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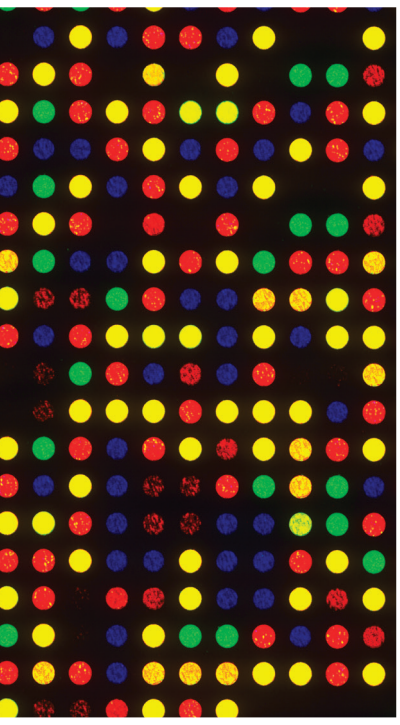
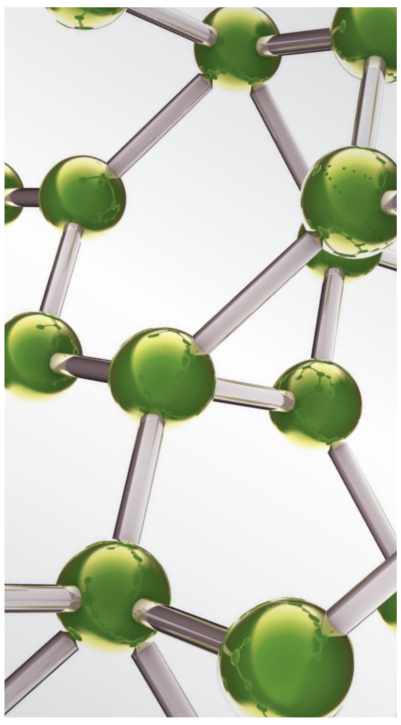
The Complementary and Alternative Medicine for Antithrombosis

Guest Editors: Feng-Qing Yang, Chun-Su Yuan, Swee Ngin Tan, and Jian-Li Gao



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Editorial Board

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Editorial

The Complementary and Alternative Medicine for Antithrombosis

Feng-Qing Yang,[1] Chun-Su Yuan,[2] Swee Ngin Tan,[3] and Jian-Li Gao[4]

[1]*School of Chemistry and Chemical Engineering, Chongqing University, No. 174, Shazhengjie Street, Shapingba, Chongqing 400030, China*
[2]*Tang Center for Herbal Medicine Research, The Pritzker School of Medicine, University of Chicago, 5841 South Maryland Avenue, MC 4028, Chicago, IL 60637, USA*
[3]*Natural Sciences and Science Education Academic Group, Nanyang Technological University, 1 Nanyang Walk, Singapore 637616*
[4]*Zhejiang Chinese Medical University, No. 548 Binwen Road, Binjiang, Hangzhou, Zhejiang 310053, China*

Correspondence should be addressed to Feng-Qing Yang; fengqingyang@cqu.edu.cn

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Blood stasis syndrome (BSS) or blood stagnation is often understood in terms of hematological disorders such as thrombosis, congestion, hemorrhage, and local ischemia (microclots). In reality, BSS or thrombosis is considered to be closely related to senile diseases such as atherosclerosis, ischemic heart disease, and stroke, as well as rheumatoid arthritis, hyperuricemia, and various inflammatory conditions. Therefore, more studies have been undertaken to focus on preventing thrombosis for the treatment of those thrombotic diseases. It is well known that the complementary and alternative medicines (CAMs) such as traditional Chinese medicines (TCMs) have a long history for treating thrombosis or BBS. This special issue is focused on the evidence-based research of the CAMs in antithrombotic therapies and therefore brings more attention from readers and researchers on complementary and alternative therapies for thrombosis.

The formation of thrombosis is highly complex. Understanding the molecular functions of thrombosis network is helpful for predicting potential targets for the treatment of thrombosis. X. Kong et al. retrieved pathways and reaction information related to thrombosis from the Reactome database (a peer-reviewed pathway database), then split the complex into separate single proteins, reconnected splitting proteins and detected the relations between the proteins with reacting directions, and finally mapped the integrative and comprehensive molecular network of thrombosis by combining platelet signaling, the coagulation cascade, and natural clot dissolution systems. Moreover, the topological characteristics of the network including the centralities of nodes, network modules, and network robustness were analyzed. However, further pharmaceutical experiments are necessary for eventual validation of network results.

In order to reveal the antiatherosclerotic molecular mechanisms of Astragaloside IV (AsIV) from Huangqi (*Astragalus membranaceus*), H. Qin et al. investigated the effects of AsIV on blood lipids, CD40-CD40L signal system, and SDF-1/CXCR4 biological axis in high-fat diet ApoE$^{-/-}$ mice. Their results proved that AsIV can alleviate the extent of atherosclerosis in aorta of ApoE$^{-/-}$ mice. And AsIV can significantly downregulate PAC-1, CD40L, and CXCR4 expression on platelet surface in blood, as well as mRNA and protein level of SDF-1, CXCR4 in thoracic aorta of the model mice. On the other hand, AsIV could downregulate TG, TC, and LDL-C levels and upregulate HDL-C levels in model mice's blood. Therefore, they made a conclusion that the protective effects of AsIV in atherosclerotic injury may be related to regulating blood lipids, CD40-CD40L system, and SDF-1/CXCR4 biological axis.

Safflower Yellow Injection (SYI) has been reported for the treatment of acute cerebral infarction. L.-J. Li et al. investigated the effects of SYI on the patients with acute cerebral infarction. Their results indicated that the scores of National Institute of Health Stroke Scale significantly decreased in the SYI treated group (after treatment for 7 and 14 d) as compared

to the control group (placebo injection). Furthermore, the hemorheological index including index of red blood cell (RBC) deformation and aggregation were significantly different before and after treating with SYI. Meanwhile, the value of prothrombin time (PT) increased, and the value of fibrinogen (FIB) decreased in the SYI treated group. In addition, the serum levels of TNF-α, IL-1β, and IL-6 in the SYI group decreased as compared to the control group. The data suggests that SYI therapy may be beneficial for the patients with acute cerebral infarction.

In another study, S. Mahmud et al. investigated the antithrombotic properties (clot lysis effects) of cold methanol extracts of five Bangladeshi plants *in vitro*. Seven secondary metabolites including alkaloids, flavonoids, steroids, tannins, saponins, phlobatannins, and cardiac glycosides were identified in the experimental extracts by phytochemical screening. Among those herbal extracts, *Leea macrophylla* showed the most prominent antithrombotic effects, while *Andrographis paniculata* possessed moderate activity. Moreover, *in silico* docking simulation showed that glycosides molecule fit the best to the activation of tissue plasminogen activator, which suggested that glycosides of these plants are to be the major components contributing to the observed thrombolytic effects.

There is considerable interest in the role of natural products and their bioactive components in the prevention and treatment of thrombosis related disorders. In the review paper by C. Chen et al., the mechanisms of thrombus formation were briefly described on three aspects including coagulation system, platelet activation and aggregation, and change of blood flow conditions. And the natural products for antithrombosis by anticoagulation (inhibition of tissue factors and the coagulation pathways), antiplatelet aggregation (inhibition of platelet membrane receptors functions and granules secretion, impacting on nucleotide and arachidonic acid systems), and fibrinolysis were summarized, respectively.

It is believed that advances in the understanding of both the mechanisms of thrombus formation and the antithrombotic functions of CAMs will provide new insights to promote human health by preventing or treating thrombotic disorders.

Feng-Qing Yang
Chun-Su Yuan
Swee Ngin Tan
Jian-Li Gao

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Research Article

The Value of Safflower Yellow Injection for the Treatment of Acute Cerebral Infarction: A Randomized Controlled Trial

Le-Jun Li,[1,2] Yu-Mei Li,[1] Ben-Yu Qiao,[1] Shan Jiang,[1] Xin Li,[1] Hong-Ming Du,[3] Peng-Cheng Han,[2] and Jiong Shi[2]

[1] *Department of Neurology, Affiliated Lianyungang Hospital of Nanjing University of Chinese Medicine, No. 160 W. Chaoyang Road, Lianyungang 222000, China*
[2] *Barrow Neurological Institute, St. Joseph's Hospital and Medical Center, Phoenix, AZ 85013, USA*
[3] *Changchun Sanzhen Industry Co., Ltd., Changchun 130000, China*

Correspondence should be addressed to Le-Jun Li; lilejjun999@163.com

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Background. Safflower Yellow Injection has been reported as a treatment for acute cerebral infarction in recent studies in China. However, there is a lack of availability of the evidence for the efficacy and safety of Safflower Yellow Injection for the treatment of acute ischemic stroke. So we investigated the effects of Safflower Yellow Injection for the treatment of acute cerebral infarction. *Method*. All subjects were randomly divided into Safflower Yellow Injection group given Safflower Yellow Injection (80 mg) and control group given placebo (0 mg) injection by intravenous drop once daily for 14 days. National Institute of Health Stroke Scale (NIHSS); hemorheological detection; coagulation function; and serum inflammatory markers, tumor necrosis factor-alpha (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6), were used to investigate the effects before and 14 days after the treatment. *Results*. The scores of NIHSS were decreased on day 7 and day 14 after treatment. The hemorheological index of RBC deformation and RBC aggregation were significantly improved, prothrombin time (PT) increased, and fibrinogen (FIB) and TNF-α, IL-1β, and IL-6 were decreased in patients treated with Safflower Yellow injection on day 14 after treatment ($P < 0.05$). *Conclusion*. Data suggests that Safflower Yellow Injection therapy may be beneficial for acute cerebral infarction.

1. Introduction

Stroke is a leading cause of death and disability worldwide [1]. Despite improvements in acute stroke care-stroke unit care, thrombolysis in appropriately selected patients and early and sustained antiplatelet therapy-many patients only makes a partial or poor recovery after stroke and the major burden of stroke is chronic disability [2]. Therefore, there is a need for treatments that would further improve the efficacy. Clinical research in China, based on traditional Chinese medicine (TCM), has the potential of introducing new treatments for cerebral infarction.

Safflower Yellow Injection was certificated in China by the State Pharmaceutical Administration of China (SPAC) in 2002 (number 2002ZL0176) after being evaluated in clinical trials. The constituent of Safflower Yellow Injection is Safflower Yellow, hydroxysafflor yellow A is a major component of Safflower Yellow [3]. The protective effect of hydroxysafflor yellow A in ischemic stroke has been investigated in previous studies [4], which have shown effects of antithrombosis [5, 6], anticoagulation [7], antioxidation [8], anti-inflammation [9], and anticalcium dysregulation [10].

Safflower Yellow Injection has been reported as a treatment for acute cerebral infarction in recent studies in China [11, 12]. However, there is a lack of good-quality and convincing supporting evidence for the efficacy and safety of Safflower Yellow Injection for the treatment of acute cerebral infarction [4]; no conclusions about efficacy or safety could be drawn [13]. Therefore, our study hypothesis was that patients who underwent a treatment with Safflower Yellow Injection

for acute cerebral infarction would have a better therapeutic effect compared to those who did not receive this therapy. So the aim of our study was to assess if giving Safflower Yellow Injection can improve effects of acute cerebral infarction patients in China.

2. Materials and Methods

2.1. Study Design. The study was a prospective, single-blinded, and randomized controlled trial. All eligible patients included in the study agree to participate and signed the informed consent form and the study procedures were approved by the Ethical Committee of the Affiliated Lianyungang Hospital of Nanjing University of Chinese Medicine. Full compliance with the Helsinki Declaration was applied during the study. Patients were recruited from the Neurology Department of the Affiliated Lianyungang Hospital of Nanjing University of Chinese Medicine between January 2004 and December 2012.

2.2. Inclusion and Exclusion Criteria. All patients meeting the World Health Organization's definition of ischemic stroke were enrolled during the study. Diagnostic criteria of acute cerebral infarction were diagnosed according to diagnostic criteria of various types of cerebrovascular disease [14] and China Guideline for Cerebrovascular Disease Prevention and Treatment. The main inclusion criteria of the trial were diagnosed ischemic stroke patients within 6 h to 14 days of onset; a score of 4–24 points on the National Institute of Health Stroke Scale (NIHSS); adults between 35 and 80 years old; and signed informed consent form. The main exclusion criteria were as follows: after 14 days from onset; a history of previous stroke; hemorrhagic stroke and transient ischemic attacks; other severe diseases such as heart or kidney failure, tumors, and gastrointestinal hemorrhage; and pregnant or lactating women.

2.3. Control and Intervention Groups. All patients of both groups were hospitalized for 14 days; we provide baseline treatment for all subjects to keep the respiratory tract unobstructed, preventing and treating aspiration pneumonia, monitoring and treating arrhythmia and ischemic heart disease, normalizing the blood pressure, and controlling blood sugar.

All patients were given conventional antiplatelet aggregation drugs (oral aspirin tablet, 100 mg, once daily) and neuroprotective agent (oral Piracetam tablet 400 mg, tid) therapy, based on conventional treatment; the Safflower Yellow Injection (80 mg) was given to the Safflower Yellow Injection group and placebo (0 mg) injection was given to the control group by intravenous drop once daily for 14 days.

The constituent of Safflower Yellow Injection is Safflower Yellow. The investigational drug Safflower Yellow Injection was supplied as Nankin freeze-dried powder. Each unit contains 80 mg Safflower Yellow. The placebo drug was supplied as blank colorless freeze-dried powder, where each unit contains no Safflower Yellow. Investigational drug and placebo were provided by Changchun Sanzhen Industry Co., Ltd. The Safflower Yellow Injection group intravenously received 80 mg Safflower Yellow dissolved in 250 mL 0.9% sodium chloride or 5% glucose once per day. The control group was given a placebo. For two groups, the course of treatment lasted for 14 days.

2.4. Objectives and Outcome Measures

2.4.1. Measurement of NIHSS Score. The NIHSS was measured for stroke severity. The primary outcome measures were the differences in patients' scores on this scale among baseline, day 7, and day 14. The score was assessed by an independent clinical investigator.

2.4.2. Measurement of Hemorheological Detection. Blood samples were collected from ulnar vein before and after the treatment, the index of hemorheolgy included the whole blood viscosity, reduced viscosity, plasma viscosity, hematocrit, and the index of red cell deformity.

Keep 6 mL blood sample in an anticoagulation cuvette mixed with Heparin 100 μL, with LG-R-80 type blood viscosity tester and LG-B-190 type cytomorphosis/aggregation tester, at 37°C ± 0.1°C and detect the whole blood hypsitomy coefficient of viscosity (shear rate 150 s^{-1}), hypo-tomy coefficient of viscosity (shear rate 5 s^{-1}), plasma viscosity, and index of red blood cell deformation and aggregation. The HCT determination used decigram method centrifugation in common temperature with rotary speed 3000 r/min for 30 min.

2.4.3. Measurement of Coagulation Function. Blood samples were collected from ulnar vein before and after the treatment, kept 6 mL blood sample in an anticoagulation cuvette mixed with Heparin 100 μL, centrifuged the sample immediately in common temperature with rotary speed 3000 r/min for 15 minutes and then collected serum and froze it at −80°C to measure. The values of prothrombin time (PT), activated partial thromboplastin time (APTT), fibrinogen (FIB), and international normalized ratio (INR) were detected by using blood coagulation method before and after the treatment.

2.4.4. Measurement of Serum Inflammatory Markers TNF-α, IL-1β, and IL-6. Peripheral venous blood (10 mL) was collected into sampling tubes without EDTA. Blood was centrifuged at 3500 rpm for 5 minutes to separate the serum. Serum was stored at −80°C until analysis. All assays were performed in a blinded fashion on coded samples. Serum levels of TNF-α, IL-1β, and IL-6 were measured in duplicate by a sandwich-type enzyme-linked immunosorbent assay (ELISA) technique by using kits from R&D Systems, TNF alpha DTA00C (range of detection: 15.6–1,000 pg/mL), IL-1b DLB50 (range of detection: 3.9–250 pg/mL), and IL-6 D6050.

2.5. Statistical Analysis. Statistical analysis was performed using SPSS 12.0 statistics software. Date was presented as mean ± S.D.; baseline variables were analyzed using student's t-test. For efficacy variables, they were analyzed using analysis of covariance (ANOVA); comparisons were made between

the two groups at baseline, day 7, and day 14. The two-group t-test was used separately for each comparison. A level of $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Baseline Characteristics. 150 patients were approached and screened for their eligibility to enroll in this study. 108 patients consented and were eligible for the study and signed their informed consent for participation. Patients were randomized by a central stochastic system (2 : 1) to either Safflower Yellow Injection group ($n = 72$) or control group ($n = 36$). Seven patients were rejected during the treatment period for not meeting the inclusion criteria (four from the Safflower Yellow Injection group and three from the control group) and another four patients dropped out for adverse events (two from the Safflower Yellow Injection group and two from the control group). Ninety-seven patients completed the trial, 66 patients in the Safflower Yellow Injection group and 31 patients in the control group (see Figure 1). The characteristics of the Safflower Yellow Injection and control groups are not significantly different at baseline and are given in Table 1.

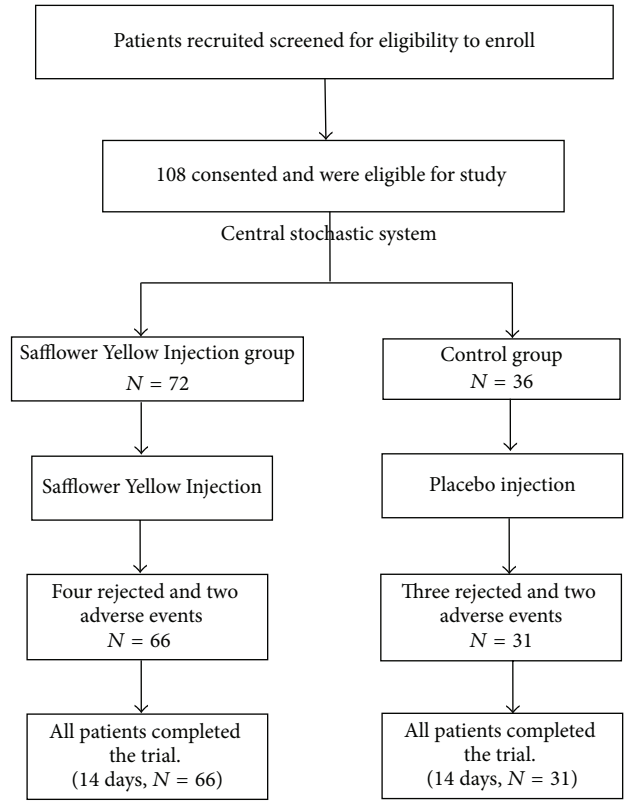

Figure 1: Study design and CONSORT diagram showing the flow of participants.

3.2. Efficacy Results. The scores of NIHSS are all differently reduced after treatment for 7 and 14 days. This improvement becomes more apparent with time. After subtracting the baseline effect, the between-group total score is statistically significant ($P < 0.01$) after treatment for 7 and 14 d.

There were no significant differences in the values of hemorheological index between the two groups before the treatment ($P > 0.05$). Safflower Yellow Injection group hemorheological index of RBC deformation and index of RBC aggregation; there were significant differences before and after the treatment ($P < 0.05$). In the control group, only the whole blood viscosity was significantly different before and after the treatment ($P < 0.05$); the remaining hemorheological indices were not significantly different before and after treatment. Safflower Yellow Injection group's whole blood viscosity and plasma viscosity were significantly different after treatment between the two groups ($P < 0.05$). Results are listed in Table 2. The reason may be that Safflower Yellow Injection can improve the values of hemorheological index.

There were no significant differences in the PT, APTT, INR, and FIB values between the two groups before the treatment ($P > 0.05$). The value of PT increased in the Safflower Yellow Injection group after treatment ($P < 0.05$); there was significant difference between the two groups ($P < 0.05$). After the treatment, the values of FIB decreased in the Safflower Yellow Injection group after treatment; there was significant difference between the two groups ($P < 0.05$). Results are listed in Table 3. The reason may be that Safflower Yellow Injection can inhibit both intrinsic and extrinsic coagulations.

There were no significant differences in the serum levels of TNF-α, IL-1β, and IL-6 between the two groups before the treatment ($P > 0.05$). The serum levels of TNF-α, IL-1β, and IL-6 in the Safflower Yellow Injection group decreased after treatment ($P < 0.05$); there were significant differences between the two groups ($P < 0.05$). Results are listed in Table 4. The reason may be that Safflower Yellow Injection has anti-inflammatory effects.

3.3. Safety and Tolerability. Safflower Yellow Injection for the treatment acute cerebral infarction was well tolerated. Four adverse events were reported during the whole trail (two in the Safflower Yellow Injection group and two from the control group). In the Safflower Yellow Injection group, one patient suffered a transfusion reaction and the other was diagnosed of poststroke depression. Two patients in the control group suffered a recurrence of stroke. All adverse events were considered not to be related to the Safflower Yellow Injection.

4. Discussion

Stroke is a sudden onset and acute cerebral vascular disease commonly characterized by focal neurological deficit. It is the most common neurological disease with high incidence, morbidity, mortality, and recurrence. The disease is more common in the elderly, together with myocardial infarction and cancer; these are the three major causes for senile fatality [15].

This clinical study showed that Safflower Yellow Injection is safe and effective for acute cerebral infarction. It effectively

Table 1: Baseline characteristics of study patients.

Characteristics		Control group	Safflower Yellow Injection group	P value
$n = 97$		$n = 31$	$n = 66$	
Male		9	28	0.595
Mean age and range, years		58.84 ± 10.18 (30–70)	59.47 ± 10.92 (31–70)	0.564
Body height/cm	Male	170.00 ± 6.69	168.04 ± 6.07	0.573
	Female	159.19 ± 4.82	159.86 ± 4.86	0.554
Body weight/kg	Male	67.56 ± 9.48	67.34 ± 9.85	0.563
	Female	58.73 ± 8.18	60.92 ± 8.44	0.612
Weight index/(kg/m^2)		23.19 ± 2.49	23.79 ± 2.76	0.963
Disease time/d		5.00 ± 3.55	3.91 ± 2.77	0.925
NIH stroke scale score		7.65 ± 2.32	8.53 ± 3.30	0.556

Values are mean ± S.D.; no significant changes between control group and Safflower Yellow Injection group.

Table 2: Comparison of hemorheological index before and after treatment between 2 groups ($\overline{x} \pm s$).

Variables	Control group $n = 31$		Safflower Yellow Injection group $n = 66$	
	Pretreatment	Posttreatment	Pretreatment	Posttreatment
Whole blood viscosity (mPa·s/150 s^{-1})	5.95 ± 0.80	5.34 ± 0.83$^{\#}$	5.92 ± 0.82	5.14 ± 0.85$^{*\#}$
Whole blood viscosity (mPa·s/5 s^{-1})	12.66 ± 2.52	12.57 ± 2.24	12.69 ± 2.64	10.05 ± 2.48$^{*\#}$
Plasma viscosity (mPa·s)	1.82 ± 0.23	1.88 ± 0.26	1.78 ± 0.21	1.42 ± 0.23$^{*\#}$
HCT (%)	49.76 ± 4.80	47.64 ± 4.95	48.53 ± 5.30	47.61 ± 4.77
Index of RBC deformation	0.51 ± 0.01	0.50 ± 0.01	0.51 ± 0.07	0.59 ± 0.03*
Index of RBC aggregation	1.21 ± 0.16	1.36 ± 0.19	1.38 ± 0.19	1.21 ± 0.16*

Data are expressed as mean ± S.D., $^{*}P < 0.05$, compared with the pretreatment, $^{\#}P < 0.05$, compared with control group.

Table 3: Changes of coagulation and fibrolysis indexes in the patients before and after treatment between 2 groups ($\overline{x} \pm s$).

Variables	Control group $n = 31$		Safflower Yellow Injection group $n = 66$	
	Pretreatment	Posttreatment	Pretreatment	Posttreatment
PT (s)	10.64 ± 0.58	10.85 ± 0.57	11.02 ± 0.65$^{\#}$	13.38 ± 0.71$^{*\#}$
APTT (s)	29.43 ± 4.76	29.21 ± 4.83	29.84 ± 4.81	29.06 ± 5.02
INR	1.04 ± 0.13	0.94 ± 0.12	1.04 ± 0.15	0.94 ± 0.16
FIB (g/L)	3.35 ± 0.61	3.54 ± 0.70	3.57 ± 0.73	2.07 ± 0.19$^{*\#}$

Data are expressed as mean ± S.D., $^{*}P < 0.05$, compared with the pretreatment, $^{\#}P < 0.05$, compared with control group.

Table 4: Comparison of serum levels of TNF-α, IL-1β, and IL-6 before and after treatment between 2 groups ($\overline{x} \pm s$).

Variables	Control group $n = 31$		Safflower Yellow Injection group $n = 66$	
	Pretreatment	Posttreatment	Pretreatment	Posttreatment
TNF-α	39.45 ± 5.62	40.24 ± 1.55	39.82 ± 4.71	30.85 ± 3.10$^{*\#}$
IL-1β	44.16 ± 4.32	40.07 ± 1.44	44.69 ± 4.24	28.85 ± 1.68$^{*\#}$
IL-6	669.92 ± 61.93	711.28 ± 31.86	689.88 ± 48.92	558.82 ± 38.03$^{*\#}$

Data are expressed as mean ± S.D., $^{*}P < 0.05$, compared with the pretreatment, $^{\#}P < 0.05$, compared with control group.

improved the stroke symptoms and significantly improved the patient's life competency. The NIHSS is widely used in nearly all large clinical stroke trials to document baseline and outcome severity of neurological impairment [16]. In our study, we found that the scores of NIHSS were significantly decreased in patients treated with Safflower Yellow on day 7 and day 14 after treatment. These results suggest that Safflower Yellow is beneficial in reducing the severity of stroke.

Hemorheological detection is important in the course of disease, including nosogenesis, development, prognosis, and turnover as well as curative effect assessment, especially for prevention of cardiocerebrovascular disease. Brain

microcirculation slowdown or stagnation is the main pathological change at the beginning of cerebral ischemia [17]. In our study, we found that the hemorheological index of RBC deformation and RBC aggregation were significantly improved in patients treated with Safflower Yellow Injection on day 14 after treatment. These results suggest that Safflower Yellow Injection has protective effect; the reason may be that Safflower Yellow Injection can reduce whole blood viscosity and plasma viscosity, improve index of RBC deformation, reduce index of RBC aggregation, improve microcirculation, and improve brain following ischemic reperfusion in the acute cerebral infarction; the protective effect of hydroxysafflor yellow A in ischemic stroke has been investigated in previous studies [18].

Coagulation function detection is important in the course of disease, including nosogenesis and curative effect assessment of acute cerebral infarction. In our study, we found that the value of PT increased in the Safflower Yellow Injection group after treatment and the values of FIB decreased in the Safflower Yellow Injection group after treatment, and there were significant differences between the two groups. These results suggest that Safflower Yellow Injection can inhibit both intrinsic and extrinsic coagulations, significantly the prothrombin time and clotting time, and reduce platelet adhesion, thrombosis, and fibrin cross-linking process. Recent studies show that Safflower Yellow inhibits platelet activation factor-induced platelet aggregation, antagonizes platelet activation factor-induced capillary permeability [19, 20], and protects against antithrombin III injuries originated from free radicals [21].

Researchers have demonstrated that the following brain insult cytokine levels are elevated as a result of increased production from inflammatory cells [22] with IL-1, IL-6, and tumor necrosis factor-alpha (TNF-α) and being the most studied for stroke [23, 24]. IL-1β and TNF-α have been associated with exacerbation of injury in stroke while IL-6 has been found to be neuroprotective [25]. In our study, we found that the serum levels of TNF-α, IL-1β, and IL-6 were significantly decreased in patients treated with Safflower Yellow Injection on day 14 after treatment. These results suggest that Safflower Yellow Injection is beneficial in reducing the serum levels of TNF-α, IL-1β, and IL-6 after treatment. The reason may be that Safflower Yellow Injection has anti-inflammatory effects; it is considered to be the main source of antioxidant defense in the brain following ischemic reperfusion; the protective effect of hydroxysafflor yellow A in ischemic stroke has been investigated in previous studies [12].

Our data suggest that Safflower Yellow Injection therapy may be beneficial for acute cerebral infarction. In the Chinese studies, Safflower Yellow Injection exhibits a favorable safety profile; there were no serious adverse events recorded and only 2 cases adverse events. This low rate of adverse events may be due to a combination of the fact that the patients were recruited during their recovery phase when their clinical condition had stabilized and due to the method of collection of adverse events in China.

However, there are still some limitations in our study. For example, the size (108 subjects) was not sufficient. The period of observation lasted for only 14 days. To further studies with larger sample size and long-term observations are required. Therefore, we could not rule out the expectation bias because neither the patients nor the therapists were blinded. Some future double-blinded studies would be feasible to be carried out in the future by using Safflower Yellow Injection.

5. Conclusions

In summary, our data suggest that Safflower Yellow Injection therapy may be beneficial for acute cerebral infarction. The results presented here invite for further studies with larger sample size, long-term observation, and strict blinding to confirm the efficacy of Safflower Yellow Injection.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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Research Article

Effects of Astragaloside IV on the SDF-1/CXCR4 Expression in Atherosclerosis of apoE$^{-/-}$ Mice Induced by Hyperlipaemia

Hewei Qin,[1] Ping Liu,[1] and Shengchao Lin[2]

[1] *Longhua Hospital Affiliated to Shanghai University of Traditional Chinese Medicine, 725 South Wanping Road, Shanghai 200032, China*
[2] *East China University of Science and Technology, Shanghai 200237, China*

Correspondence should be addressed to Ping Liu; liupinglonghua@126.com

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Astragaloside IV (AsIV) is the major effective component extracted from the Chinese herb *Astragalus membranaceus,* which has been widely used to treat cardiovascular disease. Recent studies have shown that AsIV can potentially protect the arteries from atherosclerosis; however the mechanisms underneath are unknown. The aim of this study was to investigate the effects of AsIV on blood lipids, CD40-CD40L signal system, and SDF-1/CXCR4 biological axis in high-fat diet apoE$^{-/-}$ mice and reveal the molecular mechanisms of AsIV against atherosclerosis. Here, we showed that AsIV alleviated the extent of atherosclerosis in aorta of apoE$^{-/-}$ mice. And AsIV can significantly downregulate PAC-1, CD40L, and CXCR4 expression on platelet surface in blood of high-fat diet apoE$^{-/-}$ mice. AsIV also can significantly downregulate mRNA and protein level of SDF-1 and CXCR4 in thoracic aorta. Consistent with aorta CXCR4 expression, CXCR4 in bone marrow-derived endothelial progenitor cell (EPC) was also reduced. Meanwhile biochemical analysis showed that AsIV could downregulate TG, TC, and LDL-C levels and upregulate HDL-C level in blood of high-fat diet apoE$^{-/-}$ mice. We concluded that the protective effects of AsIV in atherosclerosis injury may be related to regulating blood lipids, CD40-CD40L system, and SDF-1/CXCR4 biological axis. SDF-1/CXCR4 biological axis is probably one of the main targets of intervening atherosclerosis.

1. Introduction

Huang qi (*Astragalus membranaceus*) is a traditional Chinese herbal medicine that has been widely used in stroke patients in China for decades; astragaloside IV (AsIV) is a major active component of *Astragalus membranaceus*; its molecular structure, pharmacokinetics, and pharmacological actions have been well studied. Studies showed that AsIV plays a potential role in protecting the heart from myocardial ischemia, which may interpret its beneficial effects to treat cardiovascular diseases in Chinese herb medicine [1]. The mechanism of its action may be related to reducing lipid peroxidation, improving energy metabolism and inhibiting production of free radicals, and upregulation of several KATP channel subunits and facilitation of KATP currents [2].

Studies have suggested that platelets play a vital role in inflammatory reactions. Resting platelets can be activated when they are exposed to stimulants, damaged vessel wall, or extravascular tissue, accompanied by change in platelet shape and ability of adhesion, as well as aggregation. Platelet activation is an ordered sequence of events which begins with the interaction between the adhesive protein (agonist) and its receptor; the activation of platelets leads to secretion of a variety of mediators which can lead to progress of atherosclerosis, such as cytokines, chemokines, growth factors, adhesion molecules, and coagulation factors [3–5].

There are markers that can be used for platelet activation, such as integrin αIIbβ3 (PAC-1) and CD40 ligand (CD40L). CD40L is one of the receptors which translocate to the platelet surface upon platelet activation and constitutively expresses on platelets; CD40-CD40L interactions play a central role in immune responses and inflammation; PAC-1 is key part in platelet aggregation through interaction with fibrinogen. Also researchers reported that stromal cell-derived factor 1

(stromal cell-derived factor-1, SDF-1) was upregulated upon platelet activation; SDF-1 and its receptor CXCR4 (CXC chemokine receptor 4) composed SDF-1/CXCR4 axis which is related to the formation of atherosclerosis; expression of SDF-1 is enhanced on platelet surface in atherosclerosis plaques [6–8]. AMD3100 is an antagonist of CXCR4, which can disrupt binding of SDF-1 to CXCR4 by competing for the binding site, thus blocking the physiological function of SDF-1/CXCR4 axis. Moreover, SDF-1/CXCR4 axis has been shown to play critical roles in stem cell mobilization, migration, and homing and in immunoregulation, inflammatory disease, and autoimmune disorder [9, 10].

In the present study, we measured the level of biomarkers of platelet activation, PAC-1, CD40L, and CXCR4 in platelet-rich plasma, so as to investigate the effect of AsIV on platelet activation. We also examined the expression of SDF-1/CXCR4 biological axis in apoE$^{-/-}$ mice. The effects of astragaloside IV on atherosclerosis in high-fat diet apoE$^{-/-}$ mice may be through platelet activation and SDF-1/CXCR4 biological axis.

2. Materials and Methods

2.1. Animals. 30 healthy male apoE$^{-/-}$ mice at 8 weeks old were provided by Model Animal Research Center of Nanjing University, specific pathogen-free grade, weighing 20 ± 2 g. Another 12 healthy male inbred C57BL/6 mice at 8 weeks old were obtained from Shanghai SLAC Laboratory Animal Company, specific pathogen-free grade, weighing 20 ± 2 g. All animals were housed on a 12 : 12 light/dark cycle in the experimental animal center of Longhua Hospital Affiliated to Shanghai University of Traditional Chinese Medicine.

2.2. Drugs and Reagents. Astragaloside IV (purity > 98%, CAS: 84687-43-4) was purchased from Nanjing Spring & Autumn Biological Engineering Co. (Nanjing, China). AMD3100 octahydrochloride hydrate (A5602-5MG, P-code: 1001580646–1001580659) was from Sigma-Aldrich Co. LLC (St. Louis, MO, USA).

Immunohistochemical kit was purchased from Beijing Zhongshan Reagent Co. Ltd. Kit of SABC (SA2002) and kit of DAB chromogenic (AR1022) were purchased from Wuhan Boster Company (Wuhan, China).

2.3. Animal Model and Grouping. Mice in blank control group were given normal diet. All apoE$^{-/-}$ mice were given high-fat western diet whole diet (21% fat, cholesterol, 0.15%) till 2 weeks old, three of them were chosen randomly to be sacrificed in order to observe the success of modeling. apoE$^{-/-}$ mice were randomly divided into three groups: model group, AMD3100 groups, and AsIV group; C57BL/6 mice were used as the control group.

Mice in AsIV group were given 40 mg/kg·d^{-1} astragaloside by oral gavage. Mice in AMD3100 group were given 2.5 mg/kg·2d^{-1} AMD3100 by intraperitoneal injection. Mice of model group and the normal group were given 0.9% sodium chloride solution by oral gavage. Mice of each group continued to be fed following the original feeding, within all 12 weeks.

2.4. Collection of Venous Blood and Aorta. After 12 weeks of drug administration, mice were fasted for 12 hours, and venous blood was collected through the eyeball under sterile condition. Then open the chest and abdominal cavity quickly, and peel total length of the aorta along the aorta valve to the iliac artery branch.

2.5. Detection of Blood Lipid Index. After 30-minute standing, venous blood was obtained and centrifuged at 3000 rpm for 15 min. Serum was isolated. Total cholesterol [11], triacylglycerol (TG), high-density lipoprotein (HDL-C), and low-density lipoprotein (LDL-C) levels were detected by automatic biochemical analyzer. Kit of total cholesterol, Kit of TC and TG was purchased from Wenzhou Dongou Ma Jin Biotech Co., Ltd (Wenzhou, China). Kit of HDL-C and LDL-C was purchased from Beijing North of Conde Clinical Reagent Co. (Beijing, China). Kit of apolipoprotein A I (ApoA I) and apolipoprotein B (ApoB) was purchased from Shanghai Rongsheng Pharmaceutical Co. (Shanghai, China).

2.6. Histological Analyses. Thoracic aorta was isolated, fixed, and embedded in paraffin for histopathological analysis. Hematoxylin and eosin (H&E) staining was performed. Imaging from aorta tissues was detected with microscope. Detection using Image Pro Plus: luminal area (LA), intimal thickness (IMT), plaque area (PA), fibromuscular component (FS), a lipid center area (CA), and minimum fibrous cap thickness (FCT).

2.7. Detection of Platelet Activation. Platelet-rich plasma was prepared from flesh blood of the mice by density gradient centrifugation as described by Hoffman's report [12]. Each sample was then analyzed by FACScan (BD Bioscience) to detect PAC-1, CD40L, and CXCR4 level on platelet surface. FITC-conjugated PAC-1 antibody, PE-conjugated CD40L antibody, and APC-conjugated CXCR4 antibody were all purchased from GeneTex Co.

2.8. Immunohistochemical Stain. Immunohistochemical staining was performed as described [13] with the following antibodies: anti-SDF-1 (GeneTex, GTX45117) and anti-CXCR4 (GeneTex, GTX53457). Antigen granular or diffuse coloring, tan or brown pigmented cells are regarded as positive cells. The results analyzed using Image-Pro Plus image analysis system. In each section, five positive coloring regions were randomly selected and the average optical density of cells was determined.

2.9. Isolation and Culture of Bone Marrow-Derived Endothelial Progenitor Cells (EPC). Bone marrow-derived EPC were isolated from femurs of apoE$^{-/-}$ mouse bone marrow (Bm) progenitor cells and cultured in endothelial basal medium-2 (EBM-2) supplemented with growth factors (EBM-2; Lonza, catalogue number CC-3156) and 10% FCS as previously reported [14]. All experiments were performed with day 10 EPC cultures.

2.10. RNA Extraction and Quantification. Total RNA of bone marrow-derived endothelial progenitor cells and aorta tissue

were extracted with the TRIzol reagent (Invitrogen Corporation, California, USA) reverse transcription system (TaKaRa Biotechnology Ltd., Shandong, China). Real-time PCR was performed using SYBR Green SuperMix with an iCycler thermal cycler (Bio-Rad Laboratories Inc., California, USA). Primer sequences of SDF-1 and CXCR4 are listed (SDF-1: F: 5′-CCTGTGTGTCATGCCCTCTT-3′ and R: 5′-AGTCCAGCCTGCTATCCTCA-3′; CXCR4: F: 5′-GTCAACCTCTACAGCAGCGT-3′ and R: 5′-CTATCGGGGTAAAGGCGGTC-3′). The data were collected and analyzed using the comparative Ct (threshold cycle) method. GADPH RNA was used as internal control.

2.11. Western Blot Analysis. Proteins of bone marrow-derived endothelial progenitor cells and aorta tissue were extracted and separated in SDS-PAGE gels, and western blot analyses were performed according to standard procedures as previously described [15]. Protein concentrations were determined using the BCA assay (Pierce Biotechnology Inc., Rockford, USA). Equal amounts of proteins (30 μg) were prepared for western blotting assay. β-Actin was used as loading controls. The primary antibodies against SDF-1 and CXCR4 were all purchased from GeneTex Co.

2.12. Statistical Method. Data were denoted by mean value ± standard deviation ($\overline{x} \pm s$). Variance analysis was adopted. Comparison between two groups was carried out by SNK method. $P < 0.05$ indicated significant differences; and statistical calculation was accomplished by SPSS18.0 software.

3. Results

3.1. Effects of AsIV on Animal Blood Lipid. The results of TC, TG, HDL-C, and LDL-C levels of mouse in model group, AMD3100 groups, and AsIV group were shown in Table 1. Serum TC, LDL-C, and TG levels were increased and HDL-C was reduced more significantly in model group, compared with control group (all $P < 0.01$). In AsIV group, levels of TC, TG, and LDL-C were lower than those in model group and AMD3100 group (all $P < 0.05$), while level of HDL-C in AsIV group was significantly higher than that in model group and AMD3100 group ($P < 0.05$).

3.2. Histopathological Assessment. To assess the extent of atherosclerosis in thoracic aorta of high-fat diet apoE$^{-/-}$ mice after AMD3100 or AsIV treatment, aorta cross-section pathological damage was detected by HE staining. Histopathological specific data analysis (Tables 2, 3, and 4) suggested that, compared with the model group, aorta pathology of AsIV group and AMD3100 group showed that lumen areas (LA) were larger, intima medium thickness (IMT) was thinner, plaque area (PA) was smaller, fiber structure (FS) was smaller, cholesterol area (CA) was smaller, fiber cap thickness (FCT) was thinner, PA/LA was smaller, CA/PA was larger, and CA/FS was larger, with all data showing significant differences ($P < 0.05$). Compared with the AMD3100 group, aorta pathology of AsIV group showed that LA was larger, IMT was thinner, PA was smaller, FS was smaller, CA was smaller, FCT was thinner, PA/LA was smaller, CA/PA was larger, and CA/FS was larger, with all data showing significant differences ($P < 0.05$). Examples of each group were showed in Figure 1.

3.3. Effects of AsIV on PAC-1, CD40L, and CXCR4 Expression of Platelet Surface. To investigate the effect of AsIV on the activation of platelet, biomarkers of platelet activation were measured by flow cytometry. Results showed that expression of PAC-1, CD40L, and CXCR4 was significantly higher in the model group than in the control group ($P < 0.05$). Compared with the model group, the expression of PAC-1, CD40L, and CXCR4 was significantly decreased in AsIV group ($P < 0.05$). Compared with the AMD3100 group, the expression of PAC-1, CD40L, and CXCR4 was significantly decreased in AsIV group ($P < 0.05$). Figure 2 showed the results.

3.4. Effects of AsIV on SDF-1 and CXCR4 Levels in Mice Aorta Wall. Immunohistochemical staining was applied to investigate the effect of AsIV on SDF-1/CXCR4 biological axis in aorta wall of the high-fat diet apoE$^{-/-}$ mice. Figure 3(a) illustrated that expression of SDF-1 and CXCR4 in model group was significantly higher than that of control group ($P < 0.05$). However, in AMD3100 group and AsIV group, SDF-1 and CXCR4 had lower expression in the aorta smooth muscle cells than in model group. Compared with the AMD3100 group, average optical density values of SDF-1 and CXCR4 in AsIV group were higher, but the difference was not statistically significant ($P > 0.05$). Examples of each group were showed (Figures 3(b) and 3(c)).

3.5. Effects of AsIV on Expression of SDF-1 and CXCR4 in mRNA Level and Protein Level. We further examined the expression of SDF-1 and CXCR4 in mRNA level and protein level in aorta by quantitative PCR and western blotting analysis. Quantitative real-time PCR demonstrated that mRNA level of SDF-1 and CXCR4 was significantly lower in AMD3100 group and AsIV group than in model group (Figure 4(a)). However, compared with the AMD3100 group, the mRNA levels of SDF-1 and CXCR4 were higher in the AsIV group, but the difference was not statistically significant ($P > 0.05$).

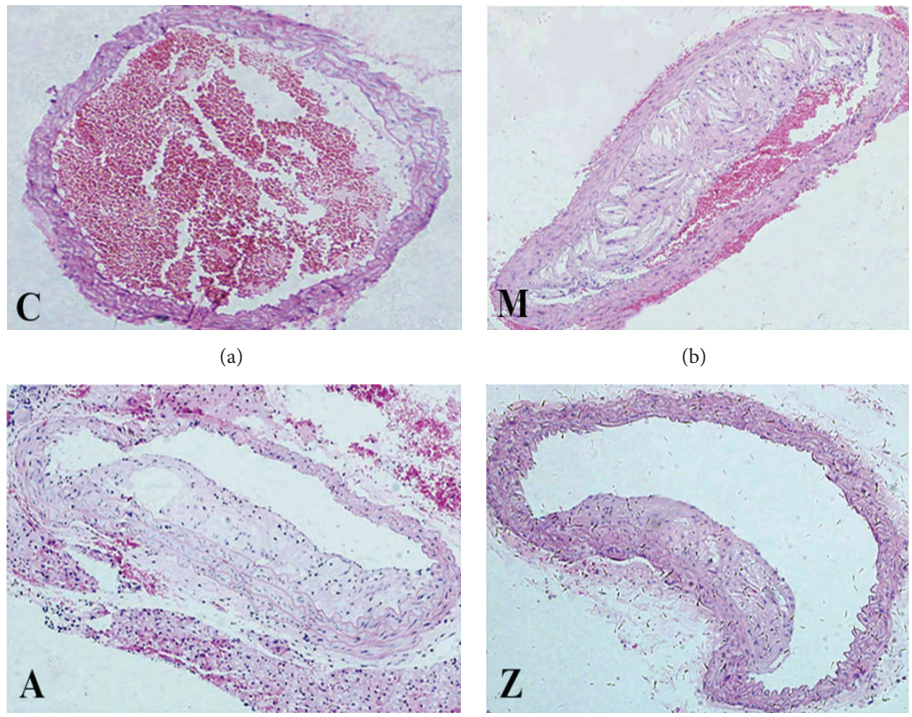
Moreover, western blotting demonstrated that proteins level of SDF-1 and CXCR4 was significantly lower in AMD3100 group and AsIV group than in model group. However, compared with the AMD3100 group, the proteins levels of SDF-1 and CXCR4 were higher in the AsIV group, but the difference was not statistically significant ($P > 0.05$) (Figures 4(b) and 4(c)). The expression of SDF-1 and CXCR4 proteins showed highly consistence with the mRNA level after AMD3100 and AsIV treatment.

3.6. Effects of AsIV on CXCR4 Expression in Bone Marrow-Derived EPC from apoE$^{-/-}$ Mice. To further verify the effect of AsIV SDF-1/CXCR4 biological axis associated with atherosclerosis, bone marrow-derived EPC were isolated and cultured (Figure 5). CXCR4 mRNA and protein levels in bone marrow-derived EPC were then detected. Quantitative real-time PCR and western blot demonstrated that the expression

Table 1: Comparison of blood lipid (mmol/L, $\overline{x} \pm s$, $n = 10$).

Group	TC	TAG	LDL-C	HDL-C
Control group	4.16 ± 1.59	1.47 ± 0.18	1.11 ± 0.23	2.59 ± 0.12
Model group	16.12 ± 0.95*	12.75 ± 2.65*	6.48 ± 0.81*	1.12 ± 0.13*
AMD3100 group	16.10 ± 0.93	12.64 ± 2.25	6.28 ± 0.91	1.13 ± 0.13
AsIV group	10.96 ± 1.32$^{\triangle\blacktriangle}$	4.78 ± 0.86$^{\triangle\blacktriangle}$	4.30 ± 0.76$^{\triangle\blacktriangle}$	2.90 ± 0.39$^{\triangle\blacktriangle}$

Note: compared with control group: $^{*}P < 0.05$; compared with model group: $^{\triangle}P < 0.05$; compared with AMD3100 group: $^{\blacktriangle}P < 0.05$.



(a) (b) (c) (d)

Figure 1: Hematoxylin and eosin stained histological sections. Note: C: control group; M: model group; A: AMD3100 group; Z: AsIV group (magnification ×200).

Table 2: Comparison of LA, IMT, and PA ($\overline{x} \pm s$, $n = 10$).

Group	LA (mm^2)	IMT (μm)	PA (mm^2)
Model group	0.255 ± 0.007	0.159 ± 0.047	0.158 ± 0.016
AMD3100 group	0.447 ± 0.042$^{\triangle}$	0.074 ± 0.013$^{\triangle}$	0.076 ± 0.018$^{\triangle}$
AsIV group	0.532 ± 0.017$^{\triangle\blacktriangle}$	0.028 ± 0.011$^{\triangle\blacktriangle}$	0.012 ± 0.013$^{\triangle\blacktriangle}$

Note: compared with model group: $^{\triangle}P < 0.05$; compared with AMD3100 group: $^{\blacktriangle}P < 0.05$.

Table 3: Comparison of FS, CA, and FCT ($\overline{x} \pm s$, $n = 10$).

Group	FS (mm^2)	CA (mm^2)	FCT (μm)
Model group	0.065 ± 0.002	0.093 ± 0.012	13.724 ± 0.443
AMD3100 group	0.031 ± 0.005$^{\triangle}$	0.041 ± 0.011$^{\triangle}$	4.136 ± 0.672$^{\triangle}$
AsIV group	0.002 ± 0.003$^{\triangle\blacktriangle}$	0.013 ± 0.012$^{\triangle\blacktriangle}$	2.306 ± 0.558$^{\triangle\blacktriangle}$

Note: compared with model group: $^{\triangle}P < 0.05$; compared with AMD3100 group: $^{\blacktriangle}P < 0.05$.

of CXCR4 mRNA and protein was significantly higher in the model group than in the control group ($P < 0.05$). Compared with the model group, the expression of CXCR4 mRNA and protein was significantly decreased in the AMD3100 group and AsIV group ($P < 0.05$). Compared with the AMD3100 group, the expression of CXCR4 mRNA and protein in AsIV group was higher, but the difference was not statistically significant ($P > 0.05$) (Figures 6(a) and 6(b)).

4. Discussion

The pathology of atherosclerosis development is a comprehensive long-term process. Due to its complex pathogenesis, there is no existing preventive and control strategy. "Injury-response theory" and the "inflammatory reaction theory" are the most popular explanation theories [16].

Table 4: Comparison of PA/LA, CA/PA, and CA/FS ($\overline{x} \pm s$, $n = 10$).

Group	PA/LA	CA/PA	CA/FS
Model group	2.718 ± 0.386	0.581 ± 0.150	1.493 ± 0.290
AMD3100 group	0.690 ± 0.130$^{\triangle}$	0.776 ± 0.070$^{\triangle}$	2.516 ± 0.317$^{\triangle}$
AsIV group	0.041 ± 0.023$^{\triangle\blacktriangle}$	0.797 ± 0.116$^{\triangle\blacktriangle}$	3.546 ± 0.913$^{\triangle\blacktriangle}$

Note: compared with model group: $^{\triangle}P < 0.05$; compared with AMD3100 group: $^{\blacktriangle}P < 0.05$.

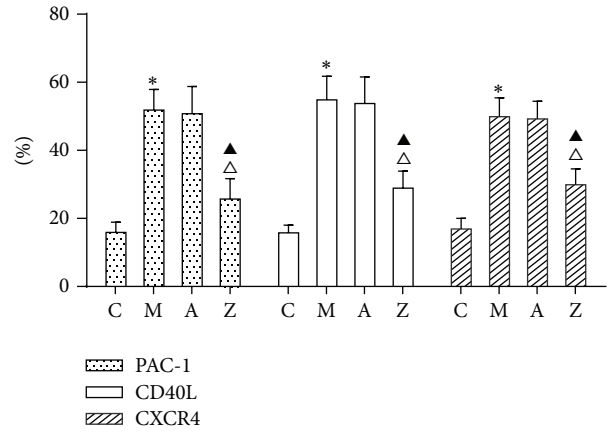


Figure 2: PAC-1, CD40L, and CXCR4 expression of platelet surface. Note: C: control group; M: model group; A: AMD3100 group; Z: AsIV group. Compared with control group: $^{*}P < 0.05$; compared with model group: $^{\triangle}P < 0.05$; compared with AMD3100 group: $^{\blacktriangle}P < 0.05$.

Targeting the key component in the atherosclerosis inflammatory response network can interrupt the formation of atherosclerosis and reduce the degree of injury. Recently, an increasing number of reports show that Chinese medicine has extraordinary effects on the treatment of atherosclerosis, which gradually draw people's attention to Chinese medicine. Huang qi (*Astragalus membranaceus*) is described as the dried root of leguminous plants in the Chinese Pharmacopoeia, it can help regeneration, and it also has effect on cardiovascular disorders, hepatitis, kidney disease, and skin diseases. Astragaloside IV is a major active component of the native *Astragalus membranaceus*. During decade, several researches have focused on studying extraction separation, pharmacokinetics, and pharmacological activities of astragaloside IV by separation and pure. It has been reported that astragaloside IV can promote zebrafish *in vivo*, which is closely associated with increase in the expression of vascular endothelial cell growth factor and its receptor, thereby activating the pathway of protein kinase B and phosphoinositide 3-kinase, as well as regulation of the expression of hypoxia inducible factor protein [17]. With regard to protection of endothelial function, researches showed that astragaloside IV can resist lipoprotein-induced injury to endothelial cells and increase the level of malondialdehyde (MDA) and SOD [18].

Recently, the role of CD40/CD40 ligand (CD40L) interactions in atherothrombosis, in the response of the immune system to pathogens and in thrombosis is now widely accepted. CD40-CD40L interactions have been identified in atherosclerosis, and such interactions can destabilize atherosclerotic plaques by inducing the expression of cytokines, chemokines, growth factors, matrix metalloproteinases, and procoagulant factors. Many literatures report that activated platelets can lead to overexpression of SDF-1; moreover, conglutination between SDF-1 and chemokine receptor CXCR4 can regulate cell migration, tissue targeting, and homing [19]. Based on the above evidence, our assumption is that astragaloside IV can target CD40-CD40L and therefore intervenes in atherosclerosis.

A latest study shows that atherosclerosis was related to SDF-1/CXCR4 biological axis. SDF-1, also known as CXCL12, belonged to CXC subfamily of chemokines. It was first discovered in cytokines secreted by mouse bone marrow stromal cells. After platelet activation, SDF-1 can be abundantly expressed and combined with chemokine receptors, CXCR4. SDF-1/CXCR4 biological axis consisting of SDF-1 and its receptor CXCR4 induced CD34+ stem cells to differentiate into macrophages and foam cells, which later caused the atherosclerosis. CXCR4 is widely expressed on surfaces of hematopoietic stem cells and bone marrow stromal cells; it is also expressed in AS lesions. Interaction of SDF-1 and CXCR4 control cell migration and issue-selective homing, which cause blood cells adhere to endothelial cells more conducive. Treating endothelium with chemokines suggests that SDF-1/CXCR4 axis plays an important role in the development of atherosclerosis [20]. On the other hand, SDF-1/CXCR4 biological axis is associated with bone marrow-derived endothelial progenitor cells during the development of atherosclerosis. Later on, Asahara et al. isolated and cultured progenitor cells from peripheral blood progenitor cells [21]. Werner et al. found that endothelial progenitor cells have independent predictive value for atherosclerotic disease [22]. On the basis of all these studies, we strongly assume that SDF-1/CXCR4 biological axis is targeted by astragaloside IV which explains how it intervenes in atherosclerosis.

apoE knockout (apoE$^{-/-}$) mice were used as the animal model in our study. apoE$^{-/-}$ mice fed by high-fat diet had the lesion characteristics of serious dyslipidemia and atherosclerosis, which were relatively good animal model for the research on atherosclerosis. In this research, C57BL mice had the characteristics of short life time and generation time, which were a kind of experimental animal commonly used in geriatric medicine.

In our research, we observed the effects of AsIV on blood lipids, CD40-CD40L signal system, and SDF-1/CXCR4 biological axis in high-fat diet apoE$^{-/-}$ mice; the research results showed that TG, TC, HDL-C, and LDL-C levels in AsIV group were significantly better than those in model group ($P < 0.05$), AsIV significantly downregulated PAC-1, CD40L, and CXCR4 expression of platelet surface in blood of high-fat diet apoE$^{-/-}$ mice. The extent of atherosclerosis in aorta of apoE$^{-/-}$ mice in AsIV group was significantly lighter than that in model group, and the SDF-1 and CXCR4 expression of aorta reduced, showing statistical significance ($P < 0.05$).

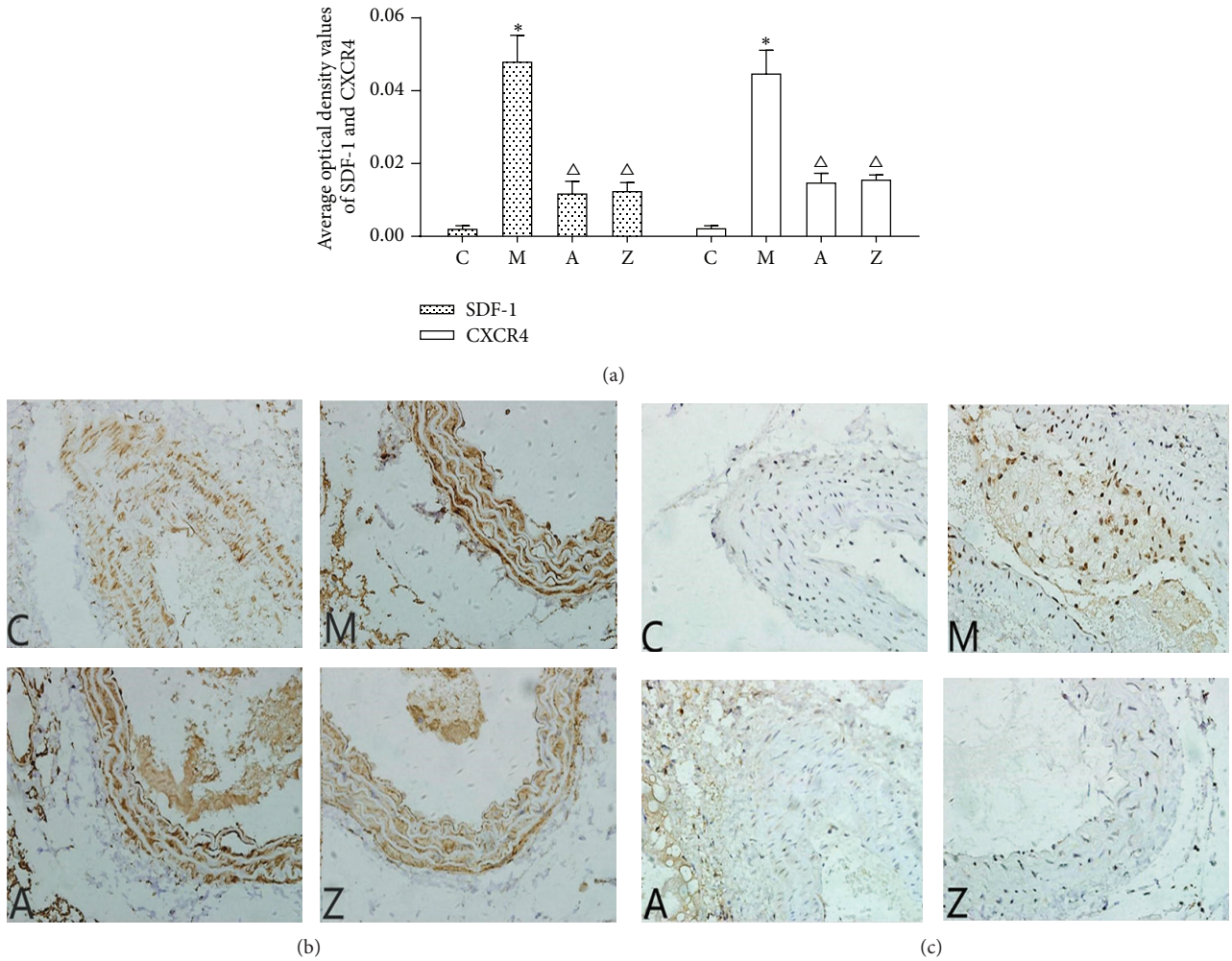


Figure 3: (a) The expression of SDF-1 and CXCR4 (optical density value). Compared with control group: $^{*}P < 0.05$; compared with model group: $^{\triangle}P < 0.05$. (b) Expression of SDF-1 (magnification ×400). (c) Expression of CXCR4 (magnification ×400). Note: C: control group; M: model group; A: AMD3100 group; Z: AsIV group.

Western blotting and real-time PCR demonstrated that astragaloside IV significantly downregulated protein and mRNA expression of SDF-1 and CXCR4 ($P < 0.05$ versus model group), showing statistical significance. Consistent with this, astragaloside IV significantly downregulated protein and mRNA expression of CXCR4 of bone marrow-derived endothelial progenitor cell ($P < 0.05$ versus model group).

5. Conclusions

By above results, we can clearly know that platelet activation can induce SDF-1/CXCR4 biological axis imbalance. The protective effects of AsIV in atherosclerosis injury may be related to AsIV downregulation of CD40L, PAC-1, and CXCR4 expression by blocking the CD40-CD40L system. In addition, AsIV significantly down-regulated mRNA and protein level of SDF-1, CXCR4 in thoracic aorta. SDF-1/CXCR4 biological axis is probably one of the main targets of intervening atherosclerosis. Therefore, astragaloside IV plays a role in atherosclerosis of high-fat diet apoE$^{-/-}$ mice by regulating blood lipids, CD40-CD40L system, and SDF-1/CXCR4 biological axis probably. The research provides new approach for treatment of atherosclerosis and related diseases.

Conflict of Interests

The authors declare that they have no conflict of interests.

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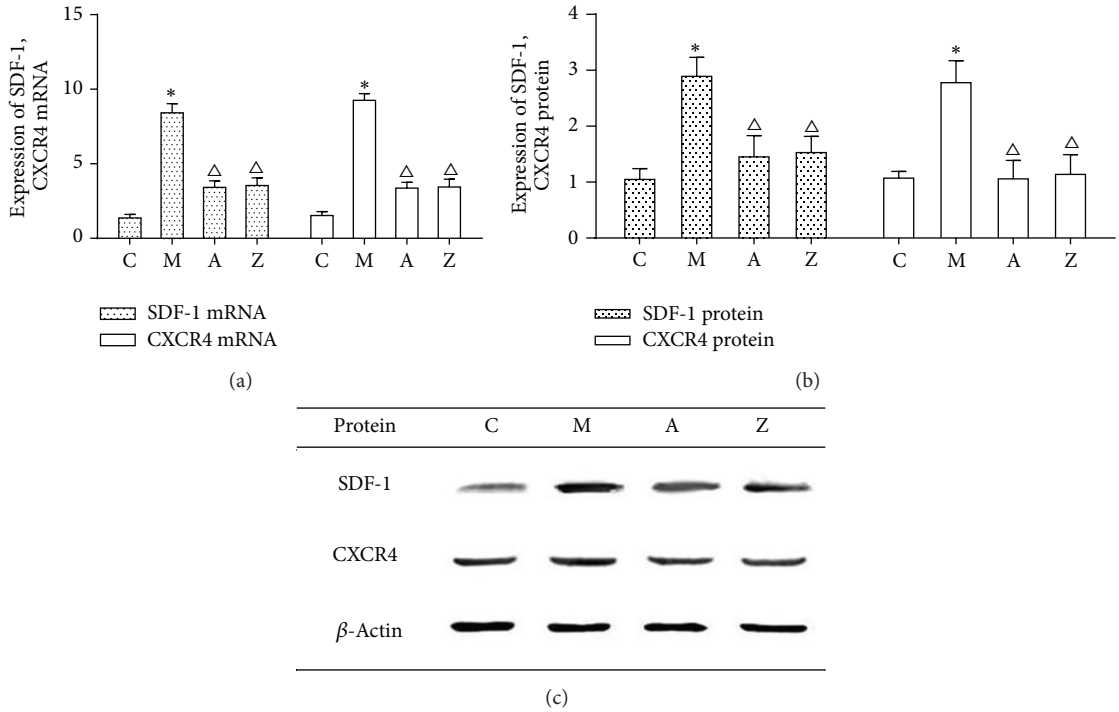

Figure 4: (a) The expression of SDF-1, CXCR4 mRNA. (b, c) The expression of SDF-1, CXCR4 protein. Note: C: control group; M: model group; A: AMD3100 group; Z: AsIV group. Compared with control group: $^{*}P < 0.05$; compared with model group: $^{\triangle}P < 0.05$.

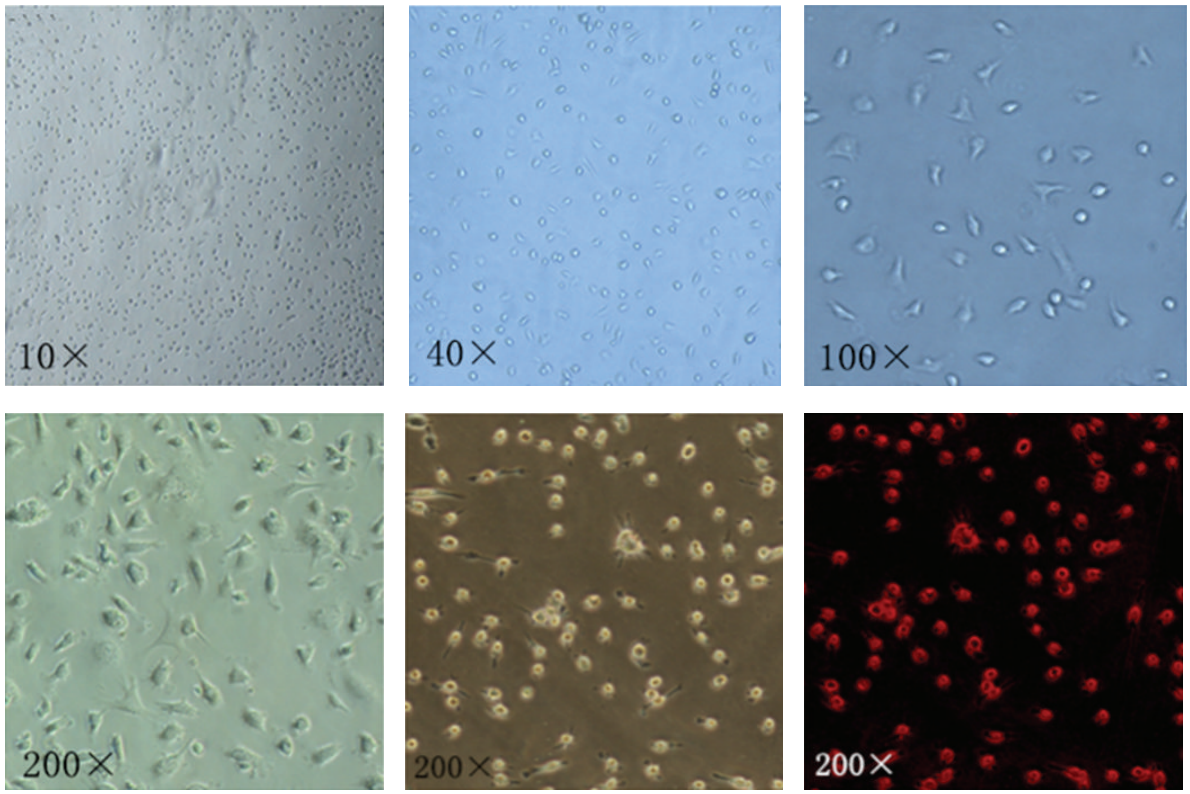

Figure 5: Identification of bone marrow-derived EPC (magnification ×200, VEGF-R positive cells, white shot; PE labeled VEGF-R antibodies stimulate green shoot).

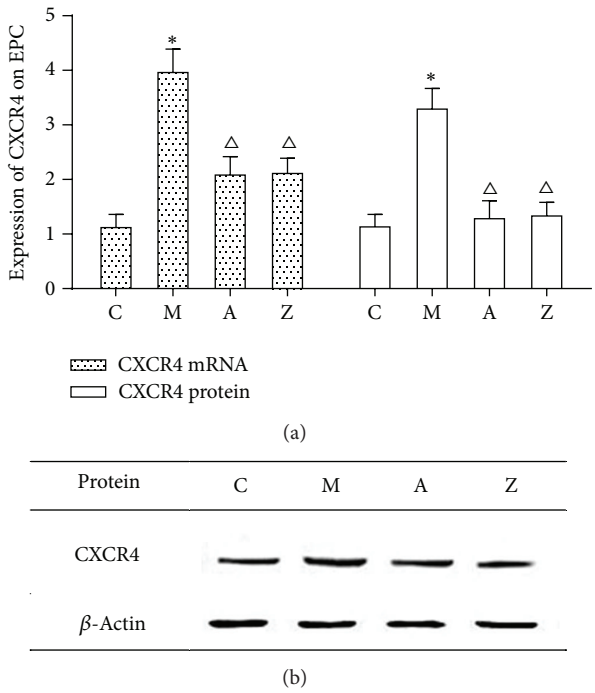

Figure 6: The expression CXCR4 on EPC. Note: C: control group; M: model group; A: AMD3100 group; Z: AsIV group. Compared with control group: $^{*}P < 0.05$; compared with model group: $^{\triangle}P < 0.05$.

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Research Article

Antithrombotic Effects of Five Organic Extracts of Bangladeshi Plants *In Vitro* and Mechanisms in *In Silico* Models

Sakib Mahmud,[1] Samina Akhter,[1] Md. Atiar Rahman,[1] Jannatul Aklima,[1] Shaheen Akhter,[2] Syeeda Rayhana Merry,[2] S. M. Rakibul Jubair,[2] Raju Dash,[3] and Talha Bin Emran[3]

[1]*Department of Biochemistry and Molecular Biology, University of Chittagong, Chittagong 4331, Bangladesh*
[2]*Bangladesh Forest Research Institute, Chittagong 4000, Bangladesh*
[3]*Department of Pharmacy, BGC Trust University, Chittagong 4000, Bangladesh*

Correspondence should be addressed to Md. Atiar Rahman; atiarh@yahoo.com

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This research was carried out to investigate the thrombolytic effects of the methanolic extracts of five Bangladeshi plants. Phytochemical metabolites of those plants have been identified to elucidate whether the plant-derived metabolites are linked with the thrombolytic effects. Potential computer aided models were adopted in this study to find out a structure-function correlation between the phytochemical constituents and thrombolytic effects using the secondary metabolites as ligands and tissue plasminogen activator (t-PA) as receptor for the best fit ligand-receptor interaction.

1. Introduction

Thrombosis is a fatal disease which is characterized by the formation of blood clots (thrombus) in the circulatory system because of the imbalance of homeostatic system of physiological procedures. This is a critical event in the arterial diseases connected with acute coronary disorders such as pulmonary emboli, deep vein thrombosis, strokes, heart attacks, and venous thromboembolic disorders that account for sudden morbidity and mortality. Thrombosis leads to vascular blockade and while recovering it causes fatal consequences, such as cerebral or myocardial infarction and even death [1].

Thrombolytic agents that include tissue plasminogen activator (t-PA), alteplase, anistreplase, urokinase (UK), and streptokinase (SK) are widely used throughout the world for the treatment of thromboembolic diseases although streptokinase and urokinase are the first choices in Indian regions due to the easy reach and lower cost [2, 3] as compared to other thrombolytic drugs. But the weak substrate specificity of these first-generation drugs (streptokinase and urokinase) commonly leads to some major side effects such as anaphylactic reaction, systemic fibrinolysis, and hemorrhage [4]. Immunogenicity is another important issue which restricts the multiple treatments of a given patient with streptokinase [5]. Because of the setbacks of the available thrombolytic drugs, attempts are underway to develop improved recombinant variants of these drugs [6, 7]. Thrombolytic therapy with recombinant t-PA is effective in acute myocardial infection, but the treatment is limited by a fairly slow reperfusion rate and frequent early reocclusions. Moreover, the platelet-rich thrombi are highly resistant to lysis by t-PA [8]. Due to the limited scopes of almost all the synthetic and recombinant antithrombotic drugs, the mergence for alternatives is highly necessitated. Previously we reported few Bangladeshi plants showing mild to potent thrombolytic effects [9]. This research aims to investigate the thrombolytic effects of some other five Bangladeshi medicinal plants such as *Ocimum tenuiflorum*, *Andrographis paniculata*, *Adhatoda vasica*, *Leea macrophylla*, and *Litsea glutinosa*. These plants have been chosen based on their traditional uses by local communities, herbalists, or traditional healers. Researchers reported their common uses in cardiovascular diseases, atherosclerosis, blood impurities, and other relevant disorders. *Ocimum tenuiflorum* has also been shown to counter metabolic stress through normalization of blood glucose, blood pressure and lipid levels, and psychological stress through positive effects on memory and

cognitive function and through its anxiolytic and antidepressant properties [10]. *Andrographis paniculata* has long been used in cardiovascular diseases. It is a spontaneous hypotensive agent. Different components of this plant are also involved in heart diseases [11]. *Adhatoda vasica* is used as a blood purifier [12]. *Leea macrophylla* is reported to be used in effusion of blood [13]. *Litsea glutinosa* leaf extract is used in cardiovascular activities [14]. In spite of their uses in relevant disorders, there is little or no scientific evidence to be used as therapeutically proven drugs. This research, therefore, investigated not only their thrombolytic effects but also the probable mechanism, through *in silico* docking model, of how they are involved in such biological action.

2. Materials and Methods

2.1. Plant Collection and Identification. The plants *Ocimum tenuiflorum*, *Andrographis paniculata*, and *Adhatoda vasica* were cultivated and harvested from Rangamati, Khagrachari, and Bandarban hill tracts area, respectively. They were harvested in the month of July-August (2013) and they were preserved in Bangladesh Forest Research Institute, Chittagong, at low temperature (16–20)°C. *Leea macrophylla* was collected from the cultivated area of Bangladesh Council of Scientific and Industrial Research (BCSIR), Rajshahi, whereas *Litsea glutinosa* leaves were collected from Chittagong University Campus, Bangladesh. *Leea macrophylla* and *Litsea glutinosa* were also preserved at low temperature (16–20)°C in the Laboratory of Phytomedicine, Department of Biochemistry and Molecular Biology, University of Chittagong. The plants were authenticated as *Ocimum tenuiflorum*, *Andrographis paniculata*, *Adhatoda vasica*, *Leea macrophylla*, and *Litsea glutinosa* by Dr. Shaikh Bokhtear Uddin, Taxonomist and Associate Professor, Department of Botany, University of Chittagong.

2.2. Chemicals and Reagents. To the commercially available lyophilized streptokinase (SK) vial (Incepta Pharma. Co. Ltd., Dhaka, Bangladesh) of 1500000 I.U., 5 mL sterile distilled water was added and mixed properly. This suspension was used as a stock from which 100 μL (30,000 I.U.) was used for *in vitro* thrombolysis. Hexane (99.5%) and methanol (99%) were purchased from the local sources and they were of reagent grade.

2.3. Preparation of Extracts. Plant materials were washed properly and chopped into small pieces to make a semished sun-dry for seven days. After drying, plant materials were powdered with mechanical grinder (Willey mill). The resulting powder was defatted through hexane followed by soaking into methanol for eight days with an occasional stirring while the filtrate was collected after every two days using cheesecloth and Whatman filter paper number 1. The filtrate was concentrated under reduced pressure at the temperature below 50°C using rotatory evaporator (RE 200, Bibby Sterling Ltd., UK) to find crude extract in glass Petri dishes (90 × 15 mm, Pyrex, Germany). The crude was allowed to dry for the complete evaporation of solvent at 37°C [15]. The crude methanol fractionation was obtained using protocol designed by Kupchan et al. [16]. These concentrated extracts were used to investigate antithrombotic effect of the mentioned medicinal plants.

2.4. Sample Preparation and Qualitative Phytochemical Screening. The crude methanol extracts of *Ocimum tenuiflorum*, *Andrographis paniculata*, *Adhatoda vasica*, *Leea macrophylla*, and *Litsea glutinosa* were undertaken for phytochemical screening in order to detect the presence (or absence) of alkaloids, flavonoids, steroids, tannins, saponins, phlobatannins, and glycosides. 100 mg of each of the extracts was suspended in 10 mL distilled water and the suspension was shaken vigorously on a vortex mixer. The suspension was kept overnight and decanted to remove the soluble supernatant, which was filtered through a 0.22 μm syringe filter. 100 μL of this aqueous preparation was added to the micro centrifuge tubes containing the clots to check thrombolytic activity. This experimental approach was carried out using standard techniques as described below [17–19].

2.4.1. Test for Alkaloids. 5 mg of extract was taken in a test-tube. 2.0 mL of 1% HCl is added to the test-tube. Mixture was stirred on a steam bath. After that, cooling and filtering were done. A few drops of Mayer's reagent were added to it. A creamy precipitation indicates the presence of alkaloids.

2.4.2. Test for Flavonoids (NaOH Test). 1 mL of extract of each plant species was taken in test-tubes. A few drops of 1% HCl were added to the test-tubes. Then 2 mL of NaOH was added. A canary yellow color indicates the presence of flavonoids.

2.4.3. Test for Steroids (Salkowski's Test). 1 mL of each plant extract was taken in test-tubes. 1 mL of concentrated sulfuric acid (H_2SO_4) was added to the test-tubes. Appearance of a clear reddish brown color ring at the interface confirms the presence of steroids.

2.4.4. Test for Tannins. 1 mL of each plant extract was taken in test-tubes. Two drops of freshly prepared $FeCl_3$ were added to it. Dark black color precipitation can be observed which gives green black to blue black color on dilution which indicates the presence of tannins.

2.4.5. Test for Saponins. 1 mL of each plant extract was taken in test-tubes. 2.5 mL of distilled H_2O is added to the test-tubes. Test-tubes with solution were vigorously shaken and allowed to stand for few minutes at room temperature. A persistent frothing indicates the positive results for saponins.

2.4.6. Test for Phlobatannins. 1 mL of each plant extract was taken in test-tubes. 2.5 mL of 2% HCl was added to it. Formation of precipitation indicates the positive results for phlobatannins.

2.4.7. Test for Cardiac Glycosides. 2 mL of each plant extract was taken in test-tubes. 1 mL of glacial acetic acid was added. After that, 1 drop of liquid $FeCl_3$ was added and then 0.5 mL of

concentrated H_2SO_4 was added to the test-tube. Brown ring at the interface indicates the presence of glycosides.

2.5. Antithrombotic Effects

2.5.1. Blood Specimen. From twenty healthy human volunteers, six milliliters of the whole blood was drawn without a history of oral contraceptive or anticoagulant therapy using a protocol approved by the Institutional Ethics Committee of Chittagong University, Faculty of Medicine. An earlier consent, approval number HET-CU2013/3, was taken from the Faculty of Medicine, University of Chittagong, for collection of blood samples from human volunteers. 500 μL of blood was transferred to each of the seven previously weighed micro centrifuge tubes to form clots.

2.5.2. Consent of Informed Donor. A consent form mentioning research project title, name, and details of investigators contacts as well as the purpose of the research was supplied to the volunteer donors. They were also supplied with the detailed description of the inclusion and exclusion criteria of the donors, whether donors will receive any therapy or not, volume of blood to be taken, possible discomfort of the puncture sites, and time required for blood sampling. Explanation was made on if future use of the research data beyond the current study is anticipated, whether this is a focus group if so the Principal Investigator should put a procedure in place in which the researchers caution people against the limit on confidentiality. Access to research information regarding who would have access to the collected sample and information regarding retention of sample and schedules for their disposal was also detailed. It was indicated in the consent form that the volunteers might refuse to donate blood at any time. Donor whether could withdraw his sample data was disclosed. The sample that was restricted for that individual study not for future research projects was presented in the consent form. Possible complications, for example, the possibility of bruising or swelling while giving blood, or some other discomforts at the site where blood is drawn, and that there might be minimal chance of infection and that these discomforts were brief and transient, were also informed. The potential benefits of this study, not directly of the donors but the society in general or individuals with a similar condition that might be benefitted from the results of the study, were explained. Confidentiality statement was included in the consent form in the way that "confidentiality will be respected and no information that discloses the identity of the participant will be released or published without consent unless required by law of states. The legal obligation includes a number of circumstances, such as suspected child abuse and infectious disease, expression of suicidal ideas where research documents are ordered to be produced by a court of law and where researchers are obliged to report to the appropriate authorities. In those rare instances where it will not be possible to assure complete confidentiality," the limits on this obligation were carefully explained. The signatures with date of the donors were also included in the consent form.

2.5.3. Determination of Clot Lysis. Clot lysis approaches were carried out as reported earlier [20]. 6 mL venous blood drawn from the healthy volunteers was distributed in 10 different preweighed sterile micro centrifuge tubes (0.5 mL/tube) and incubated at 37°C for 45 min. After clot formation, serum was completely removed without disturbing the clot and each tube having clot was again weighed to determine the clot weight (clot weight = weight of clot containing tube − weight of tube alone). 100 μL of methanol extracts of *Ocimum tenuiflorum*, *Andrographis paniculata*, *Adhatoda vasica*, *Leea macrophylla*, and *Litsea glutinosa* was added separately. As positive and negative controls, 100 μL of SK and distilled water, respectively, was added separately. All the tubes were then incubated at 37°C for 90 min and observed for clot lysis. Released fluid was removed and tubes were again weighed to observe the difference in weight after clot disruption. Difference obtained in weight taken before and after clot lysis was expressed as percentage of clot lysis. The experiment was repeated with the blood samples of the informed donors.

2.5.4. Molecular Docking Analysis for Thrombolytic Mechanism. For molecular docking studies, the data from databases used in this study include PDB (Protein Data Bank) [21] and Pubchem [22]. For docking analysis, the protein file was prepared through the receptor preparing wizard in FlexX [23] which includes Flexx (LeadIT 2.1.6). FlexX, a fully automated docking program available on LeadIT 2.1.6 package, was used to dock compound into the active site of the enzymes. FlexX is a fragment based docking algorithm, which builds putative poses of the ligands using an incremental construction approach. FlexX considers ligand flexibility by changing the conformations of the ligand in the active site while making the protein rigid [23].

(1) Docking with FlexX. FlexX (which is now a part of LeadIT) is a flexible docking method that uses an incremental construction (IC) algorithm and a pure empirical scoring function similar to the one developed by Böhm and coworkers to place ligands into the active site [24]. IC algorithms first dissect each molecule into a set of rigid fragments according to rotatable bonds and then incrementally assemble the fragments around the binding pocket [23]. For docking studies, a receptor description file was prepared through the FlexX graphic interface. An active site was defined by selecting the residue of the protein. The active site includes protein residues around 10 Å radius sphere centered on the center of mass of the ligand. Based on energy values, top ten ranked poses for each ligand in data set were selected for further analysis.

The free binding energy ΔG of the protein-ligand complex is given by

$$\Delta G = \Delta G_0 + \Delta G_{\text{rot}} \times N_{\text{rot}} + \Delta G_{\text{hb}} \sum_{\text{neutral H bonds}} f(\Delta R, \Delta\alpha) + \Delta G_{\text{io}} \sum_{\text{ionic int.}} f(\Delta R, \Delta\alpha)$$

Table 1: Methanol extracts of *Ocimum tenuiflorum*, *Andrographis paniculata*, *Adhatoda vasica*, *Leea macrophylla*, and *Litsea glutinosa* and their physical properties.

Plant name	Solvent	Powder (g)	Crude extract (g)	Yield (%)	Crude physical appearance
Ocimum tenuiflorum	Methanol	320	25.67	8.02	Deep green with presence of arbitrary shaped crystals
Andrographis paniculata	Methanol	880	83.34	9.47	Deep green gummy mass
Adhatoda vasica	Methanol	921	31.97	3.47	Green with reddish gummy mass
Leea macrophylla	Methanol	600	33.6	5.6	Greenish semisolid
Litsea glutinosa	Methanol	450	20	4.4	Black

Table 2: Qualitative phytochemical screening of *Ocimum tenuiflorum*, *Andrographis paniculata*, *Adhatoda vasica*, *Leea macrophylla*, and *Litsea glutinosa*.

Chemical constituent	Name of the species				
	Ocimum tenuiflorum	*Andrographis paniculata*	*Adhatoda vasica*	*Leea macrophylla*	*Litsea glutinosa*
Alkaloids	—	—	+	—	++
Flavonoids	+++	++	+	++	++
Steroids	+++	++	++	++	++
Tannins	+++	-	++	—	++
Saponins	++	+++	+	—	++
Phlobatannins	—	—	—	+	—
Glycosides	++	+	+	+	++

Note: "+," "++," and "+++" indicate the mild, moderate, and strong presence, whereas "—" indicates the absence of secondary metabolites in respective extract.

$$+ \Delta G_{\text{ar}} \sum_{\text{aro int.}} f(\Delta R, \Delta\alpha) + G_{\text{lipo}} \sum_{\text{lip ocont.}} f * (\Delta R). \quad (1)$$

Here, $f(\Delta R, \Delta\alpha)$ is a scaling function penalizing deviations from the ideal geometry and N_{rot} is the number of free rotatable bonds that are immobilized in the complex. The terms ΔG_{hb}, ΔG_{io}, ΔG_{ar}, and ΔG_0 are adjustable parameters. ΔG_{lipo} is lipophilic contact energy [23, 25].

2.6. Statistical Analysis. The calculated significance between the percentages of clot lysis by SK and plant extracts of *Ocimum tenuiflorum*, *Andrographis paniculata*, *Adhatoda vasica*, *Leea macrophylla*, and *Litsea glutinosa* was tested by the paired *t*-test analysis using the software SPSS, version 18.0 (SPSS for Windows, Version 18.0, IBM Corporation, New York, USA). Data are expressed as mean ± SD. The mean difference between positive and negative controls was considered significant at *P* values that were less than 0.05.

3. Results and Discussion

3.1. Results. Physical properties and yields of the crude extracts are summarized in Table 1. Qualitative phytochemical screening of the extracts revealed the presence of different secondary metabolites (Table 2). The presence of flavonoids, steroids, and cardiac glycosides was consistently noted in all the plant extracts. Alkaloids were present in *Adhatoda vasica* and *Litsea glutinosa* but absent in others. Only *Leea macrophylla* showed the phlobatannins. Tannins and saponins were absent in *Leea macrophylla*. Tannins were not present in *Andrographis paniculata.*

In antithrombotic approach with human blood sample, addition of 100 μL streptokinase, a positive control (30,000 I.U.), to the clots and subsequent incubation for 90 minutes at 37°C showed 71.14 ± 6.91% clot lysis. On the other hand, distilled water was treated as negative control which showed only 8.89 ± 2.22%, a negligible clot lysis. The mean difference in clot lysis percentage between positive and negative controls was very significant (*P* values less than 0.001). *Leea macrophylla* showed the highest significant (47.47 ± 6.65%) clot lysis activity among all the extracts (*P* values < 0.001). *Andrographis paniculata* showed 35.74 ± 6.76% of clot lysis and its *P* value was less than 0.001. *Ocimum tenuiflorum* and *Litsea glutinosa* showed 26.08 ± 5.12% and 27.25 ± 3.97% of clot lysis, respectively. *Adhatoda vasica* showed 22.86 ± 3.61% clot lysis and the value was very significant (*P* values < 0.001). Percentages of clot lysis obtained after treating the clots with different organic extracts and appropriate controls are shown in Table 3 and their comparison was represented in Figure 1.

Presence of three common and major metabolites in all the experimental extracts was considered as the basis to undertake the metabolites for molecular docking analysis. Molecular docking is an effective and fast computational technique to estimate the binding affinity of a ligand (drug candidate) in the macromolecular target site (receptor). The active site was identified and considered the reference ligand binding in the position of Lys-698 residues (SK binding site) shown in Figures 2(a) and 2(b). However, after docking simulation done by flexX simulation, it was found that only α-d-glucopyranose (CID79029 and CID64689) and β-d-glucopyranose considered as glycoside skeleton showed

Table 3: *In vitro* clot lysis activity of *Ocimum tenuiflorum*, *Andrographis paniculata*, *Adhatoda vasica*, *Leea macrophylla*, and *Litsea glutinosa*.

Herbs/drugs	Fractions	% of clot lysis (mean ± SD)	P value (two-tailed) when compared to negative control (water)
Negative control (water)	Methanol	8.89 ± 2.22	—
Positive control (streptokinase)	Methanol	71.14 ± 6.91	$P < 0.001$
Ocimum tenuiflorum	Methanol	26.08 ± 5.12	$P < 0.001$
Andrographis paniculata	Methanol	35.74 ± 6.76	$P < 0.001$
Adhatoda vasica	Methanol	22.86 ± 3.61	$P < 0.001$
Leea macrophylla	Methanol	47.47 ± 6.65	$P < 0.05$
Litsea glutinosa	Methanol	27.25 ± 3.97	$P < 0.001$

Values are mean ± SD ($n = 20$); $^{*}P < 0.05$, $^{**}P < 0.001$, Dunnett test as compared to control (positive and negative). Statistical representation of the effective clot lysis percentage by herbal preparations, positive thrombolytic control (streptokinase), and negative control (sterile distilled water) processed by paired t-test analysis (Dunnett test).

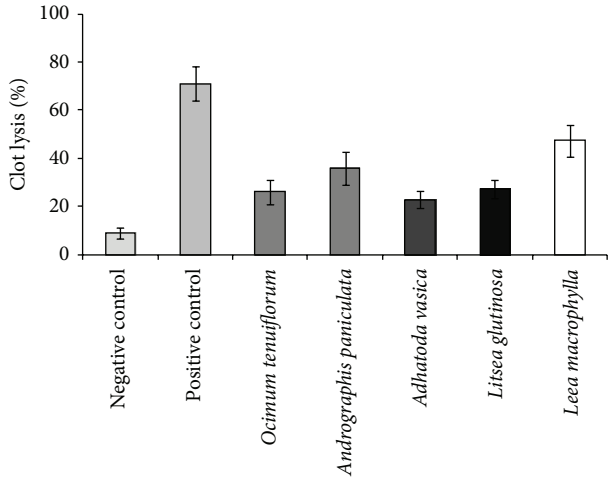

Figure 1: Comparative clot lysis by streptokinase, water, and methanol extract of *Ocimum tenuiflorum*, *Andrographis paniculata*, *Adhatoda vasica*, *Leea macrophylla*, and *Litsea glutinosa*.

the maximal binding energies which were −14 kj/mol and −15 kj/mol, respectively. The postdocking analysis suggests that α-d-glucopyranose was involved in the formation of six salt bridges with Cys^{737}, Gly^{739}, Glu^{687}, Trp^{685}, and Gln^{738} (Figure 2(c)) and other residues such as Gly^{686}. In case of β-d-glucopyranose (Figure 2(d)), six hydrogen bonds with Gly^{564}, Gln^{738}, Trp^{685}, Gly^{739}, Glu^{687}, and Lys^{698} residues were formed. However, Lys^{698} was found to act as a contributor of hydrophobic bond. Ligand efficiency of α-d-glucopyranose was 0.27 and that of β-d-glucopyranose was 0.30. No binding efficiency was observed for the basic skeleton of flavonoids and steroids.

3.2. Discussion. Advances in phytochemistry and identification of plant compounds, which are effective in curing certain diseases, have renewed the interest in herbal medicines. About 30% of the pharmaceuticals are prepared from plants worldwide [26, 27]. Phytochemical analysis conducted on the plant extracts revealed the presence of constituents which are known to exhibit medicinal as well as physiological activities [17]. In this research, all the experimented plant extracts exhibited flavonoids, steroids, and cardiac glycosides. The activities of flavonoids are due to their ability to form complex with extracellular and soluble proteins [28]. They are also effective as antioxidant and antiplatelet [29–33]. Glycosides are known to lower the blood pressure according to many reports [34].

A number of studies have been conducted by various researchers to find out the herbs and natural food sources and their supplements having antithrombotic (anticoagulant and antiplatelet) effect and there is evidence that consuming such food leads to prevention of coronary events and stroke [35–37]. Herbal preparations, if taken in appropriate dose, can lead to a better option for curing various ailments. In our thrombolytic assay, the comparison of positive control with negative control clearly demonstrated that clot dissolution does not occur when water was added to the clot. When compared with the clot lysis percentage obtained through SK and water, a significant ($P < 0.05$) thrombolytic activity was observed after treating the clots with the extracts. Methanol extract of *Leea macrophylla* showed the highest and *Adhatoda vasica* showed the lowest thrombolytic effects. *Andrographis paniculata* showed thrombolytic effects close to those of *Leea macrophylla* although the extent of glycosides in *Andrographis paniculata* is much lower than that of the latter one. The phenomenon could be explained as a fact that the active constituent in both the glycosides might be different while the active glycosidic ingredient of *Andrographis paniculata* could be manifolds stronger than that of *Leea macrophylla* suggesting that not only the extent of an individual type of metabolite but also the active ingredient of that sort of metabolite is also important for biological activity. And we investigated the type and extent of secondary metabolites in our research but not the active principle in the extract.

As discussed earlier, all the experimental plant extracts exhibited flavonoids, steroids, and glycosides. So, it is our interest to know which secondary metabolite is particularly involved in the activation of tissue plasminogen activator because as we know, tissue plasminogen activator (t-PA) is a serine protease that converts plasminogen (Pg) to plasmin and can trigger the degradation of extracellular matrix

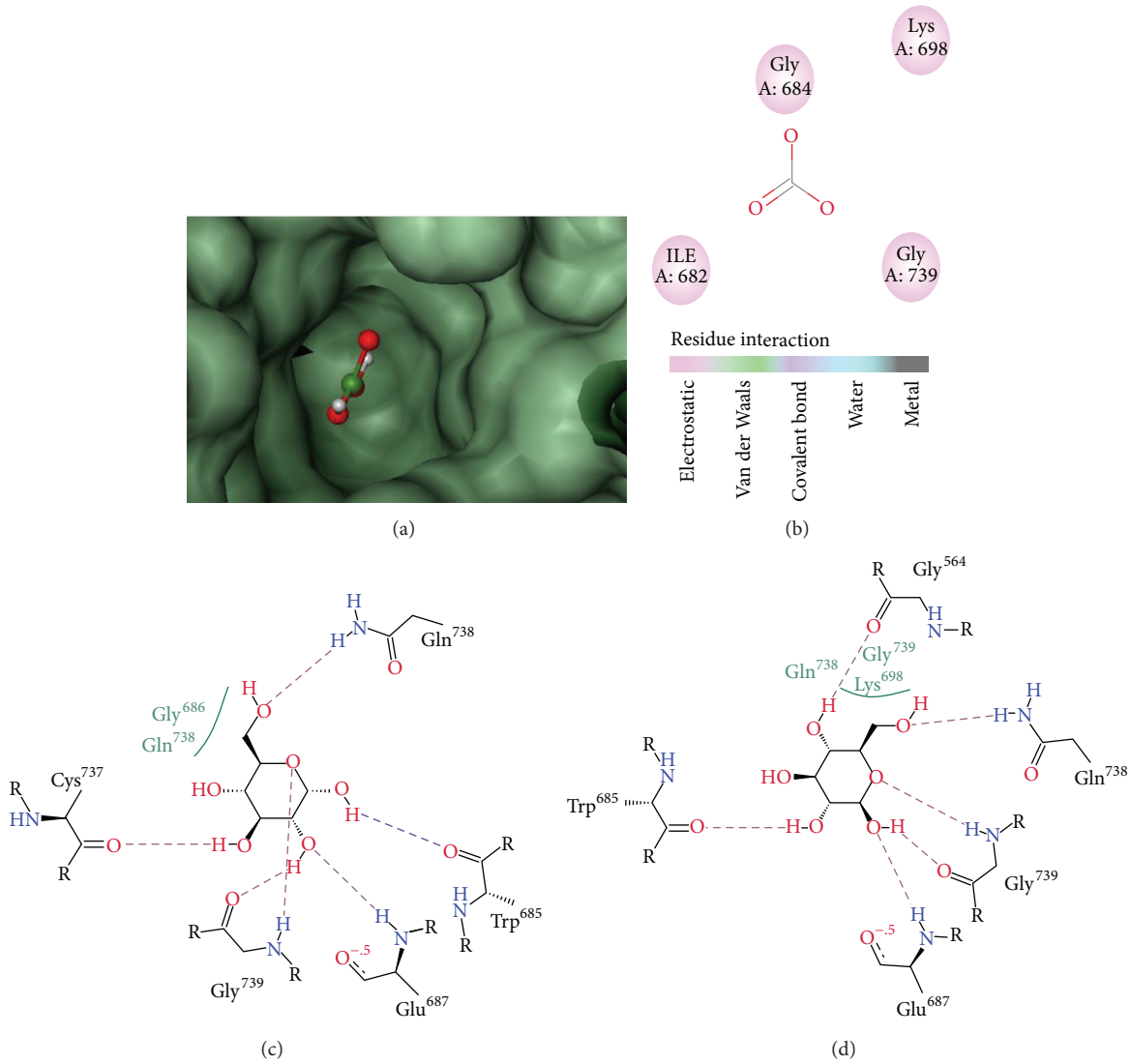

Figure 2: (a) Receptor cavity of reference ligand of tissue plasminogen (Pg, pdb id: 4DUR), (b) active site residues of SK binding site. Interaction of (c) α-d-glucopyranose and (d) β-d-glucopyranose with the tissue plasminogen protein.

proteins or clots and thus exerts thrombolysis [38, 39]. It is that the standard drug streptokinase (SK), a three-domain protein, is involved in the activation of tissue plasminogen and forms a tight stoichiometric complex with Pg, changing the zymogen proteolytic specificity from fibrin to the activation of other Pg molecules [40]. It was anticipated that Lys-698 (156) in human Pg plays a key role in the contact activation mechanism [41, 42]. This lysine residue is located in the activation pocket of Pg shielded from bulk solvent by the flexible autolysis loop. Molecular modeling suggests that it is feasible for the lysine side chain to reach a position from which it forms a salt bridge bond with Asp-740 (194) under the influence of the cofactor SK, thus triggering active site formation during contact activation [41]. Regarding this, we undertook the general skeleton of flavonol, steroid, and cardiac glycoside to find out their interaction on the SK binding site of Pg which was done by molecular docking simulation.

Molecular docking is an effective and fast computational technique to estimate the binding affinity of a ligand (drug candidate) in the macromolecular target site (receptor). A scoring system is used to detect the ideal docking configuration. Scoring systems usually use entropy maximization strategies, which generally depend on electrostatic attraction forces, Van der Waal's forces, and hydrophobic interactions [43]. In this scoring system, the crystal structure of tissue plasminogen was downloaded from protein data bank (pdb id: 4DUR) and the 3D structure of general skeleton of flavonol, glycoside (α-d-glucopyranose and β-d-glucopyranose), and steroid was obtained from Pubchem databases. The protein file was prepared through the receptor preparing wizard in FlexX [23]. The active site was identified and Lys-698 residues (Sk binding site) were considered as the reference ligand binding [44]. However, after docking analysis by flexX simulation through incremental search, only alpha-d-glucopyranose (CID79029 and CID64689) and

beta-d-glucopyranose were found to show the maximal free binding energies, −14 kj/mol and −15 kj/mol, respectively. A positive value binding energy for flavonoids was recorded while no docking confirmation for steroid was noted. The postdocking analysis suggests that alpha-d-glucopyranose was involved in the formation of six salt bridges with Cys^{737}, Gly^{739}, Glu^{687}, Trp^{685}, Gln^{738}, and Gly^{686}. Out of these six salt bridges, Gly^{686} was involved in hydrophobic interaction and it possesses moderate ligand efficiency 0.27. In contrast, the ligand protein complex for beta-d-glucopyranose formed six hydrogen bonds with Gly^{564}, Gln^{738}, Trp^{685}, Gly^{739}, and Glu^{687} residues. However, Lys^{698}, in that case, was found to act as a contributor of hydrophobic bond. And ligand efficiency of beta-d-glucopyranose was 0.30. The docking simulation study, on the basis of the above result, suggested that only glycoside is responsible for thrombolytic mechanism as it has the interaction on the activation site of tissue plasminogen activator that converts plasminogen to plasmin and can trigger the degradation of extracellular matrix proteins or clots and thus exerts thrombolysis.

4. Conclusion

Phytochemical screening revealed the presence of flavonoids, steroids, and glycosides in all the extracts. From this study, thrombolytic activity of *Leea macrophylla* and *Andrographis paniculata* methanol extracts has been found to show promising *in vitro* clot lysis activity whereas *Ocimum tenuiflorum*, *Adhatoda vasica*, and *Litsea glutinosa* were found to show moderate-to-mild thrombolytic activity. Molecular docking analysis suggested the glycosides of these plants to be the major molecules that contribute to the observed thrombolytic/antithrombotic effects. However, other metabolites might have the contribution in these effects but that is not revealed at least in our experimental design.

Conflict of Interests

The authors have declared that there is no conflict of interests regarding the publication of this paper.

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Review Article

Natural Products for Antithrombosis

Cen Chen,[1] Feng-Qing Yang,[1] Qian Zhang,[1] Feng-Qin Wang,[1] Yuan-Jia Hu,[2] and Zhi-Ning Xia[1]

[1]*School of Chemistry and Chemical Engineering, Chongqing University, Chongqing 400030, China*
[2]*State Key Laboratory of Quality Research in Chinese Medicine, Institute of Chinese Medical Sciences, University of Macau, Macau*

Correspondence should be addressed to Feng-Qing Yang; fengqingyang@cqu.edu.cn

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Thrombosis is considered to be closely related to several diseases such as atherosclerosis, ischemic heart disease and stroke, as well as rheumatoid arthritis, hyperuricemia, and various inflammatory conditions. More and more studies have been focused on understanding the mechanism of molecular and cellular basis of thrombus formation as well as preventing thrombosis for the treatment of thrombotic diseases. In reality, there is considerable interest in the role of natural products and their bioactive components in the prevention and treatment of thrombosis related disorders. This paper briefly describes the mechanisms of thrombus formation on three aspects, including coagulation system, platelet activation, and aggregation, and change of blood flow conditions. Furthermore, the natural products for antithrombosis by anticoagulation, antiplatelet aggregation, and fibrinolysis were summarized, respectively.

1. Introduction

The hemostatic system, which comprises platelet aggregation, coagulation, and fibrinolysis, is a host defense mechanism that preserves the integrity of the high pressure closed circulatory system in mammals after vascular damages [1]. Under normal physiological conditions, the thrombi formation, controlled by the regulatory system, is temporary and spatial [2–5]. However, when pathological processes overwhelm the regulatory system of hemostasis or a shift in the hemostatic balance towards the procoagulant side, thrombosis is initiated [6]. Under this hypercoagulable state, excessive quantities of thrombi will be formed, which will ultimately lead to parts or total blockage of blood vessels [7, 8]. The development of clots in the artery, vein as well as microvascular circulation is the most frequent cause of morbidity and mortality worldwide [9, 10]. The formation of thrombi in the arterial circulation usually occurs in individuals at high risk of cardiovascular diseases [11] and coronary myocardial infarction and ischemic stroke are the main results of atherosclerosis and thrombosis in the coronary arteries [12]. Furthermore, peripheral arterial diseases including mesenteric artery embolism and limb arterial thrombosis are also closely related to the arterial thrombosis. Venous thromboembolism (VTE), consisting of deep vein thrombosis (DVT) and its complication, pulmonary embolism (PE), is a relatively common condition that associated with serious symptoms [13, 14]. In reality, venous thrombosis is the second leading cause of death in patients with cancer. In addition, disseminated intravascular coagulation and microangiopathy hemolytic anemia (thrombotic thrombocytopenic purpura (TTP) and hemolytic uremic syndrome (HUS)) are associated with microvascular thrombotic disorders [6]. Therefore, more and more studies have been focused on preventing thrombosis for the treatment of those thrombotic diseases.

In recent years, antithrombotic drugs, which can be classified into three major categories including anticoagulation, antiplatelet aggregation, and fibrinolysis, have been intensively studied and developed as potential therapeutic approaches for arterial and venous thrombosis [15, 16]. Among these clinical used drugs, heparin [17], warfarin [18], and their derivates are mainly applied in inhibition of the blood coagulation factors, while plenty of antiplatelet drugs such as aspirin (ASP), clopidogrel, and abciximab have been

used in reducing the risk of cardiovascular diseases [19–22]. Furthermore, fibrinolytic agents, such as streptokinase, tissue plasminogen activator (t-PA), and reteplase, are engaged to remove and dissolve the formed blood clots [23, 24]. Despite intense investigation over the last 40 years into the discovery and development of more effective antithrombotic drugs, the effect of these therapies on mortality rates still remained small [25]. And this situation will probably become more challenging in the future as the incidences of obesity, diabetes, and the metabolic syndromes rapidly increase. The reasons of low cure rates of these drugs mainly lie in drug resistance, limited efficacy in some patients, and side effects such as higher bleeding risk and gastrointestinal dysfunctions [26]. A study in United Kingdom, researchers indicated that the responsible drug for over 60% of the deaths caused by adverse drug reactions is ASP [27]. The side effects of ASP include bleeding, gastrointestinal toxicity, and thrombocytopenia. Cilostazol, a potent inhibitor of cyclic adenosine monophosphate-(cAMP-) phosphodiesterase 3 (PDE_3), has serious side effects such as headache and palpitation [28]. Apixaban is an oral selective direct factor Xa (FXa) inhibitor and its most common adverse event is bleeding [29], and other adverse events reported are hypersensitivity reactions, syncope, nausea, dizziness, and so forth. Therefore, there is a rising urgent need for novel therapeutic approach to reduce current adverse effects of antithrombotic drugs without impairing their efficacy.

Nowadays, much effort has been focused on the discovering of natural products as effective supplements or even substitutes to those currently used antithrombotic drugs [30]. These natural products, composing of natural plants [31–33], traditional Chinese medicines (TCMs) [34, 35], and functional foods [36–38] as well as some special animal materials [39], have been found to possess remarkable antithrombotic property both in experimental and clinical stages. It is known to all that TCMs have a long history for treating many kinds of human diseases including thrombotic diseases and blood stasis syndromes. In reality, in Shennong's Classic of Materia Medica (Shennong Bencao Jing in Chinese) [40], 83 of 365 TCMs were recorded with the function of "HuoXueHuaYu," which means to promote blood circulation for removing blood stasis. Nowadays, there are some natural products that have been used in clinic for the treatment of thrombotic diseases. For example, Shimotsu-To, which is a combined prescription of four herbal extracts, *Paeonia lactiflora*, *Rehmannia glutinosa*, *Angelica sinensis*, and *Ligusticum chuanxiong*, has been used in clinic for improving abnormal blood coagulation, fibrinolysis, and atherosclerosis [41]. Kang naoxueshuan (in Chinese) tablet, which consists of *Flos Carthami*, *Radix Angelicae Sinensis*, *Hirudo*, and so forth, can protect cerebral ischemia through antiplatelet aggregation and reduction of blood viscosity [42]. Besides, *Ginkgo biloba* leaves tablets are widely used in treating ischemic cerebrovascular diseases [43]. The main reasons for applying natural products to the treatment of thrombotic diseases are that they comprise multiple constituents and each constituent may have multiple targets; they may exert pleiotropic and synergistic effects that have positive functions for increasing the therapeutic efficacy. Besides, the constituents of natural products usually have less side effects on the gastrointestinal system [44].

This review will provide an overview on the formation mechanisms of thrombosis and the antithrombotic properties exerted by natural products and describe the pathways by which their activities may contribute to reduce thrombotic risks.

2. The Formation of Thrombosis

Thrombus can be classified into four groups based on different positions and constituents [45]: (1) pale thrombus, mainly occurs in fast-flowing blood with numerous platelets; (2) red thrombus, constituting of fibrin and erythrocyte in slow-flowing blood; (3) mixed thrombus, a continuous process of thrombus formation; (4) hyaline thrombus (also called microthrombus), the formation of cellulose in microcirculation small vessels. On the other hand, venous thrombosis, arterial thrombosis, and microvascular thrombosis are more likely to be distinguished depending on different blood vascular systems [46].

Thrombus formation, including platelet adhesion, activation, secretion, and aggregation as well as tissue factor (TF) initiating thrombin generation and fibrin formation, is highly complex [1]. When the vessel wall is breached or the endothelium is disrupted, collagen, and TF become exposed to the flowing blood, thereby initiating formation of a thrombus. Exposed collagen triggers the accumulation and activation of platelets, whereas exposed TF initiates the generation of thrombin, which not only converts fibrinogen to fibrin but also further activates platelets [8]. In this paper, the formation of thrombi is described in brief on three aspects, including coagulation system, platelet activation, and aggregation, and the change of blood flow conditions.

2.1. Coagulation System. Blood coagulation and platelet adhesion and activation are critical for cessation of blood loss at sites of vascular injury in the high-pressure closed circulatory system [47]. Upon vessel injury, coagulation system can be activated via either the contact activation (or intrinsic) pathway or by the TF (or extrinsic) pathway and converge on a common (intrinsic + extrinsic) pathway, which starts at the level of factor X (FX) to lead to thrombin and fibrin formation [48]. The extrinsic pathway is initiated by excessive exposure of TF which is a 263-residue membrane-bound glycoprotein [49] and as receptor and cofactor for factor VII (FVII) and its active form VIIa (FVIIa) [3, 50, 51]. On binding of FVIIa to TF, complex (TF-FVIIa) acquires catalytic activity and converts factors IX (FIX) and X (FX) to their active derivatives factors IXa (FIXa) and Xa (FXa), respectively [52]. Simultaneously, the intrinsic pathway begins with formation of the primary complex on collagen by high-molecular-weight kininogen, prekallikrein, and FXII. FXII firstly becomes FXIIa; and FXIIa converts FXI to FXIa. FXIa activates FIX, which with its cofactor FVIIIa forms the tenase complex and then activates FX to Fxa [53]. In the common pathway, FXa derived from both intrinsic and extrinsic processes with FVa on membrane surface in complex with prothrombinase complex activates thrombin

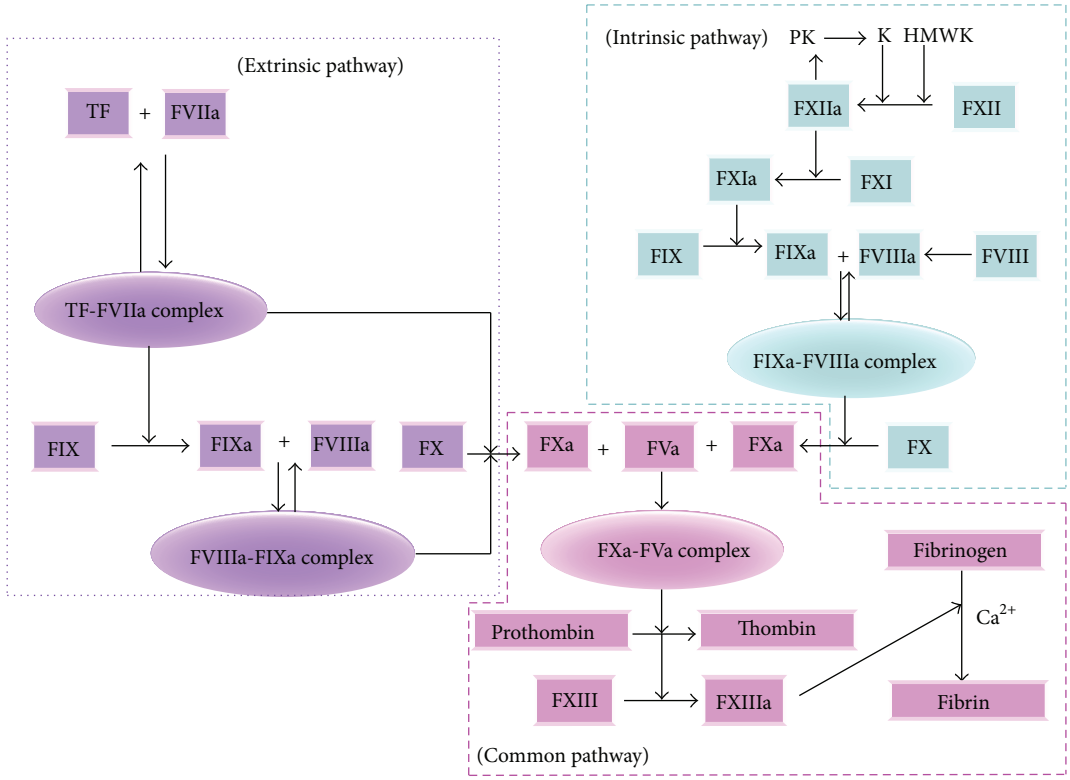

FIGURE 1: Extrinsic, intrinsic, and common pathways of blood coagulation during hemostasis and thrombosis. PK: prekallikrein; K: kallikrein; HMWK: high molecular weight kininogen.

formation which finally converts fibrinogen to fibrin polymers [54, 55] (Figure 1).

2.2. Platelet Activation and Aggregation. The intact vascular endothelium is a semipermeable barrier that controls the diffusion of plasma molecules, regulates vascular tone and inflammatory, and releases gaseous signal molecule including nitric oxide (NO) and prostacyclin (PGI_2) as well as endothelial CD_{39} to prevent platelet aggregation or dilate blood vessels under physiological conditions. However, dysfunctional or impaired endothelium is characterized by the loss of such antiplatelet properties and tends to mediate and accelerate thrombosis. The exposure binding sites of collagen and von Willebrand factor (vWF), a multimeric plasma glycoprotein, allow the platelet membrane glycoprotein (GPIb-IX-V or GPVI) to adhere on it in the first place. After the initial adhesion of platelets to the extracellular matrix, platelets undergo shape change and the activation process requires a rapid response to autocrine and paracrine mediators, including adenosine diphosphate (ADP), thrombin (THR), epinephrine, and thromboxane A2 (TXA_2) [56]. Furthermore, platelet granule secretions lead to the local release of ADP/adenosine triphosphate (ATP), 5-hydroxytryptamine (5-HT), Ca^{2+}, adhesion proteins (e.g., fibrinogen, fibronectin, thrombospondin, vitronectin, P-selectin, and GPIIb/IIIa), and coagulation factors (factor V, factor XI, plasminogen activator inhibitor type 1, plasminogen, and protein S), all of which contribute to perpetuate and amplify the thrombotic response [57]. These platelet agonists binding to specific membrane receptors (e.g., collagen binds to GPVI or $\alpha_2\beta_1$, THR interacts with protease activated receptors, and ADP binds at least two ADP receptors on platelets) [58–60] activate phospholipase Cβ (PLCβ), resulting in the production of diacylglycerol (DAG) and inositol trisphosphate (IP_3). DAG and IP_3 activate protein kinase C (PKC) and mobilize cytoplasmic Ca^{2+}, respectively. Then TXA_2 is produced as a consequence of increased cytoplasm Ca^{2+}-levels and the high concentration of Ca^{2+} is necessary for the activation of PLA2 through phosphorylation by p-38-mitogen-activated protein kinase (MAPK) [61]. Platelet aggregation is regulated in the final part of the pathway by activation of the platelet heterodimer GPIIb/IIIa receptor, the most abundant proteins on the platelet surfaces. Fibrinogen, the main ligand for the GPIIb/IIIa receptor, binding to GPIIb/IIIa also triggers an "inside out" signaling, causing amplification of the initial signal and further platelet activation. In the final phase of thrombus formation, fibrinogen is converted to fibrin by thrombin, leading to the stabilization of the platelet aggregates with more platelets and blood cells (leukocytes and red blood cells), thus getting trapped and contributing to growth of thrombus [62].

2.3. Change of Blood Flow Conditions. Physiologically, plasma separates blood vessel from the tangible components such as erythrocyte, leukocyte, and platelet in blood. Once the blood flow slows down, platelet will move to the edge of blood

vessel as well as adhere to the impaired endometrial, coagulator factors will be activated, and thrombin accumulates and amounts to a high concentration to facilitate thrombus formation. Furthermore, the blood viscosity [63], which will result in a lower erythrocytic deformability and a stronger platelet aggregation, will increase under slow blood flow condition. This cycling process between increasing erythrocytic deformability and slowing down blood flow finally promotes the adherence and aggregation of platelet. As a result, it is easy to form thrombus in vein with slow blood flow, where the concentration of coagulation factors and thrombin are very high locally [64, 65]. On the contrary, in artery where coagulation factors and thrombin can be scattered by fleet blood flow and it is less likely to achieve effective concentrations, so the thrombus formation in artery mainly relies on the adherence, activation, and aggregation of platelet rather than the impacts of coagulation factors and thrombin [66].

3. Antithrombotic Effects of Natural Products

Studies have demonstrated that natural products become increasingly crucial in reducing the thrombotic risks and treating various cardiovascular diseases. As previously mentioned, drugs for treating thrombosis can be divided into three categories: (1) anticoagulants, which prevent the coagulation system and interfere with further plaque expansion; (2) antiplatelet agents, which decrease platelet aggregation and inhibit thrombus formation; (3) fibrinolytic drugs, which dissolve the formed thrombus directly [67].

3.1. Anticoagulation. The extrinsic and intrinsic coagulation systems are initiated after vascular disruption via TF and collagen, respectively [8]. In clinical treatment, inhibition of coagulation system is an effective way to prevent the pathological thrombus formation.

3.1.1. Inhibition of Tissue Factors. TF as a membrane protein and the main initiator of the coagulation cascade is essential for thrombus formation [68]. TF expression in endothelial cells is induced by different inflammatory mediators including tumor necrosis factor- (TNF-) α [69], interleukin- (IL-) 1β [70], or histamine [71]. In reality, reducing TF expression significantly impairs thrombus formation, and agents focused on inhibition of TF activation become increasingly used effective clinical methods to treat coagulation diseases.

It has been reported that *Chaenomeles sinensis* has antithrombotic and antiplatelet aggregation activities [72]. Thirteen components were isolated and purified from the fruits of *C. sinensis* and five of them including hovertrichoside C (IC_{50} = 14.0 μg), luteolin-7-O-β-D-glucuronide (IC_{50} = 31.9 μg), hyperin (IC_{50} = 20.8 μg), avicularin (IC_{50} = 54.8 μg) and quercitrin (IC_{50} = 135.7 μg) can inhibit the TF expression of rat plasma after the addition of $CaCl_2$ *in vitro*. Furthermore, the TF inhibitory activity of the C-ring pentacyclic flavonol was evidently stronger than that of C-ring hexacyclic flavonol [73]. Rhizoma Ligustici Chuanxiong (with the main active component ligustrazine) is widely used in treating cardiovascular diseases, pulmonary hypertension, chronic renal failure and liver cirrhosis [74]. Shang et al. reported the inhibitory effects of ligustrazine on the expression of TF and vWF in human blood induced by THR *in vitro*. The result showed that ligustrazine suppressed TF expression not only in quiescent condition but after being induced by THR, and also decreased vWF formation after being induced by THR. These results provide a scientific basis for Rhizoma Ligustici Chuanxiong to be used as an antithrombotic agent [75]. In addition, a sesquiterpene glycoside (3-O-α-L-rhamnopyranosyl-(→4)-α-L-rhamnopyranosyl-(1 → 2)-α-L-(4-trans-feruloyl)-rhamnopyranosyl-(1 → 6)-β-D-glucopyranosyl) isolated from the leaves of *Eriobotrya japonica* Lindley (Rosaceae) showed a strong TF inhibitory activity (IC_{50} = 2 μM) *in vitro* and another component ferulic acid illustrated a weak inhibitory activity (IC_{50} = 369 μM). This active sesquiterpene glycoside was composed of three parts including nerolidol, carbohydrate and feruloyl moieties, and the nerolidol moiety was mainly responsible for the inhibitory effect against TF [76].

In addition, estrogen replacement therapy could protect cardiovascular system and decrease the incidence of related diseases [77]. α-Zearalanol (ZAL), which is one of the natural phytoestrogens usually found in beans and grain, could decrease the contents of TF and its expression on vascular endothelium in rat plasma *ex vivo* with similar to or better than that of positive drug 17β-estradiol [78].

3.1.2. Inhibition of the Coagulation Pathways. The pathways of the coagulation system mainly consist of two distinct cascades (intrinsic and extrinsic coagulation pathways) ultimately contributes to the formation of the key protease thrombin which in turn converts fibrinogen into fibrin to stabilize the formed platelet-rich plug. In experiment models, activated partial thromboplastin time (APTT), prothrombin time (PT) and thrombin time (TT) are tested to indicate the activation of intrinsic, extrinsic and their common (intrinsic + extrinsic) pathway, respectively [79]. The anticoagulation effects by inhibition of the coagulation pathways of natural products are summarized in Table 1.

The green algae *Monostroma arcticum* (MA), with polysaccharide as its important bioactive substance, is widely distributed in China. A polysaccharide HAF0 (average molecular weight of 9.36 kDa) isolated from MA showed the inhibition effect on the intrinsic and/or common coagulation pathway with prolonging APTT and TT [80]. *Polygala fallax* Hesml. (PFH) is used as a folk medicine for antiaging, preventing myocardial ischemia and regulation of immune system. The anticoagulation and antithrombotic effects of the total saponins from PFH was mainly contributed to the inhibition of intrinsic coagulation system by prolonging APTT, plasma recalcification time (RT) as well as THR-induced fibrinogen clotting time, but did not impact on PT [81]. In reality, the anticoagulation mechanisms for most of the drugs mainly rely on inhibition of both intrinsic and extrinsic, or common coagulation pathways. Hyperoside, isolated from the leaves of *Rhododendron brachycarpum*, was observed *ex vivo* in mice with dose-dependent prolongation of the APTT and PT as well as inhibited platelet aggregation induced by THR and collagen *in vitro*, ADP *in vivo* [82]. Polysaccharide from *Umbilicaria esculenta* inhibited the thrombus formation

Table 1: Inhibition on the coagulation pathways of natural products.

Natural products	Experimental models	Pathways	Effects	Reference
Polysaccharide HAF0 of *Monostroma arcticum*	Human blood (*in vitro*)	IN & CO	Prolonging APTT and TT, but without PT	[80]
Total saponin of *Polygala fallax* Hesml.	Rabbit blood (*in vitro*)	IN	Prolonging APTT and RT and fibrinogen clotting time, but without PT	[81]
Borneol	Rat blood (*ex vivo*)	EX & CO	Prolonging PT and TT and inhibition of arteriovenous shunt as well as venous thrombosis	[97]
Withaferin A of *Withania somnifera*	Human blood (*ex vivo*)	IN & CO	Prolonging APTT and PT and inhibition of thrombin, FXa formation, and TNF-α induced PAI-1 production as well as extending *in vivo* and *ex vivo* bleeding time	[86]
Saline extract of *Hirudinaria manillensis*	Rat blood (*ex vivo*)	IN, EX & CO	Prolonging APTT, PT, and TT	[98]
Total glycosides of paeony	Rabbit blood (*in vitro*)	IN, EX & CO	Prolonging APTT, TT, and PT	[90]
95% ethanol extract of *Ferula lehmannii* Boiss.	Rat blood (*ex vivo*)	IN, EX & CO	Prolonging APTT, TT, and PT	[99]
Dilinoleic acid, safflower yellow, and compatibility preparation	Rat blood (*ex vivo*)	IN & EX	Prolonging APTT, TT, CT, and BT	[100]
Aqueous extract of *Whitmania pigra* Whitman	Rat blood (*in vitro*)	IN & EX	Prolonging APTT as well as TT and suppression of fibrinogen formation	[101]
Phlorotannins STP-1 and STP-2 of *Sargassum thunbergii* Kuntze	Rabbit blood (*in vitro*)	IN, EX & CO	Prolonging APTT, TT, PT, CT, and BT	[102]
Sulfated polysaccharides of *Hizikia fusiformis*	Rat/Rabbit blood (*in vivo*/*in vitro*)	IN	Prolonging rats BT, CT *in vivo,* and rabbits APTT *in vitro*	[103]
Hyperoside of *Rhododendron brachycarpum*	Rat blood (e*x vivo*)	IN & EX	Prolonging APTT and PT	[82]
Polysaccharide of *Umbilicaria esculenta*	Rat blood (i*n vitro*)	IN, EX & CO	Prolonging APTT, PT, and TT	[83]
Sulfated (1 → 3)-β-L-arabinan of *Codium vermilara*	Human blood (*in vitro*)	IN, EX & CO	Prolonging APTT, PT, and TT	[104]
Wogonin and wogonoside of *Scutellaria baicalensis* Georgi	Human blood (*in vitro*)	IN & EX	Prolonging APTT and PT and inhibition of the activities and production of THR and FXa	[88]
Crude extracts of *Erigeron canadensis* L.	Human blood (i*n vitro*)	IN & EX	Prolonging APTT and PT	[89]

IN, EX, and CO represent for intrinsic, extrinsic, and common coagulation pathways, respectively; APTT: activated partial thromboplastin time; TT: thrombin time; PT: prothrombin time; RT: recalcification time; CT: coagulative time; BT: bleeding time.

in a dose-dependent manner using an arteriovenous shunt thrombosis model in rats, and the more prolongation of APTT suggested a more obvious inhibition of the intrinsic than the extrinsic coagulation systems [83]. Withaferin A (WFA), an active compound from *Withania somnifera*, is widely studied on its effects on inflammatory, cardiovascular and central nervous system [84]. It is reported that WFA significantly prolonged APTT as well as PT, inhibited the activities and production of thrombin and FXa following extending *in vivo* and *ex vivo* bleeding time, and inhibited the production of TNF-α induced plasminogen activator inhibitor type 1 (PAI-1), an important component of the coagulation system that down-regulates fibrinolysis in the circulation [85]. Those results indicated that WFA possessed antithrombotic activities and might be developed as a new anticoagulant agent [86]. Wogonin (WGN) as well as its metabolite wogonoside (WGNS) is the flavonoids from *Scutellaria baicalensis* Georgi [87]. Treatment with WGN and WGNS resulted in prolonging APTT and PT as well as inhibition of the activities and production of THR and FXa in tumor necrosis factor- (TNF-) α activated human umbilical vein endothelial cells [88]. Pawlaczyk et al. studied the anticoagulant and antiplatelet activities of different fractions of *Erigeron canadensis* L. The mixture parts of polysaccharide-polyphenolic macromolecules inhibited both intrinsic and extrinsic coagulation pathways, as well as platelet aggregation

induced by collagen *in vitro*. While in the carbohydrate part, only glucuronic acid and galacturonic acid showed weak anticoagulant activity [89]. In addition, the anticoagulant effect of total glycosides of paeony included prolonging APTT, PT, and TT *in vitro* confirmed that intrinsic, extrinsic, and common coagulation pathways were all inhibited [90].

3.2. Anti-Platelet Aggregation. The inhibition of platelet function has been widely studied for a long time in an effort to prevent and treat thrombosis, especially in antiplatelet aggregation. Andrographolide, the active component of *Andrographis paniculata*, could inhibit PAF-induced human blood platelet aggregation in a dose-dependent manner ($IC_{50} \approx 2\,\mu M$) [91]. Bupleurumin from the aerial parts of *Bupleurum falcatum* showed an 8-fold potent inhibitory effect ($IC_{50} = 47.5\,\mu M$) compared to that of ASP ($IC_{50} = 420\,\mu M$) on collagen-induced platelet aggregation, and comparable inhibitory effects as ASP on AA-induced platelet aggregation [92]. In Maione's study, Tanshinone IIA (TIIA) selectively inhibited rat platelet aggregation induced by reversible ADP stimuli ($3\,\mu M$) in a concentration-dependent manner (0.5–$5\,\mu M$). Nevertheless, TIIA was less active against the aggregation induced by irreversible ADP ($10\,\mu M$) and collagen ($10\,\mu g/mL$) stimuli [93]. Apart from single bioactive component, studies have also provided evidences for antiplatelet aggregation effects of crude extracts of natural products. The 80% aqueous-ethanol extract of *Abies webbiana* was found to inhibit both ADP- and epinephrine-induced human platelets aggregation, thereby suggesting therapeutic potential of this plant against thromboembolic conditions [94]. In Gadi's study, crude aqueous extract (CAE) of parsley was evaluated for its antiplatelet aggregation activity in rats *in vitro* and *ex vivo*. CAE dose-dependently inhibited platelet aggregation *in vitro* induced by THR, ADP, collagen and epinephrine. The oral administration of CAE (3 g/kg) significantly ($P < 0.001$) inhibited platelet aggregation *ex vivo* and prolonged bleeding time ($P < 0.001$) without changes of the platelet amount [95]. In terms of the mechanisms for antiplatelet therapies, they are mainly composed of platelet membrane protein inhibitors, impacting nucleotide and arachidonic acid system as well as inhibition of platelet granules secretion.

3.2.1. Inhibition of Platelet Membrane Receptors. Development of definite platelet receptor inhibitors contributed to clinical treatment of antiplatelet aggregation, for example, ADP $P2Y_{12}$ receptor antagonists include ticlopidine and clopidogrel; GPIIb/IIIa antagonists include abciximab, tirofiban, and eptifibatide [96]. Based on the variety of protein structures, functions and ligand properties, platelet receptors can be classified into three groups include integrin, adhesion and agonist receptors. A large number of natural products and their constituents are reported as platelet receptors antagonists (Table 2).

GPIIb/IIIa, a heterodimeric receptor of the integrin family expressed at high density (50000–80000 copies/cell) on the platelet membrane, determines the final process during platelet aggregation. So many new antiplatelet aggregation drugs mainly focus on inhibition of this dominant receptor [151]. *Spatholobus suberectus* is a widely used TCM to promote blood circulation for the treatment of diseases related to the blood stasis syndromes [152]. It has been demonstrated that 95% ethanol extract of *S. suberectus* significantly inhibited ADP- and collagen-induced platelet aggregation in human platelet by inhibiting fibrinogen binding to the GPIIb/IIIa receptor and further suppressing the formation of TXA_2 [106]. Garlic is a common used spicy food all over the world, and a garlic preparation aged garlic extract (AGE) is reported to have inhibition effect of platelet aggregation [153]. Allison et al. [113] investigated the antiplatelet aggregation mechanism of AGE by testing their adhesion to fibrinogen using Rose Bengal and ^{51}Cr uptake, fluorescence activated cell sorting (FACS) analysis and measurement of intracellular cAMP contents in human platelet after induced by ADP. The results showed that AGE at concentrations of 3.12% to 12.5% (v/v) can inhibit the binding of platelets to fibrinogen by approximately 40% in the Rose Bengal assay ($P < 0.05$) as well as 61.5%~72% in the ^{51}Cr experiments ($P < 0.05$), and significantly decrease the amount of PAC-1 binding to GPIIb/IIIa by approximately 72% in the FACS analysis with increasing platelet cAMP ($P < 0.01$) level. These findings suggested that AGE inhibits platelet aggregation via inhibition of the GPIIb/IIIa receptor and an increase of cAMP level. In Jeon's study, two bioactive compounds isomaltol and pentagalloyl glucose were separated from bark of *Rhus verniciflua* Stokes, and their antiplatelet mechanism were evaluated using receptor expression on platelet membranes, including GPIIb/IIIa (CD41), GPIIb/IIIa-like expression (PAC-1) and P-selectin (CD62), and intracellular calcium mobilization responses. The results indicated that pentagalloyl glucose had a significant inhibitory effect on the expression of P-selectin, but isomaltol had no such effect. Furthermore, isomaltol and pentagalloyl glucose decreased the expression of GPIIb/IIIa, which appeared to have anti-GPIIb/IIIa activity [118].

Adhesion receptors, which mainly refer to collagen receptors, mediate the platelet binding to injury endothelium including $\alpha_2\beta_1$ (GPIa/IIa) and GPVI. Glaucocalyxin A (GLA) is a biologically active ent-kauranoid diterpenoid isolated from *Rabdosia japonica* var. *glaucocalyx*, a traditional Chinese medicinal herb. GLA can significantly inhibit platelet aggregation in response to most of the platelet agonists including collagen, THR and ADP [154]. The inhibitory effect of GLA on collagen-stimulated platelet aggregation was notably potent, even occurred at as low as $0.01\,\mu g/mL$. GLA inhibited platelet aggregation induced by collagen-related peptide (CRP), a GPVI specific agonist in a dose-dependent manner and reduced collagen-induced phosphorylation of three major molecules, tyrosine kinase Syk, LAT, and phospholipase Cγ2 in GPVI signaling pathway. Therefore, GLA can be developed and used as an collagen receptor antagonist for antiplatelet aggregation [108]. Salvianolic acid B (SB) is an active component isolated from Danshen (*Salvia miltiorrhiza*), a TCM widely used for the treatment of cardiovascular disorders. Ma et al. demonstrated that $\alpha_2\beta_1$ might be one of the direct target proteins of SB on platelets, and the signal cascade network of SB after binding with integrin $\alpha_2\beta_1$ might include regulation of intracellular Ca^{2+} level, cytoskeleton-related proteins such as coronin-1B and

Table 2: Inhibition of platelet membrane receptors of natural products.

Natural products	Experimental models	Possible mechanisms	Reference
2,3,5,4′-Tetrahydroxystilbene-2-O-β-D-glucoside of *Polygonum multiflorum*	Human blood (*in vitro*); agonist: collagen	Inhibition of FcγRIIa, Akt (Ser473), and GSK3β (Ser9) phosphorylation	[105]
95% ethanol extract of *Spatholobus suberectus*	Human blood (*in vitro*); agonist: collagen	Blockage of fibrinogen binding to the GP IIb/IIIa, suppression of TXA_2 formation	[106]
A new tripeptide (AAP) of *Agkistrodon acutus* Venom	Rabbit blood (*in vitro*); agonist: ADP, PAF-acether, collagen and THR	Inhibition of fibrinogen binding to GP IIb/IIIa	[107]
Glaucocalyxin A of *Rabdosia japonica* (Burm. f.) var. *glaucocalyx* (Maxim.) Hara	Human blood (*in vitro*); agonist: collagen	Inhibition of tyrosine phosphorylation of Syk, LAT, phospholipase Cγ2, and P-selectin secretion	[108]
Salvianolic acid B of *Salvia miltiorrhiza*	Rat blood (*in vitro and ex vivo*); agonist: collagen	Exerting binding affinity to $\alpha_2\beta_1$, decreasing of intracellular Ca^{2+}, and impacting on cytoskeleton-related proteins level	[109]
Indole-3-carbinol of cruciferous vegetables	Human blood (*in vitro*); agonist: collagen	Inhibition of fibrinogen binding to GP IIb/IIIa and decreasing the levels of TXB_2, prostaglandin E_2	[110]
II-3,I-5,II-5,II-7,I-4′,II-4′-Hexahydroxy-(I-3,II-8)-flavonylflavanonol and acacetin of *Garcinia nervosa* var. *pubescens* King	Rabbit blood (*in vitro*); agonist: PAF	Possessing strong PAF antagonistic activity	[111]
Essential oils of five *Goniothalamus* species	Human blood (*in vitro*); agonist: ADP, AA, and collagen	Possessing strong PAF antagonistic activity	[112]
15–20% ethanol extract of aged garlic	Human blood (*in vitro*); agonist: ADP	Inhibition of fibrinogen binding to GP IIb/IIIa and increasing the level of cAMP	[113]
Tetramethylpyrazine of *Ligusticam wallichii* Franch	Human blood (*in vitro*); agonist: ADP, collagen, and U46619	Inhibition of fibrinogen binding to GP IIb/IIIa and the levels of intracellular Ca^{2+} as well as TXB_2	[114]
Aqueous extract of *Agrimonia pilosa*	Human blood (*in vitro*); agonist: ADP	Inhibition of fibrinogen binding to GP IIb/IIIa and decreasing the level of P-selectin	[115]
N-butanol extract of *Toona sinensis* Seed	Human blood (*in vitro*); agonist: THR	Inhibition of fibrinogen binding to GP IIb/IIIa and decreasing the level of intracellular Ca^{2+}	[116]
Eryloside F of Erylus formosus	Human blood (*in vitro*); agonist: THR, SFLLRN, and U-46619	Possessing strong THR antagonistic activity	[117]
Isomaltol and pentagalloyl glucose of *Rhus verniciflua* Stokes	Human blood (*in vitro*); agonist: ADP, AA, and collagen	Decreasing the expression of GPIIb/IIIa	[118]
Piperlongumine of *Piper longum* L.	Rabbit blood (*in vitro*); agonist: U4619 and THR	Inhibition of U46619-induced phosphatidylinositol hydrolysis as well as the binding of (^{3}H)SQ29548 to TXA_2 receptor	[119]
Hot-water extract of modified Je-Ho-Tang (Mume Fructus, Amomi Tsaoko Fructus, Santali Albi Lignum, and Amomi Fructus)	Human blood (*in vitro*); agonist: collagen	Inhibiting adhesion and decreasing the activation of GPIIb/IIIa-like expression and P-selectin monoclonal, Ca^{2+} mobilization	[120]
Pomolic acid of *Licania pittieri*	Human blood (*in vitro*); agonist: ADP	Competitive antagonism of ADP-induced platelet aggregation	[121]

ADP: adenosine diphosphat; PAF: platelet activating factor; THR: thrombin; AA: arachidonic acid; SFLLRN: thrombin receptor activating peptide; GP IIb/IIIa: Glycoprotein IIb/IIIa; TXA_2: thromboxane A_2; TXB_2: thromboxane B_2; cAMP: cyclic adenosine monophosphate; (^{3}H)SQ29548: TXA_2 receptor antagonist.

cytoskeleton structure of platelets [109]. A traditional Korean formula called modified Je-Ho-Tang (MJHT), which is composed of Mume Fructus, Amomi Tsaoko Fructus, Santali Albi Lignum and Amomi Fructus, could promote blood flow and eliminate blood stasis. The hot-water extract of MJHT dose-dependently inhibited collagen-induced whole blood aggregation and adhesion by shear stress in flow conditions. Besides, the extract significantly inhibited the conformational change of GPIIb/IIIa (PAC-1), the activation of P-selectin and mobilization of platelet Ca^{2+} [120].

Once adhere to the sites of vascular injury, platelets are involved in the process of activation and aggregation by releasing of agonists such as ADP, 5-HT, TXB_2 to amplify the thrombus. Therefore, inhibition of the agonist' receptor can attenuate the formation of thrombus. Two active components, acacetin and II-3,I-5,II-5,II-7,I-4′,II-4′-hexahydroxy-(I-3,II-8)-flavonylflavanonol from the leaves of *Garcinia nervosa* var. *pubescens* King, showed strong inhibitory effects on platelet-activating factor (PAF) receptor [111]. Another agonist receptor of THR could be strongly inhibited by Eryloside F, a novel steroidal disaccharide metabolite of *Erylus formosus*, and finally leaded to inhibit human platelet aggregation *in vitro* [117]. *Piper longum* L. has been used as a crude drug to improve intestinal disorder as well as the activity of peripherally poor blood circulation in Asia [155]. Piperlongumine, a constituent of *P. longum*, could concentration-dependently inhibited platelet aggregation induced by TXA_2 receptor agonist U46619, but slightly inhibited THR-induced aggregation. Piperlongumine also inhibited U46619-induced phosphatidylinositol hydrolysis and the binding of (^{3}H)SQ29548 (TXA_2 receptor antagonist) to TXA_2 receptor, so it is assumed that piperlongumine act as a TXA_2 receptor antagonist to inhibit platelet aggregation [119]. Pomolic acid (PA), triterpenoid isolated from *Licania pittieri*, has shown a potent ability to inhibit ADP- and epinephrine-induced human platelet aggregation. According to the mechanism study, PA could be a potent competitive antagonist of $P2Y_{12}$ receptor [121].

3.2.2. Impacting on Nucleotide System. cAMP plays a modulatory role in PLC-mediated secretion and aggregation of human platelets. The levels of cAMP are tightly controlled and dependent on both its synthesis rate by adenylate cyclase (AC) and its hydrolysis rate by PDE [156]. In addition, cAMP levels may be increased by peroxisome proliferator-activated receptors (PPARs) activation [157]. Intracellular cyclic guanosine monophosphate (cGMP) levels are rapidly increased by soluble guanylyl cyclase (sGC), which modulates multiple signaling pathways, including cGMP-dependent receptor proteins, cGMP-regulated PDE and cGMP-dependent protein kinases. The increasing in cGMP levels is accompanied by a decrease in intracellular Ca^{2+} mobilization while the decrease in Ca^{2+} levels inhibits the conformation change of GPIIb/IIIa into its active form and thus decreases platelet binding to fibrinogen [158]. In a word, the increasing in cAMP and cGMP levels may exert a strong platelet inhibitory effect by decrease of intracellular Ca^{2+} levels.

Cordycepin (3′-deoxyadenosine), the major active component in *Cordyceps militaris*, had significant inhibition effect on human platelet aggregation. Cordycepin may increase cAMP and cGMP levels and subsequently inhibit the intracellular Ca^{2+} as well as TXA_2 but without affecting on PLC-γ2 or IP3 [159]. In another study, cordycepin-enriched-(CE-) WIB801C from *Cordyceps militaris* dose-dependently inhibited ADP-induced platelet aggregation with IC_{50} of 18.5 μg/mL. The possible inhibition mechanism was that CE-WIB801C elevated cAMP involved in IP_3RI (Ser^{1756}) phosphorylation to inhibit Ca^{2+} mobilization and VASP (Ser^{157}) phosphorylation to inhibit α_{IIb}/β_3 activation [160]. The ancient plant *Ginkgo biloba* possesses many biological activities such as radical scavenging, blood flow improvement and vasoprotection. Ginkgolide C, one of the active components in *G. biloba*, can significantly increase the formation of cAMP and cGMP as well as suppressing the level of intracellular Ca^{2+} and TXA_2. In addition, zymographic analysis confirmed that pro-matrix metalloproteinase-9 (pro-MMP-9, 92-kDa) released from human platelets can be activated by Ginkgolide C to form an activated MMP-9 (86-kDa), which can significantly inhibit platelet aggregation stimulated by collagen [161]. Furthermore, another active component of *G. biloba*, quercetin prevented platelet aggregation by inhibition of PDE_3 [162]. It should be mentioned that PDEs can limit the intracellular levels of cyclic nucleotides by catalyzing the hydrolysis of cAMP and cGMP, thus regulating platelet function. The inhibition of PDEs may therefore exert a strong platelet inhibitory effect [163]. Oligoporin A from *Oligoporus tephroleucus*, an edible mushroom cultivated in Korea, inhibited collagen-induced platelet aggregation in a concentration-dependent manner, but not affecting ADP- and THR-induced platelet aggregation. Further study revealed that oligoporin A can induce the dynamic increase of cAMP and cGMP in platelet. Rat blood *in vitro* pretreatment with oligoporin A significantly blocked collagen-induced ERK2 phosphorylation as well as diminished the binding of fibrinogen to its cognate receptor, integrin α_{IIb}/β_3 [164].

3.2.3. Inhibition of Platelet Granules Secretion. Platelet granules mainly consist of α-granules, dense granules and lysosomes which serve an essential role in promoting platelet aggregation by releasing numerous activated factors such as Ca^{2+}, 5-HT, ATP, ADP, P-selectin, and so forth [165]. Inhibitions of platelet granules secretion by natural products are summarized in Table 3.

The concentration of cytosolic Ca^{2+} plays a fundamental role in mediating dense granule release and platelet aggregation. Crocetin, a major ingredient of saffron, against platelet aggregation were mainly contributed to inhibiting Ca^{2+} mobilization via reducing both intracellular Ca^{2+} release and extracellular Ca^{2+} influx, as well as inhibiting secretion of 5-HT, an independent risk factor for platelet aggregation and for thrombus formation [122]. Geiji-Bokryung-Hwan (GBH), Korean traditional formulation, consisting of Cinnamomi Ramulus, Poria Cocos, Mountan Cortex Radicis, Paeoniae Radix and Persicae Semen. GBH potently inhibited thrombin, CRP, U46619 (a TXA_2 mimic), ADP, or SFLLRN

Table 3: Inhibition of the platelet granules secretions of natural products.

Natural products	Experimental models	Possible mechanisms	Reference
Crocetin of Saffron	Rat blood (*ex vivo*); agonist: ADP	Inhibition of Ca^{2+} mobilization via reducing both intracellular Ca^{2+} release and extracellular Ca^{2+} influx as well as 5-HT secretion	[122]
Aqueous extract of Soshiho-tang	Rat blood (*in vitro*); agonist: collagen, THR and AA	Inhibition of 5-HT and TXA_2 formation	[123]
Geiji-Bokryung-Hwan (*Cinnamomi Ramulus, Poria Cocos, Mountan Cortex Radicis, Paeoniae Radix,* and *Persicae Semen*)	Human blood (*in vitro*); agonist: THR and CRP	Inhibition of IP3-mediated Ca^{2+} mobilization	[124]
20% ethanol extract of black soybean	Human blood (*in vitro*); agonist: collagen	Attenuating 5-HT secretion and P-selectin expression, and inhibiting TXA_2 formation	[125]
Magnolol of magnolia bark	Rabbit blood (*in vitro*); agonist: collagen	Inhibition of 5-HT secretion	[126]
Ligustrazine ferulate of Rhizoma Ligustici Chuanxiong	Rat blood (*ex vivo*); agonist: THR	Reduction of the expression of platelet P-selectin as well as suppression of platelet adhesion to neutrophil	[127]
Dihydroxybenzyl alcohol of *Gastrodia elata* Blume.	Rabbit blood (*in vitro*); agonist: AA	Inhibition of Ca^{2+} mobilization via reducing both intracellular Ca^{2+} release and extracellular Ca^{2+} influx	[128]
Rhynchophylline	Rabbit blood (*in vitro*); agonist: ADP and THR	Inhibition of Ca^{2+} mobilization via extracellular Ca^{2+} influx rather than intracellular Ca^{2+} release	[129]
Salvianolic acid B of *Salvia miltiorrhiza*	Human blood (*in vitro*); agonist: ADP and THR	Inhibition of P-selectin and CD40L releasing	[130]
Guanosine of *Solanum lycopersicum*	Human blood (*in vitro*) agonist: ADP and collagen	Inhibition of CD40L and ATP secretion	[131]
Curdione of Rhizoma Curcumae	Human blood (*in vitro*) agonist: THR, PAF, ADP and AA	Inhibition of P-selectin expression, intracellular Ca^{2+} mobilization and increasing the cAMP levels in PAF-activated platelets	[132]

ADP: adenosine diphosphate; THR: thrombin; AA: arachidonic acid; CRP: collagen-related peptide; 5-HT: 5-hydroxytryptamine; IP3: inositol-1,4,5-trisphosphate; TXA_2: thromboxane A_2.

(a thrombin receptor agonist peptide) induced platelet aggregation by acting on a certain step of the signal transduction pathway. Park et al. confirmed that GBH inhibited IP3-mediated Ca^{2+} mobilization without altering tyrosine phosphorylation of PLC-γ2 [124]. Magnolol was isolated from Magnolia bark for the treatment of anxiety, neural and cardiovascular disorders [166], the antiplatelet aggregation mechanism of magnolol contribute to an inhibitory effect on 5-HT releasing [126]. Curdione, one of the major sesquiterpene compounds from Rhizoma Curcumae, had a potent protective effect on acute liver injury in mice and potentially to be an active constituent for strengthening the anti-inflammatory or cancer chemo-preventive capacity [167]. In the antiplatelet aggregation test, curdione preferentially inhibited PAF- and THR-induced platelet aggregation in a concentration-dependent manner (IC_{50} = 60–80 μM). Curdione can inhibit P-selectin expression, intracellular Ca^{2+} mobilization as well as causing an increase of cAMP levels in PAF-activated platelets [132].

P-selectin, shows a crucial function in mediating platelet adhesion to the damage vessels, is localized in the α-granules and released when activation of platelet. Black soybean (BB) significantly inhibited collagen-induced platelet aggregation by attenuating 5-HT secretion and P-selectin expression, as well as inhibiting TXA_2 formation *in vitro* [125]. Ligustrazine ferulate, the main active component of *Rhizoma Ligustici Chuanxiong* had distinct antithrombotic effect. Ligustrazine ferulate reduced the expression of platelet P-selectin as well as suppression of platelet adhesion to neutrophil [127]. Soshiho-tang (SH), which consists of seven herbal drugs, had antithrombotic and antiplatelet activities. Lee et al. reported that SH significantly inhibited various agonist-induced platelet aggregations and completely inhibited 5-HT secretion and TXA_2 formation. Furthermore, SH presented antithrombotic activity by prolonging the occlusion time of thrombus formation when applied in a $FeCl_3$-induced thrombus formation model [123]. Fuentes et al. demonstrated for the first time that guanosine from *Solanum lycopersicom*

possessed antiplatelet (secretion, spreading, adhesion and aggregation) activity induced by ADP as well as collagen *in vitro* and inhibited platelet inflammatory mediator of atherosclerosis (sCD40L), while depression of CD40L expression can prevent thromboembolic-related disorders [131].

3.2.4. Impacting on Arachidonic Acid System. TXA_2, intensely induces platelet activation and vasoconstriction, is generated from arachidonic acid (AA) which released when membrane phospholipids are broken down by diverse agonists such as collagen, thrombin and ADP. The enzymes related to TXA_2 production are cyclooxygenase (COX-1) and thromboxane synthase (TXAS), which are located at microsomes. COX-1 produces prostaglandin (PGG_2) from substrate AA, TXAS produces TXA_2 from PGH_2 that oxidized from PGG_2 by endoperoxidase. Therefore, inhibition of COX-1 or TXAS is a very useful marker to evaluate the antiplatelet effect of compound. For instance, COX-1 inhibitor aspirin and TXAS inhibitor ozagrel are being used as antiplatelet agents [168]. Another metabolic pathway of AA is the lipoxygenase (LOX) pathway that forms hydroxyeicosatetraenoic acids (HETE) and leukotrienes. TXB_2 and 6-keto-$PGF1_\alpha$ are the stable metabolites of TXA_2 and PGI_2, respectively. When the ratio of TXA_2/PGI_2 is above normal conditions, thrombus formation will occur. On the other hand, when the ratio of TXA_2/PGI_2 is lower than normal conditions, the processes of platelet aggregation or thrombus formation will be self-limited and a bleeding tendency may occur. A variety of natural products (Table 4) including berberine [138], hesperetin [139] and ethyl acetate extract of *Caesalpinia sappan* L. [145] inhibited platelet aggregation by keeping balance of TXA_2 and PGI_2.

As mentioned above, interference of the activation of the associated enzymes such as COX-1, COX-2, TXAS and LOX during arachidonic acid pathway is regarded as an effective way to inhibit platelet aggregation. Obovatol, a major biphenolic component of *Magnolia obovata* leaves, presented antiplatelet activity by inhibiting COX-1 and LOX activities to suppress production of TXB_2, PGD_2 and 12-HETE [136]. Morroniside, extracted and purified from *Cornus officinalis* Sieb.et Zucc, significantly inhibited the activation of COX as well as TXB_2 generation, and had a selective antiplatelet effect on ADP-induced aggregation [146, 147]. Coy et al. isolated 26 neolignans (14 bicyclooctane-type and 12 benzofuran-type) from three Lauraceae species (*Pleurothyrium cinereum*, *Ocotea macrophylla*, and *Nectandra amazonum*) and evaluated their antiplatelet aggregation property *in vitro* through inhibition of COX-1, COX-2, 5-LOX and agonist-induced aggregation of rabbit platelets. The results showed that benzofuran neolignans were found to be the COX-2 selective inhibitors, whereas bicyclooctane neolignans selectively inhibited the PAF-action as well as COX-1 and 5-LOX. The neolignan 9-nor-7, 8-dehydro-isolicarin B, and cinerin C were found to be the most potent COX-2 inhibitor and PAF-antagonist, respectively. In addition, nectamazin C (bicyclooctane-type neolignan) exhibited dual 5-LOX/COX-2 inhibition [148]. Abe et al. screened for inhibitors of human platelet aggregation and human 5-LOX from the Myoga (*Zingiber mioga* Roscoe) extracts. Experimental results indicated that miogatrial, miogadial, sesquiterpene and polygodial were potent inhibitors of human platelet aggregation and human 5-LOX, and their 3-formyl-3-butenal structure was essential for the activities [149]. In addition, Ginsenoside Rk1 from white ginseng decreased the 12-HETE level involved in AA pathway, which is related to 12-LOX translocation resulting from the decreased of Ca^{2+} levels [150].

3.3. Fibrinolysis. The conversion of fibrinogen to fibrin and the consequent formation of a stable fibrin clot are the ultimate events in the coagulation and thrombotic cascades [169]. The agents available for clinical treatment on fibrinolysis can be classified into two groups: plasmin-like proteases which can directly hydrolyse fibrin, for example, nattokinase and lumbrokinase; and plasminogen activators, for example, tissue type plasminogen activator (t-PA) and streptokinase [170]. In recent years, some effective thrombolytic agents have been purified and characterized from foods or animal materials such as Japanese natto, douche (a traditional Chinese soybean food) [171] and earthworm [172].

In 1983, a high fibrinolytic active enzyme named lumbrokinase was firstly separated from artificial breeding earthworm in Japan [173]. This fibrinolytic enzyme had a dual functions included dissolving fibrin directly and activate plasminogen. Furthermore, Mihara et al. [172] isolated a strong fibrinolytic enzyme from *Lumbricus rubeulls* which contained abundant asparagine and aspartic acid with little proline or lysine. In addition, Xiong et al. separated and purified a fibrinolytic enzyme (33 kDa) with strong fibrinolysis effects and proteolytical activity from *Eisenia foelide* [174].

Nattokinase (27.3 kDa to 35 kDa) is a kind of serine proteases which is produced in the fermentation process of *Bscillus natto* or *Bacillus subtilis* var. *natto*. Nattokinase possesses a significant fibrinolytic property and the main mechanisms were to dissolving fibrin directly as well as activating plasminogen to increase the intrinsic plasmin formation. In the expectation to be developed as a new generation of fibrinolytic agents and health food, nattokinase has lots of advantages such as high safety, low cost and fast acting [175, 176]. Another serine protease (31 kDa with a single polypeptide chain) with fibrinolytic activity named CSP was purified from the culture supernatant of the fungus *Cordyceps sinensis*. CSP was found to be a plasmin-like protease, but not a plasminogen activator through preferentially cleaving the Aα chain of fibrinogen and the α-chain of fibrin [170].

Pinus densiflora, an evergreen needle-leafed tree indigenous to Asia Pacific, has been used for the treatment of multiple ailments such as cardiovascular disease, cancer, diabetes and antihypertension. It was reported that pine needle extract would facilitate fibrinolysis, decrease the blood plasma cholesterol and triglyceride in cholesterol fed rat, and it's helpful in removing blood clots [177]. On the other hand, Huang et al. screened for the fibrinolytic activities of 6 kinds of authentic medicinal materials from Guangxi (China) by fibrin plate method *in vitro*. As a result, *Pueraria lobata*, *Trichosanthes kirilowii*, *Lonicera japonica*, and *Desmodium styracifolium* showed fibrinolytic activity, and in particular the fibrinolytic activity of *D. styracifolium*

Table 4: Impacting on the arachidonic acid system of natural products.

Natural products	Experimental models	Possible mechanisms	Reference
Epigallocatechin-3-gallate of green tea leaves	Rat blood (*in vitro*); agonist: collagen	Inhibiting the activation of COX-1 and TXAS, with a stronger selectivity in COX-1 inhibition than TXAS inhibition	[133]
Jujuboside B of seeds of *Zizyphus jujuba*	Rat blood (*in vitro*); agonist: collagen	Inhibition of TXA_2 production	[134]
Alditol and monosaccharide of sorghum vinegar	Human blood (*in vitro*); agonist: AA, collagen, ADP, and THR	Inhibition of COX-1 and TXAS and attenuating TXA_2 production	[135]
Diacetylated obovatol of *Magnolia obovata* leaves	Rabbit blood (*in vitro*); agonist: collagen and AA	Inhibition of COX-1 and LOX activities and decreasing in cytosolic Ca^{2+} mobilization and 5-HT secretion	[136]
Ethanol extract, eupatilin, and jaceosidin of *Artemisia princeps* Pampanini	Human blood (*in vitro*); agonist: AA	Inhibition the generation of 5-HT and TXA_2	[137]
Berberine of berberine sulfate injection	Rabbit blood (*ex vivo*); agonist: ADP, AA, and collagen	Suppressing of TXA_2	[138]
Hesperetin of grapefruits and oranges	Rabbit blood (*in vitro*); agonist: AA and collagen	Inhibition of PLC-γ2 phosphorylation, COX-1 activity, and decreasing of Ca^{2+} as well as TXA_2	[139]
Green tea catechins of *Camellia sinensis*	Rabbit blood (*in vitro*); agonist: AA, collagen, and U-46619	Inhibition of AA liberation, TXA_2 synthesis, PGD_2, and ATP formation	[140]
Hydroxychavicol of betel quid	Rat blood (*in vitro*); agonist: AA, collagen, and THR	Inhibition of COX-1/COX-2 enzyme activity and decreasing TXA_2 and ROS production as well as Ca^{2+} mobilization	[141]
Tetrandrine and fangchinoline of *Radix Stephaniae Tetrandrae*	Human blood (*in vitro*); agonist: PAF, THR and AA	Suppression of TXA_2 formation, but without inhibiting the binding of PAF to PAF-receptor	[142]
Isorhynchophy lline of *Uncaria sinensis* (Oliv.) Havil.	Rabbit blood (*in vitro*); agonist: collagen	Inhibition of TXA_2 formation	[143]
Genistein	Rabbit blood (*in vitro*); agonist: PAF	Inhibition of TXA_2 formation and increasing PGI_2 generation	[144]
Ethyl acetate extract of *Caesalpinia sappan* L.	Rat blood (*ex vivo*); agonist: ADP	Inhibition of TXA_2 formation and increasing PGI_2 generation	[145]
Morroniside of *Cornus officinalis* Sieb.et Zucc	Rabbit blood (*in vitro*); agonist: ADP	Inhibition of COX activation and decreasing TXB_2 generation	[146, 147]
Neolignans of three Lauraceae species (*Pleurothyrium cinereum, Ocotea macrophylla, and Nectandra amazonum*)	Rabbit blood (*in vitro*) agonist: PAF, ADP and AA	Inhibition of COX-2 by Benzofuran neolignans; inhibition of PAF-action, COX-1, 5-LOX by bicyclooctane; inhibition of COX-2, PAF-action by neolignan 9-nor-7,8-dehydro-isolicarin B and cinerin C; inhibition of 5-LOX/COX-2 by Nectamazin C	[148]
Extracts of Myoga (*Zingiber mioga* Roscoe)	Human blood (*in vitro*) agonist: ADP and AA	Inhibition of 5-LOX by miogatrial, miogadial, sesquiterpene and polygodial	[149]
Ginsenoside Rk1 of white ginseng	Rat blood (*in vitro*) agonist: AA	Decreasing of 12-HETE, 12-LOX, and Ca^{2+} levels	[150]

AA: arachidonic acid; ADP: adenosine diphosphate; THR: thrombin; PAF: platelet activating factor; COX-1: cyclooxygenase-1; COX-2: cyclooxygenase-2; TXAS: thromboxane synthase; LOX: lipoxygenase; TXA_2: thromboxane A_2; TXB_2: thromboxane B_2; 5-HT: 5-hydroxytryptamine; PLC-γ2: phospholipase C-γ2; PGD_2: prostaglandin D_2; ATP: adenosine triphosphate; ROS: reactive oxygen species; PGI_2: prostacycline 2; 12-HETE: 12-hydroxy-5,8,10,14-eicosatetraenoic acid.

was similar to that of positive drug urokinase [178]. In addition, two components (1-palmitoyl-2-oleoyl-3-*O*-α-D-glucopyranosylglycerol and 1-myristoyl-2-oleoyl-3-*O*-α-D-glucopyranosylglycerol) were purified from *Sargassum fulvellum* and the fibrinolytic effect was identified *in vitro* [179].

4. Conclusion

Thrombosis remains a final pathway to disease and death in some of our most common diseases such as myocardial infarction and stroke. Although substantial progress has been made in understanding the biology of thrombus formation and the pathophysiology of thrombosis, all the pharmacological agents available for prevention or treatment have been in use for decades or have been replaced with newer variants that offer a modest incremental improvement. Natural products have been reported with apparent inhibitory activity on thrombotic diseases both in experimental and clinical stages, which provide a useful preventive approach or an adjunct to current pharmacological treatments for thrombotic diseases. Advances in the knowledge of both the mechanisms of thrombus formation and of the biological functions of natural products will provide new insights to promote human health.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

An Integrative Thrombosis Network: Visualization and Topological Analysis

Xiangjun Kong,[1] Wenxia Zhou,[2] Jian-Bo Wan,[1] Qianru Zhang,[1,3] Jingyun Ni,[1] and Yuanjia Hu[1]

[1] *State Key Laboratory of Quality Research in Chinese Medicine, Institute of Chinese Medical Sciences, University of Macau, Macau*
[2] *Institute of Pharmacology and Toxicology, Academy of Military Medical Sciences, Beijing 100850, China*
[3] *Pharmacy School, Zunyi Medical College, Zunyi, Guizhou 563000, China*

Correspondence should be addressed to Yuanjia Hu; yuanjiahu@umac.mo

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A comprehensive understanding of the integrative nature of the molecular network in thrombosis would be very helpful to develop multicomponent and multitarget antithrombosis drugs for use in traditional Chinese medicine (TCM). This paper attempts to comprehensively map the molecular network in thrombosis by combining platelet signaling, the coagulation cascade, and natural clot dissolution systems and to analyze the topological characteristics of the network, including the centralities of nodes, network modules, and network robustness. The results in this research advance understanding of functions of proteins in the thrombosis network and provide a reference for predicting potential therapeutic antithrombotic targets and evaluating their influence on the network.

1. Introduction

Thromboembolic disorders are a major cause of death and disability and affect millions worldwide. Thrombosis can occur in either the arterial or the venous circulation and results in different clinical symptoms, such as pulmonary emboli, deep vein thrombosis, strokes, and heart attacks.

While antithrombotic drugs, including anticoagulants, antiplatelet drugs, and thrombolytic drugs, have been widely used for the prevention and treatment of arterial and venous thrombosis, new targets, more effective agents against existing targets, as well as new therapeutic strategies still need to be developed for overcoming resistance to current drugs, suppressing the stimulus in platelet activation, and regulating the anticoagulation effect more conveniently [1].

Traditional Chinese medicine (TCM), especially HuoXueHuaYu Chinese medicines, has long been used to treat thrombosis. The significant efficacy of TCM in treating thrombosis has been reported in the literature and in recent pharmacological experiments [2–4]. Thus, TCM seems to offer a possible route to the discovery of new targets, agents, and therapeutic strategies for the treatment of thrombosis. It is widely believed that the mechanism of multicomponent and multitarget may be of great essence for TCM to exert integrative treatment effects [5–7]. To better understand the potential of TCM in the treatment of thrombosis, the molecular network involved in the disease needs to be elucidated.

Moreover, several studies have been so far conducted to evaluate the efficacy of different compounds against platelet aggregation, exhibit formula-target relations, and develop model to predict coagulation response [8–10]. However, little is known about the system-wide effects of molecules of thrombosis from a holistic perspective with the comprehensive consideration between efficacy and safety referring to the balance of antithrombosis and bleeding.

On the other hand, the rapid progress of bioinformatics and systems biology has provided not only a systems-level understanding of biological processes and disease complexity but also an efficient and promising approach, such as network analysis, for integrative drug development [5, 11]. Csermely et al. presented a comprehensive review of analytical tools

of network topology and dynamics and advances in applications for drug discovery [12]. Moreover, potential targets were identified by detecting key nodes in a disease-specific network with important topological properties [13, 14].

In this context, this research attempts to comprehensively map the thrombosis molecular network and analyze topological characteristics of the network from several perspectives, including the centralities of nodes, network modules, and network robustness. This research is of significance to improve the understanding of molecular functions in the thrombosis network and further predict potential targets for the treatment of thrombosis by evaluating their influence on the network.

2. Methods

2.1. Network Construction. Reactome is a curated and peer-reviewed pathway database that functions as a data-mining resource and electronic textbook, with the focus on *Homo sapiens* [15]. Details of pathways, such as constituent reactions and participating complexes and relationships, are elucidated in Reactome. We retrieved pathways and reaction information from the Reactome database that were relevant to thrombosis. These included (1) platelet activation, signaling, and aggregation, (2) the clotting cascade, and (3) the dissolution of fibrin clots. We organized these pathways and reactions as elementary reactions that contained one reactant and its corresponding product, regardless of small molecules. In this step, protein complexes were involved in the majority of elementary reactions. To identify potentially effective therapeutic targets against thrombus, we split the complexes in the elementary reactions into separate single proteins forming reactant group and product group. Then, the splitting proteins are reconnected from each reactant to each different product, except self-connections. Finally, the relations between the proteins with reacting directions were detected. Gephi software (http://gephi.github.io/) was then used to construct an evidence-based and integrative thrombosis network (Figure 1) [16].

In this network, nodes represent proteins related to thrombosis, and edges with direction between nodes indicate their interacting connections. The direction of the edges denotes the reaction stream, from the node at the start of the arrow to the node downstream at the end of the arrow. The edge of a double-headed arrow denotes the bidirectional reaction of a protein pair. Based on the principle of network generation, the double-headed arrows imply that the proteins function in complexes.

2.2. Centrality Analysis. The centrality definition of a node in a network is related to the concept of importance. Dozens of centrality measures have been developed to understand network structure, and these have been widely used to find central nodes in various biological systems [12, 17]. In this research, we examined the node degree, betweenness centrality, and closeness centrality of the nodes to shed light on key druggable proteins that might serve as targets in thrombosis. The centralities are calculated based on the algorithms referred by Gephi [18].

The degree of node v, $C_{\text{deg}}(v)$, is calculated by the following equation:

$$C_{\text{deg}}(v) = \sum_{u \in V, u \neq v} d(v, u), \tag{1}$$

where $d(v, u)$ is 1, if and only if node v and node u are connected by an arrow, no matter where v positions are (the start or the end of the arrow); otherwise, it is 0. Nodes u and v are different nodes from node set V of network. Thus, degree is limited in the scope of nodes that are directly connected to a node, but not including the indirect connections.

We used k to denote a node's degree in thrombosis network. Then, we tested the degree distribution $p(k)$ of the network, giving the fraction of nodes with degree k, ($k = 1, 2, \ldots$) (Figure 2). We performed a goodness-of-fit test to determine the degree distribution of the constructed thrombosis network whether it follows power-low. The hub of a network refers to a node with a much higher degree than the average. The network hubs are listed by degree order from high to low in Table 1.

The betweenness centrality of node v, $C_{\text{bet}}(v)$, measures the number of shortest paths that pass through the node:

$$C_{\text{bet}}(v) = \sum_{u,w \in V, u \neq v \neq w} \frac{\sigma_{u,w}(v)}{\sigma_{u,w}}, \tag{2}$$

where $\sigma_{u,w}(v)$ is the number of shortest paths from node u to node w that pass through the node v; $\sigma_{u,w}$ is the number of shortest paths between node u and node w. A node with high betweenness centrality serves as a bridge between other nodes in the whole network. Thus, the communication between other nodes becomes more dependent on this node in the network.

Unlike the degree, closeness centrality of a node examines the direct and indirect links connected to the nodes. The closeness centrality of node v, $C_{\text{clo}}(v)$, is the mean shortest path of the node connecting to all other nodes in the network:

$$C_{\text{clo}}(v) = \frac{\sum_{u \in V, u \neq v} \text{dis}(v, u)}{\sum_{u \in V, u \neq v} n(v, u)}, \tag{3}$$

where $\text{dis}(v, u)$ denotes the distance between nodes v and u, that is, the minimum length of any path connecting v and u in network. In this equation, $n(v, u)$ is 1, if there is a path linking node v and node u; otherwise, it is 0. For an isolated node, its closeness centrality is 0.

2.3. Identification of Network Module. Network modules are classical measures of mesoscopic network structures. A group of nodes that is connected more closely to group members than others outside this group is regarded as a module or a community that has fewer connections between modules. In this paper, the module detection Louvain algorithm incorporated in Gephi was used to explore the modularity structure of the network [19]. The modular function was then analyzed to shed light on the complex relationship among the modules. The modular hubs (i.e., the nodes with a higher degree than the other nodes in the same module) are listed in Table 2.

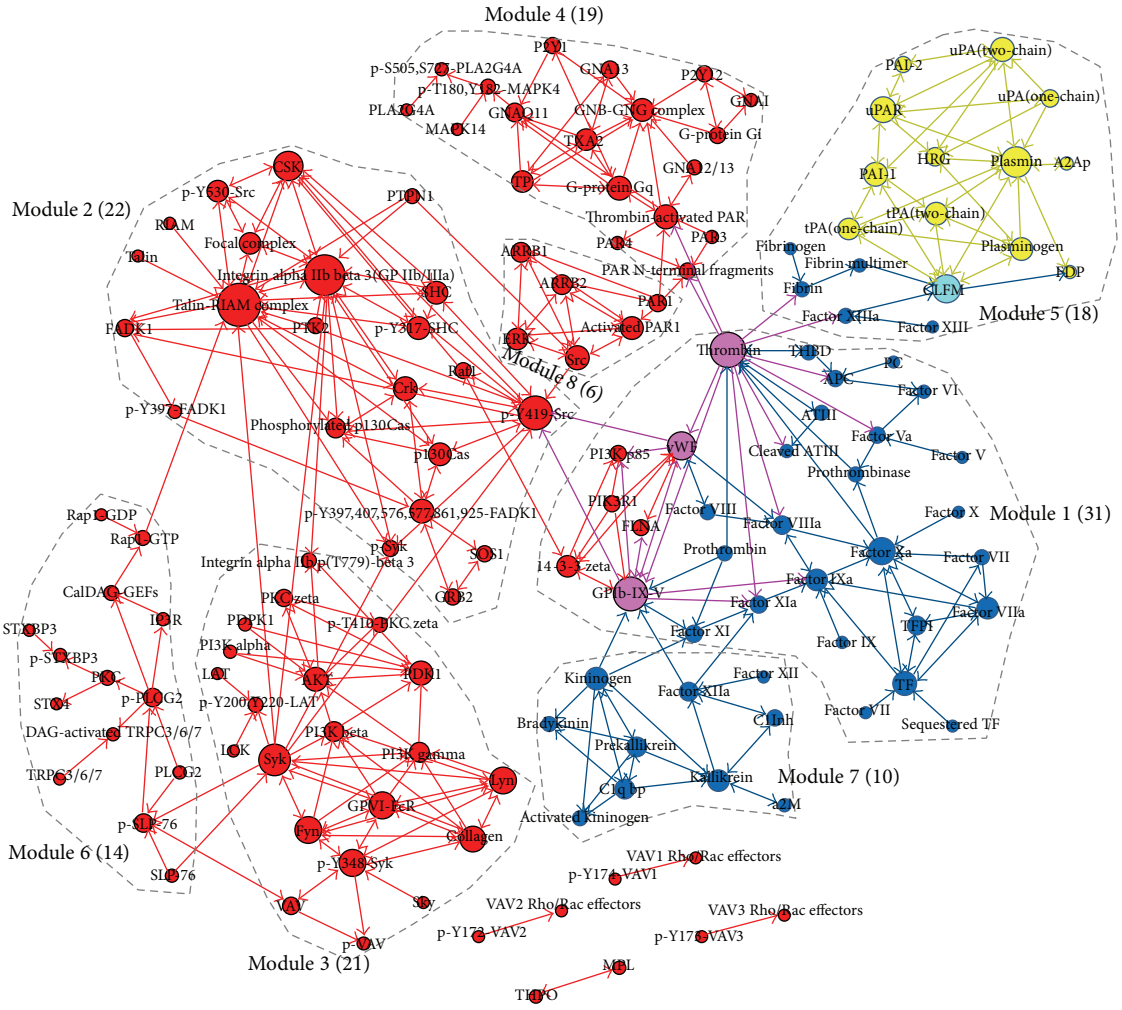

FIGURE 1: Visualized thrombosis network. The colors of the different nodes represent their involvement in diverse functions in thrombosis, as determined from the Reactome pathway analysis. The red node means protein taking part in platelet activation, signaling, or aggregation, which owns most participants. The blue and yellow represent function of clotting cascade and fibrin clot dissolution process, respectively. The size of node corresponds to its degree. Nodes involved in a module are marked within the largest component, and modules are sorted by number of involved nodes.

2.4. Analysis of the Robustness of the Network. The robustness of a network reflects the tolerance of a network to failures or its ability to withstand attacks. Robust networks maintain the stability of system function against failures or attacks. Drug action often fails or generates serious side effects due to high network robustness or hitting unexpected points of networks [20–22]. Here, in order to identify potential drug targets, we investigated the robustness of a thrombosis network under the simulation of random failure or a deliberate attack. Random failures of cellular network are usually caused by the oxidative damage, the indirect effect from somatic mutations, and complex influence of ageing [23, 24], while deliberate attacks refer to drug-driven influence to network. As introduced by Albert et al., we used the indicator S and $\langle s \rangle$ to evaluate the network robustness and fragmentation process [25]. When a fraction, f, of all the network nodes was removed randomly (failure) or removed as degree order (hub attack) or betweenness centrality order (bridge attack) of nodes, we calculated the fraction of the size of the largest component comparing to the total system size, S. Then, we detected the average size $\langle s \rangle$ of the isolated components (all the components except the largest one) when the same fraction of nodes was removed. The behavior of the network, with an increasing f, is presented in Figure 4.

3. Results and Discussion

3.1. Visualization of the Network. We constructed a human thrombosis network by combining serial signal pathways of activating and recruiting platelets initiating blood coagulation and generating thrombi and fibrin. These events occur concomitantly (Figure 1). The resulting thrombosis network provides a visual and relatively integrative perspective to understand thrombosis in various diseases.

There are 149 proteins and 414 relations in the network, which is made up of one large component and four small

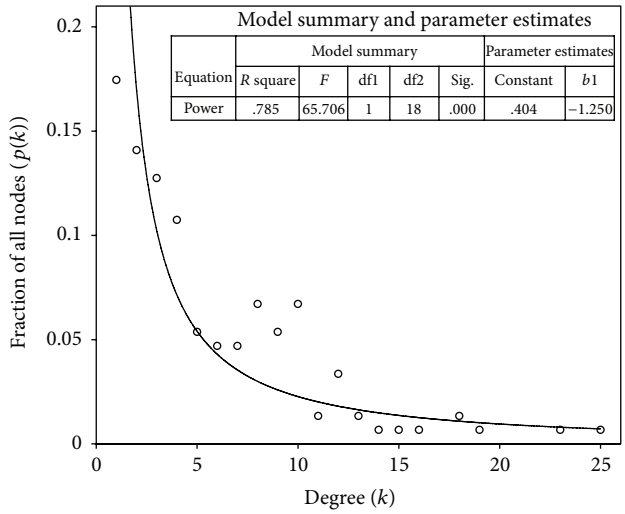

Figure 2: The degree distribution of the thrombosis network (scatter spot and fitting curve in power law). The horizontal axis denotes the number of connections of the nodes and the vertical denotes the fraction of nodes with a specific degree. The distribution fitted a power law, with $\gamma = 1.25$, $p < 0.001$.

separated components. The network is composed of the three parts of functions that connect with each other. It includes most of the receptors and enzymes involved in these three factors, such as integrin alpha IIb beta 3 (GP IIb/IIIa), antithrombin III (ATIII), glycoproteins of the Ib, IX, and V complex (GPIb-IX-V), von Willebrand factor (vWF), thrombin, proteinase-activated receptor 1 (PAR1), P2Y12, P2Y1, tissue pathway factor inhibitor (TFPI), plasminogen activator inhibitor-1 (PAI-1), and plasminogen activator inhibitor-2 (PAI-2). Interestingly, four nodes exhibited two diverse functions: GPIb-IX-V, vWF, and thrombin were particularly important contributors to both platelet signaling and the coagulation cascade; the cross-linked fibrin multimer (CLFM) was the common target of the coagulation and natural clot dissolution system. We consider the four proteins (GPIb-IX-V, vWF, thrombin, and CLFM) as multifunctional proteins. The average degree of each node was 2.78, and the average shortest path length was 5.37.

The degree distribution $p(k)$ is an important measure of the topological features of the network (Figure 2) [26]. The degree distributions of most real-world networks, including biological networks, follow a power law, $p(k) \sim Ak^{-\gamma}$, where γ is the power-law exponent. The degree distribution in network generated in this way obeys the following power law: $p(k) = Ak^{-1.25}$, $p < 0.001$. The degree distribution of the thrombosis network was approximately scale free (when $2 < \gamma < 3$). As confirmed by the power law, most of the nodes in this network only influenced a limited number of other nodes, and a small number of nodes interacted with many other nodes. These nodes are likely to play key roles in the functional system [27].

3.2. Identification of Key Targets. Hubs with a high degree of centrality occupy a critical position in a network, although they house only a small number of all the nodes in a network. If hubs are attacked, the integrity of the network deteriorates more rapidly than nonhubs, which makes hubs attractive drug targets [25]. Thus, it is useful to study the key proteins contributing to thrombus formation as network hubs. Therefore, hubs with degrees larger than 10 and their topological properties were extracted (Table 1).

Table 1 shows 27 hubs with diverse functions including the four multifunctional proteins. The locations of multifunctional proteins indicate the mutually influential relation among the three functions in formation of thrombi. Among these hubs, many have been well developed as effective antithrombotic targets, involving U.S. Food and Drug Administration- (FDA-) approved therapeutic targets and preclinical developing targets. Thrombin, factor Xa, GP IIb/IIIa, PAI-1, and urokinase plasminogen activator receptor (uPAR) are typical targets of popular clinical medicines [28, 29]. However, there are plenty of proteins with a high degree, such as the Rap1-interacting adaptor molecule (RIAM) complex, that are not suitable for drug development [30].

On average, the targets of FDA-approved drugs tend to have more connections than most peripheral nodes but do not cover all the hubs [31]. Hub connectors, such as factor XIa, factor IXa, and 14-3-3 zeta, that connect GPIb-IX-V, ATIII, and factor V with thrombin and connect plasminogen with CLFM are linked to major hubs and provide very interesting targeting options [24].

Different centrality measures indicate different importance of nodes in the network. Nodes with high betweenness centrality indicate their particular targeting potential for antithrombosis due to their bottleneck positions in the thrombosis network. It should be noted that 16 out of 27 hubs also have high betweenness centrality. Moreover, high degree and betweenness centralities exhibit essential topological significance in thrombosis network by serving as network hubs and bridges.

On the other hand, a substantial number of key proteins in Table 1 are enzymes related to cell survival, growth, and metabolism and activate or promote the development of thrombosis signaling series, such as Src family kinases, the PI3K/AKT pathway, and Syk. The topological positions of enzymes in network highlight their potential roles as therapeutic targets. Enzyme signal pathways are increasingly recognized as targets of antithrombosis drugs. The activation of Src family kinases (SFKs), a family containing eight structurally related tyrosine kinases, namely, Lyn, Fyn, Src, Fgr, Blk, Hck Yes, and Lck, is an important event downstream of integrin adhesion signaling that is involved in initiating and amplifying signals in platelets [32, 33]. Research on mice has provided preliminary but important implications for exploring inhibitors targeting individual SFKs, in particular, Lyn [32]. The central role of Syk identified by both high degree and betweenness centrality in numerous signaling cascades also highlights its promise in the development of novel antithrombotic therapeutics [34]. All these appear to be consistent with the prediction derived from network centrality implication exhibited in Table 1. However, as these enzymes have multiple roles in other biological processes, an appropriate drug-delivery system is needed that specifically targets the thrombus system.

Table 1: Topological information on key proteins in the thrombosis network, ranked by degree.

Rank	Nodes	Degree	Betweenness	Closeness	Functions[a]
1	Talin-RIAM complex	25	689	6.2	Platelet signaling
2	GP IIb/IIIa	23	215	6.2	Platelet signaling
3	Thrombin[b]	19	3089	2.7	Multifunction
4	GPIb-IX-V[b]	18	2818	2.9	Multifunction
5	p-Y419-Src	18	2866	4.8	Platelet signaling
6	Syk	16	686	4.7	Platelet signaling
7	Plasmin	15	294	1.5	Clot dissolution
8	CSK	14	151	6.3	Platelet signaling
9	vWF[b]	13	542	3.4	Multifunction
10	CLFM[b]	13	992	1.7	Multifunction
11	Fyn	12	133	5.6	Platelet signaling
12	GPVI-FcR	12	133	5.6	Platelet signaling
13	Lyn	12	133	5.6	Platelet signaling
14	p-Y348-Syk	12	288	6.4	Platelet signaling
15	Factor Xa	12	1032	3.5	Coagulation
16	Collagen	11	9	6.4	Platelet signaling
17	uPAR	11	145	1.5	Clot dissolution
18	Src	10	396	5.4	Platelet signaling
19	PDK1	10	21	1.0	Platelet signaling
20	AKT	10	21	1.0	Platelet signaling
21	PAI-1	10	177	1.7	Clot dissolution
22	Crk	10	502	5.5	Platelet signaling
23	p-Y397, 407, 576, 577, 861, 925-FADK1	10	414	6.4	Platelet signaling
24	TF	10	213	4.4	Coagulation
25	Thrombin-activated PAR	10	803	1.8	Platelet signaling
26	G-protein Gq	10	338	1.5	Platelet signaling
27	uPA (two-chain)	10	53	1.6	Clot dissolution

[a]Functions refer to platelet signaling, coagulation, clot dissolution, or multifunction identification summarized from Reactome.
[b]Thrombin, GPIb-IX-V, vWF, and CLFM are multifunctional proteins serving as two functions. The first three combining functions are in platelet signaling pathways and coagulation cascades, while CLFM is in coagulation and clot dissolution system.

Table 2: Modularity of the thrombosis network and the identification of modular hubs.

Modules	Number of nodes	Modular hubs	Mechanisms
1	31	Thrombin, GPIb-IX-V, vWF, factor Xa	Platelet adhesion signaling and classic coagulation cascade system
2	22	Talin-RIAM complex, GP IIb/IIIa, p-Y419-Src, CSK	Platelet activation and aggregation through GP IIb/IIIa
3	21	Syk, Fyn, GPVI-FcR, Lyn, p-Y348-Syk	Platelet activation through GPVI-FcR and Syk signal
4	19	Thrombin-activated PAR, G-protein Gq, GNB-GNG complex	Accumulation of soluble agonists for platelet recruitment
5	18	Plasmin, CLFM, uPAR, PAI-1	Fibrin formation and dissolution events
6	14	p-PLCG2, p-SLP-76	Signalosome formation for promoting full platelet activation through PLCG2
7	10	Kininogen, kallikrein, C1q bp, prekallikrein	Kallikrein-kinin system
8	6	Src, activated PAR1	Typical platelet activation signal via ERK

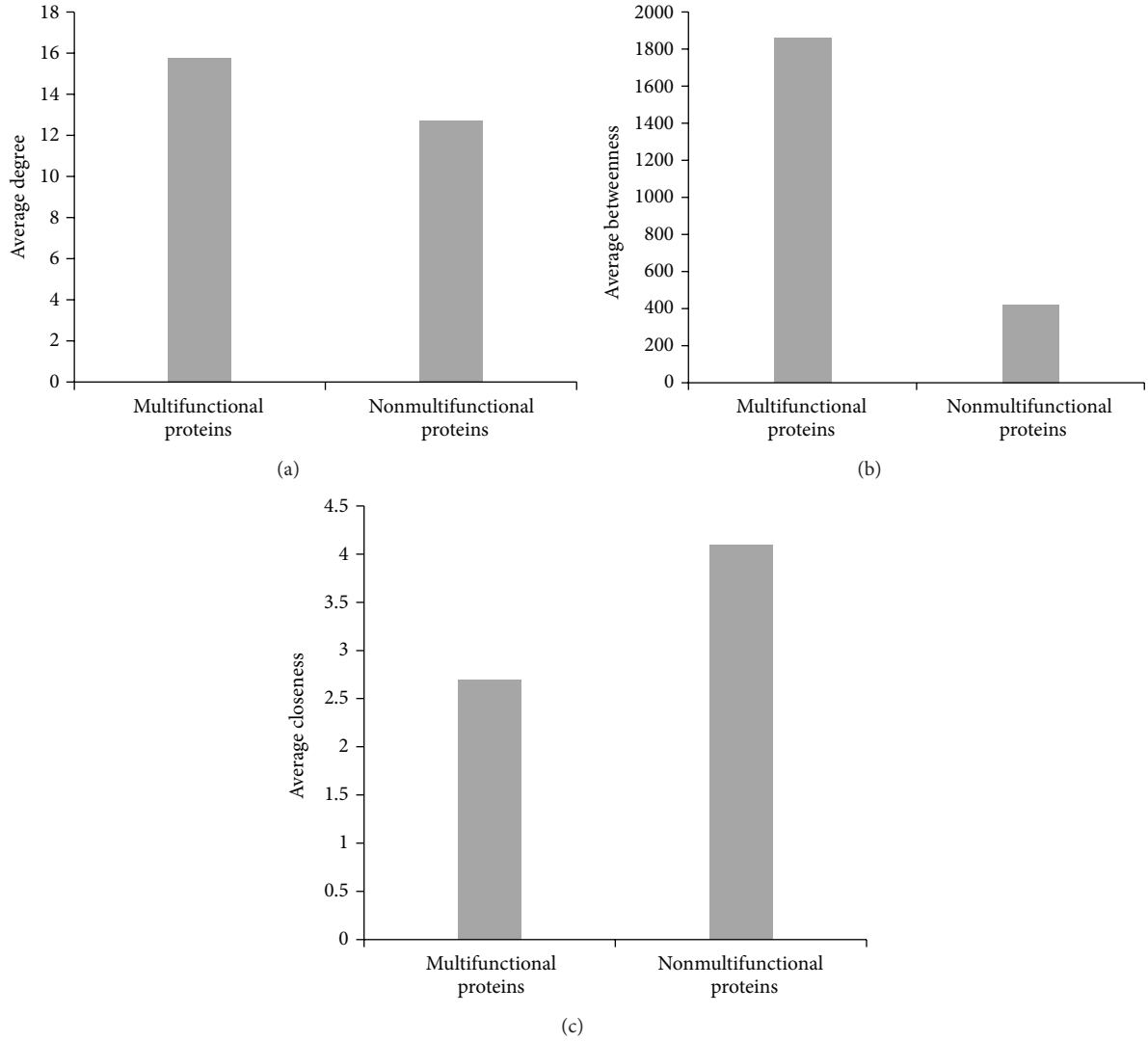

Figure 3: The centralities of multifunctional and nonmultifunctional proteins: (a) average degree; (b) average betweenness; (c) average closeness.

Nodes with overlapping function are key determinants of network cooperation. Overlapping nodes occupy specific network positions and can provide more subtle regulation. As shown in Figure 1 and Table 1, four multifunctional proteins cross-linking coagulation cascade and platelet signaling or clot dissolution affect both sides broadly, due to their high degree and betweenness centralities. The potential of GPIb-IX-V and vWF as antiplatelet adhesion targets has been investigated in mounting evidence from basic research and clinical evaluations for antiplatelet agents identification [35, 36]. Inhibitors against thrombin are also the focus of much research to improve the treatment of thrombus [37].

We compared the average degree, betweenness centrality, and closeness centrality of multifunctional proteins with those of the nonmultifunctional proteins shown in Table 1 to identify their specific topological characteristics. Figure 3 shows the difference in the betweenness and closeness centrality of these multifunctional and nonmultifunctional proteins. Multifunctional nodes bridging the three components of thrombosis (i.e., platelet signaling, the coagulation cascade, and the natural clot dissolution system) show much higher betweenness and interact with other nodes closely. These likely contribute to such encouraging performance of functional overlaps as attractive targets for antithrombotic treatment.

3.3. Implications of Network Modularity. To facilitate the interpretation of the complex relationships in the thrombosis system, the modular structure of the system was explored. We marked eight modules positioned in the largest component in Figure 1 and sorted them by the number of involved nodes in Table 2. The mechanism of each module and the functional interdependencies among the modules are illustrated in Table 2. The findings provide insight into the complex biological process of thrombosis corresponding to the functional modules' network positions.

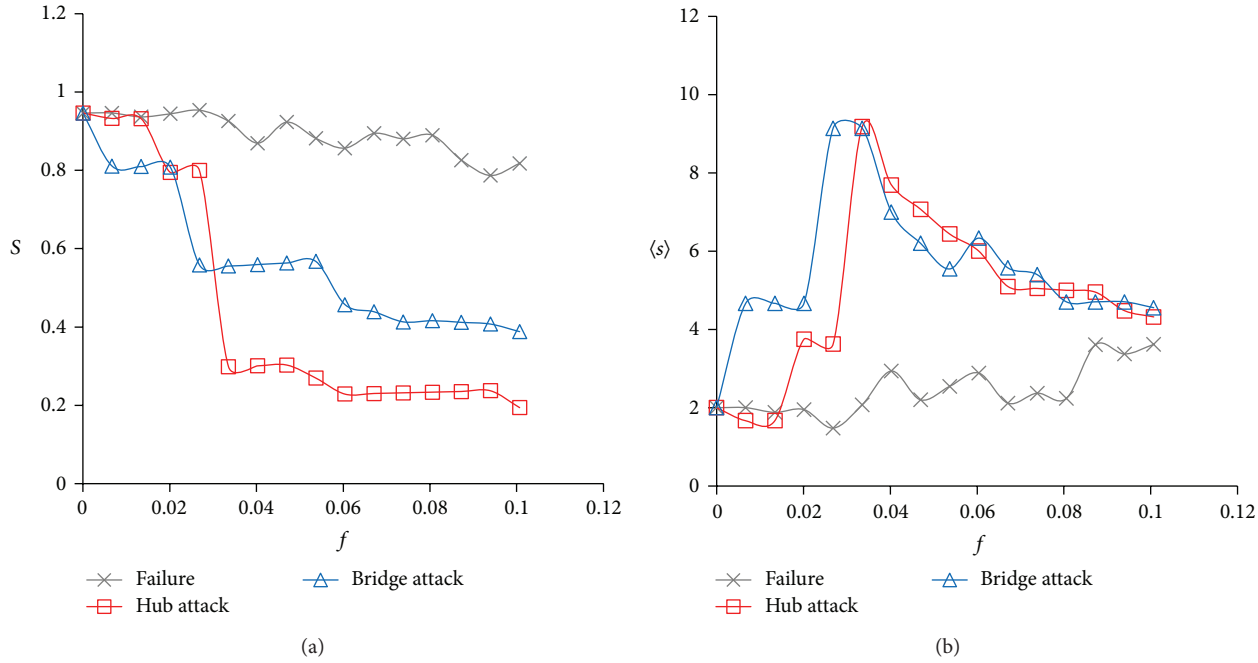

Figure 4: Network fragmentation under random failures, hub attacks, and bridge attacks measured by (a) the relative size of the largest cluster S and (b) the average size of the isolated components $\langle s \rangle$.

In module 1, GPIb-IX-V and vWF not only were important components of platelet adhesion but also strongly associated with the classic coagulation cascade by factor XI and factor VIII [38]. In addition, thrombin, as the most potent platelet agonist, coordinates the process of platelet activation and aggregation with coagulation [1]. Together with factor Xa [39], they serve as modular hubs and are considered important targets in antithrombotic treatment. Network hubs are scattered throughout diverse modules. Most are modular hubs.

Otherwise, modular hubs which are not network hubs should also be emphasized in view of their local influence on some specific functions. For example, phosphorylated phospholipase C gamma 2 (p-PLCG2), as a hub of module 6, and kininogen and prekallikrein as hubs in the kallikrein-kinin system have been demonstrated by previous studies to show the potential as antithrombotic targets [40–43]. Another study also showed that hub-related properties significantly affected modular functions, making them attractive network drug targets when partial modulating against specific thrombosis processes [44]. The aforementioned suggests that putative targets can be identified by their modular status as well.

3.4. Analysis of Network Robustness. Robustness is an intrinsic property of networks. It refers to the ability of a network to continue functioning in the face of various perturbations. The action of drugs can be perceived as a disease network perturbation modulating disordered network towards a functional state [45, 46]. Drugs that target a single node destroy the connections between that node and other nodes. In this context, a network approach can shed light on the effect of different drugs on various targets.

Due to advances in the theoretical understanding of network structure, it is possible to quantitatively describe a network with graph concepts. As the degree distribution of the thrombosis network conforms to power law, and the network is relatively scale free (Section 3.1), it is likely resistant to random damage but sensitive to the targeted removal of nodes [25]. Networks have a number of vulnerable points, such as hubs and bridges, and they can be attacked at any of these. In this paper, we simulated hub attacks and bridge attacks to examine the robustness of a thrombosis network (Figure 4).

As shown in Figure 4, the response of the thrombosis network to attacks and failures differed. When nodes were removed continuously from the network (up to $f = 0.1$), the size of the largest component S remained the dominating position under random failure but fell apart to moderate size obviously when hubs or bridges were attacked (Figure 4(a)). When one or two nodes were removed, only bridge attacks had much of an effect on the network, pointing to the importance of targeting nodes with high betweenness when developing single-target agents. As f increased, the size of the network largest component decreased more rapidly under hub attacks than bridge attacks. When hubs were attacked, S displayed threshold-like behavior. At $f \approx 0.03$ (about five nodes were removed), $S \approx 0.2$, and the network experienced catastrophic fragmentation. As shown in an earlier study, the fragmentation would break off continuously but less severely when larger fraction than 0.1 of nodes was removed [25].

The fact that the average size $\langle s \rangle$ of the isolated components increased slowly indicated that increasing failure level led to the isolation of single nodes, not large components

(Figure 4(b)). In the attack mode, the system was sensitive to the removal of key nodes and was separated into certain size of components, which explains the rapid increased $\langle s \rangle$ for the small f. Similar threshold of $\langle s \rangle$ was detected in attack mode, where the main component broke into small pieces and also led to the size of fragments peaks. As we continued to remove nodes, the isolated components became deflated, leading to a descending $\langle s \rangle$. The aforementioned behavior provides evidence that the thrombosis network shows topological stability against random failures but that it fragments in response to attacks on a small number of nodes. Obviously, bridge attacks are more sensitive than attacks on hubs, and hub attacks cause more serious fragmentation of the network. These observations of the global influence of network attacks could provide clues for seeking fragile targets and designing multitarget therapeutic strategies against thrombosis.

4. Conclusions

Network analysis has the advantage of providing system-level perspectives on complex issues. Topological analysis can help to extract valuable information hiding in large-scale and complex experimental data. In summary, on the foundation of evidence-based data, we constructed an integrated thrombosis network composed of platelet signaling, the coagulation cascade, and the natural clot dissolution system and conducted various network topological analyses. The degree distribution followed a power law, and the network was relatively scale free. With this in mind, local topology analysis was conducted to identify central nodes that could be putative drug targets. The results showed that targets can also be predicted from their modular position by modularity analysis. The analysis of the robustness of the thrombosis network demonstrated that it was highly resistant to random failure but sensitive to hub and bridge attacks. Such studies can elucidate the function of proteins in thrombosis network, help discover new targets for the treatment of thrombus using TCM, and contribute to the development of new targets of TCM and multitarget strategies.

Network analysis seems to provide a valuable prediction of therapeutic targets, but it is still insufficient to validate the effectiveness of targets. Further pharmaceutical experiments are necessary for eventual validation of network results. Network approach can serve as a valuable complement to the experimental efforts, while a combination between simulated and experimental studies is of great significance for effective drug discovery in future.

Conflict of Interests

The authors declare that they have no conflict of interests to disclose.

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