Modulation of CD4$^+$ T Cell Subsets by *Euphorbia microciadia* and *Euphorbia osyridea* Plant Extracts

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ABSTRACT

Background: Euphorbia plants are traditionally used in folk medicine for infections, inflammation, and cancer. **Objectives**: To investigate the effects of the butanolic extracts of *Euphorbia micorociadia* and *Euphorbia osyridea* on specific transcription factors and cytokines expression of T cell subsets. **Methods:** Activated mouse splenocytes were cultured in the presence of non-cytotoxic concentrations of the extracts. Cells were evaluated for the gene expressions of T cell transcription factors and cytokines of T helper (Th)1 [T-bet and interferon gamma (IFNγ)], Th17 [retinoic acid receptor related orphan receptor (RORγt) and interleukin (IL)-17], and T regulatory (Treg) cells [forkhead box P3(Foxp3), IL-10, and Transforming growth factor (TGF)-β] using real-time PCR. The cytokine secretions were evaluated by ELISA and Foxp3 protein expression by flow cytometry. **Results:** Both *E. osyridea* and *E. microciadia* extracts at 0.1 μg/ml increased T-bet expression [>1.73 relative fold change (RFC), $p<0.05$] and IFNγ production (>1195 pg/ml, $p<0.001$). Both decreased Foxp3 (<0.41 RFC, $p<0.05$) expression. At the higher concentration both extracts significantly reduced T-bet mRNA as well as IFNγ, IL-17, IL-10, and TGF-β cytokines and Foxp3 at the mRNA and protein levels. **Conclusion:** These data showed the immunomodulatory effects of *E. osyridea* and *E. micorociadia* extracts on T cell-mediated responses. The extracts caused upregulation of Th1 and downregulation of Treg cells at a low concentration which suggested their possible therapeutic value in tumor models and infectious diseases. The observed immunosuppressive effects at the higher concentration potentially make these plants candidates for identification of active components and studying their mechanisms of action.

Namdari H, et al. Iran J Immunol. 2017; 14(2):134-150.

Keywords: *Euphorbia microciadia*, *Euphorbia osyridea*, T cell subsets

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INTRODUCTION

T helper (Th) cells are the most critical of the immune effector cells. It is essential to modulate the activation and/or inhibition of these cells in various diseases such as infections, tumors, and immune-mediated disorders. Th1 and Th17 are two main subsets of Th cells that are critical in inflammatory and cellular immune responses (1). Th1 cells express the transcription factor T-bet and are characterized by the production of interferon gamma (IFNγ). Th1-dominated responses are particularly important in host defence aginst intracellular infectious agents (2).

Th17 cells produce interleukin (IL)-17 and express the transcription factor retinoic acid receptor related orphan receptor (ROR)γt. Th17 responses are useful for protection against fungi and extracellular pathogens. When Th responses are exhaustively prolonged, host pathology may result (3).

Th1 cells are considered the primary Th cell subset important in anti-tumor responses. These cells are associated with anti-tumor responses in humans and mouse models, which is achieved in part by their production of IFNγ (4-6). The role of Th17 cells in the tumor microenvironment is not well understood and is controversial. Different studies show negative roles of these cells in tumor immunity (7,8). In contrast, some new studies have suggested an anti-tumor role for Th17 cells (9,10).

Uncontrolled Th1 responses against self-antigens can cause the development of autoimmunity. Several lines of evidence point to Th1 cells as the major effector T cells responsible for inducing experimental autoimmune encephalomyelitis (EAE) and potentially multiple sclerosis (11). In addition, Th17 cells are mainly suited for the promotion of autoimmunity due to the significant role of IL-17 in inducing tissue inflammation (12). Accordingly, high levels of IL-17 have been detected in several autoimmune diseases such as multiple sclerosis, rheumatoid arthritis and psoriasis (12-14).

While effector T cells promote inflammation, T regulatory (Treg) cells control it. Treg cells are mainly characterized by expression of the transcription factor forkhead box P3 (Foxp3). They play an extremely important role in autoimmune pathogenesis by sustaining self-tolerance in addition to controlling expansion and activation of autoreactive $CD4^+$ effector T cells. The function of Treg cells is partially mediated by the production of inhibitory cytokines such as IL-10 and transforming growth factor (TGF)-β (15,16).On the other hand, one consequence of the modulation of excessive immune responses by Treg cells is enhanced pathogen survival and, in some cases, long-term persistence (16).

During past centuries, numerous countries worldwide have used medicinal plants in traditional medicine (17). Studies have evaluated the immunomodulatory properties of numerous medicinal plants (18). Plants and related natural products which possess immunomodulatory activities are good candidates for the treatment of various illnesses such as autoimmune disorders and tumors, as well as infections (19).

Plants of the Euphorbia genus (*Euphorbiaceae*), known as ‘‘Farfion (Shirsag)’’ in Persian, include over 1000 species located throughout temperate and tropical areas. A total of 17 out of 70 reported species are endemic to Iran. Euphorbia is used in traditional medicine as treatment of inflammations, infections, and tumors (20). The *Euphorbia microciadia* [*E. microciadia* (Farfioun-e Esfahani)] and *Euphorbia osyridea*

[*E. osyridea* (Farfioun-e Khashbi)] plants are widely distributed throughout central and southern Iran, with less in the northern areas (21). There are a few reports of the chemical compositions and biological effects of these plants. Several flavonol glycosides from *E. microciadia* have reported immunomodulatory activities (22). These flavonoids primarily inhibited lymphocyte proliferation when used at a high concentration (50 μg/ml) (22). Our previous study showed the immunomodulatory effects of the butanolic extract of these plants (21). In the present study we have examined their effects on mice Th1 and Th17 cell subsets considered to be important in tumor immunity, infection, and inflammatory diseases. We also researched the effects of the extracts on Treg cells as one of the most important components for regulation of the immune system and assistance with avoiding immune-mediated pathology.

MATERIALS AND METHODS

Reagents. Dimethyl sulfoxide (DMSO), trypan blue, propidium iodide and Roswell Park Memorial Institute 1640 (RPMI 1640) culture medium were obtained from Sigma St. Louis, MO. Fetal bovine serum (FBS) was obtained from Roche (Germany), 5-bromo-2′-deoxy-uridine (BrdU) kit from Gibco (Ashland, KY) and Lymphodex from Inno-Train Diagnostic (Kornberg, Germany). Phosphate-buffered saline (PBS) was purchased from Lonza (Switzerland) and Concanavalin A (Con A) from Fluka (Germany). Anti-CD3 and Anti-CD28 monoclonal antibodies were purchased from BD Biosciences (Pharmingen, San Diego, CA). RNXTM-plus solution kit for RNA extraction was obtained from Sinagen (Tehran, Iran). High-capacity cDNA reverse transcription kit was purchased from Applied Biosystems (Foster City, CA) and SYBR® Green qPCR SuperMix-UDG with ROX from TaKaRa (Shuzo, Kyoto, Japan). IL-10, IFNγ, TGF-β and IL-17 enzyme-linked immuosorbent assay (ELISA) kits was obtained from eBioscience (San Diego, CA). Reagents used for flow cytometry including PE-conjugated anti-Foxp3 antibody, FITC-conjugated anti-CD4 antibody, APC-conjugated anti-CD25 antibody and fixation/permeabilization kit were purchased from Becton Dickinson (BD) Biosciences, San Jose, CA). Mouse naïve $CD4^+$T cell isolation kit was obtained from Miltenyibiotec, Germany, Serum free culture medium X-vivo-15 from Invitrogen, MO, TGF-β and IL-2 from Life Technologies, MO. Other chemicals and solvents were of reagent grade and available.

Animals. BALB/c female mice, at 6–8 weeks of age (25–30 g body weight) were obtained from Center of Experimental and Comparative Medicine of Shiraz University of Medical Sciences. The animals were housed under standard environment and maintained on a standard mouse chow and drinking water *ad libitum*. All protocols for animal care and treatment were accepted by the ethics committee of Shiraz University of Medical Sciences.

Preparation of Plant Extracts. Euphorbia aerial parts were collected in May from Fars Province and identified by Dr. Khosravi from Biology Department of Shiraz University. A sample from each plant was preserved in the local herbarium. Methanol was used as the initial extractant and maceration was carried out at room temperature (RT) for two days (22). After filtration and concentration of the methanol extracts, the resultants were suspended in 0.5 liter of water and re-extracted with butanol. We prepared the butanol extracts because these extracts had shown the highest modulatory effects in the previous study (22). The extracts were concentrated under vacuum using a rotavap and then

lyophilized. For the experiments, samples were dissolved in DMSO and then they were diluted in the RPMI 1640 culture medium to obtain a 20 mg/ml solution.

Splenocyte proliferation assay. After the mice sacrificed, spleens were quickly dissected and rinsed in sterilized PBS and washed four times with RPMI 1640 medium. Spleens were smashed using the plunger end of a 3 ml syringe until completely dissociated. Mononuclear cells were then separated by centrifugation over Lymphodex cell separation medium and after washing twice with PBS, they were counted using 0.4% trypan blue stain. Cells were seeded into 96-well culture microplates (10^5 cells/well/100 µl) to determine the effects of the extracts on splenocytes proliferation using a BrdU assay as previously described (24). Briefly, cells were treated with various concentrations of the extracts (0.01– 50 µg/ml) in the presence of Con A (2 µg/ml) for 72 h. The positive control was cells treated only with Con A and the negative control was those cells with no treatment (neither the extract nor Con A). Control wells contain DMSO as the solvent at the highest concentration used in the test wells (0.05%). After labeling with BrdU, DNA was denatured and the cells were incubated with peroxidase-conjugated anti-BrdU monoclonal antibody for detecting incorporated BrdU. The substrate solution was added and then the optical density (OD) of the samples was measured in a microplate reader at 450 nm and a reference wavelength of 690 nm. The OD of cells treated with the extracts to the OD of positive control was calculated and considered as the proliferation index (PI). Experiments were done in triplicate and each experiment was performed at least three times.

Cell viability assay. To determine the non-cytotoxic concentrations of the extracts, their effect on the viability of stimulated splenocytes was evaluated using propidium iodide staining by flow cytometry. Cells were seeded into 96-well culture microplates (10^5 cells/well/100 µl) and treated with various concentrations of the extracts in the presence of Con A (2 µg/ml) as mentioned above. Cisplatin, a cytotxic drug (50 µg/ml) was added in triplicate wells as positive control. The negative control was cells treated only with Con A. Control wells had DMSO as the solvent at the highest concentration used in the test wells. Cells were incubated at 37°C in humidified incubator with 5% of CO_2 atmosphere for 72 h. After incubation time, propidium iodide assay was performed according to the method previously described with some modifications (25). Cells were collected and after centrifugation to remove media, they were resuspended in PBS containing 5 µg/ml propidium iodide. During 5 min the fluorescence intensity was analyzed using a FACSCalibur flow cytometer (Becton Dickinson Biosciences, San Jose, CA). Cell death was expressed as the percentage of high red fluorescence for each treatment versus the negative control. Dot plots and statistics were designed with the FlowJo software Version 7.6.5 (TreeStar Inc, Ashland, OR).

Real time-polymerase chain reaction (PCR) analysis of transcription factors and cytokines. To determine the effects of the extracts on mRNA expression of T cell specific transcription factors and cytokines, real time-PCR was performed on splenocytes treated with 0.1 and 1 µg/ml of the extracts. These non-cytotoxic concentrations were selected based on the results of BrdU cell proliferation and propidium iodide cell viability assessments. In this step we used anti-CD3 and anti-CD28 monoclonal antibodies to clone and expand mouse T cells. Splenocytes at a density of 5×10^5 cells/well were cultured in 24-well culture microplates coated with

anti-CD3 monoclonal antibody (2.5 µg/ml/well). After addition of anti-CD28 monoclonal antibody (2 µg/ml/well) and the extracts, cells were incubated at 37°C in a humidified incubator with 5% of CO2 atmosphere. Cells treated only with antibodies without the extracts were considered as positive control and cells without any treatment (neither antibodies nor extracts) were considered as negative control. After 72h, well supernatants were collected and stored at -80°C for cytokines assessment and the cells were used for real time-PCR analysis. Cells were washed with cold PBS and then RNA was extracted using RNA extraction kit RNXTM-plus solution (CinnaGen Inc., Iran) according to the manufacturer's instructions. For determination of the concentration and quality of the extracted RNA, the relative absorbance of OD260/OD280 was measured and the banding pattern of RNA in agarose gel electrophoresis was visualized under UV illumination. Then, cDNA synthesis was carried out using a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems) with the use of random primers. Primer designing was carried out using Primer Express™ software (Applied Biosystems, Foster City, CA). The sequences of primers are shown in Table 1.

Table 1. Primer sequences used for real-time PCR.

Primer name	Primer sequence	Gene accession number
GAPDH	F 5′-CGGTGTGAACGGATTTGGC-3′	NM_001289726.1
	R 5′-GTGAGTGGAGTCATACTGGAAC-3′	
T-bet	F 5′- TAAGCAAGGACGGCGAAT -3′	NM_019507.2
	R 5′-GGTGGACATATAAGCGGTTC-3′	
RORγt	F 5′- AAGAGAAGAGGAGAGTGGAA-3′	NM_011281.3
	R 5′- GTGGAGGTGCTGGAAGAT -3′	
Foxp3	F 5′- AATAGTTCCTTCCCAGAG-3′	NM_001199347.1
	R 5′- GATTTCATTGAGTGTCCT -3′	
IL-17	F 5′- AACACTGAGGCCAAGGACTTC -3′	NM_010552.3
	R 5′- GTCTTCATTGCGGTGGAGAGT-3′	
IFNγ	F 5′- GAGGAACTGGCAAAAGGATGGT -3′	NM_008337.4
	R 5′- CGCTTATGTTGTTGCTGATGGC -3′	
IL-10	F5′-GCAGGACTTTAAGGGTTACTTGGG-	NM_010548.2
	3′ R5′- GATTTCTGGGCCATGCTTCTC -3′	
TGF-β	F 5′- GCAACAACGCCATCTAT-3′	NM_011577.2
	R 5′- AAGGTAACGCCAGGAAT -3′	

The real time-PCR was performed in duplicate with the SYBR® Green qPCR SuperMix-UDG with ROX on ABI thermocycler (Foster City, CA). Reaction mixes were prepared with 200 nM of each primer pair and the cDNA (10 ng) added last. Each reaction consisted of 1 cycle of 95°C for 30 s, followed by 40 cycles of 95°C for 5 s, annealing temperature (specific for each primer) for 18 and 72°C for 30 s. Results of

target mRNA levels were normalized against GAPDH mRNA in each sample. All target genes results were shown as relative fold change (RFC) to positive control.

Cytokine assay. Total levels of cytokines including IFNγ, IL-17, IL-10 and TGF-β in the supernatants of cultures were evaluated using mice ELISA commercial kits. In brief, the appropriate capture antibodies were coated onto the plates and after an overnight incubation at 4°C, the blocking solution was added. Then, samples and standards were added and the plates were incubated at 4°C for 24 h. The plates were washed and after addition of detection antibodies, they were allowed to be in RT for 1 h. After removing the supernatant, avidin-HRP solution was added and the plates were incubated at RT for 30 min. Then, substrate was added and the procedure was terminated by using stop solution. The OD was read at the recommended wavelength (450 nm) in a microplate reader (Biotek, Winooski, VT). The amount of each cytokine was quantified by extrapolation from a standard curve. The sensitivity of kits was 2 pg IFNγ/ml, 7 pg IL-17/ml, 32 pg IL-10/ml and 8 pg TGF-β/ml.

$CD4^+$ naïve T cells isolation and induction of Treg cell differentiation. Splenocytes were washed three times with PBS and $CD4^+$ naïve T cells were separated using mouse naïve $CD4^+$ T cell isolation kit according to the manufacturer's instruction. Briefly, $CD44^+$ activated/memory T cells and non-$CD4^+$ T cells were depleted by indirect magnetic labeling using a cocktail of biotin-conjugated antibodies against CD8a, CD11b, CD11c, CD19, CD25, CD45R (B220), CD49b (DX5), CD105, anti-MHC class II, Ter-119, and TCRγ/δ, and anti-biotin micro-beads. Isolated cells (3×10^5 cells/well in x-vivo 15 culture medium) were activated with 5 μg/ml plate-bound anti-CD3, 2 μg/ml soluble anti-CD28, 2 ng/ml TGF-β and 100 Unit/ml IL-2 in the presence (1 μg/ml) and absence of the extracts in 96-well culture plates. After 4 days of incubation, cells were stained with FITC-conjugated anti-CD4 and APC-conjugated anti-CD25 antibodies. The cells were fixed and made permeable with Foxp3 staining buffer set, and then stained with PE- labeled anti-Foxp3 antibody. Samples were run in a FACSCalibur flow cytometer and analyzed using FlowJo software.

Statistical analysis. All data were expressed as mean ± standard error of at least three independent experiments. The differences between treatments for different tests were analyzed using Student's t-test and one-way analysis of variance (ANOVA) at significance level of $p<0.05$, using SPSS (Abaus Concepts, Berkeley, CA) and GraphPad Prism 5 (San Diego, CA) softwares.

RESULTS

Effects of the extracts on splenocyte proliferation.

We used the BrdU incorporation assay to analyze the effects of *E. microciadia* and *E. osyridea* butanolic extracts on proliferation of stimulated splenocytes. As shown in Figure 1, *E. microciadia* extract at 0.1 μg/ml (PI, 1.47 ± 0.08) and 1 μg/ml (PI, 1.43 ± 0.06) significantly increased splenocyte proliferation ($p<0.01$). Similar augmentation was observed when cells were treated with 0.1 μg/ml (PI, 1.23 ± 0.09) and 1 μg/ml (PI, 1.28 ± 0.04) of *E. osyridea* extract ($p<0.01$). These two plant extracts significantly decreased cell proliferation at concentrations greater than 10 μg/ml ($p<0.01$).

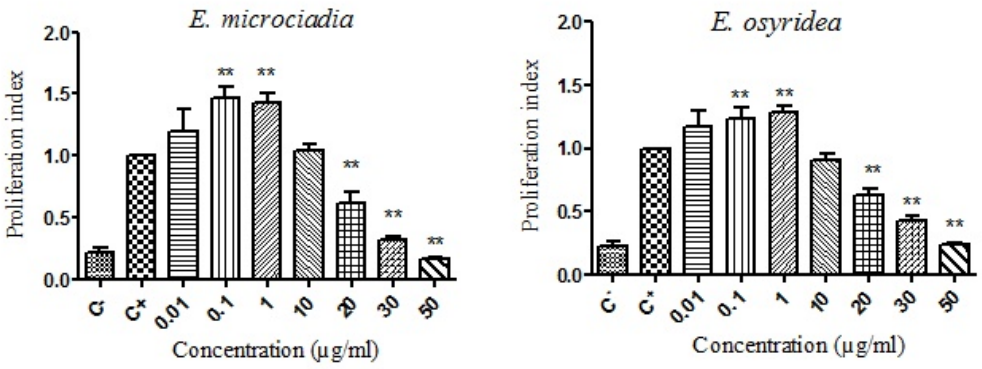

Figure1. Effects of *E. microciadia*, and *E. osyridea* extracts on splenocyte proliferation. Mice splenocytes were stimulated with Con A and cultured in the presence of various concentrations of *E. microciadia* and *E. osyridea* extracts for 72 h, after which BrdU incorporation assay was performed. Con A-only treated cells considered as positive control (C+) and cells with no treatments as negative control (C-). Control wells had DMSO as the solvent at the highest concentration used in the test wells (0.05%). The proliferation index was determined by dividing the optical density of treated cells to that of positive control. Data represent mean ± standard deviation of three experiments. **P < 0.01 compared to positive control.

Effects of the extracts on splenocyte viability.

Based on the results of the cell proliferation assay, we considered four concentrations of the extracts in order to evaluate their effects on cell viability by analyzing the propidium iodide influx. As seen in Figure 2, flow cytometry results showed that as the concentration of the extracts increased there was an augmented fluorescent intensity in cells exposed to *E. microciadia*. There was a significantly greater percentage of dead cells in cultures treated with equal or greater than 20 µg/ml of this extract compared to the negative control ($p<0.001$). The number of dead cells following treatment with different concentrations of *E. osyridea* extract also increased in a concentration-dependent manner. We observed significant levels of dead cells which were positive for the propidium iodide labeling compared to the negative control (3.4 ± 0.8%), as well as for the 20 µg/ml (10.7 ± 2.7%, $p<0.01$) and 30 µg/ml (15.9 ± 0.7%, $p<0.001$) concentrations of this extract. Dot plots that represent the results from one experiment of the propidium iodide staining are shown in Figure 3. These results indicated that the inhibitory effects of both extracts at concentrations greater than 10 µg/ml were due to cell death. Therefore, the 0.1 and 1 µg/ml concentrations of the extracts which had significant stimulatory effects compared to the control were used for the next experiments.

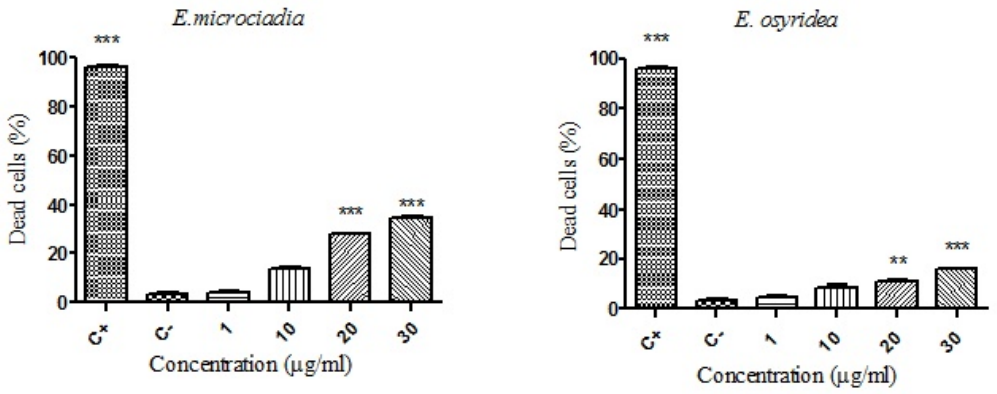

Figure 2. Effects of *E. microciadia* and *E. osyridea* extracts on splenocyte viability. Mice splenocytes were activated with Con A and cultured in the presence of various concentrations of *E. microciadia* and *E. osyridea* extracts for 72 h to examine the viability of cells by propidium iodide staining using flow cytometry. Cells treated with cisplatin considered as positive control and Con A-only treated cells as negative control. Control wells had DMSO as the solvent at the highest concentration used in the test wells (0.05%). The data represents a mean ± standard deviation of four independent experiments. **P < 0.01, ***P < 0.001 compared to negative control.

Effects of the extracts on gene expression of T cell transcription factors.

The mRNA levels of T-bet, RORγt, and Foxp3 in the extract treated-splenocytes were measured via real time-PCR. As shown in Figure 4, cells treated with 0.1µg/ml of *E. microciadia* extract significantly amplified T-bet gene expression (2.49 ± 0.3 relative fold change (RFC), $p<0.05$), whereas cells treated with 1 µg/ml markedly diminished the level of this transcription factor (0.01 ± 0.002 RFC, $p<0.001$). This extract at 1 µg/ml reduced RORγt gene expression (0.3 ± 0.1 RFC, $p<0.001$). Treatment of cells with *E. microciadia* concentrations significantly decreased Foxp3 mRNA levels to less than 0.23 RFC ($p<0.01$). The mRNA levels of these transcription factors in *E. osyridea* treated splenocytes are shown in Figure 4. Similar to *E. microciadia*, this extract induced T-bet mRNA expression at 0.1 µg/ml (1.73 ± 0.06 RFC, $p<0.001$), whereas it reduced the level of this transcription factor at 1 µg/ml (0.51 ± 0.05 RFC, $p<0.01$). This extract showed an increase in RORγt expression at both concentrations of 0.1 µg/ml (3.04 ± 0.24 RFC, $p<0.01$) and 1 µg/ml (2.2 ± 0.64 RFC, $p<0.05$). Splenocytes showed significantly decreased Foxp3 gene expression when treated with 0.1 µg/ml (0.41 ± 0.13 RFC, $p<0.05$) and 1 µg/ml (0.36 ± 0.11 RFC, $p<0.01$) of this extract.

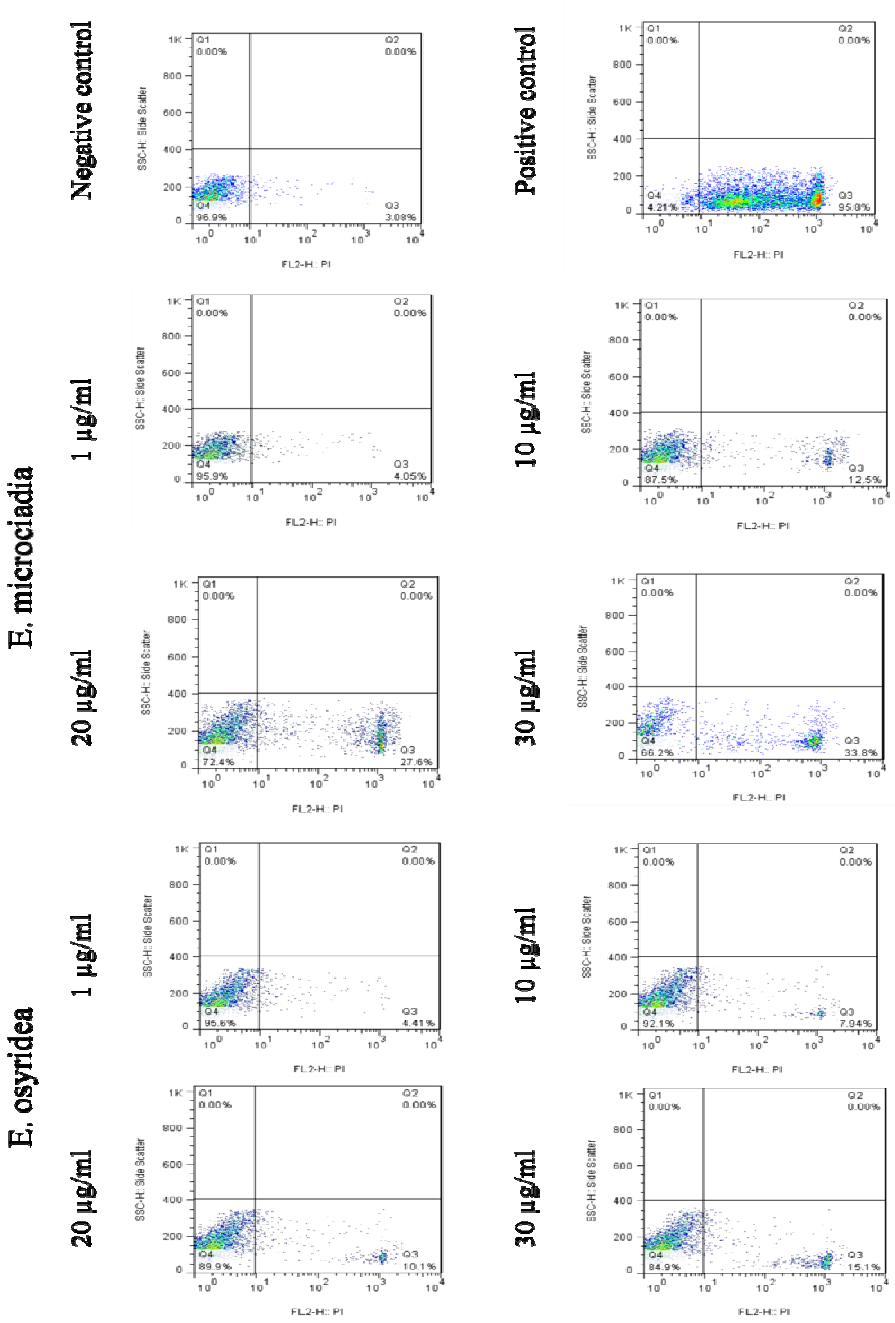

Figure 3. Propidium iodide staining by flow cytometry in activated splenocytes after 72 h treatment with the extracts. Cells treated with cisplatin considered as positive control and Con A-only treated cells as negative control. Data were analyzed by gating cell populations according to propidium iodide (PI) log fluorescence (x-axis) versus side scatter (y-axis). PI positive cells (lower right) were considered as dead cells. The results shown are one representative experiment of three independent experiments.

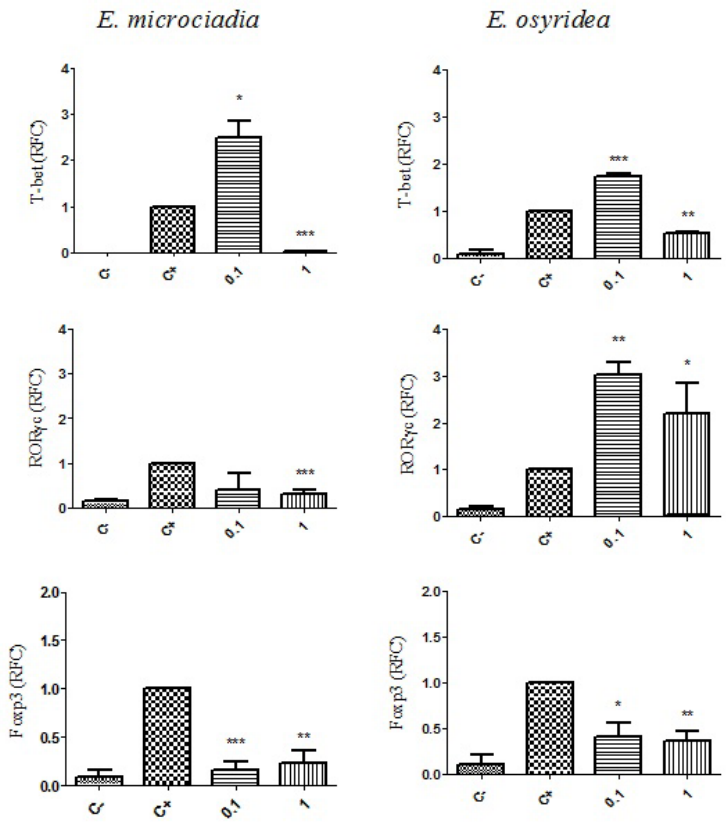

Figure 4. Effects of *E. microciadia* and *E. osyridea* extracts on the gene expressions of T cell specific transcription factors in activated splenocytes by real-time PCR. Mouse splenocytes were stimulated with plate-bound anti-CD3 and soluble anti-CD28 monoclonal antibodies and treated with 1 and 0.1 µg/ml of *E. microciadia* and *E. osyridea* for 72 h. Antibody-only treated cells considered as positive control group and cells with no treatments as negative control (C-). Total RNA was extracted from the cells and reverse-transcribed to cDNA, after which real time-PCR was performed. Results of target mRNA levels were normalized against GAPDH mRNA in each sample and are shown as relative fold change (RFC) to positive control. The values represent mean ± standard deviation of at least three independent experiments. *P< 0.05, **P< 0.01, ***P< 0.001 compared to positive control.

Effects of the extracts on cytokine gene expressions.

The influence of the extracts on gene expressions of four T cell cytokines (IFNγ, IL-17, IL-10, and TGF-β) were measured in stimulated splenocytes by real time-PCR. As illustrated in Figure 5, *E. microciadia* extract at 0.1 µg/ml showed no significant change in IFNγ gene expression, whereas addition of 1 µg/ml of this extract to the culture inhibited IFNγ gene expression (0.63 ± 0.08 RFC, $p<0.05$). Treatment of splenocytes with this extract reduced IL-17 gene expression to less than 0.3 RFC ($p<0.001$) at both concentrations. *E. microciadia* extract at 1 µg/ml decreased expressions of both IL-10 (0.02 ± 0.001 RFC, $p<0.001$) and TGF-β (0.005 ± 0.002 RFC, $p<0.001$).

With respect to *E. osyridea*, this extract caused a significant increase in IFNγ gene expression at 0.1 µg/ml (1.36 ± 0.18 RFC) and a significant decrease at 1 µg/ml (0.57 ± 0.04, RFC, $p<0.001$). Both concentrations of the extract reduced IL-17 gene expression to less than 0.47 RFC ($p<0.01$). Real time-PCR analysis of IL-10 and TGF-β transcripts in extract-treated splenocytes showed decreased cytokine mRNA levels of IL-10 (0.78 ± 0.002 RFC, $p<0.001$) and TGF-β (0.78 ± 0.03 RFC, $p<0.001$) at the 1 µg/ml concentration of extract (Figure 5).

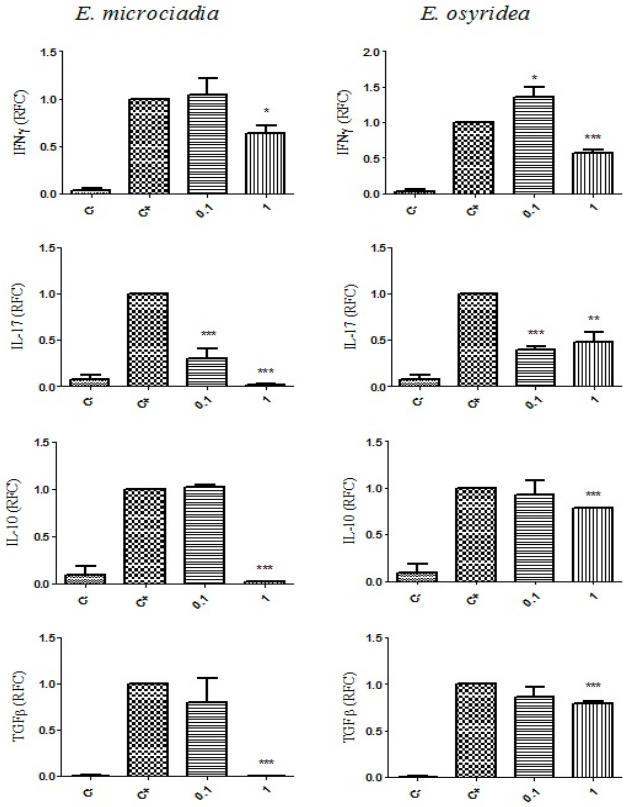

Figure 5. Effects of *E. microciadia* and *E. osyridea* extracts on the gene expressions of various cytokines in activated splenocytes by real-time PCR. Mouse splenocytes were stimulated with plate-bound anti-CD3 and soluble anti-CD28 monoclonal antibodies and treated with 1 and 0.1 µg/ml of *E. microciadia* and *E. osyridea* for 72 h. Antibody-only treated cells considered as positive control group and cells with no treatments as negative control (C-). Results of target mRNA levels were normalized against GAPDH mRNA in each sample and are shown as relative fold change (RFC) to positive control. The values represent mean ± standard deviation of at least three independent experiments. *P< 0.05, **P< 0.01, ***P< 0.001 compared to positive control.

Effects of the extracts on cytokine production.

The effect of the extracts on secretion of T cell cytokines by stimulated splenocytes was examined by ELISA. As shown in Figure 6, stimulation of splenocytes with anti-CD3/CD28 increased IFNγ production to 664 ± 6.7 pg/ml in the positive control. Treatment of the cells with 0.1 µg/ml of *E. microciadia* extract increased this level to 1195 ± 9 pg/ml ($p<0.001$). The extract at both 0.1 µg/ml (248.3 ± 1.17 pg/ml, $p<0.001$) and 1 µg/ml (348.5 ± 2.94 pg/ml, $p<0.01$) caused significant decreases in IL-17 production. Treatment of cells with *E. microciadia* extract reduced IL-10 levels at both concentrations (<162 pg/ml) compared to the positive control (279 ± 36.2 pg/ml, $p<0.05$). The *E. osyridea* extract significantly increased IFNγ production at 0.1 µg/ml (1219 ± 4.8 pg/ml, $p<0.001$; Figure 6). Treatment of cells with the extract decreased IL-17 production to less than 469 pg/ml ($p<0.05$). This extract at 1µg/ml significantly

inhibited IL-10 production (111.8 ± 1.07 pg/ml, p<0.05). Both extracts slightly decreased TGF-β production at the 1 µg/ml concentration, but this decrease was not significant.

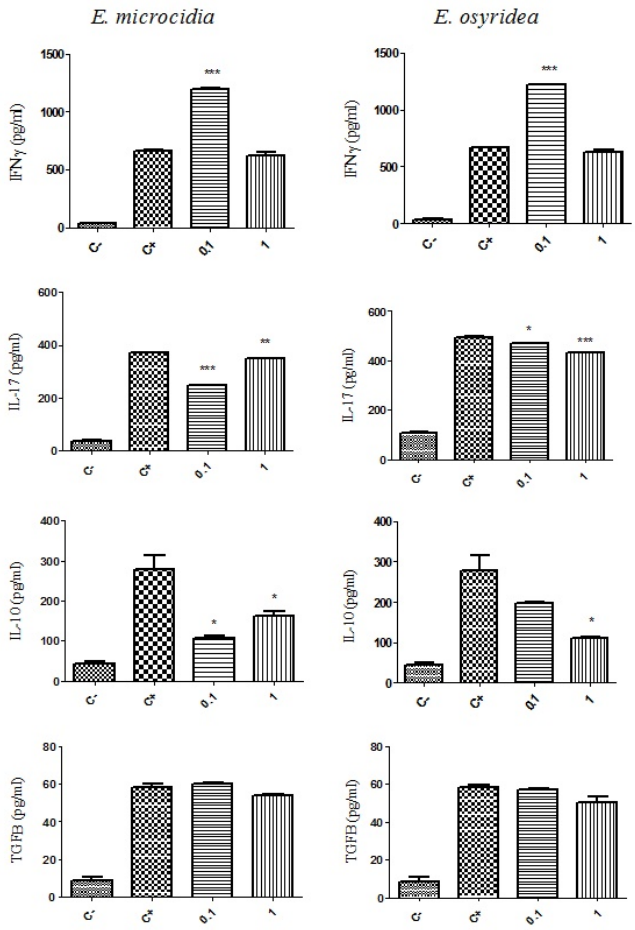

Figure 6. Effects of *E. microciadia* and *E. osyridea* extracts on the production of various cytokines by activated splenocytes. Mouse splenocytes were stimulated with plate-bound anti-CD3 and soluble anti-CD28 monoclonal antibodies and treated with 1 and 0.1 µg/ml of *E. microciadia* and *E. osyridea* for 72 h. Antibody-only treated cells considered as positive control group and cells with no treatments as negative control (C-). Cytokine levels in the supernatant of cells were measured by enzyme-linked immunosorbent assay. The values represent mean ± standard deviation of two measurements. *P< 0.05, **P< 0.01, ***P< 0.001 compared to positive control.

Effects of the extracts on Foxp3 protein induction in Naïve T cells.

To analyze the effects of the extracts on Tregs, naïve $CD4^+$ T cells were isolated from mouse splenocytes. The purity of cells was confirmed by staining of cells with labeled anti-CD4 and anti-CD25 antibodies (Figure 7A). Naive T cells were cultured in the presence of antiCD3/antiCD28 antibodies and the extracts, to detect $Foxp3^+$ T cells in $CD4^+CD25^+$ cell population by flow cytometry. We found that *E. microciadia* (15.55 ± 1.35%, p< 0.05) and *E. osyridea* (17.45 ± 1.30%, p< 0.05) significantly decreased Treg levels compared to the control (24.54 ± 0.13) (Figure 7B).

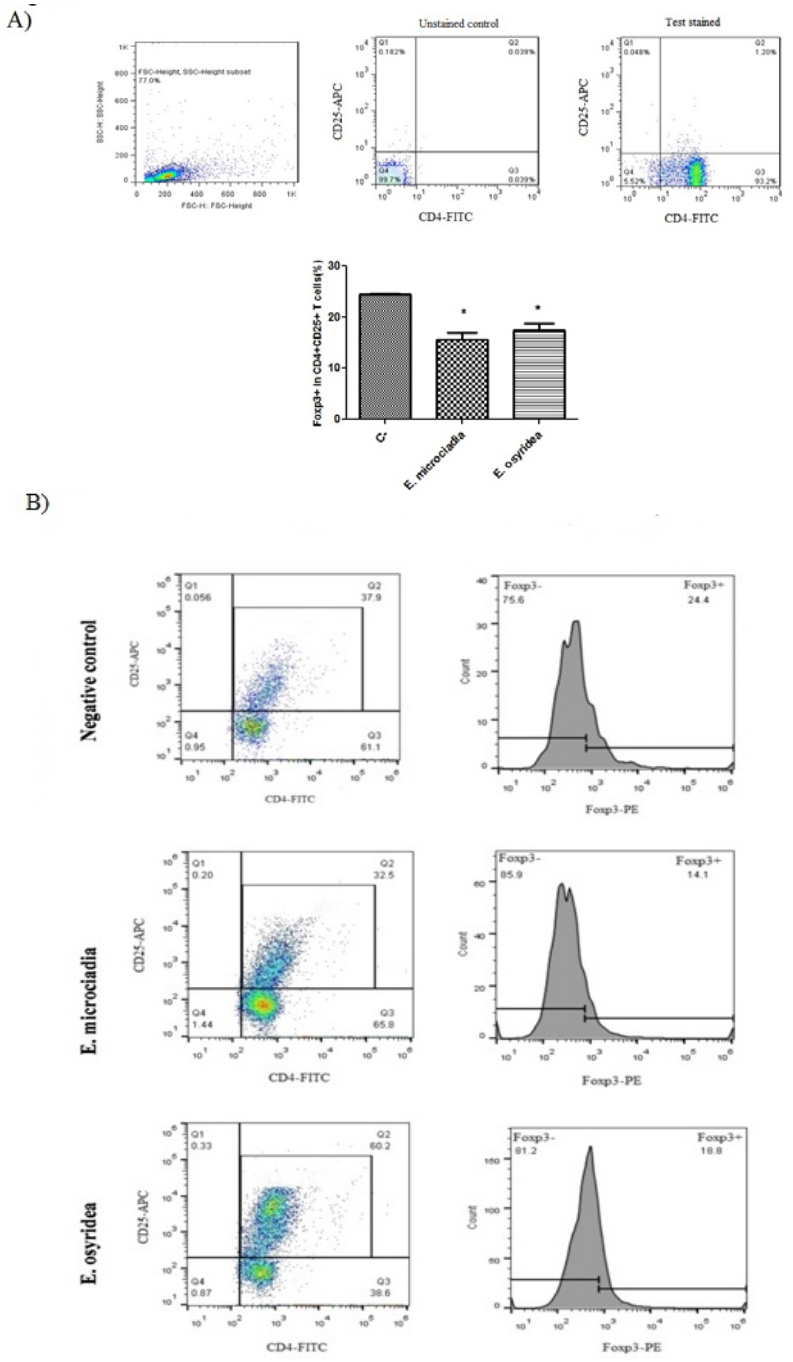

Figure7. Effects of *E. microciadia* and *E. osyridea* extracts on Foxp3 protein expression analyzed by flowcytometry. Naïve CD4$^+$ T cells were isolated from mouse splenocytes. A) Isolation of highly pure naive CD4$^+$ CD25- T cells is shown after immune staining with FITC-conjugated anti-CD4 and APC-conjugated anti-CD25 antibodies. B) Naive CD4$^+$ T cells were stimulated with anti-CD3, anti-CD28 monoclonal antibodies, TGFβ and IL-2 cytokines and treated with 1 µg/ml of the extracts for 4 days. Negative control was stimulated cells without the extract. The percentage of Foxp3$^+$ Treg cells was determined by gating of Foxp3+ cells in CD4+CD25+ population. The values represent mean ± standard deviation of two experiments. *P<0.05, compared to negative control.

DISCUSSION

In the present study, we investigated the immunomodulatory effects of two butanolic extracts from the Euphorbia species on mice T cell subsets. We first evaluated the effects of *E. microciadia* and *E. osyridea* extracts on mouse splenocyte proliferation.

As the results showed, both the extracts at concentrations of 0.1 and 1 μg/ml increased proliferation of the activated splenocytes, which indicated their immunostimulatory effects on T cell responses. The extracts at higher concentrations showed a dose-related decrease in cell proliferation. These results supported our previous observation regarding the stimulatory effects of the extracts on human peripheral blood lymphocytes at low concentrations and inhibitory effects at higher concentrations (26). Ghanadian *et al.* reported a decrease in cell proliferation of human peripheral blood lymphocytes upon treatment with high concentrations of two isolated flavonoids from *E. microciadia* butanolic extracts (23). In order to find whether the observed inhibitory effects of the extracts at higher concentrations were due to the cell suppression or their toxicity, we performed propodium iodide staining. Our data showed that increased concentration of the extracts caused increased numbers of dead cells. These results indicated the toxicity of the extracts at higher concentrations and therefore we selected non-cytotoxic concentrations of the extracts (0.1 and 1μg/ml) for evaluation of their effects on T cell responses. In order to determine the effect of the extracts on T cell subsets, real time-PCR for specific transcription factors and cytokine expressions was performed. The results have indicated that both the extracts at 0.1 μg/ml significantly increased T-bet gene expression. T-bet is a specific and key transcription factor for Th1 development. This transcription factor controls the expression of IFNγ as the hallmark Th1 cytokine. In cytokine gene analysis, *E. osyridea* at 0.1 μg/ml increased IFNγ expression at both gene and protein expression levels which suggested the ability of *E. osyridea* extract at this concentration to deviate T cell responses toward a Th1 response. This extract at the same concentration increased RORγt gene expression but failed to increase IL-17 gene and protein expressions. This inconsistency between RORγt and IL-17 expressions might be a result of transcriptional and/or post-transcriptional regulation of IL-17 expression. Although, RORγt is believed as an IL-17-specific transcription factor, new evidences have shown other factors that are effective on the up- and/or down-regulation of this cytokine (27). The v-ets erythroblastosis virus E26 oncogene homolog 1 (ETS-1), a T-bet interacting transcription factor, has been shown to prevent IL-17 production without affecting RORγt (28). Enforced growth factor independent-1 (Gfi-1) expression in Th17 cells have also been shown to suppress IL-17A, but not RORγt expression (29). With respect to the effects of *E. microciadia*, this extract at 0.1 μg/ml increased both T-bet mRNA in splenocytes and the level of IFNγ production. This extract at the same concentration showed no significant effects on RORγt expression. At the 1 μg/ml concentration, similar to the same concentration of *E. osyridea*, we observed reductions in all studied transcription factors and cytokine gene expression levels. These data suggested a general suppressive effect of both the extracts at higher concentrations on Th cell subsets. This effect could be helpful for inhibition of T cell over-activation which is observed in various immune-mediated diseases.

We have also investigated the effect of the extracts on Treg cells by measuring the changes in Foxp3 mRNA levels in treated splenocytes as well as its protein expression in naïve $CD4^+$ T cells. Foxp3 is indispensible for the differentiation and function of Treg cells. Our results have shown decreased Foxp3 gene and protein expression levels at both concentrations of the extracts. This decrease in Treg transcription factor expression was accompanied with increased expansion of activated T cells as noted by the higher

percentage of $CD4^+CD25^+$ cells in cultures treated with *E. osyridae* compared to the negative control.

We observed diminished IL-10 and TGF-β gene expression levels at 1 μg/ml concentration which might suggest the capacity of the extracts to inhibit Treg cells. Treg cells play a critical role in tumor immunity and contribute to tumor development and progression by blocking anti-tumor effector T cells, thus significantly impacting the outcome of cancer patients (30-32). Latest data have shown that a major outcome of immune suppression by Treg cells is evasion of tumor from the immune system which could be a main hindrance to a successful tumor immunotherapy (33). On the other hand, Th1 cells and their produced cytokines (IFNγ) are strongly associated with good clinical outcome for all cancer types (34). Therefore natural products with the ability to increase Th1 responses and decrease Treg cells might be valuable as anti-tumor agents when combined with conventional chemotherapy. As our results showed both the extracts at 0.1 μg/ml had the ability to increase Th1 and decrease Treg related factors. These plants have previously shown a capacity to inhibit tumor cell growth (26). Such features might provide evidence to conduct studies to additionally evaluate these plants in terms of their usefulness as natural anti-tumor products with desirable immuomodulatory effects. The increased Th1/Treg ratio is desirable for enhancement of cellular immune response against cancer. In addition, it is also important for treatment of chronic and persistent infections in which Treg cells negatively impact host defense and allow pathogens to infect the host by suppressing immune system activation (35). The traditional usefulness of Euphorbia species in treatment of infections makes a study of the antimicrobial effects of these extracts attractive from the combined immunomodulatory and anti-infective point of view.

As shown, both concentrations of the extracts had inhibitory effects on Treg cells. Of note, the results have shown that these extracts at a narrow range of concentration had different Th1 stimulatory and inhibitory effects. This finding is proposed to be attributed to the presence of various compounds with different modes of action in these extracts. A bioassay-guided fractionation would help to isolate and identify the active compounds responsible for their observed immunomodulatory effects.

In conclusion, our data demonstrated that *E. microciadia* and *E. osyridea* extracts have immunomodulatory effects on T cell specific transcription factors and cytokines. These extracts at a low concentration increased the expression of T-bet, augmented IFNγ production, and decreased Foxp3 gene expression which suggested their capacity at this concentration to increase the Th1/Treg ratio. This effect on upregulation of Th1 and downregulation of Treg cells might be beneficial for treatment of cancer and infectious diseases. Both extracts at the higher concentration showed suppressive effects on Th1 and Th17 cell subsets. This effect combined with other conventional drugs could be more studied with regards to the usefulness for treatment of immune-mediated diseases with overactive T cells. Whether these various effects are due to similar or different compound/s can be determined after isolation of the main constituents of these extracts and studying their immunological activities.

ACKNOWLEDGEMENTS

The present article was extracted from the thesis written by H. Namdari. We would like to express our specific thanks to the Deputy of Research Affairs of Shiraz University of Medical Sciences (grant no. 7611) for financial support.

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