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Antitumor Activity of *Ziziphus Jujube* Fruit Extracts in KG-1 and NALM-6 Acute Leukemia Cell Lines

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ABSTRACT

Background: *Ziziphus jujube Mill*. belongs to the *Rhamnaceae* family. It has been reported to have a variety of biological activities such as antitumor, antioxidant, and anti-inflammatory effects. This study investigates the antiproliferative effect of *Ziziphus jujube* on KG-1 and NALM-6 acute leukemia cell lines.

Materials and Methods: In this experimental study, the aqueous, ethyl acetate, and hydroalcoholic extracts of the *Ziziphus jujube* were prepared. Total phenolic and flavonoid components were detected because the presence of these compounds is associated with antioxidant and anticancer effects. Different concentrations of extracts were prepared, and KG-1 and NALM-6 cell lines were treated with them at 12, 24, 36, and 48 hours. Cell viability and IC_{50} values of the extracts were calculated using MTT assays. BD Cycle TEST PLUS DNA Kit was used for cell cycle progression analysis. Bcl_2, Bax, and caspase-3 mRNA expressions were also assessed.

Results: Cell viability decreased in a concentration-dependent manner. The best efficacy belonged to the ethyl acetate extract. Investigation of cell cycle progression demonstrated that the number of G0/G1 cells enhanced and the number of G2/M cells decreased when the ethyl acetate extract was applied in its IC_{50} concentration. A considerable increase in *Caspase-3* and *Bax* and a decrease in *Bcl2* gene expression were detected in molecular examination.

Conclusion: According to our research, *Ziziphus jujube* ethyl acetate extract has antitumor properties on KG-1 and NALM-6 cell lines, possibly through induction of apoptosis and cell cycle regulation.

Keywords: Ziziphus jujube; Acute leukemia; KG-1; NALM-6; Cell cycle; Caspase-3; Proto-oncogene proteins; c-bcl-2; Bax

INTRODUCTION

Acute leukemia is a large group of leukemia. Based on the cellular manifestations of primary stem cell defects on the maturation and differentiation of common myeloid precursors or common lymphoid precursors, they are divided into two categories: acute myeloid leukemia (AML) and acute lymphoid leukemia (ALL)[1]. ALL is a hematologic disorder associated with the malignant proliferation of cancer cells and the accumulation of immature and malfunctioning hematopoietic cells in the bone marrow. This disorder is due to somatic acquired genetic mutation in lymphoid precursor in one of the maturation stages, leading to the formation of malignant clones. These cells exhibit a high capacity for self-renewal and resistance to apoptosis due to the cessation of differentiation that prevents them from developing into adult cells[1]. This neoplasm is not common in adults and is more common in men than women. In children, it is responsible for 30% of all cancers and 80% of all leukemia. Therefore, it is more common in children, with a peak prevalence of

2 to 5 years. Survival rates are higher in children than in adults[2].

The essential characteristics of AML include the ability for continuous proliferation and maturation arrest. High cell proliferation can be the result of mutations in growth factors, growth factor receptors, messenger pathway components, and transcription factors that affect the genes involved in cell survival and proliferation. More than half of AML cases have cytogenetic abnormalities, most of which are balanced bilateral chromosomal translocations at the locus of transcription factor genes[1]. AML is the most common cause of acute leukemia during the first few months of life. However, it is responsible for only about one-third of acute leukemia cases in childhood and adolescence (15-20% of acute leukemia)[3,4.] Treatment for this type of leukemia usually involves chemotherapy drugs and monoclonal antibodies. One type of these drugs is purine analogs such as fludarabine, which inhibits DNA synthesis, repairs, and activates the apoptotic pathway. At present, the optional treatment of leukemia is bone marrow transplantation[5]. The utilization of medicinal plants to treat various diseases, particularly cancer, has witnessed a rise in recent years. This trend is driven by the cost-effectiveness and improved accessibility of medicinal plants and the desire to mitigate the adverse effects of chemotherapy drugs[6,7].

Ziziphus jujube Mill. (Z. *jujube)* is a medicinal plant with many uses. It belongs to the *Rhamnaceae* family and contains various chemical constituents, including triterpenic acids, flavonoids, saponins, and alkaloids, among others. It is reported to have a variety of biological activities such as antitumor, antioxidant, and anti-inflammatory effects. Many studies have shown that *Z. jujube* exerts anticancer activities on several tumor cell lines[8-19]. *Z. jujube* extract inhibits the growth of HeLa cervical cancer cells and decreases the growth of A549 lung cancer cells (Suk-Hyun Choi et al., 2012)[9]. Chloroform fraction from *Z. jujube* has anticancer activities in human liver cancer cells (X Huang et al., 2009)[10]. This extract reduces the viability of Hela and MAD-MB-468 cells significantly and concentration-dependently (Abbas Jafarian et al., 2014)[11]. *Z. jujube* delays colon cancer progression (Srinivasan Periasamy et al., 2015)[12]. *Z. jujube* water extract induces apoptosis in HEp-2, HeLa, and Jurkat cell lines (Fatemeh Vahedi et al., 2008)[13]. It has a cytotoxic effect on the HEp-2 cell line[14-16]. *Z. jujube* fruit extracts exert antiproliferative and apoptotic effects in human breast cancer cells[17]. It can be useful in colorectal cancer treatment (Xiaolong Ji et al., 2020)[18] and induce apoptosis cell death in human cancer cells through mitochondrial reactive oxygen species production[19].

The present study aimed to evaluate the antiproliferative effects of *Z. jujube* on KG-1 and NALM-6 cell lines since no article was found to have investigated the effect of *Z. jujube* on these cell lines. We also aimed to study the cell cycle progression and caspase-3, Bcl_2, and Bax gene expression under the influence of Z. jujube. If this substance demonstrates positive outcomes in animal studies and clinical trials, it may serve as a supplementary treatment to chemotherapy drugs.

MATERIALS AND METHODS

Cell culture

NALM-6 and KG-1 acute leukemia cell lines (ALL and AML, respectively), which were provided by (the Pastor Institute of Iran,) were grown and subcultured in RPMI1640 containing 20 mM HEPES-buffer and 1% GlutaMAX (Biosera, France) supplemented with 10% heat-inactivated FBS (fetal bovine serum) (Gibco, USA) and 100 µg/ml penicillin/streptomycin (Biosera). The cultures were incubated at 37°C with 5% CO_2 and 95% humidity. The medium was changed every 2-3 days.

Preparation of extracts

At first, the dried jujube kernels were separated, and the remainder of the fruit was pulverized to obtain a homogeneous powder. In order to prepare an aqueous extract, 50 grams of the powder was weighed and macerated in boiling distilled water for 30 minutes. The mixture was filtered, and then the samples were lyophilized. The dried aqueous extracts were subsequently diluted in RPMI medium and prepared at different concentrations[22]. One hundred grams of dried powder was extracted by 300 ml of ethyl acetate using the maceration method. The residue was subjected to more

extraction using 300 ml of 60% methanol to prepare hydroalcoholic extract[11]. The extracts from each step were filtered and transferred to a rotary balloon (Heidolph, Germany) and concentrated at 100 rpm at 40°C.

Total phenol assay

Three test tubes were prepared for each extract; 1 ml of the extract with a concentration of 1 mg/ml was added to each tube, followed by 1.5 ml of folin-ciocalteu's phenol reagent (1%). After 10 minutes, 1.5 ml of sodium bicarbonate (7%) was added to each tube, and the tubes were kept in the dark for 30 minutes. Moreover, a blank tube was prepared containing 1 ml of methanol, 1.5 ml folin-ciocalteu's phenol reagent, and 1.5 ml of sodium bicarbonate. After 30 minutes, the samples were transferred to special cuvettes and read at 765 nm with a spectrophotometer. Gallic acid solutions with concentrations of 6.25, 12.5, 25, 50, 100, and 200 µg / ml were prepared in methanol solvent to draw the gallic acid standard curve. 1.5 ml of the folin-ciocalteu's phenol reagent solution was added in two stages to 200 µl of the gallic acid solutions and the sample separately. After 5 minutes, 1.5 ml of sodium bicarbonate solution was added to the above mixtures. The absorbance of the samples was read after 2 hours by a spectrophotometer at 760 nm. The adsorption diagram was plotted against the gallic acid concentration, and the line equation was obtained. Once the gallic acid calibration curve was plotted, the amount of total phenol in the extract was calculated by placing the extract's adsorption value in the linear equation of the standard curve[23].

Total flavonoid assay

This is a colorimetric method using aluminum chloride. Three test tubes were prepared for each extract. First, 0.5 ml of the extract and 150 µl of sodium nitrite were added to each tube. After 6 minutes, 150 µl of aluminum chloride was added to all tubes. After six minutes, 2 ml of sodium bicarbonate was added, and the total volume of the test tube was increased to 5 ml. Lastly, distilled water was used to increase the volume. Simultaneously with the preparation of these three tubes, a blank tube was also prepared, which contained 0.5 ml of methanol, 150 µl of sodium nitrite, and 2 ml of sodium bicarbonate. The blank was prepared following all necessary time steps. The absorbance of the samples was recorded at 510 nm using a spectrophotometer.

Quercetin was used to draw the standard curve, and the results were expressed in mg of quercetin equivalent per gram of extract. At first, standard solutions with a concentration of 0-500 µg/ml quercetin in absolute methanol were prepared. It was subsequently added to 0.2 ml of plant extract or 0.2 ml of aluminum chloride solution plus 0.1 ml of 33% aqueous acetic acid and mixed well. Finally, the mixture volume was increased to 5 ml with 90% ethanol, and the tubes were kept at room temperature for 30 minutes. Their optical absorption at 510 nm was read, and the total flavonoids were obtained using a standard curve[23].

MTT assay

In order to check cell viability, the 3-(4, 5-dimethylthiazol-2-yl)-2, 5 di-phenyl tetrazolium bromide (MTT) assay was used. The cells were seeded at a concentration of 1×10^6 cells/well in a 96-well plate. Both cell lines were treated with aqueous, methanol, and ethyl acetate extracts of the *Z. jujube* at 0.125, 0.25, 0.5, and 1 mg/ml concentrations for 12, 24, 36, and 48 h. Following 4h incubation with 50µl MTT (Sigma, USA), the supernatant was removed, and 100µl DMSO (Sigma-Aldrich, USA, Biologic Grade) was added to dissolve MTT. After 15 minutes of incubation, the OD was read with an ELISA plate reader (BioTek ELx808, USA) at 492 nm. The assay was performed at least three times. The viability percentages of each cell line and IC_{50}s of all extracts were calculated using the Excel 2013 software. The optimum time and the best IC_{50}s were considered for further analysis.

Cell cycle analysis

For cell cycle analysis, tumor cell populations were exposed to the extract and subsequently stained with propidium iodide using BD Cycle TEST PLUS DNA Kit (BD Biosciences); flow cytometry was then performed with a BD FACS Calibur Flow Cytometry Machine (BD Biosciences, USA). Data were collected

using BD Cell FIT software, and cell cycle progression was analyzed using the ModFit software (BD Biosciences). The number of cells gated in each phase was reported as %.

RT-PCR

RT-PCR was performed to identify *Bcl2*, *Bax*, and *caspase-3* gene expressions. For this purpose, the cells were collected after 48 hours of treatment with the extract that had the best IC50 value on the MTT test, and their total RNA was extracted using the Trizol (Qiagen, Germany) method. Then, according to the manufacturer's instructions, 1µg total RNA was converted to cDNA using the PrimeScript 1st strand cDNA Synthesis kit (Takara, Japan). RQ-PCR assay was performed to investigate the level of *Bcl2*, *Bax*, and *caspase-3* gene expression in drug-treated and control groups using primers in Table 1. β-2 microglobulin (B2M) was used as a housekeeping gene for normalization of RT-qPCR data. It should be noted that all tests were performed in triplicate. The fold change *Bcl2*, *Bax*, and *Casp3* mRNA in treated cells in comparison with untreated cells was computed by the $2^{-\Delta\Delta CT}$ method.

Table 1: Bcl2, Bax, caspase-3, and β-2 microglobulin primers

Gene	Primer(5′-3′)	PCR product size (bp)
Casp3 (Forward)	TCTGGTTTTCGGTGGGTGTG	137
Casp3 (Reverse)	CGCTTCCATGTATGATCTTTGGTTC	
B2M (Forward)	CTCCGTGGCCTTAGCTGTG	69
B2M (Reverse)	TTTGGAGTACGCTGGATAGCCT	
Bcl_2 (Forward)	CTGCACCTGACGCCCTTCACC	119
Bcl_2 (Reverse)	CACATGACCCCACCGAACTCAAAGA	
Bax (Forward)	GTGCACCAAGGTGCCGGAAC	205
Bax (Reverse)	TCAGCCCATCTTCTTCCAGA	

Statistical analysis

All experiments were performed in duplicate and repeated three times. The data were expressed as mean±SD for all experiments. IC_{50} value was calculated using the Excel 2013 software, and cell cycle analysis was done using the BD Cell FIT software. GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA) was used to detect the significant differences between the control and treated groups. Statistical significance levels were defined at **P*<0.05, ***P*<0.01, and ****P*<0.001 compared to the corresponding controls.

RESULTS

Calculation of different IC_{50} extracts and cell viability using the MTT assay

Based on the cell treatments with the *Z. jujube*'s aqueous, hydroalcoholic, and ethyl acetate extracts at 0.125, 0.25, 0.5, 1 mg/ml concentrations for 12, 24, 36, and 48h, the best IC_{50}s belonged to the ethyl acetate extract of the *Z. jujube* with 48 h treatment in both cell lines.

IC_{50} values of the aqueous extract were 0.582±1.76 and 8.719±2.87 mg/ml; IC_{50} values of the hydroalcoholic extract were 0.446±2.36 and 5.337±1.43 mg/ml; and those of the ethyl acetate extract were 0.242±3.12 and 0.665±2.57 mg/ml at 48 h for KG1 and Nalm6 cell lines, respectively (Figure 1). It was shown that cell proliferation was inhibited in a concentration-dependent manner with all extracts (Figure 2).

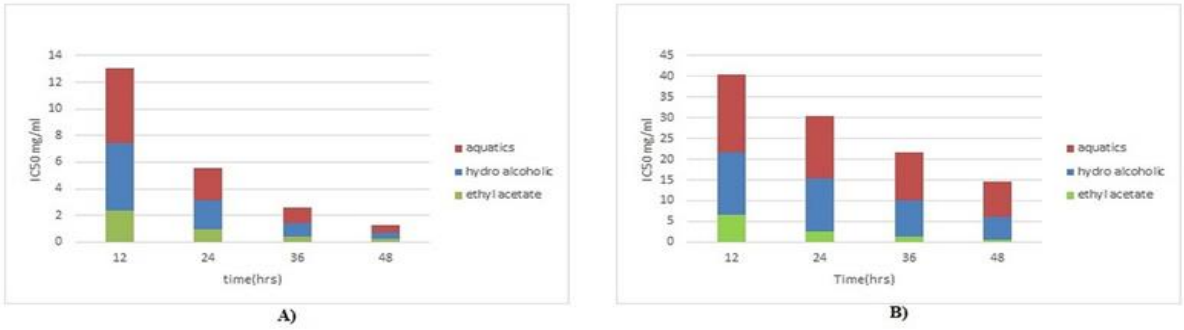

Figure 1. Aqueous, hydroalcoholic, and ethyl acetate extract IC_{50} values for A) KG-1 and B) NALM-6 cell lines at 12, 24, 36, and 48 h, showing that the best IC_{50} belonged to the ethyl acetate extract of the *Z. jujube* semen in all three-cell lines with 48 h treatment.

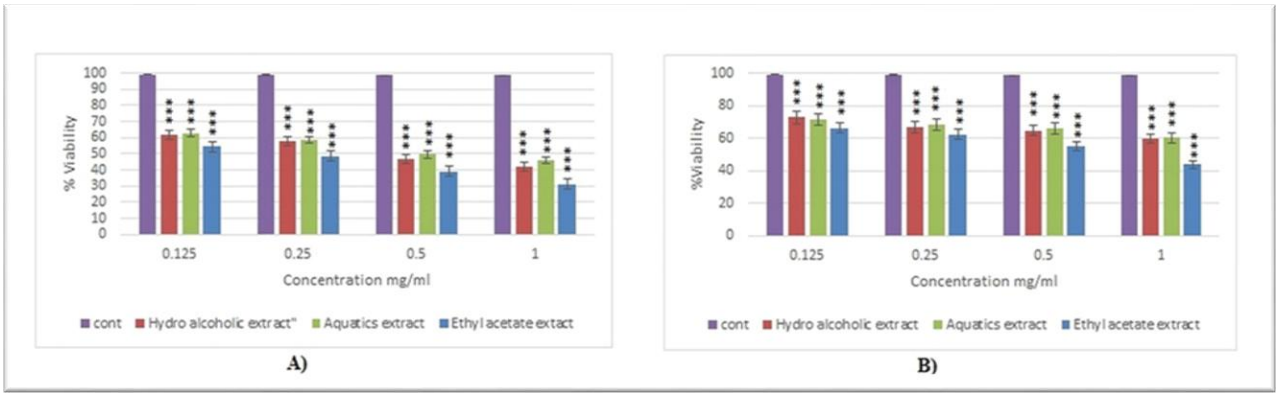

Figure 2. Effects of *Z. jujube*'s aqueous, methanol, and ethyl acetate extracts on the viability of KG-1 and NALM-6 cells treated with different concentrations of extracts at 48 h (the optimum time). Following the addition of 50 µl of MTT-labeling reagent, the cells were incubated for 4 hours prior to the addition of 100 µl of DMSO solution. The experiments were repeated at least three times. The data represent the mean (± standard deviation, SD) of the three independent experiments, each performed in triplicate and presented relative to the controls: (A) KG-1 and (B) NALM-6. *** represents $P<0.001$ with respect to the controls.

Cell cycle progression

Z. jujube modulated cell cycle progression dependent on the tumor cell line. Applying ethyl acetate extract to KG-1 increased the number of G0/G1 (P=0.0253) cells and reduced the number of G2/M cells (P=0.0171). In NALM-6 cells, the number of G0/G1-phase cells increased (P=0.0228), but the number of cells in the G2/M-phase decreased (P=0.0437) compared to the controls (Figure 3).

Effect of the ethyl acetate extract of *Z. jujube* on Bcl_2, Bax, and caspase3 gene expression

Molecular examination 48 hours after the treatment of KG-1 and NALM-6 cell lines revealed a significant increase in Bax and caspase-3 mRNA expression and a noticeable reduction in Bcl2 mRNA in test samples compared to the controls (Figures 4-6).

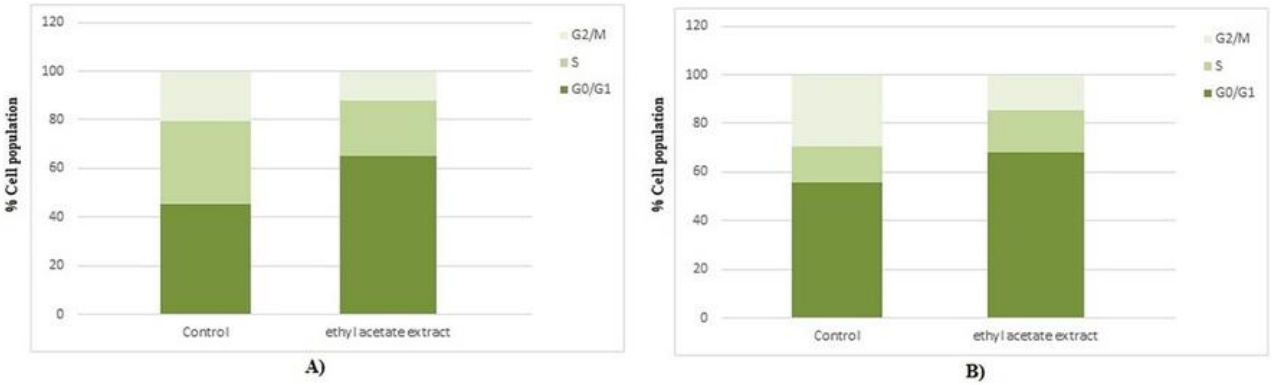

Figure 3. Cell cycle analysis of A) KG-1 and B) NALM-6 cultures pretreated with *Z. jujube* at 48 h (Controls remained untreated). The cell population is expressed as a percentage of the total cells analyzed. One representative experiment of the three is shown.

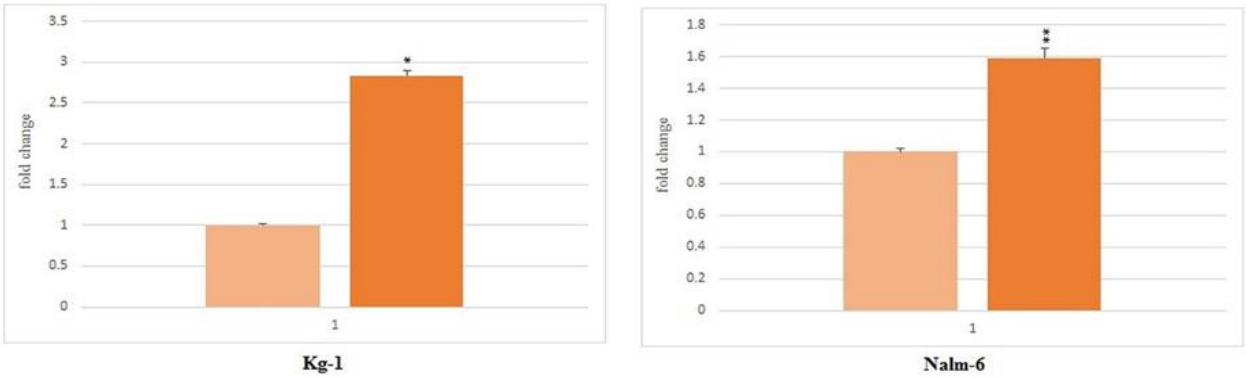

Figure 4. RT-PCR analysis results of the mRNA levels of Bax. The following formula was used to calculate the fold change. Fold difference = $2^{-\Delta\Delta Ct}$. Results showed a significant increase in Bax mRNA expression. *P<0.05* in KG-1 and *P<0.01* in NALM-6 cell lines, respectively.

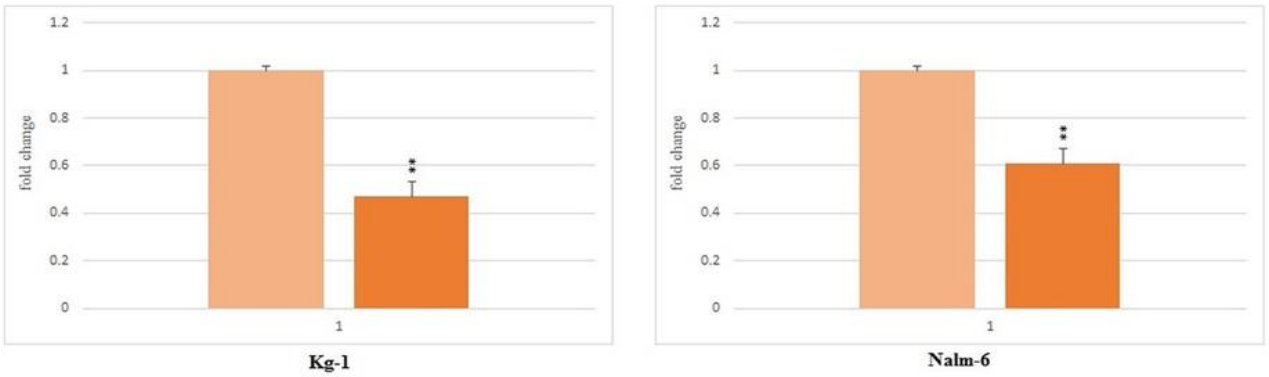

Figure 5. RT-PCR analysis results of the mRNA Levels of Bcl-2. The following formula was used to calculate the fold change. Fold difference = $2^{-\Delta\Delta Ct}$. A noticeable decrease was detected in Bcl2 mRNA in test samples compared to the control group. *P<0.01* in both KG-1 and NALM-6 cell lines.

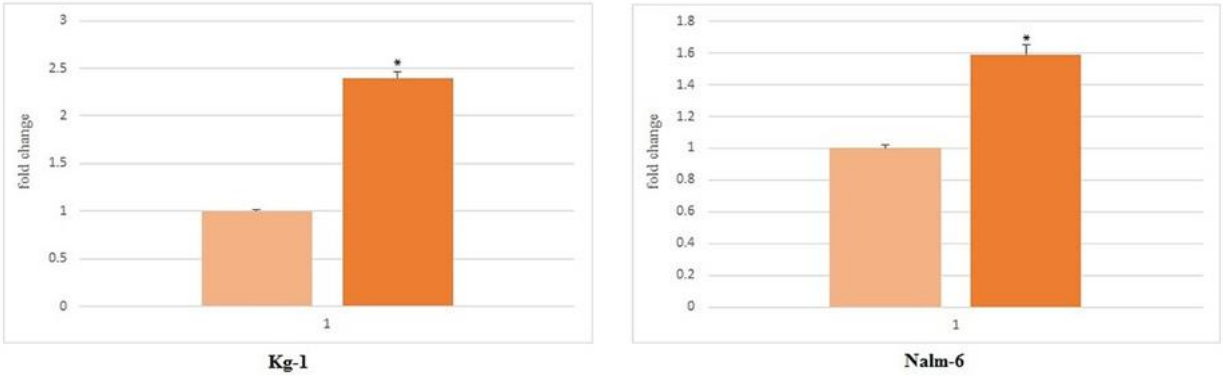

Figure 6. RT-PCR analysis results of the mRNA levels of caspase3. The following formula was used to calculate the fold change. Fold difference = $2^{-\Delta\Delta Ct}$. A significant increase was observed in caspase3 mRNA expression. *P<0.05* in both KG-1 and NALM-6 cell lines.

Secondary metabolites measurement

Based on the results of the MTT test, the best IC_{50} was related to the ethyl acetate extract. Phenolic compounds are known to possess antioxidant and anticancer properties. Therefore, it is plausible to expect the presence of phenolic compounds in ethyl acetate fractions. For this reason, this fraction was measured for phenolic and flavonoid compounds.

After determining the adsorption of the samples, using the equation obtained from the standard curves, the total phenolic content equivalent to the amount of gallic acid and the flavonoid content equivalent to the amount of quercetin were calculated. Each experiment was repeated three times, and the results were reported as Mean ± SD. Moreover, the amounts of phenolic compounds (mg of equivalent to gallic acid per gram of sample) and flavonoids (mg of equivalent to quercetin per gram of sample) were obtained.

The ethyl acetate extract contained 151.64 ± 0.03 mg/g of total phenol and 1.21±0.02 mg/g of total flavonoids (Figures 7 and 8).

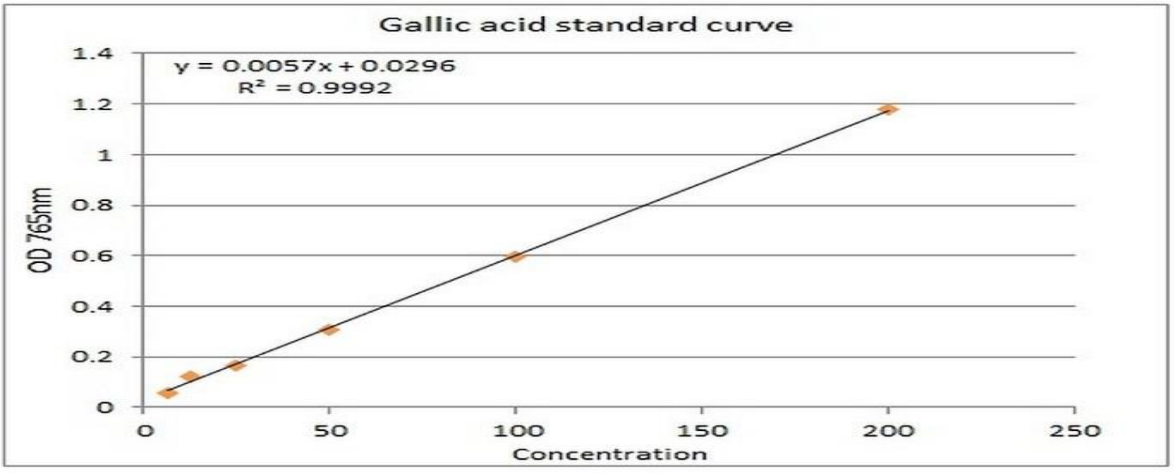

Figure 7. Standard curve of absorption against gallic acid concentration. The ethyl acetate extract contains 151.64 ± 0.03 mg/g of total phenol. Y=0.0057X+0.0296; R^2 =0.9992

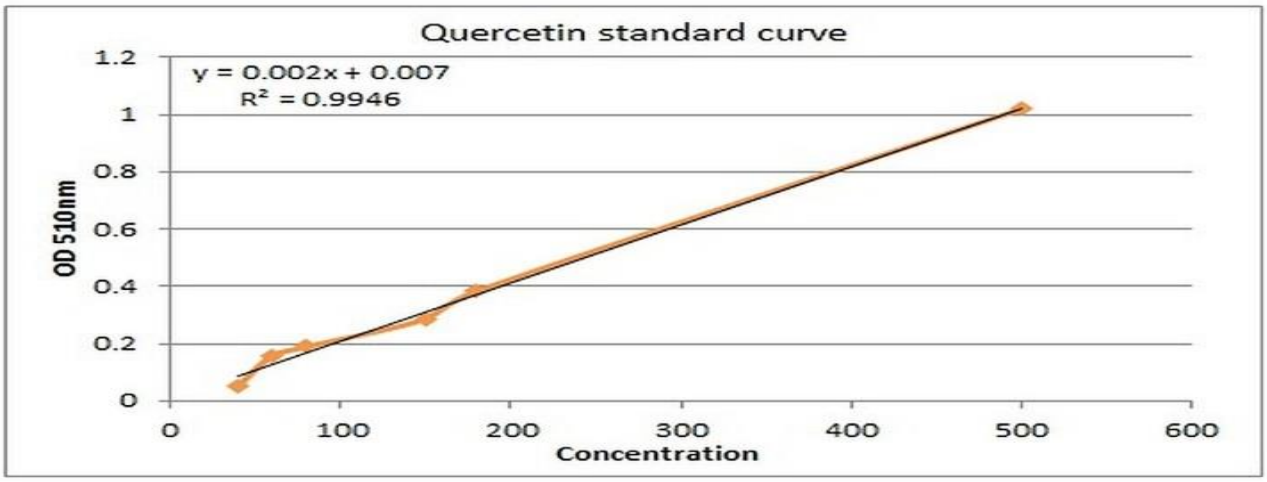

Figure 8. Standard curve of absorption against quercetin concentration. The ethyl acetate extract contains 1.21±0.02 mg/g of total flavonoids. Y=0.002X+0.007; R^2= 0.9946

DISCUSSION

The main purpose of this study was to evaluate the antiproliferative effect of *Z. jujube* extract on acute leukemia. Accordingly, cell viability, cell cycle progression, and Bcl_2, Bax, and caspase-3 mRNA expressions were investigated. Results indicated that cell viability decreased as the extract concentration increased in both KG-1 and NALM-6 cell lines, implying that tumor cell survival was concentration-dependent. The best efficacy belonged to the ethyl acetate extract. The application of ethyl acetate extract of *Z. jujube* in its IC50 concentration was found to increase the number of G0/G1 cells and decrease the number of G2/M cells, as observed in the investigation of cell cycle progression. The molecular examination 48 hours after treating KG-1 and NALM-6 cell lines revealed a considerable increase in caspase-3 and Bax and a decrease in Bcl2 gene expression compared to the controls.

Natural compounds have garnered significant interest in cancer treatment[20] due to their higher accessibility, lower cost, and potential to mitigate chemotherapy drugs' adverse effects[6,7]. They accomplish this via their capacity to act as antioxidants and to induce apoptosis[21]. Many studies showed that *Z. jujube* exerts anticancer activities on several tumor cell lines[8-19].

In the present study, cell viability decreased in both KG-1 and NALM-6 cells in a concentration-dependent manner, as demonstrated by the MTT assay results. Hoshyar et al., in 2015, showed the concentration- and time-dependent inhibitory effect of *Z. jujube* aqueous extracts on the MDA-MB-468 cell growth[22]. In 2012, Choi et al. demonstrated that all growth stages of *Z. jujube* inhibited HeLa cervical cancer cells concentration-dependently[9]. Jafarian et al. reported that *Z. jujube* significantly and concentration-dependently reduced the viability of Hela and MAD-MB-468 cells[11] *Z. jujube* is shown to have cytotoxic effects on the HEp-2 cell line[14-16]. Moreover, *Z. jujube* fruit extracts exert antiproliferative and apoptotic effects in human breast cancer cells[17].

In our study, the cell cycle analysis of KG-1 and NALM-6 after treatment with the IC_{50} concentration of *Z. jujube*'s ethyl acetate extract showed an increase in the number of G0/G1 cells and a reduction in the number of G2/M cells in both cell lines. In 2009, Huang et al. examined the changes in cell cycle dynamics. Their results showed that CHCl3-F of *Z. jujube* increased the cell numbers in the G1

cell cycle region[10]. They also found that the CHCl3-F and green tea extract combination effectively induced G1 phase arrest, while it did not induce apoptosis[14]. Huang et al.'s study showed that the application of *Z. jujube* in the HepG2 cell line would lead to the accumulation of the G1 cell cycle region and a decrease in the S phase, suggesting that the cell cycle was arrested at the G2/M phase[15]. The same results were reported when human hepatoma cells were treated with *Z. jujube* and green tea extracts[16]. All in all, these findings indicate that *Z. jujube* can have antitumor properties by regulating cell cycle progression.

In the current study, molecular examination detected a considerable increase in caspase-3 and Bax and a decrease in Bcl2 gene expression compared to the controls 48 hours after KG-1 and NALM-6 cell lines were treated. Similarly, Plastina et al. (2012) observed elevated Bax levels in MCF-7 and SKBR3 breast cancer cells following treatment with *Z. jujube* extracts, as compared to the control group[17]. Therefore, it can be stated that *Z. jujube* induces apoptosis by increasing pro-apoptotic proteins and decreasing anti-apoptotic proteins.

Since phenolic compounds are associated with antioxidant and anticancer effects, the ethyl acetate extract of Z. jujube with the best IC_{50} value in the MTT test was measured for phenolic and flavonoid compounds. The results confirmed the presence of 151.64 ± 0.03 mg/g of total phenol and 1.21±0.02 mg/g of total flavonoids in this extract. Likewise, Choi et al.'s study determined total phenolic and flavonoid content. They pointed out that *Z. jujube* fruit is a great source of flavonoids. Nonetheless, the maturity, variety, geographic location, soil, and climate environments cause variations in the content of these compounds[9]. In 2015, Jafarian et al. noticed that because of the presence of chemical agents such as alkaloids, flavonoids, terpenoids, saponin, and phenolic compounds, Ziziphus plants are important sources of cytotoxic compounds[11]. Hamood Al-Saeedi et al., in 2016, studied the phenolic content of different *Z. jujube* extracts. They showed that *Z. jujube* ethyl acetate extract had the highest content of phenols and flavonoids[23].

CONCLUSION

According to our study, *Z. jujube*'s ethyl acetate extract contains large amounts of phenolic compounds. The ethyl acetate extract of *Z. jujube* has been found to possess antitumor properties on KG-1 and NALM-6 cell lines. This is likely due to the presence of phenolic compounds, which are associated with antioxidant properties. The extract appears to induce apoptosis and regulate the cell cycle by increasing the expression of pro-apoptotic proteins, decreasing anti-apoptotic proteins, and promoting cell cycle arrest in the G1 phase while reducing the number of cells in the G2 phase. Among the limitations of this study was that the in vitro effect of extracts and serum antioxidant activity were not investigated. Therefore, it is recommended that future studies focus on the isolation of the effective ingredients of the ethyl acetate extract and evaluate their apoptotic molecular mechanisms in animal and human cancer cell lines.

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CONFLICTS OF INTEREST

None declared.

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