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# Immunoinhibitory Effect of Teuclatriol a Guaiane Sesquiterpene from *Salvia mirzayanii*

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## ABSTRACT

**Background:** *Salvia mirzayanii*, a native plant to Iran, is shown to have immunomodulatory effects on lymphocyte proliferation. **Objective:** To identify the bioactive immunomodulatory compound(s) present in *S. mirzayanii*. **Methods:** The crude extract was fractionated to five fractions in two steps using different solvents. The fractions were subjected to bioassay-guided fractionation. All the fractions were tested for bioactivity on human activated-peripheral blood lymphocytes (PBLs) using cell proliferation assay. **Results:** The methanol fraction (Fr. M) showed the highest inhibitory effect on PBLs compared to other fractions. Fr. M was applied on a gravity column chromatography for further fractionation. Resultant fractions, demonstrated inhibitory effects at higher concentrations. Fr. 4 with an  $18.9 \pm 0.2\%$  inhibitory activity at  $200 \mu\text{g/ml}$  and with the highest quantity was applied on preparative TLC plates for further purification. The final purified compound was identified as teuclatriol, a guaiane sesquiterpene, by NMR analysis. This compound showed a significant anti-proliferative effect on human activated-peripheral blood lymphocytes ( $\text{IC}_{50}$ ,  $72.8 \pm 5.4 \mu\text{g/ml}$ ). **Conclusion:** Teuclatriol was found to be one of the compounds responsible for the immunoinhibitory effect of *Salvia mirzayanii*. We suggest further studies on teuclatriol, exploring its mechanism of action as an immunomodulatory compound.

**Keywords:** Immunomodulatory, *Salvia mirzayanii*, Sesquiterpenes

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## INTRODUCTION

Study of natural products for discovering new bioactive compounds can offer better structural diversity than usual synthetic chemistry and will provide a better chance in discovering new low molecular weight bioactive compounds (1). Bioassay-guided fractionation methods have a crucial role in finding naturally occurring therapeutic agents. In this procedure, the crude plant extract is purified and tested for bioactivity step by step until the compound(s) responsible for the effect is isolated. Bioactivity-guided fractionation of the medicinal plant extracts has led to identifying important compounds such as taxol and camptothecin that are widely used for the treatment of cancer (2,3).

A variety of studies have demonstrated the immunomodulatory effect of *Salvia* species. For example, ethanol extract of *Salvia miltiorrhiza* inhibited cyclooxygenase-dependent phases of prostaglandin D<sub>2</sub> formation and leukotriene C<sub>4</sub> production in bone marrow-derived mast cells (4). Tanshinone pigments of this plant protect against immune-mediated liver injury through activation of T cell subsets and regulation of cytokines (5). Crude polysaccharides from the aerial parts of *Salvia officinalis* showed significant immunomodulatory effects on rat thymocytes (6).

*Salvia mirzayanii* belonging to the Labiatae family grows in the southern parts of Iran (7). In folk medicine, it is used for the treatment of diarrhea, stomach ache, headache, hypercholesterolemia and diabetes, and also for wound healing (8). Several studies have shown the various biological activities of this plant including its antibacterial properties (9), free radical scavenging activity (10), antioxidant and antiglycating activities and neuroprotective effects (11). There are limited reports concerning *Salvia mirzayanii* constituents. In our previous study, we showed the presence of spathulenol, a sesquiterpene with immunomodulatory effects, in this plant (12). Javidnia et al. reported  $\delta$ -cadinene, linalool,  $\alpha$ -terpinyl acetate,  $\alpha$ -cadinol and spathulenol, as major components of the essential oil of *Salvia mirzayanii* (13). Salvimirzacolide, a sesterterpene, is another constituent of this plant (14). In our previous study, various concentrations of the methanol extract of *Salvia mirzayanii* demonstrated inhibitory effects on human lymphocyte proliferation which is a critical event leading to the initiation and development of inflammation (15). In the current study, the bioassay-guided fractionation was performed for the purification and structural identification of the responsible compound(s).

## MATERIALS AND METHODS

**Preparation of the Extract.** The aerial parts of *Salvia mirzayanii* were collected from Bastak in Hormozgan Province, south of Iran in the period of flowering in June. Mr. Azizollah Jafari from the Department of Botany, Research Center for Natural Resources and Animal Husbandry in Yasuj, Iran, identified the plant, and a voucher sample was deposited there. Plants were air dried in shade, powdered and defatted with petroleum ether (40–60°C) for 4 hours. Using maceration of the plant in 3 × 1500 ml methanol for 48 hours at room temperature, a methanol extract was obtained. The methanol extract was filtered, dried under reduced pressure with a yield of 2.2% (w/w). A stock solution from the dry extract at a concentration of 20 mg/ml in dimethyl sulfoxide (DMSO) was prepared. Appropriate dilutions of the extract in RPMI 1640 culture medium were prepared.

**Lymphocyte Proliferation Assay.** Human peripheral blood lymphocytes (PBLs) were isolated using gradient centrifugation with Ficoll-Hypaque from healthy male individuals who had provided informed consent. The trypan blue exclusion dye test was applied to determine the cells that represented more than 98% viability. The effects of crude extract and the resultant fractions and the pure compound on the proliferation of mitogen-activated lymphocytes were assessed using 5-Bromo-2-deoxyuridine (BrdU) proliferation assay kit (Roche, Germany). This assay is based on quantification of BrdU incorporated into the newly synthesized DNA of replicating cells. The assay was performed according to the manufacturer's instructions. Briefly, 100  $\mu$ l PBLs ( $5 \times 10^4$  cells/well) were seeded in 96-well culture plates (Nunc, Germany) containing a suboptimal dose of phytohemagglutinin (PHA) 1/1500 (Gibco, USA). The extract, the fractions and the pure compound were separately added to the wells at final concentrations of 0.1-200  $\mu$ g/ml. Two series of wells were considered as controls. The first set of controls contained cells treated with PHA and DMSO in a final concentration equal to the highest concentration of the test wells (extract-untreated cells) and the second were treated with dexamethasone instead of the extract (Sigma, St Louis, USA) as a positive control. The plates were incubated at 37°C in an atmosphere of humidified air containing 5% CO<sub>2</sub>. After 48 hours, BrdU was added to each well and the plates were incubated again at 37°C for 18 hours. Then, the plates were centrifuged, the supernatants were removed and the cells were incubated with anti-BrdU antibody. Following the addition of the substrate and the stopping solution, absorbance was measured at 450 nm with a reference filter at 630 nm. At least three independent experiments in triplicate were carried out. The proliferation index for each sample was calculated and compared with the untreated control taken as 100%.

**Bioassay-Guided Fractionation for the Purification of the Bioactive Compound.** In order to fractionate the crude methanol extract; it was first subjected to solvent partitioning by ethyl acetate and water. Water fraction (Fr. W) was partitioned using butanol to extract the less polar soluble components. An inter phase narrow layer appeared between the water and the butanol phases (Fr. I). Ethyl acetate fraction was then dissolved in methanol (Fr. M) and extracted with hexane (Fr. H). The inhibitory effect of these five fractions on the PBLs was examined by cell proliferation assay. The methanol fraction (Fr. M) with the highest inhibitory effect, was further fractionated a column packed with silica gel 60 (230-400 mesh, Merck, Germany) eluted with a gradient mixture of chloroform and methanol (100:0 - 70:30, v/v) which resulted in 38 fractions (250 ml each). Thin layer chromatography (TLC) analysis was carried out on plastic-backed silica gel TLC plates with a 254 nm fluorescent indicator, medium pore 60 Å diameter and a thickness of 0.2mm (Fluka, Switzerland) using the same solvent system as the mobile phase, and the spots were visualized by spraying the plates with ethanol-sulfuric acid reagent (16). According to the TLC pattern, some of the fractions were then pooled in seven new fractions as Fr. 1 to Fr. 7, six of which showed significant inhibitory activities. Among these fractions, Fr. 4 that demonstrated strong anti-proliferative effect on PBLs and was available in the highest quantity (400 mg), was selected for further isolation using preparative TLC. Preparative TLC was performed on silica gel 60 F254, 1mm 15 PLC Plates (Merck, Germany) and the mobile phase was a combination of chloroform and methanol (90:10, v/v). A colorless compound was obtained from Fr. 4. All solvents used were of analytical grade and we repurchased from Merck, Germany.

**Compound Spectroscopy.** The NMR spectra were recorded in a BRUKER DRX 500

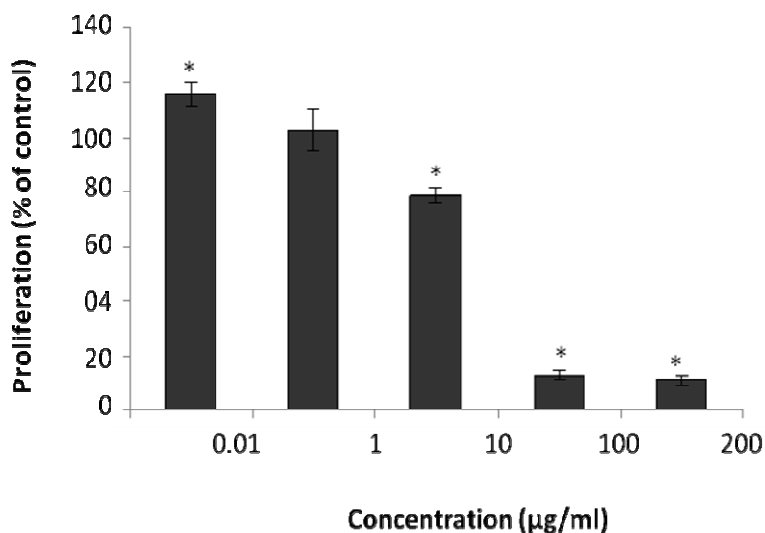
spectrometer ( $^1\text{H}$  NMR: 500 MHz,  $^{13}\text{C}$  NMR: 125 MHz) in  $\text{CDCl}_3$  relative to  $\text{CHCl}_3$  at  $\delta_{\text{H}}$  7.24.  $^{13}\text{C}$  multiplicities were determined using the DEPT pulse sequence. The assignments were taken from 2D NMR spectra, recorded as gsCOSY, gsHSQC and gsHMBC experiments.

Teuclatriol (17). Oil,  $^1\text{H}$  NMR: 4.13 (*dd*,  $J = 9, 4.5$  Hz, H-6), 1.94 (*dd*,  $J = 12, 6.5$  Hz, H-9a), 1.85 (*m*, H-7), 1.77 (*m*, H-2a), 1.73 (*m*, H-5), 1.68 (*m*, 2H, H-3), 1.56 (*m*, H-2b), 1.38 (*m*, 2H, H-8), 1.36 (*m*, H-9b), 1.26 (*s*, 3H, H-15), 1.24 (*m*, H-11), 1.23 (*s*, 3H, H-14), 1.08 (*m*, H-1), 0.96 (*s*, 3H, H-12);  $^{13}\text{C}$  NMR: 81.12 (*s*, C-4), 75.46 (*s*, C-10), 71.41 (*d*, C-6), 55.25 (*d*, C-5), 52.04 (*d*, C-1), 48.02 (*t*, C-9), 45.48 (*d*, C-7), 41.13 (*t*, C-3), 29.56 (*d*, C-11), 23.17 (*t*, C-2), 23.09 (*q*, C-15), 22.16 (*q*, C-14), 21.50 (*q*, C-13), 21.14 (*q*, C-12), 20.51 (*t*, C-8).

**Statistical Analysis.** The data was analyzed using SPSS version 14.0 software. All tests were performed at least in three independent experiments. The data were presented as mean  $\pm$  standard deviation. The Student's t-test determined whether the results had statistical significance. The level of significance was set at  $p < 0.05$ .

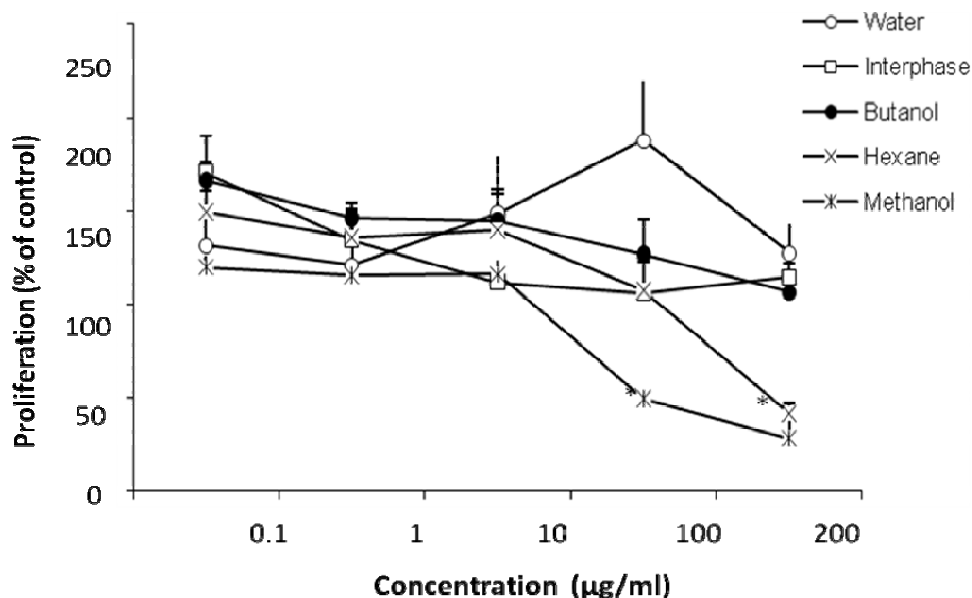
## RESULTS

**Effect of Crude Extract on Lymphocyte Proliferation.** Treatment of PHA-stimulated PBLs with different concentrations (0.1 to 200  $\mu\text{g}/\text{ml}$ ) of the methanol extract of *Salvia mirzayanii* decreased the proliferation of lymphocytes in a dose dependent manner, the percentage of proliferation compared to untreated cells at concentrations of 0.1 to 200  $\mu\text{g}/\text{ml}$  ranged from  $116 \pm 4.6$  to  $11 \pm 1.7$ ; indicating a mild immune-stimulatory effect of this extract at 0.1  $\mu\text{g}/\text{ml}$  concentration and strong inhibitory effects at higher concentrations (Figure 1).



**Figure 1.** The effect of various concentrations of the methanol extract of *Salvia mirzayanii* on the human peripheral blood lymphocyte proliferation. Lymphocytes were stimulated with suboptimal concentration of phytohemagglutinin in the presence or absence of the extract. Control was extract-untreated lymphocytes containing the solvent in a final concentration equal to the highest concentration in the test wells. Results were calculated as the mean  $\pm$  standard deviation of the percentage of cell proliferation compared to the control. Asterisks represent statistical significance ( $p < 0.001$ ).

**Effects of Fractions on Lymphocyte Proliferation and Identification of Purified Compound.** Five fractions including Fr. W, butanol (Fr. B) and interphase (Fr. I) derived from water partitioning and hexane (Fr. H) and Fr. M from ethyl acetate partitioning were examined for their immunomodulatory activity on activated-PBLs. Fr. W, Fr. B and Fr. I presented mild immune-stimulatory effect on activated-PBLs, while Fr. M and Fr. H exhibited strong inhibitory effect (Figure 2).



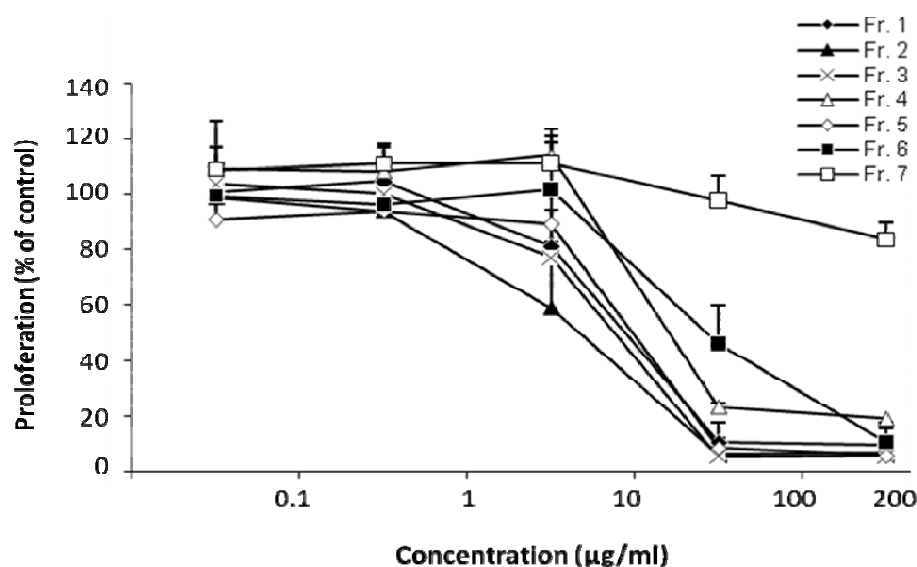
**Figure 2.** Treatment of human peripheral blood lymphocytes with various concentrations of the water, butanol, interphase, hexane and methanol fractions of *Salvia mirzayanii*. Control was fraction-untreated lymphocytes containing mitogen and the solvent in the final concentration equal to the highest concentration in the test wells. Data show mean  $\pm$  standard deviation of the percentage of proliferation compared to the control. Asterisks represent statistically significance inhibitory effects of the fractions ( $p < 0.001$ ).

**Table 1. Effect of different fractions of *Salvia mirzayanii* on the proliferation of peripheral blood lymphocytes.**

| Fraction<br>( $\mu\text{g/ml}$ ) | Proliferation (% of control) |                    |                   |                     |                   |
|----------------------------------|------------------------------|--------------------|-------------------|---------------------|-------------------|
|                                  | 0.1                          | 1                  | 10                | 100                 | 200               |
| Fr. B                            | 166.2 $\pm$ 9.9***           | 146.0 $\pm$ 5.2*** | 144.9 $\pm$ 16.5* | 126.8 $\pm$ 18.2    | 106.4 $\pm$ 10.6  |
| Fr. H                            | 149.2 $\pm$ 11.1*            | 135.8 $\pm$ 18.0   | 139.6 $\pm$ 19.2  | 107.6 $\pm$ 15.4    | 41.6 $\pm$ 5.9*** |
| Fr. I                            | 170.4 $\pm$ 10.8***          | 134.4 $\pm$ 15.0*  | 111.9 $\pm$ 0.9   | 106.2 $\pm$ 2.8     | 114.5 $\pm$ 7.4   |
| Fr. M                            | 119.8 $\pm$ 6.0              | 115.5 $\pm$ 10.0   | 116.0 $\pm$ 7.9   | 49.6 $\pm$ 2.6***   | 28.2 $\pm$ 9.9*** |
| Fr. W                            | 131.5 $\pm$ 22.4             | 121.0 $\pm$ 29.8   | 148.9 $\pm$ 30.0* | 187.7 $\pm$ 31.7*** | 127.5 $\pm$ 31.1  |

Data represent mean  $\pm$  SD. Fractions (Fr.), \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$

A lower percentage of proliferation was observed in cells treated with 200  $\mu\text{g/ml}$  Fr. M ( $28.2 \pm 9.9\%$ ) and the highest percentage was appeared in cells treated with 100  $\mu\text{g/ml}$  Fr. W ( $187.7 \pm 31.7\%$ ) (Table 1). After fractionation of Fr. M on a gravity column chromatography, the resultant fractions were pooled and evaluated for their effects on PBLs. The activity of the fractions is presented in Figure 3.



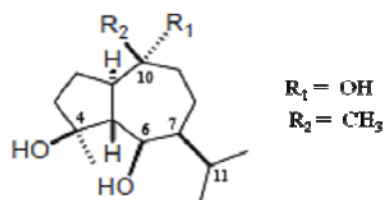
**Figure 3.** The inhibitory effect of different concentrations of fractions Fr 1 to 7 obtained from methanol fraction on the proliferation of stimulated-lymphocytes. Control was fraction-untreated lymphocytes containing mitogen and the solvent. Data exhibit mean  $\pm$  standard deviation of the percentage of proliferation compared to the control. Fr. 4 with strong anti-proliferative effect and the highest quantity was selected.

**Table 2. Anti-proliferative effect of fractions isolated from fraction M on human peripheral blood lymphocytes.**

| Fraction<br>( $\mu\text{g/ml}$ ) | Proliferation (% of control) |                       |                      |
|----------------------------------|------------------------------|-----------------------|----------------------|
|                                  | 10                           | 100                   | 200                  |
| Fr. 1                            | $81.3 \pm 7.3^*$             | $10.4 \pm 7.1^{***}$  | $9.4 \pm 6.3^{***}$  |
| Fr. 2                            | $59.1 \pm 23.6^{**}$         | $6.1 \pm 2.4^{***}$   | $6.4 \pm 2.8^{***}$  |
| Fr. 3                            | $77.2 \pm 23.2^{***}$        | $5.2 \pm 1.7^{***}$   | $5.4 \pm 1.9^{***}$  |
| Fr. 4                            | $114.1 \pm 9.5$              | $23.2 \pm 1.0^{***}$  | $18.9 \pm 0.2^{***}$ |
| Fr. 5                            | $89.4 \pm 4.7$               | $8.5 \pm 3.4^{***}$   | $5.6 \pm 3.9^{***}$  |
| Fr. 6                            | $101.9 \pm 19.5$             | $45.9 \pm 14.1^{***}$ | $10.6 \pm 6.7^{***}$ |
| Fr.7                             | $111.4 \pm 7.2$              | $97.8 \pm 9.2$        | $83.7 \pm 6.0$       |

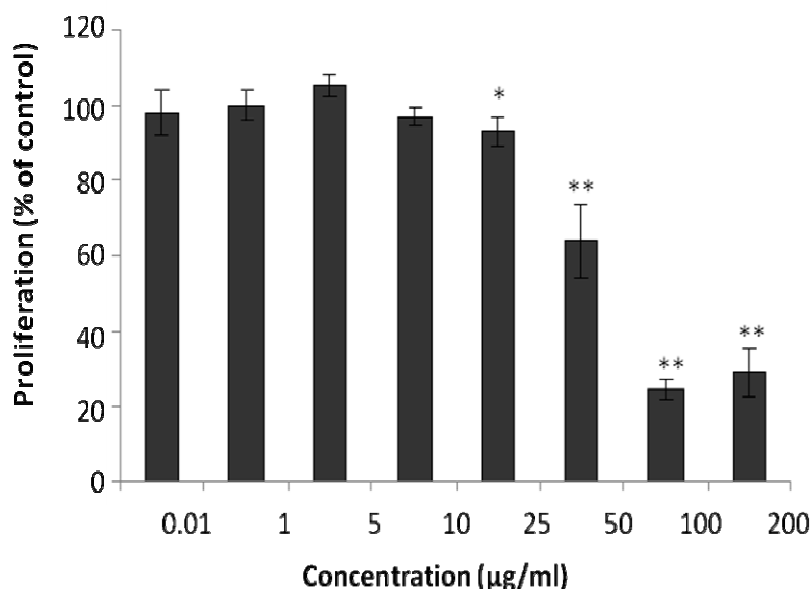
Data represent Mean  $\pm$  SD. Fractions (Fr.), \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$

None of them had a significant effect at 0.1 and 1  $\mu\text{g/ml}$ . Fr. 4 with a strong anti-proliferative effect ( $< 23.2\%$  of control,  $p < 0.001$ ) (Table 2) at concentrations of 100 and 200  $\mu\text{g/ml}$  and the highest quantity (400 mg) was selected for application on preparative TLC plates for further purification of the active compound(s). The quantity of other fractions was not sufficient to continue purification except for Fr. 2 that its immunomodulatory effect was reported previously (12). Comparing of the  $^1\text{H}$  and  $^{13}\text{C}$  NMR data of the purified compound (see materials and methods) with those from literature, resulted in identification of a sesquiterpene known as teuclatriol (17) (Figure 4).



**Figure 4.** Structure of teuclatriol; formula:  $\text{C}_{15}\text{H}_{28}\text{O}_3$ .

**Anti-Proliferative Effect of the Pure Compound.** Treatment of human PHA-activated PBLs with various concentrations of teuclatriol demonstrated the ability of the compound to down regulate the activation of PBLs (Figure 5). The percentage of proliferation in cells treated with different concentrations of teuclatriol compared to the untreated cells (100%) ranged from  $93.0 \pm 3.6$  at 25  $\mu\text{g/ml}$  ( $p < 0.05$ ) to  $28.9 \pm 6.5$  at 200  $\mu\text{g/ml}$  ( $p < 0.001$ ) ( $\text{IC}_{50}$ ,  $72.8 \pm 5.4$   $\mu\text{g/ml}$ ).



**Figure 5.** The Effect of various concentrations of teuclatriol on the proliferation of activated-peripheral blood lymphocytes. Control was untreated lymphocytes containing mitogen and the solvent. Data exhibit mean  $\pm$  standard deviation of the percentage of proliferation compared to the control. \* $p < 0.05$ , \*\* $p < 0.001$ .



## DISCUSSION

Medicinal plant extracts provide a broad spectrum of pharmacological and biological specificities including anti-cancer, cytoprotective, anti-inflammatory, immunomodulatory, anti-microbial and antioxidant activities (18). There has been a tendency toward using traditional medicine to treat different diseases (19). However, because of some unknown adverse effects they could offer a safer and more efficient platform if their bioactive components were identified. In the present study, we used bioassay-guided fractionation based on the anti-proliferative actions on mitogen-activated PBLs to identify more active fractions and immunoinhibitory compound(s) from *Salvia mirzayanii*. Mitogen activation is similar to antigen-induced activation (20) and activation of lymphocytes is associated with proliferation. The rate of DNA synthesis, which correlates well with the number of stimulated cells, is the most commonly used measure of lymphocyte activation and proliferation. To provide a nonradioactive proliferation measurement, BrdU has been used in conjunction with an immunoassay (21).

In traditional folk medicine, *Salvia mirzayanii* is used for anti-inflammatory purposes. The immunomodulatory effect of this plant on human PBLs was reported in a previous study (12). In the current study, re-examination of the crude extract of *Salvia mirzayanii* showed its significant immunostimulatory effect on PHA-activated PBLs at low concentration and its significant immunoinhibitory effect at higher concentrations.

To identify the active compound (s) within the extract, fractionation of the crude extract using different solvents with various polarities was performed. This resulted in separation of immunostimulatory compounds in water and immunoinhibitory compounds in fractions derived from hexane and methanol. Thin layer chromatography of Fr. W and detection with anisaldehyde in sulfuric acid reagent (22) revealed the possibility of the presence of saponins in this fraction (data not shown). In several previous studies the presence of saponins in other species of *Salvia* such as *Salvia officinalis* and *Salvia miltiorrhiza* have been reported (23,24). Saponins and some polysaccharides, soluble in water, have been shown to have stimulatory effects on immune cells (25,26). It requires further investigations to identify responsible compounds responsible for the stimulatory effect of *Salvia mirzayanii* on human PBLs. Further fractionation of Fr. M using column chromatography resulted in new fractions with immunoinhibitory effects, among which Fr. 4 with the highest quantity and strong anti-proliferative effect was selected to apply on preparative TLC plates for further purification of the active compound(s). Based on the  $^1\text{H}$  and  $^{13}\text{C}$  NMR data a sesquiterpene known as teuclatriol was identified in the purified fraction (17). Sesquiterpenes are rare compounds in *Salvia* species (27) and the presence of teuclatriol in *Salvia* genus has not been reported before.

Treatment of PHA-stimulated PBLs with various concentrations of teuclatriol shown that of this compound down regulates the activity of PBLs in a dose dependent manner. The biologic function of teuclatriol had not been reported so far. Lymphocyte activation is a critical event leading to the initiation and development of inflammation. Therefore, it is reasonable to assume that the anti-inflammatory use of *Salvia mirzayanii* in folk medicine may be partly due to the presence of teuclatriol.

Various experiments have shown the immunomodulatory effect of sesquiterpenes. For example, sesquiterpenes isolated from the root of *Ferula fukanensis* inhibited nitric oxide production and inducible nitric oxide synthase gene expression by a murine macrophage

-like cell line (28). A number of sesquiterpenes containing lactone group, have shown anti-inflammatory and anti-cancer properties (29). Various studies have revealed that sesquiterpene lactones most likely induce their immunoinhibitory effect through apoptosis (30-32) and/or NF- $\kappa$ B inactivation (33). Teuclatriol does not have a lactone, or a ketone group, and whether induces its anti-proliferative effect through apoptosis or other pathways are unknown. Further studies are proposed to find out the underlying mechanism of action and the relation of its molecular structure with its activity.

Using bioassay-guided fractionation, it appeared that teuclatriol is one of the compounds responsible for the immunoinhibitory effect of *Salvia mirzayanii*. The effect of this guaiane sesquiterpene on the proliferation of activated lymphocytes demands to perform further investigations to find its precise immunomodulatory activity.

## ACKNOWLEDGMENTS

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