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Bioassay-directed fractionation of a blood coagulation factor Xa inhibitor, betulinic acid from *Lycopus lucidus*

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Abstract: Thrombosis is a major cause of morbidity and mortality worldwide and plays a pivotal role in the pathogenesis of several cardiovascular disorders, including acute coronary syndrome, unstable angina, myocardial infarction, sudden cardiac death, peripheral arterial occlusion, ischemic stroke, deep-vein thrombosis, and pulmonary embolism. Anticoagulants, antiplatelet agents, and fibrinolytics can reduce the risks of these clinical events. Especially, the blood coagulation factor Xa (FXa) inhibitor is a proven anticoagulant. Promoting blood circulation, using traditional Chinese medicine (TCM), for the treatment of these diseases has been safely used for thousands of years in clinical practice. Therefore, highly safe and effective anticoagulant ingredients, including FXa inhibitors, could be found in TCM for activating the blood circulation. One FXa inhibitor, a pentacyclic triterpene (compound **1**, betulinic acid) characterized by IR, MS and NMR analyses, was isolated from the ethyl acetate fraction of *Lycopus lucidus* by bioassay-directed fractionation. Compound **1** exhibited an inhibitory effect on FXa with IC_{50} 25.05 μmol/L and reduced the thrombus weight in an animal model at 25-100 mg/kg. These results indicate that betulinic acid could be the potential for anticoagulant therapy.

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Keywords: Natural anticoagulat; Pentacyclic triterpene; Thrombosis; *Lycopi Herba*.

Abbreviations

FXa blood coagulation factor Xa
VKA vitamin K antagonists
DOAC direct oral anticoagulants
VTE venous thromboembolism
EtOAc ethyl acetate
n-BuOH normal butanol
DMSO dimethyl sulfoxide
IVC inferior vena cava

1 Introduction

Abnormal blood clotting is responsible for several thromboembolic diseases, including myocardial infarction, unstable angina, deep vein thrombosis, pulmonary embolism, and ischemic stroke. Anticoagulants are used for the prevention and treatment of venous and arterial thromboembolic disorders. Vitamin K antagonists (VKAs) have been available for several years. Moreover, until recently, they were the only therapeutic option available as the oral anticoagulation treatment. Direct administration of oral anticoagulants (DOAC) became a critical alternative to VKA for the prevention and treatment of venous thromboembolism (VTE) and the prevention of systemic embolism and ischemic stroke. DOACs are often termed as novel oral anticoagulants or target-specific oral anticoagulants. DOAC and VKA exert different mechanisms of action. VKAs reduce the hepatic synthesis of vitamin K-dependent coagulation factors, such as factor II, VII, IX, and X, whereas DOACs are direct inhibitors of the activated factor II (dabigatran) or activated factor

X (Rivaroxaban, Apixaban, Edoxaban) [1]. Factor X has been known to play a pivotal role in hemostasis [2], and FXa is crucial in the blood coagulation pathway acting as a catalyst in the production of thrombin, which leads to clot formation and wound closure [3]. The activity of Factor X can be markedly suppressed without affecting hemostasis, as the existing thrombin is not affected. An ideal anticoagulant would prevent thrombosis without inducing systemic hypocoagulation, thereby avoiding the complications, such as unintended bleeding. Therefore, an FXa inhibitor potentially exhibits the properties of an anticoagulant [4] and has emerged as an attractive target for new anticoagulants.

Traditional Chinese Medicines (TCM) have been used in China for a prolonged period in promoting blood circulation and removing blood stasis. Thus, safe and effective anticoagulant ingredients might be found in TCM. Previously, we found a small, natural and direct inhibitor against FXa from the root of *Glycyrrhiza glabra* L. [5]. In the present study, we focused on another TCM for removing blood stasis, which was screened out from 18 types of TCM extracts using enzyme-based assay [6]. *Lycopus lucidus* Turcz.var. *hirtus* Regel (Lamiaceae, Zelan in Chinese), an herbaceous perennial plant, the aerial part is commonly used for improving blood rheology in clinical practice, as recorded in the chinese famous original herbal classic "ShenNongBenCaoJin" for thousands of years ago [7~9]. Pharmacological research showed that the water decoction of *L. lucidus* reduced the blood viscosity, fibrinogen content, and hematocrit, shortened the erythrocyte electrophoresis time, and inhibited the erythrocyte and platelet aggregation and thrombosis formation [7~9]. It also resisted coagulation and thrombosis, thereby improving the microcirculation and regulation of metabolism of blood lipids [7,9]. Therefore, to investigate the mechanisms of the plant activity, bioassay-directed fractionation was undertaken using blood coagulation FXa activity *in vitro* as a reference. This study led to the isolation of a pentacyclic triterpene, betulinic acid, as the active component. Betulinic acid is also found in various plants (*Ziziphus jujuba* Mill.var. *spinosa*, *Syzygium jambos* (L.) Alston, and *Betula platyphylla* Suk). Its anticoagulant activity has been reported in other study [10], and was verified in our study. Furthermore, our study also revealed that one of the mechanisms of its anticoagulation activity was inhibition of FXa.

2 Materials and methods

2.1 Chemicals and reagents

Purified FXa (HYPHEN BioMed, France, Batch # 090825C-PK:1), chromogenic substrate BIOPHEN CS-11(22) (HYPHEN BioMed, Batch # 93803-1-PK:3), and Rivaroxaban (Bayer AG, Germany, Batch # BXFD8J1) were purchased.

Column chromatography and thin layer silica gel (Qingdao Marine Chemical Factory, China), Sephadex LH-20 gel (Pharmacia Biotech Products, USA), and silica gel GF254 thin layer of prefabricated plates (Yantai chemical industrial Research Institute, China) were utilized.

Petroleum ether, ethyl acetate, normal butanol, methanol, ethanol, chloroform, acetone, and other organic reagents were of analytical grade.

2.2 Plant material

Chinese herbal pieces from aerial part of *Lycopus lucidus* was purchased from Anhui Hujiao Pharmaceutical Co., Ltd.(batch #20101128). The plant was first identified by a senior research scientist, Shihui Qian of Jiangsu Province Academy of Traditional Chinese Medicine, and voucher specimens deposited in the Chemistry Laboratory of Jiangsu Province Academy of Traditional Chinese Medicine (No.20110614).

2.3 Bioassay-directed extraction and isolation by assessment of the inhibitory effect on FXa in vitro

Pieces of *Lycopus lucidus* aerial part (5 kg) were extracted three times with 85% ethanol (3×50 L) under reflux for 3 h each time. The extract was evaporated under reduced pressure. The residue (830 g) was suspended in H_2O (3 L) and partitioned sequentially with an equivalent volume of ethyl acetate (EtOAc, 118 g) and normal butanol (*n*-BuOH, 180 g). Subsequently, 1 g of each fraction was solubilized in dimethyl sulfoxide (DMSO) to obtain the concentration of 15 mg/mL for further experiments.

The assay of FXa activity was slightly modified from the previously reported method [11,12]. The reaction system included 20 μL of the phosphate-buffered saline (PBS; pH=8.34, containing 0.3 M NaCl, 0.065 M Na_2HPO_4, and 0.00167 M KH_2PO_4), 20 μL of FXa solution (2.5 μg/mL), 0.2 μL of sample solution (15 mg/mL), and 20 μL of the substrate solution (2.5 mg/mL) was added after

preincubating it for 30 mins at 37 °C. DMSO was added to the blank control group instead of the sample solution. The absorbance was measured at 405 nm at an interval of 0.5 min for a total of 5 min after the start of the reaction. The regression curve of the absorbance (OD) was plotted against time (T) using the continuous rate method. The slope of the regression curve represented the average speed of action, the activity of the enzyme, and the effect of the sample on the enzyme as compared to the slope of the blank control group. The inhibitory rate of the sample (I%) was calculated as follows:

$$I\% = (V_0 - V_i) / V_0 \times 100\%$$

Where V_0 is the slope of the regression curve to the blank control group; V_i is the slope of the regression curve to the sample group.

According to the results of assessment, the EtOAc soluble part (110 g) was further separated by silica gel column chromatography (1000g,100×12cm) eluting with a gradient of $CHCl_3$/MeOH (100:1, 50:1, 25:1, 10:1, 5:1, v/v) to obtain seven fractions ZL1-7. The ZL5 (6.7 g) was subjected to silica gel column chromatography (106g, 40×3cm) eluting with a gradient of petroleum ether /EtOAc (20:1, 15:1, 10:1, 5:1, 3:1, v/v) to obtain five fractions ZL5.1-5.5. The ZL5.3 was subjected to Sephadex LH-20 column chromatography eluting with MeOH/H_2O 80:20 to yield the active compound (compound **1,** 68.7 mg). These fractions resulted in assay inhibitory effect on FXa. The extraction and isolation process of *Lycopus lucidus* is shown in Figure 1.

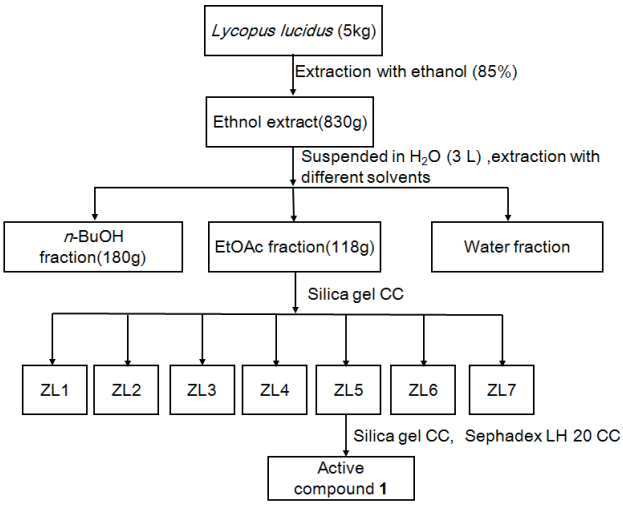

Figure 1: Extraction and isolation process of *Lycopus lucidus*.

2.4 Assessment of half maximal inhibitory concentration (IC_{50})

Compound **1** and Rivaroxaban were solubilized in DMSO, respectively, to obtain different concentrations for assessing the inhibitory effect on FXa at each concentration. The conditions of the reaction and the method of assessment were the same as that for the inhibitory effect on FXa *in vitro*. Consequently, the IC_{50} was calculated by establishing the regression curve of inhibition rate of the sample vs. the concentration.

2.5 Bleeding time test and inferior vena cava (IVC) thrombosis experiments

The experiments were performed as reported previously [5]. A total of 36 male Sprague–Dawley(SD) rats (220±20 g) were purchased from DongChuang Laboratory Animal Service Department (Changsha, China) and acclimatized for 3 days with free access to food and water. All rats were randomly divided into 6 groups (n=6) and fasted overnight before dosing; however, free access to water was allowed. For the *p.o.* administration, betulinic acid was dissolved in a mixture containing 6% PEG400, 9.8% (v/v), and 4.4% (v/v) ethanol. Different groups of rats were orally administered 20, 50, 75, 100 mg/kg betulinic acid and 8.6 mg/kg Rivaroxaban; the control group received blank mixture solvent. After 90 min, the animals were anesthetized with chloral hydrate (*i.p.* 350 mg/kg), a 2 mm tail portion was cut and placed in physiological saline, and the bleeding time recorded. After normally bred for 3 days, the drugs were administered orally to the rats. Then, the animals were anesthetized, the abdomen opened, IVC isolated, and two surgical sutures placed under it. Subsequently, thromboplastin (0.5 mg/kg) was injected into the femoral vein, and after 15 s, the IVC was subjected to ligature by surgical sutures (segment length 12mm). 20 min later, the thrombosis in the IVC segment was carefully dissected and weighted after drying in the oven at 60°C.

2.6 Data and statistical analysis

All experiments *in vitro* were performed at least in triplicate, and the results were expressed as mean±SD. The results of animal experiments expressed as mean±SEM. Statistical analysis was performed using SPSS 17.0 software. $P < 0.05$ was considered statistically significant.

Ethical approval: All rat experiments were performed in accordance with the Institutional Animal Care and Use Committee at the Hainan Medical University (Haikou,

China), as well as the Guidance for Ethical Treatment of Laboratory Animals (The Ministry of Science and Technology of China, 2006).

3 Results

3.1 Effects of extract and fractions on the activities of FXa

The inhibitory effects of Ethanol extract, EtOAc fraction, *n*-BuOH fraction and Water fraction of *L. lucidus* on FXa (Table 1). The results showed that the ethanol extract of *L. lucidus* exerted an inhibitory effect against FXa. Moreover, the EtOAc fraction exhibited a maximal inhibitory effect in the other three extractive fractions. The dose-effect relationship of EtOAc fraction was studied. The inhibitory activity of six different concentrations (1, 3, 6, 12.5, 15, and 25 mg/mL) EtOAc fraction on FXa were as follows: 6.73±4.81%, 46.93±9.92%, 59.01±4.08%, 72.67±3.29%, 74.45±3.48%, and 74.46±2.81% (Figure 2) which increased with increasing concentration. On the other hand, the trend of enhancement declined and saturated at a high concentration, thereby indicating that EtOAc fraction contained direct inhibitors of FXa. The EtOAc fraction was further subjected to column chromatography and obtained fractions ZL1-7, ZL5.1-5.5, compound **1** (betulinic acid). In Table 2, indicated that compound **1** is the active compound to inhibit FXa.

Table 1: Inhibitory effects of different fractions of ethanol extract from *Lycopus lucidus* on FXa.

Fraction	Concentration (mg/mL)	Inhibition rate (%)
Ethanol extract	15	92.71±5.92
EtOAc fraction	15	74.45±3.48
n-BuOH fraction	15	44.54±4.58
Water fraction	15	34.57±2.73

Note: Inhibition rate was expressed as mean±SD, n=5. The positive control was Rivaroxaban with the inhibition rate of 99.16% at the concentration of 50 μg/mL.

Table 2: Inhibitory effects of different fractions from EtOAc fraction on FXa.

Fraction	Concentration (mg/mL)	Inhibition rate (%)
ZL1	15	17.03±2.11
ZL2	15	10.10±1.09
ZL3	15	52.08±5.22
ZL4	15	50.10±4.19
ZL5	15	86.12±2.99
ZL6	15	51.48±1.01
ZL7	15	36.44±1.25
ZL5.1	15	22.19±1.54
ZL5.2	15	50.78±2.31
ZL5.3	15	89.92±4.62
ZL5.4	15	56.98±4.31
ZL5.5	15	40.23±2.57
Compound **1**	15	85.89±4.10

Note: Inhibition rate was expressed as mean±SD, *n*=3. The positive control was Rivaroxaban with the inhibition rate of 99.16% at the concentration of 50 μg/mL.

3.2 Identification of the structure of the purified compound

The compound **1** (betulinic acid) was obtained as white crystals. UV (MeOH) λ_{max}: 206.8nm. Optical rotation: [α] D+8°. The IR spectrum showed absorption bands for OH (3441 cm^{-1}), associated hydroxyl (2500-3080cm^{-1}), and COOH (1710–1735 cm^{-1}) groups. ESI-MS m/z: 457 $[M+H]^+$. ^{1}H-NMR (DMSO-*d6*, 300 MHz) δ ppm: 4.68 (1H, s, 29-H), 4.56 (1H, s, 29-H), 1.64 (3H, brs, 30-H), 0.65, 0.76, 0.87, 0.93 (15H, s, Me×5). ^{13}C-NMR (DMSO-d_6, 75 MHz) δppm: 37.5 (C-1), 27.1 (C-2), 76.7 (C-3), 38.2 (C-4), 54.8 (C-5), 18.9 (C-6), 33.9 (C-7), 38.7 (C-8), 49.9 (C-9), 36.7 (C-10), 20.4 (C-11), 25.0 (C-12), 38.4 (C-13), 42.0 (C-14), 29.1 (C-15), 31.7 (C-16), 55.4 (C-17), 48.5 (C-18), 46.6 (C-19), 150.3 (C-20), 30.1 (C-21), 36.3 (C-22), 28.0 (C-23), 15.7 (C-24), 15.7 (C-25), 15.9 (C-26), 14.3 (C-27), 177.9 (C-28), 109.5 (C-29), 17.9 (C-30). The detailed analysis NMR and MS data and comparison with the reference data [13,14] indicated that the compound was betulinic acid. The chemical structure of the compound **1** is shown in Figure 3.

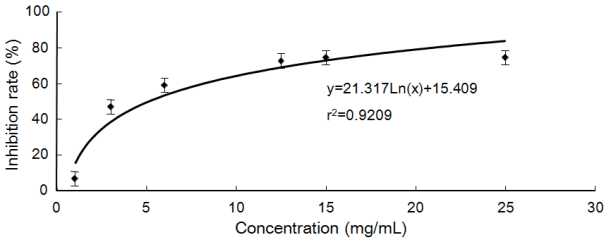

Figure 2: Inhibition curves of different concentrations of EtOAc fraction to FXa.

Table 3: IC_{50} of betulinic acid on FXa.

Compound	Concentration (mg/mL)	Final Concentration (μmol/L)	Inhibition rate (%)	IC_{50} (μmol/L)
Betulinic acid (**1**)	1	7.29	12.52±5.66	25.05
	2.5	18.21	38.51±7.73	
	5	36.43	65.59±4.10	
	10	72.86	86.98±2.72	
	15	109.28	85.89±4.95	
	20	145.71	92.52±3.04	
Rivaroxaban	5×10^{-4}	3.8×10^{-3}	24.90±6.86	1.04×10^{-2}
	1×10^{-3}	7.6×10^{-3}	42.30±9.43	
	2×10^{-3}	15.2×10^{-3}	56.91±4.04	
	3×10^{-3}	22.7×10^{-3}	73.58±7.91	
	4×10^{-3}	30.5×10^{-3}	76.10±9.29	
	5×10^{-3}	38.1×10^{-3}	81.00±6.54	

Note: Inhibition rate was expressed as mean±]SD, *n*=5.

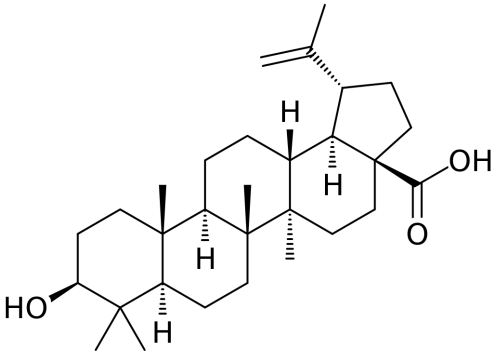

Figure 3: Chemical structure of betulinic acid (compound **1**) isolated from *Lycopus lucidus*.

3.3 Effects of betulinic acid on the activities of FXa

The inhibitory effect of betulinic acid on FXa was measured at the concentration of 15 mg/mL in DMSO. The inhibitory activity was 85.89±4.95% (n=5, P < 0.01). Thus, betulinic acid is a direct inhibitor of blood FXa. The IC_{50} of betulinic acid against FXa was measured in the presence of rivaroxaban as a positive control (Table 3). The IC_{50} of betulinic acid and Rivaroxaban were found to be 25.05 μmol/L and 1.04×10^{-2} μmol/L, respectively.

3.4 Effects of betulinic acid on the bleeding time and thrombosis of IVC

Bleeding in rats was induced by tail trisection. The thrombus model was prepared by intravenous injection of thromboplastin (0.5 mg/kg) and ligation of the IVC. As shown in Figure 4A, betulinic acid did not have a significant influence on bleeding time at a concentration of 25–100 mg/kg. As shown in Figure 4B, betulinic acid can significantly reduce the weight of thrombosis at 25–100 mg/kg.

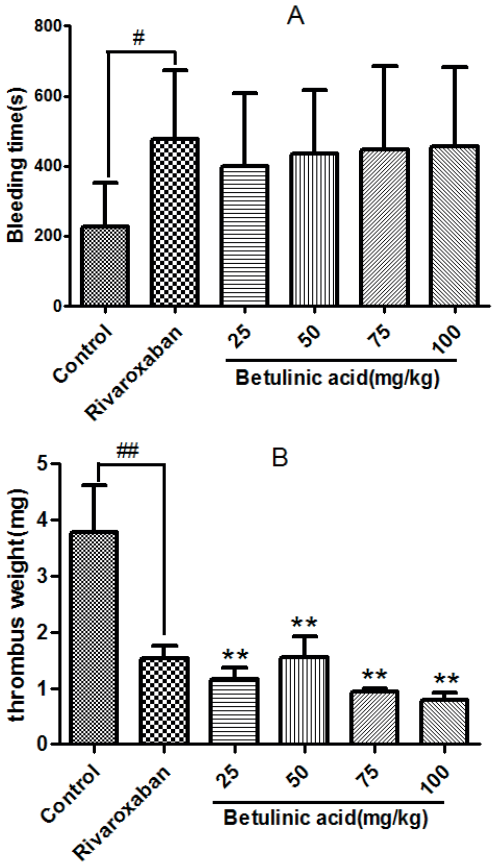

Figure 4: Effects of betulinic acid on bleeding time (A) and thrombosis of IVC (B).Values are mean±SEM (*n*=6), compared with control group ** P< 0.01, # P< 0.05.

4 Discussion

We found that betulinic acid has direct inhibitory effect on FXa, this discovery which may partially revealed to be material basis and mechanism of anticoagulant activity of *L. lucidus*. Although the inhibitory effect of betulinic acid was significantly weaker than control compound rivaroxaban, it's inhibitory effect was similar comparing with oxazolidinones (IC_{50}=20μmol/L), the lead compound of rivaroxaban [15]. This indicated, possible development of more potent compounds from natural product betulinic acid by chemical structural modification. Various biological properties of betulinic acid have been reported in other studies, including anti-tumor anti-inflammatory, and antiviral effects [16,17]. In the present study, we found that betulinic acid inhibited FXa *in vitro* directly and reduced the thrombus weight in an animal model; however, the bleeding times were not significantly affected. These characteristics indicated that betulinic acid might be used as a relatively safe anticoagulant with favorable efficacy/bleeding ratio. We, also previously, reported that glycyrrhetinic acid directly inhibited FXa [5]. Together, these studies indicated the presence of natural inhibitors of FXa in medicinal plants. These results proved that medicinal plants contain anticoagulant ingredients. However, when using anticoagulants clinically, patients should not use these plants at the same time to avoid the risk of bleeding.

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Conflict of interests: The authors declare no conflict of interest.

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