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*Original Article*

# Pterostilbene suppresses vascular adhesion molecule expression in TNF-α-stimulated vascular muscle cells

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**Abstract:** Atherosclerosis is a chronic inflammatory disease and the expression of adhesion molecules on vascular smooth muscle cells (VSMCs) contributes to the progress of the disease. Pterostilbene (PT), a natural dimethylated analog of resveratrol from blueberries, has been shown to have a variety of biological activities including anti-inflammatory activity. However, the role of PT in vascular inflammation remains unknown. Therefore, in this study, we investigated the effect of PT on vascular inflammation. In this study, we demonstrated that PT inhibits the expression of vascular cell adhesion molecule-1 (VCAM-1) and intracellular adhesion molecule-1 (ICAM-1) in tumor necrosis factor-α (TNF-α)-stimulated VSMCs. In addition, we found that PT prevented TNF-α-induced NF-κB p65 protein expression and phosphorylation of MAPKs in VSMCs. Taken together; our results show that PT inhibits the expression of VCAM-1 and ICAM-1 induced by TNF-α in VSMCs through suppressing the NF-κB and MAPK signaling pathways. Therefore, PT is proposed as an effective anti-inflammatory agent that may have a potential therapeutic use for preventing the advancement of atherosclerotic lesions.

**Keywords:** Pterostilbene (PT), anti-inflammation, cell adhesion molecule, NF-κB, MAPK

## Introduction

Atherosclerosis (AS) is the most common type of coronary artery disease and the leading cause of morbidity and mortality worldwide. It is characterized by the accumulation of lipids in the vessel wall of arteries [1]. Several lines of studies support a crucial role of vascular smooth muscle cell (VSMCs) at various process of atherosclerosis [2-4]. As the disease progresses, VSMCs are in proximity to and physically interact with inflammatory leukocytes, which play a critical role in exacerbating the disease [5], and leukocyte adhesion to VSMC is primarily mediated by cell adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1) and intracellular adhesion molecule-1 (ICAM-1). In addition, it has been reported that inflammatory cytokines and/or chemokines such as tumor necrosis factor-α (TNF-α), interleukin-8 (IL-8) and IL-6 play a critical role in the mediation of inflammatory of vascular smooth muscle cells (VSMC) and the pathogenesis of atherosclerosis following balloon injury [6]. Therefore, inhibiting the expression of these adhesion molecules on VSMCs may be an important therapeutic approach for atherosclerosis.

Several studies have demonstrated that natural products, especially medicinal plants, may represent an ideal source to develop safe and effective agents for the management of atherosclerosis. Pterostilbene (PT) is a natural dimethylated analog of resveratrol from blueberries. It exerts a broad range of important biological actions including anti-cancer activities against colon [7], breast [8] and hepatocellular carcinoma [9] as well as anti-inflammatory [10], antioxidant [11] and neuro-protective properties [12]. Recently, one study showed that PT significantly inhibited the DNA synthesis and proliferation of platelet-derived growth factor BB (PDGF-BB)-stimulated VSMCs in a dose-dependent manner [13]. Another study demonstrated that PT protects vascular endothelial cells

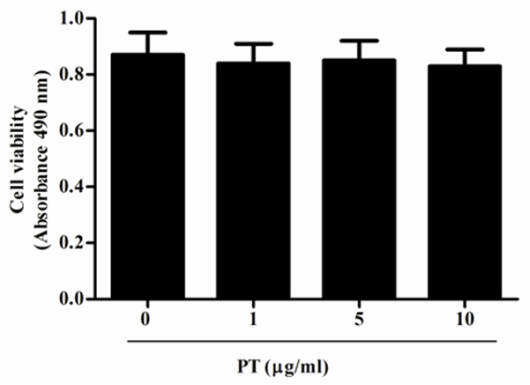


**Figure 1.** Effect of pterostilbene (PT) on VSMC viability. Cell viability was determined by MTT assay, PT did not have cytotoxic effects on VSMCs at the concentration range of 1-10 μg/ml. All experiments were repeated at least three times and all data are reported as means ± SD.

against oxidized low-density lipoprotein-induced apoptosis *in vitro* and *in vivo* [14]. However, the role of PT in vascular inflammation remains unknown. Therefore, in this study, we investigated the effect of PT on vascular inflammation.

## Materials and methods

### *Materials*

Pterostilbene was synthesized according to the method reported by Pettit et al [15]. The purity of pterostilbene was determined by high-performance liquid chromatography (HPLC) as >99.2%. pGL3-NF-κB and the luciferase assay system were purchased from Promega (Madison, WI). pCMV-β-gal was purchased from Lonza (Walkersville, MD). Polyclonal antibodies specific for phospho-p38, phospho-ERK, phospho-JNK, p38, ERK1/2, JNK and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The anti-VCAM-1, anti-ICAM-1, anti-NF-κB p65 and anti-NF-κB antibodies were purchased from Invitrogen (Carlsbad, CA, USA). All other chemicals and reagents were purchased from Sigma (St. Louis, MO, USA).

### *Cell culture*

Rat aortic VSMC were prepared from thoracic aortas of Sprague-Dawley rats as previously described [16]. Cells were cultured in DMEM containing 10% FBS, 100 units/ml penicillin and 100 mg/ml streptomycin at 37°C in a humidified atmosphere of 5% $CO_2$ in air. For all experiments, rat aortic VSMC from passages 3 to 8 were used.

### *Cell viability assay*

Cell proliferation was assessed by the MTT assay [17]. For MTT measurement, VSMCs were seeded in 96-well plates ($1\times10^4$ cells per well) and then treated with various concentrations (0, 1, 5 and 10 μg/ml) of PT. After 24 h, MTT (5 mg/ml) was added to each well and incubated for 4 h. The formazan crystals were solubilized in 100 μl of dimethyl sulphoxide (DMSO). Absorbance at 490 nm was measured using a microplate reader (Takara Biotechnology, Dalian, China). All experiments were conducted in triplicate.

### *Real-time quantitative PCR (qRT-PCR)*

Total RNA was extracted with QIAzol reagent according to the manufacturer's instructions. One microgram of total RNA was converted to cDNA by Super Script reverse transcriptase and then amplified by Platinum Taq polymerase using the Super Script One-Step RT-PCR kit (Invitrogen, Carlsbad, CA, USA). For PCR experiments, the forward and reverse primers corresponding to rat VCAM-1 were 5'-CAAAGGTGGATCAGATTCAAG-3' (forward), 5'-GGTGAGCATTATCACCCAGAA-3' (reverse); ICAM-1 were 5'-CAAAGGTGGATCAGATTCAAG-3' (forward), 5'-GGTGAGCATTATCACCCAGAA-3' (reverse); β-actin were 5'-GATCATTGCTCCTCCTGAGC-3' (forward), and antisense, 5'-ACTCCTGCTTGCTGATCCAC-3' (reverse). The PCR cycling program was 95°C for 3 min, then 40 cycles of 94°C for 20 s, 58°C for 30 s and 72°C for 20 s, and a final extension at 72°C for 5 min. For relative quantification, the levels of individual gene mRNA transcripts were firstly normalized to the control β-actin. Subsequently, the differential expression of these genes was analyzed by the ΔCt method and expressed as the fold changes.

### *Transfection and reporter assays*

VSMCs ($5\times10^4$ cells/ml) were plated into a 6-well plate and transiently co-transfected with the plasmids, pGL3-NF-κB and pCMV-β-gal using Lipofectamine Plus according to the manufacturer's instruction. In brief, a transfection mixture containing 0.5 μg pGL3-NF-κB and 0.2

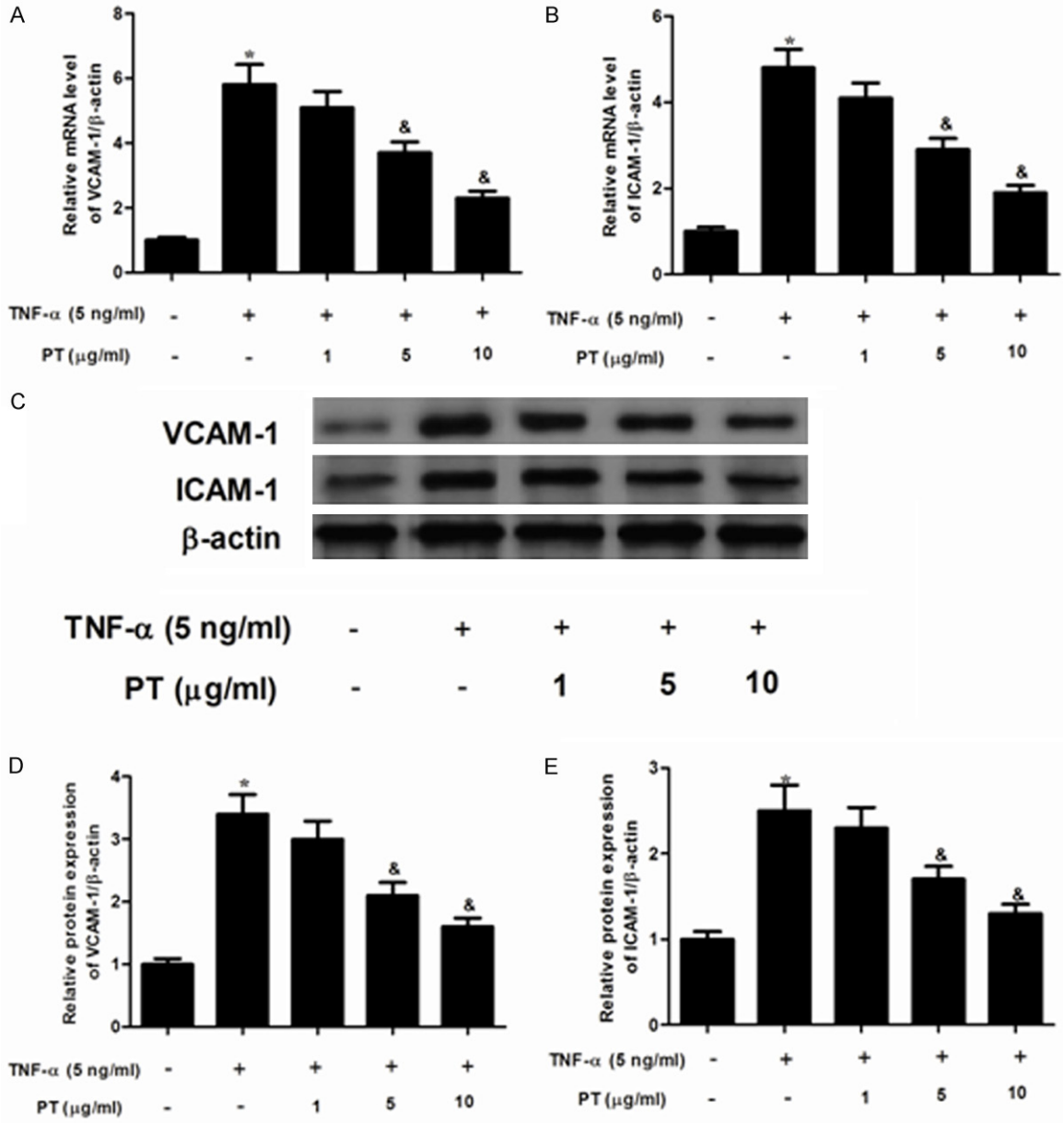


**Figure 2.** Effect of PT on adhesion molecule expression in TNF-α-stimulated VSMCs. VSMCs were preincubated with various concentrations (0, 1, 5 and 10 μg/ml) of PT for 2 h and stimulated with TNF-α (5 ng/ml) for 4 h. A and B. VCAM-1 and ICAM-1 mRNA expression was determined by qRT-PCR. C. VCAM-1 and ICAM-1 protein expression was determined by Western blot. D and E. Relative protein expression of VCAM-1 and ICAM-1 was analyzed with Image-Pro Plus 6.0 software and normalized with β-actin. All experiments were repeated at least three times and all data are reported as means ± SD. $^{*}P < 0.05$ vs. control group, $\&P < 0.05$ vs. TNF-α group.

μg pCMV-β-gal was mixed with the Lipofectamine Plus reagent and added to the cells. After 4 h, the cells were pretreated with various concentrations of PT for 2 h followed by the addition of TNF-α for 4 h. Cell extracts were prepared, then luciferase substrate was added to cell extracts, and luminescence was measured in a microplate luminometer. The value of luciferase activity was normalized to transfection efficiency monitored by the co-transfected β-galactosidase expression vector luciferase and β-galactosidase activities were measured.

*Western blot*

After treatment with PT, cells were washed twice with PBS and lysed in lysis buffer. Protein concentration was determined using the BCA protein assay kit (Beyotime, Nantong, China). Equal amounts of protein from each sample

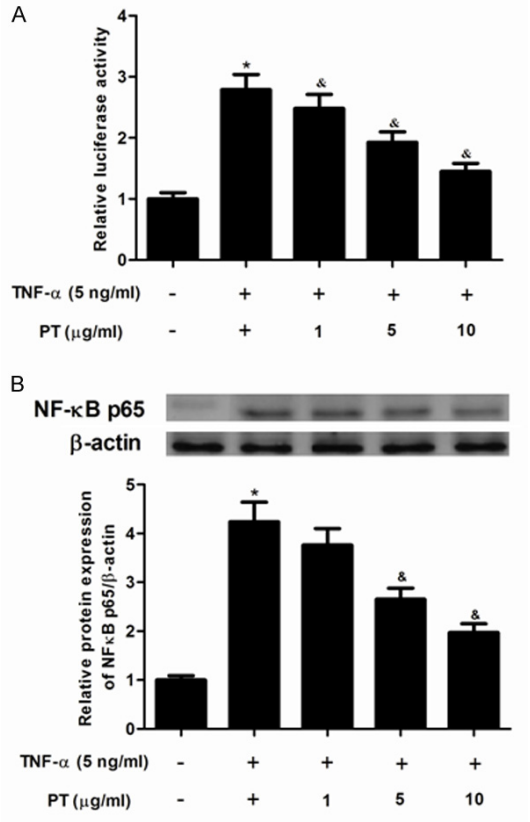


**Figure 3.** Effects of PT on NF-κB activation in TNF-α-stimulated VSMCs. A. VSMCs were transfected with a pGL3-NF-κB-Luc reporter plasmid and pCMV-β-gal, pretreated with various concentrations (0, 1, 5 and 10 μg/ml) of PT for 2 h, and stimulated with TNF-α for 4 h. B, VSMCs were preincubated with or without various concentrations of PT for 2 h, then treated with TNF-α for 4 h. The protein expression level of NF-κB p65 was detected by Western blotting. All experiments were repeated at least three times and all data are reported as means ± SD. $^{*}P < 0.05$ vs. control group, &$P < 0.05$ vs. TNF-α group.

were electrophoresed on a 10% polyacrylamide SDS gel, followed by transfer onto polyvinylidene difluoride membranes (Millipore, Boston, MA, USA). The membranes were then blocked with 5% non-fat dried milk in Tris buffered saline (TBS) and subsequently probed with the primary antibodies overnight. After three washes, the blots were subsequently incubated with horseradish peroxidase conjugated secondary antibodies for 1 h. Expression was visualized by using ECL Western blotting detection reagent (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

*Statistical analysis*

Quantitative data are reported as the mean ± standard deviation (SD). Statistical evaluation was performed using a Student's t-test or one-way ANOVA followed by Dunnett's test. A $P < 0.05$ was considered to indicate statistical significance.

## Results

*Effect of PT on VSMC viability*

To exclude the possibility that reductions in the levels of adhesion molecule occurred due to direct toxicity of PT on VSMCs, we evaluated cell toxicity of various concentrations of PT (0, 1, 5, and 10 μg/ml). As shown in **Figure 1**, the viabilities of VSMCs were unaffected by PT. These results showed that PT did not have cytotoxic effects on VSMCs at the concentration range of 1-10 μg/ml.

*Effect of PT on adhesion molecule expression in VSMCs*

Then, we investigated the effect of PT on adhesion molecule expression in VSMCs. As shown in **Figure 2A** and **2B**, after stimulation with TNF-α, the mRNA expression of VCAM-1 and ICAM-1 was significantly elevated; however, PT significantly inhibited this effect in a concentration-dependent manner. Similarly, Western blot analysis also demonstrated that PT markedly suppressed TNF-α-induced protein expression of VCAM-1 and ICAM-1 in VSMCs (**Figure 2C**).

*Effect of PT on TNF-α-induced NF-κB activation in VSMCs*

Because activation of NF-κB is critical for the expression of adhesion molecule [18], therefore, we examined the effect of PT on NF-κB transcriptional activation. As shown in **Figure 3A**, stimulation with TNF-α (5 ng/ml) markedly stimulated NF-κB luciferase activity. The increased luciferase activity was prevented by PT in a dose-dependent manner. Furthermore, we investigated the effect of PT on NF-κB p65 protein in TNF-α-stimulated VSMCs. As shown in **Figure 3B**, TNF-α significantly increased the expression of NF-κB p65 protein, as compared with the control group, while PT prevented TNF-α-induced NF-κB p65 protein expression. These results suggest that PT inhibits TNF-α-induced NF-κB activation.

*Effect of PT on TNF-α-induced phosphorylation of MAPKs in VSMCs*

The MAP kinase (MAPK) pathways play important role in regulating the expression of adhe-

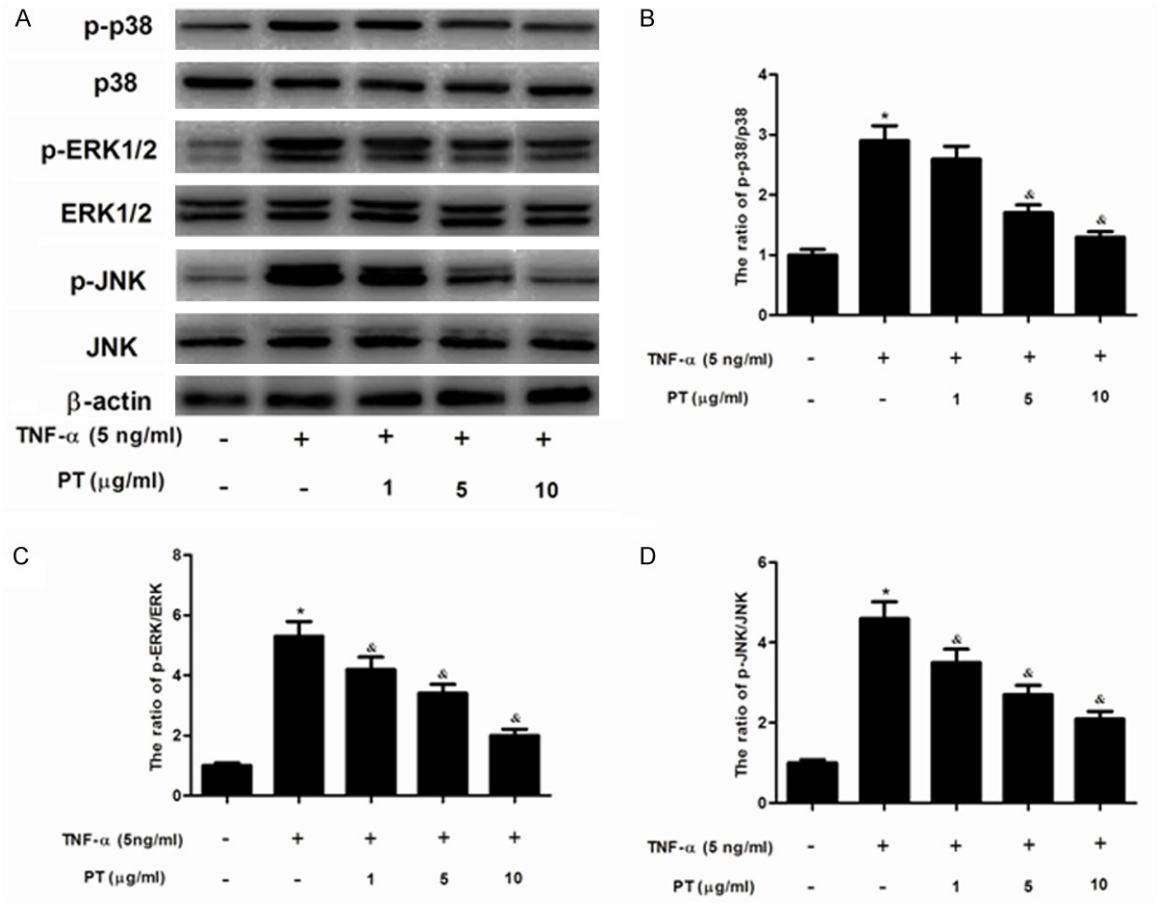


**Figure 4.** Effect of PT on TNF-α-induced phosphorylation of MAPKs in VSMCs. VSMCs were pretreated with various concentrations (0, 1, 5 and 10 μg/ml) of PT for 2 h and then incubated with TNF-α (5 ng/ml) for 30 min. The p38, ERK and JNK phosphorylation was determined by immunoblotting using phospho-p38, ERK and JNK specific antibody, respectively. B-D. The relative protein expression levels of p-p38, p-ERK and p-JNK were quantified using Image-Pro Plus 6.0 software and normalized to β-actin. All experiments were repeated at least three times and all data are reported as means ± SD. $^{*}P < 0.05$ vs. control group, &$P < 0.05$ vs. TNF-α group.

sion molecules. Therefore, we investigated the effect of PT on TNF-α-induced phosphorylation of MAPKs in VSMCs. As shown in **Figure 4**, TNF-α significantly increased the levels of activation of p38 MAPK, ERK1/2 and JNK, while, PT prevented TNF-α-induced phosphorylation of MAPKs in VSMCs.

## Discussion

Adhesion molecules mediated cell-cell adhesion play an important role in inflammatory processes such as atherosclerosis. Thus, pharmacological agents that inhibit the expression of these adhesion molecules have the potential to treat atherosclerosis. In this study, we demonstrated that PT inhibits the expression of VCAM-1 and ICAM-1 in TNF-α-stimulated VSMCs. In addition, we found that PT prevented TNF-α-induced NF-κB p65 protein expression and phosphorylation of MAPKs in VSMCs.

Atherosclerosis is accompanied by increased levels of adhesion molecules such as VCAM-1 or ICAM-1 [19]. In addition, the expression of adhesion molecules was evoked by inflammatory cytokines in the atherosclerotic lesion [20]. In line with these results, in the present study, we observed that TNF-α significantly increased the expression of VCAM-1 and ICAM-1 in VSMCs, while, PT could prevent the TNF-α-induced expression of VCAM-1 and ICAM-1. Our data suggest that PT has an inhibitory effect on adhesion molecule expression in TNF-α-stimulated VSMCs.

It is known that NF-κB signaling pathway plays a critical role in the regulation of inflammatory

response [21, 22]. NF-κB activation was associated with the phosphorylation and degradation of IκB-α and the nuclear translocation of p65 [23]. PT has been shown to suppress production of inflammatory products in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells through suppression of NF-κB activation [24]. In addition, it has been reported that activation of transcription factor NF-κB by TNF-α is required for the transcriptional activation of muscle cell adhesion molecules. Moreover, previous studies showed that natural extracts inhibits cell adhesion molecules via the suppression of NF-κB activation under pathophysiological conditions [25]. Liu et al reported that acetylbritannilactone suppresses lipopolysaccharide-induced VSMC inflammatory response through blockade of NF-κB activity [26]. Byeon et al indicated that stereocalpin A inhibited TNF-α-induced adhesion of THP-1 monocytic cells and expression of VCAM-1 and ICAM-1 through inhibition NF-κB signaling pathway [27]. Consistent with these results, in the present study, we observed that PT inhibited TNF-α-induced NF-κB activation through inhibition of NF-κB p65 in VSMCs, suggesting that PT inhibits TNF-α-induced adhesion molecule expression at least partially through inhibition of NF-κB activation.

MAPK signaling pathway plays an important role in regulating cell adhesion molecules expressed on cells in response to external stimuli [28]. TNF-α increased ICAM-1 and VCAM-1 expression via JNK, ERK1/2 and p38 MAPK. Furthermore, various phytochemicals have been shown to inhibit the expression of adhesion molecules in VSMCs. For instance, diosgenin inhibits the adhesive capacity of VSMC and the TNF-α-mediated induction of ICAM-1 and VCAM-1 in VSMC by inhibiting the MAPK signaling pathway [29]. Ohioensin et al also suppresses TNF-α-induced adhesion molecule expression by inactivation of the MAPK signaling pathway in VSMCs [30]. Interestingly, we observed that PT inhibited phosphorylation of p38 MAPK, JNK and ERK1/2 induced by TNF in VSMCs, suggesting that PT inhibits TNF-α-induced adhesion molecule expression through inhibition of MAPK activation.

In conclusion, our results show that PT inhibits the expression of VCAM-1 and ICAM-1 induced by TNF in VSMCs through suppressing the NF-κB and MAPK signaling pathways. Therefore, PT is proposed as an effective anti-inflammatory agent that may have a potential therapeutic use for preventing the advancement of atherosclerotic lesions.

## Disclosure of conflict of interest

None.

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