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RESEARCH ARTICLE

Immunomodulatory effect of Parsley (*Petroselinum crispum*) essential oil on immune cells: Mitogen-activated splenocytes and peritoneal macrophages

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Abstract

Introduction: Parsley (*Petroselinum crispum*) has been traditionally used for the treatment of allergy, autoimmune and chronic inflammatory disorders. The present study aims to investigate the suppressive effects of parsley essential oil on mouse splenocytes and macrophages cells.

Methods and Materials: Parsley essential oil was harvested. It was treated on splenocytes and phytohemagglutinin (PHA) (5 μg/mL) and lipopolysaccharide (LPS) (10 μg/ mL) activated splenocytes in different concentrations (0.01–100 μg/mL); then, proliferation was assayed by methyl tetrazolium (MTT) method. Treatment was also performed on the macrophages and LPS-stimulated macrophages (10 μg/ml) and the nitrite levels were measured using the diazotization method based on the Griess reaction and MTT assay for evaluation of the viability of the macrophages.

Results: Proliferation of splenocytes in all the treated groups was suppressed. In PHA-stimulated splenocytes, the suppression was seen in all the examined concentrations (0.01–100 μg/mL), while in the unstimulated and LPS-stimulated groups suppression was relatively dose dependent and in high concentration (10 and100 μg/mL).The viability of the macrophages in all groups was the same and in the unstimulated groups; NO suppression was significant in all the concentrations but in LPS-stimulated groups, it was significant in the three higher concentrations (1, 10, and100 μg/mL).

Conclusion: The results of this study indicate that parsley essential oil may be able to suppress the cellular and humoral immune response. It can also suppress both NO production and the functions of macrophages as the main innate immune cells. These results may suggest that parsley essential oil is a proper suppressant for different applications.

Keywords: Parsley, splenocyte, macrophage, nitric oxide, immunomodulation

Introduction

In most of the cultures around the world, herbal medicine is a way to treat different diseases. Identification of the active components and the mechanisms of traditional medicines on the immune system are highly desirable.[1] During recent years, a variety of plant extracts have been studied for their immunomodulatory effects on the lymphocytes.[2] Due to the enormous variety of higher plant species, their potentiality as the new drug sources has not been completely explored. Up to now, about 5000 species have been phytochemically studied, but their pharmacological and biological activities have been rarely evaluated. Among the extracts of plants with immunomodulatory effects, some plants of Umbelliferae family can be seen. For example an ethanol extract of *Centella asiatica* (CA) inhibited human peripheral blood mononuclear cells (PBMC) mitogenesis

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and the production of IL-2 and TNF-α.[4] in another study, ethanol extract of CA stimulated neutrophil phagocytic function.[3] Parsley (*Petroselinum crispum*) has been applied in foods, pharmaceutical products, perfume, and cosmetic industry.[5] For example, it is used as a medicinal plant to treat arterial hypertension,[6] diabetes, cardiac[6,7] and renal diseases.[7] Phytochemical screening of parsley has revealed the presence of flavonoids (apiin, luteolin, and apigenin-glycosides),[8] carotenoids,[9] ascorbic acid,[10] tocopherol,[11] volatile compounds (myristicin, apiole), coumarines (bergapten, imperatorin),[8] phthalides, furanocoumarins, and sesquiterpenes.[12] A study showed that the parsley extract inhibits *in vitro* and *ex vivo* platelet aggregation and prolongs the bleeding period in rats.[13] Also, some previous studies have reported the antioxidant properties of parsley in chemical assays.[14,15] In spite of different studies on parsley and its various uses, there is no sufficient data on its effect on the immune system. It is proposed that the traditional benefits of parsley are related to its immunomodulatory effects and this study is an attempt to investigate the above probability. To do this, we evaluated the effect of parsley essential oil on splenocytes in the presence or absence of phytohemagglutinin (PHA) and lipopolysaccharide (LPS) to analyze T and B lymphocyte activity as the main effector cells in the cellular and humoral immune system. Moreover, the influence of this oil on peritoneal macrophages as an important arm of the innate immune system and their nitric oxide production were explored.

Methods

Sample and preparation of essential oil

The leaves of *Petroselinum crispum* were collected from the plants cultivated in the Center of Medicinal Plants Research 25 km north of Tehran, Iran, and confirmed by the Center of Agricultural Research, Tehran, Iran.

Dried powdered leaves of parsley (50 g) were placed in a Clevenger distillation apparatus with 1 L of distilled water and hydrodistilled for 3 h (hydrodistillation method). Then, the oil was removed and stored at 4°C until use. During the experiment, the obtained essential oil was diluted using Tyrode's buffers to prepare various concentrations.[16]

Animals

Eight- to ten-week-old Balb/c mice were purchased from the Pasteur Institute of Iran, (Tehran, Iran). They were kept in the animal house of Tarbiat Modares Universit, and were given standard mouse chow sterilized water throughout the study.

Splenocyte isolation and treatment

Under sterile conditions, Balb/c mice spleens were removed and minced using a pair of scissors and homogenized in RPMI-1640 (Sigma, USA) by homogenizer and then passed through a fine steel mesh to obtain a homogeneous cell suspension. The erythrocytes were osmotically lysed with 0.75%NH_4Cl in Tris buffer (0.02%, pH = 7.2). After centrifugation (360×*g* at 4°C for 10 min), the pelleted cells were washed three times with phosphate-buffered saline (PBS) and resuspended in RPMI-1640 complete medium supplemented with 11 mM sodium bicarbonate, 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% fetal bovine serum. After tripan-blue staining and counting, cell viability was more than 95%. Splenocyte treatment and proliferation was assayed as follows: 5×10^6 cell/mL of the cells was seeded into each well of a 96-well flat-bottom plate (Nunc, Denmark) in complete medium. Then PHA (final concentration 5 μg/mL), or LPS (final concentration 10 μg/mL) or Tyrode buffer with parsley essential oil (final concentration 0.01–100.0 μg/mL) was added giving a final volume of 200 μL (tetraplicates). The obtained mixture was incubated for 40 h at 37°C and 5% CO_2 and then methyl tetrazolium (MTT) assay was performed.

MTT assay

After 40 h of incubation, 20 μL of MTT (5 mg/mL in PBS) was added to 200 μL wells and incubated for 4 h at 37°C and 5% CO_2. Then, the supernatants were gently removed and added to100 μL of acidic isopropanol (0.04 M HCl in isopropanol) in order to dissolve the formazan crystals. The absorbance was evaluated in an ELISA reader at 540 nm. The test results were expressed as a Stimulation Index (SI), which is A_{540} value for mitogen-cultures/the absorbance value for nonstimulated cultures.

Macrophage culture and stimulation

The macrophages were harvested by the lavage of the peritoneal cavity with 10 mL of RPMI-1640 (Sigma, USA) from Balb/c mice. The cells were centrifuged at 200×*g* and washed. Then, they were adjusted to 1.5×10^6 cells/mL in RPMI-1640 (supplemented with 11 mM sodium bicarbonate, 2 mM L-glutamine, 100U/mL penicillin, 100 μg/mL streptomycin, and 5% fetal bovine serum). The 3×10^5 cell suspensions were plated (200 μL/well) onto 96-well flat-bottomed plates (Nunc, Denmark) and they were incubated for 4 h to adhere in a humidified atmosphere with 5% CO_2 at 37°C. The nonadherent cells were removed by washing the wells with PBS three times. The adherent cells contained macrophages and were incubated for 48 h in RPMI medium. For stimulation, 10μg/mL LPS was added to a group of macrophages and just RPMI-1640 was added to another group. Tyrode buffer was applied as the negative control for both groups. Different concentrations of parsley essential oil (0.01–100 μg/mL) were added to both groups giving a final volume of 200 μL (triplicate). Interferon-γ (IFN-γ: 50 IU/mL) was used as positive control. They were incubated for 40 h at 37°C and 5% CO_2. After incubation, MTT assay was performed as described above.

Measurement of nitrite concentration

After 40 h of incubation, macrophages culture supernatants were collected and stored at –20°C for nitrite

assay. Nitric oxide was measured using Stuehr & Nathan method by the standard Griess reagent.[17] In brief, 50 μL of the test solution (supernatants of macrophage culture) was mixed with 50 μL of Griess reagent in a 96-well flat-bottomed plate (triplicate). After 15 min, the absorbance was measured in a Multiskan MS microplate reader at 540 nm. Nitrite concentration was determined by means of a standard curve of sodium nitrite.[18]

Endotoxin assay

The parsley essential oil used was analyzed for the presence of LPS by multitest limulus amebocyte lysate (LAL) assay (Endotoxin Detection Kit, BioWhittaker, Walkersville, MD, USA). All the material and reagents were prepared according to the procedure of the kit. Control standard endotoxin and LAL reagent water were used in the same manner as positive and negative controls, respectively. The positive reaction was indicated by a firm gel that remains intact momentarily when the test tube is inverted 180° following 1 h of incubation at 37°C.

Statistical analysis

Statistical analysis was performed using SPSS version 15 (SPSS Inc., Chicago, IL, USA) for windows software. For multiple comparisons, data were analyzed by one-way analysis of variance (ANOVA) and followed by LSD test. The *p*-value less than 0.05 was considered as significant. The results are expressed by mean ± standard deviation (SD).

Results

The effect of parsley essential oil on mitogen-stimulated splenocyte proliferation in vitro

The effects of parsley essential oil on the proliferation of mitogen-stimulated splenocyte *in vitro* are shown in Figure 1. Proliferation of the splenocytes in all the

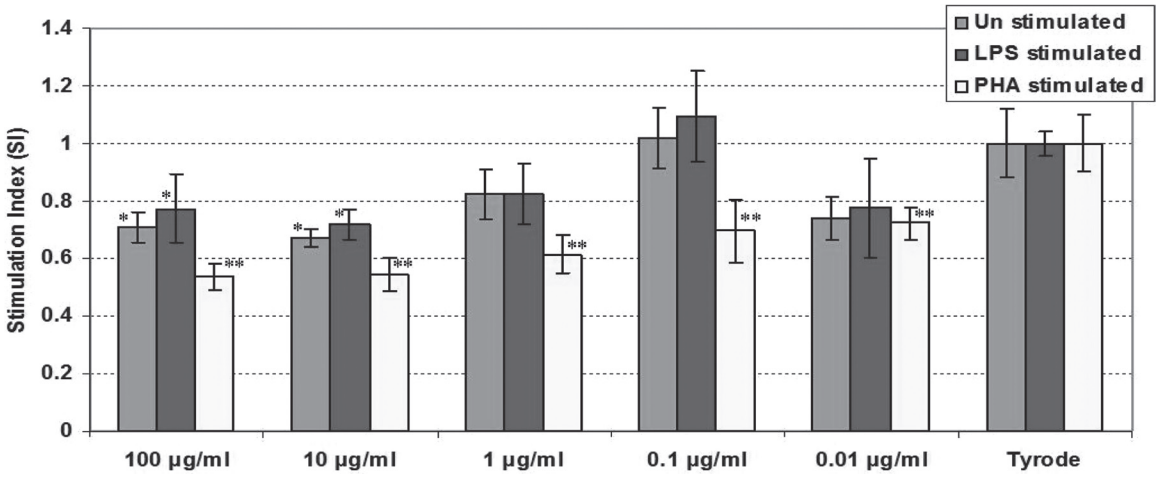

Figure 1. Effect of parsley essential oil (0.01–100 μg/mL) on the proliferation of mitogen-stimulated splenocyte *in vitro*. Tyrode buffer was the negative control. The values are presented as means ±SD. Significant differences were designated as *($P<0.05$), and ** ($P<0.01$). (See colour version of this figure online at www.informahealthcare.com/ipi)

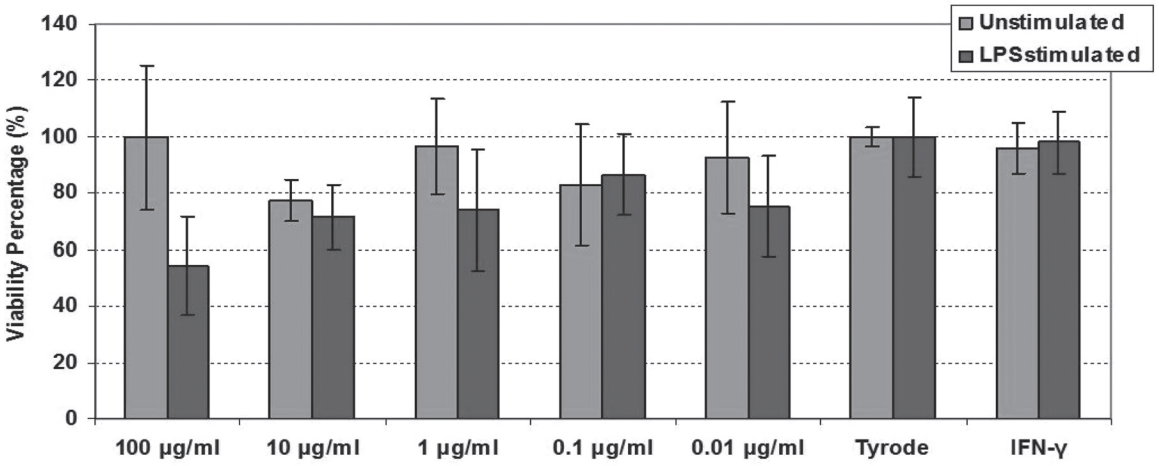

Figure 2. The values (mean ± SD) of MTT reduction by peritoneal macrophages treated with various concentrations of parsley essential oil. In all groups, MTT reduction was the same ($P>0.05$). ($P=0.528$ for unstimulated, $P=0.078$ for LPS-stimulated macrophages). Interferon-γ (IFN-γ: 50 IU/mL) was used as positive control. (See colour version of this figure online at www.informahealthcare.com/ipi)

parsley oil treated groups was decreased compared to untreated splenocytes ($P = 0.012$ splenocytes without mitogen; $P = 0.001$ LPS-stimulated and $P < 0.0001$ PHA-stimulated splenocytes). In all the concentrations, the proliferation of PHA-stimulated group was significantly lower than that of the negative control ($P < 0.001$). The effect of parsley essential oil on two other groups was dose dependent. The unstimulated and LPS-stimulated splenocyte proliferations were reduced by parsley essential oil in 10 μg/mL ($P = 0.017$, $P = 0.012$, respectively) and 100 μg/mL ($P = 0.031$ and $P = 0.035$, respectively).

The effect of parsley essential oil on macrophages MTT assay

The percentage of the viability of the macrophage cells was pretreated with various concentrations (0.01–100 μg/mL) of parsley essential oil, as shown in Figure 2. In all the concentrations of parsley essential oil, both unstimulated and LPS-stimulated macrophages showed the same viability as their controls ($P = 0.528$ unstimulated, $P = 0.078$ LPS-stimulated macrophages).

The effects of parsley essential oil on macrophages NO production

For both groups of unstimulated and LPS-stimulated macrophages treated with parsley essential oil, NO production was determined by detection of nitrite concentrations in the cell supernatants (Figure 3). The results indicated that production of NO by both groups of macrophages was reduced by essential oil ($P < 0.001$). In comparison with their control ($P < 0.001$), unstimulated group showed the decreased NO production in all concentrations. Suppression in LPS-stimulated group was dose dependent and in three concentration of 1, 10, and 100 (µg/ mL), the differences with the control group were significant ($P < 0.01$). Positive control in both series was higher than the negative control ($P < 0.001$).

The effects of parsley essential oil are not related to endotoxin or β-D-glucan contamination

The results of multitest LAL assay showed that the level of LPS in PPD preparation was < 0.125 EU/mL, the lower detection limit of the kit. The same test also rules out the presence of β-D-glucan contamination as will be discussed later.

Discussion

The health-promoting effects of natural compounds have now attracted attention. Plant extracts have long been used as traditional medicines for the treatment of a wide variety of ailments and diseases.[19,20] So many immunosuppressive drugs have now been adopted to control the unwanted immune responses, particularly for allergies, autoimmune disease, and transplant rejection.[21] Some immunosuppressive drugs as cyclosporine, tacrolimus, and mycophenolic mofetil are in use for organ transplantation and treatment of some autoimmune diseases. However, these drugs have some disadvantages and side effects. For example, they have a narrow therapeutic index, they exhibit a high degree of inter-individual and intra-individual pharmacokinetic, pharmacodynamic variability, and a number of serious, most notorious, side effects such as nephrotoxicity, hepatotoxicity, induction of diabetes, induction of hypertension and neurotoxicity.[22,23] So, new immunosuppressants with lower side effects and other probable advantages are demanded which have become a subject of scientific investigations recently. In this study, we evaluated the effect of parsley essential oil on PHA-stimulated splenocytes for T cells and LPS-stimulated for B cells analysis as the main effector cells in adaptive immune systems. Our results showed that in general parsley essential oil could modulate the immune responses. In all the examined concentrations, parsley essential oil inhibited the proliferation of the splenocytes stimulated with PHA.

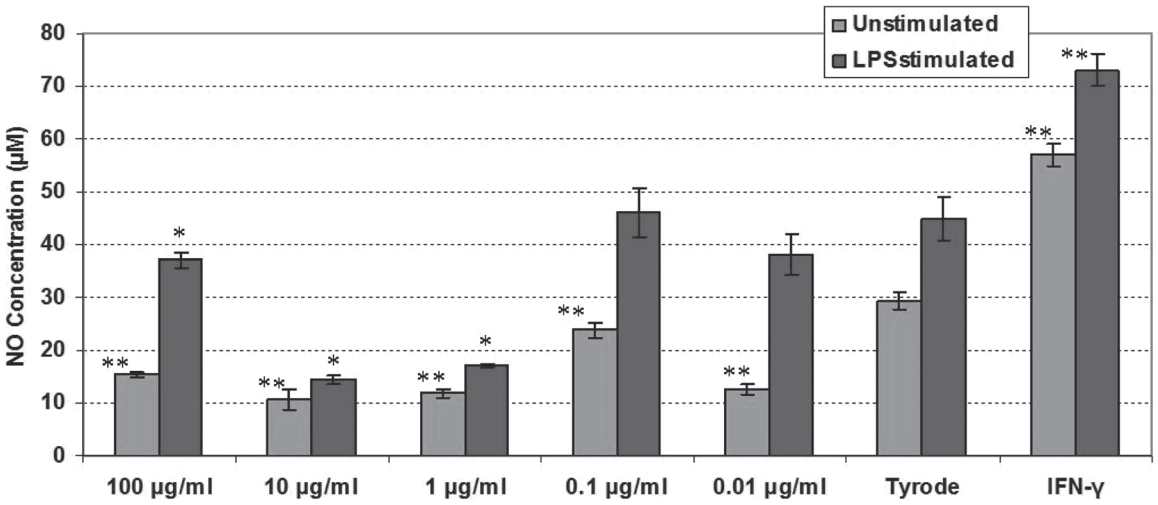

Figure 3. The values (mean ± SD) of NO production by two groups of unstimulated and LPS-stimulated peritoneal macrophages treated with various concentrations of parsley essential oil. Interferon-γ (IFN-γ: 50 IU/mL) was used as positive control. Significant differences were designated as *($P < 0.01$), ** ($P < 0.001$). (See colour version of this figure online at www.informahealthcare.com/ipi)

Proliferation was prevented in the unstimulated and LPS-stimulated groups only in high concentrations of parsley essential oil. The above results may be due to the inhibition effect of parsley essential oil on the proliferation. This is concluded by the fact that T-cell proliferation to mitogen is excessive, while unstimulated splenocytes and B cells have a lower proliferation rate. In other words, parsley essential oil may have a specific inhibitory effect on T cells. On the whole, the results showed its more inhibitory effects on T cells and as a result on the cellular immune responses. These data can explain the beneficial traditional application of parsley for diabetics, rheumatoid arthritis, and other autoimmune and inflammatory diseases.[24] It should be mentioned that some previous studies have shown the immunosuppressive activity of other herbal components as *Achillea talagonica*,[25] *Plantago ovata*,[26] *Boerhaavia diffusa*,[27] *Acorus calamus*,[28] *Pollen Typhae*,[22] and *Prunella vulgaris*.[29] We also examined the cytotoxic effect of parsley essential oil on peritoneal macrophages. The results of MTT assay showed that essential oil did not have any significant cytotoxic effect on macrophages. NO production was assayed in two groups of intact macrophages and activated macrophages. In both groups, NO release was suppressed, but the rate of suppression in LPS-activated macrophages was less effective; also, this suppression was dose dependent in higher doses. So, we can conclude that parsley essential oil can inhibit the activity of the macrophages. However, when macrophages become active, more essential oils are needed. With respect to the inhibitory effect of parsley essential oil on NO production of macrophages, it may be suggested that parsley essential oil can modulate macrophages without adverse toxic effect. As a result, this component can be used in inflammatory responses of different conditions although its doses should be managed. Previous studies demonstrated the antioxidant activity of parsley,[14,15] and in this research we showed its effect on NO production of macrophages. In other studies, the inhibitory effect of some other components as *Cocoa*,[30] *Tamarindus indica*,[31] *Polygonum tinctorium*,[32] and *Scrophularia striata*[33] extracts have also been demonstrated. Excess NO production has been associated with many diseases such as autoimmunity, rheumatoid arthritis, multiple sclerosis, systemic lupus erythematosus, inflammatory bowel disease, transplantation, and septic shock.[34,35] We showed the suppressive activity of the parsley essential oil on NO production from macrophages activated by LPS. Bacterial infections are accompanied by some ailments such as burns, injuries particularly in the eyes and other sites which may also initiate autoimmunity, solid tumors, and organ transplantation.[35,36] In these conditions, the LPS released from the bacteria could bear some adverse effects on septic shock. As a results a suitable component like parsley essential oil can be helpful for regulation of the immune responses, inflammation, and also bacterial infection. In another research, further activity of the parsley essential oil was studied and the antibacterial effect of this component was demonstrated.[37] In conclusion, we showed the immunomadulatory effect of parsley essential oil on splenocytes, especially PHA-activated splenocytes considered as T cells. Parsley essential oil also modulated macrophages NO production and function without any cytotoxic effect. These influences of parsley essential oil will candidate it as a natural herbal component to treat a variety of autoimmune and allergic diseases, transplantation, and also other ailments. Further specific immune evaluations, *in vivo* tests and analysis of parsley essential oil ingredients will clarify its further applications.

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Declaration of Interest

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