Pharmacogn. Mag.
A multifaceted peer reviewed journal in the field of Pharmacognosy and Natural Products
www.phcog.com | www.phcog.net

ORIGINAL ARTICLE

Anti-Inflammatory Effects of *Malus toringoides* Extract in Lipopolysaccharide-Induced Human Umbilical Vein Endothelial Cells

Chengde Fan[1], Zhuoma Dongzhi[2,3], Linsha Dong[1], Ruiying Yuan[2,4], Jule Wang[2], Bin Li[1], Shan Huang[1]

[1]Department of Pharmacy, Key Laboratory of Pharmaceutical Research for Metabolic Diseases, Qingdao University of Science and Technology, Qingdao, [2]Department of Medicament, College of Medicine, Tibet University, Lhasa, China, [3]Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat-Yai, Songkhla, Thailand, [4]Center of Tibetan Studies (Everest Research Institute), Tibet University, Lhasa, China

Submitted: 16-Aug-2020 Revised: 12-Oct-2020 Accepted: 13-May-2021 Published: 11-Nov-2021

ABSTRACT

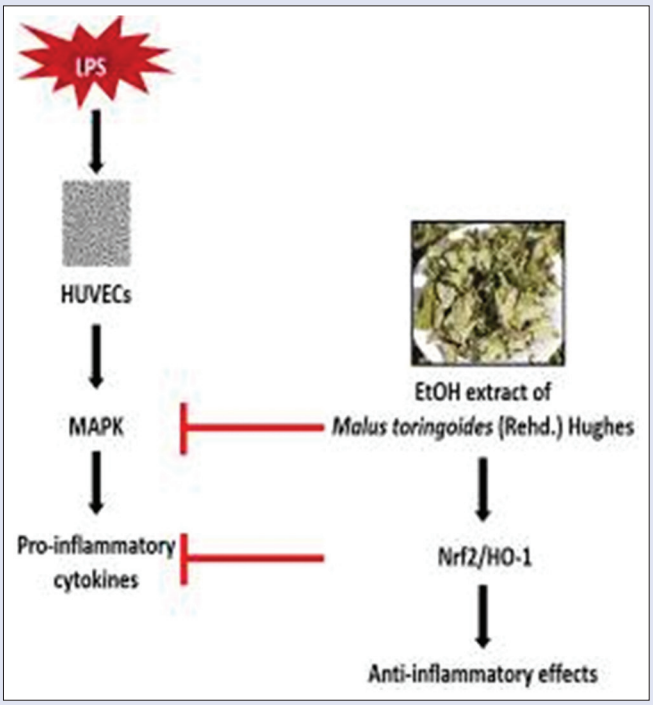
Background: *Malus toringoides* (Rehd.) Hughes is a traditional Tibetan medicine. It demonstrates significant hypoglycemic and hypolipidemic potential. However, the protective effects of *M. toringoides* extracts on endothelial cells and the mechanisms that underlie their activity have not yet been reported. **Objectives:** The aim of the study was to explore the anti-inflammatory effects and cellular mechanisms of extracts of *M. toringoides* (CBTM-E375) in lipopolysaccharide (LPS)-induced human umbilical vein endothelial cells (HUVECs). **Materials and Methods:** HUVECs were exposed to LPS, and the level of proinflammatory mediators was measured by enzyme-linked immunosorbent assay. Furthermore, the activation of heme oxygenase-1 (HO-1), nuclear factor erythroid 2-related factor 2 (Nrf2), and mitogen-activated protein kinase (MAPK) was examined by Western blot and immunofluorescence analysis. **Results:** CBTM-E375 significantly downregulated the levels of inflammatory mediators and upregulated the expression of HO-1 by modulating Nrf2 translocation in HUVECs. The transfection of HO-1 small interfering RNA into HUVECs actively reversed the effects of CBTM-E375 in suppressing the expression of proinflammatory cytokines. Furthermore, MAPK activation in response to LPS was also blocked by CBTM-E375. **Conclusion:** CBTM-E375 exerts anti-inflammatory effects, possibly by modulating the translocation of Nrf2 and expression of HO-1, and inhibiting the phosphorylation of MAPK signaling pathway.

Key words: Human umbilical vein endothelial cells, *Malus toringoides* (Rehd.) Hughes, mitogen-activated protein kinases, nuclear factor erythroid 2-related factor 2/heme oxygenase-1

SUMMARY

- The anti-inflammatory effects of extracts from the traditional Tibetan plant *Malus toringoides* in lipopolysaccharide-induced human umbilical vein endothelial cells have been confirmed in this study, thus making this product a suitable prospect for the future development of functional beverages.

Abbreviations used: JNK: C-Jun-N-terminal kinase; ERK: Extracellular signal-regulated kinase; HUVECs: Human umbilical vein endothelial cells; HO-1: Heme oxygenase-1; IL-8: Interleukin-8; IL-6: Interleukin-6; LPS: Lipopolysaccharide; MAPK: Mitogen-activated protein kinase; Nrf2: Nuclear factor E2-related factor 2; TNF-α: Tumour necrosis factor-α; HPLC: High-performance liquid chromatography.



Correspondence:

Prof. Shan Huang,
Department of Pharmacy, Key Laboratory of Pharmaceutical Reaearch for Metabolic Diseases, Qingdao University of Science c and Technology, Qingdao, 266042, China.
E-mail: huangshan@qust.edu.cn
DOI: 10.4103/pm.pm_357_20

Access this article online
Website: www.phcog.com
Quick Response Code:

INTRODUCTION

Cardiovascular Disease (CVD) is characterized by a high rate of mortality and disability. The earliest manifestation of CVD is vascular endothelial damage, which is responsible for vascular dysfunction, which eventually leads to CVD.[1] Vascular inflammation, caused by the inflammatory response of endothelial cells, is the primary cause of vascular endothelial

Cite this article as: Fan CD, Dongzhi ZM, Dong LS, Yuan RY, Wang JL, Li B, *et al*. Anti-Inflammatory Effects of *Malus toringoides* Extract in Lipopolysaccharide-Induced Human Umbilical Vein Endothelial Cells. Phcog Mag 2021;17:518-24.

damage and is regarded as the main cause of other serious diseases including atherosclerosis, cardiac failure, and diabetes mellitus.[2,3]

By forming a barrier between the vessel and the surrounding tissue, endothelial cells maintain control over vasoactive factors such as permeability, adhesiveness, and integrity.[4] However, several proinflammatory stimuli, such as high serum levels of lipopolysaccharide (LPS), induce the secretion of numerous proinflammatory mediators in endothelial cells. These proinflammatory cytokines can damage the organs and arteries and promote the inflammatory process. In the case of CVDs, this inflammatory response is maintained, which will aggravate the disease condition.[5] Therefore, inhibiting the inflammatory response of the endothelial cells inflammation can be considered as an important target for the prevention and treatment of vascular inflammation and its complications.

Nuclear factor erythroid 2-related factor 2 (Nrf2), a member of the leucine zipper activator of transcription family and heme oxygenase-1 (HO-1), which is a downstream transcription factor of Nrf2, together constitute a classic antioxidant defense system. In addition to reducing oxidative stress, Nrf2/HO-1 signaling pathway also plays a significant role in inhibiting the inflammatory response.[6] Mitogen-activated protein kinases (MAPKs) are common in most cells including endothelial cells, and it is easily activated by LPS. Under external stimuli, MAPK signaling pathway regulates the release of proinflammatory cytokines. MAPK signaling pathway contains three major classes of molecules in mammals, namely, p38, Jun-N-terminal kinase (JNK), and extracellular signal-regulated kinase (ERK).[7] The recruitment of inflammatory cells is initiated by p38. Neutrophils and leukocytes then increase the levels of transcription factors and the secretion of proinflammatory mediators to further aggravate the inflammatory processes. At present, studies on JNK mainly focus on cell proliferation and apoptosis. However, some studies found that the activation of JNK can lead to the secretion of numerous proinflammatory factors and then induce the production of inflammatory cytokines and the infiltration and aggregation macrophages.[8] Moreover, some studies have verified that the phosphorylation of ERK is positively correlated with the occurrence of inflammation, and the recovery of ERK resting-state often contributes to the alleviation and disappearance of cellular inflammation.[9]

Malus toringoides (Rehd.) Hughes is a traditional Tibetan medicine and is used by local doctors, in combination with ghee, the treatment of hyperglycemia, hyperlipidemia, hypertension, and some liver diseases. Phytochemical studies have shown that *M. toringoides* contains flavonoids, polysaccharides, fatty acids, and amino acids. Previous studies have demonstrated that *M. toringoides* is rich in flavonoids, such as phlorizin and phloretin, which are generally considered to possess significant antioxidant and antiapoptotic activities.[10,11] In addition, the extract shows significant anti-inflammatory, hypolipidemic, and hypoglycemic activity in high-fat-diet-induced diabetic rats.[12-14] However, so far, there are studies conducted to test the bioactivity and the underlying mechanisms of *M. toringoides* against inflammation in endothelial cells. Therefore, in this study, we aimed to study the anti-inflammatory effects of *M. toringoides* in LPS-induced human umbilical vein endothelial cells (HUVECs) and analyze the underlying mechanisms of its activity.

MATERIALS AND METHODS

Chemicals and reagent

Dulbecco's modified eagle's medium (DMEM), fetal bovine serum (FBS), and other cell culture reagents were purchased from GIBCO BRL Co.(Grand Island, NY, USA). Cobalt protoporphyrin IX (CoPP; HO-1 inducer) and si-RNA targeting HO-1 were purchased from Invitrogen (Carlsbad, CA, USA). Nuclear protein extraction kit was purchased from Solarbio Technology Co. Ltd.(Beijing, China). Primary antibodies, mouse/rabbit anti-HO-1, anti-Nrf2, and secondary antibodies were purchased from Affinity Biologicals, Inc. (Cincinnati, OH, USA). Enzyme-linked immunosorbent assay (ELISA) kits for tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-8 were obtained from RandD Systems, Inc.(Minneapolis, MN, USA). Other chemicals were purchased from Sigma Chemical Co.(St. Louis, MO, USA).

Preparation of *Malus toringoides* extracts

The collection and preparation of *M. toringoides* extract has been described previously.[14] Briefly, pulverized leaves of *M. toringoides* were boiled twice with 70% ethanol and refluxed in the same solvent for 1.5 h. The extract was filtered with filter paper, and the residue was extracted again by petroleum ether and the petroleum ether extracts were discarded; the other solution was purified on a polyamide column and initially eluted with 2000 mL of water and then evaporated *in vacuo* at 60°C. The obtained extract of *M. toringoides* (CBTM-E375) was stored in the Component Bank of Tibetan Medicine (Lhasa, China). In each experiment, the extract was dissolved in dimethyl sulfoxide (DMSO) whose concentration was not more than 0.05% in the medium.

Material analysis of CBTM-E375

The operating condition for high-performance liquid chromatography (HPLC) has been described previously.[13] The mobile phase consisted of methanol: 2% phosphoric acid in a ratio of 20:80 (v/v), and Hypersil BDS C_{18} column (4.6 mm × 250 mm, 5 μm) was used for chromatographic separation. Following were the chromatographic conditions: Column temperature was maintained at 30°C with a flow rate of 1 mL/min. The chromatograms were captured by the UV detector.

Cell culture and viability assay

HUVECs was purchased from the China Center for Type Culture Collection (Wuhan, China). The cells were maintained in DMEM containing 10% FBS and routinely cultured in a humidified incubator with 5% CO_2 at 37°C. HUVECs (8×10^3 cells/well) were incubated in 96-well plates and pretreated with different concentrations of CBTM-E375. The cells were grown for 48 h and the cell viability was examined using 50 μL 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, 2.5 mg/mL) to each well. After 4 h, DMSO (150 μL) was added to each well, then the absorbance of the crystals in 96-wells was detected at 490 nm.

Proinflammatory factors *a*ssay

HUVECs were seeded in 6-well plates and then were treated with different concentrations of CBTM-E375 for 12 h, before 24 h-incubation with 1 μg/mL of LPS. The levels of IL-6, TNF-α, and IL-8 in the culture medium were analyzed using ELISA.

Small interfering RNA transfection

According to the transfection protocol provided by the manufacturer (Invitrogen), HUVECs were transfected with the small interfering RNA (siRNA) targeting HO-1 inserted in the negative control siRNA plasmid for 12 h by using Opti-MEM reduced serum medium (Gibco, Grand island, NY, USA) with lipofectamine followed by recovery in DMEM. HUVECs were then cultured together with LPS for 24 h to detect the release of proinflammatory cytokines by ELISA. Subsequently, the cell samples were prepared and further analyzed by Western blot.

Preparation of nuclear and cytosolic samples

Nuclear protein extraction kit (20191022, Solarbio, Beijing, China) was used to separate the nuclear and cytosolic sections of the cells. After transferring the cells into PBS, HUVECs was centrifuged at 650 × g for 6 min at 4°C. Cytosolic extraction reagent was added to the cell

sediment, which was then resuspended and kept on ice or at 0°C for 9 min to accelerate cell lysis. The mixture was centrifuged at 15,000 × g for 15 min. The supernatant as the cytosolic fraction was collected. After adding the nuclear extraction reagent to cell pellets, the supernatant as the nuclear fraction was collected under the condition that it is centrifuged at 15,000 × g for 10 min. Analysis of the protein content of the two fractions was performed using bicinchoninic acid (BCA) protein assay kit (Thermo Scientific, Rockford, USA).

Immunofluorescence microscopy

For examining the localization of the Nrf2, cells were inoculated onto Lab-Tek chamber slides for growth. The cells were treated with CBTM-375 and were placed in 4% formalin and permeabilized with cold acetone. The osmotically treated HUVECs were probed with Nrf2 antibody and fluorescein isothiocynate-labeled secondary antibody (Invitrogen, Carlsbad, CA, USA). For visualizing the nuclei, HUVECs were treated with 4',6-diamino-2-phenylindole (DAPI, 5 μg/mL) for 5 min, washed thrice with cold PBS and treated with 50 μL VectaShield (Vector Laboratories, Burlingame, CA, USA). The fixed cells were visualized and photographed using a fluorescence microscope (Provis AX70, Olympus Optical Co., Tokyo, Japan).

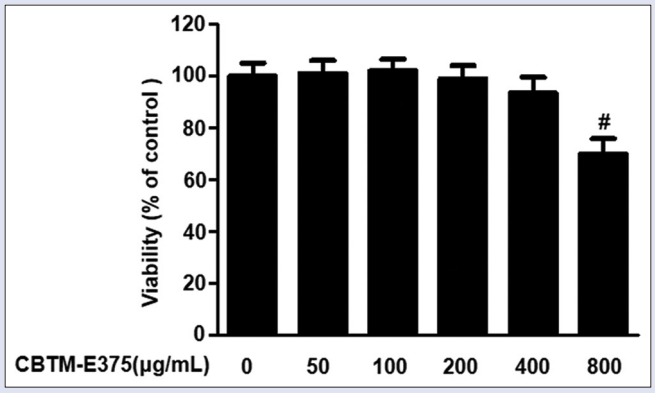


Figure 1: Effects of CBTM-E375 on cell viability. Human umbilical vein endothelial cells were incubated for 48 h with various concentrations of CBTM-E375 (50-800 μg/mL). Cell viability was determined as described in materials and methods. Bar represents the mean±standard deviation of 3 independent experiments. $^{\#}P < 0.05$ versus the group without CBTM-E375 was considered to be statistically significant

Western blot analysis

The total cell protein was extracted using RIPA buffer and quantified using the BCA Assay Kit (Thermo Scientific, Rockford, USA) according to the manufacturer's instructions. The prepared samples were separated on 6%–12% gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto a PVDF membrane. The membranes were transferred into Tris-buffered saline Tween-20 (TBST) solution containing 5% dried milk for 2.5 h and washed thrice with TBST after every 10 min. Next, the PVDF membranes were incubated with an appropriate concentration of diluted primary antibody for 2 h. After washing thrice with TBST, the membranes were incubated with the indicated primary and secondary antibodies coupled with horseradish peroxidase for 2 h, and then, the membrane was washed again. The blots were followed by visualization with the ECL Western blot substrate (Amersham Bioscience, Buckinghamshire, UK). The intensity of each band was analyzed using a ChemiDoc image analyzer (Tanon 4600, Tanon, China).

Statistical analysis

Data are expressed as the mean ± standard deviation for each experimental group. The differences between groups was analyzed by one-way analysis of variance followed by Student's *t*-test. Statistical significance is represented as $^{\#}P < 0.05$ versus control group; $^{\#\#}P < 0.01$ versus control group; $^{*}P < 0.05$ versus LPS group; $^{**}P < 0.01$ versus LPS group; $\delta P < 0.05$ versus CBTM-E375 + siRNA control group; $\delta\delta P < 0.01$ versus CBTM-E375 + siRNA control group; and $^{\dagger}P < 0.05$ versus LPS + CBTM-E375 group.

RESULTS

High-performance liquid chromatography analysis of CBTM-375

According to the results of HPLC analysis, phloridzin and phloretin were the primary bioactive components in CBTM-375, which is basically consistent with our previous experimental results.[14]

Effects of CBTM-E375 on cell toxicity in *human umbilical vein endothelial cells*

After the cells were treated with 50–800 μg/mL of CBTM-E375 for 48 h, the toxicological activities of extracts were evaluated through MTT assay.

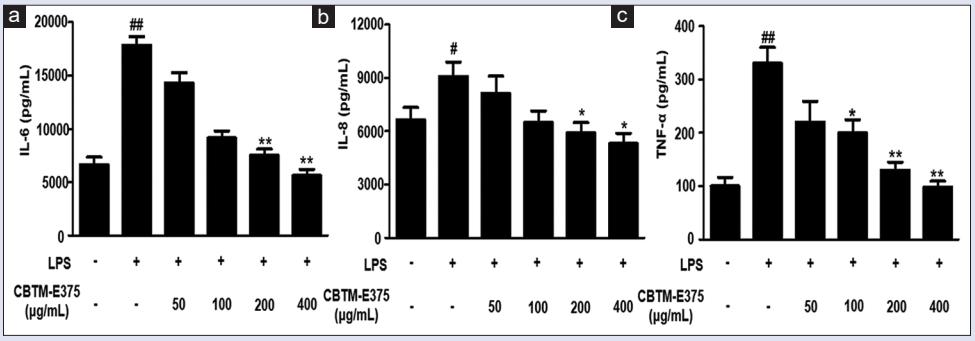


Figure 2: Effects of CBTM-E375 on LPS-induced TNF-α, IL-6, and IL-8 levels in HUVECs. HUVECs were pretreated for 12 h with the indicated concentrations of CBTM-E375 and stimulated for 24 h with LPS (1 μg/mL). The concentration of IL-6, IL-8, and TNF-α (a-c) were determined as described under materials and methods. Data represent mean values of 3 experiments ± standard deviation. $^{\#}P < 0.05$ versus control group; $^{\#\#}P < 0.01$ versus control group; $^{*}P < 0.05$ versus LPS group; $^{**}P < 0.01$ versus LPS group; was considered to be statistically significant. HUVECs: Human umbilical vein endothelial cells; TNF-α: Tumour necrosis factor-α; IL-8: Interleukin-8; IL-6: Interleukin-6

As shown in Figure 1, 800 μg/mL of CBTM-E375 exhibited significant cytotoxic effects. Therefore, we selected 50–400 μg/mL of CBTM-E375 for further experiments.

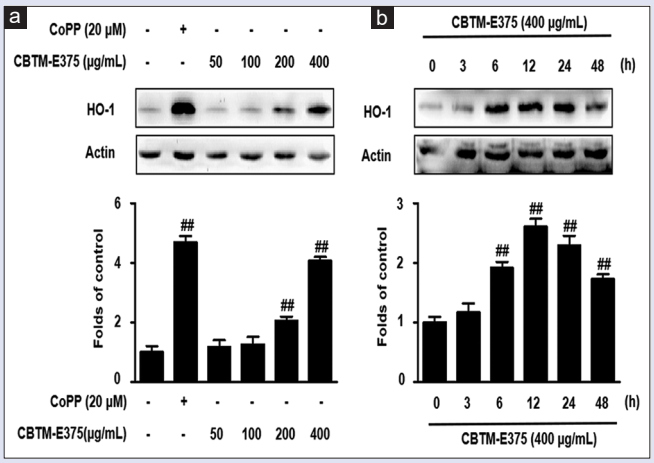


Figure 3: Effects of CBTM- E375 on HO-1 expression. HUVECs were incubated for 12 h with the indicated concentration of CBTM-E375 (a) and periods with 400 μg/mL of CBTM-E375 (b). The HO-1 inducer CoPP increased HO-1 expression at 20 μM (a). Data represent the mean values of 3 experiments±standard deviation. $^{\#\#}P < 0.01$ compared to the control group was considered to be extremely statistically significant. HUVECs: Human umbilical vein endothelial cells; HO-1: Heme oxygenase-1

Effects of CBTM-E375 on the level of inflammatory cytokines in lipopolysaccharide-induced *human umbilical vein endothelial cells*

To determine whether CBTM-E375 was associated with released level of inflammatory cytokines, after treating HUVECs with 1 μg/mL of LPS in the absence or presence, the cells were incubated with different concentrations (50–400 μg/mL) of CBTM-E375 for 24 h. As indicated in Figure 2, compared with the control group, LPS prompted the production of inflammatory cytokines, and CBTM-E375 markedly decreased the production of TNF-α, IL-6, and IL-8 compared to that of LPS-induced group.

Effects of CBTM-E375 on the expression of *heme oxygenase-1* and *nuclear factor erythroid 2-related factor 2* in human umbilical vein endothelial cells

The results of Western blot analysis indicated that CBTM-E375 elevated both the expression of HO-1 and the nuclear translocation of Nrf2 in HUVECs. As shown in Figure 3a, CBTM-E375 dose-dependently upregulated the expression of HO-1. CoPP (20 μM) was used as a positive control in this experiment. Moreover, the expression level of HO-1 in cells was increased in a time-depentent manner with CBTM-E210 treatment. Treatment with CBTM-E375 (400 μg/mL) for 6 h markedly upregulated the expression of HO-1, reaching a peak at 12 h ($P < 0.01$) and gradually decreasing after 24 h [Figure 3b].

Furthermore, we examined whether treatment of HUVECs with CBTM-E375 stimulated the nuclear translocation of Nrf2. As shown in Figure 4a, CBTM-E375 gradually promoted the translocation of Nrf2 from

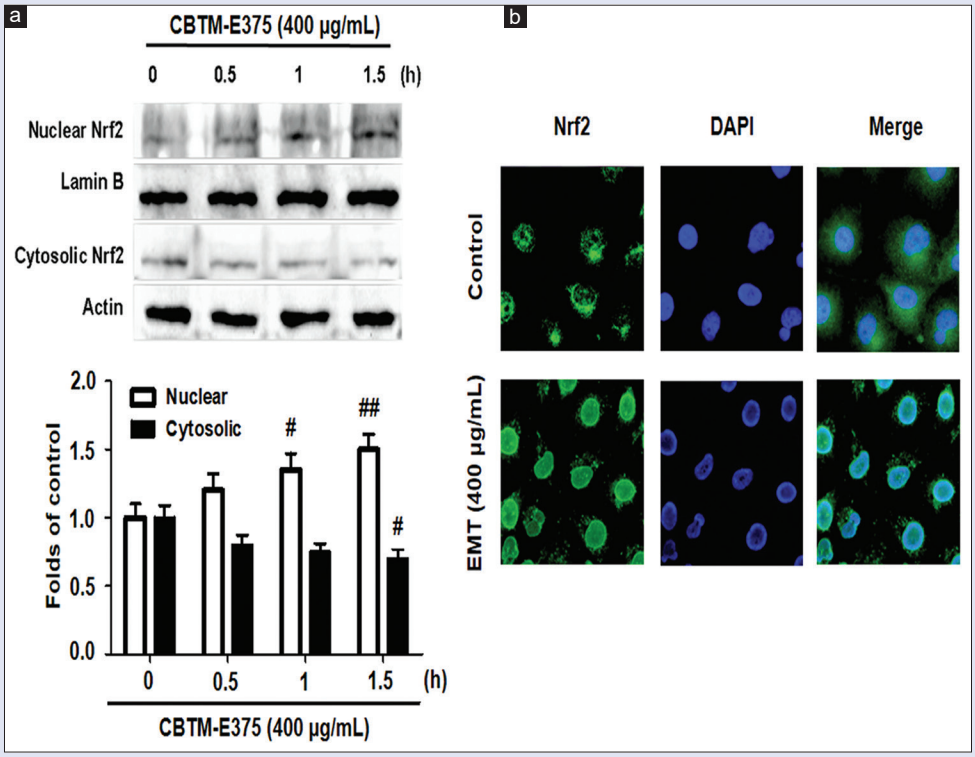


Figure 4: Effects of CBTM-E375 on expression of the nuclear translocation of nuclear factor E2-related factor 2 in human umbilical vein endothelial cells. Human umbilical vein endothelial cells were treated with 400 μg/mL of CBTM-E375 for 0.5 h, 1 h and 1.5 h (a), and Nrf2 translocation at 1.5 h was photographed by immunofluorescence (b). Data represent the mean values of 3 independent experiments±standard deviation. #P < 0.05 compared to the control group was considered to be statistically significant, $^{\#\#}P < 0.01$ compared to the control group was considered to be extremely statistically significant

cytoplasm to nucleus. The localization of Nrf2 by immunofluorescence can be observed more clearly under a microscope [Figure 4b].

Effects of upregulation of heme oxygenase-1 after treatment with CBTM-E375 in inhibiting the inflammation

To verify whether the upregulated expression of HO-1 was crucial for inhibiting LPS-stimulated inflammatory cytokines, the cells were transfected with siRNA-targeting HO-1 and then stimulated with LPS in the 12 h-presence or absence of CBTM-E375 (400 μg/mL). According to the results, CBTM-E375 suppressed the inflammatory effects of increased levels of HO-1 [Figure 5a]. As shown in Figure 5b-d, transfection of siRNA-HO-1 blocked the inhibitory effects of CBTM-E375 toward TNF-α, IL-8, and IL-6.

Effects of CBTM-E375 on the regulation of mitogen-activated protein kinase signaling pathway in lipopolysaccharide-induced human umbilical vein endothelial cells

It has been reported that the phosphorylation of MAPK is involved in endothelial inflammation; therefore, in this study, we studied whether CBTM-E375 affects the activation of MAPK signaling pathways. Our results showed that the phosphorylation of p38, JNK, and ERK was activated by LPS compared to that of the control group. However, CBTM-E375 dose-dependently downregulated the phosphorylation of MAPK with statistical significant difference compared to that of LPS-induced group [Figure 6].

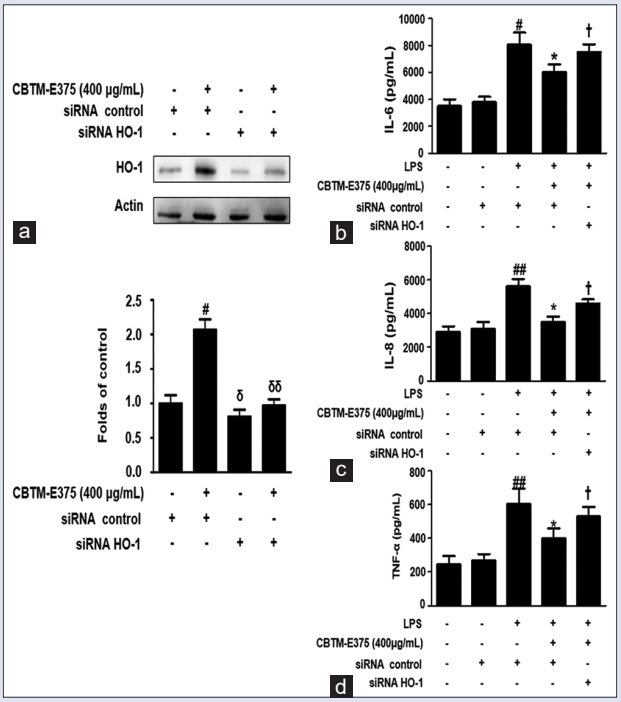


Figure 5: HO-1 mediates the suppressive effects of CBTM-E375 on LPS-stimulated pro-inflammatory cytokines production. HUVEC cells were pretreated for 3 h with CBTM-E375 (400 μg/mL) in the presence or absence of small interfering RNA HO-1(40 μM) and stimulated for 24 h with LPS (1 μg/mL), the HO-1 expression was measured (a) and the levels of IL-6 (b), IL-8 (c) and TNF-α (d) were detected by ELISA. Data represent mean values of 3 experiments ± standard deviation. $^{\#}P < 0.05$ versus control group; $^{\#\#}P < 0.01$ versus control group; $^{\delta}P < 0.05$ versus control group; $^{\delta\delta}P < 0.01$ versus CBTM-E375 group $^{*}P < 0.05$ versus LPS group; $^{\dagger}P < 0.05$ versus LPS + CBTM-E375 group was considered to be statistically significant. HUVECs: Human umbilical vein endothelial cells; HO-1: Heme Oxygenase-1; LPS: Lipopolysaccharide

DISCUSSION

M. toringoides is a traditional Tibetan medicine; its pharmacological activities have not been comprehensively and systematically studied in recent years. From our previous experiments, we had a preliminary understanding of the components and pharmacological effects of CBTM-E375.[11,12] HPLC analysis showed that CBTM-E375 is rich in phloridzin and phloretin, which are potential antioxidants. The flavonoids purified from CBTM-E375 were found to significantly inhibit the inflammatory reactions in LPS-induced macrophages cells.[13] In addition, CBTM-E375 effectively decreased the blood lipid levels and inhibited hepatic oxidative stress in rats fed with high-fat diet.[14]

Some studies have shown that inflammatory response driven by cytokines is the initiator of CVDs.[1,15,16] LPS, one of the strongest stimulating factors in vascular endothelium, can trigger the "waterfall" production of inflammatory cytokines, further accelerating the inflammatory response.[17] TNF-α is a cytokine with a variety of biological effects. It has been shown to progress vascular disorders and can initiate the encoding of inflammatory molecules associated with vascular tone and remodeling, thereby causing endothelial dysfunction.[18] TNF-α can bind to specific receptors, which leads to a series of reactions that cause vascular damage. This, in turn, leads to the production of a variety of adhesion factors and chemokines, which recruit inflammatory cells, aggravate the inflammatory response in the vascular wall, and promote the exacerbation of vascular endothelial damage. These continuous effects may lead to the formation of vascular events such as atherosclerosis.[19] Under inflammatory conditions, the levels of TNF-α and ILs persistently increase, which maintains the inflammatory state and contributes to the organs and arterial damage. The elevated levels of several proinflammatory cytokines such as ILs and several other chemotactic cytokines induced by TNF-α eventually aggravate inflammation. IL-6 is an inflammatory mediator that cannot be ignored in the process of vascular inflammation. When subjected to external stimuli or other signal trends, IL-6 is expressed in large quantities immediately, whereas the sustained synthesis and release level of IL-6 is positively correlated with various systemic syndromes and chronic immune-mediated diseases.[20,21] IL-8, belonging to the CXC chemokine family,[22] is a proinflammatory chemokine, which has been evaluated to be the primary marker in patients with atherosclerosis who are at high risk of developing CVD.[23,24] In this experiment, CBTM-E375 treatment effectively reduced the secretion of TNF-α, IL-6, and IL-8 in LPS-induced HUVECs and the anti-inflammatory effects are positively correlated with the concentrations of CBTM-E375.

The Nrf2/HO-1 pathway has been regarded as an important mechanism in cellular antioxidative and anti-inflammatory defense.[25] Increasing evidences indicate that a large number of phytochemicals protect vascular function by upregulating the translocation and expression of Nrf2/ARE, which can trigger the transcription of many antioxidants such as HO-1, superoxide dismutase, and glutathione peroxidase to decrease the high level of oxidative stress and the invasion of the inflammatory cytokines.[26] In the resting state, the complex formed by Keap1 and Nrf2 is inactive. However, under the stimulation of oxidative stress or inflammatory factors, Keap1 and Nrf2 no longer bind with each other, resulting in the activation and nuclear translocation of Nrf2, leading to the transcription and expression of HO-1 and other anti-inflammatory enzymes.[27] Thus, the activation of Nrf2/HO-1 exerts remarkable effects in maintaining the normal physiological function of endothelial cells. The results of

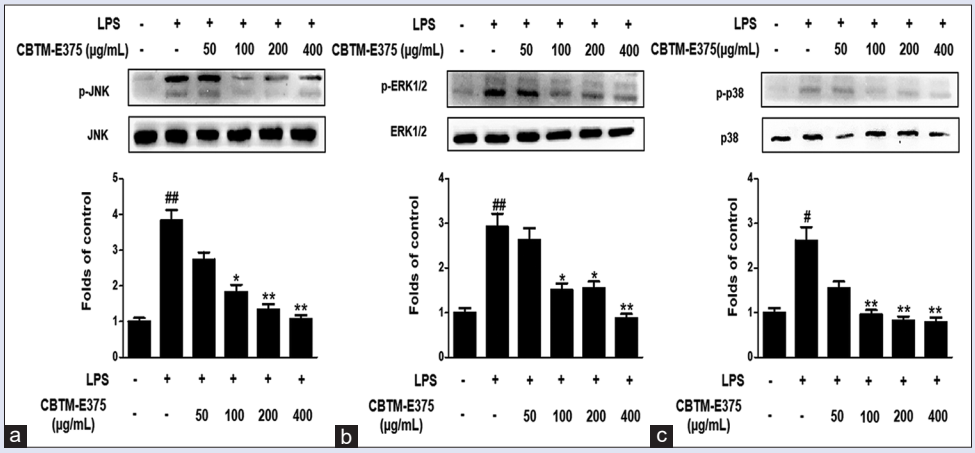


Figure 6: Effects of CBTM-E375 on MAPK (p38, ERK1/2, JNK) phosphorylation. HUVECs were pretreated for 12 h with the indicated concentrations of CBTM-E375 and stimulated for 24h with LPS (1 μg/mL), the expression of JNK (a), ERK (b) and p38 (c) were detected as described in materials and methods and representative blots of 3 independent experiments are shown. Data shown represent the mean values of 3 experiments ± standard deviation. $^{\#}P < 0.05$ versus control group; $^{\#\#}P < 0.01$ versus control group; $^{*}P < 0.05$ versus LPS group; $^{**}P < 0.01$ versus LPS group; was considered to be statistically significant. HUVECs: Human umbilical vein endothelial cells; LPS: Lipopolysaccharide; ERK: Extracellular signal- regulated kinase; JNK: c-Jun-N-terminal kinase; MAPK: Mitogen- activated protein kinase

this study indicate that CBTM-E375 promotes the translocation of Nrf2 from the cytoplasm to the nucleus, which upregulates the expression of HO-1. Furthermore, we demonstrated that CBTM-E375 may increase the expression of HO-1, as in the anti-inflammatory effect of the si-RNA targeting HO-1 experiment. The experiment demonstrated that siRNA targeting HO-1 reversed the anti-inflammatory effect of CBTM-E375.

MAPK signaling pathway is composed of a series of serine/threonine protein kinases activated in cascade. It exhibits highly conserved form in the whole evolution process. The primary function of MAPK signaling pathway is to transform extracellular stimuli into cellular response and transmit extracellular signals to adjacent cells and even nuclei in stages.[28,29] It has been proved that there are three main MAPK cascades. ERK pathway is mainly activated by mitogen, whereas JNK and p38 pathway are mainly activated by environmental and genotoxic stress; therefore, they are also called stress-activated protein kinases. The MAPK signaling pathway participates in the process of many diseases, such as atherosclerosis, cancer, and neuroinflammation, which inevitably involves in multiple cellular processes including cell apoptosis, proliferation, and differentiation, as well as regulating the development of inflammation.[30,31] Thus, MAPK signaling pathways are involved in multiple forms of cell-to-cell communication, which especially is closely related to the development of inflammation. Many studies have shown that inhibiting the phosphorylation of MAPK is beneficial to the reduction and disappearance of inflammation.[32,33] In this study, CBTM-E375 treatment not only inhibited one kind of MAPK phosphorylation, but also effectively blocked all three kinds of the phosphorylation induced by LPS, which suggests that an improvement in vascular inflammation is related to an inhibition of phosphorylation in the MAPK signaling pathway associated with CBTM-E375 treatment.

CONCLUSION

In conclusion, *M. toringoides* exhibited anti-inflammatory activity possibly through the upregulation of HO-1, reducing the release of proinflammatory mediators, and inhibiting the activation of MAPK signaling pathways.

Financial support and sponsorship

The work was supported by National Science Foundation for Distinguished Young Scholars of China (No. 31500288), the National Natural Science Foundation of China (No. 81760779) and (No. 81360686).

Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Mak A, Kow NY. Imbalance between endothelial damage and repair: A gateway to cardiovascular disease in systemic lupus erythematosus. Biomed Res Int 2014;2014:178721.
2. Incalza MA, D'Oria R, Natalicchio A, Perrini S, Laviola L, Giorgino F. Oxidative stress and reactive oxygen species in endothelial dysfunction associated with cardiovascular and metabolic diseases. Vascul Pharmacol 2018;100:1-19.
3. Sitia S, Tomasoni L, Atzeni F, Ambrosio G, Cordiano C, Catapano A, *et al.* From endothelial dysfunction to atherosclerosis. Autoimmun Rev 2010;9:830-4.
4. Lertkiatmongkol P, Liao D, Mei H, Hu Y, Newman PJ. Endothelial functions of platelet/endothelial cell adhesion molecule-1 (CD31). Curr Opin Hematol 2016;23:253-9.
5. Golia E, Limongelli G, Natale F, Fimiani F, Maddaloni V, Pariggiano I, *et al.* Inflammation and cardiovascular disease: From pathogenesis to therapeutic target. Curr Atheroscler Rep 2014;16:435.
6. Park SY, Jin ML, Yi EH, Kim Y, Park G. Neochlorogenic acid inhibits against LPS-activated inflammatory responses through up-regulation of Nrf2/HO-1 and involving AMPK pathway. Environ Toxicol Pharmacol 2018;62:1-10.
7. Yeung YT, Aziz F, Guerrero-Castilla A, Arguelles S. Signaling pathways in inflammation and anti-inflammatory therapies. Curr Pharm Des 2018;24:1449-84.
8. Kwok KH, Cheng KK, Hoo RL, Ye D, Xu A, Lam KS. Adipose-specific inactivation of JNK alleviates atherosclerosis in apoE-deficient mice. Clin Sci (Lond) 2016;130:2087-100.
9. Li D, Hu J, Wang T, Zhang X, Liu L, Wang H, *et al.* Silymarin attenuates cigarette smoke extract-induced inflammation via simultaneous inhibition of autophagy and ERK/p38 MAPK pathway in human bronchial epithelial cells. Sci Rep 2016;6:37751.
10. Li D, Peng C, Xie X, Mao Y, Li M, Cao Z, *et al.* Antidiabetic effect of flavonoids from *Malus toringoides* (Rehd.) Hughes leaves in diabetic mice and rats. J Ethnopharmacol 2014;153:561-7.
11. Fan Z, Wang Y, Yang M, Cao J, Khan A, Cheng G. UHPLC-ESI-HRMS/MS analysis on phenolic compositions of different E Se tea extracts and their antioxidant and cytoprotective activities. Food Chem 2020;318:126512.

12. Ye Q, Guo L, Liu HM, Liu YS. Optimization of ultrasound-assisted extraction on antioxidative activity of *Malus toringoides* using response surface methodology. Processes 2019;7:270.
13. Chang BQ, Li B, Yuan RY, Zhuoma DZ, Huang S. Separation and purification of flavonoids in *Malus toringoides* (Rehd.) Hughes leaves with macroporous resins and evaluation of anti-inflammation activities. Drug Cosmet Mater 2019;6:1149-58.
14. Huang S, Liu H, Meng N, Li B, Wang J. Hypolipidemic and antioxidant effects of *Malus toringoides* (Rehd.) Hughes leaves in high-fat-diet-induced hyperlipidemic rats. J Med Food 2017;20:258-64.
15. Haybar H, Shokuhian M, Bagheri M, Davari N, Saki N. Involvement of circulating inflammatory factors in prognosis and risk of cardiovascular disease. J Mol Cell Cardiol 2019;132:110-9.
16. Berg AH, Scherer PE. Adipose tissue, inflammation, and cardiovascular disease. Circ Res 2005;96:939-49.
17. Seeley JJ, Ghosh S. Molecular mechanisms of innate memory and tolerance to LPS. J Leukoc Biol 2017;101:107-19.
18. Saha P, Smith A. TNF-α (Tumor necrosis factor-α). Arterioscler Thromb Vasc Biol 2018;38:2542-3.
19. Gao W, Liu HB, Yuan J, Wu CN, Huang D, Ma YJ, *et al*. Exosomes derived from mature dendritic cells increase endothelial inflammation and atherosclerosisviamembrane TNF-α mediated NF-κB pathway. J Cell Mol Med 2016;20:2318-27.
20. Tanaka T, Narazaki M, Kishimoto T. IL-6 in inflammation, immunity, and disease. Cold Spring Harb Perspect Biol 2014;6:a016295.
21. Hunter CA, Jones SA. IL-6 as a keystone cytokine in health and disease. Nat Immunol 2015;16:448-57.
22. Ghasemi H, Ghazanfari T, Yaraee R, Faghihzadeh S, Hassan ZM. Roles of IL-8 in ocular inflammations: A review. Ocul Immunol Inflamm 2011;19:401-12.
23. Russo RC, Garcia CC, Teixeira MM, Amaral FA. The CXCL8/IL-8 chemokine family and its receptors in inflammatory diseases. Expert Rev Clin Immunol 2014;10:593-619.
24. Chen L, Liu X, Pan Z, Liu S, Han H, Zhao C, *et al*. The role of IL-8/CXCR2 signaling in microcystin-LR triggered endothelial cell activation and increased vascular permeability. Chemosphere 2018;194:43-8.
25. Ge M, Yao W, Yuan D, Zhou S, Chen X, Zhang Y, *et al*. Brg1-mediated Nrf2/HO-1 pathway activation alleviates hepatic ischemia-reperfusion injury. Cell Death Dis 2017;8:e2841.
26. Loboda A, Damulewicz M, Pyza E, Jozkowicz A, Dulak J. Role of Nrf2/HO-1 system in development, oxidative stress response and diseases: An evolutionarily conserved mechanism. Cell Mol Life Sci 2016;73:3221-47.
27. Song Y, Huang L, Yu J. Effects of blueberry anthocyanins on retinal oxidative stress and inflammation in diabetes through Nrf2/HO-1 signaling. J Neuroimmunol 2016;301:1-6.
28. Lee Y, Kim YJ, Kim MH, Kwak JM. MAPK cascades in guard cell signal transduction. Front Plant Sci 2016;7:80.
29. Martínez-Limón A, Joaquin M, Caballero M, Posas F, de Nadal E. The p38 pathway: From biology to cancer therapy. Int J Mol Sci 2020;21:1913.
30. Huang P, Han J, Hui L. MAPK signaling in inflammation-associated cancer development. Protein Cell 2010;1:218-26.
31. Wu W, Zhang J, Zhao C, Sun Y, Pang W, Yang G. CTRP6 regulates porcine adipocyte proliferation and differentiation by the AdipoR1/MAPK signaling pathway. J Agric Food Chem 2017;65:5512-22.
32. Deng GL, Wang LL, He WY, Zhou W, Chen BW, Bing S. Anti-inflammatory effects of neferine on LPS-induced human endothelium via MAPK and NF-κB pathways. Pharmazie 2018;73:541-4.
33. Stark R, Choi H, Koch S, Lamb F, Sherwood E. Monophosphoryl lipid A inhibits the cytokine response of endothelial cells challenged with LPS. Innate Immun 2015;21:565-74.