



Original article

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## Immunomodulatory effects of barberry's (*Berberis integerrima*) ingredients on macrophages: an *in-vitro* study

Shirin Fateh, Shaghayegh Pishkhan Dibazar, Saeed Daneshmandi\*

Department of Immunology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran

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### ABSTRACT

**Objective:** To investigate suppressive effects of barberry ingredients on macrophage.

**Methods:** Barberry alcoholic and aqueous extracts were obtained and tested on macrophages and lipopolysaccharide-stimulated macrophages. Nitric oxide levels were determined using Griess method and MTT assay which were done for evaluation of macrophage viability. Supernatant tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-6 (IL-6) and interleukin-12 (IL-12) were measured by ELISA kits.

**Results:** MTT reduction capability was the same in all groups, unless the group with higher doses of extracts ( $P < 0.05$ ). Both extracts suppressed nitric oxide production ( $P < 0.05$ ). TNF- $\alpha$  production were suppressed by low doses of both extracts and induced by aqueous extract ( $P < 0.05$ ). IL-6 release was suppressed and IL-12 was induced ( $P < 0.05$ ).

**Conclusions:** This study showed anti-inflammatory functions of barberry's ingredients on macrophages, and at the same time displayed a low cytotoxic effect. Changes in cytokine production was various as data shows decrease in TNF- $\alpha$  and IL-6 levels and induction in IL-12 released by macrophages. These data propose diverse medical use of barberry in treatment of different disorders but with more precision.

## 1. Introduction

The identification of active component and action mechanisms of traditional medicines is highly desirable[1]. Natural dietary agents including fruits, vegetables, and spices have drawn a great deal of attention from both the scientific community and the general public due to their various health-promoting effects and use for treatment of different disorders[2]. Moreover, plant extracts could be used for immune system modulation, but their specific effects on immune system and cells haven't been completely explored. Barberry is an evergreen shrub that belongs to *Berberis* species (Berberidaceae family)[3,4]. It has yellow-brown bark, red colored fruits, and thick and woody roots covered with a brittle bark[5,6]. Some of *Berberis*

sp. are available in Iran, including *Berberis vulgaris* L. (*B. vulgaris*), *Berberis orthobotrys* Bienert, *Berberis crataegina* D.C, *Berberis integerrima* (*B. integerrima*), and *Berberis khorasanica* Browicz[3,7]. Some barberry's components which are responsible for this plant effects are alkaloids such as berberine, oxyacanthine, berbamine, and palmatine[5,8]. *Berberis* species are worldwide used for various purposes to alleviate central nervous system disorders[9], Alzheimer[10], liver disorder, bronchial diseases, and urinary discomforts[11]. It has also been used for the treatment of diarrhea[12], lowering blood glucose in type 2 diabetes mellitus[13]. Barberry could be beneficial in cancer treatment and modulation of immune functions. This plant is useful in wound healing and it's been suggested for multiple pharmacological actions including anti-inflammatory effects and immunomodulatory properties[14]. For example, berberine's immunoregulatory potential has been demonstrated by inhibiting HIV protease inhibitor induced tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin-6 (IL-6) production in macrophages[15], and barberry effectively inhibits neuroinflammatory responses

\*Corresponding author: Saeed Daneshmandi, Department of Immunology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran.

Tel: +982182884529

E-mail: daneshmandi2006@yahoo.com

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which might contribute to its beneficial neuroprotective effects in central nervous system[16]. Application of barberry's ingredients in autoimmune disorders such as diabetes[17] and experimental autoimmune encephalomyelitis[18] has been shown. Despite these studies have shown some modulation influence on immune system, there are no appropriate data on the effect of barberry on immune cells and immune elements. In this research, we tried to analyze effects of barberry extracts on macrophages as important linkers between innate and adaptive immune systems.

## 2. Materials and methods

### 2.1. Animals

The study was performed on female inbred BALB/c mice which were purchased from the Pasteur Institute of Iran, Tehran, Iran. Animals were housed in a ventilated room under a 12 h light/12 h dark cycle at  $(24 \pm 2) ^\circ\text{C}$  and had free access to standard water and food throughout the study. All animal experiments were carried out in accordance with acts of Ethical Committee, Tarbiat Modares University of Medical Sciences.

### 2.2. Plant material

The fruits of *B. integerrima* were collected from 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.

### 2.3. Preparation of extracts

Barberry alcoholic and aqueous extracts were harvested from dried fruits. Preparation of aqueous and ethanol extracts was done as following steps: the fruits of barberry were powdered by mechanical grinder; for aqueous extract, the powder (25 g) was macerated in 100 mL distilled water, and for ethanol extract, 25 g of barberry powder was soaked in 100 mL of 96% ethanol and solutions were mixed on a rotary for 48 h. Then the mixture was filtered with Whatman No. 1 filter paper and filtrates were lyophilized. A stock of 100 mg/mL was prepared for both extracts, sterilized using 0.22  $\mu\text{m}$  filter and used in assays.

### 2.4. Macrophage culture and stimulation

Macrophages were obtained from BALB/c mice by lavage of the peritoneal cavity with 10 mL of Roswell Park Memorial Institute (RPMI) 1640 (Sigma Chemical Co., LTD.). The cells were centrifuged at 1500 r/min and washed, then adjusted to

$1.5 \times 10^6$  cells/mL in RPMI medium (supplemented with 11 mmol/L sodium bicarbonate, 2 mmol/L L-glutamine, 100 IU/mL penicillin, 100  $\mu\text{g}/\text{mL}$  streptomycin and 10% fetal bovine serum). The cell suspensions ( $3 \times 10^5$  cells/well) were plated (200  $\mu\text{L}$ /well) onto 96-well flat-bottomed plates (Nunc) and incubated for 4 h with 5%  $\text{CO}_2$  at  $37 ^\circ\text{C}$  to make a humidified atmosphere. The nonadherent cells were removed by washing the wells with phosphate buffer solution (PBS) (pH = 7.2) 3 times. Adherent cells included macrophages were incubated for 48 h in an RPMI medium. Macrophages were divided into two groups: a group was treated with 10  $\mu\text{g}/\text{mL}$  lipopolysaccharide for stimulation, the other group of macrophages were cultured in RPMI medium with no stimulation. Different concentrations of barberry extracts were added into both groups (final concentrations of 0.001–1000  $\mu\text{g}/\text{mL}$ ) giving a final volume of 200  $\mu\text{L}$  (triplicated wells) and incubated for 48 h in  $37 ^\circ\text{C}$  with 5%  $\text{CO}_2$ . After incubation, supernatant was obtained then NO and cytokine production were assayed. Besides we did MTT assay on the cells. Positive control for anti-inflammatory functions was dexamethasone (10  $\mu\text{mol}/\text{L}$ ).

### 2.5. NO measurement

The cells were plated at a density of  $3 \times 10^5$  cells/well in 200  $\mu\text{L}$  of culture medium per well in a flat-bottomed, 96-well plate. After 48 h of incubation and treatment, the amount of nitrite produced by mouse macrophages was measured in cell culture supernatant. NO is unstable and rapidly converts to nitrite and nitrate. We quantified the level of NO synthesis by measuring the amount of nitrite accumulation in the macrophages culture media using the method of Stuehr and Nathan[19]. Briefly, 50  $\mu\text{L}$  of supernatant were mixed with the same volume of Griess reagent [stock-I: 0.2% N-(1-naphthyl) ethylenediamine-HCl; stock-II: 2% sulfanilamide in 5%  $\text{H}_2\text{PO}_4$ ]. After 15 min, absorbance was read at 540 nm using an automated microplate reader. The concentration of nitrite was estimated from a  $\text{NaNO}_2$  standard curve.

### 2.6. MTT reduction assay

The MTT reduction assay is quantitative and reliable colorimetric assay used to measure the viability, proliferation and activation of cells. If the metabolic rate is constant, the technique can be employed to count living cells in a sample, so we evaluated the macrophage's viability by this test[20]. In brief, after 48 h of macrophage treatment, MTT (5 mg/mL in PBS) in one tenth of total volume were added to wells and incubated for 4 h at  $37 ^\circ\text{C}$  and 5%  $\text{CO}_2$ . In this time, formazan crystals will be generated by viable cells. After incubation, culture supernatants were gently removed and 100  $\mu\text{L}$  of acidic isopropanol (0.04 mol/

L HCl in isopropanol) were added in order to dissolve formazan crystals. The optical density (OD) of each well was measured at 540 nm. The result of this test was expressed as a survival index, which is OD at 540 nm of the test samples/OD at 540 nm of negative control.

### 2.7. Cytokines assay

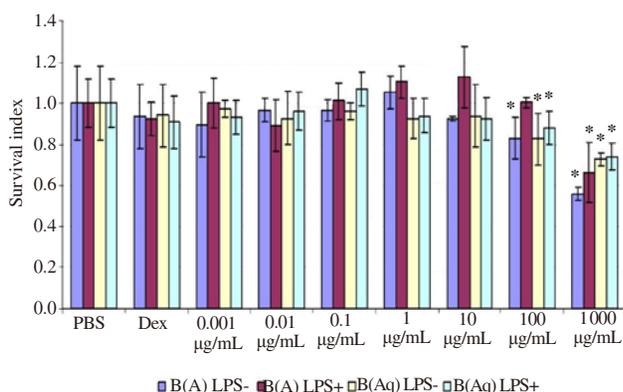
The amounts of interleukin-12 (IL-12), IL-6 and TNF- $\alpha$  in the cell culture supernatant were measured using an ELISA kit (eBioscience, Germany), according to the manufacturer's instructions.

### 2.8. Statistical analysis

Results were expressed as mean  $\pm$  SD. Statistical analysis was performed using the One-way ANOVA and least significant difference test. The  $P$ -values less than 0.05 were considered significant differences.

## 3. Results

Cytotoxicity of barberry extracts on macrophages was determined by MTT assay. MTT reduction capability was almost the same in all groups, unless in groups with higher doses of extracts (Figure 1). The concentrations at 100 and 1000  $\mu\text{g/mL}$  of both extracts caused reduced viability of macrophages ( $P < 0.05$ ) and other concentrations showed almost the same effect as of that negative control ( $P > 0.05$ ).

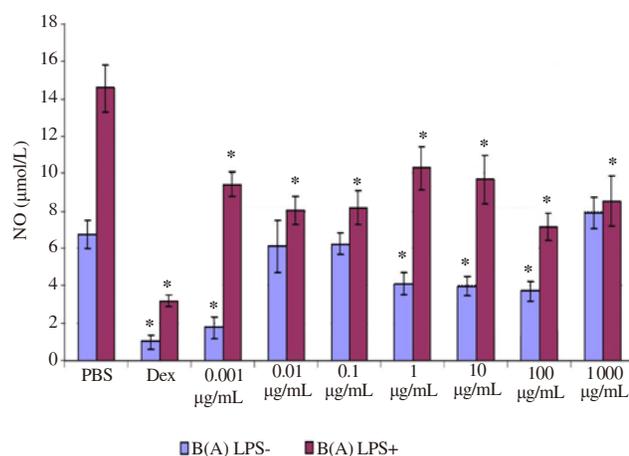


**Figure 1.** MTT reduction (mean  $\pm$  SD) by peritoneal macrophages treated with various concentrations of barberry extracts.

Dex: Dexamethasone; B(A) LPS-: Cells treated with alcoholic extract and without lipopolysaccharide stimulation; B(A) LPS+: Cells treated with alcoholic extract and lipopolysaccharide stimulation; B(Aq) LPS-: Cells treated with aqueous extract and without lipopolysaccharide stimulation; B(Aq) LPS+: Cells treated with aqueous extract and lipopolysaccharide stimulation; \*: Significant differences ( $P < 0.05$ ).

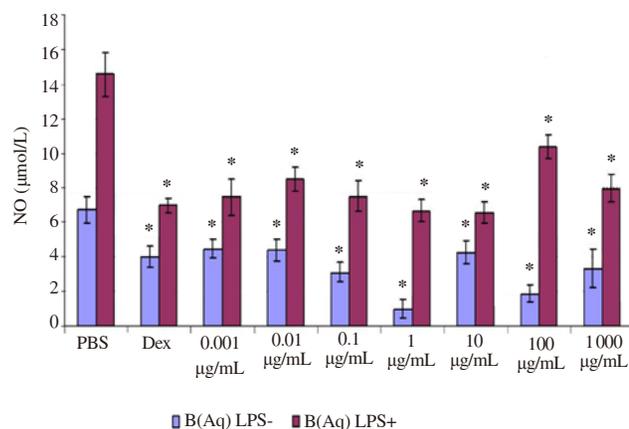
NO release by macrophages was determined using Griss reagent (Figures 2 and 3). Results showed that barberry alcoholic

extract in all examined concentrations suppressed NO production from lipopolysaccharide stimulated macrophages ( $P < 0.05$ ) and it showed inhibitory effect on unstimulated macrophages at 0.001, 1, 10 and 100  $\mu\text{g/mL}$  ( $P < 0.05$ ). Barberry aqueous extract also showed inhibitory effect on NO production at all examined concentrations ( $P < 0.05$ ). Cytokine assay on treated macrophages supernatant showed that 2 concentrations of 0.001 and 0.01  $\mu\text{g/mL}$  of both extracts significantly reduced TNF- $\alpha$  production ( $P < 0.05$ ) while 0.1  $\mu\text{g/mL}$  of alcoholic extract and 0.01–1000  $\mu\text{g/mL}$  of aqueous extract significantly induced TNF- $\alpha$  production ( $P < 0.05$ ) (Figure 4). In case of IL-6, all examined concentrations of both extracts have decreased IL-6 production ( $P < 0.05$ ) (Figure 5). IL-12 production were induced by both extracts in range of concentration from 0.1 to 1000  $\mu\text{g/mL}$  ( $P < 0.05$ ) (Figure 6).



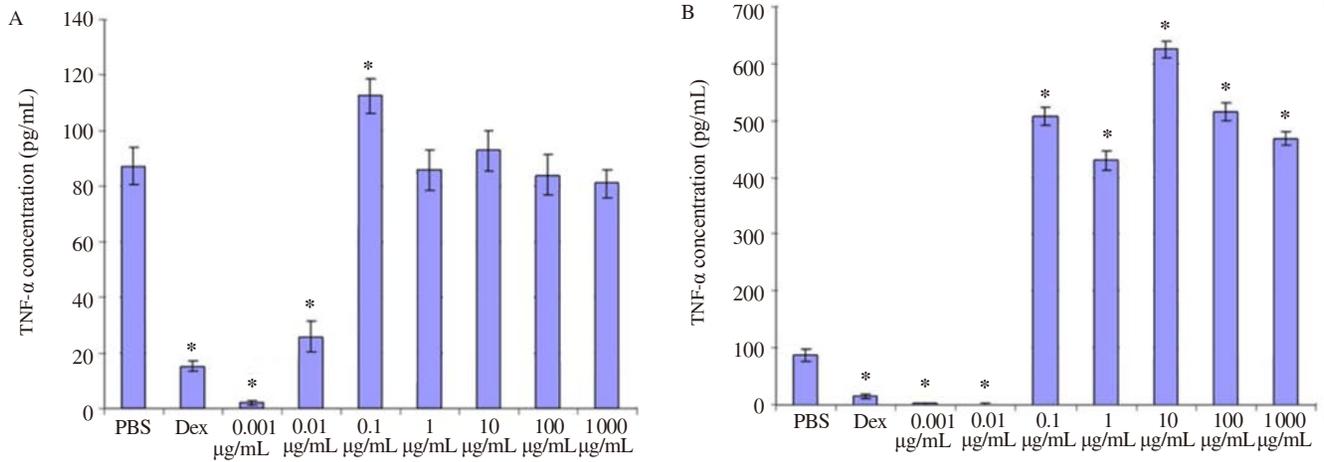
**Figure 2.** NO production (mean  $\pm$  SD) peritoneal macrophages treated with barberry alcoholic extracts.

Dex: Dexamethasone; B(A) LPS-: Cells treated with alcoholic extract and without lipopolysaccharide stimulation; B(A) LPS+: Cells treated with alcoholic extract and lipopolysaccharide stimulation; \*: Significant differences ( $P < 0.05$ ).

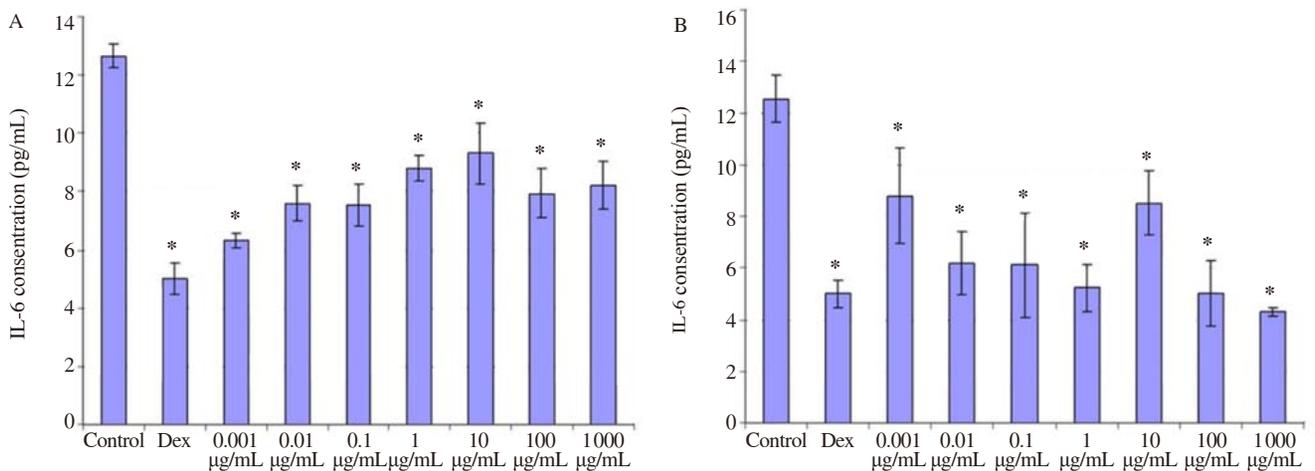


**Figure 3.** NO production (mean  $\pm$  SD) by peritoneal macrophages treated with barberry aqueous extracts.

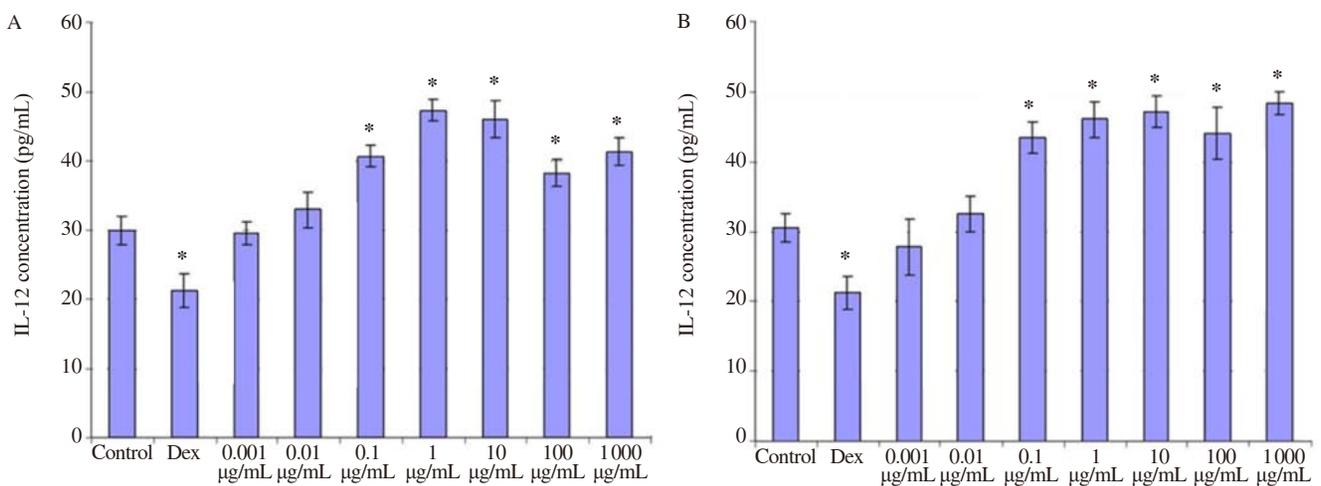
Dex: Dexamethasone; B(Aq) LPS-: Cells treated with aqueous extract and without lipopolysaccharide stimulation; B(Aq) LPS+: Cells treated with aqueous extract and lipopolysaccharide stimulation; \*: Significant differences ( $P < 0.05$ ).



**Figure 4.** TNF- $\alpha$  production by peritoneal macrophages treated with various concentrations of barberry alcoholic (A) and aqueous (B) extracts. Dex: Dexamethasone; \*: Significant differences ( $P < 0.05$ ).



**Figure 5.** IL-6 production by peritoneal macrophages treated with various concentrations of barberry alcoholic (A) and aqueous (B) extracts. Dex: Dexamethasone; \*: Significant differences ( $P < 0.05$ ).



**Figure 6.** IL-12 production by peritoneal macrophages treated with various concentrations of barberry alcoholic (A) and aqueous (B) extracts. Dex: Dexamethasone; \*: Significant differences ( $P < 0.05$ ).

#### 4. Discussion

Barberry (*B. integerrima*) is one of the representatives of Berberidaceae family. Extracts of *Berberis* are examined thoroughly these days, revealing its different medicinal properties. These plants are rich sources of tannins, waxes,

mucus, mineral salts and pectines. The roots and barks of *Berberis* species are used in traditional Chinese, Korean and Japanese medicine for treatment of various inflammatory diseases. High concentration of isoquinoline alkaloids (major active constituents) with berberine (their main representative) affects barberry's activity. Currently, protoberberine alkaloids

are known for their ability to block the synthesis of DNA and to inhibit cellular permeability[21,22]. The chief constituents of fraction A are *n*-nonacosane (11.91%), linoleic acid (7.26%) and 6,10,14-trimethyl-2-pentadecanone (5.85%). Fraction B contains primarily: *n*-nonacosane (11.91%), *n*-heptacosane (5.79%) and 4-hydroxy-3-methoxy-benzaldehyde (4.23%)[22,23]. There is a low number of studies on barberry. Some studies have been done on berberine that is also found in other plants such as barberry (*B. vulgaris*), coptis (*Coptis chinensis*), and Oregon grape (*Mahonia aquifolium*). Extracts of *Berberis* species are characterized by analgesic, antibacterial, antiviral, antiarrhythmic, anti-inflammatory, cholagogic and ossific properties[24,25]. A broad spectrum of its properties encourages to do further research on it. In the present study, we evaluated effect of alcoholic and aqueous extracts of barberry on viability and functions of macrophages. Cytotoxic evaluation showed that MTT reduction capability was the same in all groups, unless groups with higher doses of extracts (100 and 1000 µg/mL) of both extracts that caused reduced viability of macrophages. The cytotoxic effect of high doses of barberry extracts may be because they interrupt with culture media conditions or it may be due to barberry ingredient effect on macrophage cells. In previous studies on mouse pancreatic islets treated with berberine, it was indicated that this alkaloid acts as anti-apoptosis in these cells[26], and in other studies, berberine affected the cell cycle and apoptosis of the human colorectal adenocarcinoma cell line[27]. Evaluation of NO production by treated macrophages showed that all examined concentrations of aqueous extract suppressed NO production from both stimulated and unstimulated macrophages and also alcoholic extracts inhibited NO release from stimulated macrophages, but in unstimulated cells, just some concentrations (0.001, 1, 10 and 100 µg/mL) of barberry alcoholic extract were effective. In this case, the high antioxidant capacity of barberry fruit is primarily due to the high levels of total phenolic flavonoids such as anthocyanin and other polyphenolic compounds found in this fruit, especially, barberry fruits with high amount of anthocyanin and phenolic content. In addition, high antioxidant capacity may increase its popularity among the other wild fruits[28]. A study demonstrated that components of barberry as 13-methylberberine and 13-ethylberberine reduced NO production by inhibition of induced NO synthase protein expression in RAW 264.7 cells stimulated by lipopolysaccharide[29]. Some other barberry's ingredients are organic acids such as malic and citric which indicate that barberry fruits have rich source of natural antioxidant substances that were confirmed by antioxidant activity on macrophage cells too[28]. Non-significant data for low concentrations of alcoholic extracts may be because those cells were not stimulated, and suppressor effects were not demonstrated. Cytokine assay on treated macrophages showed that 0.001 and 0.01 µg/mL of both extracts were crucial concentrations and reduced TNF- $\alpha$  production. On the other hand, higher concentrations of aqueous

extract (0.1–1000 µg/mL) induced TNF- $\alpha$  production. We showed a concentration dependent function in TNF- $\alpha$  production from macrophages and dose-dependency that may explain previous contradictory reports. Some study indicated the inhibition of TNF- $\alpha$  intracellular pathways as activator protein-1 and also nuclear factor of activated T-cells[30] by berberine and indicated TNF- $\alpha$  suppression, and on the other hand, other studies mentioned that berberine does not influence on macrophages for production of TNF and interleukin-1[31] or study that showed the expression of cyclooxygenase-II and TNF- $\alpha$  mRNA as well as their protein was not affected by berberine[29]. The best explanation for these results is shown in our study as dose dependency and critical concentrations for their functions of TNF- $\alpha$ . As for IL-6, both extracts have decreased IL-6 production ( $P < 0.05$ ) by all examined concentrations. These results have been confirmed by another *in vivo* study and reduction of IL-6 from barberry treated lymphocytes of mice[29]. Dose dependency function was observed in IL-12 production as well; 0.1-1000 µg/mL of both extracts enhanced IL-12 production. Another study on barberry ingredient of barberry showed IL-12 p40 increases in macrophage cells[29]. Our results are verified by other studies but the point is that they studied on berberine (one of barberry's ingredients). Maybe berberine could be the major component and effective molecule but other ingredients will have their important role in barberry's effects.

In conclusion, the results of this study indicated that barberry fruit's alcoholic and aqueous extracts do not have cytotoxic effect on macrophages, while the extracts have a significant antioxidant effect on macrophages by suppression of NO production. Barberry's extracts affect macrophages in a dose dependent manner for cytokine production as inhibition of TNF- $\alpha$  in lower concentrations and inhibition of IL-6 in all concentrations. Extracts can induce TNF- $\alpha$  and IL-12 in high concentrations. Therefore, using barberry for treating diseases or developing functional foods should be carefully considered. This study provides additional evidence of the immunomodulatory properties of barberry.

### Conflict of interest statement

We declare that we have no conflict of interest.

### Acknowledgments

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