



## The effect of hydroalcoholic extract of *Otostegia persica* (Burm.) Boiss. against H<sub>2</sub>O<sub>2</sub>-induced oxidative stress in human endothelial cells

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### Abstract

**Background and objectives:** *Otostegia persica* (Burm.) Boiss. is an endemic plant of Iran with various applications in traditional medicine which contains of several antioxidant constituents. This research was aimed to investigate the effect of hydroalcoholic extract from *O. persica* aerial parts in human umbilical vein endothelial cells (HUVECs) using hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) as an inducer of oxidative damage. **Methods:** The total phenolics content of the extract was estimated using Folin-Ciocalteu method. The probable cytotoxicity of *O. persica* extract and also its cytoprotective effect on HUVEC cells against oxidative stress was assessed using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The intra and extra-cellular hydroperoxides concentration and ferric reducing antioxidant power (FRAP) were determined in pretreated cells. **Results:** Total phenolics content was found to be 42.41±0.1 mg tanic acid equivalent/g of the dried extract. No cytotoxic effect was observed from *O. persica* extract in the range of 25-250 µg/mL. Pretreatment of HUVECs with *O. persica* extract with the concentrations of 50-250 µg/mL significantly reduced the cytotoxic effect of H<sub>2</sub>O<sub>2</sub>. *Otostegia persica* extract attenuated the concentration of hydroperoxides and increased FRAP value in intra- and extra-cellular fluids at different concentration ranges. **Conclusion:** This study indicated the antioxidant and cytoprotective activities of *O. persica* extract against H<sub>2</sub>O<sub>2</sub>-induced oxidative stress in HUVEC cells; however, more researches are required for finding the precise mechanism and assessing its clinical value.

**Keywords:** antioxidant, HUVEC, MTT assay, *Otostegia persica*, oxidative stress

### Introduction

Plants as sources of pharmacologically active constituents have shown beneficial effects in human health [1]. Supplementation with herbal antioxidants has been associated with reduction

in the risk of cardiovascular diseases (CVDs) [2]. Phytochemical compounds with antioxidant capacity are able to protect endothelium against oxidative stress through inhibition of reactive

oxygen species (ROS) production, scavenging of ROS or improvement of antioxidant capacity [3,4]. Oxidative stress has a pivotal role in the endothelial dysfunction and therefore in the pathogenesis of a variety of CVDs including hypertension, dyslipidemia, diabetes mellitus, atherosclerosis, coronary artery diseases and heart failure [5]. Decreased nitric oxide (NO) bioavailability, oxidation of low density lipoprotein (LDL), inflammation and apoptosis of endothelial cells are subsequent events following oxidative damage in the vasculature system [6,7]. Antioxidant, anti-inflammatory, anti-atherosclerotic and anti-apoptotic activities have been proven for various herbal bioactive compounds [8,9].

*Orostegia persica* (Burm.) Boiss. which belongs to the Lamiaceae family, is an endemic plant widely growing in south and southeast of Iran. The aerial parts of this plant is traditionally used for treatment of various diseases including fever, cough, arthritis, headache, stomachache, toothache, gastric discomfort, cardiac distress, palpitation, hypertension and diabetes [10]. Pharmacological studies have proven antioxidant, antimicrobial, anti-diabetic, anti-inflammatory, antihypertensive and hepatoprotective properties for *O. persica* [11-13].

Significant antioxidant, anti-lipid peroxidation and radical scavenging activities have been reported for several extracts and essential oils of the aerial parts of *O. persica* [14,15]. This study was conducted to investigate the antioxidant and cytoprotective effects of the hydroalcoholic extract from *O. persica* aerial parts against oxidative stress induced by H<sub>2</sub>O<sub>2</sub> in human umbilical vein endothelial cells (HUVEC cells).

## Experimental

### Plant material

The aerial parts of *O. persica* were collected from Jiroft Mountains (Kerman province, Iran) in June 2013 and identified by the Botany Department, Faculty of Sciences of Isfahan University, Isfahan, Iran. A voucher specimen (No. 2835) was deposited at the Herbarium of the School of Pharmacy and Pharmaceutical Sciences, Isfahan, Iran.

### Preparation of the extract

The air-dried aerial parts of *O. persica* were powdered and extracted with ethanol 70% for 48 h using percolation method, with rate of 18 drops per min at room temperature. Then the extract was filtered under reduced pressure and ethanol was removed by a rotary evaporator at 50 °C. The residue was freeze-dried and stored at -20 °C. The yield of the plant extract was 15.6 % (w/w). Different concentrations of the extract were dissolved in dimethyl sulfoxide (DMSO) 1% and diluted with cell culture medium for using in cellular experiments.

### Determination of total phenolics content

The total phenolics content of *O. persica* extract was determined using Folin-Ciocalteu method [9]. Briefly, the sample of the plant extract was mixed with sodium carbonate (20%). Then the mixture was treated with diluted Folin-Ciocalteu's phenol reagent (Merck, Germany). After 2 h, the absorbance was measured at 765 nm. The total phenolics content was estimated using a standard curve obtained from various concentrations of tannic acid.

### Cell culture

HUVEC cells were obtained from National Cell bank of Iran (Pasteur Institute, Tehran, Iran) and maintained as monolayer culture grown in 75 cm<sup>2</sup> flasks during a humidified atmosphere of 5% CO<sub>2</sub> at 37 °C in Dulbecco's modified Eagle's medium (DMEM), (GIBCO BRL Life Technologies, USA). The medium was supplemented with 10% fetal bovine serum (FBS), (Bioidea Company, Iran) and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin).

### Cell viability assay

The viability of HUVEC cells was determined using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) kit (Bioidea Company; Tehran, Iran). This assay is based on the reduction of the tetrazolium salt by mitochondrial dehydrogenases of viable cells [16]. MTT method was firstly used for evaluation of the probable cytotoxicity of *O. persica* extract

on HUVEC cells viability at normal condition (5% CO<sub>2</sub> at 37 °C). In Brief, the cell monolayer was harvested in exponential growth (the period of cell line growing with maximal density which was attained after 24 h incubation) and  $1.5 \times 10^5$  cells/mL was seeded in each well of the 96-well plates. Twenty four hours after plating, HUVECs were treated with different concentrations of freshly prepared *O. persica* extract (25 to 1000 µg/mL) and incubated for another 24 h at 37 °C in 5% CO<sub>2</sub>. Then the medium of each well was discarded and new medium and MTT reagent was added and re-incubated for 3 h at 37 °C. After addition of DMSO for dissolving the formazan crystals resulting from MTT reaction with living cells, the absorbance was measured at 570 nm by a microplate reader (BioTek Instruments, USA).

The cytoprotective effect of *O. persica* extract on HUVEC cells against H<sub>2</sub>O<sub>2</sub>-induced oxidative stress was also evaluated by MTT assay. The cells were pre-incubated with *O. persica* extract (25 to 500 µg/mL) for 24 h at 37 °C and after washing out with PBS, the cells were exposed to 0.5 mM H<sub>2</sub>O<sub>2</sub> for 2 h. The rest of the experiment was done as mentioned above. The viability of treated samples was estimated by comparison of the absorbance of each sample with negative control (the cells without exposure to the extract or H<sub>2</sub>O<sub>2</sub>) and each experiment was assessed in triplicate.

#### *Measurement of extra- and intra-cellular hydroperoxides concentration*

The effects of pretreatment with *O. persica* extract on intra- and extra-cellular hydroperoxides level were detected using ferrous ion oxidation by xylenol orange (FOX1) kit (Hakiman Shargh Research Co., Iran). This method estimates the hydroperoxides based upon oxidation of reagent Fe<sup>+2</sup> to Fe<sup>+3</sup> by oxidizing agents and formation of a color complex through its binding to xylenol orange in an aqueous medium containing sorbitol [17].

After pretreatment of HUVEC cells with different concentrations of *O. persica* extract and exposure to H<sub>2</sub>O<sub>2</sub>, 10 µL of supernatant of the cells or the cell lysates from each well was added

to 190 µL of reagent and incubated for 30 min at 40 °C. Absorbance was measured at 540 nm with a microplate reader/spectrophotometer. The hydroperoxides content of the samples were assessed using a standard curve of H<sub>2</sub>O<sub>2</sub> concentrations and expressed as H<sub>2</sub>O<sub>2</sub> equivalents.

#### *Measurement of intra- and extra-cellular ferric reducing antioxidant power (FRAP)*

For evaluation of total antioxidant capacity of *O. persica* extract, the ferric reducing antioxidant power (FRAP) was estimated using a commercial kit (Hakiman Shargh Research Co., Iran). This assay is based on the reduction of ferric-tripyridyltriazine complex to ferrous form using a reagent containing tripyridyltriazine/ferric chloride/acetate buffer [18]. Ten 10 µL of sample was added to 200 µL of FRAP reagent. The samples consisted of supernatant of the cells or the cell lysates as mentioned above. After incubation of the mixture of sample and reagent for 40 min at 40 °C, the absorbance was measured at 570 nm using a microplate reader/spectrophotometer. The FRAP value of the samples were calculated using the standard curve of FeSO<sub>4</sub>.7H<sub>2</sub>O and were expressed as µM of Fe<sup>2+</sup> equivalents.

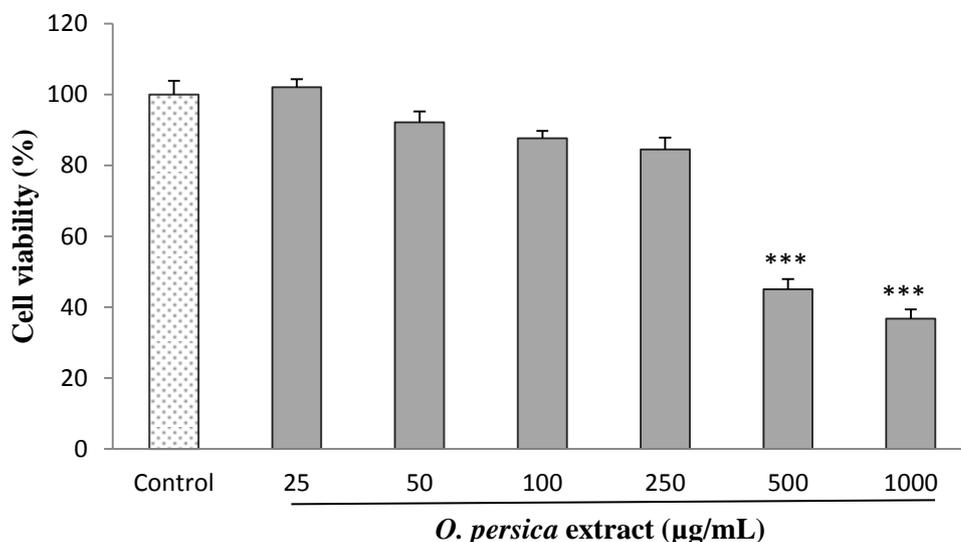
#### *Statistical analysis*

The data were presented as mean ± standard error of mean (SEM) and analyzed using SPSS software (version 16.0) by one-way analysis of variance (ANOVA) followed by Tukey post-hoc test. The results were considered significantly different when *p* was <0.05.

#### **Results and Discussion**

The total phenolics content was estimated as 42.41±0.1 mg tannic acid equivalent/g of the dried plant extract which indicated high amount of phenolics in *O. persica* extract.

MTT assay for evaluation of probable cytotoxicity of *O. persica* extract on HUVECs showed no inhibitory effect on HUVECs viability after exposure to the concentrations of 25-250 µg/mL of the extract for 24 h; however, cytotoxic effects were observed in the concentration range



**Figure 1.** Effect of different concentrations of *Otostegia persica* extract on HUVEC cells viability determined by MTT assay. Values are means±SEM from three independent experiments in triplicate. \*\*\*  $p < 0.001$  versus control (untreated cells)

of 500-1000 µg/mL of *O. persica* extract (figure 1). The presence of some toxic compounds such as labdane type diterpenoids may be the possible reason for this cytotoxic activity at higher concentrations [19].

In the present study, H<sub>2</sub>O<sub>2</sub> was used for triggering oxidative stress in human endothelial cells as a precursor of other ROS. This small non-free radical is believed to be a contributor to different vascular physiologic and pathologic conditions such as growth, inflammation and apoptosis [20]. As shown in figure 2, oxidative damage induced by H<sub>2</sub>O<sub>2</sub> (0.5 mM for 2 h) caused significant reduction in HUVEC cells viability ( $p < 0.001$ ). Pretreatment of HUVEC cells with *O. persica* extract at the concentrations of 50-250 µg/mL significantly reduced the cytotoxicity resulted from exposure to H<sub>2</sub>O<sub>2</sub>.

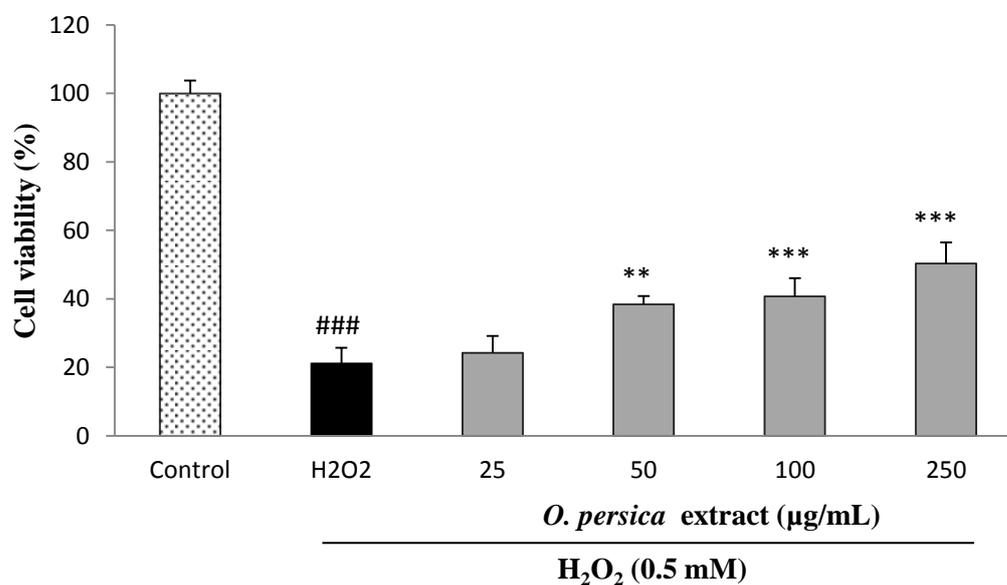
Figure 3 shows the effects of pretreatment with *O. persica* extract on intra- and extra-cellular hydroperoxides concentration in HUVEC cells after exposure to H<sub>2</sub>O<sub>2</sub>. During 2 h incubation with H<sub>2</sub>O<sub>2</sub> (0.5 mM), the intra- and extra-cellular hydroperoxides concentration considerably increased ( $p < 0.001$ ). Incubation of HUVECs with *O. persica* extract significantly reduced the intra- and extra-cellular hydroperoxides at the

concentrations of 100-250 µg/mL compared to the control group.

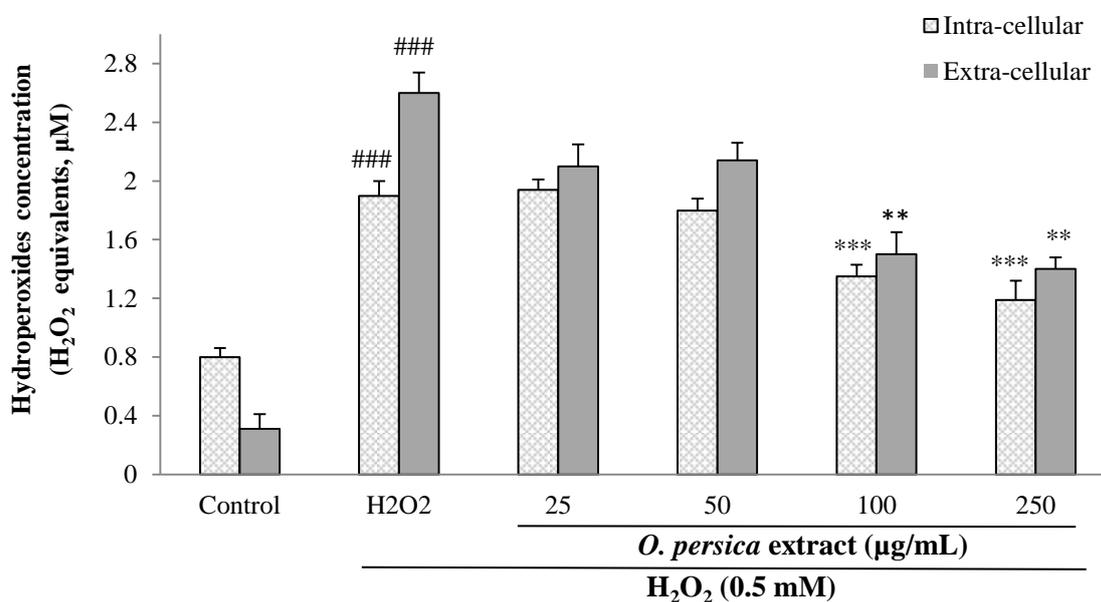
Pretreatment of HUVEC cells with *O. persica* extract significantly increased the FRAP levels in intra-cellular fluid at the concentration of 250 µg/mL ( $p < 0.05$ ) and in extra-cellular fluid at the concentration of 25-250 µg/mL (figure 4) in a concentration dependent manner.

Results of the present study revealed that hydroalcoholic extract of *O. persica* effectively protected HUVEC cells against H<sub>2</sub>O<sub>2</sub>-induced toxicity in the concentration range of 50-250 µg/mL. Pretreatment with *O. persica* extract also showed antioxidant effects through decreasing hydroperoxides concentration and increasing FRAP value in intra- and extra-cellular fluids.

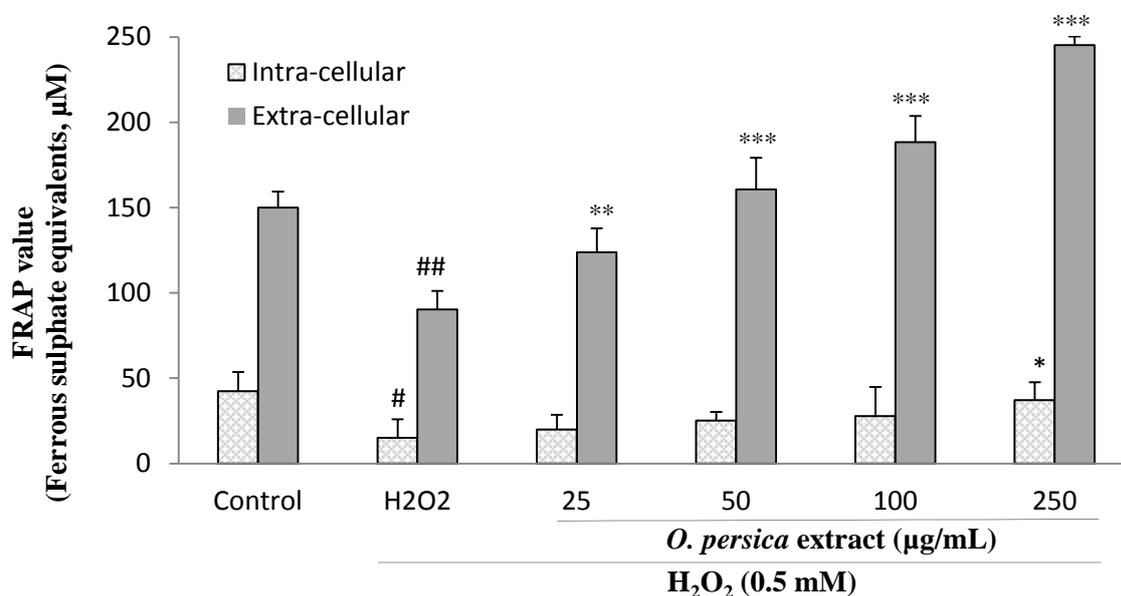
The observed cytoprotective effect of *O. persica* extract against oxidative injury of HUVECs may be due to the high phenolics content and antioxidant activity of its components. Phytochemical analysis has revealed the presence of flavonoids, terpenoids and essential oils in the aerial parts of *O. persica*. Quercetin, morin, kaempferol, caffeic acid, trans-cinnamic acid, *p*-hydroxy benzoic acid, β-sitosterol and isovitexin are the main phenolic compounds isolated from this plant [13,14].



**Figure 2.** Effect of different concentrations of *Otostegia persica* extract on H<sub>2</sub>O<sub>2</sub>-induced oxidative stress in HUVEC cells determined by the MTT assay. Values are means±SEM from three independent experiments in triplicate. ###*p*<0.001 versus control (untreated cells), \**p*<0.05, \*\**p*<0.01 and \*\*\**p*<0.001 versus H<sub>2</sub>O<sub>2</sub> stimulated cells.



**Figure 3.** Effects of different concentrations of *Otostegia persica* extract on intra- and extra-cellular hydroperoxides concentration in HUVEC cells as H<sub>2</sub>O<sub>2</sub> equivalents determined by FOX1 method. Values are means±SEM from three independent experiments in triplicate. ###*P* < 0.001 versus control (untreated cells), \**p* < 0.05, \*\**p* < 0.01 and \*\*\**p* < 0.001 versus H<sub>2</sub>O<sub>2</sub> stimulated cells



**Figure 4.** FRAP values of different concentrations of *Otostegia persica* extract determined as ferrous sulphate equivalents. Values are means±SEM from three independent experiments in triplicate.

The major terpenoids of the plant are  $\beta$ -amyrin, campesterol, stigmasterol and some new diterpenes, and its important essential oil components include  $\alpha$ -pinene, diisooctyl phthalate, hexadecanoic acid, caryophyllene oxide,  $\beta$ -sinesal and verbenol [21,14]. It has been found that flavonoids are able to protect cells against oxidative stress by stimulation of cellular defense pathways [22]. Quercetin has shown high cytoprotective capacity against H<sub>2</sub>O<sub>2</sub>-induced oxidative damage in melanocyte and neuroblastoma cell types [23,24]. This prototypic flavonoid has significant antioxidant effects through radical scavenging activity, increasing the intracellular antioxidant level and reducing lipid peroxidation [25]. Quercetin has also exhibited many useful activities in vascular system including vasodilatory effects, reduction of adhesion molecules and other inflammatory markers, inhibition of platelet aggregation and improvement of endothelial function [26]. Morin and Kaempferol are other flavonoids of *O. persica* with antioxidant properties. Cytoprotective effect of morin has been

established against oxidative damage through activation of extracellular-regulated kinase (ERK) and nuclear factor erythroid 2-related factor 2 (Nrf2) signaling cascades and consequently up-regulation of heme oxygenase-1 [22]. Kaempferol has also protective effects against endothelial injury by reduction of ROS formation and improvement of nitric oxide production [27]. Caffeic acid, another antioxidant phenolic compound, can inhibit lipid peroxidation and decrease the glutathione depletion and therefore improves resistance of the cells against oxidative damage [28]. Cytoprotective activity of *trans*-cinnamic acid against glucose oxidase-induced oxidative stress has been reported in mouse fibroblast cells and human keratinocyte cells [29]. It has been also reported that isovitexin may prevent cellular ROS damage by inhibition of xanthine oxidase [30]. Regarding the reduction of hydroperoxides concentration and increasing FRAP value in intra- and extra-cellular fluids by *O. persica*, the cytoprotective effect of this herbal extract may be mediated by direct scavenging of ROS and

indirect antioxidant action by induction of total antioxidant capacity.

In conclusion, the hydroalcoholic extract of *O. persica* aerial parts were found to protect HUVECs against oxidative damage induced by H<sub>2</sub>O<sub>2</sub>. However, to elucidate the precise mechanisms further investigations are required.

#### Acknowledgments

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#### Declaration of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the content of the paper.

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