



# In Vitro Effects of Olive Vegetation Water on IFN- $\gamma$ and IL-10 Secretion in Multiple Sclerosis Patients

Mahboubeh Baheri<sup>1</sup>, Narges Baharifar<sup>2</sup>, Abolfazl Sheikh<sup>3</sup>, Mohammadreza Dayer<sup>1</sup> and Abdolkarim Sheikhhi<sup>2,\*</sup>

<sup>1</sup>Biology Department, Shahid Chamran University of Ahvaz, Ahvaz, Iran

<sup>2</sup>Department of Immunology, School of Medicine, Dezful University of Medical Sciences, Dezful, Iran

<sup>3</sup>Islamic Azad University of Dezful, Dezful, Iran

\*Corresponding author: Department of Immunology, School of Medicine, Dezful University of Medical Sciences, Dezful, Iran. Email: sheikhi@queensu.ca

Received 2021 February 21; Revised 2021 March 14; Accepted 2021 March 14.

## Abstract

**Background:** Multiple Sclerosis (MS) is an autoimmune and inflammatory disorder of the central nervous system (CNS), which is associated with the imbalance of pro- and anti-inflammatory cytokines. Evidence indicates that nutritional interventions have some immunomodulatory impacts.

**Objectives:** In this study, we investigated the effect of olive vegetation water (OVW) on IFN- $\gamma$  and IL-10 secretion by peripheral blood mononuclear cells (PBMCs) of MS patients.

**Methods:** In this study, PBMCs of MS patients were separated by Ficoll-Hypaque centrifugation. The cytotoxicity of OVW was assessed by the MTT assay. The treatments were performed for 48 and 72 hours, and IFN- $\gamma$  and IL-10 were measured by ELISA.

**Results:** No cytotoxicity was observed for OVW. Besides, OVW showed a significant inhibitory effect on IFN- $\gamma$  secretion but augmenting effect on IL-10 secretion by PBMCs dose-dependently.

**Conclusions:** This study indicated that OVW could have immunoregulatory effects on inflammatory reactions in MS patients.

**Keywords:** Olive Vegetation Water, Multiple Sclerosis, IL-10, IFN- $\gamma$

## 1. Background

Multiple sclerosis (MS) is a central nervous system (CNS) myelogenous disease and is still the most common immune-mediated disorder affecting the CNS. The onset of MS is most commonly seen in people aged 20 to 40 years (1). Pathophysiological processes in MS patients include chronic inflammation, oxidative stress, and axonal demyelination (2, 3). The myelin destruction of axons is revealed by blocking the impulse conduction and the process of permanent inflammation (4).

Although the pathogenesis of MS is not yet fully understood, CNS axonal damage is likely to be directly related to the exacerbation of inflammatory processes and oxidative stress (5). According to evidence, a lot of inflammatory and oxidative stress mediators, including cytokines and chemokines, are secreted in MS. On the other hand, it has been indicated that oxidative stress is involved in inflammatory and autoimmune processes. Therefore, antioxidant therapy could help reduce the progression of MS (6).

Cytokines represent as critical mediators of the autoimmune process. Studies show a correlation between MS symptoms and increased production of inflammatory cytokines (7). Accordingly, cytokine expression is elevated in serum, cerebrospinal fluid, and cultured mononuclear cells of MS patients. Some of these augmented cytokines include tumor necrosis factor (TNF), interferon  $\gamma$  (IFN- $\gamma$ ), interleukin 12 (IL-12), and interleukin 1 (IL-1) (8, 9).

Produced by T-helper cells, IFN- $\gamma$  has several effects, such as the induced expression of major histocompatibility complex (MHC).

Many of these features happen in MS brain lesions and could thus show a relationship between this cytokine and the disease (10). The other types of cytokines that participate in this regulation are anti-inflammatory cytokines such as transforming growth factor (TGF $\beta$ ), IL-10, and IL-4. As known, IL-10 is the most important anti-inflammatory cytokine in the human immune system. After connecting to its receptor, it inhibits monocyte/macrophage pro-inflammatory cytokine production, including IFN- $\gamma$  and

TNF- $\alpha$  (11-13).

Olive is a fruit rich in flavonoids. One of the most important flavonoids extracted from olive is hydroxytyrosol (HT) (3,4-dihydroxyphenyl)ethanol, with antioxidant and anti-inflammatory activities. Besides, HT has been detected in the olive pulp, vegetation water, and pomace; however, it has not been found in virgin olive oil, as shown in previous work (14-16).

## 2. Objectives

This study aimed to evaluate the probable inflammatory or anti-inflammatory effects of olive vegetation water (OVW) on peripheral blood mononuclear cells (PBMCs) collected from MS patients. We chose IFN- $\gamma$  as a pro-inflammatory and IL-10 as an anti-inflammatory cytokine, which works in the opposite direction.

## 3. Methods

### 3.1. Subject Selection

In this study, 20 MS patients (aged  $32 \pm 14$  years) were selected among those referring to Ganjavian Hospital in Dezful city (Khuzestan province, Iran) between April 2016 and June 2018 based on their medical history and status, confirmed by specialist physicians according to the clinical findings and McDonald criteria (17). Peripheral blood samples were collected before receiving any therapy. Patients with other disorders such as liver diseases, alimentary tract, infections, and other complications were excluded from the study. This study was evaluated and approved by the Ethics Committee of Dezful University of Medical Sciences.

### 3.2. Sample Preparation and Cell Culture

Blood samples were collected after 8 to 10 hours of fasting in heparinized tubes containing 200 IU/mL of heparin (Sigma Chemical Co., Munich, Germany). Then, PBMCs were isolated by using Ficoll-Hypaque gradient centrifugation (Sigma Chemical Co., Munich, Germany). Washing of the isolated cells was done by PBS, and they were resuspended in RPMI 1640 medium (Gibco) containing 10% heat-inactivated FBS, 100 U/mL penicillin, and 100  $\mu$ g/mL streptomycin (Gibco), cultured at 37°C in a humidified 5% CO<sub>2</sub> incubator as indicated before (18-21). Next, a density of  $2 \times 10^5$  cells/well of suspensions was prepared in a 96-well microplate. Finally, PBMCs from each patient were treated with OVW for 48 h and 72 h.

### 3.3. Solutions and Reagents

First, Taarom olive fruit, collected in the 2015-2016 crop year, was pitted, and then the seedless pulp was mechanically pressed to give a liquid phase mixture including olive oil, vegetation water, and solids. The solids were separated from the liquid phase mixture by filtration and olive oil, and water fractions were separated by centrifugation at 8,000 g for 40 min. Then, PBMCs were incubated with 0, 10, 20, or 50  $\mu$ L of the extracted aqueous fraction for 48 h and 72 h.

### 3.4. Cytotoxicity Assay

The cytotoxicity of OVW on PBMCs in MS patients was examined using a standard MTT assay with 4, 5-dimethylthiazoyl-2, 5-diphenyltetrazolium bromide (Sigma). In this stage,  $5 \times 10^4$  cells were cultured in the presence of 0, 10, 20, or 50  $\mu$ L of OVW for 72 h. After the incubation time passed, 10  $\mu$ L of the MTT reagent (0.5 mg.mL<sup>-1</sup>) was added to each well and incubated for 4 h. Then, 100  $\mu$ L of acidified isopropanol was added to the wells. After the generation of purple formazan crystals, the absorbance of the samples was measured using an ELISA reader (Awareness, USA) at 570 nm and 630 nm as the reference wavelength.

### 3.5. Cytokine Measurement

After incubating, the supernatants were aspirated. Then, using the sandwich ELISA (Quantikine, R&D Systems, Minneapolis, MN) technique, the concentrations of INF- $\gamma$  and IL-10 were measured. Standard curves were generated at the same time with each set of samples. All the tests were performed in duplicate.

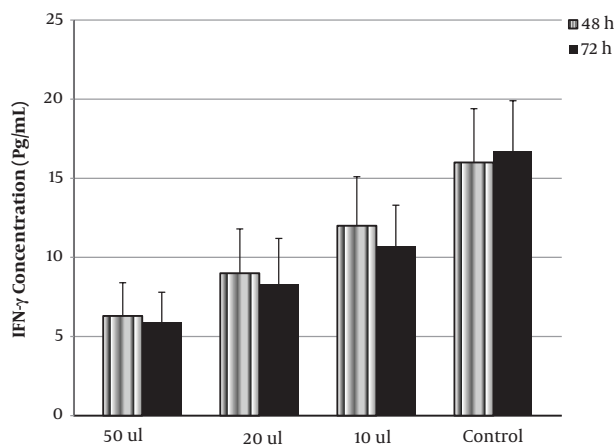
### 3.6. Statistical Analysis

Statistical analyses were performed using SPSS (version 19). One-way ANOVA and student *t*-test were used to check for statistical differences in INF- $\gamma$  and IL-10 secretions in the presence and absence of OVW. The results are presented as mean  $\pm$  SD, and the level of significance was considered as *P* value < 0.05.

## 4. Results

The viability percent of PBMCs, measured by the MTT assay, after co-culture with OVW (10, 20, or 50  $\mu$ L) did not change in comparison with the control (all concentrations showed more than 97.5% viability compared to the control at 72 h incubation time). Thus, OVW had no detrimental effect on PBMCs viability at the concentrations used for treatment in this study.

Also, INF- $\gamma$  and IL-10 concentrations were tested by ELISA. As shown in Figure 1, OVW significantly decreased INF- $\gamma$  dose-dependently ( $P < 0.05$ ). The amount of secreted INF- $\gamma$  in the presence of 10, 20, and 50  $\mu\text{L}$  of OVW was  $10.7 \pm 2.6$ ,  $8.3 \pm 2.3$ , and  $5.9 \pm 1.9$  pg/mL (compared to control with  $16.7 \pm 3.2$  pg/mL) in 72 h, respectively. The difference between the effect of OVW on INF- $\gamma$  secretion at 48 h and 72 h was not significant.



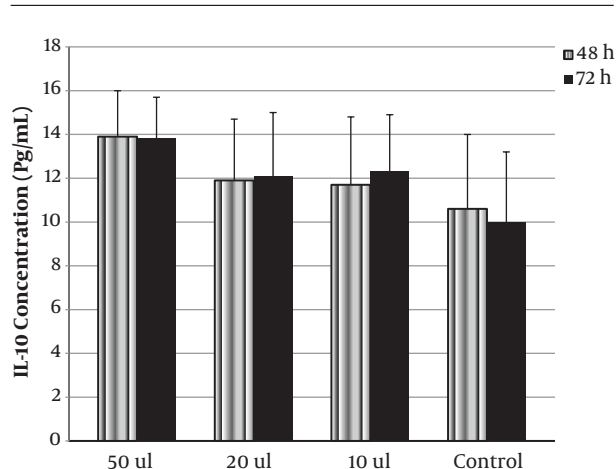
**Figure 1.** Mean and SD of IFN- $\gamma$  secretion from PBMCs of MS patients after treating the cultured PBMCs with zero (control), 10, 20, or 50  $\mu\text{g/mL}$  of olive vegetation water (OVW) in vitro for 48/72h. OVW decreases IFN- $\gamma$  dose-dependently.

OVW at 10, 20, and 50  $\mu\text{L}$  increased IL-10 significantly from  $10 \pm 3.2$  to  $12.3 \pm 2.6$ ,  $12.1 \pm 2.9$ , and  $13.8 \pm 1.9$  pg/mL, respectively ( $P < 0.05$ ) (Figure 2). The difference between the effect of OVW on IFN- $\gamma$  and IL-10 secretion at 48 h and 72 h was not significant.

### 5. Discussion

MS is one of the most common autoimmune diseases among young people. The pathological process is still unknown, which makes it more difficult to find a cure. There is much research investigating new ways and compounds to exert an anti-inflammatory effect on the disease. The autoimmunity concept has lead studies to work on the immune system and suppressing the pro-inflammatory cytokines. The immune pathway cytokines that work in are complicated and partly unknown, so further studies are necessary to find out how they can affect the disease process.

The same happens for olive products and OVW that was used in this study. A decrease in IFN- $\gamma$ , a pro-inflammatory cytokine, showed the anti-inflammatory effect of OVW. The



**Figure 2.** Mean and SD of IL-10 secretion from PBMCs of MS patients after treating the cultured PBMCs with zero (control), 10, 20, or 50  $\mu\text{g/mL}$  of OVW in vitro for 48/72h. OVW increases IL-10 dose-dependently.

effect was seen dose-dependently, which showed a direct correlation between the concentration of OVW and the reduction of IFN- $\gamma$ . On the other hand, the treatment caused a significant increase in IL-10. As known, IL-10 is a cytokine that suppresses the effect of pro-inflammatory cytokines, making it an anti-inflammatory cytokine. Increasing IL-10 can be a sign of suppressing the inflammatory condition in MS patients. It is in agreement with data published by Bitler et al., which showed OVW had anti-inflammatory effects in addition to antioxidant activity (22).

There are several polyphenols with potential health benefits in olive fruit that are retained in the olive vegetation wastes during virgin olive oil processing. Some of them, due to their hydrophilic properties, are concentrated in OVW. Among them, 3,4-dihydroxyphenethyl alcohol (hydroxytyrosol) has been well recognized for its health beneficial activities, including antioxidant and anti-inflammatory activities. It should be noted that the concentration of hydroxytyrosol is more than 10 times higher in OVW than in the corresponding virgin olive oil due to its high hydrophilic properties. Therefore, hydroxytyrosol, as one of the main known constituents of OVW, could be the reason for the anti-inflammatory effect of OVW (23-26).

Although in this study, we demonstrated the effects of the aqueous olive extract on PBMCs obtained from MS patients, it would be good to continue the study by employing healthy controls and/or patients with other immune-mediated diseases. Furthermore, to confirm the anti-inflammatory effects of OVW, we propose to further investigate the effect of different polyphenols extracted from OVW by testing the above-mentioned cytokines and some

other ones. This study could be a starting point for future studies on the effects of the aqueous extract of olive on the cytokine profile of MS patients.

## Acknowledgments

This work was financially supported by Dezful University of Medical Sciences, Dezful, Iran.

## Footnotes

**Authors' Contribution:** MB, NB, AS, MRD, and AS contributed to writing. MRD and AS contributed to the supervision of the project.

**Conflict of Interests:** The authors declare no conflicts of interest.

**Ethical Approval:** This study was evaluated and approved by the Ethics Committee of Dezful University of Medical Sciences (DUMS-139).

**Funding/Support:** This work was financially supported by Dezful University of Medical Sciences, Dezful, Iran.

**Informed Consent:** Patients consented to participate in the study.

## References

- Bielekova B, Martin R. Development of biomarkers in multiple sclerosis. *Brain*. 2004;**127**(Pt 7):1463–78. doi: [10.1093/brain/awh176](https://doi.org/10.1093/brain/awh176). [PubMed: [15180926](https://pubmed.ncbi.nlm.nih.gov/15180926/)].
- Ohl K, Tenbrock K, Kipp M. Oxidative stress in multiple sclerosis: Central and peripheral mode of action. *Exp Neurol*. 2016;**277**:58–67. doi: [10.1016/j.expneurol.2015.11.010](https://doi.org/10.1016/j.expneurol.2015.11.010). [PubMed: [26626971](https://pubmed.ncbi.nlm.nih.gov/26626971/)]. [PubMed Central: [PMC7094520](https://pubmed.ncbi.nlm.nih.gov/PMC7094520/)].
- Goldenberg MM. Multiple sclerosis review. *P T*. 2012;**37**(3):175–84. [PubMed: [22605909](https://pubmed.ncbi.nlm.nih.gov/22605909/)]. [PubMed Central: [PMC3351877](https://pubmed.ncbi.nlm.nih.gov/PMC3351877/)].
- Motavaf M, Sadeghizadeh M, Babashah S, Zare L, Javan M. Dendrosomal nanocurcumin promotes remyelination through induction of oligodendrogenesis in experimental demyelination animal model. *J Tissue Eng Regen Med*. 2020;**14**(10):1449–64. doi: [10.1002/term.3110](https://doi.org/10.1002/term.3110). [PubMed: [32750189](https://pubmed.ncbi.nlm.nih.gov/32750189/)].
- Bendszus M, Storch-Hagenlocher B. Multiple sclerosis and other demyelinating diseases. In: Hähnel S, editor. *Inflammatory diseases of the brain*. Berlin, Heidelberg: Springer Berlin Heidelberg; 2013. p. 3–18. doi: [10.1007/174\\_2012\\_787](https://doi.org/10.1007/174_2012_787).
- Miller ED, Dziedzic A, Saluk-Bijak J, Bijak M. A review of various antioxidant compounds and their potential utility as complementary therapy in multiple sclerosis. *Nutrients*. 2019;**11**(7). doi: [10.3390/nu11071528](https://doi.org/10.3390/nu11071528). [PubMed: [31284389](https://pubmed.ncbi.nlm.nih.gov/31284389/)]. [PubMed Central: [PMC6682972](https://pubmed.ncbi.nlm.nih.gov/PMC6682972/)].
- Mahon BD, Gordon SA, Cruz J, Cosman F, Cantorna MT. Cytokine profile in patients with multiple sclerosis following vitamin D supplementation. *J Neuroimmunol*. 2003;**134**(1-2):128–32. doi: [10.1016/S0165-5728\(02\)00396-x](https://doi.org/10.1016/S0165-5728(02)00396-x). [PubMed: [12507780](https://pubmed.ncbi.nlm.nih.gov/12507780/)].
- Ghaffari SA, Nemati M, Hajghani H, Ebrahimi H, Sheikhi A, Jafarzadeh A. Circulating concentrations of interleukin (IL)-17 in patients with multiple sclerosis: Evaluation of the effects of gender, treatment, disease patterns and IL-23 receptor gene polymorphisms. *Iran J Neurol*. 2017;**16**(1):15–25. [PubMed: [28717429](https://pubmed.ncbi.nlm.nih.gov/28717429/)]. [PubMed Central: [PMC5506751](https://pubmed.ncbi.nlm.nih.gov/PMC5506751/)].
- O'Shea JJ, Ma A, Lipsky P. Cytokines and autoimmunity. *Nat Rev Immunol*. 2002;**2**(1):37–45. doi: [10.1038/nri702](https://doi.org/10.1038/nri702). [PubMed: [11905836](https://pubmed.ncbi.nlm.nih.gov/11905836/)].
- Link J, Soderstrom M, Olsson T, Hojeborg B, Ljungdahl A, Link H. Increased transforming growth factor-beta, interleukin-4, and interferon-gamma in multiple sclerosis. *Ann Neurol*. 1994;**36**(3):379–86. doi: [10.1002/ana.410360309](https://doi.org/10.1002/ana.410360309). [PubMed: [8080246](https://pubmed.ncbi.nlm.nih.gov/8080246/)].
- Hulshof S, Montagne L, De Groot CJ, Van Der Valk P. Cellular localization and expression patterns of interleukin-10, interleukin-4, and their receptors in multiple sclerosis lesions. *Glia*. 2002;**38**(1):24–35. doi: [10.1002/glia.10050](https://doi.org/10.1002/glia.10050). [PubMed: [11921201](https://pubmed.ncbi.nlm.nih.gov/11921201/)].
- Moore KW, O'Garra A, de Waal Malefyt R, Vieira P, Mosmann TR. Interleukin-10. *Annu Rev Immunol*. 1993;**11**:165–90. doi: [10.1146/annurev.iy.11.040193.001121](https://doi.org/10.1146/annurev.iy.11.040193.001121). [PubMed: [8386517](https://pubmed.ncbi.nlm.nih.gov/8386517/)].
- Ahmed KM. The effect of olive leaf extract in decreasing the expression of two pro-inflammatory cytokines in patients receiving chemotherapy for cancer. A randomized clinical trial. *Saudi Dent J*. 2013;**25**(4):141–7. doi: [10.1016/j.sdentj.2013.09.001](https://doi.org/10.1016/j.sdentj.2013.09.001). [PubMed: [24371380](https://pubmed.ncbi.nlm.nih.gov/24371380/)]. [PubMed Central: [PMC3871392](https://pubmed.ncbi.nlm.nih.gov/PMC3871392/)].
- Bianco A, Mazzei RA, Melchioni C, Romeo G, Scarpati ML, Soriero A, et al. Microcomponents of olive oil—III. Glucosides of 2(3,4-dihydroxyphenyl)ethanol. *Food Chem*. 1998;**63**(4):461–4. doi: [10.1016/S0308-8146\(98\)00064-8](https://doi.org/10.1016/S0308-8146(98)00064-8).
- Sugiyama M, Kikuchi M. Studies on the constituents of osmanthus species. X. Structures of phenolic glucosides from the leaves of osmanthus asiaticus nakai. *Chem Pharm Bull*. 1992;**40**(2):325–6. doi: [10.1248/cpb.40.325](https://doi.org/10.1248/cpb.40.325).
- Visioli F, Caruso D, Plasmati E, Patelli R, Mulinacci N, Romani A, et al. Hydroxytyrosol, as a component of olive mill waste water, is dose-dependently absorbed and increases the antioxidant capacity of rat plasma. *Free Radic Res*. 2001;**34**(3):301–5. doi: [10.1080/10715760100300271](https://doi.org/10.1080/10715760100300271). [PubMed: [11264904](https://pubmed.ncbi.nlm.nih.gov/11264904/)].
- Polman CH, Reingold SC, Banwell B, Clanet M, Cohen JA, Filippi M, et al. Diagnostic criteria for multiple sclerosis: 2010 revisions to the McDonald criteria. *Ann Neurol*. 2011;**69**(2):292–302. doi: [10.1002/ana.22366](https://doi.org/10.1002/ana.22366). [PubMed: [21387374](https://pubmed.ncbi.nlm.nih.gov/21387374/)]. [PubMed Central: [PMC3084507](https://pubmed.ncbi.nlm.nih.gov/PMC3084507/)].
- Sheikhi A, Saadati K, Jafarzadeh A, Karimi H, Mousavinasab N. Augmenting the expression of NKp44 molecule and the natural killer activity in peripheral blood mononuclear cells from patients with malignant colorectal carcinoma. *Drug Res*. 2014;**64**(6):281–6. doi: [10.1055/s-0033-1358465](https://doi.org/10.1055/s-0033-1358465). [PubMed: [24154937](https://pubmed.ncbi.nlm.nih.gov/24154937/)].
- Sheikhi A, Saadati K, Salmani R, Yahaghi N, Sheikhi A, Siemens DR. In vitro modulation of natural killer activity of human peripheral blood mononuclear cells against prostate tumor cell line. *Immunopharmacol Immunotoxicol*. 2011;**33**(4):700–8. doi: [10.3109/08923973.2011.561437](https://doi.org/10.3109/08923973.2011.561437). [PubMed: [21425925](https://pubmed.ncbi.nlm.nih.gov/21425925/)].
- Sheikhi A, Nazarian M, Khadem-Al-Melleh A, Nasab NM, Esmailzadeh A, Yahaghi N, et al. In-vitro effects of Mycobacterium bovis BCG-lysate and its derived heat shock proteins on cytokines secretion by blood mononuclear cells of rheumatoid arthritis patients in comparison with healthy controls. *Int Immunopharmacol*. 2008;**8**(6):887–92. doi: [10.1016/j.intimp.2008.01.034](https://doi.org/10.1016/j.intimp.2008.01.034). [PubMed: [18442794](https://pubmed.ncbi.nlm.nih.gov/18442794/)].
- Amirghofran Z, Sheikhi AK, Kumar PV, Saberi Firouzi M. Soluble HLA class I molecules in malignant pleural and peritoneal effusions and its possible role on NK and LAK cytotoxicity. *J Cancer Res Clin Oncol*. 2002;**128**(8):443–8. doi: [10.1007/s00432-002-0371-0](https://doi.org/10.1007/s00432-002-0371-0). [PubMed: [12200601](https://pubmed.ncbi.nlm.nih.gov/12200601/)].
- Bitler CM, Viale TM, Damaj B, Crea R. Hydrolyzed olive vegetation water in mice has anti-inflammatory activity. *J Nutr*. 2005;**135**(6):1475–9. doi: [10.1093/jn/135.6.1475](https://doi.org/10.1093/jn/135.6.1475). [PubMed: [15930455](https://pubmed.ncbi.nlm.nih.gov/15930455/)].

23. Scoditti E, Carpi S, Massaro M, Pellegrino M, Polini B, Carluccio MA, et al. Hydroxytyrosol modulates adipocyte gene and miRNA expression under inflammatory condition. *Nutrients*. 2019;**11**(10). doi: [10.3390/nu11102493](https://doi.org/10.3390/nu11102493). [PubMed: [31627295](https://pubmed.ncbi.nlm.nih.gov/31627295/)]. [PubMed Central: [PMC6836288](https://pubmed.ncbi.nlm.nih.gov/PMC6836288/)].
24. Yangui A, Abderrabba M. Towards a high yield recovery of polyphenols from olive mill wastewater on activated carbon coated with milk proteins: Experimental design and antioxidant activity. *Food Chem*. 2018;**262**:102–9. doi: [10.1016/j.foodchem.2018.04.091](https://doi.org/10.1016/j.foodchem.2018.04.091). [PubMed: [29751896](https://pubmed.ncbi.nlm.nih.gov/29751896/)].
25. Richard N, Arnold S, Hoeller U, Kilpert C, Wertz K, Schwager J. Hydroxytyrosol is the major anti-inflammatory compound in aqueous olive extracts and impairs cytokine and chemokine production in macrophages. *Planta Med*. 2011;**77**(17):1890–7. doi: [10.1055/s-0031-1280022](https://doi.org/10.1055/s-0031-1280022). [PubMed: [21830187](https://pubmed.ncbi.nlm.nih.gov/21830187/)].
26. Lesage-Meessen L, Navarro D, Maunier S, Sigoillot JC, Lorquin J, Delatre M, et al. Simple phenolic content in olive oil residues as a function of extraction systems. *Food Chem*. 2001;**75**(4):501–7. doi: [10.1016/S0308-8146\(01\)00227-8](https://doi.org/10.1016/S0308-8146(01)00227-8).