16). As tumour progression is closely related to oxidative stress, compounds that have antioxidant properties can act as anti-carcinogens.

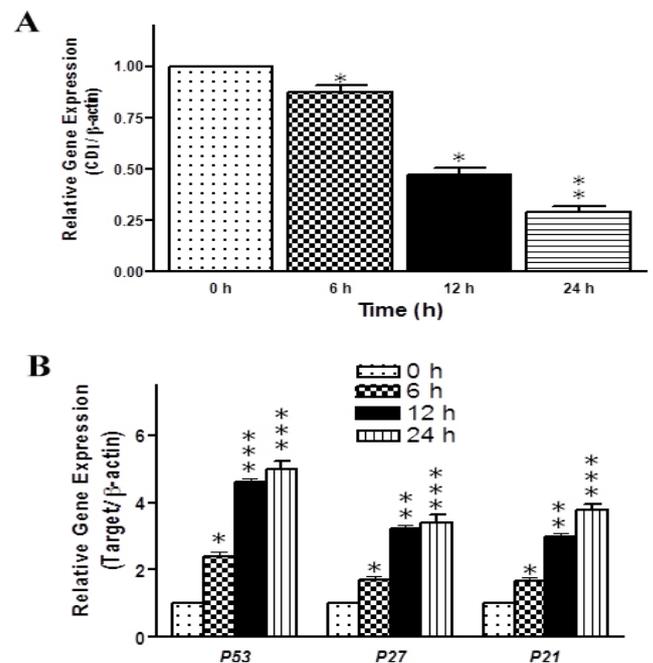


Figure 2. OV2008 cells treated with 1.2 mg/ml *Jujube* for 0-24h. (A) *Jujube* decreased the gene expression of cyclin D1 (B) *Jujube* increased the gene expression of P53, P21, P27 in cells. Data represents relative gene expression (Target/ β -actin) mean \pm SEM of three experiments (n=3). Values are statistically significant at at * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$ vs. respective control group (One-way ANOVA followed by Tukey's post hoc test).

Cell cycle dysregulation resulting in uncontrolled cell proliferations is one of the most frequent alterations occurs during tumor development. In this study we investigated alteration of cell cycle regulating genes expression by *Jujube* in OV2008 cells. P53 is one of the most important tumour suppressor genes and is responsible for protecting cells from tumorigenic alterations, and modulates various cellular functions such as apoptosis and cell cycle arrest. Numerous studies indicated that low levels of P53, P27 and P21 expressions as tumor suppressors and high level of cyclin D1 expression as an oncogene correlated with carcinogenesis in different cancer cell lines such as OV2008 (17,18). Our in vitro findings like previous investigations demonstrated that P53 expression remained significantly unregulated during cell cycle arrest induced by herbal medicines and was followed by induction of P21 and P27 (19).

In conclusion, *Jujube* significantly decreased the proliferation of OV2008 cells via up-regulating the expression of P53, P21 and P27, as well as down-regulating cyclin D1 expression. Therefore, the therapeutic potential of this herb in the treatment of cervical cancer is worth pursuing in future studies.

Acknowledgments

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