

Research Paper:

Immunomodulatory Effects of *Origanum Vulgare* L. and *Origanum Majorana* L. Ethanolic Extracts in Vitro



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ABSTRACT

Background: Many plants possess therapeutic properties against infectious and non-infectious diseases, including cancer and inflammatory diseases. Plants may have immunomodulatory properties, too. In this study, we evaluated the immunomodulatory effects and toxic properties of ethanolic extract from the Lamiaceae family on macrophage, lymphocyte, and B Cell Leukemia (BCL-1) in vitro.

Materials and Methods: Plant samples were purchased, and the ethanolic extract from their leaves was prepared by dissolving them in ethanol (maceration) for 48 hours. The obtained ethanolic extract was dried at room temperature. The normal lymphocytes and macrophages extracted from BALB/c mice and BCL-1 were cultured with different concentrations of the extracts for 24 hours. MTT assay was performed to evaluate proliferation, and Lactic Dehydrogenase (LDH)-based cytotoxicity was measured.

Results: We found that ethanolic extracts at a concentration of 1 mg/mL significantly increased lymphocyte proliferation (47% by *Origanum vulgare* and 95% by *Origanum majorana* compared to the control group). Also, the activity of macrophages increased 35% after *O. vulgare* administration and 23% after *O. majorana* administration at a dose of 1 mg/mL. Furthermore, LDH results prove that *O. vulgare* and *O. majorana* (1 mg/mL) had no toxic effect on normal lymphocytes and macrophages. The finding of the MTT assay showed that extracts of *O. vulgare* (with IC₅₀ of 1.04 mg/mL) and *O. majorana* (with IC₅₀ of 1.6 mg/mL) could significantly suppress the growth of BCL-1.

Conclusion: The *O. vulgare* and *O. majorana* extract with immunomodulatory effects increased the proliferation and activities of lymphocytes and macrophages in a dose-dependent manner. Also, the tested extracts were showed significant anticancer activity.

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1. Introduction

Plants have always been used for their healing properties, and there is now an increasing scientific interest in their biological and pharmacological properties [1-3]. The use of herbal medicine to strengthen and regulate the immune system is a critical approach to complement and replace chemical drugs. So today researchers are working to extract the most effective herbal compounds with immunomodulatory properties [4-6]. Plants of the Lamiaceae family are known to have various medicinal effects and are found in different parts of the world, such as North America, Europe, and Asia. Origanum is also from this family and has two species, *O. vulgare*, and *O. majorana*. This plant is used medicinally in Iran [7, 8]. The therapeutic effects of these two species include anti-inflammatory, antimicrobial, and antioxidant properties. The features mentioned belong to hydroalcoholic, methanolic, ethanolic extracts, and essential oil of *O. vulgare*, and *O. majorana* [9-11]. For example, the anti-inflammatory properties of *O. vulgare* are seen in reducing the production of nitric oxide and inflammatory cytokines and increasing anti-inflammatory compounds. The majority of the studies were carried out to assess antimicrobial and antioxidant properties of these plants, while few studies have investigated their immunomodulatory effects [12-15].

Lymphocytes and macrophages are the main cell types of acquired and innate immunity. After identifying the foreign antigen, these two cell types are activated and begin to proliferate, secrete cytokines, and produce antibodies [16, 17]. Given the importance of lymphocytes and macrophages in the immune process, examining the status of these cells can be an excellent start to assess the effects of a plant product on the response of the innate and acquired immune system.

Several investigations have reported the positive effect of *O. vulgare* and *O. majorana* to treat diseases such as rheumatoid arthritis, type 1 diabetes, and cancer [18-21]. However, there are limited findings to evaluate the effects of *O. vulgare*, and *O. majorana* extracts on the immune response. Although the two species of *O. vulgare* and *O. majorana* are similar, they may have different pharmacological effects [22, 23]. Therefore, we investigate the immunomodulatory effects of *O. vulgare*, and *O. majorana* extracts on lymphocytes and macrophages. Also, to ensure the preservation of anticancer effects mentioned in other studies, we examined the toxic effects of these two extracts on the cancer line BCL-1 (B Cell Leukemia) [20, 21].

This study aimed to evaluate and compare the immunomodulatory properties of different concentrations of plant extracts *O. vulgare* and *O. majorana* in vitro. The assessment of anticancer properties and proliferation of lymphocytes and macrophages were studied by measuring cell viability with the MTT assay. Compounds with medicinal properties, despite beneficial functions, can cause cell damage, so the possible toxic effect of the extracts on the normal cell of the immune system was assessed by the Lactic Dehydrogenase (LDH) test.

2. Materials and Methods

Identification and preparation of extract

O. vulgare and *O. majorana* species were purchased from the Institute of Medicinal Plants, ACECR, Karaj City, Iran. After preparing standard herbarium samples, the plants were identified according to Flora Iran by the authors. Finally, the voucher specimens were deposited in the Institute of Medicinal Plants Herbarium (IMPH). The voucher numbers of *O. vulgare* and *O. majorana* were 7093 and 7094, respectively.

Before the preparation of ethanolic extract Origanum herb, 40 g of dried leaf powder was extracted with 400 mL of 80% (v/v) ethanol (48 hours), and then the prepared extract was filtered with Whatman filter paper. To evaporate ethanol, we poured the extracts into several plates and placed them in the room. The solvent evaporated at room temperature, and the extract stored at 4°C. The weight of extracts powder was measured. The yield of the extracts for *O. vulgare* was 4.5 g, 11.25% w/w, and for *O. majorana* 4 g, 10% w/w from the dried sample. The extracted powder was dissolved in RPMI-1640 and filtered through 0.2- μ m sterile syringe filters. Then, the desired dilutions were prepared from the stock solution.

High-Performance Liquid Chromatography (HPLC) analysis

To compare the compounds of *O. majorana* and *O. vulgare* extract, we performed High-Performance Liquid Chromatography (HPLC) analysis. Identification of phenolic and flavonoid compounds in the *O. vulgare* and *O. majorana* extracts was achieved by comparing their Retention Times (RT) and visible spectra with that of the authentic standards (Figure 1). HPLC analysis has been carried out using the waters chromatography system (Waters, 600E, USA) on a Phenomenex Gemini NX C18 column (250 \times 4.6 mm, 5 μ m). A calibration curve for each of the standard phenolic and flavonoid compounds were constructed, and compound concentration was calculated.

HPLC conditions for determination of compounds

The two elution solvents were exchanged, i.e., solvent A (deionized water) and solvent B (acetonitrile). The pH of the deionized water was adjusted to 2.5 using trifluoroacetic acid. The following linear gradient elution profile was used: 85% A/15% B–0 min, and 15% A/85% B–50 min. The flow rate was 1 mL/min, and phenolic and flavonoid compounds were detected at a wavelength of 280 nm [24].

Mice

Inbred female BALB/c mice (8–12 weeks old and 25–30 g weight) were maintained in specific pathogen-free conditions prepared from the Center for Animal Resources and Research Institute Pasteur. The lymphocyte and macrophage cells were extracted from the spleen and peritoneum, respectively. All protocols were approved by the Ethics Committee of Shahed University.

Preparation of macrophage and culture

The mice were anesthetized, and the skin of the chest and abdomen of mice was carefully dissected without opening the peritoneum. Peritoneal cells were obtained from each mouse using a lavage method in which 5 mL of cold normal saline was twice injected intraperitoneally. The abdomen was massaged, and 90%–95% of the injected volume was recovered. The cells were centrifuged, washed, and re-suspended in RPMI medium 1640 (RPMI 1640; BioIdea, Iran) medium supplemented with 10% Fetal Bovine Serum (FBS; BioIdea, Iran) and were counted using Trypan blue dye (DNAbiotech, Iran) to detect dead cells. (it penetrates the cell membrane of the dead cells, but not the living cells.) Then, 2×10^5 cells/well were cultured in 96-well microplates and incubated at 37°C and 5% CO₂ for 2 h. The non-adherent cells were removed by washing the plate with normal saline (37°C), and the adherent cells were incubated for 24 h. All procedures were conducted under aseptic conditions [25].

Culture of splenic lymphocytes

The spleens of mice were separated and placed in 5 mL RPMI/FBS. So, the cell suspension was prepared. Then, red blood cells were lysed by suspending cells in 155 mM of 0.83% NH₄Cl (MERCK) and 130 mM of 10% Tris-HCl buffer (MERCK) (2–3 min at room temperature). RBC lysis was stopped with Fetal Bovine Serum (FBS). The cell suspensions were centrifuged (3000 rpm for 10 min in 4°C) and re-suspended gently in RPMI 1640 with 10% FBS. The splenic cells were counted and cultured

in 96-well flat-bottomed microtiter plate in 2×10^5 cells/well and incubated at 37°C and 5% CO₂. Finally, *O. vulgare* and *O. majorana* extracts in final concentrations of 0.01, 0.1, 1, and 10 mg/mL were added into the wells. (The experiments were performed in triplicate wells for each group). The supernatants were collected after 24 h and stored at -70°C for LDH assay.

Cell line

Anticancer activity was assayed on cancer cell lines of BCL-1 clone 5B1b. The cells were purchased from Institute Pasteur and cultured in RPMI supplemented with 10% FBS and 1% antibiotics penicillin–streptomycin. The BCL-1 cells were counted and cultured in 96-well flat-bottomed microtiter plate in 2×10^4 cells/well and incubated at 37°C and 5% CO₂. Then, the extracts in the final concentration of 0.01–10 mg/mL were added to the wells.

Cell viability by MTT assay

Effects of *O. vulgare* and *O. majorana* extracts on the viability of lymphocytes, macrophages, and BCL-1 were determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. The MTT (DNAbiotech, Iran) is a yellow dye, which is metabolized by living cells to purple formazan crystals. After 24 h incubation of cells with serial dilutions of the *O. vulgare* and *O. majorana* extracts, MTT was assayed in triplicate. The cells treated with the medium were served as a control group. About 5 mg/mL solution of MTT was prepared in (medium) RPMI-1640, filtered, and stored at -20°C. Then, the solution was added to each well as 10% of its volume. The microplates were incubated at 37°C and 5% CO₂ for 4 h. After removing the supernatants, the formazan crystals were resolved in acidic isopropanol (MERCK) (0.04 M HCL in isopropanol), and the absorbance of each well at 492 nm was read [26, 27].

The activity results are expressed as a fold increase concerning the negative control group, which was considered to exhibit a 1-fold increase. Fold increase refers to activity index and was obtained using the Formula 1. The results of anticancer activity are reported with a percentage of growth inhibition of the cancer line.

$$1. \text{ Fold increase} = \frac{\text{mean OD test}}{\text{mean OD control}}$$

$$\text{Growth inhibition(\%)} = \frac{\text{mean OD control} - \text{mean OD test}}{\text{mean OD control}} * 100$$

Measurement of cell cytotoxicity

Lactate Dehydrogenase (LDH) (Roche, Germany) is a stable cytoplasmic enzyme present in all cell types and is rapidly released into the cell culture supernatant upon damage to the plasma membrane. A common method for determining cytotoxicity is the LDH assay.

The cell cytotoxicity assay with the modified protocol

Four sets of control for each condition were used: first high control (5% Triton X-100 [DNAbiotech, Iran]) and second for low control (normal cell without extract). Third, 100 μ L of medium (RPMI-FBS) without cells were used as the negative control (blank), and the fourth was the extract control. After 24 hours of treatment, 100 μ L of supernatant was transferred from the top of the samples culture plate to the assay plate. Then, the wells were treated with 100 μ L of LDH reaction mix and incubated at room temperature in the dark for 90 minutes. The samples were finally analyzed with a microplate reader at a wavelength of 492 nm. The results of LDH activity were expressed as the percentage of cytotoxicity (Formula 2).

$$2. \text{Cytotoxicity(\%)} = \frac{\text{exp.value-low control}}{\text{high control-low control}} * 100$$

Removing the interference of extracts with MTT and LDH

Some phenolic compounds lead to the nonspecific reduction of MTT solution. To evaluate the interventional effect of extracts in MTT assay, at first, the MTT solution was added to the wells containing the extracts and was incubated at 37°C under 5% CO₂ for 4 hours. Then the contents of each well were emptied, and the formazan crystals were resolved in acidic isopropanol. Eventually, absorbance at a wavelength of 492 nm was read by a microplate reader.

Data plotted in Figure 2A show that the absorbance was significantly increased in concentrations of 10 mg/mL of *O. vulgare* and 1 to 10 mg/mL of *O. majorana* extract. The extracts reduced the MTT solution. Therefore, to eliminate the interventional effect of plant extracts, after cell culture with *O. vulgare* and *O. majorana* extracts for 24 h, the supernatant was removed, and a new culture medium was replaced. Then MTT solution was immediately added to the wells. Figure 2B shows the results of the intervention effect of the extracts on the LDH assay. Concentrations of 1-10 mg/mL *O. vulgare* and 10 mg/

mL *O. majorana* extract significantly increase absorption (P<0.05). To remove the effect of plant extract intervention in LDH assay, the extract control group was compared to the sample group in LDH parameters, and the absorption values of the extract control groups were subtracted from all sample data.

Statistical analysis

Statistical analysis was performed by 1-way Analysis of Variance (ANOVA) followed by Tukey's multiple comparison test in SPSS v. 24. According to the results of the Shapiro-Wilk test, all variables had a normal distribution. The level of statistical significance was determined at P<0.05.

3. Result

HPLC analysis of phenolic compounds

In the present study, the ethanol extracts of *O. vulgare* and *O. majorana* were analyzed by the HPLC. HPLC analysis revealed several peaks, indicating the presence of apigenin (0.01 mg/mL, Retention Time [RT]: 10.31 min), rosmarinic acid (0.03 mg/mL, RT: 20.16 min) in the *O. vulgare* extract, and apigenin (0.12 mg/mL, RT: 10.28 min), rosmarinic acid (0.01 mg/mL, RT:17.73 min), quercetin (0.001 mg/mL, RT: 20.52 min) in the *O. majorana* extract.

The effect of various doses of *O. vulgare* and *O. majorana* extract on the activation of macrophages

As shown in Table 1, the peritoneal macrophage activity increased significantly when the *O. vulgare* and *O. majorana* extract at doses of 0.1-1 mg/mL has been added to cell culture for 24 h. Increasing the cell viability and proliferation of macrophages are seen in the effective concentration of 0.1-1 mg/mL *O. vulgare* extract by 28% to 35%. Also, *O. majorana* extract in the dose of 0.1-1 mg/mL with a 14%-23% increase in MTT enhances the activity and proliferation of macrophages, which are statistically significant (Figure 3). *O. vulgare* and *O. majorana* extracts at a concentration of 10 mg/mL have a toxic effect on cells and can kill about 50% of normal macrophage cells.

Analyzing the effects of *O. vulgare* and *O. majorana* extracts on cell growth inhibition or cell death have been an essential component of biological research. The toxic effect of different concentrations of *O. vulgare* and *O. majorana* extracts on macrophages is shown in Figure 3. The results are presented as the percentage of cell dam-

Table 1. The effect of *o. vulgare* and *o. majorana* extract in macrophage activation (MTT Assay)

Group	<i>O. Vulgare</i> Extract				Control	<i>O. Majorana</i> Extract			
Concentration (mg/mL)	10	1	0.1	0.01	-	10	1	0.1	0.01
Fold increase	0.45	1.35	1.28	1.06	1	0.35	1.23	1.14	1.04
P	0.0001*	0.001*	0.008*	0.86	-	0.0001*	0.0001*	0.006*	0.120

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The effect of 24-hour exposure of *O. vulgare* and *O. majorana* extracts with macrophage in vitro. The macrophages were cultured at 2×10^5 , and the macrophage activity and cell viability were assayed by the MTT method. Data are represented as the fold increase.

*Denoted significant differences with the control group ($P < 0.05$); Fold increase: Activity index relative to the control group.

age and death. The *O. vulgare* extract at a concentration of 10 mg/mL causes 87.4% of macrophage cell death, while at a dose of 1 mg/mL, it has no toxic effect on the normal macrophage. *O. majorana* extract does not induce macrophage mortality at all concentrations examined and has no significant toxic effect.

The effect of various doses of *O. vulgare* and *O. majorana* extract on the activation of lymphocytes

The results are presented in Table 2. In lymphocytes, with increasing the concentration of the extracts to 1 mg/mL, we observe an improvement in the lymphocyte MTT; while increasing the dose and reaching a concentration of 10 mg/mL, the measure of MTT decreases.

Lymphocyte activity was enhanced by 47.7% in the *O. vulgare* extract and by 95% following the *O. majorana* extract (dose of 1 mg/mL), which is statistically significant. The difference in the effect of the two plant extracts is seen only in the concentration of 1 mg/mL. The extract of *O. majorana* is more effective than *O. vulgare* in stimulating and activating lymphocytes ($P < 0.00$, Figure 3).

The reduction in vital activity affected by the concentration of 10 mg/mL extracts of *O. vulgare* and *O. majorana* compared to the control group was significant (-31.2% and -39.6%, respectively) ($P < 0.03$). At this

concentration, both extracts have toxic effects on lymphocytes. The most effective dose to promote lymphocyte activity is 1 mg/mL of *O. vulgare* and *O. majorana* extracts. These extracts caused a dose-dependent and significantly improve lymphocyte proliferation.

The extract of *O. vulgare* in a concentration of 10 mg/mL induces 13.8% lymphocyte damage, as well as at a lower dose such as 1 mg/mL, and it has no toxic effect on cells (Figure 3).

Anticancer activity

Results obtained showed that *O. majorana* and *O. vulgare* extracts could suppress the growth and proliferation of B cell leukemia cancer cells. The anticancer activities of the extracts were similar and statistically significant at the concentration of 1-10 mg/mL.

Among the extracts, the anticancer activity against BCL-1 was found for *O. vulgare* with IC_{50} of 1.04 mg/mL and *O. majorana* with IC_{50} of 1.6 mg/mL (Figure 4).

4. Discussion

The use of medicinal plants in communities since ancient times and ensuring no side effects encourages researchers to find plants with immunomodulatory proper-

Table 2. The effect of *o. vulgare* and *o. majorana* extract in lymphocyte activation (MTT assay)

Group	<i>O. Vulgare</i> Extract				Control	<i>O. Majorana</i> Extract			
Concentration (mg/mL)	10	1	0.1	0.01	-	10	1	0.1	0.01
Fold increase	0.6	1.4	1.1	0.6	1	0.6	1.9	1.09	0.8
P	0.014*	0.0001*	0.457	0.085	-	0.032*	0.0001*	0.805	0.721

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The effect of 24-h exposure of *O. vulgare* and *O. majorana* extracts with lymphocyte in vitro. The lymphocytes were cultured at 2×10^5 , and the lymphocyte activity and cell viability were assayed by the MTT method. Data are presented as the fold increase.

*Denoted significant differences with the control group ($P < 0.05$); Fold increase: Activity index relative to the control group.

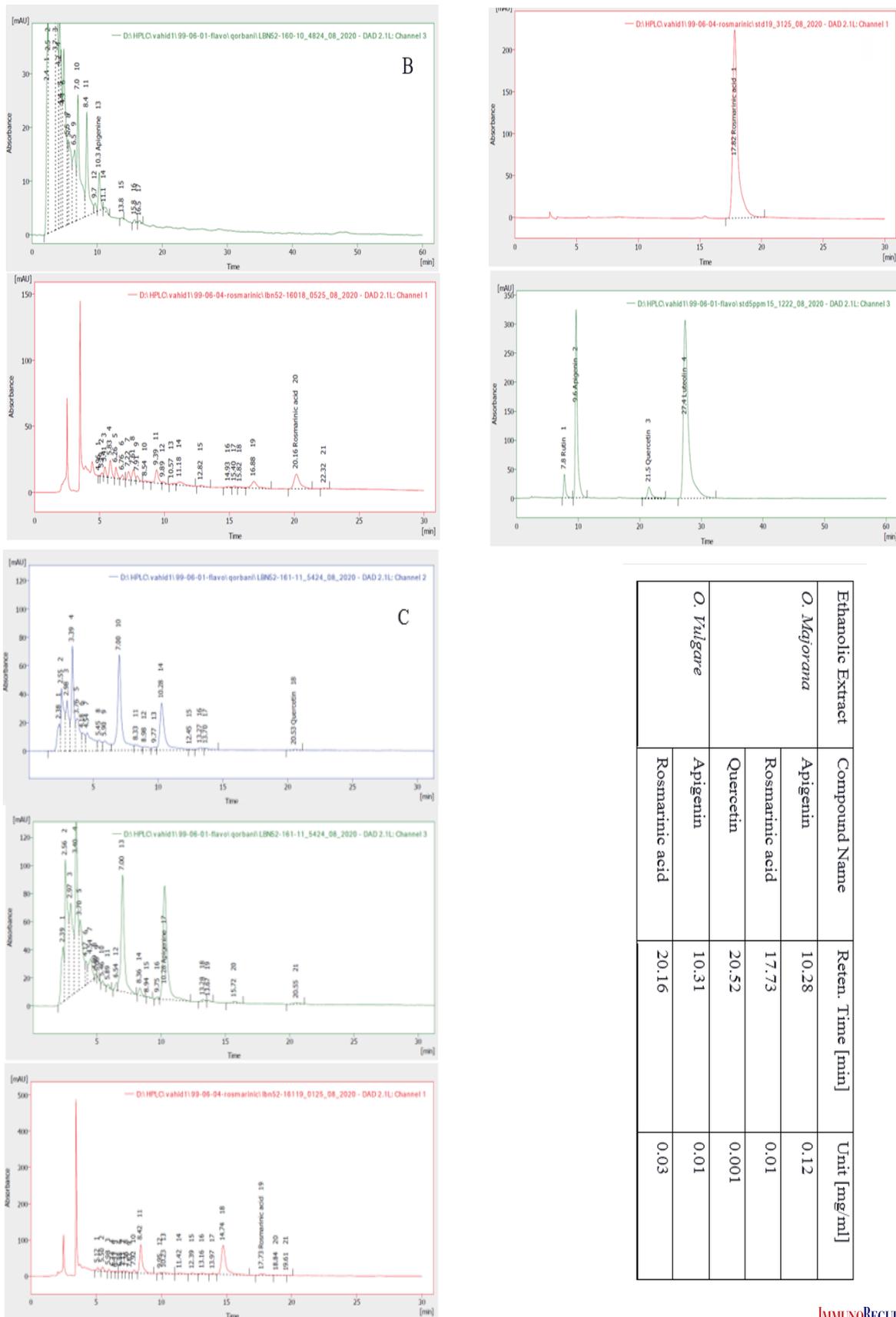
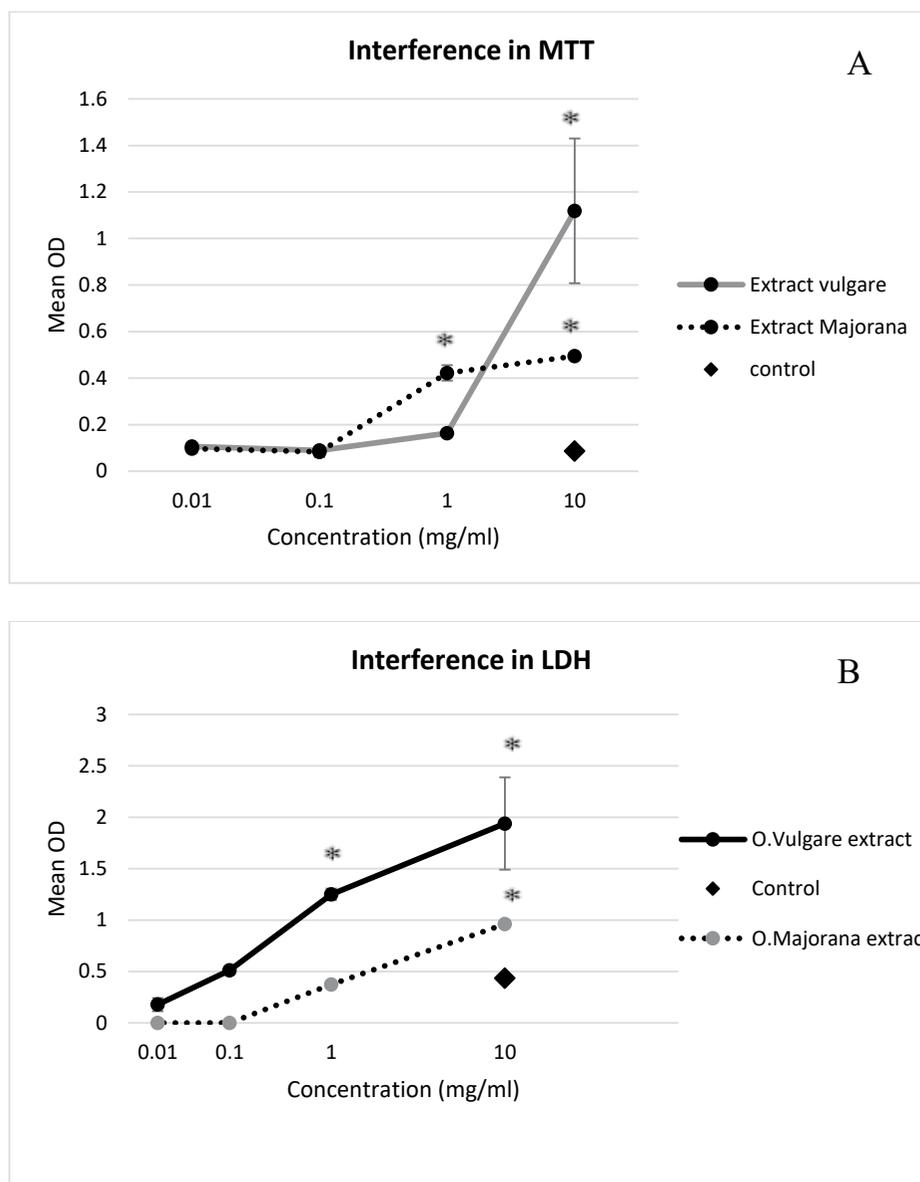


Figure 1. HPLC chromatogram of extract from *Origanum vulgare* and *Origanum majorana*
 A: Standard (Rutin, Apigenin, Luteolin, Quercetin, Rosmarinic acid); B: *Origanum vulgare*; and C: *Origanum majorana*.



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Figure 2. Interference in MTT and LDH tests by extracts, poured into a well

A: MTT solution adding to each well and microplates incubated for 4 hours, after removing the supernatants, the formazan crystals resolved in acidic isopropanol; B: The wells treated with LDH reaction mix and incubation at dark room for 90 minutes. In both tests, RPMI was considered as a control group. The absorbance at 492 nm was read by a microplate reader. Data are presented as Mean±SD. *Denoted significant differences with the control group (P<0.05).

ties for the treatment of a wide range of diseases. Among the investigated plant species, the Origanum genus exhibits promising biological activities.

This study aimed to evaluate the properties of *O. vulgare* and *O. majorana* extracts in two normal immune cells using a different method in vitro. The effect of different extract concentrations on cell viability was measured using the MTT and LDH assay. The dose of the

plant extract with the highest activation and non-toxic effects were selected. Lymphocyte proliferation after stimulation by various factors is the first step in a proper immune response to eliminate an antigen, so an increase in the proliferation of lymphocytes indicates the activation of the immune system [28]. *O. vulgare* and *O. majorana* extracts improved the immune system in a dose-dependent manner by proliferation and activity increase of lymphocyte and macrophage.

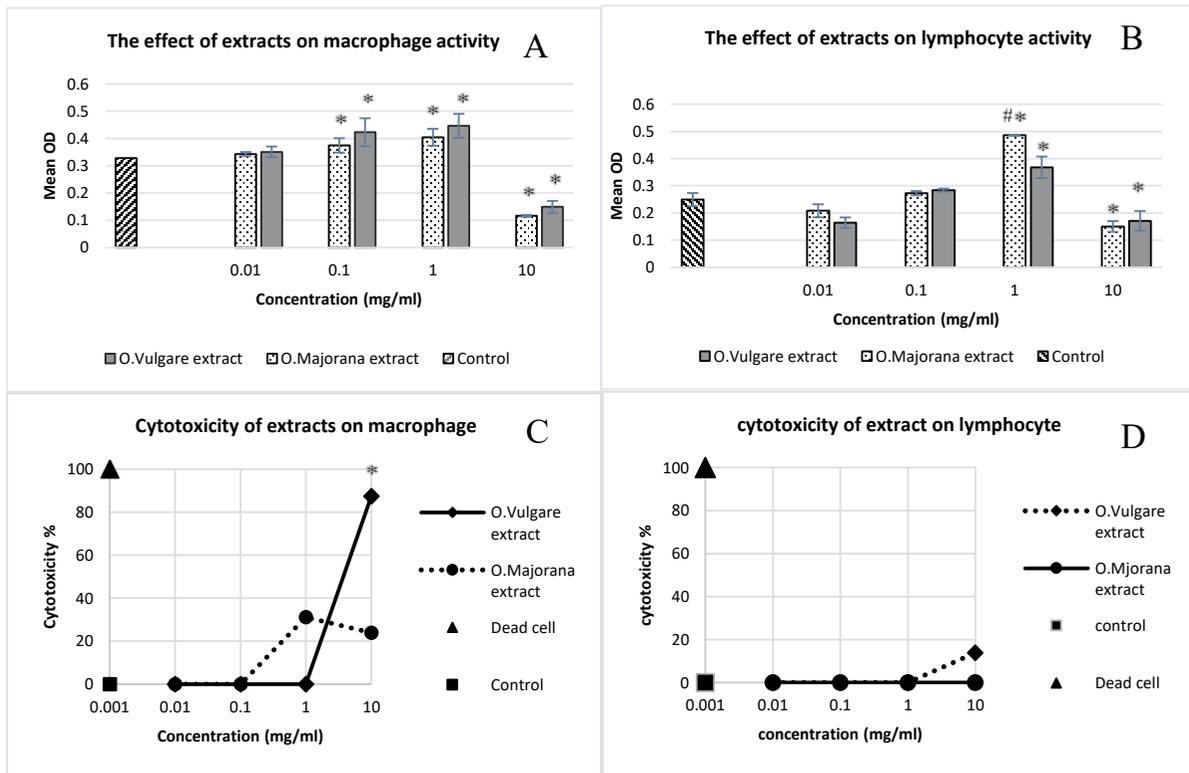


Figure 3. The effect of *O. vulgare* and *O. majorana* extracts on macrophage and lymphocyte activity in vitro
 A & B: The proliferation and viability of cells assayed by the MTT method; C & D: The cytotoxicity assayed by the LDH method
 Results are presented as a percentage of toxicity. The cells were cultured at 2×10^5 , and the extract was added to the wells at final concentrations of 10 to 0.01 mg/mL for 24 hours. All experiments were repeated in triplicate wells. All data are presented as Mean \pm SD. *Denoted significant differences with the control group; #Denoted significant differences with the *O. majorana* group ($P < 0.05$).

The amount of LDH released from lymphocytes and macrophages demonstrates the ethanolic extracts of *O. majorana* and *O. vulgare* have no toxic effect on normal immune cells and significantly improve the immune response by enhancing the activity and proliferation of lymphocytes and macrophages. Besides this, studies have shown that *O. vulgare* and *O. majorana* extract at a concentration of 1 mg/mL, despite their toxic effect on the BCL-1 cancer cell line, stimulates resting and activated lymphocytes [29].

The anticancer activity of the *Oregano* species has been studied, but in the present study, *O. majorana* and *O. vulgare* extracts have been tested to determine the anticancer activity and the activation effect of immune cells [21]. Thus, this study highlights the importance of the activation properties of the *O. vulgare* and *O. majorana* extracts. According to the result of the activation and cytotoxic properties of the extracts shown in Figure 5, the most effective concentration of the extract to stimulate and strengthen the immune system in both plants is 1 mg/mL. Comparison of the same concentration of two

plant extracts in the effective dose (1 mg/mL) shows that the immunomodulatory properties of *O. majorana* extract are significantly higher than *O. vulgare* extract.

Lymphocyte proliferation, fundamental physiological responses involved in clonal expansion after antigen challenge, could both be influenced by flavonoid exposure. *O. vulgare* and *O. majorana* extracts have immunomodulatory and anticancer properties. That means the extracts increase the activity and proliferation of lymphocytes and macrophages, causing a decrease in the viability of the cancerous cell line. Therefore, these two extracts with the anticancer activity and ability to strengthen the immune system can be considered as high-capacity compounds for broader studies in the field of immune system development. Other studies have shown an enhancement of the innate immune response due to *O. vulgare* and *O. majorana* extract [30, 31]. Comparison of HPLC analysis of two plants shows that the compounds of apigenin, rosmarinic acid, quercetin were differently observed in *O. vulgare* and *O. majorana* extracts, and the results of this study are consistent with

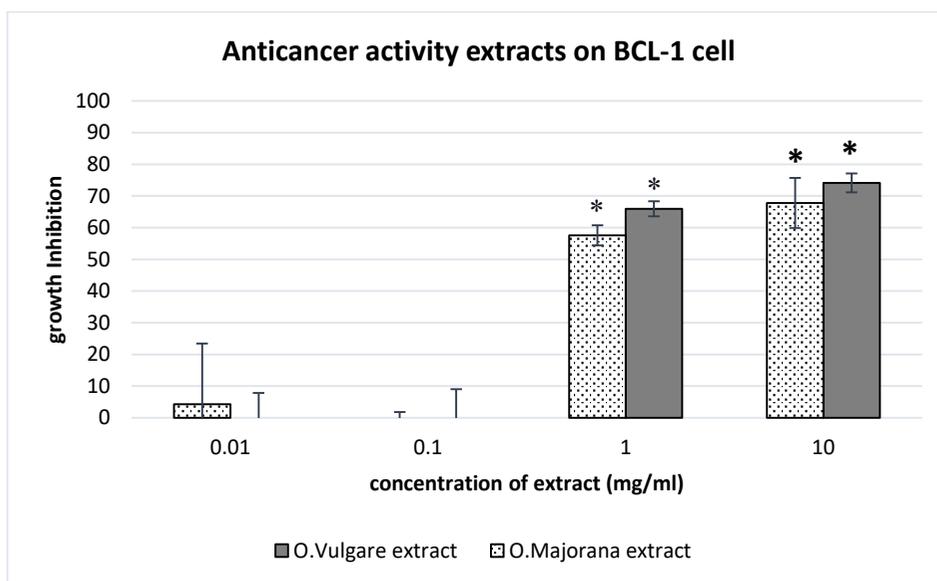


Figure 4. Activity of *O. vulgare* and *O. majorana* against B Cell Leukemia (BCL-1) cell line

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The BCL-1 was cultured at 2×10^4 , and the extract was added to the wells at final concentrations of 10 to 0.01 mg/mL for 24 hours. All experiments were repeated in triplicates. All data are presented as Mean \pm SD.

*Denoted significant differences with the control group ($P < 0.05$)

some previous studies [32, 33]. Also, in many studies, other substances such as carvacrol, thymol, and rosmarinic acid have been reported as major components [9, 32, 34]. The diversity and amounts of compounds in plants depend on many factors, including the geographical origin, soil properties, season, and growth stage. Some researchers proposed that the antioxidant and an-

ticancer, and anti-inflammatory properties of oregano species are associated with their phenolic and flavonoids content [32, 35-38]. So, the similar effects observed in *O. majorana* and *O. vulgare* extract may be related to their common substance of apigenin or rosmarinic acid. Although determining the effective compounds of plants requires further research.

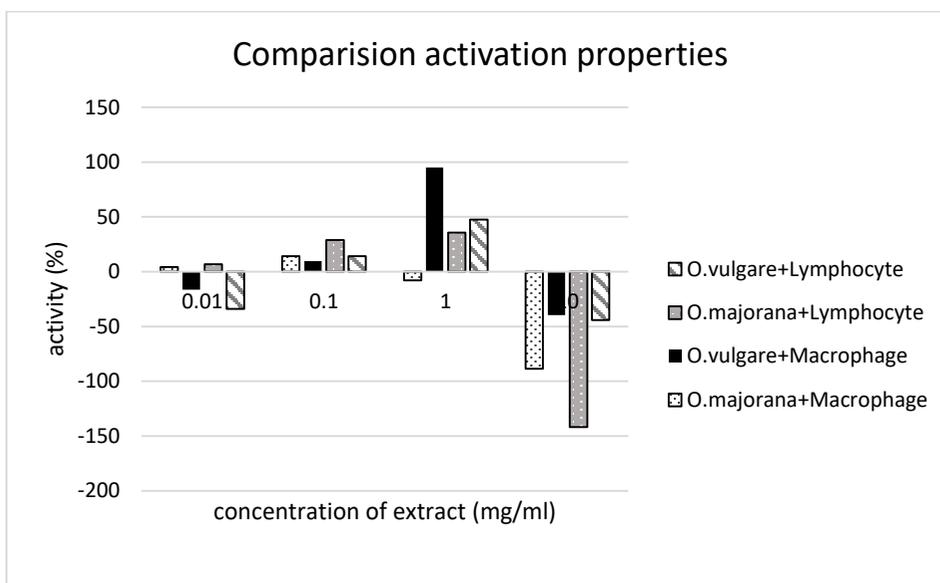


Figure 5. Comparison of the effect of *O. vulgare* and *O. majorana* extracts on lymphocyte and macrophage activities

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The results were reported by calculating the percentage of activity increase (MTT results) and decreased cell viability (LDH results).

Ethical Considerations

Compliance with ethical guidelines

The experimental animal's process was performed based on guidelines by the Research Ethics Committee of Shahed University of Medical Sciences, Iran (Code: IR.SHAHED.REC.1397.094). Also, because of in vitro study, there is no ethical guideline.

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Authors' contributions

Conceptualization, supervision: Roya Yaraee; Identification of plants: Majid Ghorbani Nohooji; Investigation, methodology, Statistic analysis, writing – original draft: Fatemeh Mashhadi; Review & editing: All authors.

Conflicts of interest

The authors declared no conflict of interest.

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