Original Article

Effect of Olive Leaf Extract on Cytokines Secreted by Macrophage

Rezvan Vajdian[1], Nariman Mosaffa[2], Seyyed Javad Seyyed Tabaei[1], Farnaz Kheirandish[3*], Mohammad Javad Tarahi[4], Zahra Arab-Mazar[5], Zahra Poursafavi[1]

[1] Department of Parasitology and Mycology, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[2] Department of Immunology, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran.
[3] Razi Herbal Medicines Research Center, Department of Parasitology and Mycology, School of Medicine, Lorestan University of Medical Sciences, Khorramabad, Iran
[4] Department of Epidemiology and Biostatistics, School of Public Health, Lorestan University of Medical Sciences, Khorramabad, Iran.
[5] Infectious Diseases and Tropical Medicine Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran

Received: 22 November, 2015; Accepted: 10 May, 2016

Abstract

Background: Herbal medicines in compared with chemical drugs have fewer side effects and can be a good medicinal alternative. The olive includes 20 different species of the family Oleaceae, with *Olea europaea* as the most recognized. Several studies have shown the immunomodulating effects of olive leaf extract. This study aimed to identify the immunoregulatory effect of olive leaf Sevillana variety on interleukins 12 and 10 which resulted from the murine macrophages *in vitro*.

Materials and Methods: In order to isolate macrophages, peritoneal macrophages BALB/C were used. To determine the cytotoxic effect of different concentrations of the olive leaf extract on macrophages, MTT assay was performed. Concentrations of 200, 100, 50, 25, 12.5, 6.25, and 3.1µg/ml in the time intervals of 12, 24, and 48 hours were evaluated. Three appropriate concentrations were selected to commence with the study of the determination of the amount of cytokines. Cell culture supernatant growth medium supernatant was collected at 12, 24, and 48 hours after adding the extract in order to examine the amount of cytokines. ELISA test was conducted using interleukins 12 and 10 measurement kits.

Results: CC50 of the olive leaf extract at 12, 24, and 48 hours was 260.3, 170.5, and 150 µg/ml, respectively. According to the results, an increase in the concentration and duration of the study resulted in observable significant differences in the production of interleukins 10 and 12. As a result, the production of IL-10 and 12 experienced decreases and increases, respectively.

Conclusion: It seemed probable that the olive leaf extract had the capability to increase the production of IL-12 through activation of the classic macrophages and also deactivate the regulatory macrophages with an increase in IL-12 and a decrease in IL-10. Therefore, this can strengthen the immune system of the host in the early stages of infection. The other immunomodulatory effects of olive leaf must be considered by appropriate research.

Keywords: Macrophage, interleukin, IL-10, IL-12, Olive leaf extract

***Corresponding Author:** Farnaz Kheirandish, Department of Parasitology and Mycology, School of Medicine, Razi Herbal Medicines Research Center, Lorestan University of Medical Sciences, Khorramabad, Iran. Email: kheirandish81@yahoo.com

Please cite this article as: Vajdian R, Mosafa N, Seyed Tabaei SJ, Kheirandish F, Tarahi MJ, Arab-Mazar Z, et al. Effect of Olive Leaf Extract on Cytokines Secreted by Macrophage. Novel Biomed. 2016;4(3):116-20.

Introduction

The olive includes 20 different species of the family Oleaceae, with *Olea europaea*as the most recognized. The ripe olive contains about 30% oil, which is a mixture of oleic acid, linoleic acid and palmitic acid. Olive contains sodium, vitamin A, vitamin E and iron. The leaves also contain glycoside, sugary materials, bitter substances, chlorophyll, gallic acid, tannin, wax and tiemannite[1].

Olive leaf is rich in a bitter substance called oleuropein. Oleuropein is the most abundant of all phenolic compounds contained in olive leaf and fruit, and its therapeutic effect as a powerful antioxidant is well known[2-5]. A total of 1500 varieties of this plant have been reported, including Manzanilla, Picual, Sevillana, Conservolia, Mission, Beldi, Kalamon[6]. Studies have shown that Sevillana and Kalamon varieties contain the highest and the lowest amounts of oleuropein, respectively[7].

The dried extract of olive leaf possesses important dose-dependent anti-inflammatory effects[8].

Olive oil also increases the internal secretion of bile and applies its laxative effects by strengthening the contraction of the intestinal muscles. It also has soothing effects on the mucosa[9].

Several studies have indicated the immunomodulatory effects of olive leaf extract. The main defensive cells against an intracellular pathogen are macrophages, which are flexible against the environmental signals and are capable of changing their phenotype and physiology in the innate and adaptive immune responses. Environmental signals can also induce changes in order to increase the immune function of macrophages. Therefore, there is a possibility that olive leaf extract can change the cytokine profiles secreted from macrophages. This study aimed to identify the effect of olive leaf Sevillana variety that was extracted using a soxhlet extraction method on interleukin 12 (IL-12) and IL 10 production which polarized from the murine macrophages *in vitro*.

Methods

Isolation & purification of murine peritoneal macrophages: BALB/c male mice, aged 6 to 8 weeks and weighing 20 g, bought from Tehran Pasteur Institute, were used for the purpose of this study. First, the mice were cervically dislocated, then immersed in 70% Betadine-alcohol mixture with 1:1 ratio, and then located in 70% alcohol. In order to peritoneal lavage 6ml of cold RPMI 1640 medium was flushed into the peritoneal cavity in order to isolate peritoneal macrophages. Cellular suspension was centrifuged in a refrigerated centrifuge at 4°C for 10 minutes with 200g. Finally, 1 ml of complete tissue culture medium [RPMI 1640 (Biowest, France) supplemented by 10% heat inactivated fetal bovine serum (Biosera, UK), 100 IU/mL penicillin and 100 µg/mL streptomycin (Biosera, UK)] was added to the obtained cellular pellet and used to count and determine the viability of cells percentage[10,11].

1×10^5 cells were added to each of the wells in 96-well flat-bottomed culture plates and incubated at 37°C in 5% CO_2 for 4 to 6 hours. Then, the non- adherent cells were removed by washing with warm PBS (37°C) (Biowest, France) and returned to the incubator. Tests were performed as duplicate.

Evaluating the cytotoxic effect of the extract on the peritoneal macrophage: To determine the cytotoxic effect of the extract on macrophages, MTT assay was performed. MTT assay is a colorimetric method used to examine the conversion rate of tetrazolium to formazan in the living cell. Twenty four hours after the macrophages culture, the non-adherent macrophages were washed with warm PBS (37°C) and then incubated in the presence of different concentrations of olive leaf hydroalcoholic extract 200, 100, 50, 25, 12.5, 6.25, and 3.1µg/ml for 12, 24, and 48 hours. The extract was not added to the number of wells considered as a control group. 100µl of MTT solution was added to the wells at time intervals of 12, 24, and 48 hours and the plates were incubated in the dark at 37°C for 4 hours. If the macrophages cells were alive, the mitochondria secrete the enzyme succinate, which converts tetrazolium to formazan. Formazan are the insoluble blue-violetdye crystals. Therefore, 100 µl of dimethyl sulphoxide (DMSO) was added to the wells as solvent, this was immediately followed by the measurement of the absorption rate in the wells using enzyme linked immunosorbent assay (ELISA) reader at a wavelength of 570 nm[12,13]. CC50 was assessed using linear regression. Also, three appropriate concentrations were selected to commence with the

study of the determination of the amount of cytokines.

Determining the concentration of cytokines: Macrophages culture was performed using the aforementioned step. In this step, three concentrations chosen in the previous step were used. In order to examine the extract effect of olive leaf on the secretion of cytokines, the cells were studied in 4 groups: A macrophage group lacking the extract and three other groups with each group containing a selected concentration. The cell culture medium supernatant was collected for 12, 24, and 48 hours after adding the extract in order to examine the amount of cytokines in micro-tubes, and maintained at 20°C until use. ELISA test was conducted using IL 12 and IL 10 measurement kits (eBioscience, USA).

Statistical analysis: Data analysis was performed using the SPSS statistical package, version 16.0 (SPSS Inc., Chicago, IL, USA). Kruskal Wallis Test and Mann-Whitney U were used to analyze the data. Differences were significant when the p-value was lower than 0.05

Results

Evaluating the toxicity of the extract on macrophage: Base on the MTT assay, CC_{50} of the olive leaf extract at 12, 24, and 48 hours was 260.3µg/ml, 170.5µg/ml and 150µg/ml, respectively. According to the results in this step, three concentrations of 6.25, 12.5 and 25µg/ml were chosen to continue the study; therefore the study groups were designed as follows:

Group1: Macrophage (free extract),

Group2: Macrophage+ concentration of 6.25µg/ml of olive leaf extracts,

Group3: Macrophage+ concentration of 12.5µg/ml of olive leaf extracts,

Group4: Macrophage +concentration of 25µg/ml of olive leaf extracts

Evaluation of the IL-10 production rate: Using Kruskal Wallis test, it was shown that the 4 study groups were significantly different in terms of production of IL-10 (P=0.001). Therefore, an increase in extract concentration resulted in a decrease in the amount of IL-10 production rate. Moreover the production of IL-10 in group 4 was

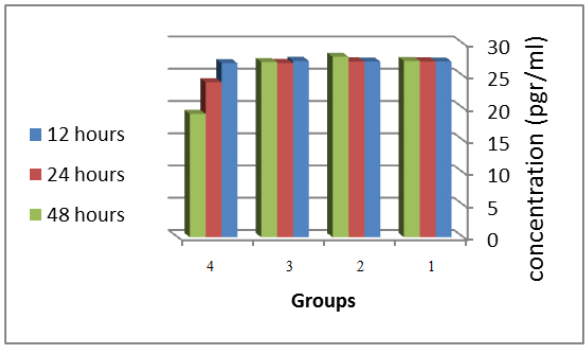

Figure 1. Comparison of IL10 production rate in the 4 studied groups.

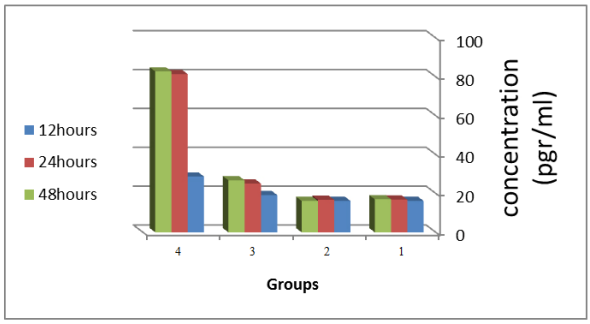

Figure 2. Comparison ofIL12production rate in the 4 studied groups.

significantly decreased by increasing the time (Figure 1). Therefore, at the concentration of 25µg/ml and time 48 hours, the lowest amount of the IL-10 production rate was observed (Figure 1).

Evaluation of the IL-12 production rate: In evaluating the amount of cytokines in cell culture supernatant, it was observed that the increased concentration of olive leaf extract led to an increase in the IL-12 production. The results of the statistical Kruskal Wallis test showed that the 4 studied groups at 12, 24, and 48 hours were significantly different (p=0.001). Therefore the production rate of IL-12 increased with an increase in extract concentration (Figure 2).This production rate increased significantly at 24 and 48 hours.

Discussion

Some information about leishmaniasis infections documented in Iran[14-17]. Leishmaniasis is endemic in many parts of Iran and is reported from at least 17 provinces of the country[18, 19].

Nowadays, the first-line chemotherapy of cutaneous leishmaniasis (CL) is antimonial drugs such as

meglumine antimoniate and sodium stibogluconate. But due to having the problems of drug resistance, side effects, and high costs researchers are looking for alternative treatment[20,21]. The effects of herbal medicines on different parasites were studied[21, 22].

Olive, with the scientific name of *Olea europaea*, has been used since 3000 years before Christ[6]. Nowadays, this tree is widely spread throughout the world. Hashemi et al. showed that a variety of Sevillana contained the highest amount of oleuropein, which is the most abundant type of phenolic compounds in olive leaves and fruit, and its therapeutic effects as a powerful antioxidant is well known[7]. The positive effects of this material on health have been widely studied. It is shown that oleuropein and related compounds such as demthyoleuropein, ligustroside, verbascosid and tyrosol work as antioxidant and reduce the risk of heart attacks and different kinds of cancers; also it possesses anti-microbial and anti-viral properties[2-5]. It was reported that oleuropein contained insect-repellent properties as well as protection against pathogens[23].

In the present study, MTT assay was used to determine the proper concentrations of hydroalcoholic extract of olive leaves to affect the macrophages. The results showed that the concentrations of 25, 12.5, and 6.25µg/ml after 12, 24, and 72 hours were the best concentrations with no cytotoxic effect on living cells, but rather improved the activity of the cells.

Several researchers, in recent years, have reported the presence of immunomodulator substances in olive plant. In a study conducted in 2012 by Rosignoli, it was shown that phenolic compounds derived from olive oil are capable of stimulating the human monocytes exposed lipopolysaccharide *in vitro* and capable of increasing the production of TNFα in monocytes [24]. In another study conducted in 2003, it was shown that the addition of olive leaf extract to the growth medium of HIV1-infected cell, apart from inhibiting the virus, it increased the expression of IL-2 and IL-2 receptor genes[25].

Studies have shown that the body's defense mechanism to control the intracellular pathogens, such as *Leishmania* parasite, is dependent on the performance of the Th1 cells. These cells, by activating macrophages, cause a lot of cytokines secretions which are essential to increase the cellular immunity activity[26]. One of the cytokines secreted from macrophages is IL-12 which is responsible for the defense against the intracellular pathogens. IL-12 activated the macrophages through affecting Th_1 in acquired immunity or with the effect on NK cell in innate immunity and finally with stimulation of IFNγ[27]. Other evidences indicated that the production of cytokines such as IL-10, IL-13 and activating Th_2 provides the immune system suppression and finally weaken the immune system[28]. Since IL-12 is the main stimulus for IFNγ secretion and has an important role in the innate immune responses, with increasing IL-10, the positive reactions are suppressed.

In this study, the immunomodulatory effect of olive leaf extract on the production of IL-10 and IL-12 secreted from macrophages was evaluated. Results showed that the production of IL-12 increased significantly when macrophages were affected by olive leaf extract. The results showed that the concentration of 25µg/ml at 48 hours were the best concentration and time suitable for the best effect of the extract, and this resulted in an increase in IL-12 and a decrease in IL-10. It seems probable that the olive leaf extract has the capability to increase IL-12 production through activating the classic macrophages and deactivating the regulatory macrophages with an increase in IL-12 and a corresponding decrease in IL-10. Therefore, it can strengthen the immune system of the host in the early stages of infection.

Conclusion

Finally, there is hope for the future, with further research that olive leaf extract as the natural, available, safe and with outside effects, could be used as a proper medicine to modulate the immune system in the early stages of infection when the immune system requires the supporting inflammatory cytokines, such as IL-12.

Acknowledgments

The authors thank sincerely all those who helped us in this research.

Conflict of Interest

The authors declare that there is no conflict of interests.

Funding

This project was supported financially by Lorestan university of medical sciences.

References

1. Joannis D. Kyriazisa, Nektarios Aligiannisb, Panagiotis Polychronopoulosb. Leishmanicidal activity assessment of olive tree extracts. J Phytomedicine. 2013;20:275-81.
2. Manna C, Angelo SD, Migliardi V, Loffredi E, Mazzoni O, Morrica P, et al. Protective effect of phenolic fraction from virgin olive oils against oxidative stress in human cells. J Agric Food Chem 2002;50:6521-6.
3. Owen RW, Giacosa A, Hull WE, Haubner R, Spiegelhalder B, Bartsch H. The antioxidant/anticancer potential of phenolic compounds isolated from olive oil. Eur J Cancer. 2000;36:1235-47.
4. Bisingnano G, Tomaino A, Lo Cascio R, Crisafi G, Uccella N, Saija A. On the in vitro antimicrobial activity of oleuropein and hydroxtyrosol. J Pharm Pharmacol. 1999;51:971-4.
5. Lo Scalzo R, Scarpati ML, Verzengnassi B,Vita G. Olea europaea chemical repellent to Dacus oleae females. J Chem Ecol. 1994;20:1813-23.
6. Andrews P, Busch JLHC, Joode TD,Groenewegen A, Alexandre H. Sensory properties of virgin olive oil polyphenols: identification of deacetoxy-ligstroside aglycon as a key contributor to pungency. J Agric Food Chem. 2003;51:1415-20**.**
7. Hashemi P, Delfan B, Ghiasvand A. The study of Oleuropein in the leaves of olive trees grown in the city of Khorramabad. J Lorestan university of Medical Sciences. 2008;4(10):99-106.
8. Eryuce N. Seasonal variation of nutrient contents in the leaves of an oil-olive variety from the Ayvalik district. Ege Universitesi Ziraat Fakultesi Dergisi. 1980;17(2):209-21.
9. Lust J, Martin JB. The Herb Book:The Most Complete Catalog of Herbs Ever Published. 2th ed. New York: Courier Corporation. 2014.
10. Scure LS, Simioni PU, Grabriel DL, Saviani EE, Modol LV, Tamashiro WM, et al. Suppression of nitric oxide production in mouse macrophage by soybean flavonoides acctumulated in response to nitroprusside and fungal elicitation. J BMC Biochem. 2004;21(2):505-50.
11. Chen LC, Pace JL, Russell SW, Morrison DC. Altered regulation of inducible nitric oxide synthase expression in macrophages from senescent mice. J Infect Immun. 1996;64(10):4288-98.
12. Dutta A, Bandyopadhyay S, Mandal C, Chatterjee M. Development of a modified MTT Sassay for screening antimonial resistant field isolates of Indian visceral eishmaniasis. J Parasitology international. 2005;54(2):119-22.
13. Denizot F, Lang R. Rapid colorimetric assay for cell growth and survival:odifications to the tetrazolium dye procedure giving improved sensitivity and reliability. Journal of immunological methods. 1986;89(2):271-7.
14. Kheirandish F,Tarahi MJ, Ezatpour B. Prevalence of intestinal parasites among food handlers in West of Iran. Rev Inst Med Trop Sao Paulo. 2014;56:111-4.
15. Badparva E, Kheirandish F, Ebrahimzade F. Prevalence of intestinal parasites in Lorestan Province, West of Iran. Asian Pac J Trop Dis. 2014;4:930- 4.
16. Kheirandish F, Tarahi MJ, Haghighi A, Nazemalhosseini-Mojarad E, Kheirandish M. Prevalence of intestinal parasites in bakery workers in Khorramabad, Lorestan Iran. Iran J Parasitol. 2011;6:76-83.
17- Kheirandish F, Badparva E, Haghighi A, Nazemalhosseini Mojarad E, Kazemi B. Differential diagnosis of Entamoeba spp. in gastrointestinal disorder patients in Khorramabad, Iran. Afr J Microbiol Res. 2011;5(18):2863-6.
18. Kheirandish F, Chegeni Sharafi A, Kazemi B, Mohebali M, Sarlak A, Tarahi MJ, Holakouee K, Hajaran H. Identification of *Leishmania* species using PCR assay on giemsa-stained slides prepared from cutaneous leishmaniasis patients. Iran J Parasitol. 2013;8(3):382-8.
19. Kheirandish F, Chegeni Sharafi A, Kazemi B, Bandehpour M, Tarahi MJ, Khamesipour A. First molecular identification of *Leishmania* species in a new endemic area of cutaneous leishmaniasis in Lorestan, Iran. Asian Pac J Trop Med. 2013;6(9):713-7.
20. Croft SL, Sundar S, Fairlamb AH. Drug resistance in leishmaniasis. Clin Microb Rev. 2006;19:11–26.
21. Kheirandish F, Delfan B, Farhadi S, Ezatpour B, Khamesipour A, Kazemi B, et al. The effect of Satureja khuzestanica essential oil on the lesions induced by Leishmania major in BALB/c mice. African Journal of Pharmacy and Pharmacology. 2011;5(5):648-53.
22. Mahmoudvand H, Saedi Dezaki E, Kheirandish F, Ezatpour B, Jahanbakhsh S, Fasihi Harandi M. Scolicidal Effects of Black Cumin Seed (Nigella sativa) Essential Oil on Hydatid Cysts. Korean J Parasitol. 2014;52(6):1-7.
23. Uccella N. Olive biophenols: biomolecular characterization, distribution and phytolexin histochemical localization in the drupes. J Trends Food Sciences. 2001;11:315-27.
24. Rosiqnoil P, Fuccelli R, Fabiani R, Servili M, Morozzi G. Effect of olive phenols on the production of inflammatory mediators in freshly isolated human monocytes. J of Nutritional Biochemistry. 2013;24:1513-9.
25. Lee-Huanq S, Zhanq l, Huanq PL, Chanq YT, Huanq PL. Anti-HIV activity of olive leaf extract (OLE) and modulation of host cell gene expression by HIV-1 infection and OLE treatment. J Biochem Biophys Res Commun. 2003;307(4):1029-37.
26. Gharavi MJ. Textbook of clinical protozoology.3th ed. Tehran: Tabib Publication. 1378. (Text in Persian).
27. Edrisian Gh, Rezaeian M, Ghorbani M, Keshavarz H, Mohebali M. In: Mohebali M editor. Medical Protozoology. 1ed. Tehran: Medical university Press; 1386.(Text in Persian)
28. Karimi A, Jahromi H, Hadipour Jahromi M. In: Jahromi H editors. Viseral Leishmania in Iran & World.1th ed. Tehran:Health & Nutrition Cultural Institute; 2008.(Text in Persian).