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Research Article
Evaluation of Anticoagulant and Antithrombotic Activities of Berberine: A Focus on the Ameliorative Effect on Blood Hypercoagulation

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Abstract

Background and Objective: Thrombosis and related diseases are the leading causes of mortality and disability worldwide. This study aimed to investigate the anticoagulant and antithrombotic activities of natural product berberine (BBR) using *in vitro* and *in vivo* models. **Materials and Methods:** In *in vitro* experiments, BBR was used to treat venous blood or plasma isolated from rabbits, with heparin sodium (HS) as a positive control. In the animal experiments, BBR was orally administered to Wistar rats fed with a regular diet or a high-fat diet (HFD) for 2 or 4 weeks, respectively. After experiments, whole blood clotting time (CT), activated partial thromboplastin time (APTT), prothrombin time (PT), thrombin time (TT), fibrinogen (FIB) and the activities of a series of coagulation factors and anti-thrombin-III (AT-III) were determined. In the HFD-feeding experiment, metabolic parameters such as blood lipids and glucose were assayed. In parallel experiments, the effect of BBR on inferior vena cava thrombus formation was determined by ligation. **Results:** The BBR significantly prolonged CT and APTT/PT/TT but decreased FIB both *in vitro* and *in vivo*. In addition, BBR significantly increased the activity of AT-III and suppressed the activities of coagulation factors participating in the intrinsic, common and extrinsic coagulation pathways. These BBR-induced changes resulted in a significant inhibition in thrombus formation in the inferior vena cava. In response to HFD-feeding, the rats developed a hypercoagulable state, as indicated by the shortening of CT and APTT/PT/TT, an increase in FIB, overactivation of coagulation factors and the deterioration of thrombosis. After treatment, BBR improved the metabolic parameters of the rats and effectively prevented the HFD-induced blood hypercoagulation. **Conclusion:** The BBR has anticoagulant activity, which is responsible for its anti-thrombotic effect. The BBR may be suitable for the intervention of hypercoagulability and thrombosis when it is used to treat metabolic diseases.

Key words: Berberine, anticoagulant activity, coagulation factors, antithrombin-III, thrombosis, hypercoagulation

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Thrombus formation is a complex pathophysiological process; its triggering factors include the dysfunction of the coagulation/fibrinolysis system, the activation of platelets, hemodynamics abnormalities and vascular endothelial damage[1-5]. Thrombus formation may have serious consequences, which include cardiovascular and cerebrovascular accidents such as myocardial infarction and stroke, as well as deep venous thrombosis (DVT) and pulmonary embolism[5]. Thrombosis and related diseases are the leading causes of mortality and disability in China and western countries and thus research and development of novel antithrombotic drugs are of scientific and clinical importance.

Berberine (BBR) is a natural compound isolated from medicinal plants such as *Coptis chinensis* Franch and has been shown to have multiple pharmacological activities and beneficial effects against a variety of human diseases, which include diseases of the endocrine and metabolic system, digestive system, nervous system, cardiovascular system and reproductive system[6-9].

Previous studies have shown that BBR inhibits platelet aggregation in experimental animals[1,10,11] as well as in clinical studies[12,13] and the inhibitory effect of BBR on platelet aggregation is comparable to that of aspirin[14,15]. The BBR has also been shown to suppress thrombus formation in preclinical studies[10,15,16]. However, the influences of BBR on coagulation function remain unclear. In this report, the effects of BBR on the coagulation system are investigated using *in vitro* and *in vivo* models and the results show that BBR has anticoagulant activity when used to inhibit thrombus formation.

MATERIALS AND METHODS

Reagents and kits: The BBR was provided by the Northeast Pharmaceutical Group (Shenyang, Liaoning, China) and heparin sodium (HS) and sodium citrate were purchased from Huayueyang Biotechnology (Beijing) Co., Ltd. (Beijing, China). Commercially available kits for determination of activated partial thromboplastin time (APTT), prothrombin time (PT), thrombin time (TT) and fibrinogen (FIB) were purchased from Beijing Succeeder Technology, Inc. (Beijing, China). Coagulation Factor (F) IX, XI, XII, X, VII and II Deficient Plasmas were purchased from Siemens Healthcare GmbH (Marburg, Germany) and the ACTICHROME® Antithrombin-III (AT-III) activity assay kit was purchased from Sekisui Diagnostics (Lexington, MA, USA). Dimethyl sulfoxide (DMSO) and adenosine 5-diphosphate (ADP) were purchased from Sigma-Aldrich Co., LLC. (St. Louis, MO, USA). Commercially available kits for assay of cholesterol (CHO), low-density lipoprotein cholesterol (LDL-c), triglyceride (TG), glucose and insulin were purchased from Beijing Strong Biotechnologies, Inc. (Beijing, China).

Instruments: The Semi-Automatic Coagulation Analyzer (model number: SF-400) and Platelet Aggregation Analyzer (model number: SC-2000) were purchased from Beijing Succeeder Technology, Inc.; the Electric Drying Oven with Forced Convection (model number: DHG-9010) was purchased from Shanghai Yiheng Instruments Co., Ltd. (Shanghai, China) and the Evolution 300 UV-Vis Spectrophotometer was purchased from Thermo Fisher Scientific (China), Inc. (Shanghai, China).

***In vitro* experiments:** All experimental protocols including animals were reviewed and approved by the Ethics Committee of the Institute of Materia Medica, Chinese Academy of Medical Sciences (CAMS) and Peking Union Medical College (PUMC). The animals were cared for according to the institutional guidelines of the CAMS and PUMC.

Male New Zealand rabbits (n = 6) weighting about 2.87±0.26 kg were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). The rabbits were fed with a regular laboratory diet and housed in an air-conditioned room with 12 h light/dark cycle. After 5 days of accommodation and overnight fasting, blood samples were taken by puncture of the auricular vein for the *in vitro* experiments.

Measurement of clotting time: To determine whole blood clotting time (CT), 1 mL of each rabbit blood sample was immediately poured into a silicified microcentrifuge tube (Corning Inc., New York, NY, USA), to which DMSO, BBR (dissolved in DMSO) or HS (dissolved in saline) at indicated concentrations were added and mixed well. The tubes were inclined every 30 sec until clotting and the CTs were recorded.

Measurement of APTT/PT/TT/FIB: After collection, the blood samples were immediately mixed with 10% sodium citrate at a ratio of 9:1; platelet-poor plasma (PPP) was prepared by centrifuging the blood at 3000 rpm for 10 min at room temperature. The PPPs were transferred into microcentrifuge tubes with 500 µL in each tube. The DMSO, BBR or HS at the indicated concentrations were then added and mixed well. The tubes were incubated at 37°C for 15 min. The APTT, PT, TT and FIB were assayed with commercially available kits in a coagulation analyzer according to suppliers' protocols, which were based on the coagulation method.

Measurement of coagulation factor activities: A pooled PPP was prepared from the six rabbits and used as a normal control and it was serially diluted with imidazole buffer according to the protocols. The APTT or PT values were determined and plotted against coagulation factor activities, which were based on the dilution ratios, to establish reference curves.

The PPPs isolated from individual rabbits were treated with DMSO, BBR, or HS as earlier described. To determine the activities of F IX, F XI and F XII, the treated PPPs were diluted with imidazole buffer and mixed with an equal volume (50 µL) of the corresponding Coagulation Factor Deficient Plasma and APTT values were determined. To determine the activities of F X, F VII and F II, the treated PPPs were diluted with imidazole buffer, mixed with an equal volume (50 µL) of the corresponding Coagulation Factor Deficient Plasma and PT values were determined. Using the tested APTT or PT values, the activities of the respective coagulation factors were calculated according to the corresponding reference curves and presented as the normal control (%).

Measurement of AT-III activity: The activity of plasma AT-III was assayed using a commercially available kit according to the supplier's protocol, which was based on the chromogenic substrate method. Briefly, the pooled rabbit PPP was serially diluted with the Assay Buffer according to the protocol. After proper reaction, the absorbance at a wavelength of 405 nm was read using a spectrophotometer. A reference curve was established by plotting the absorbance values against the AT-III activities, which were based on the dilution ratios.

After treatment with DMSO, BBR or HS, the PPPs from individual rabbits (25 µL) were diluted with the Assay Buffer (1000 µL) and 200 µL of the diluted PPPs were used for the test. Using the tested absorbance values, the AT-III activities of the samples were calculated from the reference curve and presented as the normal control (%).

***In vivo* experiments of coagulation function and thrombus formation:** Male Wistar rats (212±18.3 g) fed with a regular diet were orally treated with saline, 100 or 200 mg kg^{-1} of BBR for 14 days, respectively. On the last three days of the experiment, another group of rats was administered with HS (60 U kg^{-1}) through tail vein injection. There were 10 rats in each group.

The rats were fasted overnight before the last day of the experiment. One hour after the last drug administration, the rats were anaesthetized by intraperitoneal (i.p.) injection of 3.5% chloral hydrate (Tianjin Nankai Share Compounds Co., Ltd.) at a dose of 10 mL kg^{-1} and blood samples (about 8-9 mL from each rat) were obtained by puncture of the abdominal aorta. Approximately 1 mL of the whole blood was immediately used for the measurement of CT as earlier described and the remaining blood was immediately mixed with 10% sodium citrate (9:1). Then, 1 mL of the anticoagulated blood was used for the isolation of platelet-rich plasma (PRP) by centrifugation at 800 rpm for 10 min and the remaining blood was used to isolate PPP as earlier described.

The platelet concentration of PRP was adjusted to 4×10^8 mL^{-1} with PPP and the platelet aggregation rate was determined within 2 h of isolation. The PPPs were stored at -80°C in aliquots for the subsequent experiments. The ADP (2 µmol L^{-1})-stimulated platelet aggregation were determined using a platelet aggregation analyzer, which was based on the turbidimetric method. The platelet aggregation rate was recorded for 5 min and the maximal aggregation rate (MAR) was presented.

For the determination of plasma APTT/PT/TT/FIB, coagulation factor and AT-III activities, the frozen sample PPPs were rapidly thawed at 37°C and the experiments were performed as earlier described. A pooled PPP (isolated from abdominal aortic blood) was prepared from eight healthy Wistar rats and used as a normal control for the measurement of the activities of coagulation factors and AT-III.

In a parallel experiment on inferior vena cava thrombus formation, male Wistar rats were grouped and treated as earlier described with 16 rats in each group. An extra group of rats (n = 16) was included; these were operated on but left unligated and used for the observation of normal veins. One hour after the last drug administration, the rats were anaesthetized and an incision of about 3 cm was made on the median line of the abdomen after supine immobilization, unhairing and disinfection. The inferior vena cava was isolated carefully and ligated below the left renal vein and the abdomen was then sewed up.

Four hours later[17], the rats were anaesthetized again and sutures were removed, at 2 cm below the site of ligation, the inferior vena cava was clamped. For the eight rats in each group, the thrombi were carefully harvested after opening the veins. The thrombi were placed on pre-weighed clean filter papers and the wet weights were calculated by subtracting the weights of the filter papers. The thrombi (together with filter papers) were then dried at 60°C for 2 h in an oven and the dry weights were calculated by subtracting the weights of the filter papers. For the other eight rats in each group, the inferior vena cava (2 cm in length), together with the thrombi were harvested and immediately fixed in 10% formaldehyde. The samples were subjected to paraffin sectioning (5 µm thickness) and hematoxylin and eosin (H and E) staining, followed by observation and photography under an Olympus light microscope.

***In vivo* experiments in rats fed with a high-fat diet (HFD):** Male Wistar rats, weighing about 207±17.9 g were randomly divided into the following groups: regular diet group, HFD group which was fed with a HFD (D12492, Research Diets, Inc., New Brunswick, NJ, USA) and treated with saline, HFD+BBR 100 mg kg^{-1} group and HFD+BBR 200 mg kg^{-1} group. There were 10 rats in each group and at the same time of HFD-feeding, BBR was orally administered to the rats for 4 weeks.

Body weight and food intake of the rats were recorded twice a week. The rats were fasted overnight before the last day of experiment; 1 h after the last drug administration, approximately 1-1.5 mL of blood was collected by retro-orbital puncture. The serums were isolated and frozen at -20°C for the measurement of biochemical indexes by kits. Homeostasis model assessment-insulin resistance (HOMA-IR) was calculated as described in a previous report[18].

After anesthesia, blood samples (about 8-9 mL from each rat) were obtained from the abdominal aorta for the determination of CTs, platelet aggregation rates, APTT/PT/TT/FIB, coagulation factor activities and AT-III activities as earlier described.

In a parallel experiment, male Wistar rats were grouped (n = 16) and treated as earlier described. At the end of the experiment, thrombus formation in the inferior vena cava was determined by ligation after anesthesia. Eight rats in each group were used for the measurement of thrombus weights, whereas the other eight rats were used for the pathological examination of veins and thrombi as earlier described.

Statistical analysis: For the *in vitro* experiments, the results were expressed as the Mean±SD of six samples in each group. For the *in vivo* experiments, the results were presented as the Mean±SD of 8 or 10 animals in each group. SPSS 13.0 software was used for statistical analysis using one-way ANOVA, followed by multiple comparisons and $p<0.05$ was considered to be statistically significant.

RESULTS

Anticoagulant activity of BBR *in vitro*: As shown in Fig. 1a, compared to DMSO, whole blood CT was prolonged to about 4.73-fold ($p<0.001$) after treatment with HS at a concentration of 40 U mL^{-1}. The BBR prolonged CT in a dose-dependent manner and 2.5 μmol L^{-1} of BBR caused a significant increase in CT ($p<0.05$ vs. DMSO) and when the concentration of BBR reached 10 μmol L^{-1}, CT increased to about 1.84-fold ($p<0.01$ vs. DMSO).

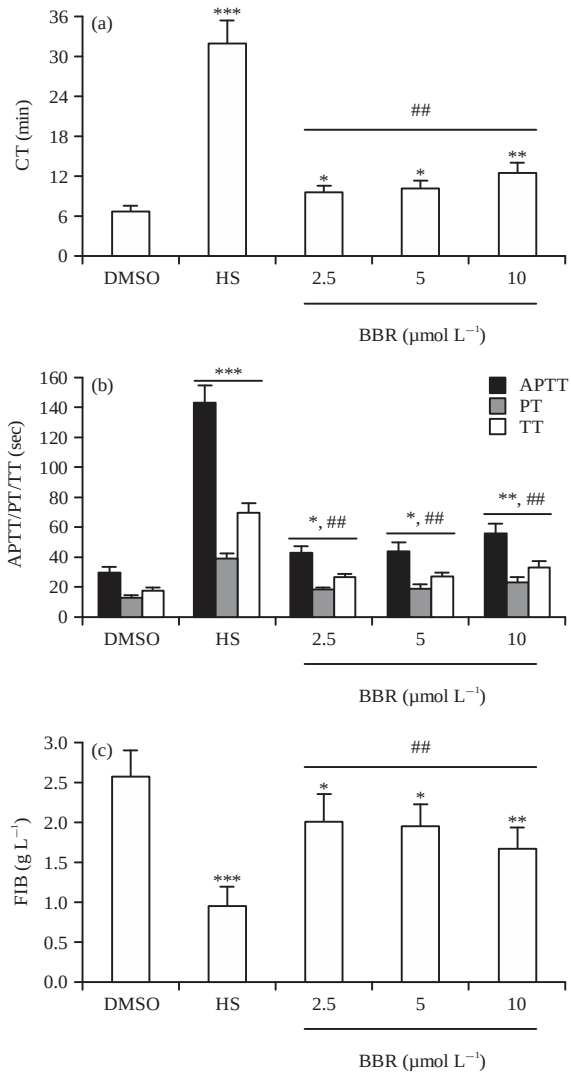

Fig. 1(a-c): Effects of BBR and HS on CT and coagulation parameters *in vitro*. Blood samples were collected from New Zealand rabbits (n = 6) by puncture of auricular vein (a) Whole blood samples were moved to silicified microcentrifuge tubes and treated with BBR or HS (40 U mL^{-1}) as indicated and CTs were recorded, (b, c) Blood samples were anticoagulated with sodium citrate, PPPs were isolated and treated as indicated at 37°C for 15 min. APTT, PT, TT and FIB were measured by using commercial kits

Values are Mean±SD of 6 samples in each group; *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs. that of DMSO, ##$p<0.01$ vs. that of HS

The BBR increased plasma APTT and PT in a dose-dependent manner ($p<0.05$ or $p<0.01$ vs. DMSO) (Fig. 1b). In addition, BBR decreased FIB concentration and significantly prolonged TT in rabbit PPPs ($p<0.05$ or $p<0.01$ vs. DMSO) (Fig. 1b, c).

Table 1: Effects of BBR and HS on the activities of coagulation factors and AT-III *in vitro* (normal control (%))

			BBR (μmol L^{-1})		
Parameters	DMSO	HS (40 U mL^{-1})	2.5	5	10
F IX	96.1±13.5	26.2±3.56***	73.0±9.15*,##	71.1±9.08*,##	61.4±8.11**,##
F XI	99.0±14.6	28.3±4.03***	73.9±8.87*,##	72.8±8.79*,##	57.4±7.08**,##
F XII	102±14.7	21.8±3.37***	74.1±9.45*,##	73.3±9.12*,##	61.2±7.89**,##
F X	104±9.9	27.8±3.91***	75.9±10.2*,##	74.0±9.05*,##	62.3±8.69**,##
F II	101±13.7	25.9±4.08***	72.8±9.58*,##	71.2±9.23*,##	58.8±7.86**,##
AT-III	106±13.1	267±35.6***	138±22.9*,##	142±24.7*,##	176±28.4**,##
F VII	98.7±11.8	61.6±9.02**	72.4±11.2*,#	70.9±10.4*,#	62.2±9.48**

Values are Mean±SD of 6 specimens in each group. *p<0.05, **p<0.01, ***p<0.001 vs. that of DMSO, #p<0.05, ##p<0.01 vs. that of HS. BBR: Berberine, HS: Heparin sodium, AT-III: Antithrombin-III, F: Factor, DMSO: Dimethyl sulphoxide

Table 2: Effects of BBR and HS on the activities of coagulation factors and AT-III in rats fed with a regular diet (normal control (%))

Parameters	Saline	HS (60 U kg^{-1})	BBR (100 mg kg^{-1})	BBR (200 mg kg^{-1})
F IX	101.0±11.5	33.7±4.55***	75.2±9.18*,##	60.7±9.02**,##
F XI	98.3±12.0	33.5±4.09***	71.8±8.79*,##	58.1±7.65**,##
F XII	103.0±13.2	25.6±2.96***	78.2±9.21*,##	61.9±8.73**,##
F X	96.9±13.8	33.0±3.98***	73.7±8.92*,##	61.0±8.79**,##
F II	105.0±14.3	28.6±4.06***	76.7±9.45*,##	62.1±9.05**,##
AT-III	97.6±12.8	236.0±30.8***	124.0±16.0*,##	151.0±19.2**,##
F VII	104.0±13.1	60.3±9.76**	79.0±11.2*,#	61.3±10.5**

Values are mean±SD of 10 rats in each group. *p<0.05, **p<0.01, ***p<0.001 vs. that of saline group, #p<0.05, ##p<0.01 vs. that of HS group. BBR: Berberine, HS: Heparin sodium, AT-III: Antithrombin-III, F: Factor

The administration of HS caused a significant reduction (by about 71.4-78.6%) in the activities of coagulation factors participating in the intrinsic coagulation pathway, which included F IX, F XI and F XII (p<0.001 vs. DMSO) (Table 1). The BBR decreased the activities of F IX, F XI and F XII significantly and dose-dependently; when the concentration of BBR reached 10 μmol L^{-1} in the plasma, their activities decreased by about 36.1-42.0% (p<0.01 vs. DMSO). The BBR also suppressed the activity of F X and F II (p<0.05 or p<0.01 vs. DMSO), which belonged to the common coagulation pathway.

The HS greatly increased plasma AT-III activity to about 2.52-fold (p<0.001 vs. DMSO) (Table 1). The efficacy of BBR on AT-III was dose-dependent; 10 μmol L^{-1} of BBR increased plasma AT-III activity to about 1.66-fold (p<0.01 vs. DMSO).

The BBR also effectively suppressed the activity of F VII (p<0.05 or p<0.01 vs. DMSO), which is essential to the extrinsic coagulation pathway. The suppressing activity of HS on F VII was comparable to that of BBR at a concentration of 10 μmol L^{-1} (Table 1).

Anticoagulant and antithrombotic activities of BBR *in vivo*: As shown in Fig. 2a, after 14 days of oral administration in Wistar rats, 100 or 200 mg kg^{-1} of BBR prolonged whole blood CT to about 1.28 and 1.59-fold, respectively (p<0.05 or p<0.01 vs. saline group). Accordingly, BBR prolonged plasma APTT, PT and TT (Fig. 2b) but decreased FIB (Fig. 2c) dose-dependently and significantly (p<0.05 or p<0.01 vs. saline group).

In accordance with the *in vitro* results, oral administration of BBR had a global suppressing effect on the *in vivo* activities of coagulation factors involved in the intrinsic, common and extrinsic coagulation pathways (p<0.05 or p<0.01 vs. saline group) (Table 2). Furthermore, 100 or 200 mg kg^{-1} of BBR treatment for 14 days increased plasma AT-III activity to about 1.27 and 1.55-fold, respectively (p<0.05 or p<0.01 vs. saline group).

Although HS had a strong anticoagulant activity after tail vein injection, it did not influence the ADP-stimulated platelet aggregation (Fig. 2d). On the contrary, 100 or 200 mg kg^{-1} of BBR suppressed ADP-stimulated platelet aggregation effectively, as indicated by the significant reduction of MAR (by about 27.5 and 38.4%) after BBR treatment (p<0.05 or p<0.01 vs. saline group) (Fig. 2d).

The vascular structure was normal and no thrombus was found in the inferior vena cava of rats left unligated (Fig. 3a, 3b). On the contrary, after 4 h of ligation, thrombi were observed in the inferior vena cava of rats treated with saline, accompanied by subintimal inflammatory cell infiltration. Injection of HS strongly inhibited thrombus formation (p<0.001 vs. saline group). The BBR was also effective in preventing thrombus formation (Fig. 3a, b), although its efficacy was not as strong as HS (p<0.01). The administration of BBR (100 or 200 mg kg^{-1}) resulted in a reduction in thrombus wet weight by about 27.0 and 56.5% and thrombus dry weight by about 27.1 and 55.0%, respectively (p<0.05 or p<0.01 vs. saline group). In addition to thrombosis, the proinflammatory response induced by ligation was also ameliorated by HS or BBR administration (Fig. 3b).

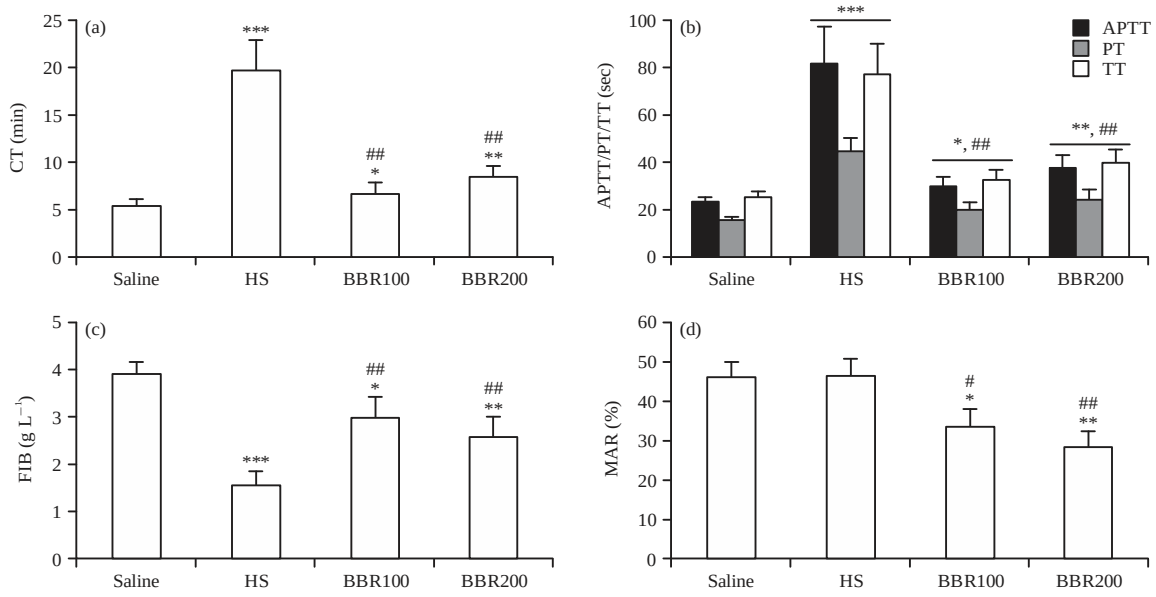

Fig. 2(a-d): Effects of BBR and HS on coagulation function and platelet aggregation in rats fed with a regular diet. Male Wistar rats were orally treated with saline, 100 or 200 mg kg^{-1} of BBR for 14 days, HS (60 U kg^{-1}) was administered through tail vein injection during the last 3 days (a) At the end of experiment, whole blood CT was determined. PPPs were isolated for the measurement of (b) APTT, PT, TT and (c) FIB. Meanwhile, PRPs were isolated for the measurement of (d) ADP-induced platelet aggregation by using a platelet aggregation analyzer

Values are Mean±SD of 10 rats in each group; *p<0.05, **p<0.01, ***p<0.001 vs. that of saline group; #p<0.05, ##p<0.01 vs. that of HS group

Table 3: Effects of BBR on body weight, food intake and metabolic parameters in rats fed with a HFD

		HFD		
Parameters	Regular diet	Saline	BBR (100 mg kg^{-1})	BBR (200 mg kg^{-1})
Body weight (g)	335.00±45.4	421.00±61.3*	352.00±49.8#	340.00±47.6#
Food intake (g day^{-1})	19.80±2.72	17.90±2.68	17.70±2.65	17.60±2.57
CHO (mmol L^{-1})	2.02±0.25	3.59±0.48**	2.76±0.36#	2.22±0.31##
LDL-c (mmol L^{-1})	0.43±0.05	0.93±0.16**	0.68±0.10#	0.53±0.09##
TG (mmol L^{-1})	1.12±0.16	2.17±0.37**	1.56±0.21#	1.21±0.18##
Glucose (mmol L^{-1})	4.58±0.31	8.61±1.21**	6.31±1.05#	4.84±0.87##
Insulin (μU mL^{-1})	12.90±1.63	21.70±3.04**	15.90±1.96#	13.10±1.68##
HOMA-IR	2.63±0.38	8.30±1.51***	4.32±0.67##	2.82±0.42###

Values are mean±SD of 10 rats in each group. *p<0.05, **p<0.01, ***p<0.001 vs. that of regular diet group; #p<0.05, ##p<0.01, ###p<0.001 vs. that of HFD+saline group. BBR: Berberine, HFD: High-fat diet, CHO: Cholesterol, LDL-c: Low-density lipoprotein cholesterol, TG: Triglyceride, HOMA-IR: Homeostasis model assessment-insulin resistance

BBR improves hypercoagulable state and suppresses thrombus formation in rats fed with a HFD: After 4 weeks of HFD-feeding, the rats developed obesity, hyperlipidemia, hyperglycemia, hyperinsulinemia and insulin resistance as compared to the regular diet group (p<0.05, p<0.01 or p<0.001) (Table 3). Blood hypercoagulation, accompanied by metabolic abnormalities was observed in the HFD+saline group, as indicated by the significant shortening of CT (Fig. 4a) and APTT/PT/TT (Fig. 4b), the increase of plasma FIB (Fig. 4c), the significant loss of AT-III activity and the overactivation of coagulation factors (Table 4) (p<0.01 vs. regular diet group). In addition, compared to the regular diet group (p<0.01), an excessive platelet aggregation was observed after HFD-feeding (Fig. 4d).

As a result, thrombosis deteriorated significantly after HFD-feeding in the parallel experiment (Fig. 5a, b). The structure of thrombus became denser (Fig. 5b) and thrombus weight significantly increased (Fig. 5a) (p<0.01) compared to the regular diet group. After ligation of the inferior vena cava, extensive destruction of the intima was observed in the HFD+saline group, which was accompanied by a large amount of inflammatory cell infiltration (Fig. 5b).

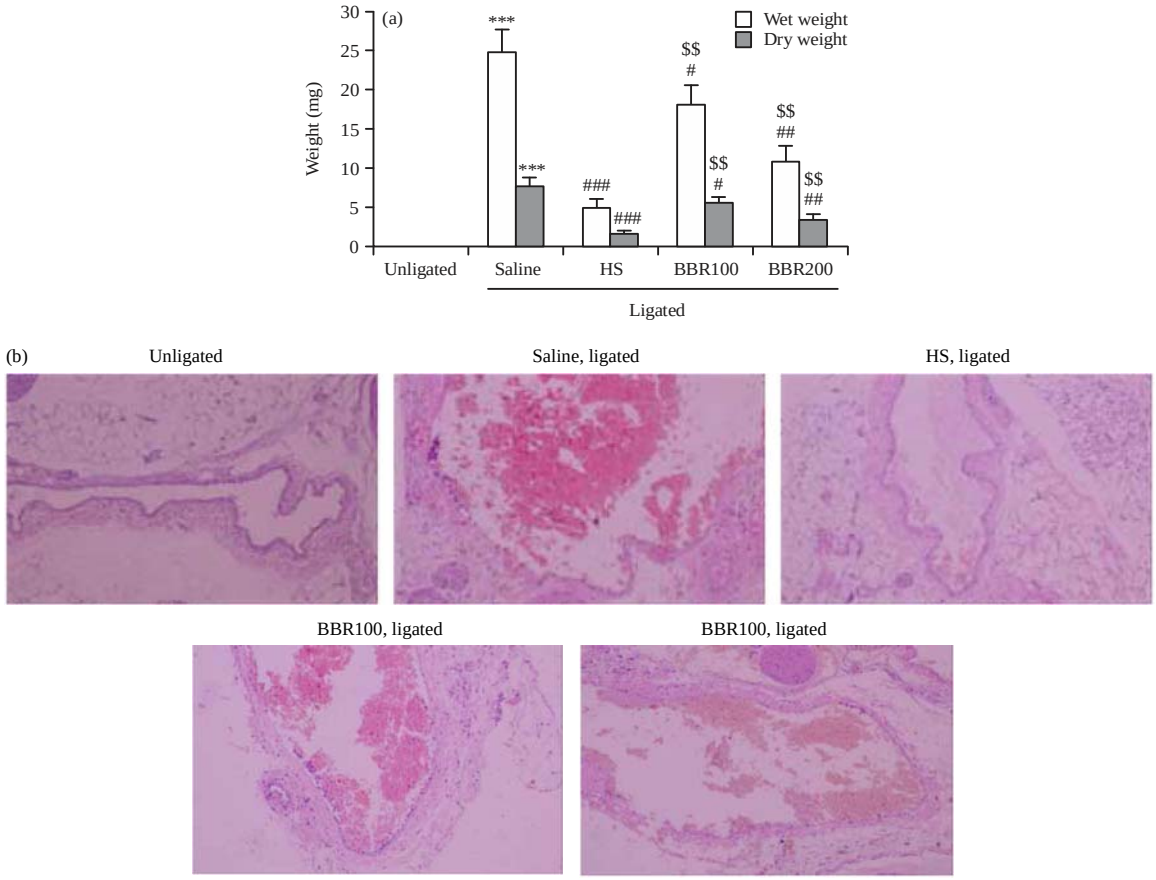

Fig. 3(a-b): Effects of BBR and HS on inferior vena cava thrombus formation in rats fed with a regular diet. Male Wistar rats were grouped (n = 16) and treated as in Fig. 2, except that an extra group (n = 16) was included and left unligated. At the end of the experiment, thrombus formation in the inferior vena cava was induced by ligation (a) For 8 rats in each group, thrombi were harvested 4 h later and the wet and dry weights were measured, (b) For the other 8 rats in each group, the inferior vena cava and thrombi were subjected to paraffin sectioning and H and E staining and typical images were presented (×100)

Values are Mean±SD; ***p<0.001 vs. that of unligated rats; $^{\#}$p<0.05, $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001 vs. that of saline-treated and ligated rats; $^{\$\$}$p<0.01 vs. that of HS-treated and ligated rats

Table 4: Effects of BBR on the activities of coagulation factors and AT-III in rats fed with a HFD (normal control (%))

		HFD		
Parameters	Regular diet	Saline	BBR (100 mg kg^{-1})	BBR (200 mg kg^{-1})
F IX	104.0±13.8	179.0±24.8**	132.0±19.1$^{\#}$	106±14.6$^{\#\#}$
F XI	107.0±12.9	183.0±23.7**	136.0±20.4$^{\#}$	108±17.0$^{\#\#}$
F XII	99.4±12.1	178.0±32.1**	134.0±21.2$^{\#}$	102±17.3$^{\#\#}$
F X	101.0±13.2	180.0±25.9**	137.0±18.8$^{\#}$	104±16.1$^{\#\#}$
F II	98.7±13.0	173.0±24.3**	128.0±18.0$^{\#}$	103±15.9$^{\#\#}$
AT-III	102.0±13.9	58.2±8.16**	73.2±10.3$^{\#}$	96.9±15.0$^{\#\#}$
F VII	99.2±12.6	176.0±23.9**	130.0±17.7$^{\#}$	101±16.2$^{\#\#}$

Values are Mean±SD of 10 rats in each group. **p<0.01 vs. that of regular diet group; $^{\#}$p<0.05, $^{\#\#}$p<0.01 vs. that of HFD+saline group. BBR: Berberine, AT-III: Antithrombin-III, HFD: High-fat diet, F: Factor

The BBR did not influence the amount of food intake (Table 3), but suppressed excessive body weight gain, lowered blood lipids, glucose, insulin levels and ameliorated insulin resistance effectively (p<0.05, p<0.01 or p<0.001 vs. HFD+saline group).

In addition to the metabolism-modulating effects, BBR overcame blood hypercoagulation and abnormal platelet aggregation, which were induced by HFD-feeding. As shown in Fig. 4a-d and Table 4, co-administration of BBR with HFD prolonged the shortened CT and APTT/PT/TT, decreased

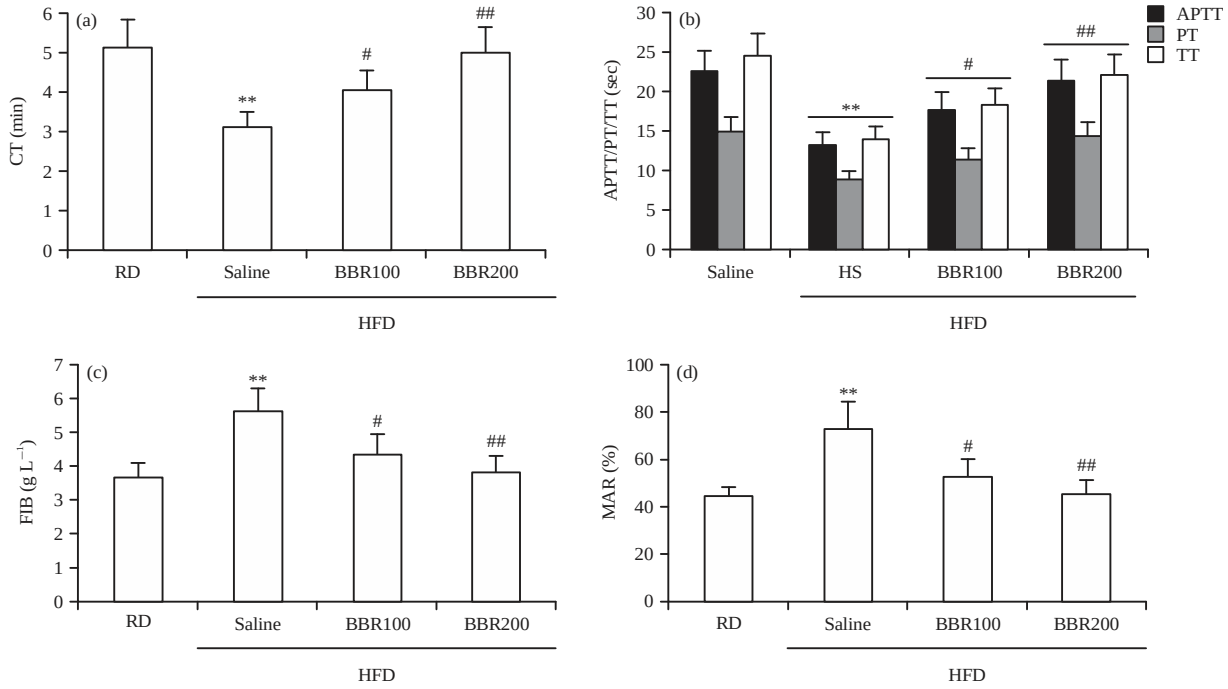

Fig. 4(a-d): Effects of BBR on coagulation function and platelet aggregation in rats fed with a HFD. Male Wistar rats were fed with a regular diet (RD) or a HFD, grouped and treated as indicated (a) After 4 weeks of treatment, whole blood CT was determined, (b) PPPs were isolated for the measurement of APTT, PT, TT, (c) FIB. Meanwhile, PRPs were isolated for the measurement of (d) ADP-induced platelet aggregation

Values are Mean±SD of 10 rats in each group; **p<0.01 vs. that of RD group; #p<0.05, ##p<0.01 vs. that of HFD+saline group

plasma FIB concentration, restored compromised AT-III activity, suppressed over-activated coagulation factors and blocked the excessive aggregation of platelets dose-dependently and significantly ($p<0.05$ or $p<0.01$ vs. HFD+saline group). Furthermore, the enhanced thrombus formation induced by HFD-feeding was inhibited by BBR administration (Fig. 5a, b). When the dose of BBR reached 200 mg kg^{-1}, the wet and dry weights of inferior vena cava thrombi almost returned to baseline levels ($p<0.01$ vs. HFD+saline group). In addition, BBR restored the vascular structure and effectively suppressed proinflammatory responses.

DISCUSSION

The present study reports that the natural product BBR has anticoagulant activity, which is also responsible for its antithrombotic effect in addition to the anti-platelet activity.

Currently, clinical commonly used antithrombotic drugs include thrombolytic drugs, anticoagulants such as heparin, low molecular weight heparin (LMWH) and oral F Xa inhibitors, as well as anti-platelet drugs such as aspirin, thienopyridine derivatives and GPIIb/IIIa antagonists[19-21]. However, the majority of the above drugs have side effects such as hemorrhagic tendency or gastrointestinal mucosal injury.

The BBR has been used in clinic for decades and no side effect of hemorrhagic tendency has been reported. This may be due to the following reasons: (1) As demonstrated in the present study, the anticoagulant activity of BBR is relatively moderate and not as strong as heparin, (2) When used to treat infectious diseases such as diarrhea, the dose of BBR is relatively low and the duration is short and (3) When used in clinical studies to treat metabolic diseases, the patients often have a hypercoagulable state[22-25].

Results of the present study showed that BBR suppressed multiple coagulation factors in the blood coagulation cascade. The coagulation factors of the intrinsic and common coagulation pathways were the targets of AT-III, a serine proteinase inhibitor[2,26,27] and BBR had a stimulating effect on its activity. Heparin could interact with AT-III directly and enhance its activity through conformational change[28]. However, the mechanism used by BBR to stimulate AT-III remains unclear and needs further investigation. In a recent report, BBR was shown to have a direct inhibitory effect on thrombin (F IIa) in an enzymatic assay[29], which was in agreement with this result. The above findings indicated that

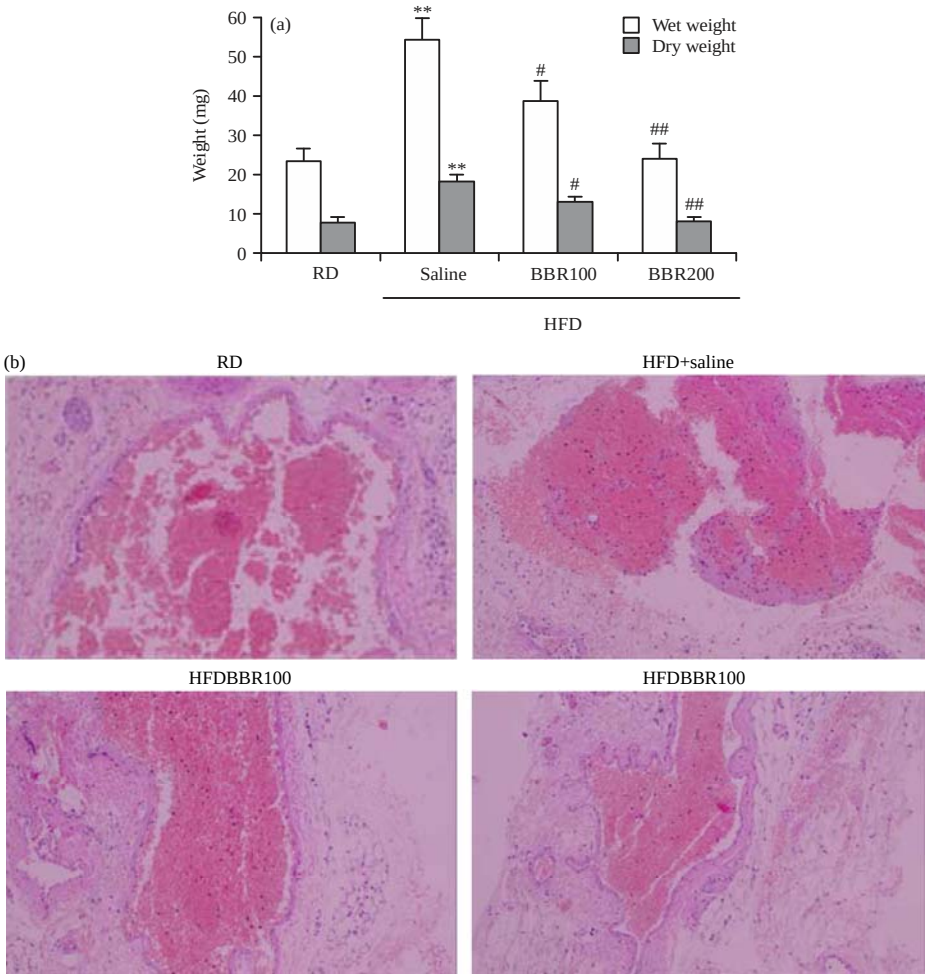

Fig. 5(a-b): Effects of BBR on inferior vena cava thrombus formation in rats fed with a HFD. Male Wistar rats fed with a regular diet (RD) or a HFD were grouped (n = 16) and treated as in Fig. 4. At the end of the experiment, thrombus formation in the inferior vena cava was induced by ligation for 4 h (a) The Wet and dry weights of thrombi were measured in 8 rats in each group and (b) For the other 8 rats in each group, the inferior vena cava and thrombi were subjected to pathological examinations by H and E staining and typical images were presented (×100)

Values are Mean±SD; **p<0.01 vs. that of RD group; #p<0.05, ##p<0.01 vs. that of HFD+saline group

BBR possibly influences the functions of coagulation factors through multiple mechanisms, which may be direct or indirect.

In addition to the intrinsic and common coagulation pathways, BBR also inhibited F VII, which belongs to the extrinsic coagulation pathway. Tissue factor (TF) is essential for the activation of F VII[1,30]. In accordance with our results, previous studies by another group have shown that BBR effectively suppressed lipopolysaccharide (LPS) and tumor necrosis factor-α (TNF-α)-induced expression and activation of TF in monocytes[16,31]. These findings also indicated that BBR may influence coagulation function through multiple targets.

In the present study, BBR suppressed coagulation factors of the intrinsic and extrinsic coagulation pathways to similar extents and in accordance, it prolonged APTT and PT similarly. For heparin, although it had a stronger inhibitory activity on the intrinsic coagulation factors than F VII, it also dramatically prolonged PT. This might be due to its strong inhibitory effects on F X and F II, which cause an increase in PT[32].

Dysregulation of coagulation and fibrinolysis induce thrombus formation. It is possible that BBR lowers plasma FIB by promoting fibrinolysis as a previous study has shown that BBR decreases the serum level of plasminogen activator inhibitor-1 (PAI-1), a major suppressor of tissue-type plasminogen activator (t-PA) in diabetic rats[33]. In addition, BBR also enhances the thrombolytic effects of urokinase and streptokinase *in vitro*[34].

Results of the present study and other reports[1,10-13,29] demonstrate that BBR significantly inhibits platelet aggregation, which may be induced by ADP, thrombin, collagen or arachidonic acid (AA). The effects of heparin on platelet are apparently dependent on different conditions. For example, when used in the clinic, heparin may induce autoimmune responses in some patients, which may cause thrombocytopenia, platelet activation and thrombus formation[35]. Heparin was also proved to inhibit thrombin-induced platelet activation and aggregation[36,37] but had no effect on ADP-induced platelet aggregation[36,37], which agrees with the results of the present study.

The present study showed that BBR effectively suppresses thrombus formation in rats and the results are consistent with previous reports. For example, in a previous report, ICR mice were orally treated with BBR at a single dose of 10 or 100 mg kg^{-1} and thrombus formation in the inferior vena cava was significantly inhibited by BBR[16]. In mice treated with AA through tail vein injection, oral administration of BBR was shown to inhibit thrombus formation in the heart and lungs[15]. In another study, i.p. injection of BBR at 20 mg kg^{-1} was shown to suppress thrombosis induced by arterio-venous shunt in a rat model of middle cerebral artery occlusion (MACO)[10].

In addition to the anti-platelet and anticoagulant activities, BBR was shown to improve hemodynamics[38]. For example, in a clinical study, BBR was used to treat patients combined with type 2 diabetes mellitus (T2DM) and nonalcoholic fatty liver disease (NAFLD)[38]. The authors found that BBR effectively improved the hemodynamic parameters of the patients, as indicated by the significant reduction of blood viscosity and erythrocyte sedimentation rate[38]. In addition, BBR was shown to protect endothelial cells from injury[39], which coincided with the vascular protection effect observed in the present study. The above beneficial effects of BBR might also contribute to its antithrombotic activity.

The most meaningful finding of the current study is that BBR prevents hypercoagulable state and thrombus formation in animals with metabolic disorders. In the clinic, patients with metabolic diseases such as T2DM, hyperlipidemia, obesity and NAFLD often have blood hypercoagulation and are prone to thrombosis[22-25]. Results of the present study indicated that BBR is expected to improve hypercoagulability and prevent thrombus formation when it is used to treat the above metabolic diseases[6,40]. In the future, clinical studies should be conducted to evaluate the anticoagulant and antithrombotic activities of BBR in order to validate its efficacy in humans.

CONCLUSION

The BBR has anticoagulant activity and suppresses inferior vena cava thrombus formation in rats. The BBR stimulates the activity of AT-III and has suppressing effects on a series of coagulation factors. In addition, BBR overcomes blood hypercoagulation induced by HFD-feeding. The findings of the present study suggested that BBR may have beneficial effects on hypercoagulability and related thrombosis when used for the treatment of metabolic diseases in the future.

SIGNIFICANCE STATEMENT

The current study reports for the first time that natural product BBR has anticoagulant activity and is effective for preventing blood hypercoagulation when used to treat metabolic disorders. Considering the serious outcomes of thrombosis and the promising future of BBR in clinics, this study is expected to bring benefits to patients with metabolic diseases, which has a large population in modern society.

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