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The Anti-Atherosclerotic Effect of *Prosopis Farcta* is Associated with the Inhibition of *VCAM-1* and *ICAM-1*

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A B S T R A C T

This study evaluated the anti-atherosclerosis effect of aqueous extract and polysaccharide-enriched fraction of *Prosopis farcta* on adhesion and inflammatory cascades in vascular endothelial cells and related molecular mechanisms. Cellular toxicities of LPS, plant extract and polysaccharide-enriched fraction were analyzed in vitro using the MTT method. The ROS accumulation, mRNA expression of *ICAM1* and *VCAM1*, and COX activity were measured in HUVEC cells. These results revealed a new underlying mechanism for anti-atherosclerosis effect of *P. farcta* and polysaccharide-enriched fraction by attenuating inflammatory cascade mediated by COX expression, modulating expression of cell-adhesion molecules including *VCAM-1* and *ICAM-1*, and diminishing oxidative stress agents.

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Introduction

Atherosclerosis is a chronic inflammatory process with increased level of oxidative stress, in which the adhesion of leukocytes to the vascular endothelium and their subsequent migration into the wall are the pivotal events [1]. Atherosclerosis is associated with ischemic heart disease as well as peripheral arterial disorders. The disease is considered as one of the top causes for premature death in adults. Oxidative stress has been associated with more than one hundred pathologies such as atherosclerosis, diabetes, cardiovascular diseases, etc. Medicinal plants can not only protect the oxidative damage, but also play a vital role in health maintenance and prevention of chronic degenerative and inflammatory diseases [2]. Plant based compounds can help to treat or prevent atherosclerosis by affecting the inflammatory and oxidative stress associated cascades [3]. The genus *Prosopis* with about 44 species is widespread in arid and semiarid regions of Asia, America, Africa and Middle East and also in south regions of Iran. Syrian mesquite (*Prosopis farcta*) is a woody perennial dwarf legume shrub, usually with 0.4-1m high. It can grow to 2-3 m where weed control is absent [4]. The plant is distributed from India to Iran. In addition, *P. farcta* has spread to the Middle East, and grows in Cyprus, Turkey, and Ukraine and along the North African coast as far as Algeria [5,6]. *P. farcta* have been traditionally used for the treatment of different diseases including diabetes, wounds and skin lesions, prostate disorders, measles, neurologic changes, urinary diseases, diarrhea and dysentery, respiratory disease, liver changes associated with diabetes, gastric ulcers, miscarriage and rheumatism [7-11]. Also, *P. farcta* can be applied to reduce cardiac or chest pain and for the management of cardiovascular disorders [12], reduction in the size of atherosclerotic plaques in aortic wall[13] and improving serum lipid profiles [13, 14].

Medicinal plants and medicinal substances from plant sources have a wide range of natural antioxidants that can be effective in treatment of diseases. Fewer side effects and reasonable price is the other reasons for the growing trend toward these drugs. In the present study, we used primary cultured human umbilical vein endothelial cells (HUVEC) to examine the therapeutic effects of aqueous extract and different fractions of *P. farcta* on the development of atherosclerosis and evaluating related molecular mechanisms.

Materials and Methods

Instrumentation and chromatographic conditions

The HPLC system consisted of two pumps (LC-10AD), a column oven (CTO-10A), a UV–vis spectrophotometer detector (SPD-10AD) operated at the wavelengths of 210, 225 and 285 nm, a degasser (DGU-3A) and a data processor (C-R4A) all from Shimadzu, Kyoto, Japan. The analytical column was a phenyl CLCODS (mz, Germany), 150 mm × 4.6 mm I.D., and particle size of 5µm. A mixture of water and methanol (90:10) was used as the mobile phase. The temperature of oven column was set at 58 °C and the mobile phase was filtered, degassed and pumped at a flow rate of 0.6 mL/min.

General Instruments and Chemicals

Silica gel 60 (0.040- 0.063 mm) was purchased from Merck (Germany). 3-(4, 5 dimethylthiazol-2yl)-2,5 - diphenyltetrazolium bromide (MTT) and rhodamine 123 were procured from Sigma Aldrich (St Louis, MO,USA).
RNA isolation kit with high purity was purchased from Roche (Mannheim, Germany). Real time polymerase chain reaction (RT-PCR) kit was supplied by TAKARA (Japan). Cyclooxygenase activity assay kits were obtained from Cayman Chemical Company (Ann Arbor, MI, USA.) Cell culture medium, penicillin-streptomycin, and fetal

bovine serum (FBS) were purchased from Gibco (Grand Island. NY, USA).

Plant Material and Extract Preparation

Roots of *P. farcta* were collected from Kermanshah province, west of Iran. Sample was identified by Dr Masoumi (Herbarium of Research Center of Agriculture College, Razi University, Kermanshah Province, Kermanshah, Iran) with the voucher number 1002 (RUH)) and kept in standard conditions. The root barks of *P. farcta* were powdered after drying. A total of 2 grams of plant powder was added to 100 mL boiling water and mixed for 5 minutes.

Fractionation of the Extract by Solid Phase Extraction (SPE)

The whole content of the mixture was first filtered through an ordinary filter paper, and the filtrate was then passed through a No.1 Whatman filtering paper. The solution was added to ethanol (1:3 volume ratios) and centrifuged for 2 min at 3000 g; then the precipitate was collected and dissolved in deionized water. Solid-phase extraction (SPE) was performed to prepare polysaccharide-enriched fraction of the aqueous extract. SPE cartridges were fitted into stop cocks and connected to a vacuum manifold. The sorbent was conditioned with 1.5mL water, followed by 1.5mL ethanol and 1.5mL water, acidic ethanol (1:3 acetic acid to ethanol volume ratio) and finally with 3mL water. The cartridge was air-dried for about 3 min. The dissolved precipitate of the extract was transferred to the SPE column reservoir. The fractionated extract was collected and injected into HPLC column using a mobile phase of water and methanol (10:90) [15, 16].

Cell Culture Conditions

Human Umblicical Vein Endothelial Cell line (HUVEC) was obtained from Pasteur Institute (Tehran, Iran) and maintained at 37 ºC in a humidified atmosphere (90%) containing 5% CO2. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM-F12) with 10% (v/v) heat-inactivated fetal bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin.

Cell Viability Assay

Cellular toxicities of the LPS, extract, and polysaccharide-enriched fraction were analyzed towards HUVEC cells using the methyl thiazoltetrazolium bromide method (MTT) assay [17]. The stock solutions of the compounds were prepared in dimethyl sulfoxide (DMSO, 1 v %). HUVEC cells were seeded in triplicate in 96-well tissue culture plates (15×10^3 cells/well) and incubated overnight. Four sets of experiments were conducted at standard culture conditions: (1) untreated control cells, (2) cells treated with different concentrations of extract and the polysaccharide-enriched fraction (3) cells treated with LPS (6, 12, 25 µg/mL), and (4) cells pretreated with different concentrations of extract and polysaccharide-enriched fraction for 2 h. Then medium was changed and cells were treated with LPS (25 µg/mL) for 24 h. Briefly, after treatment, the medium was removed and 0.1 mg/well of MTT was added to the cells, and plates were further incubated for 3 h at 37 °C, and the purple formazan crystals were dissolved in DMSO (200 µL/well). Absorbance was determined on an ELISA plate reader (BioTek, H1M) with a test wavelength of 570 nm and a reference wavelength of 630 nm to obtain the sample signal (OD570–OD630).

Determination of Intracellular ROS

Intracellular ROS level was examined using DCF-DA. DCF-DA is a non-fluorescent lipophilic ester that easily crosses the plasma membrane and

passes into the cytosol, where the acetate group is rapidly removed by unspecific esterase [18]. The oxidation of this molecule to the fluorochrome DCF results in green fluorescence. The intensity of this fluorescence is generally considered to reflect the level in which ROS are present [19]. After seeding for 24 h, HUVEC cells were washed with PBS buffer (pH 7.4). The cells pretreated with the extract and polysaccharide-enriched fraction for 2 h, and then were treated with LPS for another 24 h. After washing with PBS, the cells were incubated with 20 µL DCF-DA at 37°C for 40 min. The percentage of DMSO in solution did not exceed 0.5%. After incubation, cells were lysed with Triton X-100. The fluorescence was measured at an excitation wavelength of 488 nm and an emission wavelength of 528 nm using a fluorescence micro plate reader (BioTek, H1M, USA).

Determination of COX Activity

COX activity was measured in the proteins extracted from HUVEC cells by Cayman COX activity assay kit (Cayman Chemical Company, catalogue number 760151) according to the manufacture procedure. Cayman's COX Activity Assay Kit measures the peroxidase activity of COX. The peroxidase activity is assayed calorimetrically by monitoring the appearance of oxidized N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD) at 590 nm.

Real-Time RT-PCR Analysis of Gene expression of adhesion proteins

Total RNA from HUVEC cells were extracted using high pure isolation kit (Takara, japan) according to the manufacturer's instructions. Quality and quantity of total RNA were assessed by spectrophotometer (NanoDrop 2000, USA) and samples were stored at −80°C until use. The primers were designed to be used in this study. The performances of all the primer pairs were tested by primer concentration to determine the optimal reaction conditions. Thermal cycler conditions were: 5min at 42°C for cDNA synthesis, 10 min at 95°C followed by 40 cycles of 5s at 95°C to denature the DNA, and 30s at 60°C to anneal and extend the template. Melting curve analysis was performed to ascertain specificity by continuous acquisition from 65°C–95°C with a temperature transient rate of 0.1°C/s. All the reactions were performed in triplicate in a Corbett system (Australia). The values obtained for the target gene expression were normalized to β-actin and analyzed by the relative gene expression ΔΔCT method where ΔΔCT = (CT target − CT β-actin) Unknown − (CT target − CT β-actin) Calibrator.

Statistical Analysis

Each experiment was performed at least three times and the results were presented as mean ± S.E.M (standard error of mean). One-way analysis of variance (ANOVA) followed by Turkey's test was used to compare the differences between means. A probability value of $P< 0.05$ was considered to be statistically significant.

Results the Cytotoxicity of LPS, Extract, and polysaccharide-enriched Fraction

In order to set LPS, extract and polysaccharide-enriched fraction at concentrations which are nontoxic to cells, the cytotoxicity of the aqueous extract and fraction was examined on HUVEC cells. The results showed that both the aqueous extract and its polysaccharide-enriched fraction in the concentration range of 5-75µg/mL had no cytotoxic effect on HUVEC cells (Figure 1-a). Moreover, results showed that LPS is not able to induce cytotoxicity in this cell line up to concentration of 25µg/mL (Figure 1-b). Using the nontoxic concentrations of the extract and fraction, another experiment was performed. Cells were pretreated with different concentrations of

extract and the polysaccharide-enriched fraction (2h), then medium was changed and cells were treated with different concentrations of LPS. The pretreated HUVEC cells with the extract and polysaccharide-enriched fraction did not show any significant cytotoxicity.

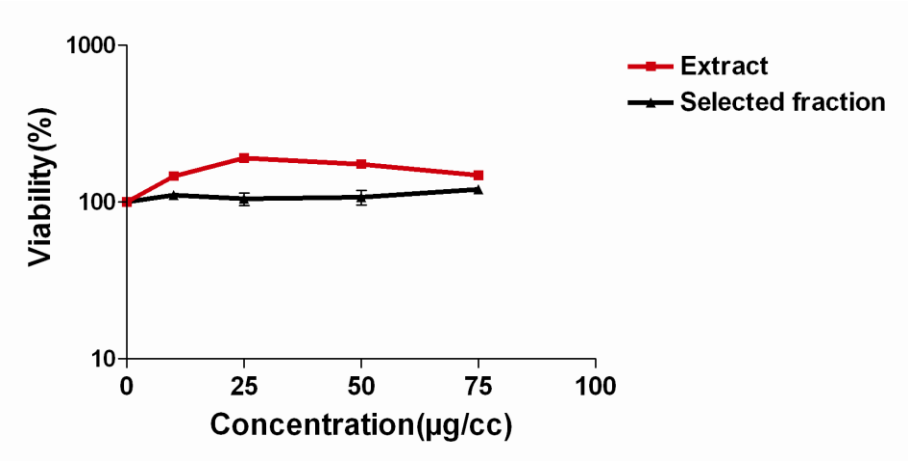

Figure 1-a

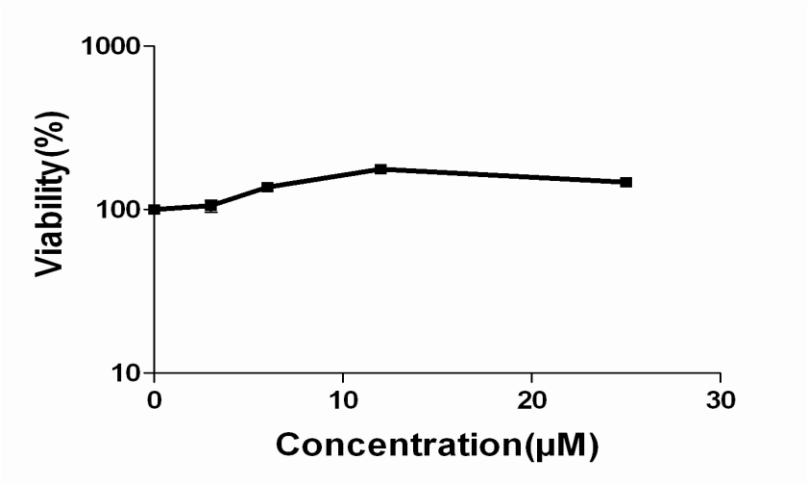

Figure 1-b

Fig. 1. Cell viability of HUVEC cells after treatment with different concentrations of aqueous extract and polysaccharide-enriched fraction (a) and with different concentrations of LPS. Cell viability was determined by MTT assay. Data are expressed as the mean ± S.E.M of three separate experiments (n=5).

Effect of the Extract and its polysaccharide-enriched Fraction on LPS-induced ROS Generation

In order to measure the oxidative stress induced by LPS, ROS accumulation was detected in HUVEC cells using the fluorescent probe DCF, which detects intracellular peroxides. Adding LPS to HUVEC cells caused a significant increase in ROS level. Then we evaluated whether the compounds protect HUVEC cells from LPS-induced oxidative damage by lowering the ROS level. As demonstrated in Figure 2, both the extract and its polysaccharide-enriched fraction exerted excellent radical scavenging activity. Pretreatment of the cells with fraction and extract significantly reduced the ROS levels to 74 and 56%, respectively.

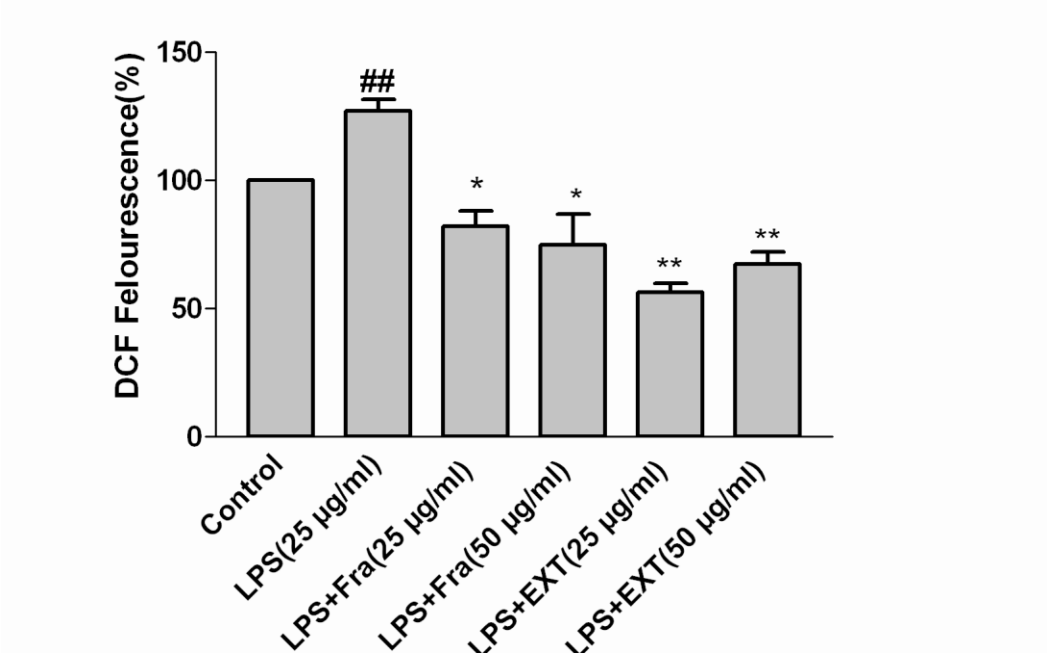

Fig. 2. The effect of aqueous extract and its polysaccharide-enriched fraction on LPS-induced ROS overproduction as detected by DCF. Cells were pretreated with extract (25, 50 µg/mL) and its polysaccharide-enriched fraction (25,50µg/mL) 24 before exposure to 25 µg/mL of LPS (## P<0.01 vs. Control, ** *P*-value < 0.02, * *P*-value <0.05 vs. LPS treated cells.

Effect of Pretreatment of HUVEC cells with the Extract and Fraction on COX activity

COX activity of the extract and polysaccharide-enriched fraction was assessed to gain further insight into anti-atherosclerosis activity of these agents. Treatment of HUVEC cells with LPS significantly increased COX-1 and COX 2 activities after 24 h of exposure, in comparison to control. As it can be observed in Figure 3, the increase in *COX-2* activity of HUVEC cells was attenuated by pretreatment with different concentrations of extract and selected fraction.

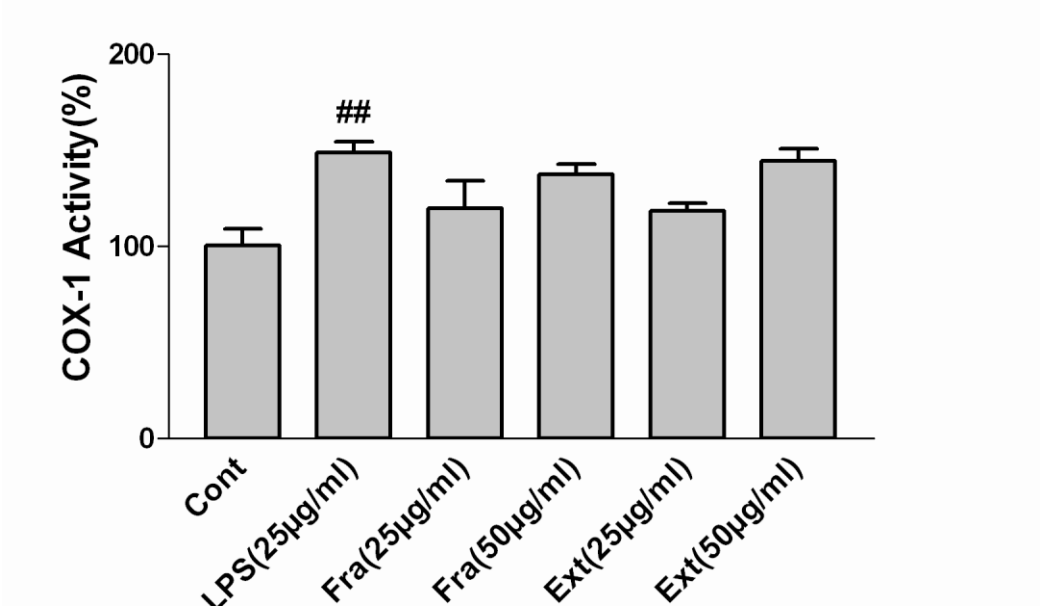

Figure 3-a

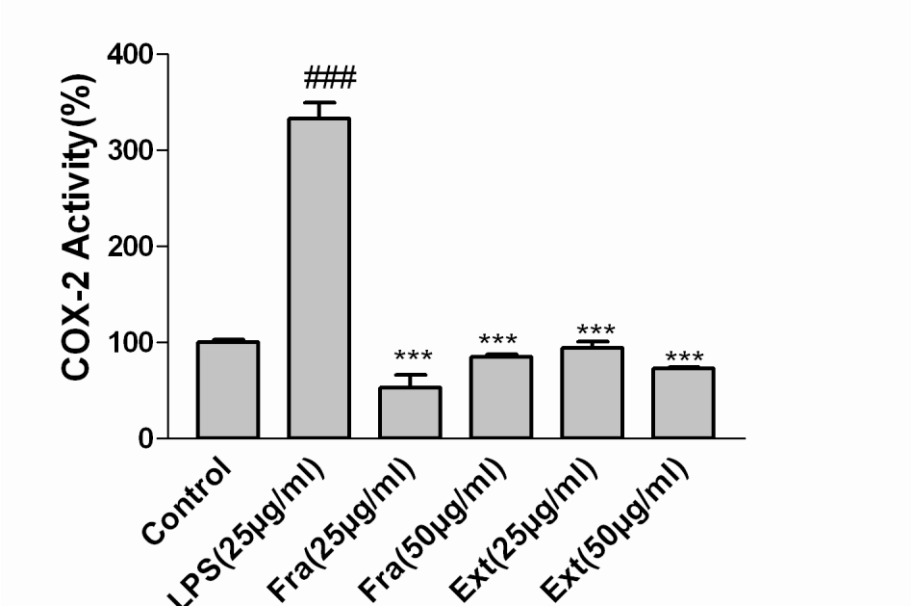

Figure 3-b

Fig. 3. Inhibition of the enzyme cyclooxygenase type 1 (a) and 2 (b) (Cox 1 and Cox 2) by the extract (25 , 50 µg/mL) and its polysaccharide-enriched fraction (25 , 50 µg/mL). Data showed as mean±SEM, (### *P*-value < 0.001 vs. , ## P<0.01 vs. Control, ***P<0.001 different from control group).

Determination of the Expression Levels of Inflammatory Genes

To investigate how the pretreatment of cells with the extract and its polysaccharide-enriched fraction decreases LPS-induced vascular inflammation, we examined the mRNA expression of the cell – adhesion molecules family (*ICAM1*, *VCAM1*) in HUVEC cells. Real time RT-PCR analysis clearly shows a significant increase in the expression level of *ICAM 1*(3.02 fold relative to control) and *VCAM* 1 (5-fold relative to control) after 24 h treatment with non-toxic concentration of LPS. As shown in the Figure 4, the extract was able to decrease significantly mRNA gene expression of *VCAM 1* and *ICAM 1* in HUVEC cells. When we examined the effect of pretreatment of cells with the selected fraction we found that it significantly alleviates the effect of LPS on *ICAM 1* and *VCAM 1* mRNA expression.

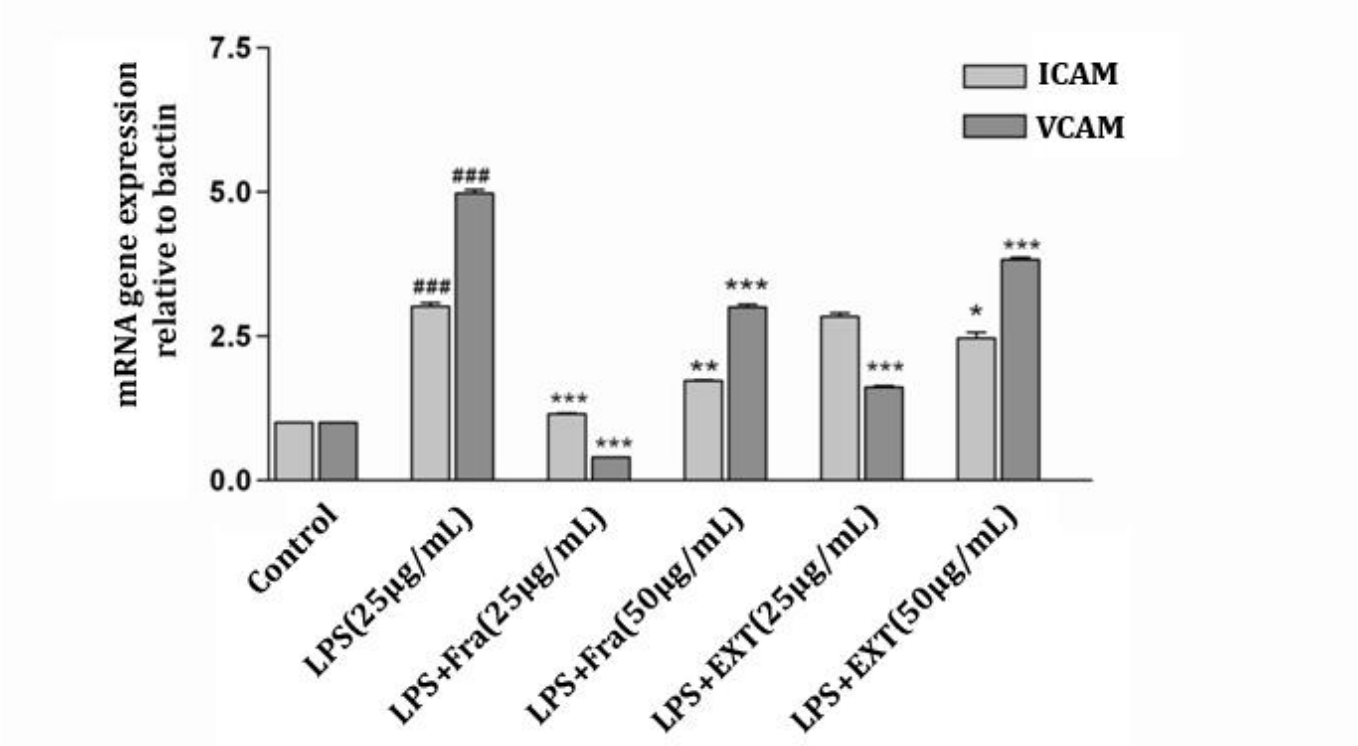

Fig. 4. The effect of aqueous extract and its polysaccharide-enriched fraction on ICAM and VCAM mRNA expression in HUVEC cells. Normalization relative to β-actin was performed. Levels of mRNA are expressed relative to control in the mean ± S.E.M values derived from three independent experiments.(###$p < 0.001$ vs. control, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. LPS treated cells).

Discussion

The current investigation demonstrated that treatment with *P. farcta* root extract can significantly diminish the inflammatory and oxidative markers of atherosclerosis in human endothelial cells induced by LPS. Atherosclerosis is a multifactorial inflammatory disease that is considered as one of the top causes of mortality all over the world. There are remarkable developments in exploring anti-atherosclerotic agents; however, a considerable number of

patients are not satisfyingly treated with the current medicinal approaches, and also numerous adverse effects are reported from the current pharmacotherapies. Thus, various researches have been conducted for the discovery of future therapeutic approaches. Natural remedies have long been used for the treatment of various pathologic conditions and their relevant complications [20]. Literature review revealed that natural drugs have beneficial effects in animal models of several cardiovascular diseases, including atherosclerosis, hypertension, endothelial dysfunction, and dyslipidemia [21, 22]. Moreover, there are various clinical trials on the efficacy of medicinal plants in patients with cardiovascular complications. *P. farcta* has long been used for the treatment of cardiovascular disorders in Iran. Preclinical studies demonstrated dose-dependent as well as endothelium-dependent relaxation action of this plant in thoracic aorta. Moreover, antihypertensive effects of hydro-alcoholic extract of *P. farcta* leaves has been reported in the animal experiments. Positive inotrope activity is another mechanism of the extract in cardiovascular diseases [12, 18]. Another species of the genus *Prosopis*, *P. julifora*, possesses modulatory effect on lipid profile of Albino rats and diminishes hyper-cholesterolemic conditions in hyper-cholesterolemic-fed diet [18]. Modulation of inflammatory processes via controlling the levels of pro-inflammatory cytokines has a pivotal role in prevention and treatment of atherosclerosis. The primary cascade of inflammatory reaction is mediated by infiltration of neutrophils and macrophages to the tissue. Inflammation can be considered as a double-edged sword, meaning it can demonstrate useful or harmful activity during the development of a disease[23]. Primary inflammation attracts the immune system factors to the damaged area that supports the improvement of lesions; while long-term presence of inflammatory parameters can result in disturbance in the functions of organs which requires therapeutic interventions to be controlled. Atherosclerosis is associated with an ongoing inflammatory reaction activated by lipid and oxLDL accumulation in the artery wall [24]. Our previous study showed that aqueous extract of *P. farcta* root has anti-inflammatory and anti-atherosclerosis properties in animal models [14]. In the present study, we demonstrated that aqueous extract *P. farcta* and its polysaccharide-enriched fraction reduced the activity of COX2 enzyme (Figure 3) of HUVEC cells, which is a human endothelial cell line derived from umbilical vein acute monocytic leukemia suggesting an inhibitory effect on one of the primary processes of inflammation involved in activating atherosclerosis. Endothelial cells can response to several stimuli such as TNF-α, IL-1β as well as LPS within inflammatory reaction and enhance the expression of cell adhesion agents such as *ICAM* and *VCAM* [25]. *P. farcta* aqueous extract and the polysaccharide-enriched fraction also revealed inhibitory effect on the mRNA expression levels of *VCAM-1* and *ICAM-1* in endothelial cells. Our results also showed that pretreatment with the extract and polysaccharide-enriched fraction can significantly cause the protective effect against inflammatory process of endothelial cells. Oxidative process has a crucial role in the progress of atherosclerosis, and establishment of oxidative-associated modification of LDL-C is suggested as the initial stage in atherogenesis. Various studies have suggested that inhibition of oxidative stress, and lipid oxidation play an essential role in the prevention and remission of atherosclerotic damage [24-27]. Results showed that the *P. farcta* extract and its polysaccharide-enriched fraction remarkably diminish free radicals and oxidants in atherosclerotic tissues (Figure 4). As it has been discussed in numerous investigations [27, 28], natural remedies possess therapeutic effects in

atherosclerosis through various mechanisms of action including modification of inflammation process, which has been mediated by reducing the levels of TNFα, INFγ and different types of interleukins. In addition, antioxidant functions of natural drugs help to diminish LDL-C oxidizability. It is suggested that oxidized LDL-C can stimulate toll-like receptor 4 (TLR-4) and result in further inflammation [29].

Conclusion

To conclude, *P. farcta* aqueous extract and the polysaccharide-enriched fraction remarkably protect endothelial cells against inflammatory and suppress protein expression of COX enzyme as well as mRNA expression of *ICAM-1* and *VCAM-1* induced by LPS. This finding may confirm traditional use of this medicinal plant in managing cardiovascular diseases particularly atherosclerosis. It could be suggested to conduct well-designed clinical studies in order to evaluate the preventive effect of *P. farcta* on atherosclerosis.

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Conflict of Interests

Authors certify that there is no actual or potential conflict of interest in relation to this article.

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