

Original Research Article

Modulation of *in vitro* proliferation and cytokine secretion of human lymphocytes by *Mentha longifolia* extracts

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Abstract

Objective: *Mentha longifolia* L. Hudson has been used in folk medicine for various purposes especially for its anti-inflammatory effects. Lymphocytes play a central role in development of inflammation. In the present study, we investigated the immunomodulatory effects of different extracts of *M. longifolia* on human peripheral blood lymphocytes (PBLs), as main players in development of inflammation.

Materials and Methods: PBLs stimulated with phytohemagglutinin (PHA) were cultured in the presence of the plant extracts. The effects of the extracts on activation of cells were determined by BrdU assay. The viability of cells was examined by flow cytometry using propidium iodide staining. Also, IFN- γ (T helper 1, TH1) and IL-4 (TH2) secretion was measured by ELISA.

Results: Except for the water extract which had a weak inhibitory effect, treatment of cells with more than 1 $\mu\text{g/ml}$ of butanol, hexane, ethyl acetate and dichloromethane extracts resulted in strong inhibition of cells proliferation (IC_{50} 4.6-9.9 $\mu\text{g/ml}$). Flow cytometry analysis showed that these extracts at $\leq 10 \mu\text{g/ml}$ were non-cytotoxic. Dichloromethane and ethyl acetate extracts at 10 $\mu\text{g/ml}$ decreased IFN- γ production in a dose-dependent manner from 919 ± 91.1 pg/ml in PHA-only-treated cells to 568 ± 22.6 pg/ml (in dichloromethane-treated cells) and 329 ± 12.3 pg/ml (in ethyl acetate-treated cells) ($p < 0.001$). At 10 $\mu\text{g/ml}$, the ethyl acetate extract increased IL-4 secretion compared to PHA-only-treated cells ($p < 0.05$). The hexane extract decreased IFN- γ level but did not affect IL-4 production.

Conclusion: Reduction of IFN- γ and augmentation of IL-4 secretion induced by the extracts suggested the potential of *M. longifolia* to inhibit TH1 inflammatory responses toward a TH2 dominant response.

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Introduction

In general, immunomodulation refers to the alteration of immune responses which

leads to increased or decreased immune reactions (Zhou et al., 2016; Amirghofran, 2009). Synthetic mediators and antibodies

have been long used as immunomodulators. These agents have some adverse effects that confine their use (Morgan and Harris, 2003). Natural herbal or mineral compounds have been employed in many systems of medicine around the world especially in traditional medicine, to alter immune responses (Ebadi et al., 2014). Many medicinal plants are used to ameliorate immunological complaints (Karimi et al., 2012; Amirghofran, 2012). Therefore, medicinal plants could potentially be used as a replacement for synthetic therapy, for treatment of a variety of immunological diseases (Kumar et al. 2012). The immunomodulatory activities of medicinal plants that could be beneficial in infections and immune-related diseases have been reported (Amirghofran et al., 2011a; Amirghofran et al., 2011b; Gao et al., 2004). Previously, we showed that medicinal plants belonging to Lamiaceae family like *Thymus vulgaris* L. and *Zataria multiflora* Boiss have the capacity to reduce the proliferation of mitogen-stimulated lymphocytes and allogenic T cells (Amirghofran et al., 2011a; Amirghofran et al., 2011b). In another study on plants of the genus *Euphorbia*, three species of the genus *Euphorbia* including *E. microciadia* Boiss, *E. osyridea* Boiss, and *E. heteradenia* Jaub. & Sp. *in vitro* immunostimulatory effects on lymphocyte activation and cytokine secretion, was reported (Ghafourian Boroujerdnia et al., 2014). Also, medicinal plants like *Calendula officinalis* L. (Asteraceae), *Cichorium intybus* L. (Asteraceae), and *Salvia mirzayanii* Rech. f. & Esfand (Lamiaceae) that grow in Iran, have shown anti-proliferative activity against phytohemagglutinin (PHA)-activated lymphocytes (Amirghofran et al., 2000; Amirghofran et al., 2010).

Plants of the genus *Mentha* from the family Lamiaceae grow in different parts of the world. Different *Mentha* preparations are used for the treatment of medical conditions like bronchitis, nausea, flatulence, colitis, liver complaints, gum, and teeth diseases, due to their anti-

inflammatory, anti-emetic, diaphoretic, antispasmodic, antibacterial and antioxidant properties (Naghbi et al., 2005). *Mentha longifolia* L. Hudson (wild mint), as a member of this genus is used in the pharmaceutical, tobacco and food industries and particularly in cosmetics. Separate parts of this plant containing its leaves, flower, stem, bark, and seeds have been widely used in folk medicine as anti-microbial, anti-inflammatory, carminative, stimulant, and anti-spasmodic as well as in the treatment of various diseases such as bronchitis, headaches and digestive disorders (Bakht et al., 2014; Gulluce et al., 2015). In our previous study, anti-inflammatory effects of *M. longifolia* extracts on macrophages were shown by reductions in the secretion of inflammatory cytokines and mediators (Karimian et al., 2013). As lymphocytes play a central role in initiation and development of inflammation, in the present study, we aimed to explore the immunomodulatory effects of different extracts of *M. longifolia* including water, butanol, methanol, dichloromethane, hexane and ethyl acetate extracts on the proliferation of peripheral blood lymphocytes (PBLs) and evaluated their effects on T lymphocytes cytokine secretion pattern, in order to better understand the mechanisms underlying the anti-inflammatory and immunomodulatory effects of this plant.

Materials and Methods

Preparation of *M. longifolia* extracts

M. longifolia collected from the south-west of Iran in June 2016, were authenticated by Mrs. Sedigheh Khademian. A sample was deposited in the Herbarium of Shiraz University of Medical Sciences (No. 1326). The aerial parts of the plants were separated, washed, dried in the shade, powdered and defatted using petroleum ether. Samples were soaked in methanol for 48 hr at room temperature (RT). The crude methanol extract (yield:13.6%) was then concentrated and

extracted by hexane (3.3%), dichloromethane (1.2%), ethyl acetate (1.8%), butanol (4.8%) and water (8.2%). Afterwards, using a rotary evaporator, the extracts were concentrated and then freeze-dried. Extracts were later dissolved in dimethyl sulfoxide (DMSO, Sigma, St. Louis, MO) and then diluted in RPMI 1640 culture medium (Sigma) to reach final concentrations of 0.1-200 μ g/ml.

Proliferation assay

Blood samples were taken from five healthy non-smoking males with no chronic diseases. The subjects did not receive any medication during three months before sampling and were within the age range of 25-35 years. The study was approved by the Ethics Committee of Shiraz University of Medical Sciences and informed consent was obtained from the subjects. PBLs were isolated by gradient centrifugation using Gibco Lymphodex (Germany). The viability of PBLs was more than 95% as assessed by trypan blue staining test. For analysis of the effect of different *M. longifolia* extracts on the proliferation of lymphocytes, PBLs were seeded (1×10^5 cells/well) in wells of a 96-well microplate in the presence of PHA (Fluka, Germany, 1/100) as the mitogen and analyzed by a 5-bromo-20-deoxy-uridine (BrdU) assay kit (Roche Diagnostics, Germany). Briefly, PBLs were treated with 0.1-200 μ g/ml concentrations of the hexane, dichloromethane, ethyl acetate, butanol and water extracts and cultured for 48 h at 37°C in a humidified atmosphere with 5% CO₂. For negative control, the cells were treated with PHA and DMSO at the highest concentration used in the test wells (i.e. 0.1%) (PHA-only treated cells). After treatment with BrdU for 18 hr, cells DNA was denatured and then anti-BrdU monoclonal antibody was added. The optical density (OD) of each sample was determined using a microplate reader (Biotek, Winooski, VT) at 450 nm reading filter using a reference filter set at 630 nm. The inhibition percentage of proliferation

was determined as follows: $100 - [(OD \text{ of treated cells} / OD \text{ of negative control}) \times 100]$. Finally, 50% growth inhibitory concentration (IC₅₀) for each extract was determined.

Viability assay

PBLs stimulated by PHA were seeded in 96-well microplates (1×10^5 cells/well) and incubated with 0.1-200 μ g/ml of each extract. Negative control was PHA-only treated cells. After 48 hr, cells viability was assessed by propidium iodide (PI) staining. The cells were harvested and washed with cold phosphate-buffered saline (PBS) twice and then re-suspended in PBS to reach 1×10^6 cells/ml concentration. Then, PI solution at a final concentration of 2 μ g/ml was added to all suspensions except the unstained tube. Cells were incubated in the dark at 4°C until analyzed by a flow cytometer (FACSCalibur, Becton Dickinson, San Jose, CA). A minimum of 1×10^4 events per test population, was analyzed. The percentage of PI positive cells was determined using FlowJo7.6 software (Tree Star, Inc., Ashland, OR). The dot plot diagrams of cells were prepared and cells were gated based on forward scatter (FSC) and side scatter (SSC) and debris was gated out. Histogram of gated cells was plotted.

Quantification of cytokines by ELISA

PBLs stimulated with PHA were seeded in 96-well microplates (1×10^5 cells/well) and incubated in the presence of non-cytotoxic concentrations of the extracts (0.1-10 μ g/ml) for 48 hr. The level of IL-4 and IFN- γ cytokines in the supernatant of cultures were measured by ELISA kits (both with the sensitivity of 4 pg/ml) obtained from eBioscience (Frankfurt, Germany). PHA-only treated cells and cells treated only with the solvent (0.01% DMSO) were used as controls. After coating the plate with capture antibody at 4°C overnight, the wells were washed with washing buffer three times and covered by bovine serum albumin (BSA) 3% as

blocking protein for 1 hr at 25°C. In the next step, standards and samples were added and the plates left at 4°C overnight. The wells were washed for the second time and cells were incubated with kit provided detected antibody for 1 hr at RT. Then, the supernatant of wells was aspirated and the avidin-horseradish peroxidase solution was added for 30 min at 25°C. Afterward, the substrate and stopping solution were added and the OD was read at 450 nm using an ELISA reader (Biotek). The level of each cytokine was proportional to the intensity of the color of each well and was quantified using a standard curve.

Statistical analysis

Data were analyzed by one-way ANOVA and Student's *t*-test using GraphPad, Prism 5 software (San Diego, CA). The level of significance was set at $p < 0.05$.

Results

Effects of *M. longifolia* extracts on PBLs proliferation in the presence of PHA

PHA-activated PBLs were treated with 0.1-200 $\mu\text{g/ml}$ concentrations of *M. longifolia* extracts and then cell proliferation was measured by BrdU incorporation assay. As shown in Figure 1, except for the water extract which had a weak inhibitory effect, other extracts at concentrations $>1\mu\text{g/ml}$ resulted in strong inhibition of cell proliferation. Calculation of the IC_{50} values for the extracts showed that the ethyl acetate extract with an IC_{50} value of $4.6 \pm 1\mu\text{g/ml}$ had the highest inhibitory effect. IC_{50} values for other extracts were as follows: butanol ($5.3 \pm 1.6\mu\text{g/ml}$), dichloromethane ($7.9 \pm 1.5\mu\text{g/ml}$), hexane ($9.9 \pm 1\mu\text{g/ml}$) and water ($159 \pm 15\mu\text{g/ml}$), respectively.

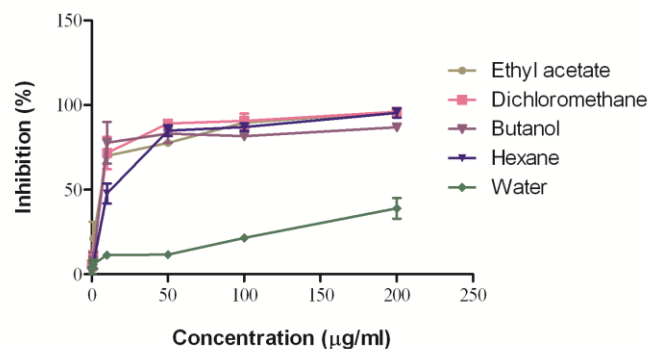
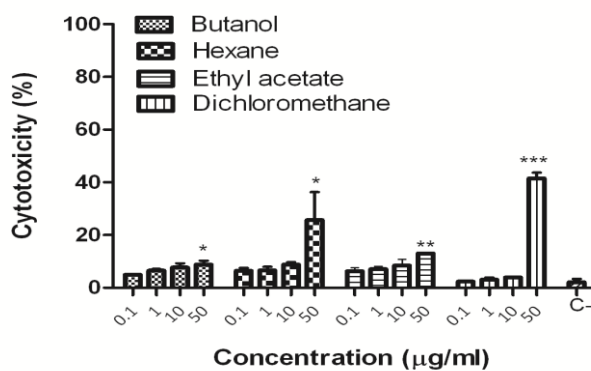


Figure 1. Effect of *Mentha longifolia* extracts on the proliferation of peripheral blood lymphocytes (PBLs) as assessed by BrdU incorporation assay. After isolation of PBLs and activation with PHA, PBLs were treated with different concentrations of the extracts for 48 hr. PHA-only treated cells were considered negative control. Data are presented as mean \pm SD of percentage of cell proliferation inhibition. EA; ethyl acetate, Dcl; dichloromethane.

Effects of *M. longifolia* extracts on PBLs viability

According to the results of cell proliferation assay, ethyl acetate, butanol, dichloromethane, and hexane extracts which had strong inhibitory effects on the lymphocytes activation, were chosen and their possible cytotoxic effects against PBLs were evaluated using PI staining method. Cytotoxicity of the extracts was determined by measuring the percentage of PI positive cells. As demonstrated in Figure 2A, treatment of cells with $50\mu\text{g/ml}$ of the ethyl acetate and butanol extracts, led to a significant increase in PI^+ cells compared to the PHA-only treated cells. The percentage of PI^+ cells after treatment with hexane and dichloromethane extracts at concentrations $\leq 10\mu\text{g/ml}$, was lower than 10%; however this value at $50\mu\text{g/ml}$ increased to $25.7 \pm 10.6\%$ (for hexane extract, $p < 0.05$) and $41.6 \pm 2.2\%$ (for dichloromethane extract, $p < 0.001$), showing that these extracts exert cytotoxic effects at this concentration. Thus, the concentration of $50\mu\text{g/ml}$ of the four extracts was excluded and other concentrations were assayed for cytokine secretion. Figure 2B shows the flow cytometry histograms obtained following PI staining.

A)



B)

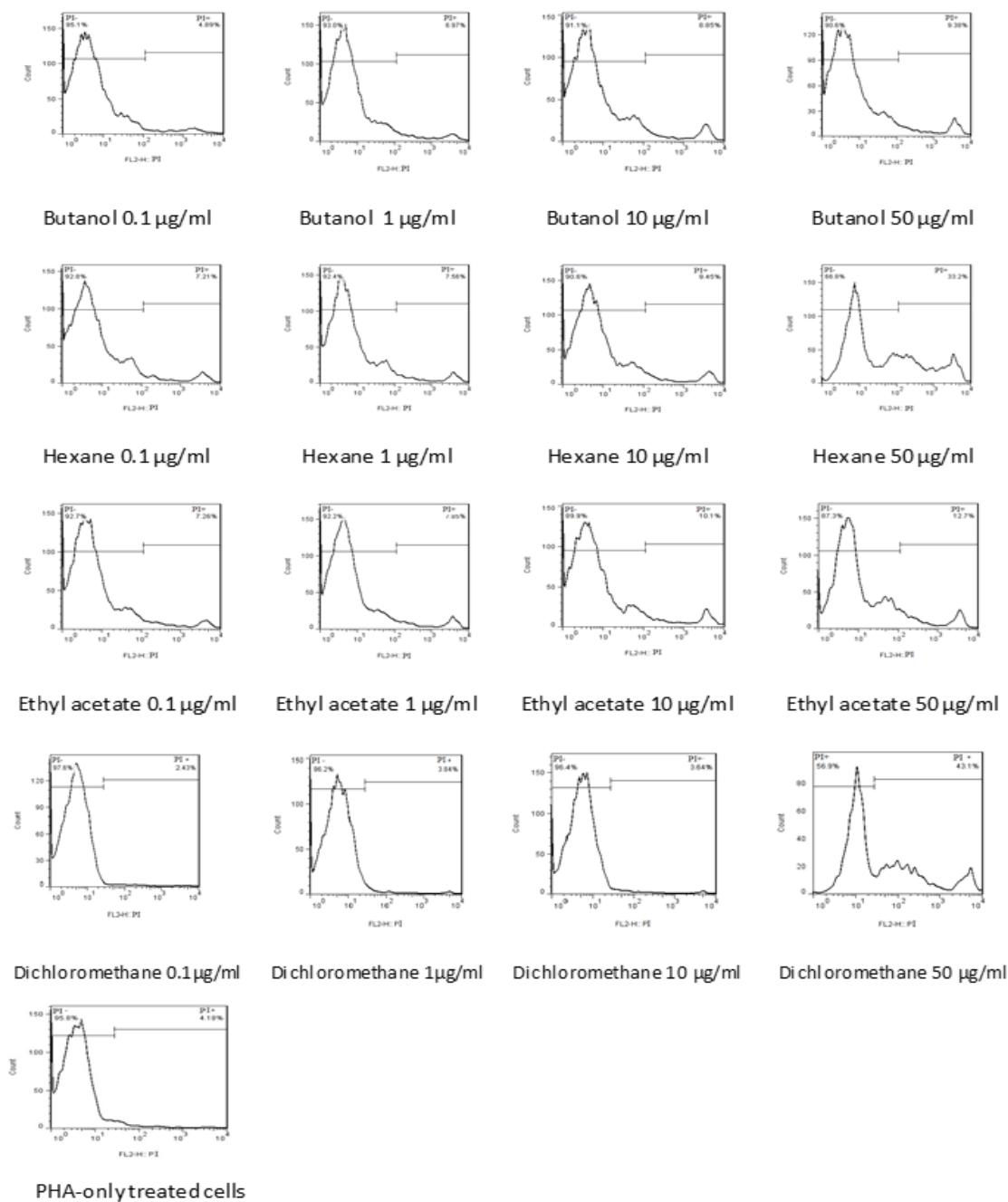


Figure 2. Effects of *Mentha longifolia* extracts on the viability of PBLs as assessed by propidium iodide (PI) staining and flow cytometry. After isolation of PBLs and activation with PHA, PBLs were treated with different concentrations of the extracts for 48 hr. Negative control (C-) was PHA-only treated cells. Cytotoxicity of the extracts was determined by measuring the percentage of PI positive cells. A) Bars are mean±SD of two different experiments. *p<0.05, **p<0.01 and ***p<0.001 compared to negative control. B) Flow cytometry histograms are representative of the patterns of PBLs treated with different concentrations of the extracts. PI⁺ are dead cells while PI⁻ are live cells.

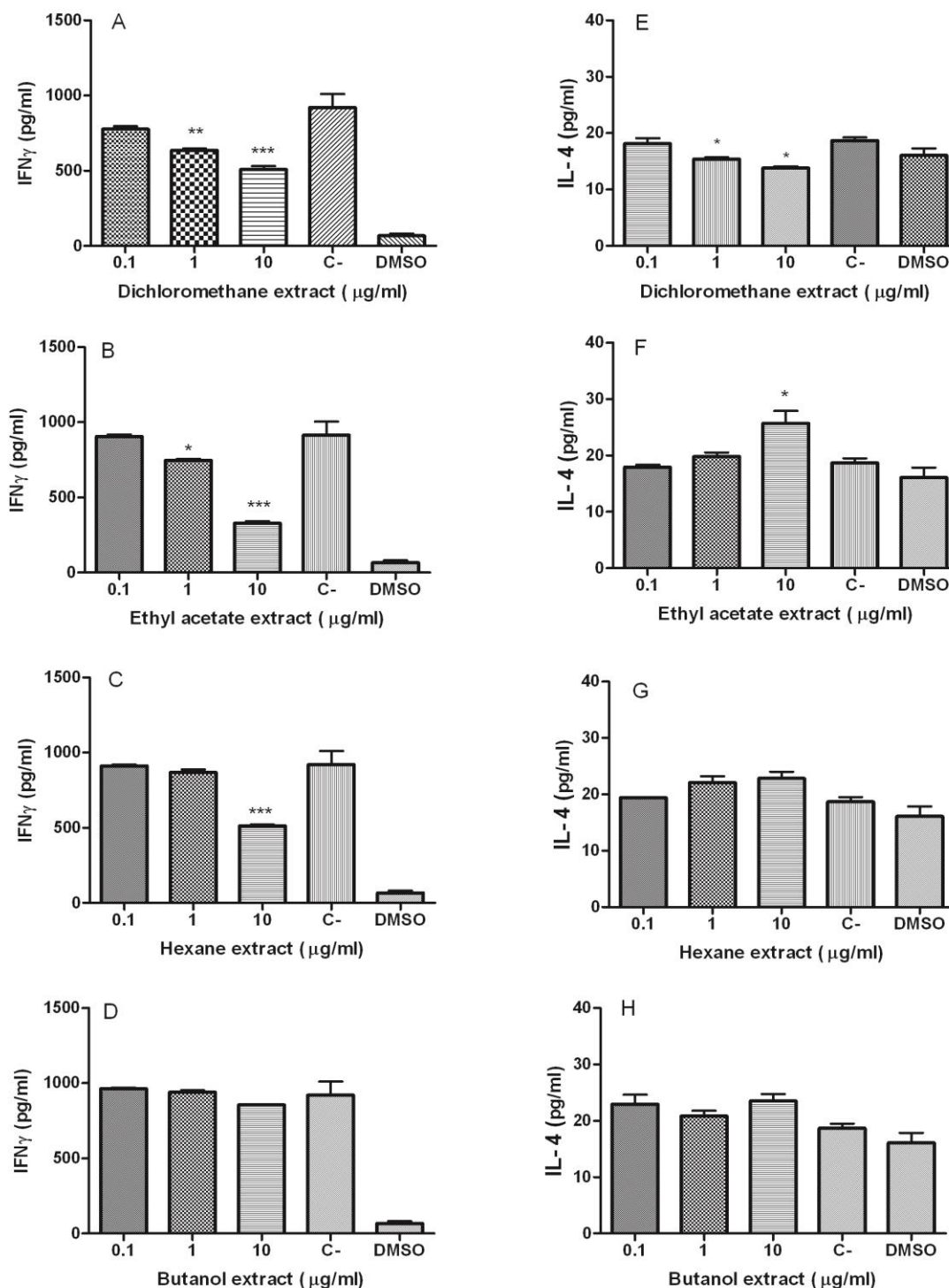


Figure 3. Effect of *Mentha longifolia* extracts on IFN-γ (A-D) and IL-4 (E-H) production as measured by ELISA. After 48 hr treatment of PBLs with the extracts in the presence of PHA, the supernatants were collected for

cytokine analysis. Controls were PHA-only treated cells (C-) and cells treated with the solvent (0.01% DMSO) without PHA and the extract. Data are presented as mean \pm SE. * p <0.05, ** p <0.01, and *** p <0.001 show statistically significance differences as compared to PHA-only treated cells.

The effect of *M. longifolia* extracts on cytokine secretion

The non-cytotoxic concentrations of the extracts were used to examine their effects on IL-4 and IFN- γ secretion. PHA-activated PBLs were cultured in the presence of 0.1-10 μ g/ml of the extracts and then the supernatants were collected for measurement of cytokine levels by ELISA. As shown in Figure 3A-D, stimulation of PBLs with PHA (C-) resulted in a marked increase in IFN- γ levels (919 \pm 91.1 pg/ml) compared to the DMSO-treated cells (67.8 \pm 14.4 pg/ml) (p <0.001). Treatment of cells with the dichloromethane and ethyl acetate extracts decreased IFN- γ production in a dose-dependent manner. The level of this cytokine in the presence of dichloromethane extract was 776 \pm 19.7 pg/ml at 0.1 μ g/ml which decreased to 568 \pm 22.6 pg/ml (p <0.001) at 10 μ g/ml (Figure. 3A). The ethyl acetate extract also reduced IFN- γ secretion from 905 \pm 11.7 pg/ml (0.1 μ g/ml) to 746 \pm 10 pg/ml (1 μ g/ml, p <0.05) and 329 \pm 12.3 pg/ml (10 μ g/ml, p <0.001) (Figure 3B). A significant difference in IFN- γ level was observed between 10 μ g/ml hexane extract (512 \pm 9.3 pg/ml) and PHA-only treated cells (p <0.001) (Figure 3C). The butanol extract showed no significant effect on IFN- γ production (Figure 3D).

The effects of the extracts on IL-4 level are shown in Figure 3E-H. Butanol extract at none of the concentrations used, had significant effect on IL-4 secretion (Figure 3H). In cells treated with the dichloromethane extract, IL-4 level was mildly reduced as the concentration of the extract increased so that at the concentration of 10 μ g/ml, the lowest level of IL-4 (13.8 \pm 0.4 pg/ml, p <0.05) was achieved (Figure 3E). In contrast to other extracts, the ethyl acetate extract significantly increased IL-4 production by stimulated PBLs (25.7 \pm 2.2 pg/ml compared

to 18.7 \pm 0.8 pg/ml in PHA-only treated cells) (Figure 3F). Although the hexane extract induced a mild increase in IL-4 level at 10 μ g/ml, but this increase was not significant (Figure 3G).

Discussion

Macrophages play a key role in chronic inflammation by producing cytokines such as IL-1 and TNF- α and mediators like NO (Gholijani et al., 2016). Lymphocytes mount up later during the inflammatory process and interact with antigen-presenting cells such as dendritic cells and macrophages, to become activated (Karimian et al., 2014). Among T cell populations, the T helper (TH) cells play a critical role in the initiation and maintenance of inflammatory process. Infiltration of TH cells into the inflamed tissue may lead to tissue damage in various diseases such as allergic and autoimmune diseases (Tahvili et al., 2015). A main characteristic of T cell activation is the production of cytokines. According to the set of cytokines they produce, several TH subpopulations such as TH1 and TH2 were defined (Hirahara and Nakayama, 2016). Various medicinal plants used in traditional medicine with immunomodulatory activity and effects on T cell subsets, have been reported (Gharagozloo and Amirghofran, 2007; Abtahi Froushani et al., 2015; Gholijani et al., 2015). *M. longifolia* is an aromatic plant with antioxidant activities (Eissa et al., 2014). This plant has been reported as a useful therapy without adverse effects to be used in various conditions such as diabetes, inflammation and amenorrhea (Mokaberinejad et al., 2012). The protective effects of moderate doses of this plant in a rat model of colitis induced by acetic acid were possibly mediated through its antioxidant and antiinflammatory

activities (Murad et al., 2016). Previously, we showed the anti-inflammatory, potent anti-oxidant and NO- scavenging activities of *M. longifolia* (Karimian et al., 2013). As the effect *M. longifolia* on the lymphocytes had not been investigated, in this study, we decided to evaluate this effect by determining the proliferation and cytokine secretion status of PHA-activated PBLs after exposure to different extracts of this plant. PHA is a lectin with the ability to act as a mitogen for T cells and trigger the activation and proliferation of these cells. Results from proliferation assay showed that all five different extracts of this plant had weak-to-strong growth inhibitory activity on human PBLs. Among the extracts, the ethyl acetate extract with an IC_{50} value of 4.6 ± 1 $\mu\text{g/ml}$ was the strongest. Dichloromethane, hexane and butanol extracts followed ethyl acetate extract in terms of growth inhibition potency and the water extract with an IC_{50} value greater than 150 $\mu\text{g/ml}$ had the lowest inhibitory activity. Assessment of the viability of mitogen-activated PBLs by PI assay, indicated that 50 $\mu\text{g/ml}$ of the ethyl acetate, dichloromethane, hexane and butanol extracts decreases the viability of cells; therefore, concentrations of 0.1, 1 and 10 $\mu\text{g/ml}$ of these extracts were used for cytokine analysis. In cytokine assays, the butanol extract showed no significant effects on IFN- γ and IL-4 production, whereas the dichloromethane extract significantly decreased the secretion of both cytokines by PHA-activated lymphocytes. Approximately 42 and 26% reductions in IFN- γ and IL-4 levels, respectively at 10 $\mu\text{g/ml}$ were observed which suggested a general inhibitory effect of this extract on TH1 and TH2 cells. At the same concentrations, the ethyl acetate extract suppressed IFN- γ secretion; however, in contrast to the dichloromethane extract, ethyl acetate extract increased the IL-4 production level at 10 $\mu\text{g/ml}$, suggesting the effect of this extract on deviation of T cells toward a TH2 pattern. The hexane extract, similar to the dichloromethane and ethyl

acetate extracts decreased IFN- γ secretion but had no significant effects on IL-4 production. In general, naive T cells treated with an antigen or a mitogen, could be activated and differentiated into two TH1 and TH2 cell types (Hirahara and Nakayama, 2016). TH1 cells principally secrete IFN- γ which is considered a pro-inflammatory cytokine, while TH2 cells produce mostly IL-4 and IL-10, which are considered anti-inflammatory cytokines (Hirahara and Nakayama, 2016). IL-4 can act in anti-inflammatory pathways in autoimmune diseases by driving naïve T cell differentiation towards the TH2 cell (Zhang et al., 2016). Moreover, TH2 cells, which produce the cytokines IL-4, IL-5, and IL-13, are involved in allergic responses and the clearance of extracellular bacteria and pathogens such as worms. IFN- γ -producing TH1 cells contribute to elimination of intracellular pathogens and they are involved in cell-mediated and delayed-type hypersensitivity responses (Gholijani and Amirghofran, 2016). Over activation of TH1 pathway could result in various autoimmune diseases such as diabetes, multiple sclerosis, rheumatoid arthritis and inflammatory bowel diseases (Zoghi et al., 2011; Yoshioka et al., 2017; Mkaddem et al., 2009). According to the results obtained in this study, dichloromethane, ethyl acetate and hexane extracts inhibited IFN- γ release and the ethyl acetate extract enhanced the levels of IL-4 as an antiinflammatory cytokine. These data are in line with our previous study regarding the antiinflammatory effects of *M. longifolia* extracts on macrophages (Karimian et al., 2013). In our study, *M. longifolia* decreased NO secretion and iNOS gene expression, and reduced the expression of TNF- α as a proinflammatory cytokine by macrophages. *M. longifolia* also showed strong antioxidant activity and various extracts of this plant at concentrations > 0.2 mg/ml had the capacity to scavenge radicals. This activity was likely due to high phenolic content and the presence of other compounds such as

flavonoids. It is known that plants-extracted antioxidants such as polyphenols play an important role in the protection of cells from oxidative damage, a process that usually comes with inflammatory conditions (Yoshioka et al., 2017). In various studies, the chemical composition of *M. longifolia* was studied and several compounds such as pulegone, isomenthone, cineole, borneol, and piperitenone oxide have been identified (Mkaddem et al., 2009). Identification of the compounds responsible for the observed immunoinhibitory effects on the PBLs and evaluation of the role of the plant antioxidant activity in these effects, need further investigations.

In conclusion, this study showed that dichloromethane, ethyl acetate and hexane extracts of *M. longifolia* had the capacity to inhibit the secretion of IFN- γ , as a main proinflammatory cytokine of TH1 subset of T cells. In addition to this effect, the ethyl acetate extract increased IL-4 antiinflammatory cytokine release as the main cytokine of TH2 cells. These data suggested the potential of *M. longifolia* plant to be used against TH1 dominant diseases.

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Conflicts of interest

The authors declare no conflicts of interest.

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