JOURNAL OF FOOD AND DRUG ANALYSIS 25 (2017) 837–844

Available online at www.sciencedirect.com

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journal homepage: www.jfda-online.com

Original Article

Physicochemical properties and anticoagulant activity of polyphenols derived from *Lachnum singerianum*

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ARTICLE INFO

Article history:
Received 22 March 2016
Received in revised form
11 August 2016
Accepted 31 August 2016
Available online 11 November 2016

Keywords:
anticoagulant activity
Lachnum singerianum
polyphenols

ABSTRACT

In this study, polyphenols (LSP) were obtained from the fermentation broth of *Lachnum singerianum*. Two fractions were isolated by Sephadex LH-20 chromatographic column, and the primary fraction (LSP-1) was collected. The comprehensive physicochemical properties of phenolic acids and polyhydroxy phenolic compounds of LSP-1 were determined by UV-visible spectroscopy, Fourier transform infrared spectroscopy, and gas chromatography–mass spectrometry. Results of anticoagulant activity assay *in vitro* showed that LSP-1 could lengthen prothrombin time, activated partial thromboplastin time, and thrombin time of mouse plasma. In addition, anticoagulant activity results *in vivo* showed that high dose of LSP-1 could significantly prolong bleeding time, coagulation time, prothrombin time, activated partial thromboplastin time, and thrombin time of hypercoagulable mice induced by adrenaline, reduce the content of fibrinogen and enhance antithrombin III activity. All results indicated that the LSP-1 could serve well as an anticoagulant, and might be used as a potential natural drug candidate for thrombosis.

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1. Introduction

Prevention and treatment of thrombotic diseases has become one of the demanding tasks in medicine as thrombosis becomes the main cause of acute myocardial infarction, cerebral infarction, and other cardiovascular diseases [1].

Formation of thrombus is mainly attributed to the damage of the cardiovascular wall, changes of blood state, and physicochemical properties [2,3]. Generally, the coagulation and anticoagulation systems are maintained in dynamic equilibrium in organisms. Inhibition of blood coagulation can be used as an important means to prevent formation of thrombosis [4,5]. Coagulation process is complex in the human body, and the key step for coagulation formation is to transform fibrinogen into fibrin, which is catalyzed by thrombin [6].

Polyphenols are widely distributed in fruits, vegetables, nuts, seeds, flowers, and saprophytic fungi [7,8]. Epidemiological studies have indicated that polyphenols could prevent the occurrence of cardiovascular diseases and maintain the

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http://dx.doi.org/10.1016/j.jfda.2016.08.011

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integrality of blood vessels [9–12]. Curcumin, one of the polyphenolic compounds, can significantly prolong activated partial thromboplastin time (APTT) and prothrombin time (PT), and suppress activity of thrombin and the production of factor Xa. Therefore, curcumin can inhibit blood coagulation and formation of thrombus by endogenous and exogenous pathway [5]. Flavonoids in purple grape juice could inhibit platelet aggregation, increase endogenous platelet-derived nitric oxide, thus reducing endogenous peroxides [13]. Polyphenols of grape seed and *Aronia melanocarpa* can prolong the blood coagulation time [APTT, PT, thrombin time (TT)] and reduce the maximal velocity of fibrous protein aggregation [14].

Lachnum is a genus of saprophytic fungi in the family Hyaloscyphaceae which is distributed throughout the world [15]. So far, more than 250 species of *Lachnum* have been reported [16] and some can produce a variety of active metabolites such as polysaccharide [17], pigment [18], and lipid [19]. Previous studies have also found that *Lachnum* could produce polyphenols, which have antioxidation, antibacterial, and other biological activities and play an important role in food quality [20,21]. The aims of the study were to evaluate the physicochemical properties, anticoagulant effect and explore the mechanism of polyphenols.

2. Materials and methods

2.1. Materials

The fruiting bodies of *Lachnum singerianum* YM296 were collected from Huangshan, Anhui, China and then separated and preserved in the Microbial Resource and Application Laboratory of Hefei University of Technology.

Fibrinogen (FIB) kit, PT kit, TT kit, antithrombin III (ATIII) kit, and APTT kit were purchased from Nanjing Jiancheng Technology Co., Ltd (Nanjing, China). Aspirin enteric-coated tablets was purchased from Shenyang Aojina Pharmaceutical Co., Ltd (Shenyang, China). Sephadex LH-20 was purchased from Sigma Chemical Co., iMark ELIAS (Bio-Rad, Hercules, CA, USA).

2.2. Experimental animals

Forty male Kunming mice, weight 25 ± 1 g, were provided by the Experimental Animal Center of Anhui Medical University (Certificate number: No. 1 license of the Medical Laboratory Animal of Anhui). The mice were kept at 23 ± 2°C with humidity of 55 ± 5%, and cultured in a 12 h:12 h light–dark cycle. All procedures involving animals were conducted in accordance with the guidelines of Regulations for the Administration of Affairs Concerning Experimental Animals of Institutional Animal Care and Use Committee (IACUC).

2.3. Extraction and purification of L. singerianum polyphenols

L. singerianum was incubated at 26°C, 160 rpm for 8 days. The composition of cultivated broth is 20 g/L sucrose, 5 g/L peptone, 0.8 g/L $MgCl_2$, 0.01 g/L tyrosine, and natural pH. After the completion of fermentation, the broth was concentrated by vacuum rotary evaporation. Three times the volume of 70% ethanol was added, and the solution was placed at 4°C for 24 hours. The supernatant was collected and evaporated to eliminate ethanol. The solution was extracted with isovolumetric ethyl acetate and evaporated to remove organic phase. The crude polyphenols were lyophilized at −40°C for 24 h and isolated by Sephadex LH-20 chromatographic column. The loading concentration of the sample was 20 mg/mL. The eluent was methanol at a flow rate of 1 mL/min and was detected at 280 nm by spectrophotometer. The main fraction was gathered, concentrated, and lyophilized to obtain *L. singerianum* polyphenol (LSP)-1 [22].

2.4. Physicochemical characterization of LSP -1

2.4.1. UV and Fourier transform infrared spectroscopy characterization

LSP-1 was dissolved in methanol and recorded by a UV spectrophotometer with scanning range of 200–800 nm.

The IR absorption of LSP-1 was measured with KBr pellet (mass ratio 1:100) by Fourier transform infrared spectroscopy (FT-IR) spectrometer Nicolet 67 with the scanning range of 400–4000/cm.

2.4.2. Gas chromatography–mass spectrometry analysis

The method of Masek et al [23] was used with minor amendments: 50 mg LSP-1 was dissolved in 2 mL methanol to make a clear solution. After being dried with N_2, 100 μL N,O-bis(-trimethylsilyl)trifluoroacetamide and 50 μL pyridine were added to the solution. Then the mixture was oscillated for 0.5 min and derived in the oven at 70°C for 45 min. Spectrogram was analyzed by the NIST 11 MS Database and MS Search Program 2.0.

Gas chromatography–mass spectrometry (GC-MS) analysis conditions are as follows: Quartz capillary column HP-5 (30 m × 0.25 mm × 0.25 μm), 1 μL of the derived solution was injected into injection port. Temperature programming was selected for column temperature which was increased from 50°C to 250°C at rate of 10°C/min. Temperature of the injection port was 280°C, while flow rate of He was 1 mL/min. The ion source was electrospray ionization and the electron bombing energy is 70 eV.

2.5. Anticoagulant activity assay in vitro

The mice were hocused with pentobarbital sodium solution before the eyeballs of mice were picked out to obtain the sample blood which had been exposed to the drug for 10 minutes. The sample blood was put into the centrifuge tube which contained 3.8% sodium citrate (whole blood: anticoagulant = 9:1, v/v), and was gently blended uniformly. After being centrifuged at 960 *g* for 10 minutes, the supernatant of the sample blood was collected and tested within 2 hours. Aliquots of 50 μL of plasma were added into 25 μL LSP-1 solutions with concentrations of 50 mg/L, 100 mg/L, 150 mg/L, and 200 mg/L. To the negative and positive control groups, other than plasma, the same amount of saline and 50 mg/L aspirin were added, respectively. APTT, TT, and PT were determined according to the instructions of the enzyme-

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