Original Article

Rhubarb Antagonizes Matrix Metalloproteinase-9-induced Vascular Endothelial Permeability

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Abstract

Background: Intact endothelial structure and function are critical for maintaining microcirculatory homeostasis. Dysfunction of the latter is an underlying cause of various organ pathologies. In a previous study, we showed that rhubarb, a traditional Chinese medicine, protected intestinal mucosal microvascular endothelial cells in rats with metastasizing septicemia. In this study, we investigated the effects and mechanisms of rhubarb on matrix metalloproteinase-9 (MMP9)-induced vascular endothelial (VE) permeability.

Methods: Rhubarb monomers were extracted and purified by a series of chromatography approaches. The identity of these monomers was analyzed by hydrogen-1 nuclear magnetic resonance (NMR), carbon-13 NMR, and distortionless enhancement by polarization transfer magnetic resonance spectroscopy. We established a human umbilical vein endothelial cell (HUVEC) monolayer on a Transwell insert. We measured the HUVEC permeability, proliferation, and the secretion of VE-cadherin into culture medium using fluorescein isothiocyanate-dextran assay, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide assay, and enzyme-linked immunosorbent assay, respectively, in response to treatment with MMP9 and/or rhubarb monomers.

Results: A total of 21 rhubarb monomers were extracted and identified. MMP9 significantly increased the permeability of the HUVEC monolayer, which was significantly reduced by five individual rhubarb monomer (emodin, 3,8-dihydroxy-1-methyl-anthraquinone-2-carboxylic acid, 1-O-caffeoyl-2-(4-hydroxyl-O-cinnamoyl)-β-D-glucose, daucosterol linoleate, and rhein) or a combination of all five monomers (1 μmol/L for each monomer). Mechanistically, the five-monomer mixture at 1 μmol/L promoted HUVEC proliferation. In addition, MMP9 stimulated the secretion of VE-cadherin into the culture medium, which was significantly inhibited by the five-monomer mixture.

Conclusions: The rhubarb mixture of emodin, 3,8-dihydroxy-1-methyl-anthraquinone-2-carboxylic acid, 1-O-caffeoyl-2-(4-hydroxyl-O-cinnamoyl)-β-D-glucose, daucosterol linoleate, and rhein, at a low concentration, antagonized the MMP9-induced HUVEC monolayer permeability by promoting HUVEC proliferation and reducing extracellular VE-cadherin concentrations.

Key words: Matrix Metalloproteinase-9; Monomer; Permeability; Rhubarb; Vascular Endothelial-cadherin; Vascular Endothelial Cell

Introduction

Capillary walls, consisting of a collagen matrix and a monolayer of endothelial cells connected through intercellular tight junction proteins, are regulators of metabolism and various substance exchanges between the blood and extracellular fluid.[1,2] Under pathological conditions, such as inflammation and trauma, the increase of capillary permeability, changes in blood flow, apoptosis of capillary cells, and down-regulation of tight junction proteins could result in capillary leakage and multiple organ dysfunction syndrome.[3-5] Therefore, protecting vascular endothelial (VE) cells is critical for targeting these pathological processes. In our previous study, we showed that intragastric administration of rhubarb, a traditional Chinese medicine, protected the intestinal mucosal capillary endothelial cells from damage in rats with metastasizing septicemia. It also increased the number of functional capillaries, promoted blood flow through intestinal mucosal

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capillaries, reduced thrombogenesis, and improved the blood and oxygen supply of the intestinal mucosa.[6] However, since rhubarb contains multiple compounds, it remains unclear which compound(s) plays a key role in capillary protection during metastasizing septicemia.

To address this question, 21 rhubarb monomers were extracted and identified. By using a Transwell permeability assay, the effects of rhubarb monomers on the permeability of human umbilical vein vascular endothelial cell (HUVEC) monolayers under inflammatory conditions was measured, and the effects of these monomers on HUVEC proliferation and secretion of VE-cadherin were examined to discuss the underlying mechanisms.

Methods

Reagents

The following reagents were used in this study: rhubarb (Shanghai Cai Tong De Tang Pharmacy, Shanghai, China); HUVEC (ATCC, Manassas, VA, USA); dimethylsulfoxide (DMSO), 3-(4,5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2-H-tetrazolium bromide (MTT), Dulbecco's modified Eagle's medium (DMEM) high-glucose culture medium, fetal bovine serum (FBS), pancreatin, penicillin, and streptomycin (Hyclone, Logan, UT, USA); dexamethasone (DEX; Zhejiang Xianju Pharmaceutical Co. Ltd., Zhejiang, China); 24-well Transwell chambers (0.4-μm aperture; Corning Incorporated, Manassas, VA, USA); fluorescein isothiocyanate-dextran (FD40, 40,000; Sigma-Aldrich, St. Louis, MO, USA); human VE-cadherin enzyme-linked immunosorbent assay (ELISA) test kit (RapidBio, Tucson, AZ, USA); and human recombinant matrix metalloproteinase-9 (MMP9; RayBiotech, Norcross, GA, USA).

Extraction of rhubarb monomers

Rhubarb (1.5 kg) was smashed and extracted with 80% ethanol (10 × volume) under heat reflux three times (2 h each time). The extracts were mixed and concentrated by decompression until there was no ethanol remaining. Then, the extracts were mixed with two liters of water to form a liquid. Petroleum ether, dichloromethane, acetic ether, and N-butyl alcohol were used sequentially to extract the rhubarb (2 L × 3 for each solvent, respectively). After decompression, four extraction fractions were obtained from each extraction liquid. Acetone, chloroform, and carbinol were used to dissolve the petroleum ether fraction, dichloromethane fraction, and acetic ether fraction, respectively. Silica gel (100–200 meshes) was added to the chromatographic column.

After the solvent was vaporized, silica gel column chromatography laminar analyses were performed (4–6 cm chromatographic column diameter, 40–100 cm length, and 100–250 g of 100–200 meshes silica gel). Petroleum ether/acetone (20:1/2:1) or dichloromethane/carbinol (50:1/10:1) was used to perform gradient elutions (50:1/5:1). A thin layer chromatography silica gel plate was used to separate the fractions. The 10% sulfuric acid ethanol coloration method was used to detect the fractions, and similar fractions were merged. Silica gel column chromatography, sephadex gel column chromatography, octadecyl silane (ODS) C18 reverse phase silica gel column chromatography, and middle chromatogram isolated (MCI) column chromatography were repeatedly used to separate and purify the compounds.[7,8] After obtaining the monomers, hydrogen-1 nuclear magnetic resonance (NMR), carbon-13-NMR, and distortionless enhancement by polarization transfer magnetic resonance spectroscopy (JEOL Ltd., Japan) were used for structural characterization of all compounds [Figure 1]. The extracted monomers were identified as chrysophanol,[9] emodin,[10] citreorosein,[11] 3,8-dihydroxy-1-methyl-anthraquinone-2-carboxylic acid,[12] catectin,[13] eriodictyol,[14] naringin,[15] resveratrol,[16] 1-O-galloyl-2-O-cinnamoyl-β-D-glucose,[17] 1-O-caffeoyl-2-(4-hydroxyl-O-cinnamoyl)-β-D-glucose,[18] caffeic acid,[19] 4-hydroxyl-cinnamic acid,[20] 4-hydroxyl-benzoic acid,[21] 9,12,13-trihydroxyoctadecadienoicacid-10(E)-acid,[22] daucosterol,[23] daucosterol linoleate,[24] 3,8-dihydroxy-1-methyl-anthraquione,[25] cassialoin,[26] aloe-emodin,[27] physcion,[28] and rhein[29] [Table 1].

Cell culture

HUVEC were cultured in DMEM media containing 10% FBS, penicillin 100 units/ml, and streptomycin 100 μg/ml at

Table 1: Identification results of rhubarb monomers

No.	Names	Molecular formula	Molecular weight (g/mol)
1	Chrysophanol	$C_{15}H_{10}O_4$	254
2	Emodin	$C_{15}H_{10}O_5$	270
3	Citreorosein	$C_{15}H_{10}O_6$	286
4	3,8-dihydroxy-1-methyl-anthraquinone-2-carboxylic acid	$C_{16}H_{10}O_5$	298
5	Catectin	$C_{15}H_{14}O_6$	290
6	Eriodictyol	$C_{15}H_{12}O_6$	288
7	Naringin	$C_{15}H_{12}O_5$	272
8	Resveratol	$C_{14}H_{12}O_3$	228
9	1-O-galloyl-2-O-cinnamoyl-β-D-glucose	$C_{22}H_{22}O_{11}$	462
10	1-O-caffeoyl-2-(4-hydroxyl-O-cinnamoyl)-β-D-glucose	$C_{24}H_{24}O_{11}$	488
11	Caffeic acid	$C_9H_8O_4$	180
12	4-hydroxyl-cinnamic acid	$C_9H_8O_3$	164
13	4-hydroxyl-benzoic acid	$C_7H_6O_3$	138
14	9,12,13-trihydroxyoctadecadienoicacid-10(E)-acid	$C_{18}H_{34}O_5$	330
15	Daucosterol	$C_{35}H_{60}O_6$	576
16	3,8-dihydroxy-1-methyl-anthraquione	$C_{15}H_{10}O_4$	254
17	Daucosterol linoleate	$C_{52}H_{88}O_7$	824
18	Cassialoin	$C_{21}H_{22}O_9$	418
19	Aloe-emodin	$C_{15}H_{10}O_5$	270
20	Physcion	$C_{16}H_{12}O_5$	284
21	Rhein	$C_{15}H_8O_6$	284

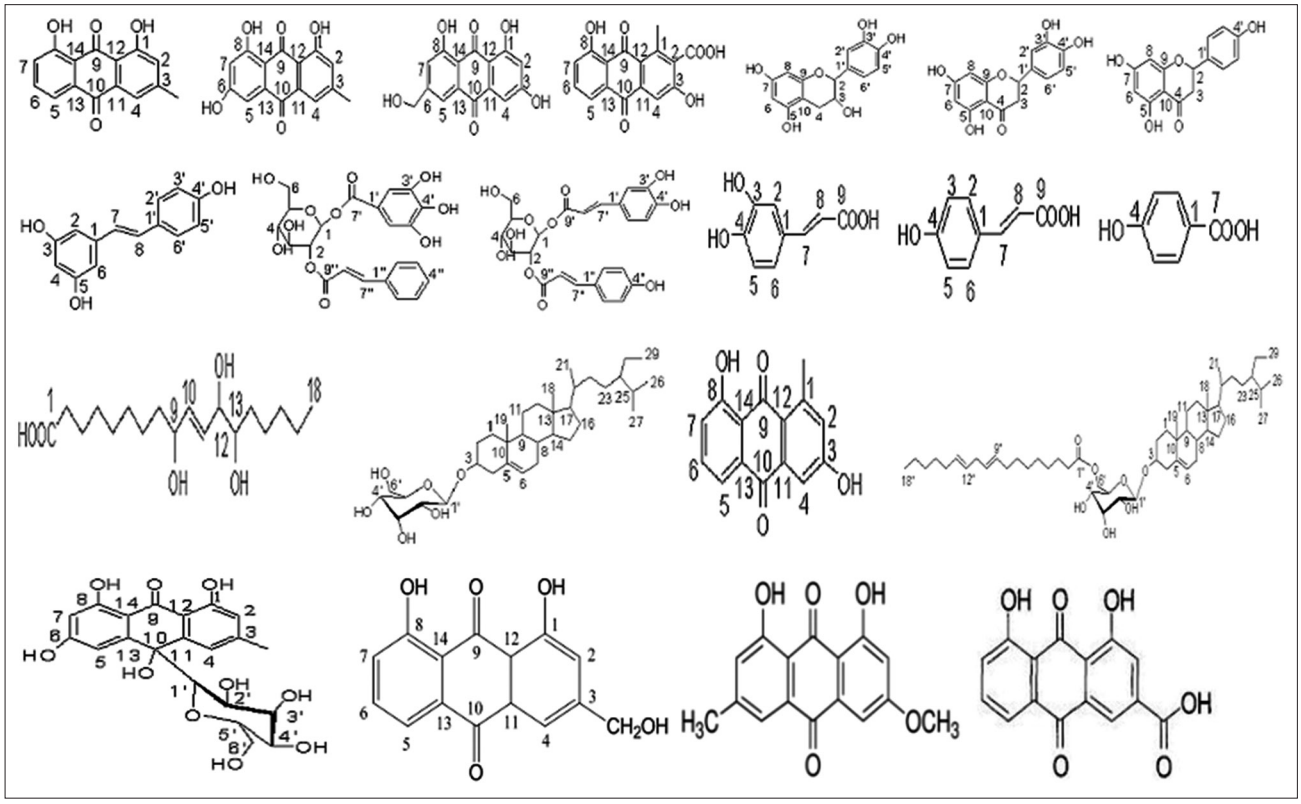

Figure 1: Molecular structures of rhubarb monomers. Row 1 contains monomers 1–7, row 2 contains monomers 8–13, row 3 contains monomers 14–17, and row 4 contains monomers 18–21.

37°C in a humidified atmosphere with 5% CO_2. The media was changed every 2 days until the cells reached 80–90% confluence. All experiments were performed using cells within twenty passages.

Human umbilical vein endothelial cell monolayer permeability assay

HUVEC (1×10^5 cells) were seeded on top of the Transwell insert in the 24-well Transwell chambers and cultured for 24 h to reach confluence. The samples were randomly divided into four groups: control group, MMP9 treated group, rhubarb monomers group, and DEX group. The upper and lower compartments were filled with media alone (control group), media containing MMP9 (1 mg/L; MMP9 group), the rhubarb monomers (1 μmol/L, 10 μmol/L, or 50 μmol/L) plus MMP9 (1 mg/L) (rhubarb monomer group), or 10 μmol/L DEX plus 1 mg/L MMP9 (DEX group). After 24 h incubation, the upper and lower layers of the culture media were collected and stored at −80°C. Phosphate buffered saline (PBS) 100 μl was used to wash the chambers twice. Then, PBS 600 μl was added to the lower layer, and PBS 100 μl containing FD40 (100 mg/L) was added to the upper layer. The Transwell chamber was incubated for 1 h at 37°C. The PBS in the lower compartments was collected and analyzed in a fluorescence spectrophotometer (Varian Inc., USA) at 547 nm and 572 nm excitation and emission wavelengths, respectively.

3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide assay on human umbilical vein endothelial cell proliferation

The MTT assay was used to detect the proliferation of HUVEC in response to different treatments. Briefly, HUVEC (4000 cells/200 μl medium per well) were seeded into a 96-well plate and cultured for 24 h. Then, the cells were treated as indicated for 24 h. For the highest concentration of rhubarb monomer group (50 mol/L) that was dissolved in 0.425% DMSO, we used the same concentration DMSO as the vehicle control. After treatment, MTT (5 mg/ml, 20 μl/well) was added to the plates and incubated for 4 h at 37°C. Then, the media was replaced with DMSO 150 μl, and the plates were agitated for 10 min to dissolve the formanzan. The plates were measured for absorbance at 490 nm.

Detection of vascular endothelial-cadherin concentration in culture supernatant

The culture media stored at −80°C was centrifuged at 846 ×*g* for 10 min to remove any cell debris, and the supernatant was collected. The concentration of VE-cadherin in the medium was detected using an ELISA assay kit (R&D, Minneapolis, MN, USA) following the manufacturer's instructions.

Statistical analysis

The SPSS version 18.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analyses. The data were expressed as a mean ± standard deviation (SD). One-way

analysis of variance (ANOVA) was used to analyze the differences among the groups. Inter-group comparisons were performed using the least significant difference test, and the nonparametric rank-sum test was used when the variance was not homogeneous. A $P < 0.05$ was considered to be statistically significant.

Results

Protect effects of rhubarb monomers on human umbilical vein endothelial cell monolayer permeability

Compared to the control group, MMP9 group significantly increased HUVEC permeability ($t = 3.77$, $P = 0.003$), which was significantly reversed by DEX group ($t = 3.30$, $P = 0.009$, as compared to the MMP9 group; Figure 2). When incubating the cells with each of the 21 rhubarb monomers (1 μmol/L) together with MMP9 for 24 h, monomer 2 ($t = 4.40$, $P < 0.001$), monomer 4 ($t = 3.40$, $P = 0.001$), monomer 10 ($t = 4.80$, $P < 0.001$), monomer 17 ($t = 2.30$, $P = 0.025$), and monomer 21 ($t = 4.20$, $P < 0.001$) [number according to Table 1] significantly decreased HUVEC permeability. When the concentration of the monomers were increased to 10 μmol/L, however, the protective effects against MMP9-induced permeability were reduced; most monomers, except for monomer 21 ($t = 2.22$, $P = 0.032$, as compared to the MMP9 group), lost the protection against MMP9-induced permeability. At 50 μmol/L, all monomers induced a higher HUVEC permeability than MMP9 alone [Figure 2].

Given that monomers 2, 4, 10, 17, and 21 at 1 μmol/L all significantly reduced HUVEC permeability in response to MMP9, we mixed these five monomers together to a final concentration of 1 μmol/L, and added them together with MMP9 to HUVEC monolayers. We found that HUVEC monolayer permeability was significantly decreased with this combination as compared to MMP9 alone ($t = 23.20$, $P < 0.001$). The five monomer mixture was also superior to DEX in protecting MMP9-induced HUVEC permeability ($t = 9.50$, $P < 0.0001$; Figure 3).

Human umbilical vein endothelial cell proliferation was increased by low concentrations of the rhubarb five-monomer mixture

Given that cell proliferation plays a critical role in endothelial permeability, we next examined the effect of the rhubarb five-monomer mixture on HUVEC proliferation. As shown in Figure 4, at a final concentration of 1 μmol/L, the five-monomer mixture increased HUVEC proliferation by 6.6% over the control group ($t = 2.04$, $P = 0.043$). However, when the total concentrations of all the five monomers were increased to 10 μmol/L ($t = -2.30$, $P = 0.021$, as compared to the control group) and 50 μmol/L ($t = -3.90$, $P < 0.001$, as compared to the control group), respectively, HUVEC proliferation was significantly suppressed (by 7.9% and 15.3%, respectively) in a dose-dependent manner. Inclusion of 0.425% DMSO did not affect HUVEC proliferation.

Vascular endothelial-cadherin secretion was reduced by rhubarb monomers

After MMP9 treatment, the culture media from the upper ($t = 5.40$, $P < 0.001$) and lower chamber ($t = 4.50$, $P < 0.001$) of the Transwell system contained increased concentrations of VE-cadherin ($P < 0.001$, as compared to the control group). Co-treatment with rhubarb monomer 2, 4, 10, 17, 21, their mixture (1 μmol/L), or DEX (10 μmol/L). It is significantly reduced the secretion of VE-cadherin into the culture media that intervened with rhubarb monomer 2 ($t = 3.20$, $P = 0.003$), monomer 4 ($t = 4.10$, $P < 0.001$), monomer 10 ($t = 2.30$, $P < 0.028$), monomer 17 ($t = 3.20$, $P = 0.003$), monomer 21 ($t = 2.60$, $P = 0.014$), their

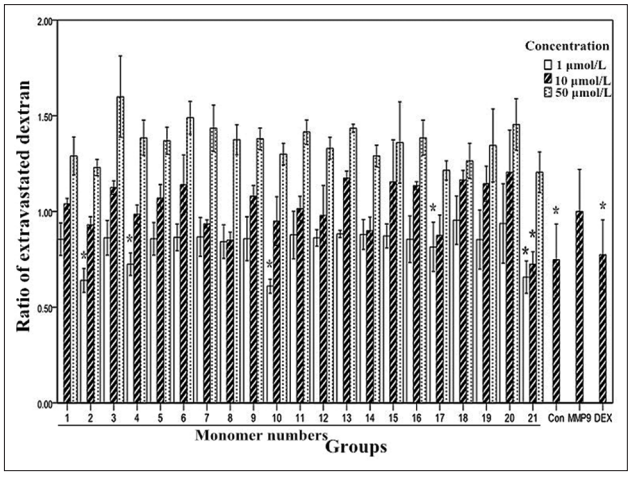

Figure 2: Effects of rhubarb monomers on MMP9-induced HUVEC permeability. MMP9 (1 mg/L) alone or together with rhubarb monomers (1 μmol/L, 10 μmol/L, and 50 μmol/L) or with DEX (10 μmol/L) were incubated with an HUVEC monolayer for 24 h, and the cell permeability was measured. *$P < 0.05$, as compared to the MMP9 group. Con: Control group; MMP9: Matrix metalloproteinase-9; DEX: Dexamethasone; HUVEC: Human umbilical vein endothelial cell.

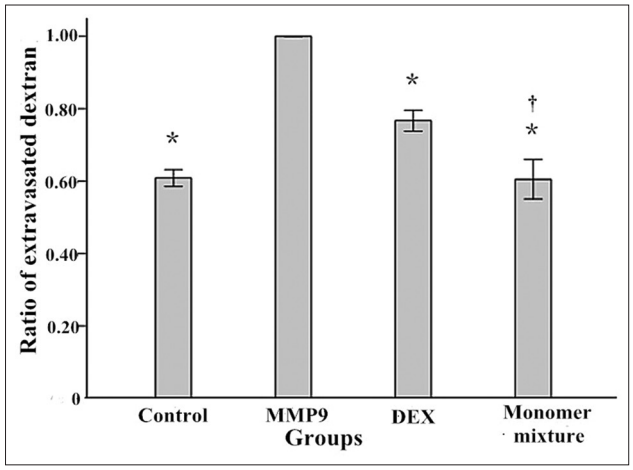

Figure 3: Effect of the rhubarb five-monomer mixture on MMP9-stimulated HUVEC permeability. MMP9 (1 mg/L) and the rhubarb five-monomer mixture (1 μmol/L, 10 μmol/L, and 50 μmol/L) or DEX (10 μmol/L) were incubated with an HUVEC monolayer for 24 h, and the cell permeability was measured. *$P < 0.05$, as compared to the MMP9 group; †$P < 0.05$, as compared to the DEX group. MMP9: Matrix metalloproteinase-9; DEX: Dexamethasone; HUVEC: Human umbilical vein endothelial cell.

mixture ($t = 3.80$, $P < 0.001$) at 1 μmol/L, or DEX ($t = 3.30$, $P = 0.002$) at 10 μmol/L, compared to the MMP9 group in the upper chamber. Moreover in the lower chamber, the concentration of VE-cadherin was also lower in the rhubarb monomer 2 ($t = 4.20$, $P < 0.001$), monomer 4 ($t = 4.30$, $P < 0.001$), monomer 10 ($t = 2.90$, $P = 0.006$), monomer 17 ($t = 3.80$, $P < 0.001$), monomer 21 ($t = 2.40$, $P = 0.02$) and their mixture group ($t = 4.20$, $P < 0.001$), and the DEX group ($t = 3.90$, $P < 0.001$), compared with the MMP9 group [Figure 5].

Discussion

In the present study, through crude extraction, chromatography analyses, and magnetic resonance spectroscopy analyses, we isolated and identified 21 monomers from rhubarb. Using a Transwell permeability analysis, we found that of the 21 monomers, emodin, 3,8-dihydroxy-1-methyl-anthraquinone-2-carboxylic acid, 1-O-caffeoyl-2-(4-hydroxyl-O-cinnamoyl)-β-D-glucose, daucosterol linoleate, rhein, and their mixture at a low concentration (1 μmol/L) protected against MMP9-induced HUVEC permeability. Mechanistically, this effect on permeability was achieved through an increase in HUVEC proliferation and a reduction in VE-cadherin secretion.

In our previous study, we demonstrated that rhubarb has specific curative effects on gastrointestinal failures in rats with metastasizing septicemia.[6,30] The intragastric administration of rhubarb powder protected the intestinal mucosal capillary endothelial cells during metastasizing septicemia, which was associated with an increasing number of functional capillaries, stimulated blood flow of capillaries, reduced thrombogenesis, and improved supply of blood and oxygen to the intestinal mucosa. Meanwhile, experiments in rat models of hemorrhagic shock and metastasizing septicemia proved that rhubarb inhibited intestinal bacteria and endotoxin translocation and improved intestinal mucosa blood perfusion.[31] Fang *et al.*[32] also confirmed that raw rhubarb reduced small intestine permeability.

In this study, we identified 21 rhubarb monomers and investigated their effects on VE cell functions. Our data showed that five rhubarb monomers had protective effects on MMP9-induced HUVEC permeability. Consistent with our results, Zhang *et al.*[33] showed that chrysophanic acid improved the permeability of the blood-brain barrier by inhibiting NALP3 inflammasome activation in rats with injury-induced cerebral ischemia reperfusion by transient middle cerebral artery occlusion. Interestingly, an *in vitro* study demonstrated that emodin-induced apoptosis in human Hep-G2 cells.[34] The mechanism might be attributed to emodin's ability to cause damage of the mitochondrial barrier and mitochondrial dysfunction in cancer cells. Our data suggested that at a low concentration, emodin promoted HUVEC proliferation. One explanation for the different results might be due to the difference in cell types. The permeability of the endothelial cell monolayer is closely related to cell proliferation, and the expression and function of intercellular connexin protein.[35-37] However, the Bcl-2 protein family, which is located in the outer mitochondrial membrane and regulates the mitochondrial permeability transition pore channel, is the main cause of the outer membrane nonspecific fracture that increases permeability and apoptosis factor release.[38-40]

Hu *et al.*[41] showed that aloe-emodin inhibited the lipopolysaccharide-induced inflammatory response in RAW264.7 macrophages. There was no cytotoxic

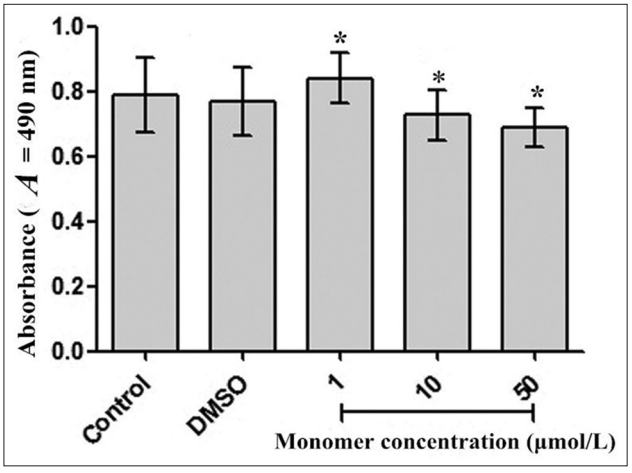

Figure 4: Effects of the rhubarb five-monomer mixture on HUVEC proliferation. HUVEC were treated as indicated and cell proliferation was measured by MTT assay. *$P < 0.05$, as compared to control group. HUVEC: Human umbilical vein endothelial cell; MTT: 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide.

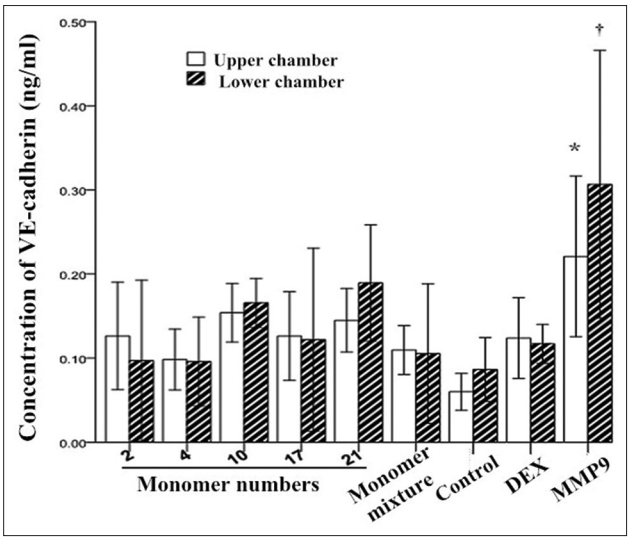

Figure 5: Effects of individual rhubarb monomers and the five-monomer mixture on VE-cadherin secretion from HUVEC. Individual rhubarb monomers (1 μmol/L), the five-monomer mixture (1 μmol/L), and DEX (10 μmol/L) were combined with MMP9 (1 μmol/L) to stimulate HUVEC for 24 h. Secretion of VE-cadherin into the medium was detected by enzyme-linked immunosorbent assay. *$P < 0.001$, †$P < 0.001$, as compared to all other groups from the upper and lower well, respectively. MMP9: Matrix metalloproteinase-9; DEX: Dexamethasone; HUVEC: Human umbilical vein endothelial cell; VE: Vascular endothelial.

effect when the aloe-emodin was added to RAW264.7 macrophages at 0.1–40 μmol/L. In contrast, Cai *et al.*[42] reported that 48 h co-culture of emodin (50 μmol/L) in four different types of pancreatic cancer cells significantly inhibited cell proliferation. The mechanism might be that emodin increases the cancer cell apoptosis. Wang *et al.*[43] revealed that a rhubarb extract of emodin, parietic acid, and gallic acid significantly inhibited the proliferation of hypertrophic scar fibroblasts. These inhibitory effects were dose-dependent between 7 μmol/L and 70 μmol/L. Our study also demonstrated that 1 μmol/L of rhubarb monomer mixture promoted HUVEC proliferation, but 10 μmol/L and 50 μmol/L inhibited it, indicating that the biological effects might be related to the concentration of the monomer. In other words, low concentrations of the rhubarb monomer mixture had no cytotoxic effects and promoted cell proliferation, whereas high concentrations are cytotoxic and significantly inhibited cell proliferation.

VE-cadherin is one of the essential components for cell adhesion. Changes to VE-cadherin structure and function cause damage to cell connections, generation of intercellular gaps, and increase of vaso-permeability.[44,45] Gao *et al.*[46] illustrated that decreased VE-cadherin may result in significantly increased permeability in microvascular endothelial cells. Soluble VE-cadherin is an extracellular component of VE-cadherin and exists in blood and tissue fluid. Cumulative studies have shown that increased soluble VE-cadherin was involved in various diseases including coronary heart disease,[47] multiple myelomas,[48] and colon cancer.[49] However, no study has demonstrated a relationship between VE-cadherin concentrations and vasopermeability during metastasizing septicemia. At present, the damaging effects of MMP9 on the extracellular matrix of VE cells and the basement membrane of blood vessels are widely known. The mechanisms may be that MMP9 degrades the basement membrane Type IV collagen, laminin, and other components of the capillary.[50,51] In our study, we found that MMP9 enhanced HUVEC monolayer permeability and stimulated these cells to secrete more soluble VE-cadherin proteins into the culture media. Rhubarb monomer treatment resulted in significantly reduced VE-cadherin protein concentrations.

In conclusion, our study suggested that the rhubarb mixture of emodin, 3,8-dihydroxy-1-methyl-anthraquinone-2-carboxylic acid, 1-O-caffeoyl-2-(4-hydroxyl-O-cinnamo yl)-β-D-glucose, daucosterol linoleate, and rhein, at a low concentration, antagonized the MMP9-induced HUVEC monolayer permeability by promoting HUVEC proliferation and reducing extracellular VE-cadherin concentrations. These effects can be utilized to protect VE cells from inflammatory damage and reduce the permeability of VE monolayers to prevent capillary leakage.

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Conflicts of interest

There are no conflicts of interest.

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