IJMCM
Winter 2020, Vol 9, No 1
DOI: 10.22088/IJMCM.BUMS.9.1.73

Original Article

Saponins from *Tribulus terrestris* L. Extract Down-regulate the Expression of ICAM-1, VCAM-1 and E-selectin in Human Endothelial Cell Lines

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Atherosclerosis is an inflammatory disease in which intracellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and E-selectin (SELE) are consistently expressed in the vascular endothelium. Several evidence support the crucial role of adhesion molecules in the development of atherosclerosis and plaque instability. Due to the anti-inflammatory activity of *Tribulus terrestris* (TT), the present study investigated the effect of aqueous extract and saponin fraction of TT on the expression of *ICAM-1*, *VCAM-1*, and *SELE* genes in endothelial cells during normal and lipopolysaccharide (LPS) induced conditions. Human umbilical vein endothelial cells (HUVEC) and human bone marrow endothelial cells (HBMEC) were cultured, stimulated by LPS, and treated with aqueous extract and saponin fraction of TT. Finally, the expression of *ICAM-1*, *VCAM-1*, and *SELE* genes were measured using quantitative real-time polymerase chain reaction. LPS-induced HUVECs and HBMECs significantly increased the expression of *ICAM-1*, *VCAM-1*, and *SELE* in comparison with control groups ($P<0.001$). Treatment of LPS-induced HUVECs and HBMECs by aqueous extract and saponin fraction of TT decreased the expression of all three mentioned genes significantly ($P<0.001$) in comparison with LPS-induced cells. Taken together, our data suggest that TT has an anti-inflammatory effect. *In vivo* study about anti-inflammatory effect of this herb may provide new insights into the development of a herbal drug for the prevention/therapy of atherosclerosis.

Key words: Atherosclerosis, *Tribulus terrestris* L., gene expression, adhesion molecules, human endothelial cells, saponin

Atherosclerosis is a chronic inflammatory disorder which initiaties by inflammatory cells in the blood stream and their migration through epithelial cells. This phenomenon, is usually facilitated by the adhesion molecules expressed on the surface of epithelial cells and also circulating leukocytes in response to an inflammatory stimulant (1, 2). Adhesion of monocytes to the endothelial cells is known as a critical step in the progression of atherosclerosis.

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[DOI: 10.22088/IJMCM.BUMS.9.1.73]

Intracellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and E-selectin (SELE), known as adhesion molecules, have key roles in this process. In addition, the role of inflammation in the initiation and progression of atherosclerosis is being increasingly identified (3). Moreover, adhesion molecules are considered to be one of the most noted biomarkers of atherosclerosis. These molecules have been detected in the early plaques of atherosclerosis and their increased expression level could be associated with the development of the disease (4, 5).

In the literature, various herbal and non-herbal medicines have been tested and used to prevent or decrease the risk of atherosclerosis among which there are herbals such as *Rhizoma polygonum*, *Panax notoginseng*, *Buddleja officinalis*, and *Salvia miltiorrhiza*. These herbs prevent atherosclerosis through their endothelial protective activities (6-9). Among the non-herbal medicines studied for the same purpose are some synthetic antioxidants such as probucol and BO-653n. These compounds function through augmentation of endothelial, repair, and also preventing the formation of fatty streaks (10-13).

The genus *Tribulus* belongs to the Zygophyllaceous family, and so far about 20 species of this genus have been identified in the world. *Tribulus terrestris* L. (TT) has long been used as a medicine for the treatment of various diseases in many countries, including China and India (14). Different parts of this plant contain various chemical compounds of clinical significance. Among these compounds are flavonoids, glycosides, steroidal saponins, and alkaloids. Studies have shown that the herbal effects of this plant are particularly due to the presence of saponin containing steroidal compounds. However, the saponins' mechanism of functioning is still not well understood (15, 16).

Currently, there are several drugs, such as steroids and non-steroidal anti-inflammatory drugs, to control and inhibit inflammation. However, most of them have been associated with side effects (17). On the other hand, herbal medicines are expanding in the clinical field. Therefore, modern medicine must prove its effectiveness through scientific methods before their practical use (18).

Inflammation plays a major role in the development and promotion of atherosclerosis (19). On the other hand, ICAM-1, VCAM-1 and SELE have been referred to as early molecular markers for atherosclerosis and predictors of coronary heart disease (20, 21). Therefore, the aim of the present study was to investigate the effect of aqueous extract and saponin fraction of TT on the expression of these markers at mRNA level in the human umbilical vein endothelial cells (HUVEC) and human bone marrow endothelial cells (HBMEC) *in vitro* during normal and lipopolysaccharide (LPS)-induced conditions.

Materials and methods

Preparation of aqueous extract and saponin fraction of *Tibullus terrestris*

After collection of TT from the western part of Iran (Kermanshah Province) and taxonomic confirmation at the Faculty of Agriculture, Razi University of Kermanshah, Iran, it was dried and cured. The obtained powder was mixed with water at a ratio of 1 to 9 (v/w) and stirred for 24 h. Subsequent steps included filtration of the extract, centrifugation (5000×g) for 20 min at 22°C, harvesting of supernatant and incubation at 45 °C for 72 h. In this study, amberlite XAD-16 resin (Merck, Germany) was used to isolate saponin fraction by hydrophobic chromatography method. To this end, dry extracts were dissolved in distilled water, and transferred to the column. Then, the column was washed with distilled water to remove the unbounded molecules. Finally, 50% ethanol was used to elute the saponin fraction.

To test the fractions, ten microliters of each of the obtained fraction and saponin standard (Merck,

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Germany) were loaded onto a suitable size of silica gel (SiO2)-coated TLC plate (Merck, Germany). The next steps included placing the plate in n-butanol: water: acetic acid (4:5:7) solution, solvent migration to 6 cm, plate drying, spraying by a fresh solution of ethanol: sulfuric acid (90:15), and heating at 110 °C. Then, the profile of separated compounds on the TLC plate was observed by a UV cabinet.

Cell culture and experimental procedure

DMEM medium (Gibco, Belgium) containing 10% FBS (Gibco, New York, USA) and other supplements (22) was used to culture the 25-26th passages of HUVEC and HBMEC cell lines under regular conditions (37 °C and 5% CO_2). Trypan blue exclusion assay was used to measure the cell viability. Approximately 7000 cells/well were transferred in a 24-well plate and exposed to different concentrations of LPS (31.6-25 Bacto) (Difco, Kansas, USA) (0.1-30 µg/ml), TT aqueous extract (20-200 µg/ml) or saponin fraction (1-60 µg/ml). After 48 h of incubation (37°C and 5% CO_2), the cells were collected and resuspended in trypan blue (Gibco, New York, USA) (0.4% in PBS). By the use of an inverted microscope, the number of viable and dead cells in each well was counted and the percent of viability was estimated.

Also, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis was used at different LPS concentrations (0.1, 1 and 10 µg/ml), and different incubation times (6, 12 and 18 h) to find optimal situations for induction of inflammation in HUVECs and HBMECs. After washing the cells with PBS and disruption by lysis buffer, the cell lysate was collected and centrifuged at 14000×g for 15 min at 4 °C to remove the cellular debris. Total protein concentration was determined by Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA). Then, equal amounts of total protein (20 µg) were boiled in SDS-PAGE sample buffer, loaded per lane and resolved by a 12.5% gel. The reagents, protocols, and conditions for SDS-PAGE analysis have been described previously (22).

The results of SDS- PAGE analysis showed that the LPS concentrations of 10 and 1 µg/ml for 6 h incubation time are the optimal ones to prime HUVEC and HBMEC cell lines, respectively. In a separate experiment, LPS- induced HUVECs and HBMECs (at the optimal concentrations mentioned above) were treated with different concentrations of TT aqueous extract (40-80 µg/ml) and TT saponin fraction (10-30 µg/ml) at 18, 24, 36 and 48 h incubation times, and their effects on protein pattern of the cells were studied by SDS-PAGE analysis.

To investigate the effects of TT aqueous extract and saponin fraction on the expression of *ICAM-1*, *VCAM-1*, and *SELE* genes, the cultured cell lines were divided into six groups: group 1 without any treatment (as negative control), group 2 treated with aqueous extract, group 3 treated with saponin fraction, group 4 only induced by LPS (as positive control), group 5 induced by LPS and treated with aqueous extract, and group 6 induced by LPS and treated with saponin fraction.

Quantitative real time PCR

RNX-Plus (SinaClon, Iran) and EUREX (EUREX, Poland) kits were used to extract total RNA and cDNA synthesis, respectively, according to the manufacturer's instructions. The concentrations and quality of RNA preparations were determined using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA) and gel electrophoresis. After reverse transcription of standardized amounts of RNA to cDNA, *ICAM-1*, *VCAM-1*, and *SELE* mRNA expression levels were assessed in duplicate for each sample by SYBR Green real-time PCR. The relative amount of gene expression, was normalized to the expression of internal control 18S rRNA (reference gene). Primer sequences used in this study are shown in Table 1. Quantitative measurements for real-time PCR were determined

[DOI: 10.22088/IJMCM.BUMS.9.1.73]

using $2^{-\Delta\Delta CT}$ method.

Statistical analysis

Results were expressed as mean ± SD, and were analyzed using either Student's t-test for comparison between two groups or by ANOVA (analysis of variance), for multiple comparisons.

Results

Preparation of TT extract and saponin fraction

The yield of aqueous extract and isolated saponin fraction of TT were 14.6 and 3.7% (w/w), respectively, relative to the initial weight of the dried material. The result of the TLC analysis showed the presence of saponin in our fraction in comparison with pure saponins as the positive control (Figure 1).

Cytotoxicity assay

According to the trypan blue exclusion assay, CC_{50} (the concentration at which 50% of the cells are dead) of TT aqueous extract and saponin fraction on HUVECs and HBMECs were estimated to be 160 and 55 µg/ml, respectively. Moreover, the obtained CC_{50} of LPS for both cell lines was estimated to be 25 µg/ml (Figure 2).

Table 1. Primers used in this study for real-time PCR analysis.

Genes	Primers sequence	
ICAM-1	Forward	5'- TGTGACCAGCCCAAGTTGTT-3'
	Reverse	5'- AGTCCAGTACACGGTGAGG-3'
VCAM-1	Forward	5'- AAACAAAGGCAGAGTACGCA-3'
	Reverse	5'- CCAAGACGGTTGTATCTCTGG-3'
E-selectin	Forward	5'- AGCTTCCCATGGAACACAAC-3'
	Reverse	5'- CTGGGCTCCCATTAGTTCAA-3'
18S rRNA	Forward	5'- GCTTAATTTGACTCAACACGGGA-3'
	Reverse	5'- AGCTATCAATCTGTCAATCCTGTC-3'

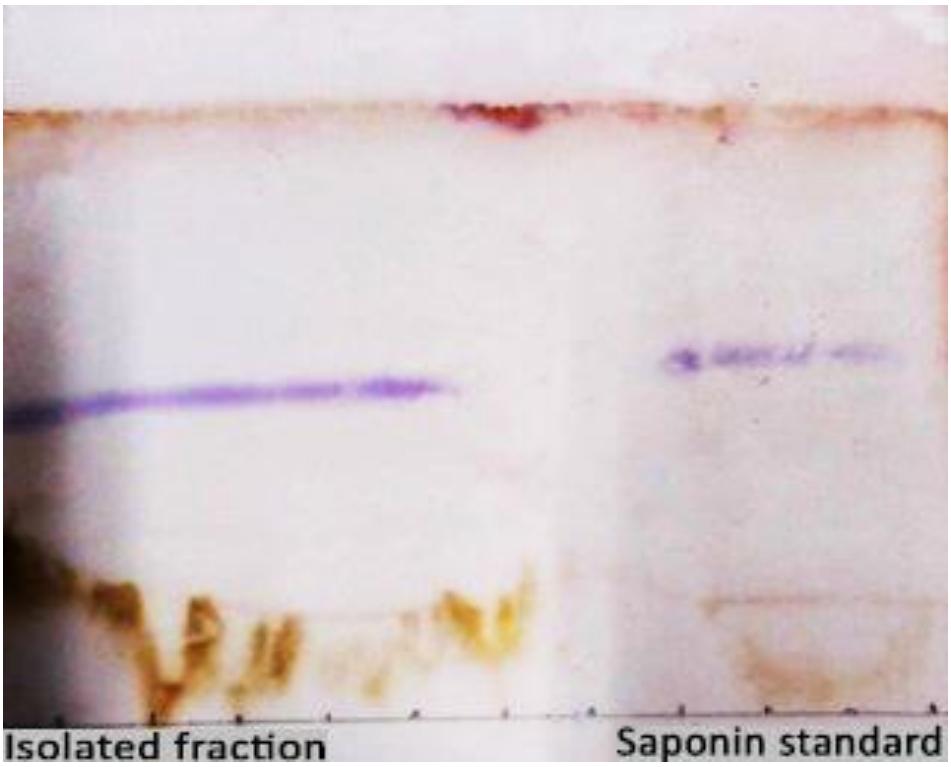

Fig. 1. Thin-layer chromatography (TLC) pattern of *Tribulus terrestris* L. saponin fraction. Isolated saponin in this study (left) compared to standard saponin (right).

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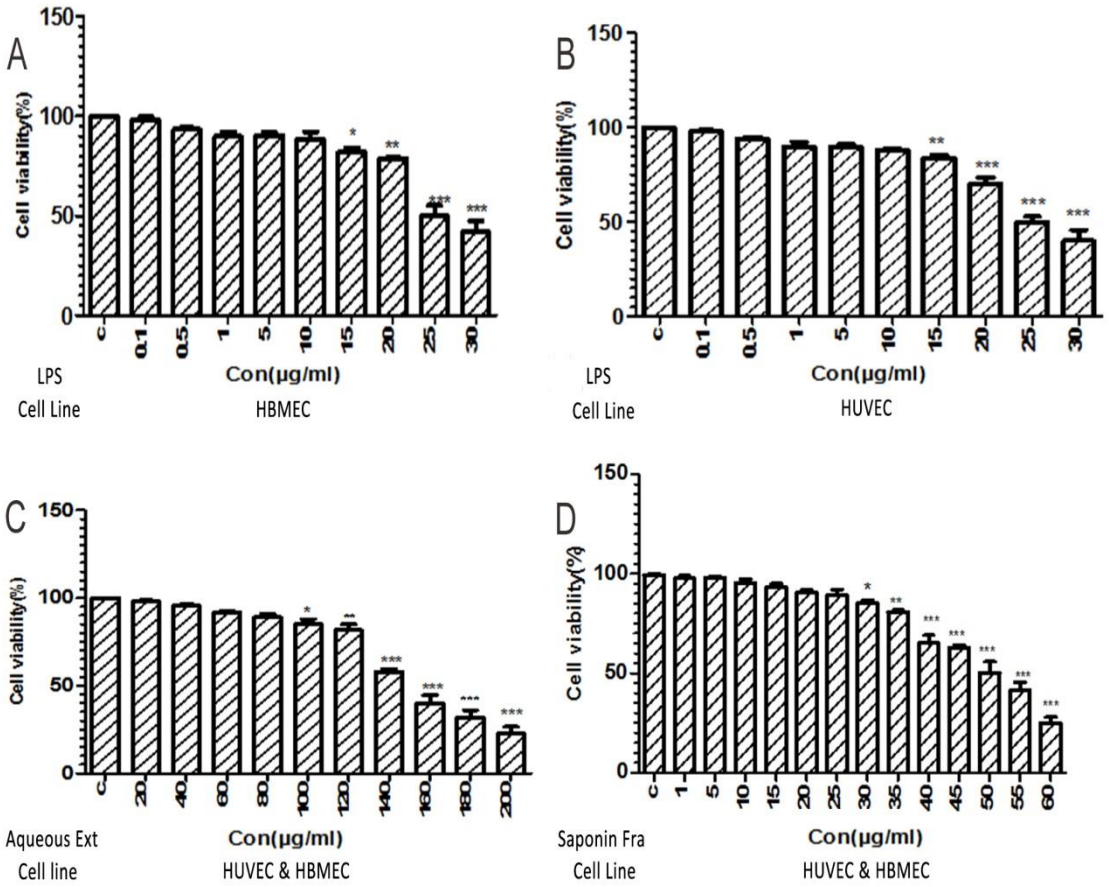

Fig. 2. Cytotoxicity of LPS, *Tribulus terrestris* L. extract, and saponin fraction on HUVEC and HBMEC cell lines. A, B: Various concentrations of LPS; C: *Tribulus terrestris* L. aqueous extract; D: Saponin fraction. LPS:lipopolysaccharide. *P<0.05; **P<0.01; ***P<0.001 compared to control group (without any treatment).

SDS-PAGE analysis

Protein pattern of HUVECs and HBMECs activated by LPS showed that the best concentrations of LPS for stimulation of the cell lines were 10 and 1 µg/ml at a 6-h incubation time, respectively (Figure 3A and B). Moreover, the selected concentrations of TT aqueous extract and saponin fraction for the treatment of LPS-induced HUVECs and HBMECs were 60 and 20 µg/ml at an 18-h incubation time, respectively (Figure 3 C and D).

Inhibition of LPS-induced expression of *ICAM-1*, *VCAM-1*, and *SELE* by TT aqueous extract and saponin fraction

Real time PCR was used to investigate the changes in the expression of *ICAM-1*, *VCAM-1*, and *SELE* genes under the influence of aqueous extract and saponin fraction of TT. The expression of each genes was similar in both cell lines due to induction of LPS. As shown in Figure 4, the LPS significantly increased the expression of *ICAM-1* (in a fold change of ~40), *VCAM-1* (in a fold change of ~4), and *SELE* (in a fold change of ~25) in comparison with control groups (P<0.001). Our data showed that treatment of LPS-induced HUVEC and HBMEC cell lines by aqueous extract and saponin fraction of TT, significantly decreased the expression of all three mentioned genes (P<0.001) in comparison with LPS-induced cells. Regarding *ICAM-1* in HUVEC, the reduction rate of expression under treatment by aqueous extract and saponin fraction was 28.5 and 22.5 folds,

[DOI: 10.22088/IJMCM.BUMS.9.1.73]

respectively. *ICAM-1* expression in HBMEC under treatment by aqueous extract and saponin fraction was reduced 13.36 and 2.78 folds, respectively. For *VCAM-1* in HUVEC, the reduction rate of expression under treatment by aqueous extract and saponin fraction was 22.7 and 5.12 folds, respectively, while in HBMEC treated with aqueous extract and saponin fraction, its expression was reduced by 7.5 and 3.75 folds, respectively. The expression of *SELE* under treatment by aqueous extract and saponin fraction was reduced 1.66 and 1.28 folds, respectively in HUVEC, and 50 and 9.07 folds, respectively in HBMEC (Figure 4).

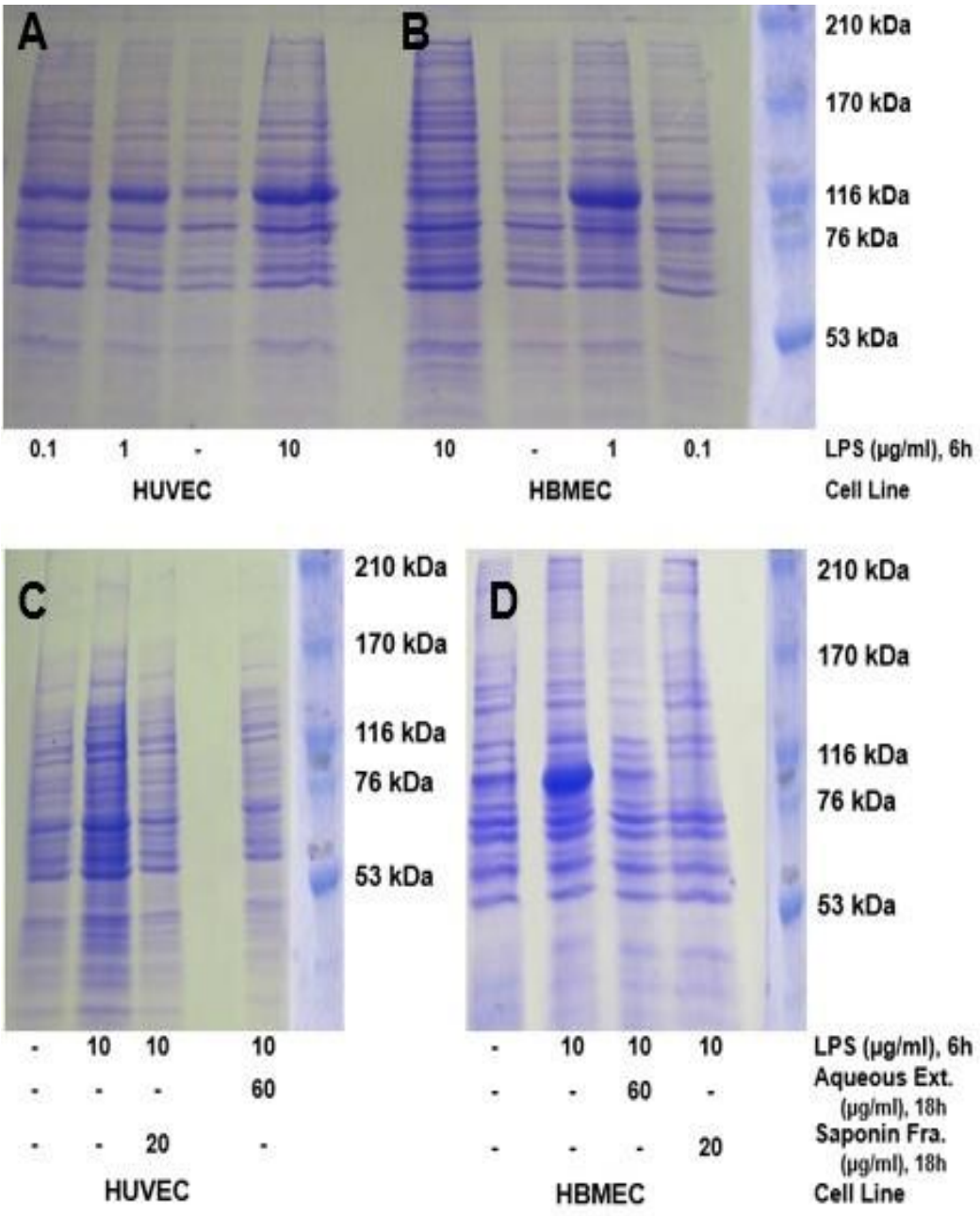

Fig. 3. SDS-PAGE analysis of total protein extracted from HUVEC and HBMEC cell lines induced by LPS, *Tribulus terrestris* L. extract, and saponin fraction. A, B: LPS; C: *Tribulus terrestris* L. aqueous extract; D: Saponin fraction. Markers: myosin (210 kDa), alpha 2-macroglobulin (170 kDa), β-galactosidase (116 kDa), transferrin (76 kDa), glutamate dehydrogenase (53 kDa). LPS: lipopolysaccharide.

[DOI: 10.22088/IJMCM.BUMS.9.1.73]

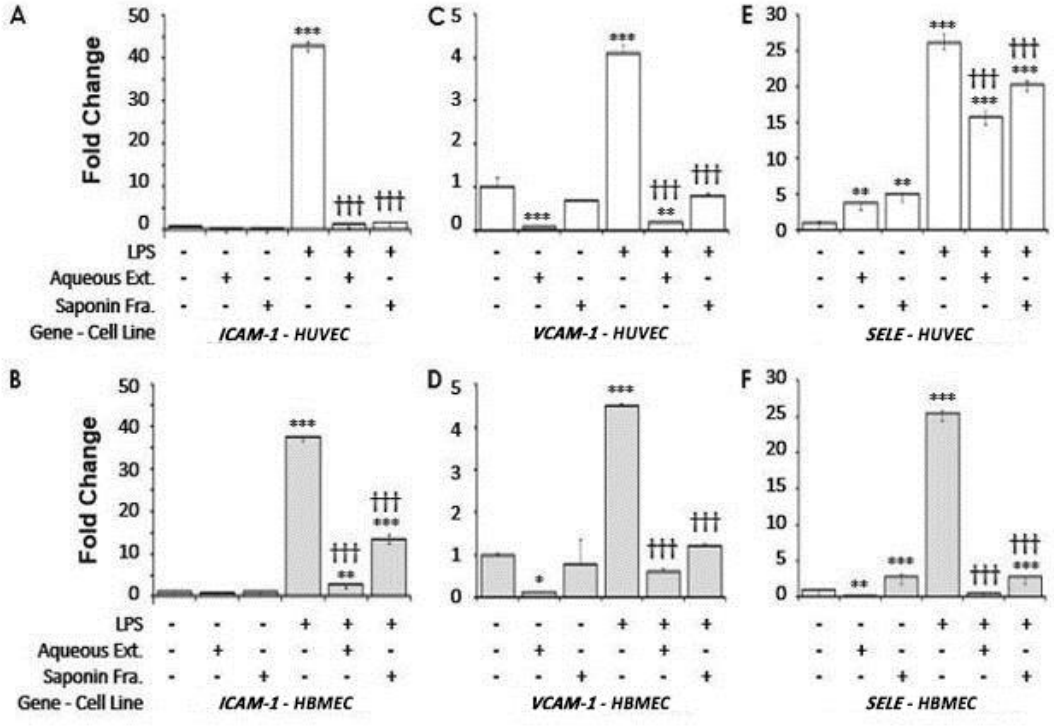

Fig. 4. Effect of *Tribulus terrestris* L. aqueous extract and its saponin fraction on LPS-induced HUVECs and HBMECs adhesion molecules expression. A, B: *ICAM-1*; C, D:*VCAM-1*; E, F:*SELE*. Each column displays the mean ± SD from two assays. *P<0.05; **P<0.01; ***P<0.001 compared to control group. †††P<0.001 compared to LPS-induced cells. LPS: lipopolysaccharide.

Discussion

Atherosclerosis is known to be a chronic inflammation. Studies show that SELE, ICAM-1, and VCAM-1 are associated with the atherogenesis as their elevated expression levels on the surface of endothelial cells result in the recruitment of the leukocytes to the arterial wall, and initiation of the atherosclerosis process (23-27).

Since ancient times, herbal medicine has long been considered for the treatment and prevention of various diseases. Due to the side effects of chemical drugs, the use of herbs for medicinal purposes are expanding in the clinical field (28).

In the present study, the effects of aqueous extract and saponin fraction of TT on the expression of adhesion molecules (ICAM-1, VCAM-1, and SELE) were investigated. For this purpose, HUVEC and HBMEC cell lines were chosen as representatives of macrovascular and microvascular vessels, respectively.

Since LPS, an endotoxin in the cell wall of gram-negative bacteria, is known as an inducer of inflammatory processes (29), we induced the HUVECs and HBMECs with LPS. SDS-PAGE analysis showed that LPS at the concentrations of 10 µg/ml (in HUVECs) and 1 µg/ml (in HBMECs) during a 6 h incubation time was able to significantly increase the expression of proteins located between the bands of 76 and 116 kDa (Figure 3 A and B). Since previous studies have shown that the molecular weight of adhesion molecules (ICAM-1, VCAM-1, and SELE) is in the range of 90-110 kDa (30, 31), we chose the above LPS concentrations for inflammation stimulation of the cell lines. In addition, SDS-PAGE analysis showed that TT aqueous extract and saponin fraction at the concentrations of 60 and 20 µg/ml (in both LPS-induced HUVECs and HBMECs) during an 18h incubation time were able to significantly decrease the expression of all proteins especially

[DOI: 10.22088/IJMCM.BUMS.9.1.73]

the proteins located between the bands of 76 and 116 kDa (Figure 3 C and D). Therefore, these concentrations were chosen as the optimal concentrations in our experiment for treatment of LPS-induced HUVEC and HBMEC cell lines.

The aqueous extract and saponin fraction of TT significantly decreased the expression of *ICAM-1*, *VCAM-1*, and *SELE* genes in both LPS-induced HUVEC and HBMEC cell lines (Figure 4). In normal (untreated) cells, treatment with the extract and fraction significantly decreased the expression level of *ICAM-1* in both cell lines. In the case of *VCAM-1*, only the aqueous extract could significantly decrease the expression level of *VCAM-1* in untreated cells. This could be due to a synergistic effect exerted by the other compounds in the extract. It is possible that treatment with the fraction at different conditions could also decrease *VCAM-1* expression on untreated cells. It is noted that the expression of *SELE* after treatment of the cell lines with the extract and fraction increased in which the reason is still unclear to us.

To the best of our knowledge, the study performed by Chang-jie et al. is one of the few studies that investigated the effects of tribusaponin from *Tribulus terrestris* (STT) on the expression of *ICAM-1* and *VCAM-1* in the atherosclerotic rats. Accordingly, STT down-regulated *ICAM-1* and *VCAM-1* in the atherosclerotic rats. Therefore, despite the difference in the type of study (*in vitro-in vivo*), our results are consistent with those reported by Chang-jie et al. (32). Previous studies have shown that nuclear factor κB (NF-κB) is masked in the cytoplasm by its inhibitor, IκBα. Induction of endothelial cells with LPS leads to the degradation of IκBα and the release of NF-kB heterodimer (p50/p65). Then, this heterodimer migrates into the nucleus and activates the expression of target genes, including the genes encoding adhesion molecules (33). Jiang et al. showed that the aqueous extract of TT decreases the expression of NF-κB p65 in the angiotensin II (Ang II)-induced HUVEC cell line (34). Therefore, the anti-inflammatory effect of TT aqueous extract and saponin fraction observed in this study is probably due to interference with NF-κB pathway.

In many studies, adhesion molecules have been targeted in clinical investigations to prevent and treat atherosclerosis using herbal and non-herbal medicnes for decreasing the expression levels of these surface molecules. Correspondingly, Lee et al. revealed that traditional Chinese medicine such as *Buddleja Officinalis*, which have similar structures and compounds to TT, could reduce the expression of VCAM-1 and 1CAM-1 molecules (35). In a similar study, Wang et al. demonstrated that the saponin fraction of the *Panax notoginseng* causes an inhibition in the expression of adhesion molecules (36).

The flavones, as natural anti-inflammatory agents, significantly suppressed nuclear translocation of NF-κB and its binding to DNA, and also other inflammatory signaling pathways. Moreover, it was shown to decrease TNF-α-induced expression of *VCAM-1*, *ICAM-1*,and *SELE* in human coronary atherosclerotic plaques(37).

In the present study, the amount of gene expression reduction rate caused by TT aqueous extract was higher than that of saponin fraction. This observation may be explained by the presence of other anti-inflammatory chemical constituents such as flavonoids in the aqueous extractof TT (38, 39). Due to the inhibitory effects of flavonoids on the expression of adhesion molecules (40, 41), it is possible that the cumulative effects of these compounds along with saponins would result in a further reduction in the expression of *ICAM-1*, *VCAM-1*, and *SELE* in the aqueous extract in comparison with the saponin fraction.

In conclusion, the present study demonstrated that TT may have an anti-inflammatory effect. Anti-inflammatory activity of TT was shown to be related to down regulation of *ICAM-1*, *VCAM-1*, and *SELE* genes. *In vivo* Study on anti-

[DOI: 10.22088/IJMCM.BUMS.9.1.73]

inflammatory effect of this herb may provide new insights into the development of an herbal drug for preventing and treating atherosclerosis.

Acknowledgments

The content of this paper is extracted from the MSc thesis NO. 94157 submitted by Z. Fereydouni. This study was supported by Medical Biology Research Center, Kermanshah University of Medical Sciences. Authors would also like to take this opportunity to express their special thanks to Dr. Keivan Moradi for his assistance during the preparation of the manuscript.

Conflict of interest

There is no conflict of interest in this study.

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[DOI: 10.22088/IJMCM.BUMS.9.1.73]