

ORIGINAL ARTICLE

Effect of *Tribulus Terrestris* L. on Expression of ICAM-1, VCAM-1, E-Selectin and Proteome Profile of Human Endothelial Cells *In-Vitro*

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ABSTRACT

Background: Atherosclerosis is a chronic inflammation that interferes with blood arteries functions due to the accumulation of low density lipids and cholesterol. **Objective:** To investigate the effect of aqueous extract and saponin fraction of *Tribulus terrestris* L. (TT) on the proteome and expression of intracellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and E-selectin in the human umbilical vein endothelial cells (HUVEC) and human bone marrow endothelial cell (HBMEC) lines. **Methods:** Two cell lines were cultured and induced with lipopolysaccharide (LPS). The primed cells were then treated with aqueous extract and saponin fraction of TT. The protein profile of the endothelial cells was assessed under normal and LPS-induced conditions using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and 2D gel electrophoresis (2-DE). The levels of VCAM-1, ICAM-1, and E-selectin were estimated by use of western blotting. **Results:** LPS-induced HUVECs and HBMECs were shown to significantly increase the expression of ICAM-1, VCAM-1, and E-selectin in comparison to control groups. Our findings revealed that TT extract resulted in significantly more reduced levels of proteome (80 spots) as well as all the three mentioned proteins compared with the effect of saponin fraction alone. **Conclusion:** TT extract and its saponin fraction exerted anti-inflammatory effects on HUVEC and HBMEC lines and reduced the expression of ICAM-1, VCAM-1, and E-selectin. However, the anti-inflammatory effect of aqueous extract was greater than that of saponin fraction. Therefore, TT could be considered as a potential candidate for the treatment or prevention of atherosclerosis.

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INTRODUCTION

Atherosclerosis is the major cause of morbidity and mortality in developing countries. Annually, more than 17 million people die from cardiovascular diseases, representing half of the deaths in these countries (1). Atherosclerosis is a complex inflammatory disorder with several important environmental and genetic risk factors (2). The first critical pathologic event in atherosclerosis is the endothelial dysfunction. In all stages of atherosclerosis, endothelial dysfunction is characterized with a disrupted balance between vasoconstrictors and vasodilators along with growth promoting and inhibiting factors (3-5). In sites of inflammation, leukocytes migrate across the activated endothelium expressing adhesion molecules. During this process, the endothelial cell adhesion molecules generate outside-in signals. Through such signal transduction, the affinity of the integrin receptor family increases, promoting the binding of endothelial cell adhesion molecules to proteins such as intracellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and E-selectin (6). Therefore, the overexpression of VCAM-1 and ICAM-1 on the surface of macrophages, endothelial, smooth muscle cells, and atheroma plaques is considered a potential biomarker in the development of atherosclerotic lesions (7). Although atherosclerosis therapies involving extensive synthetic pharmaceuticals are conducive to the treatment course of the patient, they could also cause severe side effects (8). On the other hand, over the past decades, the use of medicinal plants has been rapidly increasing due to the high efficacy of newly-driven herbal medicines in the treatment of cardiovascular diseases (8-10). The genus *Tribulus*, belonging to the family *Zygophyllaceae*, is commonly known as a traditional medicine utilized to treat various conditions such as impotence, venereal diseases, sexual dysfunction, eye problems, emission, edema, and abdominal distension. *Tribulus terrestris* L. (TT) contains valuable chemical compounds, including flavonoids, steroidal saponins, flavonol glycosides, cytoprotective lignanamides, anti-inflammatory N-trans-*p* caffeoyl tyramine, and alkaloids (11-16). To our knowledge, there exists no literature evaluating the changes in adhesion molecules under the influence of TT extract and saponin fractions. Therefore, this study aimed to investigate the effect of aqueous extract and saponin fraction of TT on the proteome and expression of ICAM-1, VCAM-1, and E-selectin in the human umbilical vein endothelial cell (HUVEC) and human bone marrow endothelial cell (HBMEC) lines under normal and LPS-induced conditions.

MATERIALS AND METHODS

Reagents. Dulbecco's Modified Eagle's Medium was supplied by Gibco (Belgium). Penicillin, streptomycin, fetal bovine serum (FBS), trypan blue, and amphotericin B were obtained from Gibco (New York, USA). Saponin standard and silica gel (SiO₂)-coated thin layer chromatography (TLC) plate were provided by Merck (Germany). Culture flasks and culture micro plates were purchased from NUNC (Roskilde, Denmark). PVDF, free essential fatty acid-bovine serum albumin (FEFA-BSA), and LPS (31.6_Bacto) were obtained from Difco (USA). Monoclonal antibodies against VCAM-1 (sc-13160), ICAM-1 (sc-107), and E-selectin (sc-137203) were achieved from Santa Cruz Biotechnology (Germany). IPGphor3 apparatus and 13 cm long IEF strips pH 4-7 were supplied by GE Healthcare (USA).

Aqueous Extract and Saponin Fraction of *Tribulus Terrestris*. *Tribulus terrestris* L. (TT) was collected from Western Iran (Kermanshah Province) and identified for genus and species at the Faculty of Agriculture, Razi University of Kermanshah, Iran. The aerial part of the plant was dried and ground. Then, nine volumes of distilled water were added to one weight of air-dried powder and stirred for 24 hours at RT. The extract was then filtered and centrifuged (5000 g) for 20 minutes at 22°C. The aqueous extract was evaporated at 45°C for 72 hours. The powder was dissolved in distilled water at a concentration of 10 percent (w/v). The saponin fraction was isolated using hydrophobic chromatography containing amberlite XAD-16 resin. The column was loaded with aqueous fraction, washed with distilled water to eliminate the unbound fractions and finally eluted with 50% ethanol (10 ml per gram of XAD-16 resin) for isolation of saponin fraction.

Thin Layer Chromatography (TLC). Ten microliters of the isolated fractions and saponin standard solution were loaded onto silica gel (SiO₂)-coated TLC plates and developed with the mobile phase of n-butanol: water: acetic acid (4:5:7) solution. In this experiment, standard saponin (Merck, Germany) was employed as the positive control. Following TLC, silica gel was slightly dried, sprayed with 15% (v/v) sulfuric acid, and heated for 15 minutes at 110°C. The spots were ultimately visualized in a UV light cabinet.

HUVEC and HBMEC Culture and Experimental Design. The HBMECs and HUVECs at the 5-8th passages were cultured in DMEM-F12 containing 10% fetal bovine albumin serum (FBS), 100 IU/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B in 75 cm² culture flasks at 37°C and 5% CO₂. For subcultures, the cells were detached through the use of 0.125% trypsin containing 0.5% ethylene diamine tetra acetic acid (EDTA). The cultured cells were divided into six groups to be further treated with TT aqueous extract or saponin fraction in order to study the changes in their proteome pattern. Group 1 (not treated) was assigned as negative control. Group 2 and 3 were treated only with aqueous extract or saponin fraction, respectively. Group 4 was considered positive control by being treated with LPS only. In Group 5, the samples were primed with LPS and treated with the aqueous extract while the LPS-primed and group 6, primed with LPS and treated with saponin fraction.

Priming with LPS and Treatment with Aqueous Extract or Saponin Fraction of TT. Here, LPS-stimulated endothelial cells were used as a model of inflammation *in vitro*. From a 1 mg/ml stock solution of LPS in DMEM/F12, the working solutions of 0.1, 1 and 10 µg/ml were prepared and stored at -4°C before use. In order to find the best concentration and incubation time for inflammation, cultured HUVECs and HBMECs were incubated at three different concentrations of LPS (0.1, 1 and 10 µg/ml) for 6, 12, and 18 h. In a separate set of experiments, the cultured HUVECs and HBMECs were also incubated with three different concentrations of TT extract (40_80 µg/ml) and different concentrations of saponins fraction (10, 20 and 30 µg/ml) for 18 and 24 h. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to find the optimal conditions for inducing inflammation in HUVECs and HBMECs. Furthermore, the parameters of cell viability (morphology, number, and trypan blue exclusion) were evaluated to select the optimal concentrations. Each experiment was performed in duplicates at least three times.

Cell Viability Using Trypan Blue Exclusion. Approximately 0.7×10^4 cells/ml were transferred to a 24-well tissue culture plate and aqueous extract of TT was added at different concentrations (20-200 µg/ml). After 48 h of incubation, the cells were

collected and re-suspended in 0.4% trypan blue. The percentage of viable cells was calculated in comparison to untreated endothelial cells using an inverted microscope. The same test was performed for saponin fraction at different concentrations (1-60 µg/ml) and LPS (0.1-30 µg/ml).

Western Blotting. Cultured cells were washed with PBS and lysed with lysis buffer (sample buffer containing 2-mercaptoethanol and cocktail). After that, the supernatant was harvested through centrifugation (14000 g for 15 minutes at 4°C), and the total protein was assayed according to the Bradford method. The extracted protein was treated with SDS-PAGE sample buffer and resolved in 12.5% polyacrylamide gel at 150 volts. The separated protein bands were transferred to poly vinylidene difluoride (PVDF) membrane using tank blotting. The PVDF membrane was blocked and incubated with anti-VCAM-1, anti-ICAM-1, and E-selectin antibodies (1:200) for an overnight at 4°C. After washing, the membrane was incubated with secondary antibody (HRP conjugate, 1:1000) and finally exposed to DAB substrate solution for detection of target proteins. The stained bands were analyzed by Image-J software. The protocols of SDS-PAGE and western blot have been described in our previous articles (17,18).

Two-Dimensional Gel Electrophoresis. For isoelectric focusing (IEF), approximately 270 µg of each protein solution (supernatant) was loaded in each rehydration channel (the rehydration buffer containing 8 M Urea, 2 M Thiourea, 4% CHAPS, 0.5% IPG buffer pH 4-7, and 65 mM DTT, DNAase, RNAase, PMSF, and a trace of bromophenol blue). Then, IPG strip (pH 3–10, 11 cm) was placed side down into the rehydration channel (15 h at 37°C) to absorb the solution. IEF was run with 500 V 1 h; 500 V 1:30 h; 5000 V 1:30 h; 5000 V 1:30 h; 9000 V 1:30 h; 9000 V 2:50 h. At the end of IEF, the strips were equilibrated in two steps (first, 25 min in 3% SDS, 30% glycerol, 6 M urea in 50 mM Tris-HCl buffer pH 8.8, and 50 mg/mL DTT and second, 25 min in the same buffer where 100 mg/mL iodoacetamide replaced DTT). The second dimension (reducing SDS-PAGE) was run in 10% polyacrylamide gel. The protein spots were stained by coomassie brilliant blue and stored in 7% acetic acid. The density of spots was determined by the Image Master 2D Platinum Software (Bio-Rad-USA) and normalized against the total gel density representing the total protein quantity in the cell group. In order to determine the quantitative changes in protein expression, the percentage volume of each stain, as a normalized value, was analyzed by ANOVA test.

Statistical Analysis. All tests were performed at least in triplicates. Results were expressed as mean ± SD and analyzed by GraphPad Prism software version 6.01, using either Student's t-test for comparing two groups or One-way ANOVA (analysis of variance) for multiple comparisons.

RESULTS

Saponin isolation and its effect on cell viability.

The yields of aqueous extract and saponin fraction isolated from TT dry materials were 14.6 and 3.7%, respectively. The presence of saponin in the fraction was confirmed by TLC method. Spots visualized after 15% sulfuric acid spray in UV cabinet were evaluated in comparison to the standard saponin. The color of standard saponin was similar to the spots of isolated saponin. These results indicated the purity and identity of the isolated saponin fraction.

Trypan blue exclusion assay showed that aqueous extract and saponin fraction of TT have no cytotoxic effects up to 80 and 20 $\mu\text{g/ml}$, respectively on HUVECs and HBMECs. In addition, based on CC50 (the concentration at which 50% of cells are dead) of LPS, 1 and 10 $\mu\text{g/ml}$ of this inflammatory compound were used to prime HUVEC and HBMEC, respectively (Figure 1).

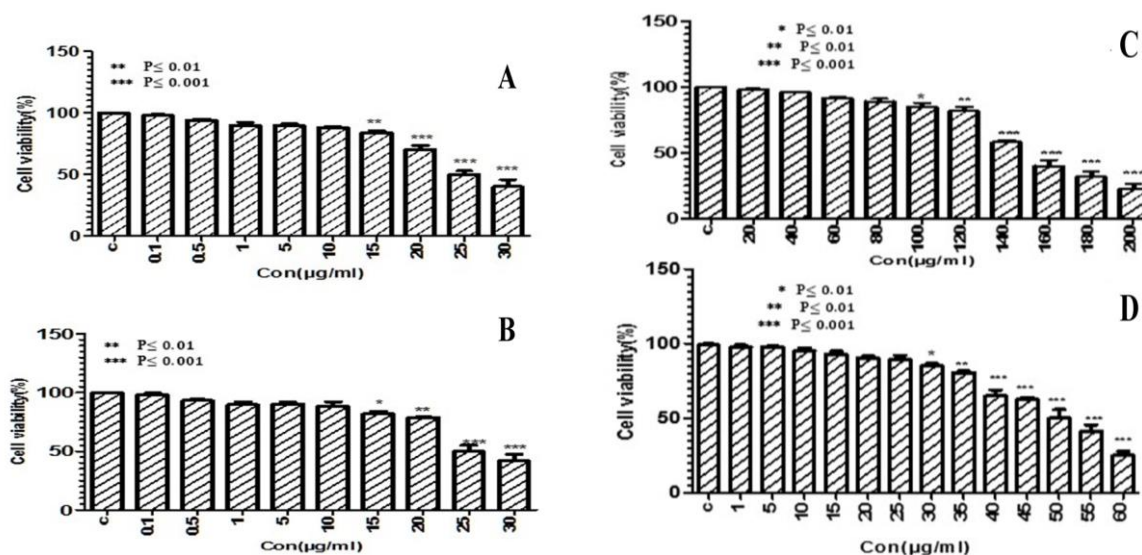


Figure 1. The results of the cytotoxic effect of different concentration of LPS (A,B), *Tribulus terrestris* L. aqueous extract (C) and saponin fraction (D) on the viability of HUVEC and HBMEC through the trypan blue dye exclusion assay.

Inhibition of LPS-induced proteins expression by TT aqueous extract and saponin fraction using SDS-PAGE and 2-DE.

The effect of LPS on the total protein of the cell lines was evaluated by SDS-PAGE. The results showed that the expression of total proteins, particularly in the range of 90 to 110 kDa, increased in HUVECs and HBMECs after 6 hours of treatment with LPS (10 and 1 $\mu\text{g/ml}$, respectively). Aqueous extract at 60 $\mu\text{g/ml}$ and saponin fraction at 20 $\mu\text{g/ml}$ significantly inhibited the effect of LPS on the protein expression at 18, 24, and 36 h. Interestingly, the reducing effect of the extract was higher than that of saponin fraction (Figure 2). Two-dimensional electrophoresis of total proteins from HUVECs and HBMECs treated with LPS presented 322 spots, approximately 50% more than the control cells (154 spots) or those treated with aqueous extract and saponin fraction of TT (130 and 150 spots, respectively) (Figure 3). In the other words, a 40% decrease was observed in total protein of both LPS-primed HUVEC and HBMEC lines after treatment with the aqueous extract and saponin fraction of TT. Among the 322 protein spots, 80 spots were expressed differentially (at least 1.5 folds, $p \leq 0.01$). Moreover, considering the optical density, these spots were also notably different between the HUVECs and HBMECs treated with aqueous extract and those treated with saponin fraction of TT (Figure 3). This difference is believed to arise from the distinct natures of the two lines of HUVEC and HBMEC.

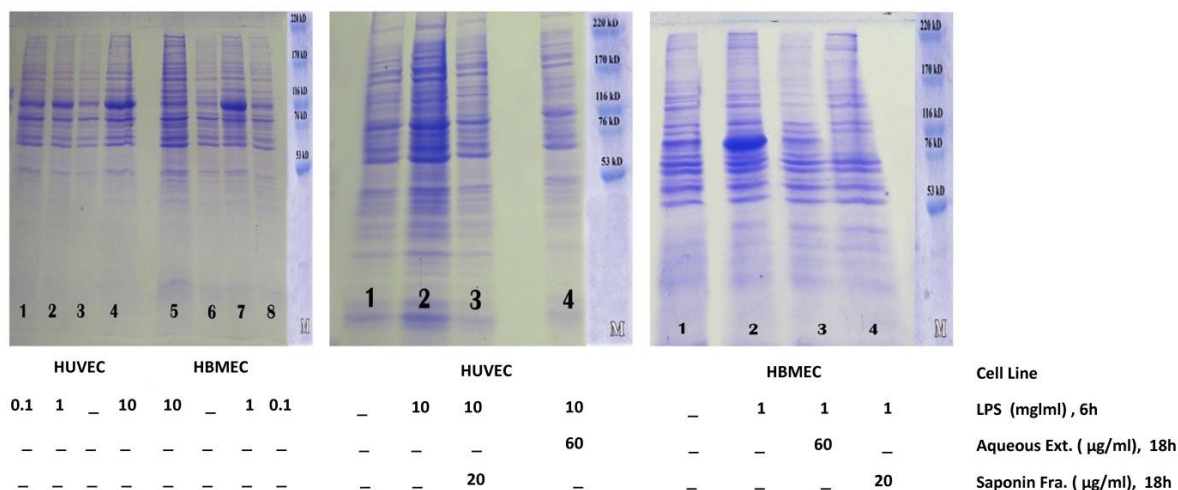


Figure 2. The effect of various amounts of LPS (6 hours), *Tribulus terrestris* L. aqueous extract and its saponin fraction (18 hours) on the protein pattern of human endothelial cells by SDS-PAGE with Coomassie blue staining. Markers: myosin (210 kDa), alpha 2-macroglobulin (170 kDa), β -galactosidase (116 kDa), transferrin (76 kDa), glutamate dehydrogenase (53 kDa).

Twenty-five and 33 up-regulated spots were the cell line specific spots (for HUVEC and HBMEC, respectively). Among those 80 protein spots mentioned earlier, 24 and 13 were significantly downregulated in HUVEC and HBMEC, respectively. The other spots only had different expressions in HBMEC and HUVEC. For instance, VCAM-1 protein had various isoforms of 75-150 KDa with a PI of about 5. Based on statistical calculations using Image-J software, spot 47 was selected in both HUVEC and HBMEC with a 2.39 fold down-regulation.

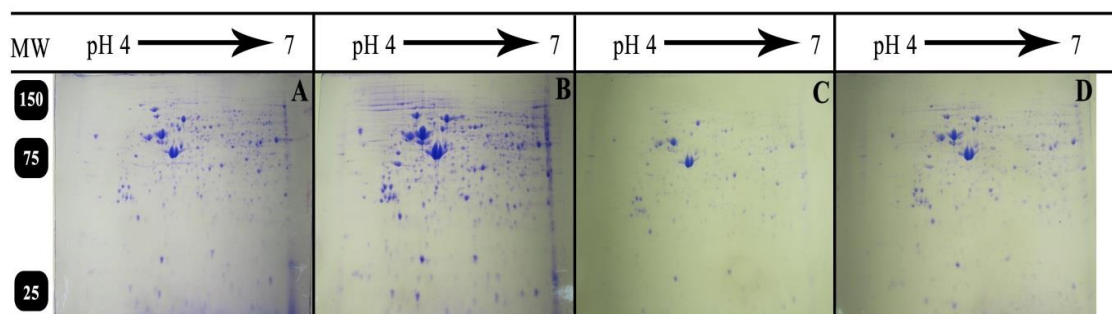


Figure 3. Two-dimensional protein profiles for HUVECs. A) patterns of protein expression in unstimulated cell lines, B) active HUVECs treated with LPS, C) active HUVECs treated with TT extract, D) active HUVECs treated with saponin fraction. Coomassie blue staining was used and proteins were separated on the basis of pI (x axis) and molecular mass (Y axis).

Effect of aqueous extract and saponin fraction on the expression of adhesion molecules.

The SDS-PAGE and 2D electrophoresis obviously revealed that the level of the adhesion molecules significantly increased in the LPS-treated HUVECs compared to the controls. In the absence of LPS, on the other hand, endothelial cells expressed VCAM-1, ICAM-1, and E-selectin at low basal levels. As shown in Figures 4 and 5, following treatment with LPS at 10 µg/ml for 6 h, HBMECs showed a noticeable increase in the adhesion molecule levels compared to the negative control.

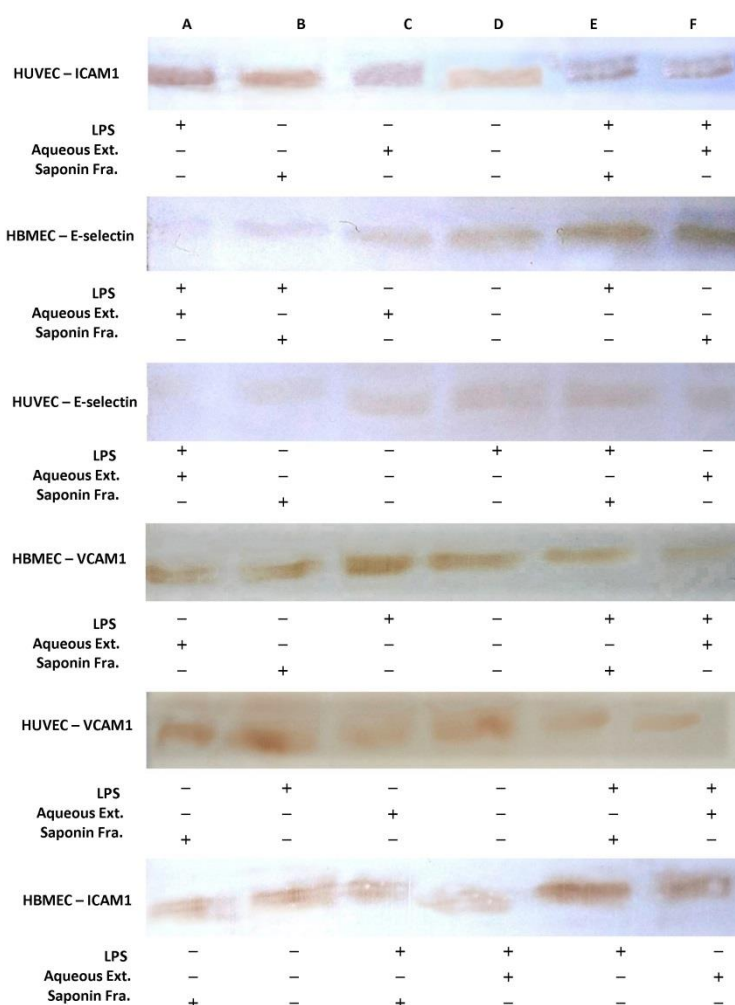


Figure 4. The effects of TT aqueous extract and its saponin fraction on LPS-treated HUVECs and HBMECs. Protein expression of ICAM-1, VCAM-1 and E-selectin visualized using western blotting.

The LPS-induced endothelial cells seemed to have the highest expression of E-selectin in 4-6 h and thereafter decreased to the basal level in the next 24 h. On the other hand, the expression of ICAM-1 and VCAM-1 reached to the highest level at 6-12 h after

stimulation. The effect of aqueous extract and saponin fraction in the absence and presence of LPS on the expression of VCAM-1, ICAM-1 and E-selectin by HBMECs and HUVECs are shown in figures 4 and 5. After priming the cell lines with LPS, the levels ICAM-1, VCAM-1 and E-selectin diminished to basal levels. Similar to the aqueous extract, saponin fraction decreased the level of ICAM-1, VCAM-1, and E-selectin; however, compared with saponin fraction, the aqueous extract caused more reduction in the expression of adhesion molecules.

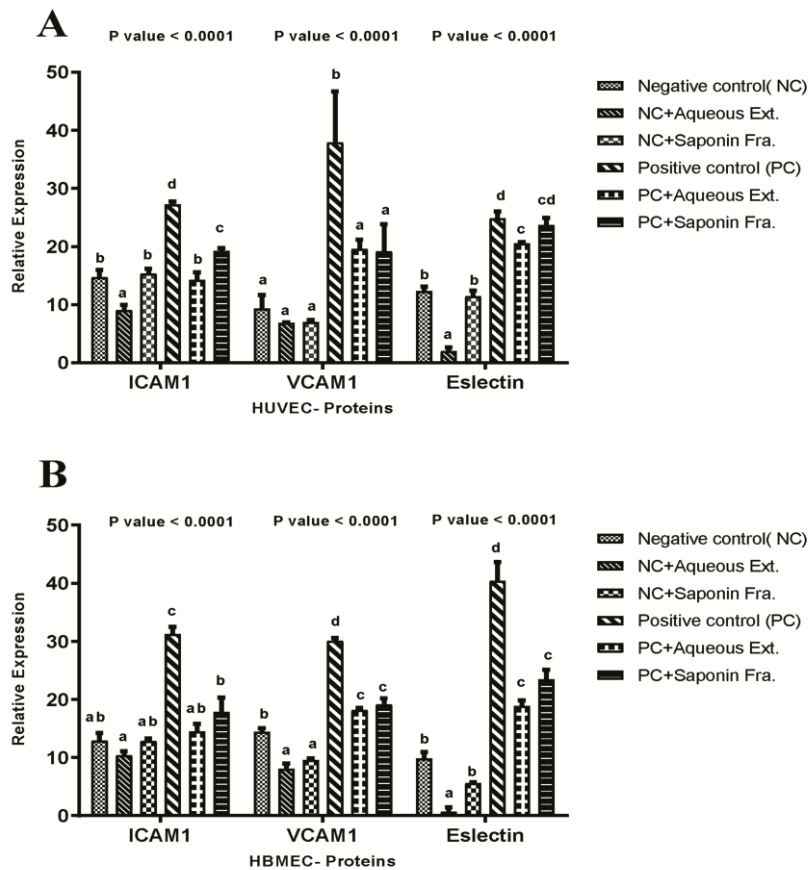


Figure 5. Aqueous extract and saponin fraction of TT downregulates ICAM-1, VCAM-1, and E-selectin protein expression in HUVECs (A) and HBMECs (B). ICAM-1, VCAM-1, and E-selectin proteins were measured in all lysates using western blotting. Untreated and LPS-treated cells were assigned as negative and positive controls, respectively.

DISCUSSION

Several studies have investigated the mechanisms and signaling pathways governing LPS-induced changes in the expression of endothelial adhesion molecules. These studies have led to the development of highly effective anti-inflammatory therapies (19-22). LPS-induced endothelial cells upregulate VCAM-1 and ICAM-1 on both mRNA and protein levels. ICAM-1, VCAM-1, and E-selectin, known as adhesion molecules, play key roles in the progression of atherosclerosis. It has been well established that the

upregulation of endothelial cells in these adhesion molecules mediates increased leukocyte migration across the endothelium, thereby leading to inflammation (17,18,21,22). To our knowledge, the current study is the first to evaluate the anti-inflammatory impact of the aqueous extract and saponin fraction from TT on the expression of ICAM-1, VCAM-1, and E-selectin in the HUVEC and HBMEC lines *in vitro*. This setting would give us the opportunity by partially mimicking the macro- and microvascular function under normal and LPS-induced conditions. Our findings showed that aqueous extract and saponin fraction had a reducing effect on the expression of the studied adhesion molecules. Notably, the anti-inflammatory effect of aqueous extract was greater than saponin fraction. It is noteworthy to mention that the changes in the expression levels of identified proteins in cell cultures might be due to various differentiation state of the cells and differently expressed proteins in the micro vascular and macrovascular cell lines. This hypothesis requires more thorough research. Since ancient times, the consumption of herbal medicines has played a leading role in preventing vascular disorders and alleviating endothelial dysfunctions (23). Several studies have reported that TT-derived saponins exert significant effects on controlling lipid metabolism, hyperglycemia in smooth muscle cell, various cancer cells of the breast, liver, malignant melanoma and ovaries, as well as treatment of atherosclerosis (24,25). The idea of the present study is in line with the report of Tuncer *et al.* (2009) who claimed that an aqueous extract of TT had a pleotropic effect on restoring the endothelial barrier and lowering serum cholesterol levels in New Zealand rabbits following a cholesterol-rich diet (10,12). According to other studies (11-16), the anti-inflammatory effect of TT aqueous extract might be due to different non-specific and/or specific compounds like flavonoids, steroidal saponins, flavonol glycosides, cytoprotective lignanamides, anti-inflammatory N-trans- ρ caffeoyl tyramine, and alkaloids found in this plant. According to the literature and the present results, TT could be more thoroughly studied, and it is recommended for the treatment of atherosclerosis. In another study in 2009, Chang *et al.* observed that a dietary intake of tribu saponin from TT (STT) was able to downregulate the gene expression of ICAM-1 and VCAM-1 in artery vessels of arteriosclerotic rats. Furthermore, STT was reported to reduce the levels of serum lipid profile in these rats (16,26). Choi *et al.* showed that *Cynanchum Wilfordii*, similar to *Tribulus terrestris* L., was able to significantly reduce the levels of endothelial adhesion molecules, including ICAM-1, VCAM-1, and E-Selectin as it contains various compounds such as flavonoids, steroids, saponins, glycosides, and alkaloids (27). *In vivo* studies indicated that STT decreases the gene expression of VCAM-1 and ICAM-1 in the coronary arteries of atherosclerotic rats (28) which is in accordance with our findings. Moreover, the beneficial effect of saponin on controlling serum lipid levels and the atherosclerosis plaque formation in rats was also reported by Marrelli *et al.* (29) and Zhao *et al.* (30). Based on the present findings, a steep increase was detected in the levels of VCAM-1 and ICAM-1 in comparison to E-selectin in the stimulated cells, which was further reduced following treatment with TT extract and its saponin fraction. Induction of ICAM-1, VCAM-1, and E-selectin by LPS was associated with cytokine secretion from inflammatory cells, which in turn might be responsible for their enhanced expression. TNF- α -activated HBMECs showed increased expressions of adhesion molecules ICAM-1, VCAM-1, PECAM, and ELAM-1. To give more details, signaling of TNF receptor 1 (TNFR1) induces the expression of two key transcription factors of nuclear factor B (NF- κ B) and activating protein-1 (AP-1) that in turn target genes including ICAM-1, VCAM-1, and E-selectin (31). According to the

results reported by Sanadgol *et al.*, ICAM-1 and VCAM-1 were upregulated in damaged endothelium. Since VCAM-1 expression is observed only in activated endothelial cells, it might be viewed as a potential marker for atherosclerosis. It is argued that the expression of sVCAM-1 occurs in the advanced phase of inflammation, while sICAM-1 is a common proinflammatory marker in healthy population (17,18). In conclusion, it is well perceived that *Tribulus terrestris* L. could alleviate inflammation in endothelial cells. We also suggest studying the anti-inflammatory activity of TT *in vivo* in order to clarify its potential in clinical prophylaxis for postponing the progression of atherosclerosis. It is noteworthy to mention that limitations to our study included the high cost of identifying the protein spots using mass spectrometry technique.

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