



# Anticancer potential of *Ferula hezarlalehzarica* Y. Ajani fraction in Raji lymphoma cell line: induction of apoptosis, cell cycle arrest, and changes in mitochondrial membrane potential

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## Abstract

**Background** Cancer is a major cause of mortality. The present study evaluates the antitumor effects of *Ferula hezarlalehzarica* Y. Ajani fractions on various cancer cell lines, including the Raji Burkitt's lymphoma cells.

**Methods** We evaluated the cytotoxic activity of various fractions of *F. hezarlalehzarica* against tumor cell lines by the MTT assay. Annexin V-PE/7-AAD and cell cycle analysis were assessed by flow cytometry. Expressions of genes associated with cell death and proliferation (Bax, Bcl-2, Fas, and c-Myc) were determined using real-time PCR. Alteration in mitochondrial membrane potential (MMP) was examined by JC-1 dye staining.

**Results** The hexane fraction of *F. hezarlalehzarica* showed the highest degree of cytotoxicity against Raji cells (IC<sub>50</sub> = 31.6 µg/ml). Flow cytometry analysis showed that 200 µg/ml of the fraction induced apoptosis in >96% of Raji cells after 24 h. In cell cycle analysis, at the same concentration, the percentage of apoptotic cells in the sub G1 phase increased to 95.25 ± 1.76% at 48 h of treatment. The fraction induced cell cycle arrest at the G0/G1 phase. Exposure to 100 µg/ml of the fraction after 48 h increased the percentage of G0/G1 cells (76.3 ± 6.08%) compared to the negative control (<50%). Treatment with 75 µg/ml of fraction reduced the expressions of Bcl-2 (0.23 ± 0.008-fold) and c-Myc (0.68 ± 0.07-fold) and increased Bax (1.75 ± 0.31-fold) and Fas (5.02 ± 0.74-fold; *p* < 0.01). We observed a decrease in MMP (≈0.4, *p* < 0.05) at ≥100 µg/ml and this effect remained almost unchanged until 48 h.

**Conclusions** The *F. hezarlalehzarica* hexane fraction induced apoptosis in Raji cells by changing the expression of apoptosis-related genes, cell cycle distribution, and MMP. These data suggested a potential effectiveness of *F. hezarlalehzarica* for inducing cell death in lymphoma cells.

**Keywords** *Ferula hezarlalehzarica* · Apoptosis · Raji cell line · Lymphoma

## Background

Apoptosis, a process of programmed cell death, happens under the control of different signaling pathways. This type of cell death is triggered by either intrinsic or extrinsic

pathways that lead to changes in cell morphology, chromatin condensation, DNA fragmentation, and generation of apoptotic bodies [1]. The intrinsic or mitochondria-mediated pathway is incited via intracellular events and conducted by the release of cytochrome C and activation of pro-apoptotic members of the Bcl-2 protein family [2]. The extrinsic pathway is triggered by the binding of extracellular death ligands such as FasL (CD178) to the related cell surface death receptor Fas (CD95) molecule [3]. Fas, a member of the tumor necrosis factor (TNF) receptor family, is expressed on many immune and non-immune cells. The aberrant activity of the Fas/FasL interaction is correlated with various diseases [4, 5]. Both pro- and anti-apoptotic molecules of the Bcl-2 family have an important role in maintaining the integrity of the mitochondrial membrane [1]. Bax, one of the driving apoptotic molecules of this family, interacts with the anion channel in the

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mitochondria to open it. This leads to loss of mitochondrial membrane potential (MMP) and cytochrome C release [6]. Bcl-2, the main member of the Bcl-2 family, prevents apoptosis by interfering with the function of pro-apoptotic members [1].

Cancer is one of the most important causes of death worldwide. Although conventional cancer treatments such as chemotherapy are used to treat different stages of malignancies, the cytotoxic drugs used in chemotherapy may also kill healthy cells and lead to unpleasant side effects in patients [7]. Therefore, there is an ongoing need for safer and newer drugs. Natural products that particularly arise from medicinal plants are sources of numerous biologically active compounds that may have potential health benefits and minimal side effects [8].

One of the related genera of medicinal plants is *Ferula*. This genus belongs to the Apiaceae family and consists of usually flowering, fragrant plants that grow in temperate zones of the Mediterranean and Central Asia [9]. From 32 species of *Ferula* that have been found, some species are endemic to Iran [10]. Among indigenous people, the *Ferula* genus is known as Koma or Kema [10]. Several species of this genus are used in traditional medicine to treat a variety of disorders. For example, *Ferula assafoetida* (*F. assafoetida*), *F. gummosa*, and *F. persica* are well known for their anticonvulsant, carminative, antispasmodic, and anti-inflammatory effects [11]. Chemical studies have shown that these plants are good sources of biologically active compounds that include derivatives of sesquiterpenes and sulfur-containing compounds [12, 13]. It has been demonstrated that a number of *Ferula* species have cytotoxic and anti-cancer effects. Valiahi et al. showed that 11 compounds extracted from various species of *Ferula* had chemosensitizing and cytotoxic effects against the CH1, A549, and SK-MEL1–28 cancer cell lines [14]. The cytotoxic effects of these compounds occur mainly via induction of apoptosis. *F. hezarlalezarica* Y. Ajani belongs to this genus and has been identified in the Hezar and Lalezar mountains of Kerman Province, Iran in 2008 [15]. To date, to the best of our knowledge, except a study performed by Hajimehdipour et al. (16) no other studies on this plant with regard to its possible cytotoxic and antitumor effects have been reported. Previous studies about the cytotoxic effects of different *Ferula* species on cancerous cell lines have encouraged us to study the cytotoxic activity of this newly discovered species. In the present study, we investigated the anti-tumor activities of the methanol, hexane, ethyl acetate, butanol, and water fractions of this plant against tumor cell lines. We have found that the hexane fraction had the strongest cytotoxicity and the Raji cell line was the most sensitive cell among the studied cell lines. Raji cells are tumor cells that originate from human Burkitt lymphoma, a malignant and metastatic form of non-Hodgkin's B cell lymphoma [16]. Burkitt lymphoma is a

highly aggressive tumor frequently observed in young adults. In most cases, this disease is associated with overexpression of an oncogene called c-Myc which leads to abnormal transcriptional regulation of various genes resulting in cell cycle changes, transformation and resistance to apoptosis [16, 17].

We chose the hexane fraction with the most activity among the *F. hezarlalezarica* fractions and examined its possible effects to induce apoptosis in Raji lymphoma cells. In this regard, we evaluated changes in the expression of apoptotic and anti-apoptotic related genes, c-Myc, the cell cycle and MMP.

## Material and methods

### Reagents

Roswell Park Memorial Institute medium (RPMI-1640) and fetal calf serum (FCS) were purchased from Gibco (Ashland, KY). Cell culture grade dimethyl sulfoxide (DMSO), 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), trypan blue dye and propidium iodide (PI) were purchased from Sigma (St. Louis, MO). PE Annexin V/7-AAD apoptosis detection kit was obtained from BD Pharmingen™ (San Diego, CA) and JC-1 MMP assay kit from Cayman Chemical (Ann Arbor, MI). RNX-Plus solution for total RNA isolation was obtained from Sinaclon (Tehran, Iran). SYBR Premix Ex Taq II and high-capacity cDNA reverse transcription kit were provided by Applied Biosystems (ABI, Foster City, CA).

### Plant material and preparation of the methanol fraction

The aerial parts of *F. hezarlalezarica* were collected from mount Hezar (Kerman province). A sample was authenticated by Mr. Ajani, Institute of Medicinal Plants (IMP) of Karaj, Iran. A voucher specimen was deposited in the herbarium of the institute (NO. 2922). Aerial parts of the plant was dried, powdered (100 g) and macerated with a 90% methanol solution for 3 days with three changes of the solution. The resulting fraction was filtered and evaporated under vacuum to get the methanol fraction of *F. hezarlalezarica* (13.4 g dry weight corresponding to 1.3%).

### Preparation of the sequential fractions

Different fractions were sequentially prepared by soaking 200 g dried and powdered aerial parts of the plant in solvents with increasingly polarity for 24 h; Hexane (1.5 L), ethyl acetate (1.5 L), butanol (1.5 L) and water (1.5 L). The resulting fractions consisted of: hexane (1.6 g dry weight corresponding to 0.8%), ethyl acetate (2.6 g dry weight

corresponding to 1.3%), butanol (2.8 g dry weight corresponding to 1.4%) and water fraction (3.1 g dry weight corresponding to 1.5%).

### Cell culture

Tumor cell lines including HeLa (cervical cancer), HepG2 (hepatocellular carcinoma), K562 (chronic myelogenous leukemia), Raji (human Burkitt's lymphoma) and EL4 (mouse T cell lymphoma) were obtained from Pasteur Institute of Iran, Tehran. RPMI-1640 medium containing 10% FCS and 1% penicillin-streptomycin were used for cell culture. The cells were grown at 37 °C in a humidified incubator with 5% CO<sub>2</sub> until reach confluence. The viability of the cells was determined by trypan blue dye staining. Cultures with more than 90% cell viability were used for the experiments.

### MTT assay

In order to determine the growth inhibitory effects of various fractions of *F. hezarlalehzarica* on different tumor cell lines, MTT assay was performed as previously described [18]. Briefly, the cell lines were seeded in a flat-bottomed 96-wells microplate to a predetermined density of  $17.5 \times 10^3$  cells/well (K562),  $7.5 \times 10^3$  cells/well (Raji and HeLa),  $5 \times 10^3$  cells/well (HepG2),  $1 \times 10^3$  cells/well (EL4) in triplicates. After that, different concentrations (0.1–200 µg/ml) of methanol, hexane, ethyl acetate, butanol and water fractions were added to a final volume of 100 µl in each well. A triplicate well of cells treated with 100 µg/ml of cisplatin instead of fractions were used as the positive control. Cells treated only with DMSO solvent (0.1%) considered as negative control. At the end of 24 h and 48 h incubation periods, 10 µl of MTT dye solution (5 mg/ml) was added to each well and incubation was continued for 4 h at 37 °C. Then, the medium was removed and 150 µl of DMSO was added to each well with shaking for 20 min to dissolve the formazan crystals. The absorbance of each well was determined with a microplate reader (BioTek, Winooski, VT) at 570 nm and 630 nm as reference wavelength. The percentage of growth inhibition in each condition was calculated by the following formula: % Inhibition =  $100 - [(absorbance\ of\ the\ test) / (absorbance\ of\ negative\ control) \times 100]$ . The half-maximal inhibitory concentration (IC<sub>50</sub>) for each fraction was determined using CurveExpert 1.4 software.

### Apoptosis detection

Raji cells were cultured at a density of  $5 \times 10^5$  cells/well in 24-well tissue culture plates in the presence of the growth inhibitory concentrations of hexane fraction (25–200 µg/ml) for 24 h. Positive control wells contained cisplatin (100 µg/ml) instead of the fraction and negative control contained 0.1%

DMSO. Induction of apoptosis was detected using Annexin-PE V/7-AAD staining kit according to the manufacturer protocol. Briefly, cells were harvested and washed in cold phosphate buffered saline (PBS) and then resuspended in binding buffer to a total density of  $1 \times 10^6$  cells/ml. Afterward, 100 µl of the cell suspension was stained with Annexin V-PE and 7-AAD solution for 15 min at 25 °C. Then, 400 µl of the binding buffer was added to the stained samples and then the percentage of early apoptotic (Annexin V-PE only positive) and late apoptotic (Annexin V-PE and 7-AAD double positive) cells was determined using a flow cytometer (BD Biosciences, San Jose, CA). Data were analyzed using FlowJo software version 7 (Tree Star, Ashland, OR).

### Cell cycle analysis by PI staining

To evaluate the effects of hexane fraction of *F. hezarlalehzarica* on cell cycle distribution, cells were seeded in 24 wells of microplates at a density of  $5 \times 10^5$ /well and exposed to 25–200 µg/ml of hexane fraction for 24, 36 and 48 h at 37 °C in a CO<sub>2</sub> incubator. The positive and negative control cells were treated with cisplatin and 0.1% DMSO, respectively. After incubation time, cells were collected and washed with cold PBS twice and treated with PI solution (50 mg/ml) containing 0.1% sodium citrate and 0.1% Triton X-100 for 1 h at 4 °C. The intensity of dye in staining cells was measured by flow cytometry in FL2 channel and the results were analyzed by FlowJo software.

### Real-time polymerase chain reaction (PCR)

Real-time PCR was performed to analyze the changes in the expression of c-Myc and apoptosis-related genes. Raji cells were cultured in 24-well plates in the presence of 25, 50 and 75 µg/ml of hexane fraction for 24 h. Cells treated only with 0.1% DMSO considered as negative control. After harvesting and washing, the number of the cells was adjusted to  $1$  to  $2 \times 10^6$  cells/ml and then total RNA was prepared with the help of a highly pure RNA extraction kit based on the manufacturer instruction. The purity and quantity of extracted RNA were determined via a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE). Then reverse transcription (RT) reaction and cDNA synthesis was carried out using a high-capacity cDNA reverse transcription kit using 10 µl RNA, dNTP, and random primers. The product of cDNA synthesis was then amplified using real-time PCR. The sequences of the primers used were as follows [19]. Bcl-2, F: 5' AAGA TTGATGGGATCGTTGC3', R: 5' GCGGAACACTTGAT TCTGGT3'; Bax F: 5' ATGCGTCCACCAAGAAGC3', R: 5' GGCGCAATCATCTCTG3'; Fas, F: 5' CACA CTCACCAGCAACAC3', R: 5' TCCTTTCTCTTCAC CCAAAC3'; c-Myc, F: 5' GCGACTCTGAGGAGGAAC3', R: 5' GCGTAGTTGTGCTGATGTG3'; β-actin, F: 5' GGCC

GCACCACCATGTACCC3', R: 5'GGAGGGGCCGGACTCGTCAT3'. The PCR mixture contained 10 µl SYBR Premix Ex Taq II, 0.3 µl ROX reference dye-2, 0.6 µl forward primer (10 pM), 0.6 µl reverse primer (10 pM), and 4.5 µl double-distilled water. The PCR procedure was carried out for a total 40 thermal cycles by using an ABI 7500 real-time PCR equipment (Foster City, CA) as follows: An initial cycle of 30 s at 95 °C, denaturation of DNA for 5 s at 95 °C, annealing of the DNA at 58 °C ( $\beta$  actin), 61 °C (Bax), 56 °C (BcL-2), 57 °C (Fas), 62.5 °C (c-Myc) for 18 s, and the extension time for 30 s at 72 °C. The expression levels were estimated by calculating  $\Delta\Delta CT$  and analyzed using  $2^{-\Delta\Delta CT}$  method. Results of target mRNA levels were normalized against  $\beta$ -actin and calculated as relative fold change (RFC) of the negative control.

### JC-1 mitochondrial membrane potential (MMP) assay

Raji cells were cultured in 24-well culture plates in the presence of 25–200 µg/ml of *F. hezarlalehzarica* hexane fraction for 24 h and then changes in MMP was assessed using a JC-1 assay kit according to the instructions of the manufacturer. Negative control cells were treated with DMSO (0.1%) alone and positive control cells were treated with 100 µg/ml of 2,4-dinitrophenylhydrazine (DNP) 3 h before the ending of the treatment period. DNP is an uncoupler that effectively causes mitochondrial depolarization and inhibition [20]. After incubation time, 10 µl of the JC-1 staining solution (1/10 in RPMI-1640 medium) was added to each well and mixed gently. The plate was incubated at 37 °C for 30 min and after centrifugation, the supernatant was removed carefully and 200 µl of the assay buffer was added to each well, mixed completely and centrifuged again. In the last step, 100 µl of assay buffer was poured into each well and then samples were immediately analyzed using a FLUOstar Omega microplate reader (BMG Labtech, Allmendgruen, Germany). The fluorescent intensity of JC-1 aggregates after excitation at 544 nm and emission at 590 nm (red), and of JC-1 monomers at excitation/emission of 485/520 nm (green) were measured. The ratio of the fluorescent intensities of aggregate to monomer dyes was determined.

### Statistical analysis

Data were reported as the mean  $\pm$  standard deviation (SD) and experiments were performed in triplicates and repeated at least three times. Results were analyzed using GraphPad Prism software version 6 (San Diego, CA). The One-way ANOVA was used for statistical significance of the results. *P* value less than 0.05 considered significant.

## Results

### Effects of various fractions from *F. hezarlalehzarica* on the growth of tumor cell lines

Figure 1 shows the effects of the methanol, hexane, ethyl acetate, butanol, and water fractions of *F. hezarlalehzarica* on different tumor cell lines after 48 h of treatment according to the MTT assay. The  $IC_{50}$  values determined for the effects of each fraction revealed that the hexane fraction had the highest inhibitory activity; hence, it showed the lowest amounts of  $IC_{50}$  against all cell types (Fig. 2). This fraction had the following  $IC_{50}$  values: 28.8 µg/ml (EL-4), 31.6 µg/ml (Raji), 57.5 µg/ml (K562), 90 µg/ml (HepG2), and 100 µg/ml (HeLa). Next, the methanol fraction had a minimal  $IC_{50}$  value of 88 against the EL-4 cell line. The ethyl acetate fraction had an  $IC_{50}$  value of 102 µg/ml against K562 cells and the butanol fraction had an  $IC_{50}$  value of 177 µg/ml against Raji cells. The aqueous fraction, with  $IC_{50}$  values of more than 1000 µg/ml against all cell lines, had almost no inhibitory activity. According to these data, the hexane fraction had the strongest effect on Raji cells. Therefore, we chose this fraction and the Raji cell line for further experiments. Figure 3 shows the growth inhibitory effect of the hexane fraction after 24 h treatment of Raji cells according to the MTT assay. The  $IC_{50}$  value obtained was 79.1 µg/ml compared to an  $IC_{50}$  value of 31.6 µg/ml at 48 h, which suggested the time dependency of this effect.

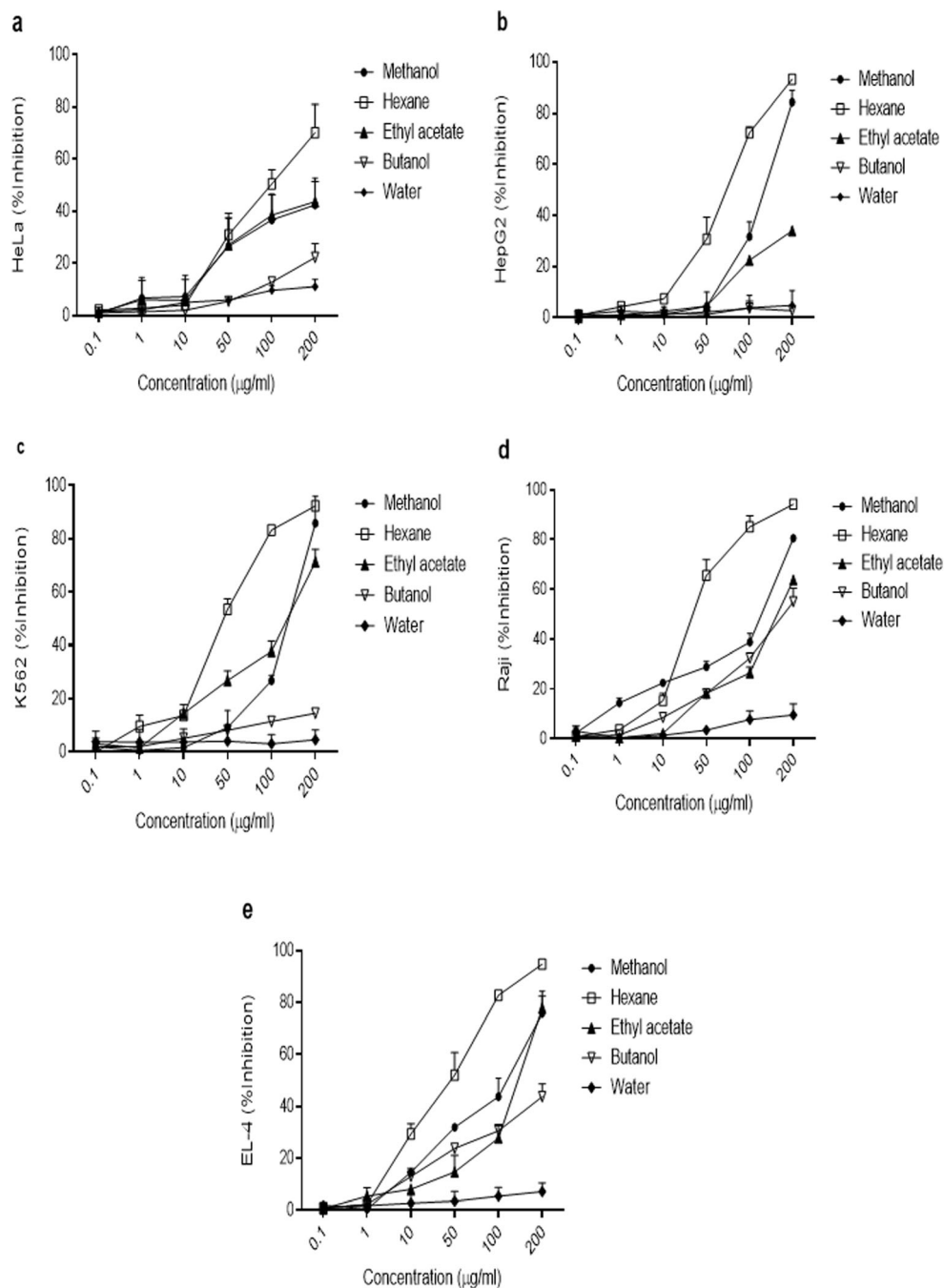
### Effects of hexane fraction on apoptosis of Raji cells

We sought to determine whether the growth inhibitory effect of hexane fraction was due to induction of apoptosis according to the Annexin V-PE/7-AAD staining method. Flow cytometry dot plots of cells treated with the fraction and the percentage of apoptotic cells are shown in Fig. 4. Treatment of cells with 50 µg/ml of the fraction increased the percentage of apoptotic cells from  $17.81 \pm 1.5\%$  to  $68.53 \pm 1.2\%$  at 100 µg/ml and  $96.93 \pm 1.45\%$  at 200 µg/ml compared to the negative control ( $5.2 \pm 2.02\%$ ), which suggested the capability of the hexane fraction to induce apoptosis in Raji cells in a dose-dependent manner.

### Effects of hexane fraction on the cell cycle of Raji cells

In order to study the cell cycle distribution of the fraction-treated cells, we used PI staining which determined the cell cycle stage based on DNA content. As shown in Fig. 5, the hexane fraction gradually decreased the percent of cells in the G2/M and S phases, yet we observed increased percent of cells in the G0/G1 and subG1 phases. This finding

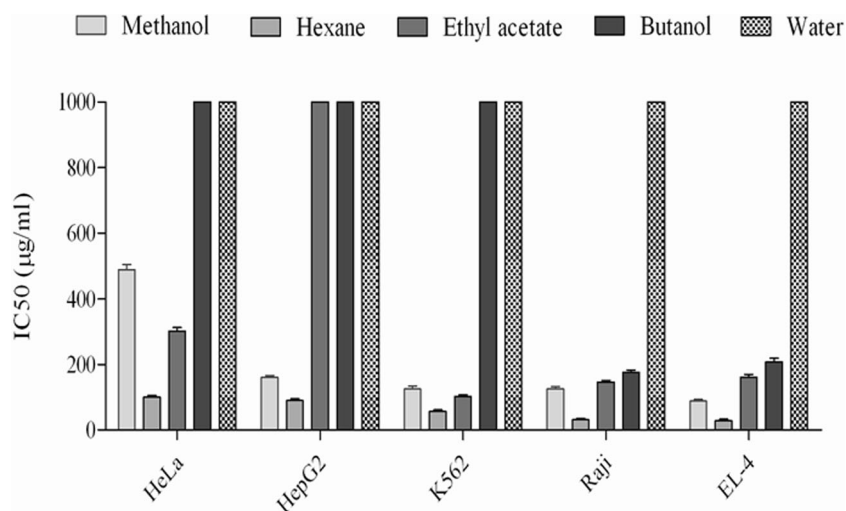
**Fig. 1** Effect of five different fractions of *F. hezarlalehzarica* on various tumor cell lines. HeLa (a), HepG2 (b), K562 (c), Raji (d) and EL-4 (e) tumor cell lines were treated with methanol, water, hexane, butanol and ethyl acetate fractions for 48 h and then the growth inhibitory effects was determined by MTT assay. Values are the mean  $\pm$  SD of three independent experiments performed in triplicate



indicated treatment induced cell cycle arrest and apoptosis which was time- and dose-dependent. Treatment with increasing amounts of fraction from 24 and 36 to 48 h shifted the main population of cells toward the G<sub>0</sub>/G<sub>1</sub> and sub G<sub>1</sub> phases. The percentage of cells accumulated in subG<sub>1</sub> phase after the 24 h treatment changed from  $3.28 \pm 1.47\%$  at  $75 \mu\text{g/ml}$  to  $51.9 \pm 3.4\%$  at  $200 \mu\text{g/ml}$  of the fraction. After 36 h,  $78.7 \pm 5.51\%$  of cells treated with  $200 \mu\text{g/ml}$  ( $p < 0.001$ ) of the fraction were in the sub G<sub>1</sub> phase. At 48 h of treatment, the percentage of apoptotic cells increased to more than  $95\%$  ( $p < 0.001$ ) at  $200 \mu\text{g/ml}$ . A comparison of the percentages of cells in the G<sub>0</sub>/G<sub>1</sub> and G<sub>2</sub>/M

stages showed considerable differences. After 24 h, the percentage of cells in the G<sub>0</sub>/G<sub>1</sub> stage increased from  $52.15 \pm 2.19\%$  (at  $25 \mu\text{g/ml}$ ) to  $79.65 \pm 13.22\%$  (at  $100 \mu\text{g/ml}$ ). The percentage of G<sub>2</sub>/M cells decreased from  $20.45 \pm 4.03\%$  to  $2.22 \pm 3.1\%$  ( $p < 0.05$ ). After 48 h, exposure to  $100 \mu\text{g/ml}$  of the fraction led to an increased percentage of G<sub>0</sub>/G<sub>1</sub> cells to  $76.3 \pm 6.08\%$  and decreased G<sub>2</sub>/M cells to less than  $0.04\%$ . Figure 6 shows a representative example of flow cytometry analysis of the cell cycle phases in the treated cells after 48 h of incubation with the fraction.

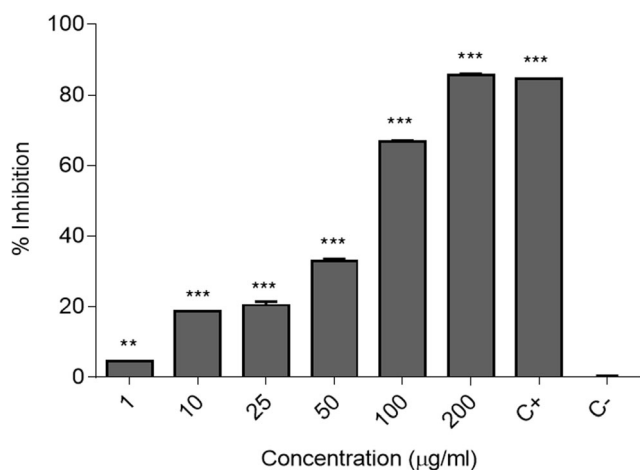
**Fig. 2** The IC<sub>50</sub> values for the effects of five different fractions of *F. hezarlalehzarica* on various tumor cell lines by MTT assay. IC<sub>50</sub> values were calculated by Curve expert



### Effects of the hexane fraction on the expression of c-Myc and apoptotic-related genes in Raji cells

We conducted real-time PCR analysis to determine the effect of the hexane fraction on the expression of cell cycle and apoptosis-related genes. Figure 7 shows 24 h treatment of Raji cells with different concentrations of the fraction led to downregulation of c-Myc and the anti-apoptotic molecule, Bcl-2. This decrease for Bcl-2 was dose-dependent with expression levels from  $0.57 \pm 0.001$  RFC ( $p < 0.001$ ) at 25  $\mu\text{g/ml}$ ,  $0.31 \pm 0.04$  RFC ( $p < 0.001$ ) at 50  $\mu\text{g/ml}$ , and  $0.23 \pm 0.008$  RFC at 75  $\mu\text{g/ml}$  ( $p < 0.001$ ; Fig. 7a). Evaluation of Bax and Fas expressions revealed significant upregulation at the

mRNA level. Similar to Bcl-2, the increased expression of Bax and Fas were dose-dependent. At the most effective concentration (75  $\mu\text{g/ml}$ ), we observed  $1.75 \pm 0.31$ -fold (Bax) and  $5.02 \pm 0.74$ -fold (Fas) increases in gene expressions compared to the negative control ( $p < 0.01$ ; Fig. 7b, c). On the other hand, the ratio of Bax to Bcl-2 expressions increased with increasing concentrations. At 75  $\mu\text{g/ml}$ , this ratio reached  $7.4 \pm 0.2$  RFC (Fig. 7e). These results showed that treatment with hexane fraction could induce a proapoptotic situation in Raji cells and supported the apoptosis-inducing activity of the hexane fraction on the Raji cell line. With respect to c-Myc, although the decrease in the mRNA level was statistically significant ( $\approx 0.7$ -fold) compared to negative control ( $p < 0.001$  for all concentrations), the effect was not dose-dependent (Fig. 7d).

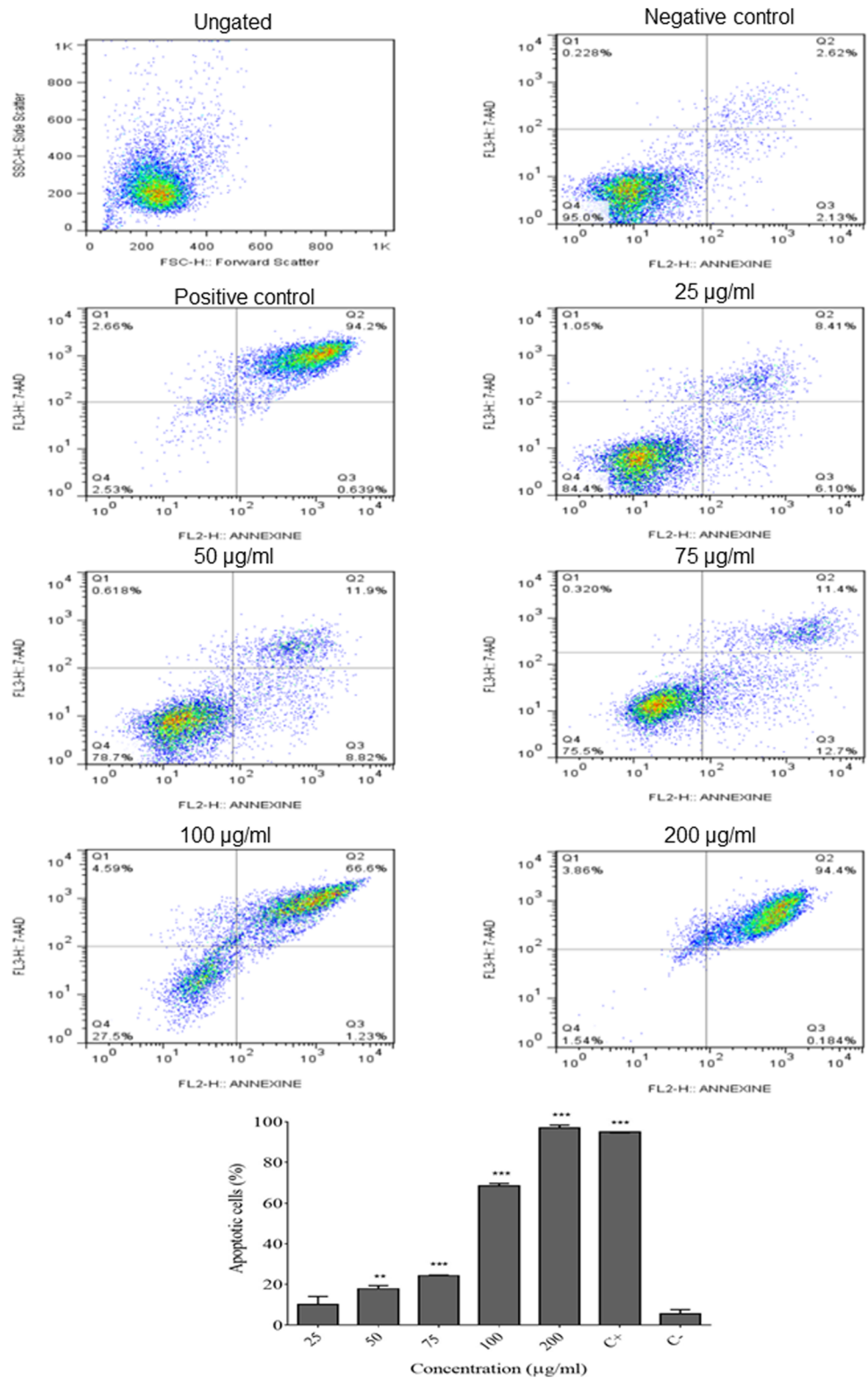


**Fig. 3** Effect of hexane fraction of *F. hezarlalehzarica* on the growth of Raji cell line after 24 h. The cells were treated with different concentrations of the hexane fraction for 24 h and then the growth inhibitory effect of the fraction was determined by MTT colorimetric assay. Positive control values were obtained in the presence of cisplatin (100  $\mu\text{g/ml}$ ). Values are the mean  $\pm$  SD of three independent experiments carried out in triplicate. \*\* $p < 0.01$ , \*\*\* $p < 0.001$

### Effects of the hexane fraction on mitochondrial membrane potential (MMP)

JC-1 fluorescence dye staining was used to assess the MMP changes of fraction-treated Raji cells. Determination of the ratio of aggregate to monomer (red/green) fluorescence which corresponded to active mitochondria showed that after 24 h treatment with 100  $\mu\text{g/ml}$  ( $0.49 \pm 0.07$ ,  $p < 0.05$ ) and 200  $\mu\text{g/ml}$  ( $0.4 \pm 0.05$ ,  $p < 0.01$ ) of the fraction, we observed a decrease in this ratio compared to the negative control (Fig. 8a). We examined the possible time-dependency of this effect by measuring the MMP changes of Raji cells after 18, 24, and 48 h of treatment with the fraction (Fig. 8b). MMP of the treated cells ( $0.97 \pm 0.01$ ) did not significantly change compared to the negative control ( $0.98 \pm 0.05$ ) after 18 h of treatment. As regards, the reduction in red/green ratio began at 24 h ( $0.49 \pm 0.05$ ,  $p < 0.01$ ) and remained at almost the same level until 48 h ( $0.46 \pm 0.12$ ,  $p < 0.01$ ).

**Fig. 4** Effects of the hexane fraction on apoptosis of Raji cells. Raji cells were treated with different concentrations of hexane fraction of *F. hezarlehzarica* for 24 h and after that, the cells were stained for Annexin V-PE/7-AAD and analyzed by flow cytometry. Negative control values were obtained in the absence of the fraction and positive control in the presence of cisplatin (100 µg/ml). Total cells in lower right quadrant was considered as early apoptotic cells (Annexin V+/7-AAD-) and the cells in upper right quadrant considered as late apoptotic cells (Annexin V+/7-AAD+). Dot plots are representative of one experiment out of three independent experiments. Significant differences from negative control are indicated by \*\* $p < 0.01$ , \*\*\* $p < 0.001$

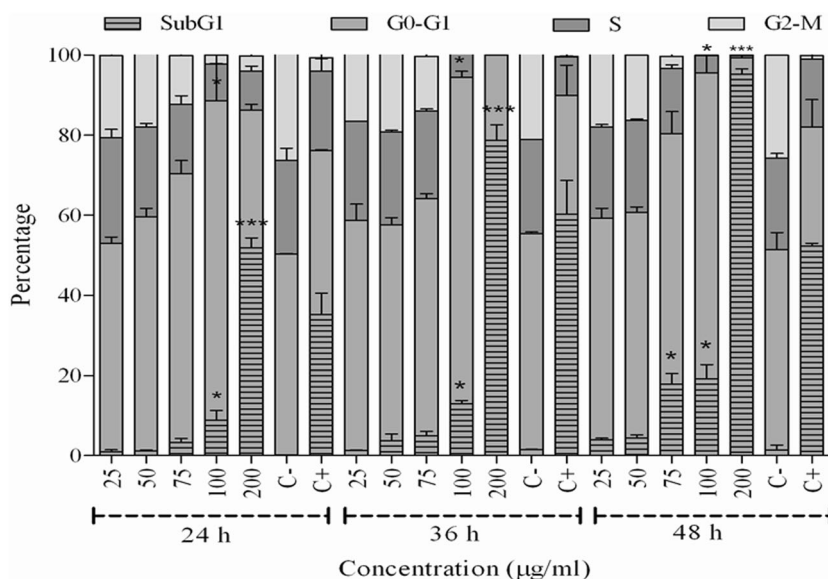


**Discussion**

Various studies reported the beneficial biological activities and antitumoral effects of several plants of the *Ferula* genus.

Bagheri et al. demonstrated the cytotoxicities of the methanolic extracts of *F. diversivittata*, *F. persia*, *F. ovena*, *F. brakema*, and *F. latisecta* on *Artemia salina*, a species of brine shrimp [21]. The anti-tumor activity of

**Fig. 5** Effect of the hexane fraction on Raji cell cycle. Raji cells were treated with different concentrations of the hexane fraction for 24 h, 36 h and 48 h and then cell cycle phases were analyzed by flow cytometry. Bars indicate the percentage of cells in each phase. Negative control values (C-) obtained in the absence of the fraction and positive control (C+) in the presence of cisplatin. Data are exhibited as means  $\pm$  SD of three separate experiments. Significant differences from negative control (C-) were indicated by \* $p < 0.05$  and \*\*\* $p < 0.001$

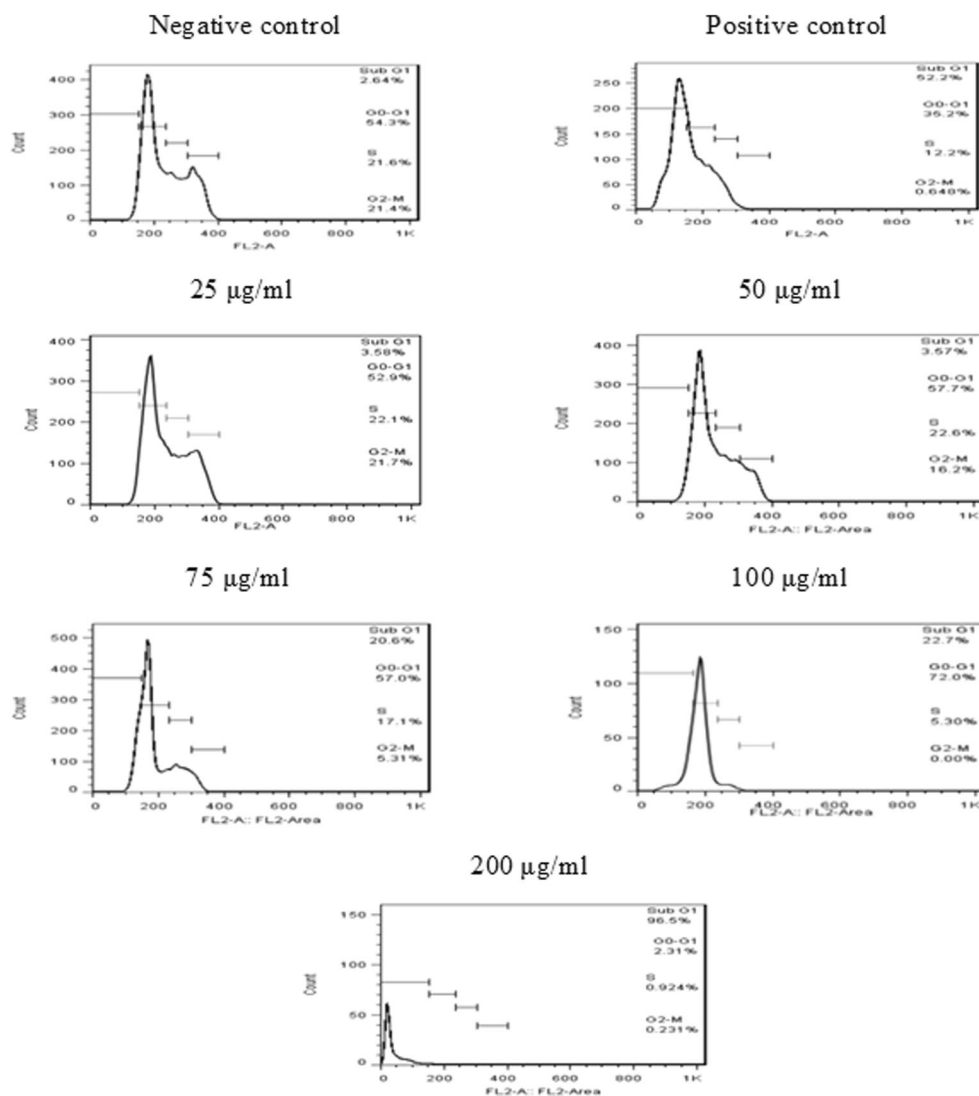


*F. asafoetida* against HepG2, Hep3B, and MCF7 cancer cell lines was reported in another study [22]. In the present study, we have examined the antitumor activities of different fractions from *F. hezarlalehzarica*. This plant is a newly discovered species of the genus *Ferula* recently reported from Iran [15]. In our primary study, the methanolic fraction of the plant showed various degrees of growth inhibitory effects on tumor cell lines. We examined other fractions of *F. hezarlalehzarica* to determine which had the strongest activity. The results showed that the hexane fraction with minimum  $IC_{50}$  values against all cell lines had the strongest anti-tumor activities, while the aqueous fraction with  $IC_{50}$  values more than 1000  $\mu\text{g/ml}$  was the weakest fraction. The butanol and ethyl acetate fractions also showed less inhibitory effects compared to the hexane fraction. The butanol fraction with an  $IC_{50}$  of 1000  $\mu\text{g/ml}$  against HeLa, K562, and HepG2 cells had the lowest cytotoxic effects after the aqueous fraction. Ethyl acetate fraction showed its lowest toxicity against the HepG2 cell line but targeted K562 cells at its highest cytotoxicity. The chemical composition of *F. hezarlalehzarica* has not been investigated; however, the strongest activity of the hexane fraction suggests that the main growth inhibitory effects of the plant are mostly attributed to the presence of non-polar and less hydrophilic compounds in this plant. These findings have corresponded to the research carried out by Hajimehdipoor et al. who showed that the hexane and chloroform extracts of *F. hezarlalehzarica* exhibited the cytotoxic activity against MCF7, HepG2, A549, and HT29 cell lines [23]. The most activity of *F. hezarlalehzarica* extract was against A549 cell line with  $IC_{50}$  value of approximately 100  $\mu\text{g/ml}$ . We observed the main effects of the hexane fraction against Raji and EL-4 cells with a less  $IC_{50}$  values ( $< 31.6 \mu\text{g/ml}$ ); therefore, we decided to continue the study on

the Raji Burkitt's lymphoma cell line to generalize the data from our experiments to the human studies. One of the main features of the highly proliferating Burkitt's lymphoma is chromosomal translocation t(8; 14) which induces excessive expression of the c-Myc oncogene that causes cell transformation, inhibition of cell cycle checkpoints and apoptosis [16]. We have first investigated the ability of the hexane fraction to induce apoptosis and cell cycle changes in Raji cells. Treatment of the cells with hexane fraction at different concentrations showed that with increasing concentrations, the percentage of apoptotic cells also increased such that at 200  $\mu\text{g/ml}$  more than 96% of the cells had undergone apoptosis. A comparison of the results of the MTT and apoptosis assays exhibited an approximately similar percentage of inhibited and apoptotic cells, which suggested that the hexane fraction could inhibit the growth of Raji cells by inducing apoptosis. Evaluation of cell cycle phases in hexane fraction-treated cells at three different time points showed that the fraction could gradually prevent cell cycle progression, such that the percentage of cells in the S and G2/M phases decreased and subsequently the cells accumulated in the G0/G1 and subG1 phases. Our observations revealed that this trend gradually increased with increasing exposure time. At 200  $\mu\text{g/ml}$  the percentage of apoptotic cells in the sub G1 phase increased from 51.9% at 24 h treatment to almost 95% at 48 h exposure. In this experiment, the percentages of sub G1 cells at different concentrations compared with the corresponding amount of apoptotic cells in the Annexin V-PE/7-AAD assay showed lower values. This could be justified by the fact that a decrease in DNA content measured by the PI cell cycle assay occurred at the end stages of apoptosis, whereas the appearance of phosphatidylserine at the cell surface measured by Annexin V was an early event [24]. Corresponding to these findings, several studies reported cell



**Fig. 6** Flow cytometry analysis of the effect of hexane fraction on Raji cell cycle. Raji cells were treated with different concentrations of the hexane fraction for 24 h, 36 h and 48 h and then cell cycle phases were analyzed by flow cytometry. Histograms are representative of one experiment out of three independent experiments for the time 48 h. Negative control values were obtained in the absence of the fraction and positive control in the presence of cisplatin. Cells are distributed throughout the four phases of the cell cycle, including subG1, G0/G1, S and G2M. SubG1 cells are considered as apoptotic cells

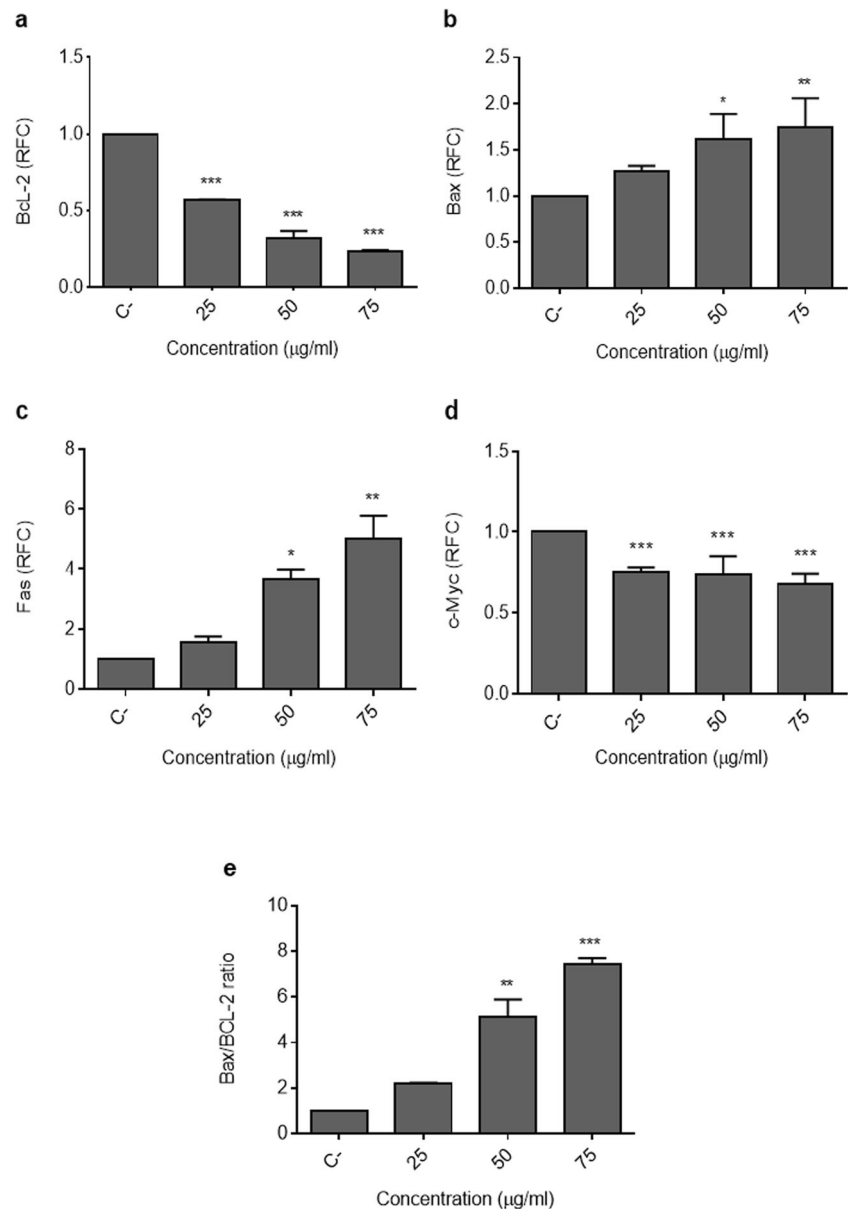


cycle arrest and apoptosis-inducing activity of some *Ferula* species. Extracts of *F. gummosa* have been shown to inhibit ACHN renal cancer cell line proliferation via apoptosis and induction of G0/G1 arrest [24] in addition to *F. assafoetida* which induced apoptosis by producing reactive oxygen intermediates (ROI) and cell cycle arrest in the MCF-7 breast cancer cell line [25].

We sought to demonstrate the apoptotic activity of hexane fraction in Raji cells at the level of gene expression. Therefore, we investigated expressions of Bax and Bcl-2, two important genes involved in the control of apoptosis. During 24 h of exposure, the fraction reduced the mRNA levels of the anti-apoptotic Bcl-2 molecule, which corresponded to increasing the concentration of fraction such that the expression of this molecule at 25 µg/ml of the fraction reduced to almost half and at 75 µg/ml decreased by  $\approx 5$  times. On the other hand, the exposure of Raji cancer cells to the hexane fraction could increase the gene expression of the Bax pro-apoptotic protein in a dose-dependent manner. Increasing the concentration of

the fraction from 25 to 75 µg/ml led to an approximately 1.7-fold increase in Bax expression. Overall, it could be proposed that the fraction induced apoptosis in treated cells by decreasing the expression of Bcl-2 and increasing Bax expression. A measurement of the ratio of Bax to Bcl-2 expressions supported this finding. We observed up to a 7.4-fold increase in this ratio in the presence of 75 µg/ml of the fraction. Evaluation of Fas gene expression in the treated cells showed increased mRNA levels to at least 3.67-fold higher after exposure to 50 and 75 µg/ml of the fraction. Bax and Bcl-2 molecules have an important role in maintaining the integrity of the mitochondrial membrane [1]. Activation of Bax could result in MMP changes that lead to cytochrome C release [26]. Fas from the extrinsic pathway could also activate the mitochondrial pathway through the cleavage of Bid by caspase 8 [27]. We investigated the effect of the hexane fraction on MMP by exposing the Raji cells to the fraction, which were further stained with cationic JC-1 dye. This fluorescent dye is monomer in the cytosol and emits green light after excitation. Under normal

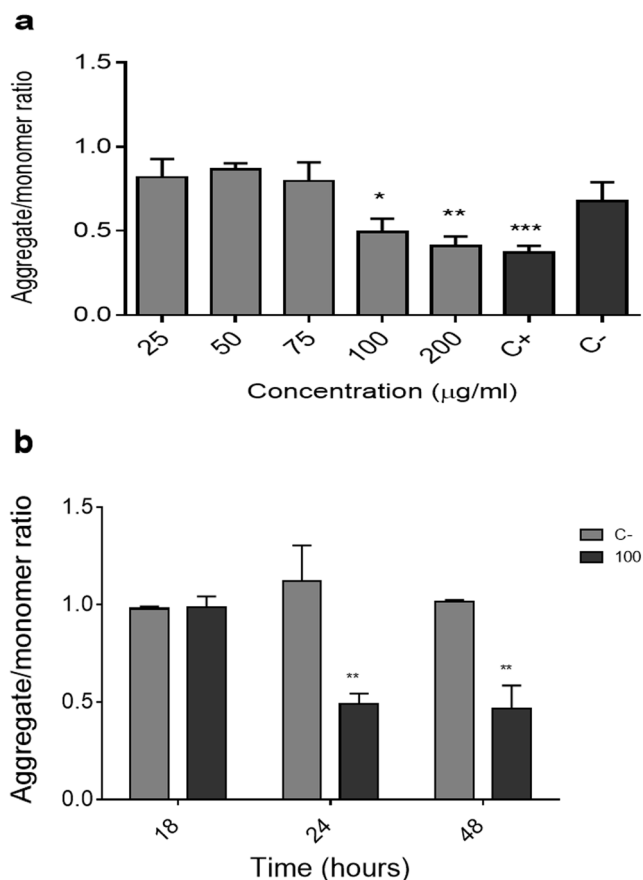
**Fig. 7** Effects of hexane fraction of *F. hezarlehzarica* on Bax, Bcl-2, Fas and c-Myc gene expressions. Raji cells were treated with hexane fraction (25, 50 and 75  $\mu\text{g/ml}$ ) for 24 h and then the gene expressions of Bcl-2 (a), Bax (b), Fas (c), c-Myc (d) and Bax/Bcl-2 ratio (e) was evaluated using real time-PCR. Negative control (C-) values were obtained in the absence of the fraction. Results are shown as mean  $\pm$  SD of three independent experiments, \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  versus negative control. RFC, relative fold-change



conditions when it enters the mitochondria aggregates and green fluorescence changed to red fluorescence. In apoptotic cells, due to loss of MMP, it remains in the cytosol in the form of monomer [28]. We have shown that the ratio of aggregate to monomer-dye, as an indication of cell activity, significantly decreased at 100 and 200  $\mu\text{g/ml}$  of the fraction. An evaluation of the exposure time-dependency of the effect showed that the fraction could exert its effects from 24 h. This effect was unchanged up to 48 h. These data suggested the capacity of the hexane fraction to change MMP, as a hallmark of apoptosis. A comparison of the results of apoptosis and the inhibition assays with MMP detection results revealed that the hexane fraction could change MMP. However, this was not the initial phenomenon of apoptosis because at the  $\leq 75$   $\mu\text{g/ml}$  concentrations we could detect apoptosis

and growth inhibition, but no changes in membrane potential of the mitochondria.

We also examined the effect of hexane fraction on c-Myc proto-oncogene expression in Raji cells. c-Myc overexpression in Raji cells could be associated with increased cell proliferation, cell cycle progression, and insensitivity to apoptotic stimuli [29]. Assessment of the gene expression of this oncogene in Raji cells showed that the fraction significantly diminished mRNA levels, though not in a dose-dependent manner. We did not find similar studies on *Ferula* species to study c-Myc; however, in various studies, the effects of several medicinal plant extracts on c-Myc expression have been investigated. For example, *Centella asiatica* juice extract induced cell death via damages to DNA and decreased expression of c-Myc in HepG2 cells



**Fig. 8** Effects of the hexane fraction on mitochondria membrane potential in Raji cells. Cells were cultured in the presence of various concentrations of hexane fraction of *F. hezarlalehzarica* for 24 h (**a**) and with 100 µg/ml of the fraction for 18 h, 24 h and 48 h (**b**) and then stained with JC-1 dye. JC-1 aggregates (red) that were indicative of active mitochondria and cell survival and JC-1 monomers (green) that were indicative of non-active mitochondria and dying cells were excited and the absorbance of aggregate and monomer dyes were analyzed by microplate reader. The ratio of red to green fluorescence is represented as bar diagram with error bars. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$  compared to negative control (C<sup>-</sup>)

[30]. Extracts of *Solanum nigrum*, *Ulmus macrocarpa*, *Euonymus alatus*, *Artemisia capillaris* and *Saururus chinensis* reduced the expression level of c-Myc in the A549 lung cancer cell line [31]. These data suggested a benefit of these plants in developing novel cancer chemoprevention agents.

## Conclusion

In total, we observed that the hexane fraction of *F. hezarlalehzarica* had the highest cytotoxicity against the Raji cell line. This effect was conducted mainly via induction of apoptosis by changing the expression of apoptosis-related genes, reducing cell cycle progression, and disturbing the MMP accompanied by downregulation of c-Myc.

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## Compliance with ethical standards

**Conflicts of interest** The authors declare no conflicts of interest.

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