Research Article

Comparison of Three Species of Rhubarb in Inhibiting Vascular Endothelial Injury via Regulation of PI3K/AKT/NF-κB Signaling Pathway

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Background/Aim. Rhubarb, a traditional Chinese medicine derived from three species, is commonly used in the prescriptions for promoting blood circulation and removing blood stasis based on its traditional effects of removing blood stasis and dredging the meridians. It has been reported that rhubarb can protect blood vessels by reducing inflammation and inhibiting vascular endothelial injury (VEI), but the effective components and mechanism of rhubarb inhibiting VEI are still unclear. This study aimed to compare the differences in chemical compositions of the three species of rhubarb and their inhibitory effect on VEI, so as to explain the material basis and select the dominant species to inhibit VEI, and to elucidate the mechanism of rhubarb’s inhibitory effect on VEI. Methods. Plant metabolomics was used to compare the chemical components of three species of rhubarb. The efficacy of three species of rhubarb in inhibiting VEI was compared through cell experiments in vitro. At the same time, combined with network pharmacology and molecular docking, the effective components and pathways of rhubarb involved in inhibiting VEI were screened. The mechanism of rhubarb inhibiting VEI was verified by molecular biology. Results. There were significant differences in the distribution of chemical components among the three species of rhubarb. We identified 36 different chemical components in the positive ion mode and 38 different chemical components in the negative ion mode. Subsequently, the results showed significant differences in inhibiting VEI among the three species of rhubarb based on the contents of inflammatory factors (such as IL-1β, IL-6, and TNF-α), ROS, and NO and confirmed that R. tanguticum had the best inhibitory effect on VEI in the light of the comprehensive efficacy, compared with R. palmatum and R. officinale. Three species of rhubarb alleviated the inflammatory response in LPS-induced EA.hy926 cells by reducing the contents of inflammatory cytokines IL-6, IL-1β, and TNF-α and decreasing expressions of PI3K, AKT, NF-κB p65, and STAT3 protein in the PI3K/AKT/NF-κB pathway and the inhibition of proteins phosphorylation. In addition, three species of rhubarb could lessen the contents of ROS and NO in EA.hy926 cells induced by LPS. All results indicated that the process of inflammation-induced cellular oxidative stress, which resulted in VEI, was obviously improved by three species of rhubarb. Conclusion. R. tanguticum was more effective among three species of rhubarb, and it had been proved that gallic acid, gallic-acid-O-gallloyl-glucoside, procyanidin B-2,3,3′-di-O-gallatein, and other potential components could reduce the contents of inflammatory factors (such as IL-1β, IL-6, and TNF-α), ROS, and NO by inhibiting the PI3K/AKT/NF-κB signaling pathway and protected the vascular endothelium and the blood vessels by improving the inflammation and oxidative stress reaction.

1. Introduction

It is a common phenomenon that a kind of traditional Chinese medicine (TCM) is derived from multiple species, such as Coptis [1] and licorice [2]. Meanwhile, for the most of TCM with multiple species, the differences in the types, contents, and efficacy of active ingredients among different species are still unclear, and there is no scientific explanation as
to whether it is reasonable to use multiple species as one TCM. Rhubarb, derived from the roots and rhizomes of *Rheum palmatum* L. (*R. palmatum*), *Rheum tanguticum* Maxim. ex Balf. (*R. tanguticum*), and *Rheum officinale* Baill. (*R. officinale*) [3], was first recorded in “Shennong’s Classic of Materia Medica” as “mainly used for lowering blood stasis and breaking up accumulation of obstruction in blood.” Nowadays, it has the effect of removing blood stasis and dredging the meridians [4], which is widely used in the field of emergency medicine and has high medicinal value [5]. Currently, the comparison of the three species of rhubarb mainly focused on properties [6], microscopy [7], chemical characteristics [8], etc., while comparative studies on pharmacological activities of three species of rhubarb were rarely reported. In addition, the indicators in the comparative study of multiple species of rhubarb were relatively single, and the methods were uneven [6, 9], and it was difficult to select the species with advantages in a certain efficacy. More importantly, the current problem with TCM research is that the studies of efficacy and chemical compositions were mostly independent of each other [10–12]. Ingredients are the basis of medicinal effects, and the correlation study between the two at the same time is more conducive to the discovery of effective ingredients and medicinal effects [13]. Therefore, it is urgent to compare the active components of different species of rhubarb and to correlate the components and functions, so as to accurately locate the functional components in different species of rhubarb.

Vascular endothelial injury (VEI) is not only an important cause of sepsis [14] and cardiovascular disease [15], but also an important inducement of blood stasis syndrome [16]. Besides, VEI would inevitably lead to the imbalance of hemagglutination-fibrinolysis system, resulting in bleeding or thrombosis. Hypoxia and inflammation are important inducements of VEI [17]. It has been reported that rhubarb had the functions of anti-inflammatory, inhibiting platelet agglutination and improving microcirculation [7], which were also the main treatment methods of relieving VEI [18, 19]. Simultaneously, VEI was an important pathological basis for inducing blood stasis syndrome [16], and rhubarb could be used to treat blood stasis syndrome [4]. Hence, whether rhubarb with the effect of “removing blood stasis and dredging the meridians” can take effect on VEI and its mechanism remains to be further studied. In this paper, based on the methods of plant metabolomics, network pharmacology, molecular docking, and cell experiment in vitro, the biological effect and material basis of three species of rhubarb on VEI were systematically investigated, which also confirmed the intrinsic ways and dominant species of rhubarb inhibiting VEI.

2. Materials and Methods

2.1. Materials and Reagents. LC-MS-grade acetonitrile were purchased from Fisher Scientific (Pittsburgh, PA, USA). Purified water from a Milli-Q purification system (Millipore, Bedford, MA, USA) was used throughout the experiments. All other chemicals were of reagent grade.

The rhubarb used in the experiment was collected from the rhubarb experiment base in Tuanjie Village (Axi Town, Zou County, Aba Prefecture, Sichuan Province, China). The samples were identified by Professor Wei Shengli of Beijing University of Traditional Chinese Medicine. The numbers a1-a8 were *R. palmatum*, the numbers t1-t12 were *R. tanguticum*, and the numbers o1-o9 were *R. officinale*. The roots of rhubarb with a diameter of more than 1 cm were cross-cut into 1-cm-thick sections. Dried raw material was crushed and sifted by 65 mesh sieve. The voucher specimens were deposited in the Resource Laboratory of Chinese Medicine, Beijing University of Chinese Medicine.

2.2. Sample Preparation. A precise 1.00 g rhubarb powder was put into an Erlenmeyer flask with 25 mL methanol and then weighed them (*w1*). It took 30 minutes to extract the active ingredients by ultrasonic wave with 40 kHz. After ultrasonic extraction, weigh again (*w2*) when the alcohol extract was cold. If *w1* was greater than *w2*, add methanol to the alcohol extract to make it equal. Then the alcohol extract was filtered through a microporous membrane with a diameter of 0.22 microns. According to the previous cell viability experiment, the IC25 of *R. officinale* was selected as the medium dose group of three species of rhubarb to inhibit VEI, and the 1/2IC25 and 2IC25 were administered as the low- and high-dose groups, respectively. Hence, the extract was dissolved in PBS at various concentrations (132.8, 265.5, and 531 μg/mL) for cell experiments.

2.3. Analysis of Chemical Constituents of Three Species of Rhubarb

2.3.1. Instrumentation. Dionex UltiMate 3000 high-pressure liquid chromatography (Thermo Fisher Scientific, USA) was used for component separation. An ACQUITY BEH C18 column (2.1 × 50 mm, 1.7 μm, Waters, Milford, MA, USA) was used with 30°C. The mobile phase consisted of acetonitrile with 0.1% formic acid (A) and acetonitrile (B) at a flow rate of 0.21 mL/min. The gradient elution procedure was as follows: 0-0.5min, 7%-10% B; 0.5-5.5min, 10%-15% B; 5.5-6.5min, 15%-15.1% B; 6.5-7.5min, 15.1%-15.2% B; 7.5-9.5min, 15.2%-15.3% B; 9.5-10min, 15.3%-15.5% B; 10-13min, 15.5%-17% B; 13-14min, 17% B; 14-15min, 17%-20% B; 15-17min, 20%-25% B; 17-20min, 25%-28% B; 20-25min, 28%-35% B; 25-26min, 35%-41% B; 26-27.5min, 41%-90%; B; 27.5-29.5min, 90%-7% B; and 29.5-30min, 7% B. The injection volume was 0.4 μL. The detection wavelength was 230 nm.

The LC system was coupled to a Q-Exact Orbitrap High-Resolution Mass Spectrometer (Thermo Fisher Scientific, USA). The mass spectrometer was operated both in positive and negative ion mode with HESI source. The parameters were as follows: N2; 3.8 KV (positive ion mode); ion source temperature 350°C; capillary voltage, 320°C; sheath gas (nitrogen) flow, 40arb; aux gas flow, 10arb; and full-scan spectra mass range, 100–1500 Da.

2.3.2. Data Processing and Multivariate Analysis. The raw mass data were analyzed with the Statistical Iterative
2.3.1. Data Analysis. Exploratory Visualization Environment (SIEVE 2.2) for peak extraction, alignment, and visualization. The data-filtered conditions were as follows: retention time was 0–30 min, the specific mass to charge ratio \((m/z)\) was 100–1500, frame time width was 2.50, and \(m/z\) width was 10 ppm. MetaboAnalyst 4.0 (https://www.metaboanalyst.ca/) was used to standardize data. Multivariate analysis was realized by introducing the resultant data to Simca14.0, and principle component analysis (PCA) and orthogonal projection to latent structures-discriminant analysis (OPLS-DA) were used to find the different components among the rhubarb samples with three species. The conditions for becoming a difference component were VIP >1 in OPLS-DA analysis and \(P < 0.05\) in one-way analysis of variance, which represented a significant difference in statistics. Then infer the structural formula information of the different components through the HMDB (http://www.hmdb.ca/) and METLIN (http://metlin.Scripps.edu/) databases.

2.4. Inhibitory Effects of Three Species of Rhubarb on VEI

2.4.1. Cell Culture. EA.hy926 cells were purchased from the National Biomedical Experimental Cell Resource Bank (Beijing, China). EA.hy926 cells were cultured in DMEM medium containing 10% fetal bovine serum and 1% penicillin/streptomycin at 37°C, 5% CO₂. The experiment was carried out with cells on logarithmic growth phase.

2.4.2. Cell Viability Assay. EA.hy926 cells were, respectively, treated with lipopolysaccharides (LPS, #L2880, Sigma, USA) at various concentrations (0.001–1000 \(\mu\)g/mL) for 24 h or the extracts from three species of rhubarb (132.8, 265.5, 531 \(\mu\)g/mL) for 48 h in 96-well plates (4.5×10³ cells). After the medium was removed, CCK8 (#MA0218, Meilunbio, China) was added and incubated for another 1 h and then determined the absorbance at 450 nm.

2.4.3. Enzyme-Linked Immunosorbent Assay (ELISA). EA.hy926 cells were treated with LPS (1 \(\mu\)g/mL) for 24 h before the addition of extracts from three species of rhubarb (132.8, 265.5, 531 \(\mu\)g/mL) for 48 h in 24-well plates. The supernatant of the medium was collected and centrifugated.

Figure 1: PCA and OPLS-DA diagram of three species of rhubarb: PCA in positive ion mode (a), PCA in negative ion mode (b), OPLS-DA in positive ion mode (c), and OPLS-DA in negative ion mode (d).
The contents of TNF-α (#KE00154), IL-6 (#KE00139), and IL-1β (#KE00021) in the supernatant were determined according to the procedure of the kit (Proteintech, USA).

2.4.4. Detection of NO Content. EA.hy926 cells were treated with LPS (1 μg/mL) for 24 h before the addition of extracts from three species of rhubarb (132.8, 265.5, 531 μg/mL) for 48 h in 24-well plates. The supernatant of the medium was collected and centrifugated. The contents of NO in the supernatant were determined according to the manufacturer’s instructions (Nanjing, China).

2.4.5. Detection of ROS. 2′,7′-Dichlorodihydrofluorescein diacetate (DCFH-DA, #HY-D0940, MCE, USA) was used
to detect the contents of ROS in cells. Extracts from three species of rhubarb (132.8, 265.5, 531 μg/mL) were added in EA.hy926 cells treated by LPS (1 μg/mL). After 48-h incubation, the cells were treated with 10 μM DCFH-DA and 8 μM Hoechst 33342 (#C0030-10, Solarbio, China) for 30 min at 37 °C, then washed the wells with PBS three times, and investigated the ROS by confocal microscopy (Olympus, Japan).

2.5. Screening Effective Components of Three Species of Rhubarb Inhibiting VEI by Component-Pharmacodynamic Correlation Analysis. The combined efficacy values \( I \) of TNF-α, IL-6, IL-1β, NO, and ROS were calculated by formulas (1) and (2). The gray correlation was applied to analyze the relative correlation between the different chemical components of three species of rhubarb and the comprehensive efficacy value. The components with correlation degree greater than 0.7 were selected as the potential effective components of three species of rhubarb in inhibiting VEI.

The combined efficacy values \( I \) = \( A \ast D^t \), \hspace{1cm} (1)
where $A$ is the content of index (TNF-α, IL-6, IL-1β, NO, and ROS) and $D'$ is the weight of index (TNF-α, IL-6, IL-1β, NO, and ROS)

$$D' = \frac{D_i}{\sum_i D_i},$$

where $D_i$ is the sum of the measured values of an index in all treatment groups and $i = 1, 2, 3, \ldots, 5$.

2.6. Network Pharmacology and Molecular Docking. What is the mechanism of rhubarb inhibiting VEI? Network pharmacology combined with molecular docking were used to predict the mechanism. We calculated the target 1 of three species of rhubarb through SuperPred and SwissTargetPredication database (C-T network) and calculated the target 2 of "vascular inflammation" through GeneCard, OMIM, and DisGeNET database (T-D network). The common targets of target 1 and target 2 were the potential targets of rhubarb inhibiting VEI. DAVID database was used to analyze the enrichment of GO and KEGG pathways, and then the "components-targets-pathways" network of rhubarb inhibiting VEI was constructed. STRING database was used to construct the protein-protein interaction (PPI) network, and the top 20 targets with the highest connectivity were screened for molecular docking with the active components using Discovery Studio 2019.

2.7. Western Blotting. Extracts from three species of rhubarb (132.8, 265.5, 531 µg/mL) were added in EA.hy926 cells treated by LPS (1 µg/mL). After 48-h incubation, the cells were lysed in RIPA buffer containing the mixture of protease inhibitor and PMSF(#C1055, Beijing, China), and homogenates were centrifuged at 12000 rpm for 5 min at 4°C, and then the supernatant was harvested. According to the protocol, the protein denaturation was carried out after the protein concentration was determined. Equal amounts of protein were subjected to 8% SDS-PAGE and transferred to PVDF membrane. The membrane was incubated with anti-p65, anti-AKT, and anti-STAT3 (1:1500, Proteintech, USA); anti-p-p65 and anti-p-PI3K(1:1000, CST, USA); anti-PI3K, anti-eNOS, and anti-iNOS (1:1000, Proteintech, USA); anti-p-AKT(1:2000, Proteintech, USA); and anti-p-STAT3 (1:2000, Abcam, USA), at 4°C overnight, and then the membrane was incubated with secondary antibody and finally added chemiluminescence solution for development.

2.8. Immuno fluorescence. The nuclear translocation of p65 and STAT3 was observed by immunofluorescence technique. Extracts from three species of rhubarb (132.8, 265.5, 531 µg/mL) were added in EA.hy926 cells treated by LPS.
Figure 3: Continued.
(1 μg/mL). After 48-h incubation, 1 mL paraformaldehyde (4%) was added and fixed at room temperature for 30 min. And then 1 mL Triton X-100 solution (0.1%) was added and permeated for 20 min. After the Triton X-100 solution was discarded, 1 mL BSA blocked for 1 h. Then, they were washed with PBS and incubated with p65 primary antibody (1:100 dilution) and STAT3 primary antibody (1:100 dilution), respectively, at 4°C overnight and, next, incubated with red fluorescent-labeled secondary antibody (1:200 dilution) at room temperature in the dark for 1 h. After maintaining DAPI (1 μg/mL) for 5 min, we investigated p65 and STAT3 by confocal microscopy.

Figure 3: The effect of three species of rhubarb with different concentrations on the release of inflammatory factors in EA.hy926 cells induced by LPS. The content of TNF-α in R. officinale (a), R. palmatum (b), and R. tanguticum (c). The content of IL-1β in R. officinale (d), R. palmatum (e), and R. tanguticum (f). The content of IL-6 in R. officinale (g), R. palmatum (h), and R. tanguticum (i). Statistical significance is labeled as below: compared with the control group, #P < 0.05; ##P < 0.01. Compared with the model group, *P < 0.05; **P < 0.01.

Figure 4: The effect of three species of rhubarb with different concentrations on the release of NO in EA.hy926 cells induced by LPS: R. officinale (a), R. palmatum (b), and R. tanguticum (c). Statistical significance is labeled as below: compared with the control group, #P < 0.05; ##P < 0.01. Compared with the model group, *P < 0.05; **P < 0.01.
Figure 5: Continued.
2.9. Statistical Analysis. All results were presented as the mean ± SD. Study data were analyzed using one-way analysis of variance (ANOVA) for significance comparison. \( P < 0.05 \) was considered as statistically significant.

3. Results

3.1. Analysis of Different Components in Three Species of Rhubarb. The model stability \((R^2 X)\) of PCA in the positive ion mode was 0.662, and the model prediction rate \((Q^2)\) was 0.335. In negative ion mode, the model stability \((R^2 X)\) of PCA was 0.683, and the model prediction rate \((Q^2)\) of PCA was 0.397 (Figure 1). The results of PCA showed that the three species of rhubarb could be well distinguished under positive and negative ion modes, indicating that the chemical components of the three species of rhubarb were significantly different.

OPLS-DA could be used to further search for different components on the basis of PCA analysis. In the scatter diagram of three species of rhubarb (in negative ion mode, \( R^2 X = 0.63 \), \( R^2 Y = 0.988 \), and \( Q^2 = 0.796 \); in the positive ion mode, \( R^2 X = 0.611 \), \( R^2 = 0.988 \), and \( Q^2 = 0.764 \)), it could be
Table 4: Relative correlation between the potential effective components of three species of rhubarb and comprehensive efficacy of inhibiting VEI.

<table>
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<tr>
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<th>Name</th>
<th>Relative correlation</th>
<th>No.</th>
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<th>Relative correlation</th>
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seen that the three species of rhubarb were obviously divided into three categories, and the discrimination of the three species of rhubarb was higher than that of PCA analysis. According to the VIP >1 of the OPLS-DA model and the Kruskal-Wallis test (P < 0.05), metabolites with different expression of three species of rhubarb were screened. The different chemical components were identified by METLIN, HMDB, MassBank, and other databases. A total of 36 differential chemical components were found in the positive ion mode and 38 in the negative ion mode (Tables 1 and 2).

3.2. Effects of LPS and Three Species of Rhubarb on the Viability of EA.hy926 Cells. The results of CCK8 showed that 0~100 μg/mL LPS had no significant effect on the viability of EA.hy926 cells (Figure 2(a)). The doses of R. officinale 1/2IC$_{25}$ (132.8 μg/mL), IC$_{25}$ (265.5 μg/mL), and 2IC$_{25}$ (531 μg/mL) were used as the low-, medium-, and high-dose groups of the three species of rhubarb, respectively. After treating EA.hy926 cells for 48 h, CCK8 experimental results indicated that, the low-, medium-, and high-dose groups of R. palmatum, R. tanguticum, and R. officinale, all could affect the viability of EA.hy926 cells in a concentration-dependent manner (Figures 2(b)–2(d)).

3.3. Effects of Three Species of Rhubarb on the Inflammatory Factors of EA.hy926 Cells Induced by LPS. When cells are stimulated by foreign substances, the immune system in the body will be activated, and the secretion of inflammatory factors, such as TNF-α, IL-6, and IL-1β will be promoted, which will directly damage endothelial cells. The results showed that the secretion of TNF-α, IL-1β, and IL-6 in the supernatant of EA.hy926 cells was significantly increased after being stimulated by 1 μg/mL LPS for 24 h, and there was a significant difference between the normal control group and the model group (P<0.01). After treating with different concentrations (132.8 μg/mL, 265.5 μg/mL, and 531 μg/mL) of R. palmatum, R. tanguticum, and R. officinale, the secretion of TNF-α, IL-1β, and IL-6 in each group had different degrees of inhibition (Figure 3). The inhibition of IL-6 secretion by three species of rhubarb showed a good dose-dependent. At the concentration of 265.5 μg/mL, the
three species of rhubarb had a significant inhibitory effect on the secretion of TNF-α, IL-1β, and IL-6.

3.4. Effects of Three Species of Rhubarb on NO of EA.hy926 Cells Induced by LPS. The massive production of NO is one of the important signs of cellular inflammatory response. After being stimulated with 1 μg/mL LPS for 24 h, the release of NO in the supernatant of EA. hy926 cells was significantly increased compared with the normal control group (P < 0.01). After treating with different
concentrations (132.8 μg/mL, 265.5 μg/mL, and 531 μg/mL) of three species of rhubarb, the release of NO in each group decreased to varying degrees (Figure 4). Among them, at the concentration of 265.5 μg/mL, three species of rhubarb had significant inhibitory effects on the release of NO in EA.hy926 cells induced by LPS, and the R. palmatum group at 265.5 μg/mL had the best inhibitory effect on NO release (Figure 4(b)).

3.5. Effects of Three Species of Rhubarb on ROS of EA.hy926 Cells Induced by LPS. ROS is an important product of cellular oxidative stress response, which can cause irreversible damage to cell membrane. The ROS content in EA.hy926 cells was significantly increased after being stimulated with 1 μg/mL LPS for 24 h. After treating with different concentrations (132.8 μg/mL, 265.5 μg/mL, and 531 μg/mL) of three species of rhubarb, ROS content in each group showed a decreasing trend to different degrees (Figure 5). The ROS content in EA.hy926 cells induced by LPS was decreased by three species of rhubarb at 132.8 μg/mL and 265.5 μg/mL.

3.6. Screening of Potential Effective Components of Rhubarb in Inhibiting VEI. Different concentrations of three species of rhubarb (132.8 μg/mL, 265.5 μg/mL, and 531 μg/mL) showed different inhibitory effects on NO, ROS, TNF-α, IL-6, and IL-1β in EA.hy926 cells induced by LPS. It is necessary to comprehensively consider the five indexes measured in each treatment group and finally obtain the dominant species and its appropriate dose. The comprehensive efficacy of groups with different rhubarb treatment was
Gallic acid
Gallic-acid-O-gallloyl-glucoside
Procyanidin B-2,3,3'-di-O-gallate
Peonidin-3-O-beta-D-glucoside
Procyanidin B-5,3,3'-di-O-gallate
Cassiaside
Glabranine
Mangiferin
5,6-Dimethoxyxanthon[2,3-b]furan-4,9-dione
Guibourtiodiol-(4alpha->6) -catechin
Resveratrol-4'-O-(6'-gallate)-b-d-glucopyranoside
Resveratrold
Ent-epicatechin-(4alpha->8) -ent-epicatechin 3-gallate
Procyanidin B-2,3,3'-di-O-gallate
7,12-Dioxo-5a-cholan-24-oic acid
Cinnamintannin A1
Cy 3-coumar-samb-5-Glc
6-(carboxymethyl)-7-hydroxy-8-methoxy coumarin
Epigallocatechin
Procyanidin G2
Emodin
6-Cinnamyl-1,2-digallloylgucose
Mesaconic acid
Pentagalloylgucose
Fermononein 7-O-glucoside
Plumbagin
Ethyl vanillate
(-)-Epicatechin gallate
4-Methocoumarin
TORACHRYSONE 8-glucoside
(-)-Epicatechin
Apiin
Procyanidin B2
5,8-Dihydroxyflavonone
Chrysophansol
Procyanidin B3
Catechin gallate
3-Hydroxy-3',4'-dihydroxyflavone
Cglycyrrhetinic acid
Aloe emodin
Vincetoxicoside A
Quercetin-3,4'-O-di-beta-glucopyranoside
5,3',4',5'-Tetrahydroxy-6,7-dimethoxyflavone
Neomangiferin

Figure 8: Continued.
calculated according to formulas (1) and (2) (Table 3). The smaller the comprehensive efficacy value was, the smaller the inflammatory factors and oxidative stress response indicators were, which represented the stronger inhibitory effect of this group on VEI. The medium-dose group of *R. tanguticum* had the best effect, second was the medium-dose group of *R. palmatum*, and the effect of low-dose group of *R. officinale* was not obvious, but the inhibition of VEI in *R. officinale* groups showed a good dose-dependent effect. After grey correlation analysis was conducted between the different chemical components in three species of rhubarb and the comprehensive efficacy value, 46 potential active ingredients in rhubarb inhibiting VEI were screened out (Table 4). Among them, gallic acid [20] and anthocyanin [21] were consistent with the results reported in the literature.

### 3.7. Construction of Rhubarb Inhibiting VEI Network

Network pharmacology combined with molecular docking were used to predict the mechanism of 46 active components in rhubarb inhibiting VEI. The 46 potential active ingredients of rhubarb had a total of 530 ingredient targets in the SuperPred and SwissTargetPrediction databases, and a total of 278 disease targets in the GeneCard, OMIM, and DisGeNET gene databases have been obtained, then 530 targets mapped to 278 targets, and finally obtained 89 potential targets for rhubarb related to VEI (Figure 6(a)). DAVID database was used to conduct enrichment analysis of GO and KEGG pathways of 89 potential targets of rhubarb in reducing VEI. According to the significance, the pathways with *P* < 0.05 were selected. GO analysis showed that the number of genes enriched in cell biological processes was relatively large, and the *P* value was low, indicating that...
Figure 9: Continued.
Figure 9: Continued.
Rhubarb played a role in inhibiting VEI mainly by regulating the following biological processes (Figure 6(c)). Moreover, 309 bioprogress (e.g., negative regulation of apoptotic process, positive regulation of gene expression, cellular response to hypoxia, positive regulation of nitric oxide biological process, etc.), 40 cell components (e.g., extracellular space, cell surface, plasma membrane, etc.), and 75 molecular functions (enzyme binding, identity protein binding, transmembrane receptor protein tyrosine kinase activity, etc.) were selected. The results were shown in Figure 6(b)(sorted from small to large by $P$ value, with the top 10).

KEGG analysis showed that a total of 30 pathways were enriched, including PI3K/AKT, TNF, MAPK, NF-kB, and p53 signaling pathway. These classical signaling pathways played an important role in rhubarb’s inhibition of VEI. According to the number of potential targets contained in the pathways, 30 pathways were sorted and visualized (Figure 6(c)). In Cytoscape 3.6.1 software, we constructed a “component-target-pathway” network diagram of rhubarb’s inhibitory effect on VEI (Figure 6(d)), which could directly observe the related effects of rhubarb active components, VEI-related targets and pathways. The same active component of rhubarb could connect to different targets, and the same target could also have related effects with different active components, which once again showed that Chinese medicine was a complex system of “multicomponent, multitarget, and multipathway.” The mechanism of rhubarb inhibiting VEI was correlated with gene expression, apoptosis, protein binding, NF-kB signaling pathway, and PI3K/AKT signaling pathway, which could reveal the characteristics of rhubarb inhibiting VEI from different perspectives.

The protein network interaction of potential targets of rhubarb inhibiting VEI was analyzed by string, and the network topology was analyzed by Cytoscape. 83 nodes and 6806 edges were obtained with an average degree of 12.4 (Figure 7(a)). The top 20 proteins were screened according to the connectivity value (Figure 7(b)). Among them, VEGFA, AKT1, TP53, IL6, SRC, STAT3, and other core targets had strong interactions with other proteins in the PPI network.

Discovery Studio2019 software was used to perform molecular docking between 46 potential active components and targets.
Figure 10: Continued.
Figure 10: Continued.
of rhubarb for inhibiting VEI and the core proteins with the top 20 connectivity values in the PPI network. The higher the molecular docking scored, the more stable the ligand and receptor bound.

In the results of molecular docking (Figure 8(a)), the more red represents the higher the relative score, and the more green represents the lower the relative score. Each active component had a relatively high score with the target receptor protein, indicating that the active component had a good structural match with the target protein receptor. Among them (Figure 8), procyanidin B-5,3,3′-dihydroxy-gallate and AKT, mangiferin and PIK3CA, neomangiferin and IL-6, formononetin 7-O-glucuronide and TNF, and procyanidin B2 and NOS3 score were higher (Figure 8). These components in rhubarb might act on the targets to inhibit VEI.

3.8. Inhibition of PI3K/AKT/NF-κB Pathway and Related Protein Expression by Three Species of Rhubarb

3.8.1. Inhibition of PI3K/AKT Pathway by Three Species of Rhubarb.

Although we screened 46 effective components of three species of rhubarb inhibiting VEI, these were still too many. Compared with the verification of the mechanism of monomer, the extract of rhubarb was more convincing to verify the mechanism of rhubarb inhibiting VEI.

PI3K are a group of signaling transduction enzymes, and AKT is the direct target protein of PI3K. Phosphorylation of PI3K and AKT can activate PI3K/AKT pathway, which plays an important role in the pathogenesis of inflammation, obesity, tumor, and immune diseases [22]. Moreover, PI3K/AKT pathway was also enriched by network pharmacology. In order to explore the effects of three species of rhubarb on PI3K/AKT pathway, the protein expressions of PI3K, AKT, p-PI3K, and p-AKT were detected by western blot. The results showed that LPS could increase the protein expressions of PI3K, AKT, p-PI3K, and p-AKT in EA.hy926 cells, and the protein expressions were significantly decreased (P < 0.05) after treated with three species of rhubarb. The difference was statistically significant (Figure 9).

3.8.2. Inhibition of NF-κB Pathway by Three Species of Rhubarb.

NF-κB is a transcriptional protein with multidirectional regulation, which usually enters the nucleus after phosphorylation. It can regulate the expression of a variety of inflammatory and immune genes and participate in the gene regulation of a variety of physiological and pathological processes such as inflammatory immune cell proliferation and apoptosis. It has an important impact on the pathogenesis of a series of inflammatory diseases involving cytokines and inflammatory mediators. Similarly, as a pathway predicted by network pharmacology, we used western blot to detect the protein expressions of NF-κB p65 and p-p65 and used immunofluorescence assay to detect NF-κB p65 phosphorylated nuclear translocation in LPS-induced EA.hy926 cells. The results of western blot showed that three species of rhubarb could significantly reduce the protein expression levels of NF-κB p65 and p-p65 (P < 0.01) (Figure 10). Immunofluorescence assay showed that NF-κB p65 was normally distributed in the cytoplasm but accumulated in the nucleus after LPS-induced phosphorylation of NF-κB p65. Moderate doses of the three species of rhubarb treatments eliminated the accumulative effect of LPS-induced phosphorylation of NF-κB p65 into the nucleus (Figure 10). Both results demonstrated that three species of rhubarb could inhibit the activation of the NF-κB pathway induced by LPS. It has been reported that NF-κB could be regulated by the PI3K/AKT pathway [23]. These results proved that three species of rhubarb might inhibit the
Figure 11: Continued.
Figure 11: Continued.
PI3K/AKT/NF-κB signaling pathway, thereby reducing the inflammatory response induced by LPS.

3.8.3. Inhibition of Inflammation-Related Proteins by Three Species of Rhubarb. STAT3 and NF-κB are two transcription factors closely linked in the process of inflammation. They share a common target gene and can activate transcription cooperatively [24]. Cytokines encoded by NF-κB, such as IL-6, are both the inflammatory factor encoded by the target gene of NF-κB and the activator of STAT3 [25]. Compared with the LPS model group, western blot results showed that the expression of STAT3 proteins in EA.hy926 cells was significantly reduced after treatment with three species of rhubarb \( (P < 0.05) \) (Figure 11). Besides, the expression of p-STAT3 proteins in LPS-induced EA.hy926 cells was significantly reduced after treatment with \( R. \) officinale high dose and \( R. \) palmatum low and medium dose \( (P < 0.05) \) (Figure 11). Immunofluorescence assay results showed that medium doses of rhubarb could slightly inhibit LPS-induced STAT3 phosphorylation nuclear translocation (Figure 11), while the inhibition of p-STAT3 protein expression and nuclear translocation by \( R. \) tanguticum and \( R. \) officinale were not obvious and STAT3 might not be the direct target of these two species of rhubarb. The results demonstrated that three species of rhubarb, in especial \( R. \) tanguticum, could reduce the inflammatory response in LPS-induced EA.hy926 cells by regulating NF-κB nuclear translocation, rather than STAT3, which was consistent with the secretion of inflammatory factors in ELISA experiment.

NO plays an important role in inflammation and immune response, and excessive production of NO may lead to endothelial injury [26]. eNOS and iNOS are the sources of excess NO in cells [27]. Western blot results showed that the protein expression levels of eNOS and iNOS in LPS model group were significantly increased \( (P < 0.01) \) and the protein expression levels of eNOS and iNOS were significantly decreased after treatment with three species of rhubarb \( (P < 0.01) \) (Figure 12). It indicated that three species of rhubarb could inhibit the protein expression of eNOS and iNOS, which was consistent with the experimental results of NO determination.

PI3K/AKT/NF-κB is a classic inflammatory injury pathway of endothelial cells. When activated, the PI3K/AKT/NF-κB pathway can promote the secretion of IL-6, IL-1β, TNF-α, and other inflammatory factors by downstream related proteins. The continuous accumulation of IL-6 and other inflammatory factors will stimulate STAT3 protein expression and its phosphorylation into the nucleus through pathways such as bypass secretion, promote the expression of inflammation-related genes, and further cause the inflammatory response in the body. At the same time, a large number of inflammatory factors will promote the expression of NOS in cells, resulting in the production of NO in large quantities, which reacts with reactive oxygen species in cells to generate peroxides, directly damaging endothelial cells.

These results suggested that the three species of rhubarb can reduce the expression levels of NF-κB p65, PI3K, and AKT in LPS-induced EA.hy926 cells; inhibit their phosphorylation; and prevent the activation of PI3K/AKT/NF-κB pathway. The protein expressions of STAT3, eNOS, and iNOS were decreased, and the contents of TNF-α, IL-6, IL-1β, NO, and ROS in the cells were decreased, which played a role in the inhibition of VEI (Figure 13).
Figure 12: Continued.
4. Discussion

The application of different species as the same TCM was one of the factors that caused the unstable curative effect of TCM. This was likely to be because of genetic differences in these species and the differences in the types, contents, and proportions of their medicinal ingredients, which in turn lead to differences in efficacy. There was still a lack of scientific explanation on whether the use of multiple species as a TCM was reasonable. Therefore, the comparison of different species of TCM had attracted attention in recent years.

The application of plant metabolomics technology to the determination of plant species has been reported in licorice [28], Bupleurum [29], Angelica [30], and so on. In this paper, the chemical components of three species of rhubarb were analyzed by UPLC-Q-Exactive Orbitrap-MS, and the inhibitory effects of three species of rhubarb on VEI were compared by cell experiments in vitro, and 46 effective components of rhubarb were screened out for their inhibitory effects on VEI, including gallic acids and anthocyanins. Gallic acids [4] and anthocyanins [31] were also the material basis of rhubarb on removing blood stasis and dredging meridians.

Through network pharmacology and molecular docking, the potential targets of the effective components of rhubarb in inhibiting VEI were analyzed. The component-target-pathway network diagram was constructed by the existing database, and relatively important targets had a good matching degree with the docking structure of component molecules. AKT1, IL6, STAT3, etc. played an important role in the efficacy of rhubarb, which further illustrated that rhubarb inhibited VEI through “multicomponent, multitarget, and multipathway” [32].

Following the enrichment analysis of GO and KEGG, PI3K/AKT and NF-κB signaling pathways were finally

![Figure 12: The effect of three species of rhubarb with different concentrations on the protein expressions of NOS in LPS-induced EA.hy926 cells. eNOS (a) and iNOS (b).](image-url)
PI3K/AKT signaling pathway, which played an important role in cell growth cycle, cell migration, cell autophagy, and other life processes [33], had also been involved in the regulation of inflammatory response in recent years [34–36]. It had been reported that phosphorylated PI3K subunits could phosphorylate AKT subunits Thr308 and Ser473 after LPS-induced endothelial cells, which lead to AKT activation [36]. Activated AKT could promote IκBα phosphorylation and degradation and activate NF-κB signaling pathways. NF-κB was a classic pathway of inflammation. It had been reported that in inflammatory response, TNF-α, IL-6, etc. could activate IKK, which induced the phosphorylation and ubiquitination degradation of the inhibitory protein IκB of NF-κB, transferring NF-κB from the resting state to the nucleus and activating gene transcription [37]. All these indicated that PI3K/AKT could regulate the NF-κB signaling pathway in oxidative responses of endotoxemia [23]. In LPS-induced EA.hy926 cells, the three species of rhubarb showed a good ability to inhibit PI3K, AKT, NF-κB, STAT3, eNOS, iNOS protein expression, and partial protein phosphorylation, exerting anti-inflammatory activity, which was again demonstrated by experimental method that the inflammatory response could be reduced through PI3K/AKT/NF-κB pathway, thus inhibiting VEI. It also illustrated the role of network pharmacology combined with molecular docking in discovering the efficacy of TCM and explaining the mechanism of action.

Generally speaking, the effect of TCM is closely related to the contents of medicinal substances. Even if a medicinal component has a high activity, but with a small content, its pharmacological effect is still weak, and its practical value is low. Therefore, the material basis of this study was found through the correlation between drug efficacy and component contents. However, in this study, only part of index components were screened for inhibiting VEI, and not all.
Still some components with high contents and strong activity were ignored. More accurate and comprehensive material basis of rhubarb inhibiting VEI should be further studied. It might be possible to expand the quantitative range of chemical components of rhubarb and associating with pharmacodynamic indicators. Additionally, this study scientifically evaluated the differences in efficacy of the same TCM with different species, which was an important basic research work for the realization of the production of “precision medicinal materials,” and provided theoretical basis for ensuring accurate symptomatic use of TCM.

5. Conclusion
In this study, the methods of plant metabolomics, network pharmacology, molecular docking, and cell experiments in vitro were combined for the first time to explore the differences of chemical components and inhibition of VEI in rhubarb with three species and clarify the mechanism of rhubarb inhibiting VEI. 36 chemical components were screened in the positive ion mode, and 38 chemical components were screened in the negative ion mode. After the correlation between chemical components and efficacy, 46 effective components were screened to inhibit VEI. *R. tanguticum* had a better inhibitory effect on VEI. Rhubarb inhibited VEI mainly by acting on P38K, AKT, NF-κB p65, STAT3, eNOS, and iNOS proteins in P38K/AKT/NF-κB signaling pathway, inhibiting protein expression and phosphorylation, and reducing the contents of TNF-α, IL-6, IL-1β, NO, and ROS in cells. This study provided an effective way for the determination of precision TCM.

Data Availability
The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest
The authors report no conflicts of interest.

Authors’ Contributions
Xin Li mainly conducted this study. Songli Huang, Bingyu Zhuo, and Jingyan Hu provided help on pharmacological studies and cell experiments. Yue Shi, Jiangyi Zhao, and Jincheng Tang assisted in the collection of experimental materials and analysis of plant metabolomics. Xiuhua Hu provided manuscript revision. Shengli Wei provided the concept and ideas support. All data were generated in-house, and no paper mill was used. All authors agree to be accountable for all aspects of work ensuring integrity and accuracy.

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