Extract from *Rostellularia procumbens* (L.) Nees Inhibits Thrombosis and Platelet Aggregation by Regulating Integrin β3 and MAPK Pathways

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ABSTRACT: Aim of study: The main objective of this study was to investigate the antithrombotic and antiplatelet effect of the extract from *Rostellularia procumbens* (L.) Nees and understand the mechanisms by which it exerts its antithrombotic and antiplatelet mechanisms. Materials and methods: The antithrombotic effective parts (RPE) were isolated using D101 macroporous adsorption resin and potential active ingredients (JAC) were isolated using the preparative liquid-phase method. The lactate dehydrogenase kit was used to determine the toxicity of RPE and JAC to platelets. The antiadhesion effect of RPE and JAC on platelets was observed by fluorescence microscopy with rhodamine phalloidin. Antithrombotic efficacy of RPE and JAC in vivo was evaluated by establishing a rat tail thrombosis model. Contents of p-selectin, TXB2, and 6-keto-PGF1α in rat serum were measured using an enzyme-linked immunosorbent (ELISA) assay, and the rat black tail rate was measured to prove the protective effect of RPE and JAC on the tail thrombus rat model. Western blot was used for detection of serum-related proteins in the tail thrombus rat model. Results: The results showed that RPE had antithrombotic and antiplatelet effects. RPE and JAC have no toxicity to platelets. In vitro experiments showed that RPE and JAC had antiadhesion effects on platelets. In vivo experiments showed that RPE significantly inhibited the increase of p-selectin and TXB2, and significantly increased the content of 6-keto-PGF1α in the serum of rats. Western blot results demonstrated that RPE and JDB significantly inhibited the phosphorylation of the MAPK protein family in the platelets of rats, and RPE also significantly inhibited the phosphorylation of β3 protein. Conclusions: RPE has antithrombotic and antiplatelet activity in vivo and vitro. Its mechanism may be via preventing integrin αIIbβ3 activation, which in turn leads to the inhibition of the phosphorylation of the MAPK family and further suppresses TXA2, which leads to the antithrombotic and antiplatelet effects.

1. INTRODUCTION

Thrombotic disease which is the general term for diseases caused by the two pathological processes of thrombosis and thromboembolism is a disease which seriously endangers human health and life.1,2 The pathogenesis of thrombotic diseases is very complex and is not very well understood. Recent studies have shown that factors such as the vascular endothelial status, platelet number, and blood coagulation play a role in the pathogenesis of thrombotic diseases.3 Antiplatelet therapy has become one of the important treatments for thrombotic diseases. Aspirin, clopidogrel, and antiplatelet aggregation drugs which are widely used in clinic often have a single mechanism of action and serious side effects. For example, when aspirin is taken for a long time, it can lead to gastrointestinal bleeding and death.4 Compared with chemical drugs, traditional Chinese medicines have great advantages. Many researchers have found that traditional Chinese medicines such as *Salvia miltiorrhiza* have strong antithrombotic activity and the potential to be developed into antiplatelet aggregation drugs compared to drugs such as aspirin.5

Information about *Rostellularia procumbens* (L.) Nees, a medicinal plant of the Acanthaceae family, was first published in Sheng Nong’s Herbal Classic. It was also included in the Chinese Pharmacopoeia (1977). It is mainly composed of lignans, flavones, and triterpenes6 which are clinically used to treat colds, fevers, flu, malaria, and hepatitis. Studies have demonstrated that multiple bioactive effects including...
Figure 1. Main active ingredients in RPE. (A) HPLC of RPE(1: 6′-hydroxy justicidin B, 2: JDB, 3: CME, 4: neojusticidin B, and 5: neojusticidin A). (B) Chemical structures of them. (a: 6′-Hydroxy justicidin B, b: justiscidin B, c: chinensinaphthol methyl ether, d: neojusticidin B, and e: neojusticidin A).

Figure 2. LDH release rate of RPE and JAC. (a) LDH release rates of RPE at different concentrations. (b) LDH release rates of JDB at different concentrations. (c) LDH release rates of CME at different concentrations.

Figure 3. Fluorescence staining results of rhodamine phalloidin. (a) Blank control with no collagen. (b) Control. (c) ASP (20.0 mg/mL). (d) JDB (0.20 mg/mL). (e) CME (0.24 mg/mL). (f) RPE-L (0.25 mg/mL). (g) RPE-M (0.50 mg/mL). (h) RPE-H (1.0 mg/mL). (i) Percentage of fluorescence staining. Fluorescence microscopy at 100 times. *P < 0.01 vs the control group, *P < 0.05 and **P < 0.01 vs the model group.
antitumor, antichronic glomerulonephritis, and bacteriostatic activities are exerted by active compositions of *R. procumbens* (L.) Nees.\(^7\)

Previous work done by our lab has demonstrated that *R. procumbens* (L.) Nees has the effect of antiplatelet aggregation at the network pharmacology level.\(^8\) The objective of this study was to verify the antiplatelet aggregation and antithrombotic efficacy of the active extract of *Rostellaria procumbent* (L.) Nees and understand the mechanism of its action at the cellular and molecular level.

2. RESULTS

2.1. Main Active Ingredients in RPE. It was found that there were five potential effective chemical components in RPE by HPLC (Figure 1A). The reference products had a purity of more than 95% found by NMR. By comparing with the reference products, we determined that the five components were 6'-hydroxyjusticidin B, justicidin B (JDB), chinensinaphthol methyl ether (CME), neojusticidin B, and neojusticidin A (chemical structures in Figure 1B).

2.2. RPE and JAC Have No Toxicity to Platelets. Results of the LDH release rate are shown in Figure 2; it was found that compared with the blank control, the concentration of RPE and JAC had no effect on the release rate of platelets, which means that the RPE and JAC have no toxicity to platelets.

2.3. RPE and JAC Resist Platelet Adhesion In Vitro. Next, the effect of RPE and JAC on platelet adhesion in vitro was evaluated. The rhodamine phalloidin fluorescence was observed, and the sections were analyzed using Image-Pro Plus 6.0 (Figure 3). There was a significant augmentation of fluorescence staining percentage in the collagen group compared with the control group (\(P < 0.01\)). In addition, there was a significant decrease in fluorescence staining percentage in JAC, RPE-M, and RPE-H groups compared with the collagen group (\(P < 0.01\)).

2.4. In Vivo Protective Effect of RPE and JAC on Tail Thrombosis in Rats. From Figure 4 and Table 1, we could see that at the 12th h after thrombus modeling, no thrombus was in the tail of the control group at 12 h post modeling. However, a blood thrombus was formed in both the model group and the treatment groups. Compared with the model group, the black tail rate of rats in the treatment groups was significantly decreased (\(P < 0.05\)).

The liver of animals in the model group and the treatment groups was swollen and the liver coefficient was increased. The liver was normal in the animals in the control group; the liver coefficient of the model group was significantly higher when compared with that of the control group (\(P < 0.05\)), while the liver coefficient of the treatment groups was significantly lower when compared with that of the model group (\(P < 0.05\)). This indicated that the drug had a protective effect on tail thrombosis.

2.5. Effects of RPE and JAC on P-Selectin Expression. P-selectin is a member of the selectin family of adhesion molecules. It is a glycoprotein on the \(\alpha\)-granule membrane of platelets. On platelet activation, P-selectin is expressed on the cell surface and released as particles, leading to adhesion to leukocytes.\(^7\) Compared with the control group, the expression of P-selectin in the model group increased significantly (\(P < 0.05\)), which was significantly alleviated in the treatment groups (\(P < 0.05\)) (Figure 5A). These results demonstrated that activation of platelets after thrombus modeling led to the release of p-selectin particles. Administration of RPE and JAC might be beneficial in reducing this increase.

2.6. Effects of RPE and JAC on TXB\(_2\) and 6-Keto-PGF\(_{1\alpha}\) Expression. TXA\(_2\) and PGI\(_2\) are a pair of antagonistic endogenous substances, which play a major role in the regulation of platelet function. We analyzed the expression of

![Figure 4](https://dx.doi.org/10.1021/acsomega.0c05227)

**Figure 4.** Tail conditions of carrageenan-induced rats. (a) Blank group. (b) Mice induced by a mixture of 1.5 mg/kg collagen and 0.5 mg/kg epinephrine were injected in the tail vein. (c) Mice induced by a mixture of 1.5 mg/kg collagen and 0.5 mg/kg epinephrine were intragastrically administered with aspirin at the dosage of 30 mg/kg. (d) Mice induced by a mixture of 1.5 mg/kg collagen and 0.5 mg/kg epinephrine were intragastrically administered with JDB at the dosage of 6.1 mg/kg. (e) Mice induced by a mixture of 1.5 mg/kg collagen and 0.5 mg/kg epinephrine were intragastrically administered with CME at the dosage of 7.4 mg/kg. (f--h) Mice induced by a mixture of 1.5 mg/kg collagen and 0.5 mg/kg epinephrine were intragastrically administered with RPE at the dosage of 7.5, 15, and 30 mg/kg, respectively.

### Table 1. Black Tail Rate and Liver Coefficient of Tail Thrombosis in Rats (Mean \(\pm SD, n = 8\))

| group       | dose (mg/kg) | black tail rate (%) | liver coefficient (%) |
|-------------|--------------|---------------------|-----------------------|
| blank control | 0\(^b\)        | 3.201 \(\pm\) 0.164 |                       |
| model       | 85.9 \(\pm\) 3.4 | 4.760 \(\pm\) 0.228\(^c\) |                       |
| ASP         | 30           | 37.4 \(\pm\) 4.3\(^c\) | 3.971 \(\pm\) 0.173\(^c\) |
| JDB         | 6.1          | 45.3 \(\pm\) 3.7\(^c\) | 4.106 \(\pm\) 0.268\(^c\) |
| CME         | 7.4          | 49.9 \(\pm\) 3.9\(^c\) | 3.835 \(\pm\) 0.312\(^c\) |
| RPE-L       | 7.5          | 71.5 \(\pm\) 5.0\(^c\) | 3.884 \(\pm\) 0.316\(^c\) |
| RPE-M       | 15           | 62.3 \(\pm\) 4.0\(^c\) | 4.002 \(\pm\) 0.312\(^c\) |
| RPE-H       | 30           | 40.4 \(\pm\) 3.9\(^c\) | 4.007 \(\pm\) 0.181\(^c\) |

\(^{a}\) \(p < 0.01\) versus the control group. \(^{b}\) \(p < 0.05\) versus model group. \(^{c}\) \(p < 0.05\) versus model group.
TXB2 and 6-keto-PGF1α in the serum because they are stable hydrolys products of TXA2 and PGI2 (Figure 5b,c).10 Compared to the model group, treatment with JDB and RPE significantly downregulated the expression of the TXB2 level and upregulated the expression of the 6-keto-PGF1α level in the serum ($p < 0.05$).

### 2.7. Reduction in $\beta_3$ Phosphorylation by RPE in Carrageenan-Induced Rats

Previous studies have demonstrated that integrin $\beta_3$ is the main receptor responsible for regulating platelet aggregation and thrombosis.11 We measured the content of $\beta_3$ proteins in rat platelet protein in order to understand the antithrombotic mechanism of RPE and JAC. Compared to the control group, there was a significant upregulation of $\beta_3$ in the model group ($p < 0.05$). Treatment with ASP, RPE-L, and RPE-M significantly decreased the expression of $\beta_3$ protein in the induced rats ($p < 0.05$). The expression of integrin $\beta_3$ was also significantly downregulated by RPE-H treatment ($p < 0.01$) (Figure 6b).

### 2.8. Reduction in MAPK Phosphorylation by RPE in Carrageenan-Induced Rats

MAPK is widely involved in various physiological processes, such as inflammatory response, oxidative stress, and apoptosis.12 In our study, after platelet activation, $\alpha_{IIb}\beta_3$ is activated, which further leads to phosphorylation of JNK, ERK, and p38 in the MAPK family. The expression of the p-MAPK family in platelet proteins in model rats was analyzed by us using western blot. Compared to the control group, the expression of $\alpha$-ERK, $\alpha$-JNK, and $\alpha$-p38 protein was notably increased in the model group ($p < 0.05$) (Figure 6c). The treatment groups decreased the $\alpha$-ERK, $\alpha$-JNK, and $\alpha$-p38 expression to some extent. Treatment with RPE-H and RPE-L significantly decreased ERK phosphorylation ($p < 0.01$). Treatment with ASP and CEM also decreased the ERK phosphorylation significantly ($p < 0.05$), while JDB had no effect on ERK phosphorylation. JNK phosphorylation was significantly downregulated by ASP, JDB, RPE-L, RPE-M, and RPE-H ($p < 0.01$). There was no effect of CME on JNK phosphorylation. ASP, RPE-M, and RPE-H significantly decreased p38 phosphorylation ($p < 0.01$), while there was no effect of JDB and CME on p38 phosphorylation.

### 3. DISCUSSION

Most cardiovascular and cerebrovascular diseases develop as a result of thrombotic diseases, such as stroke, myocardial infarction, pulmonary embolism, and so on.13 Thrombotic diseases have a complex etiology and a long course. Substantial progress has been made in the use of TCM for the treatment of thrombotic and platelet aggregation diseases. R. procumbens (L.) Nees is a traditional herbal medicine with the efficacy of detoxicating, activating blood and pain relieving, diuretic swelling, and so on. Previous studies have demonstrated its efficacy in inhibiting platelet aggregation.14 However, it is not clear if R. procumbens (L.) Nees has any protective effects on thrombotic diseases. Therefore, in this study, we extracted the antithrombotic effective parts (RPE) and potential active parts (JAC) through the proven methods in the preliminary experiment in our lab. JAC is a general term for two compounds, Justicidin B (JDB) and Chinensinaphthol methyl ether (CME). Finally, we found that all these three parts have an antithrombotic effect to a certain degree. Investigation of the potential mechanisms of action revealed that the antithrombotic effects are achieved by RPE through the inhibition of integrin $\alpha_{IIb}\beta_3$ activation, which in turn inhibits the phosphorylation of the MAPK family and further suppresses TXA2 so as to inhibit the activity of platelet aggregation.

Previous laboratory studies have found that lignans are the main antiplatelet aggregation compounds. Free lignans are lipophilic and insoluble in water. It has been reported that lignans can have metabolic reactions such as glucosaldehydation, sulfuration, hydroxylation, hydroxylation, reductive glucosaldehydation, and reductive sulfation in animals,
changing their pharmacological effects.\textsuperscript{15} Neojusticidin A, taiwanin E methyl ether, chinensinaphthol methyl ether, taiwanin E, and justicidin B have been reported to have inhibitory effects on arachidin-induced platelet aggregation in rabbits.\textsuperscript{6} However, the effect of secondary metabolites of these compounds on platelet aggregation in animals has not been reported, which deserves further study.

In the process of drug research and development, it is necessary to investigate the toxicity of drugs. In our study, the LDH release test was carried out to determine whether the RPE and JAC have toxic or side effects on platelets and to understand the way in which the RPE and JAC act, whether they directly kill platelets or induce platelet inactivation through some mechanisms.\textsuperscript{16} The results showed that RPE, JDB, and CME had no cytotoxicity, and the release rate of LDH was less than 3%. The effect of drugs on platelet adhesion was studied in this study because platelet adhesion is closely related to platelet aggregation through some mechanisms.\textsuperscript{16} In our study, rhodamine phalloidin was used to stain platelets coated with collagen to observe the adhesion of platelets to collagen. The results showed that the treatment groups had a certain platelet adhesion effect, among which the effect of RPE and JDB groups was stronger ($P < 0.01$), while that of CME was weak ($P < 0.05$).

Previous studies done by our group demonstrated that the extract had the activity of antiplatelet aggregation in vitro.\textsuperscript{8} The in vivo activity in animals was however not analyzed. Platelets often accumulate abnormally in animals with thrombotic diseases, which leads to the formation of a large number of thrombi and results in blockage of blood vessels and embolus formation.\textsuperscript{18} Therefore, our group used a rat tail model of the thrombus through intragastric administration to prove that RPE and JAC prevented platelet aggregation in animals and helped in treatment of thrombotic diseases.

P-selectin is a glycoprotein present on the platelet $\alpha$ granule membrane. After platelet activation, P-selectin is rapidly expressed on the cell surface and releases $\alpha$ particles, which leads to adhesion to leukocytes.\textsuperscript{19} In the tail thrombus rat experiment, the expression of P-selectin in the serum of the model group was increased when compared with the control group, while the expression of P-selectin in the serum of the treated group was lower when compared with the model group. This indicated that the drug reduced the expression of P-selectin in the serum of rats.\textsuperscript{20} TXA$_2$ is a thromboxane synthesized and released from platelet microvesicles. It has a
strong biological activity to promote vasoconstriction and platelet aggregation.\textsuperscript{21} PGI\textsubscript{2} is a derivative of arachidonic acid and an antagonist of thromboxane. It significantly inhibits platelet aggregation.\textsuperscript{22} TXB\textsubscript{2} and 6-keto-PGF\textsubscript{1α} are stable forms of thromboxane A\textsubscript{2} (TXA\textsubscript{2}) and prostacyclin (PGI\textsubscript{2}), respectively.\textsuperscript{23} The content of TXB\textsubscript{2} in the serum of the model group increased, while the content of 6-keto-PGF\textsubscript{1α} decreased in the tail thrombosis experiment in rats. The content of TXB\textsubscript{2} in the serum decreased and the content of 6-keto-PGF\textsubscript{1α} increased in the treatment group when compared with the model group, except for the low-dose group of RPE and CME groups. It is suggested that RPE and JDB can reduce the production of TXA\textsubscript{2} and increase the content of PGI\textsubscript{2} so as to inhibit platelet aggregation. However, antiaggregation effects are not exerted by CME in a similar manner.

Integrin \(\alpha\text{IIb}\beta3\) is a platelet membrane protein. When platelets are activated by inducers, \(\alpha\text{IIb}\beta3\) is activated. This exposes its binding site to fibrin and finally platelets aggregate (Figure 7).\textsuperscript{24} Integrin \(\alpha\text{IIb}\beta3\) consists of two type I transmembrane glycoproteins, \(\alpha\text{IIb}\) and \(\beta3\), which are connected by disulfide bonds. Integrin \(\alpha\text{IIb}\beta3\) is the main receptor regulating platelet aggregation and thrombosis.\textsuperscript{25} Previous work done by our group found through the gene chip technology that the extract affected the expression of \(\alpha\text{IIb}\beta3\) protein on platelets.\textsuperscript{14} Thus, it was suggested that the extract had the potential to develop into antiplatelet aggregation drugs. Results from the current study demonstrated via the western blot method that RPE significantly inhibited the expression of integrin \(\beta3\). After platelet activation, integrin \(\alpha\text{IIb}\beta3\) activation will further lead to the phosphorylation of JNK, ERK, and p38 in the MAPK family.\textsuperscript{26} This will result in the production of TXA\textsubscript{2} and the increase of Ca\textsuperscript{2+}. Concentration. Results from our study demonstrated that RPE significantly inhibited the phosphorylated expression of ERK and JDB and RPE. It also significantly inhibited the phosphorylated expression of JNK, JDB, and p38.

4. CONCLUSIONS

RPE exerts antithrombotic and antiplatelet activity via inhibition of integrin \(\alpha\text{IIb}\beta3\) activation, by reducing the expression of P-selectin through inhibition of the phosphorylation of the MAPK family, by suppressing production of TXA\textsubscript{2}. Antiplatelet aggregation and antithrombotic effects are also exerted by JDB and CME. Both of them could suppress production of TXA\textsubscript{2} and downregulate the phosphorylation of JNK and ERK. However, their effect on p38 phosphorylation and integrin \(\beta3\) was not significant. Our study elucidated the antithrombotic and antiplatelet mechanism of JDB, CME, and RPE by regulating integrin \(\beta3\) and MAPK pathways. It may provide a basis for the further study of \textit{R. procumbens} (L.) Nees.

5. MATERIALS AND METHODS

5.1. Plant Material. Fresh \textit{R. procumbens} (L.) Nees was collected from different regions in Hubei Province of China in October 2018 and was identified by Professor Hezhen Wu of Hubei University of Chinese Medicine. Fresh herbs are dried in the sun and stored at 25 °C, away from light and in a ventilated place. The plants were dried and powdered, and the powder was percolate-extracted with ethanol. It was then decompressed to recover the ethanol. Lignans were then enriched with macroporous adsorption resin and the effective part (RPE) was obtained at 24 °C.\textsuperscript{27} The HPLC method was used to analyze the potentially effective components in the effective part RPE. JDB and CME were obtained by liquid-phase preparation, with purity greater than 98%. We called these two parts as JAC below for convenience. Previous studies in the laboratory have proved that these three parts are nontoxic for cells and animals.

5.2. Animals. Two male New Zealand rabbits (general class, weight 2–2.5 kg) were purchased from the Hubei Provincial Center for Disease Control and Prevention; 64 male SD rats (SPF class, weight 190–230 g) were purchased from the Laboratory Animal Center of China Three Gorges University (Yichang, China). The animals were given sufficient water and feed and housed in plastic cages with the ambient temperature to 20 °C, and relative humidity was maintained between 50 and 60%. Animals were allowed a 2 week quarantine and acclimation period prior to start of the study. All experimental procedures were approved by the Animal Care and Use Committee of Institute of Materia Medica, People’s Republic of China.

5.3. Reagents and Chemicals. Aspirin enteric-coated tablets (H20160684) were purchased as positive control drugs from Bayer Healthcare. CMC-Na (C8621) and carrageenan (C8830) were purchased from Solarbio Technology Co., Ltd (Beijing, China). Thrombin (THR), collagen, and adenosine diphosphate (adenosine diphosphate, ADP) were purchased from Sigma (USA). Rhodamine phalloidin (300T) was purchased from YEASEN Co., Ltd (Shanghai, China). The LDH kit, rat P-selectin ELISA kit (E-EL-R0828c), rat thromboxane b2 (TXB2) ELISA kit (E-EL-R0965c), and 6-keto-prostaglandin F\textsubscript{1α} (6-keto-PGF\textsubscript{1α}) ELISA kit (E-EL-0054c) were purchased from Elabsciene Biotechnology Co., Ltd (Wuhan, China). The primary antibodies are as follows: \(\beta3\), ERK, p-ERK, JNK, p-JNK, P38, p-P38, and HRP anticonjugated secondary antibody were purchased from Cell Signaling Technology (MA, USA).

5.4. Toxicity Assay of RPE and JAC on Platelets. Rabbit blood was obtained from rabbit ear veins; the blood was added to a vacuum blood collection tube containing 3.8% sodium citrate to obtain anticoagulant blood. Platelet-rich plasma (PRP) was obtained by centrifugation of anticoagulant blood. Platelet-rich plasma (PRP) was obtained by centrifugation of anticoagulant blood (180g, 10 min). PRP was incubated with a blank solution and PRE (0.25–4.00 mg/mL), JDB (0.05–0.80 mg/mL), and CME (0.06–0.96 mg/mL) at 37 °C for 10 min, and then, the LDH kit was used to measure the content of LDH in each group. The following is the calculation formula of the release rate
Similar to the pulmonary embolism model, the tail thrombus model was established by injecting 0.4% carrageenan solution. Rats in the blank control were given an intraperitoneal injection of normal saline. Treatment groups were given an intraperitoneal injection of the abovementioned drugs, rats in the model group and the 20 mg/kg RPE for 7 days. One hour after the last dose of the ASP, 4.2 mg/kg JDB, 5.1 mg/kg CME, 5 mg/kg, 10 mg/kg, or intragastrically with either 0.5% CMC-Na solution, 20 mg/kg of different concentrations of drug solution and blank control solution was added to each well; the concentration of each administration was ASP (2.0 mg/mL), JDB (0.20 mg/mL), CME (0.24 mg/mL), RPE-L (0.25 mg/mL), RPE-M (0.50 mg/mL), and RPEH (1.0 mg/mL). After the drug solution and the platelets were incubated for 90 min, the round coverslips were added to each well; the concentration of each administration was ASP (2.0 mg/mL), JDB (0.20 mg/mL), CME (0.24 mg/mL), RPE-L (0.25 mg/mL), RPE-M (0.50 mg/mL), and RPEH (1.0 mg/mL). After the drug solution and the platelets were incubated for 90 min, the round coverslips were fixed with 4% paraformaldehyde for 20 min, and finally, 250 μL of rhodamine phalloidin was added for staining. The staining state was observed with a fluorescence microscope in the dark room. The percentage of fluorescent staining was calculated using Image-Pro Plus 6.0.

5.6. In Vivo Carrageenan-Induced Tail Thrombosis Model. Similar to the pulmonary embolism model, the tail thrombus model experiment was also divided into the same eight groups. The rats in the respective groups were treated intragastrically with either 0.5% CMC-Na solution, 20 mg/kg ASP, 4.2 mg/kg JDB, 5.1 mg/kg CME, 5 mg/kg, 10 mg/kg, or 20 mg/kg RPE for 7 days. One hour after the last dose of the abovementioned drugs, rats in the model group and the treatment groups were given an intraperitoneal injection of 0.4% carrageenan solution.28 Rats in the blank control were given an intraperitoneal injection of the same amount of 0.9% normal saline. The tail thrombosis length of rats was observed 12 h after thrombus modeling; the black tail rate and liver coefficient were calculated as follows. $L_A$ is the length of the black tail; $L_B$ is the body length of mice; $W_C$ is the weight of the liver; $W_D$ is the body weight of mice.

$$\text{Black tail rate (\%)} = \frac{L_A}{L_B} \times 100\%; \text{ liver coefficient(\%)} = \frac{W_C}{W_D} \times 100\%$$

5.7. Test of $p$-Selectin, $\text{TXB}_2$, and 6-Keto-PGF$_{1\alpha}$ in the Serum of Rats with Tail Thrombi. We predosed for 7 days and then injected carrageenan to make a model rat. At 12 h after the modeling, rats were anesthetized to take the blood and internal organs and finally executed. A total of 12 h after the abovementioned model rats were anesthetized with urethane, 8 mL of blood was taken from the abdominal aorta; 6 mL of blood was added with an anticoagulant for anticoagulation centrifugation, and 2 mL of blood was added without an anticoagulant for centrifugation to get the serum. The content of $p$-selectin, $\text{TXB}_2$, and 6-keto-PGF$_{1\alpha}$ from the un-anticoagulant serum was measured using an ELISA assay as per the instruction manual provided by the manufacturer.

5.8. Preparation of Platelet-Rich Plasma (PRP). The anticoagulated blood obtained from the animals as described in the previous section was then centrifuged at 180g for 10 min. Plasma containing platelets in the supernatant was taken and the remaining sample was centrifuged at 1800g for 18 min. Platelet-rich plasma (PPP) was obtained from the upper platelet plasma by adjusting the platelet plasma solubility with PPP, so that the platelet number was $3.5 \times 10^8$/mL, to obtain PRP finally.

5.9. Western Blotting. PRP was centrifuged at low speed to obtain platelet precipitation. Total proteins were extracted using RIPA lysis buffer (Beyotime Institute of Biotechnology, China). Samples were separated by SDS PAGE and electro-transferred onto the NC membrane (Millipore, USA). They were then incubated overnight at 4 °C with the specific primary antibodies. The membranes were washed three times with TBST for 10 min. After washing, the membranes were incubated with HRP conjugated secondary antibodies for 1 h. The membranes were washed in the same manner as described above. The membranes were scanned using the FluorChem FC3 system (Protein Simple, USA).

5.10. Statistical Analysis. Experimental data were presented as mean $\pm$ SD. Statistical significance was measured using one-way ANOVA. A value less than 0.05 is considered to be statistically significant.

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**Notes**

The authors declare no competing financial interest.

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REFERENCES

(1) Moake, J. L. Thrombotic microangiopathies. N. Engl. J. Med. 2002, 347, 589–600.
(2) Albany, C. J.; Trevelin, S. C.; Giganti, G.; Lombardi, G.; Scotta, C. Getting to the Heart of the Matter: The Role of Regulatory T-Cells (Tregs) in Cardiovascular Disease (CVD) and Atherosclerosis. Front. Immunol. 2019, 10, 2795.
(3) Nieswandt, B.; Pleines, I.; Bender, M. Platelet adhesion and activation mechanisms in arterial thrombosis and ischaemic stroke. J. Thromb. Haemostasis 2011, 9, 92–104.
(4) Cuzzick, J.; Otto, F.; Baron, J. A.; Brown, P. H.; Burn, J.; Greenwald, P.; Jankowski, J.; La Vecchia, C.; Meyeskens, F.; Senn, H. J.; Thun, M. Aspirin and non-steroidal anti-inflammatory drugs for cancer prevention: an international consensus statement. Lancet Oncol. 2009, 10, 501–507.
(5) Cui, J.; Hu, L.; Shi, W.; Cui, G.; Zhang, X.; Zhang, Q. W. Design, Synthesis and Anti-Platelet Aggregation Activity Study of Ginkgo1,2,3-triazole Derivatives. Molecules 2019, 24, 2156.
(6) Chen, C.-C.; Hsin, W.-C.; Ko, F.-N.; Huang, Y.-L.; Ou, J.-C.; Teng, C.-M. Antiplatelet Arylnaphthalide Lignans from Justicia procumbens. J. Nat. Prod. 1996, 59, 1149–1150.
(7) Xiong, W.; Yang, Y.; Xiong, Y.; Liu, B.; Xie, Z.; Wu, H. A New Neolignan from Justicia Procumbens. Chem. Nat. Compd. 2019, 56, 50–52.
(8) Yang, Y. F.; Wu, S. T.; Liu, B.; Xie, Z. T.; Xiong, W. C.; Hao, P. F.; Xiao, W. P.; Sun, Y.; Ai, Z. Z.; You, P. T.; Wu, H. Z. A Novel Antiplatelet Aggregation Target of Justicidin B Obtained From Rostellaria Pacomum (L.) Nees. Front. Pharmacol. 2019, 10, 688.
(9) Labelle, M.; Begum, S.; Hynes, R. O. Direct signaling between platelets and cancer cells induces an epithelial-mesenchymal-like transition and promotes metastasis. Cancer Cell 2011, 20, 576–590.
(10) Fan, H.; Li, M.; Yu, L.; Jin, W.; Yang, J.; Zhang, Y.; Han, H. Effects of Danhong Injection on platelet aggregation in hyperlipidemia rats. J. Ethnopharmacol. 2018, 212, 67–73.
(11) Legate, K. R.; Fassler, R. Mechanisms that regulate adaptor binding to beta-integrin cytoplasmic tails. J. Cell Sci. 2009, 122, 187–198.
(12) Kim, E. K.; Choi, E.-J. Pathological roles of MAPK signaling pathways in human diseases. Biochim. Biophys. Acta 2010, 1802, 396–405.
(13) Causes of Death Collaborators. Global, regional, and national age-sex specific mortality for 264 causes of death, 1980-2016: a systematic analysis for the Global Burden of Disease Study 2016. Lancet 2017, 390, 1151–1210.
(14) Wu, S.; Yang, Y.; Liu, B.; Xie, Z.; Xiong, W.; Hao, P.; Xiao, W.; Sun, Y.; Ai, Z.; Wu, H. A novel anti-platelet aggregation target of chinensinaphthol methyl ether and neojusticin B obtained from Rostellaria procumbens (L.) Nees. J. Enzyme Inhib. Med. Chem. 2019, 34, 999–1009.
(15) Li, Y.-T.; Li, M.-M.; Sun, J.; Zhu, Z.-X.; Song, Y.-L.; Pang, D.-Y.; Zheng, J.; Zhao, Y.-F.; Tu, P.-F.; Li, J. Furofuran lignan glucosides from the leaves of Vitex negundo var. cannabifolia. Nat. Prod. Res. 2017, 31, 918–924.
(16) Liu, L.; Duan, J.-a.; Tang, Y.; Guo, J.; Yang, N.; Ma, H.; Shi, X. Taoren-Honghua herb pair and its main components promoting blood circulation through influencing on hemorheology, plasma coagulation and platelet aggregation. J. Ethnopharmacol. 2012, 139, 381–387.
(17) Mahaut-Smith, M. P.; Jones, S.; Evans, R. J. The P2X1 receptor and platelet function. Purinergic Signal. 2011, 7, 341–356.
(18) Tantry, U. S.; Bleden, K. P.; Suarez, T. A.; Kreutz, R. P.; Dichiaro, J.; Gurbel, P. A. Hypercoagulability, platelet function, inflammation and coronary artery disease acuity; results of the Thrombotic Risk Progression (TRIP) study. Platelets 2010, 21, 360–367.
(19) Kolaczkowska, E.; Kubies, P. Neutrophil recruitment and function in health and inflammation. Nat. Rev. Immunol. 2013, 13, 159–175.
(20) Semple, J. W.; Italiano, J. E., Jr.; Freedman, J. Platelets and the immune continuum. Nat. Rev. Immunol. 2011, 11, 264–274.
(21) Rivera, J.; Lozano, M. L.; Navarro-Nunez, L.; Vicente, V. Platelet receptors and signaling in the dynamics of thrombus formation. Haematologica 2009, 94, 700–711.
(22) Fang, Y.-F.; Zhang, B.-H.; Lu, X.-Q.; Wang, P. Beraprost Sodium, a Stable Analogue of PG12, Inhibits the Renin-Angiotensin System in the Renal Tissues of Rats with Chronic Renal Failure. Kidney Blood Press. Res. 2018, 43, 1231–1244.
(23) Perez-Cremades, D.; Bueno-Beti, C.; Garcia-Giménez, J. L.; Ibáñez-Cabellos, J. S.; Hernégenolgo, C.; Pallardó, F. V.; Novella, S. Extracellular histones disarrange vasoactive mediators release through a COX-NOS interaction in human endothelial cells. J. Cell Mol. Med. 2017, 21, 1584–1592.
(24) Barnett, N. E.; Holbrook, L.; Jones, S.; Kaiser, W. J.; Moraes, L. A.; Rana, R.; Sage, T.; Stanley, R. G.; Tucker, K. L.; Wright, B.; Gibbins, J. M. Future innovations in anti-platelet therapies. Br. J. Pharmacol. 2008, 154, 918–939.
(25) Sinrger, T. A.; Zhu, J.; Xiao, T. Structural basis for distinctive recognition of fibrinogen gammaC peptide by the platelet integrin alphablbeta3. J. Cell Biol. 2008, 182, 791–800.
(26) Shattil, S. J.; Kim, C.; Ginsberg, M. H. The final steps of integrin activation: the end game. Nat. Rev. Mol. Cell Biol. 2010, 11, 288–300.
(27) Yang, F.-J.; Ma, C.-H.; Yang, L.; Zhao, C.-J.; Zhang, Y.; Z.; Yu, Y.-G. Enrichment and purification of deoxyshizandrin and shizandrin from the extract of Schisandra chinensis fruit by macroporous resins. Molecules 2012, 17, 3510–3523.
(28) Arslan, R.; Bor, Z.; Bektas, N.; Mericli, A. H.; Ozturk, Y. Antithrombotic effects of ethanol extract of Crataegus orientalis in the carrageen-induced mice tail thrombosis model. Thromb. Res. 2011, 127, 210–213.
(29) Ma, N.; Liu, X.-W.; Yang, Y.-J.; Li, J.-Y.; Mohamed, I.; Liu, G.-R.; Zhang, J.-Y. Preventive Effect of Aspirin Eugenol Ester on Thrombosis in kappa-Carrageenan-Induced Rat Tail Thrombosis Model. PLoS One 2015, 10, No. e0133125.
(30) Foster, T. E.; Puskas, B. L.; Mandelbaum, B. R.; Gerhardt, M. B.; Reddy, S. A. Platelet-Rich Plasma. Am. J. Sports Med. 2009, 37, 2259–2272.