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Original Article

The immunoinhibitory and apoptosis-inducing activities of *Foeniculum vulgare* on human peripheral blood lymphocytes

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Abstract

Foeniculum vulgare (*F. vulgare,* Apiaceae) is an important plant currently used in folk medicine to treat various diseases including infections and inflammatory conditions. In this study we have investigated the immunomodulatory and apoptosis-inducing activities of the fractions from this plant. We prepared dichloromethane, hexane, butanol, and water fractions from the aerial parts of the plant. We examined the growth inhibitory effects of the fractions on proliferative lymphocytes by the BrdU incorporation assay. Cell viability was determined by the propidium iodide (PI) assay. AnnexinV/PI staining and cell cycle analysis by flow cytometry was performed to investigate the apoptosis-inducing effects of the plant fractions. Cytokine levels were measured by enzyme-linked immunosorbent assay. All fractions reduced cell proliferation of the activated lymphocytes. The dichloromethane fraction with the highest inhibitory effect (IC_{50}, 19.8 µg/mL) significantly reduced cell viability. Although the butanol fraction inhibited cell growth (IC_{50}, 88.2 µg/mL) it did not affect cell viability. Annexin V/PI and cell cycle analysis showed the maximum apoptosis-inducing effect of the dichloromethane fraction at 200 µg/mL. Cytokines, that included interleukin (IL)-4 (22.8 ± 0.9 pg/mL, $P < 0.05$) and interferon (IFN)-γ (651 ± 37.5 pg/mL, $P < 0.01$) reduced the activated lymphocyte levels in cultures in the presence of 100 µg/mL concentration of butanol fraction compared to the untreated control. In conclusion, the dichloromethane fraction of *F. vulgare* had the capability of inducing apoptosis in the activated lymphocytes, whereas the butanol fraction reduced cell activation and cytokine secretion. These data suggested the potential of these examined fractions for more studies in terms of their beneficial effects on immune-mediated diseases.

Keywords: *Foeniculum vulgare*; Apoptosis; Lymphocytes; Cytokines

INTRODUCTION

Immunosuppressive agents are of significant importance for reducing immune reactions in various conditions (1). A variety of these agents induce apoptosis in lymphocytes such as methotrexate which is an efficient medication to treat rheumatoid arthritis, graft versus host disease, and other chronic inflammatory diseases (2). Apoptosis is an essential mechanism in organ development, homeostasis, and health. During apoptosis, cells completely fragment into apoptotic bodies and the phagocytes engulf these apoptotic bodies without generating inflammation (3).

Studies demonstrate that plant extracts have diverse biological activities, such as immunomodulatory activity via apoptosis induction. Examples include *Dionysia termeana* and *Linum persicum* which have apoptosis-inducing effects on human lymphocytes. The methanol extract of these plants have shown typical DNA laddering, as an indication of DNA fragmentation in apoptosis (4). *Stachys obtusicrena,* another plant with immunomodulatory effects, causes an accumulation of apoptotic cells in the sub-G1 phase of the cell cycle according to flow cytometry analysis (5).

Foeniculum vulgare Mill (*F. vulgare*) (fennel) belongs to the Apiaceae family. This plant is known as "Raziyaneh" in Persian (6).

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Fennel is native to the Mediterranean region and Southern Europe. It is used for antioxidant, antimicrobial, antipyretic, antispasmodic, and stimulator of gastrointestinal motility in traditional medicine (7-8). In the course of our screening on the immunomodulatory effects of the plants, we have determined the growth inhibitory effects of the crude extract of *F. vulgare* on human lymphocytes. In the present study we aimed to determine the fraction responsible for these growth inhibitory effects and whether this effect was due to inhibition of cell activation/proliferation or induction of apoptosis in activated lymphocytes.

MATERIALS AND METHODS

Preparation of the extract

Aerial parts of *F. vulgare* were collected from Fars province. Samples were authenticated by Mr. Asadollahi from the Medicinal and Natural Products Chemistry Research Center, Shiraz. A voucher specimen was deposited in this center (PC-MS-84-86). Methanol crude extract was obtained by maceration of the plant. The crude methanol extract was suspended in water and then sequentially partitioned with the solvents hexane, dichloromethane, and butanol. The resultant partitions and the remaining aqueous phase were dried under vacuum using a rotary evaporator to yield 10.1%, 1.6%, 7.5% and 8.1%, respectively. Samples (20 mg) were dissolved in 100 µL dimethyl sulfoxide (DMSO, Sigma, USA) and then diluted in RPMI 1640 culture medium to obtain a 20 mg/mL stock solution.

Lymphocyte isolation and proliferation assay

The effect of various concentrations of the fractions on proliferation of phyto-hemagglutinin (PHA)-activated lymphocytes was determined by BrdU incorporation assay (Roche, Germany). Ten mL heparinized blood sample was collected from 25-30 year healthy volunteers (with their consent) and peripheral blood lymphocytes (PBLs) were isolated using Ficoll-hypaque (Bahar-afshan, Iran) gradient centrifugation. After washing, lymphocytes were seeded in 96-well plates (1×10^5 cells/well). Then, 10 µL PHA (Fluka, Germany) (1/80) and various concentrations of the fractions (0.01-200 µg/mL) were added in a final volume of 100 µL. Negative control was PHA-stimulated cells treated with DMSO as the solvent at the highest concentration used in the test wells (e.g., 0.1%) (PHA-only treated cells). After addition of BrdU for 18 h, DNA was denatured and the cells were incubated with anti-BrdU monoclonal antibody. Then the optimal density (OD) was measured with an enzyme-linked immunosorbent (ELISA) microplate reader (Biotek Inc., USA) at 450 nm. The experiments were plated in triplicate wells and performed at least three times. The percentage of proliferation was determined as follows:

Percentage of proliferation = (OD of treated cells/OD of negative control) × 100

Viability assay

Peripheral blood lymphocytes (1×10^5cells/100 µL/well) were treated with inhibitory concentrations (10-200 µg/mL) of dichloromethane, hexane and butanol fractions and 10 µL PHA in triplicate and incubated for 48 h at 37°C in a 5% CO_2 incubator. Negative control was PHA-only treated cells and positive control was cells treated with cisplatin (50 µg/mL) as a cytotoxic drug. Propidium iodide (PI) dye (2 mg/mL) was added to give a final concentration of 5 µg/mL and then cells were incubated at 4 °C for 5 min. Cells were analyzed on flow cytometry (FACSCalibur, Becton Dickinson, USA). Percentage of PI positive cells was determined by WinMDI 2.8 software (Tree Star, USA). The dot plot diagrams of cells were plotted and cells were gated based on forward scatter (FSC) and side scatter (SSC) and debris was gated out. Histogram of gated cells was plotted.

Annexin V/ propidium iodide staining

Cells (1×10^5/100 µL/well) were added onto flat-bottomed 96-well culture plates in the presence of various inhibitory concentrations (10-200 µg/mL) of dichloromethane fraction in triplicate and incubated for 48 h. Negative control was PHA-only treated cells and positive control

was cells treated with cisplatin. Induction of apoptosis in the cells was quantified by a flow cytometer using fluoresce in isothiocyanate (FITC)-conjugated annexin V/PI staining kit (Q product, Netherland). According to the manufacturer's protocol, 10 µL annexin V-FITC was added to each well for 20 min at 4 ºC. After washing, 10 µL PI was added for 10 min and then cells were analyzed by flow cytometry. A total of 10000 events for each sample were acquired on a logarithmic scale for annexin V/PI fluorescences. Data were analyzed by gating cell populations according to PI log fluorescence *vs.* annexin V. Cells in early and late apoptosis were considered as apoptotic cells.

Cell cycle and DNA content analysis

Peripheral blood lymphocytes were seeded in 96-well culture plates (1×10^5 cells/100 µL/well) and treated with 10 µL PHA and 100 and 200 µg/mL of dichloromethane fraction. Negative control was PHA-only treated cells and positive control was cells treated with cisplatin. Cells were incubated for 36 and 48 h at 37 °C in a 5% CO_2 incubator and then harvested and washed with PBS. Cells were re-suspended in 1 mL freshly prepared hypotonic solution (0.1 % triton X100 and 1% sodium citrate) and then 50 µL PI (50 µg/mL) was added. After 2 h, they were analyzed by flow cytometry. Cells were gated in FL2-A *vs.* FL2-W and debris was gated out. FL2-A histogram of gated cells was plotted using WinMDI 2.8 software.

Cytokine assay

Interferon (IFN)-γ and interleukin (IL)-4 cytokines were measured in the supernatant of butanol fraction-treated cultures. As mentioned above, this fraction in contrast to dichloromethane fraction, despite of cell growth inhibitory effects did not reduce cell viability. PBLs (1×10^5 cells/100 µL) were treated with 10 µL PHA and 10-100 µg/mL of butanol fraction in triplicate and incubated for 48 h in a CO_2 incubator. Then the supernatants were collected for cytokine measurement. Controls were PHA-only treated cells and cells treated with DMSO (0.1%) without PHA and the fraction. Cytokine levels were determined by the related ELISA kits obtained from eBioscience (Germany). The sensitivity of kits was 4 and 2 pg/mL for IFN-γ and IL-4, respectively.

RESULTS

Effects of the Foeniculum vulgare fractions on activated lymphocyte proliferation

We treated the PHA-activated PBLs with 0.01 to 200 µg/mL concentrations of *F. vulgare* hexane, dichloromethane, butanol, and water fractions. Compared to other fractions, dichloromethane fraction had the highest inhibitory effect on the growth of activated lymphocytes (Fig. 1). Calculation of the half maximal inhibitory concentration (IC_{50}) showed the following results: dichloromethane (19.8 ± 9.5 µg/mL), hexane (89.8 ± 11.3 µg/mL), butanol (88.2 ± 4.3 µg/mL), and water (> 200 µg/mL).

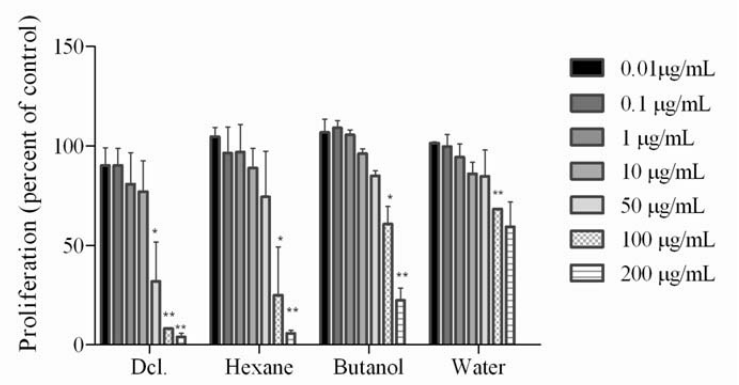

Fig. 1. Effect of *Foeniculum vulgare* different fractions on the proliferation of activated peripheral blood lymphocytes performed by Brdu assay. Peripheral blood lymphocytes were stimulated with phytohemagglutinin and cultured in the presence of various concentrations of the fractions. Negative control was phytohemagglutinin-only treated cells (100%). Data are presented as mean ± SD of three independent experiments each performed in triplicate. Dcl; dichloromethane. $*P < 0.05$, $**P < 0.01$.

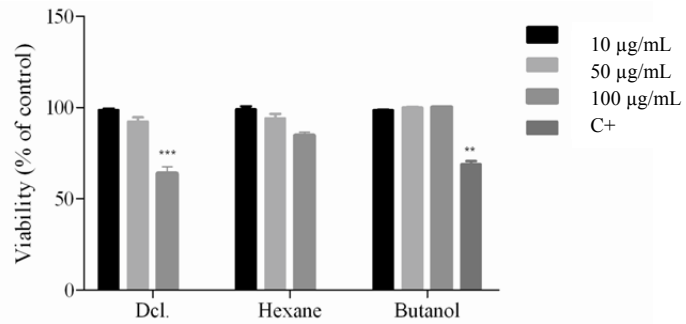

Fig. 2. Effect of *Foeniculum vulgare* different fractions on peripheral blood lymphocytes viability by propidium iodide staining. Peripheral blood lymphocytes were stimulated with phytohemagglutinin and cultured in the presence of 10-100 µg/mL concentrations of the fractions. Negative control was phytohemagglutinin-only treated cells (100% viability) and positive control (C+) was cells treated with cisplatin (50 µg/mL). Data are shown as mean ± SD of three independent experiments. Dcl; dichloromethane. $^{**}P < 0.01$ $^{***}P < 0.001$.

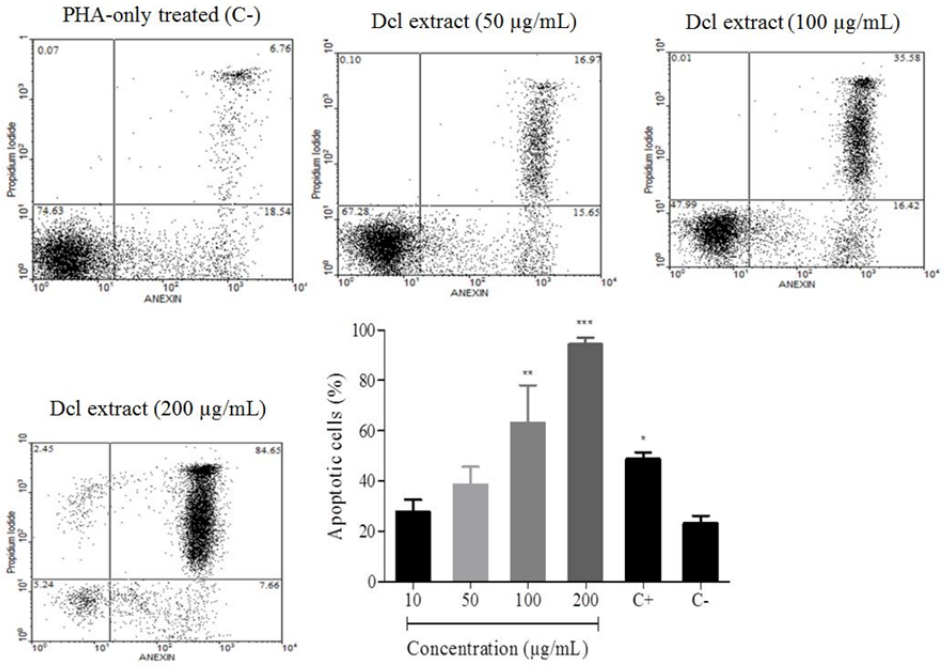

Fig. 3. Effect of *Foeniculum vulgare* dichloromethane fraction on apoptosis of phytohemagglutinin-activated lymphocytes by annexinV/PI staining. Dot plots show the result of flow cytometry analysis. (C-) negative control was phytohemagglutinin-only treated cells. Cells in upper/lower-right quadrants are considered as apoptotic cells. Percentage of apoptotic cells are shown in the histogram. Values are mean ± SD of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to negative control. (C+) positive control was cells treated with cisplatin; Dcl, dichloromethane.

Effects of different Foeniculum vulgare fractions on activated peripheral blood lymphocytes viability

We treated PBLs activated by PHA with different concentrations (10, 50, and 100 µg/mL) of the dichloromethane, hexane and butanol fractions of *F. vulgare* for 48 h. The dichloromethane fraction showed a significant cytotoxic effect on the lymphocytes. Dichloromethane fraction reduced cell viability to 64 ± 7.2% of PHA-only treated cells at the 100 µg/mL concentration ($P < 0.001$). The hexane fraction showed a slight non-significant decrease in cell viability (84.6 ± 3.2% of PHA-only treated cells). The butanol fraction had no cytotoxic effect on the activated lymphocytes (Fig. 2). According to these data, for the next step, we selected the dichloromethane fraction to examine its possible capacity to induce

apoptosis in the PBLs. We also selected the butanol fraction because of its non-cytotoxicity to determine its influence on cytokine secretion.

The effect of Foeniculum vulgare dichloromethane fraction on induction of apoptosis

The PHA-activated lymphocytes were treated by dichloromethane fraction (10 to 200 µg/mL) for 48 h and then analyzed for induction of apoptosis by annexin V/PI staining. Fig. 3 shows the representative flow cytometry dot plots for this experiment. The fraction at concentrations of 100 µg/mL (64.5 ± 12.9%, $P < 0.01$) and 200 µg/mL (94.7 ± 2.15%, $P < 0.001$) significantly increased the number of apoptotic cells (Fig. 3).

The effect of Foeniculum vulgare dichloromethane fraction on the cell cycle

The PHA-activated lymphocytes were treated with 100 and 200 µg/mL concentrations of *F. vulgare* dichloromethane fraction for 36 and 48 h. As shown in Fig. 4, after 48 h, 23.6 ± 4% of PBLs treated with 200 µg/mL of the fraction accumulated in the sub-G1 phase of the cell cycle compared with 1.43 ± 1.5% for the negative control ($P < 0.01$). In Fig. 4 the flow cytometry histogram plots from one representative experiment are shown.

The effect of Foeniculum vulgare butanol fraction on cytokine production

IFN-γ and IL-4 cytokines were measured in the supernatant of the PHA-activated lymphocytes in the presence of the butanol fraction. As shown in Fig. 5, PHA stimulation of the PBLs resulted in a slight, but significant increase in IL-4 and an extensive increase in IFNγ levels compared to the DMSO-treated cells.

The 100 µg/mL butanol fraction concentration slightly decreased IL-4 levels from 26.4 ± 0.6 pg/mL in PHA-only treated cells to 22.8 ± 0.9 pg/mL (Fig. 5A; $P < 0.05$). This fraction had a stronger dose-dependent inhibitory effect on IFN-γ production and significantly decreased its level from 807 ± 9.9 pg/mL in PHA-only treated cells to 777 ± 17 pg/mL at the 50 µg/mL concentration ($P < 0.05$) and 651 ± 37.5 pg/mL at the 100 µg/mL concentration ($P < 0.01$, Fig. 5B).

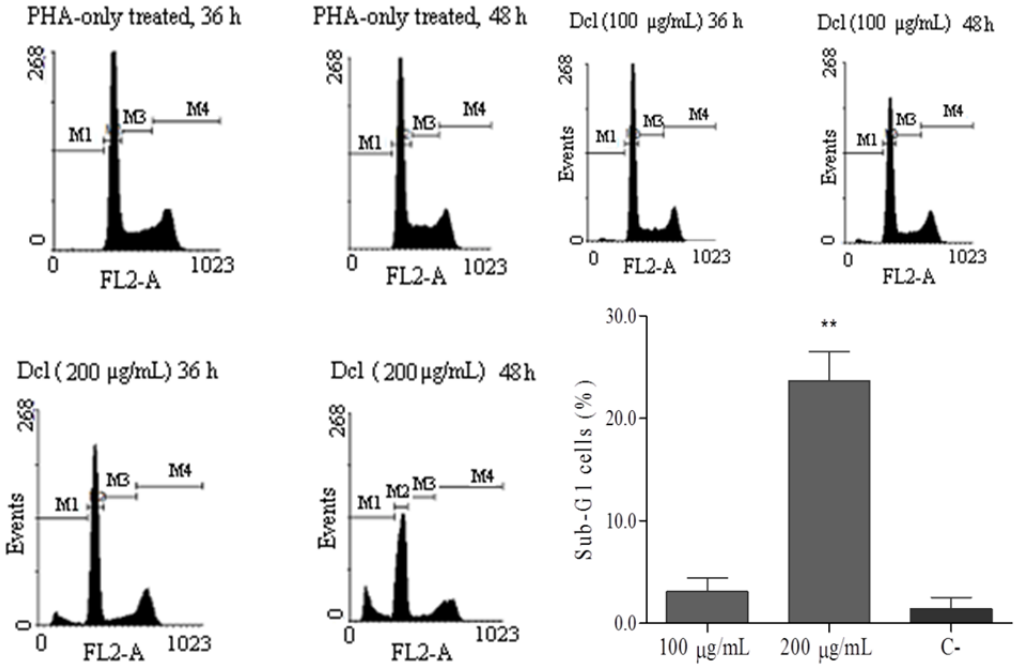

Fig. 4. Effect of *Foeniculum vulgare* dichloromethane fraction on the cell cycle of phytohemagglutinin-activated lymphocytes analyzed by flow cytometry. Representative DNA histogram plots (left) shows the effect of the fraction after 36 and 48 h. Negative control was dichloromethane-only treated cells (M1, sub-G1; M2, G0/G1; M3, S; M4, G2/M). Percentage of cells accumulated in sub-G1 phase after 48 h of treatment is shown in the right histogram. Values are mean ± SD of five independent tests. Dcl, dichloromethane; PHA, phytohemagglutinin. $^{**}P < 0.01$ compared to negative control (C-).

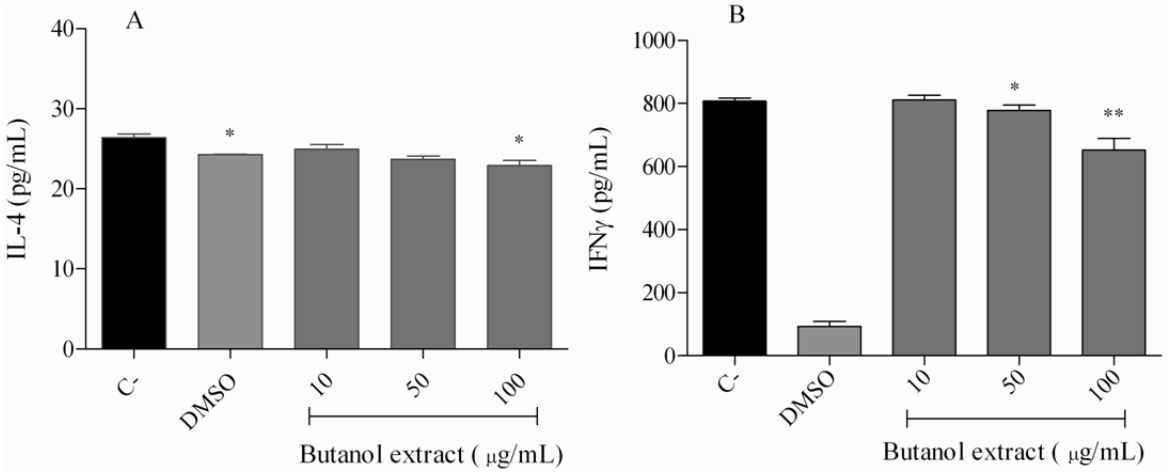

Fig. 5. The effect of *Foeniculum vulgare* butanol fraction on secretion of (A) IL-4, and (B) IFN-γ by phytohemagglutinin-activated lymphocytes. Stimulated peripheral blood lymphocytes were cultured in the presence of 10-100 μg/mL of butanol fraction for 48 h and then the culture supernatants were collected for cytokine measurement by ELISA. Controls were phytohemagglutinin-only treated cells (C-) and cells treated with DMSO (0.1%) without phytohemagglutinin and the fraction. Data showed the mean ± SD of two independent tests. **P* < 0.05, ***P* < 0.01 compared to phytohemagglutinin-only treated cells.

DISCUSSION

The capacity of medicinal plants to enhance or reduce the immune response has been shown in many studies (9). Various plants have immunoinhibitory effects through induction of apoptosis or modulation of cell proliferation and cytokine secretion (10-12). *F. vulgare,* an important medicinal plant in Iran, is currently used to treat various diseases including infections and inflammatory conditions (8). In our preliminary study, we have shown that the methanol extract of this plant had the capacity to inhibit proliferation of human lymphocytes. In this research, we sought to determine which fraction caused this inhibition. We prepared dichloromethane, hexane, butanol, and water fractions from the methanolic extract of *F. vulgare.* The effect of these fractions on cell proliferation of activated lymphocytes was examined and the results indicated that all fractions had different levels of inhibitory effects on the lymphocytes. The dichloromethane fraction had the highest level of inhibition, followed by the hexane, butanol, and water fractions. In the next step, we assessed the viability of the lymphocytes in the presence of these fractions by PI staining. According to the results of this experiment, we found that the dichloromethane fraction significantly reduced lymphocyte cell viability, whereas the butanol fraction did not affect cell viability. The hexane fraction mildly reduced cell viability. These data suggested that the mechanism of the inhibitory effects of the dichloromethane and butanol fractions might differ; possibly, the first one due to cell death and the latter by inhibition of cell activation/proliferation. Therefore, we selected the dichloromethane fraction to study its possible apoptosis-inducing effect on lymphocytes. We chose the butanol fraction for its possible effect on cytokine production. Annexin V/PI staining showed that the dichloromethane fraction, at 100 and 200 μg/mL, strongly induced apoptosis in the activated lymphocytes by ≈60%-90%. We examined the effect of this fraction on the cell cycle for the presence of sub-G_1 cells as a hallmark of apoptosis. Cell cycle analysis showed that 200 μg/mL of this fraction significantly increased the number of cells that accumulated in the sub-G1 phase after 48 h. This result supported the results of the annexin V/PI assay and the apoptosis-inducing effect of dichloromethane fraction on the activated lymphocytes. As shown, the percentage of sub-G1 cells in the dichloromethane fraction treated cells was less than the percentage of apoptotic cells according to the annexin/PI assay. This difference might have been due to our consideration of both early and late apoptotic cells in the annexin V/PI assay. Sub-G1 reflects DNA fragmentation which

occurs in the late stage of apoptosis. Hence, the number of sub-G1 cells is usually much less than other assays such as the annexin V/PI assay that detects both early and late apoptosis (13). The butanolic fraction had no cytotoxic effects on the cells. We measured the levels of two major cytokines involved in T cell responses. The ELISA results indicated that this fraction slightly decreased IL-4 levels and showed a stronger reduction of IFN-γ release. IFN-γ is the main TH1-type cytokine responsible for activation of macrophages and enhancement of antigen presentation. Overproduction of IFN-γ can lead to tissue damage and autoimmune diseases (14). IL4 is the major cytokine produced by TH2 cells. This cytokine increases the synthesis of neutralizing antibodies and is an antagonist of TH1 responses (14). To the best of our knowledge, there is no report about the apoptosis-inducing effect and decreased cytokine secretion of *F. vulgare* on human lymphocytes. A few studies have reported the immunomodulatory effects of *F. vulgare*. Mansouri, *et al*. reported that the hydroalcoholic extract of this plant had a stimulatory effect on white blood cells (15).

The acetone extract of *F. vulgare* increased NO production by peritoneal macrophages of treated male rats (16). In another study on the mouse fibrosarcoma (L929sA) cell line, Kaileh, *et al.* has reported the modulatory effect of *F. vulgare* methanol extract on nuclear factor (NF) κB, a transcription factor that plays an important role in inducible expression of genes involved in inflammatory responses and cell death (17). At present it is not clear which components of *F. vulgare* are responsible for its inhibitory effects on cytokine secretion, lymphocyte proliferation, and apoptosis. It is likely that various compounds with different modes of action are responsible for these effects. A variety of phenolic and other chemical compounds have been reported to be present in the *F. vulgare* plant (18). The major constituent of fennel oil is anethole. Yea, *et al.* have reported the immunoinhibitory effect of this component on proliferation of T lymphocytes in mice (19). Other constituents include α-pinene, α-phellandrene, β-pinene, camphene, estragole, fenchone, limonene, and p-cymene (20). Whether the phenolic compounds in the fractions or other constituents are responsible for the observed effects needs further investigation.

CONCLUSION

The results of this study showed that *F. vulgare* dichloromethane and butanol fractions might be suitable for more investigations in terms of their immune-modulatory effects on lymphocytes and their possible beneficial effects on down-regulation of immune responses in immune-mediated and autoimmune diseases.

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