Carbohydrate Polymers 105 (2014) 231–236

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Study on the antithrombotic activity of *Umbilicaria esculenta* polysaccharide

Yufeng Wang[a,*], Jiangjuan Shao[b], Shulin Yao[a], Shengsheng Zhang[a], Jingfan Yan[a], Huiqin Wang[a], Yangyang Chen[a]

[a] College of Food Science and Technology, Nanjing Agricultural University, Nanjing 210095, China
[b] College of Pharmacy, Nanjing University of Traditional Chinese Medicine, Nanjing 210046, China

ARTICLE INFO

Article history:
Received 12 October 2013
Received in revised form 21 January 2014
Accepted 24 January 2014
Available online 2 February 2014

Keywords:
Umbilicaria esculenta
Polysaccharide
Anticoagulant
Antithrombotic

ABSTRACT

Umbilicaria esculenta as a traditional food is known to have many pharmacological activities, such as cholesterol synthesis inhibition, anti-inflammation and anti-tumor. The antithrombotic activities of UEP isolated from the lichen were examined in vitro and in vivo for the first time. The in vitro anticoagulant activity of UEP was tested by its PT, APTT and TT. The more prolongation of APTT suggested a more obvious inhibition of the intrinsic coagulation systems than the extrinsic. Its antithrombotic properties were evaluated using an arteriovenous shunt thrombosis model in rats, and its inhibition of thrombus formation increased in a dose-dependent manner. It also caused a dose-dependent increase in tail transection bleeding time. Oral administration of UEP also showed a significant dose dependent preventive effect against thrombotic death or paralysis. UEP has a potent antithrombotic effect in vitro and in vivo, which may be used as a novel, effective and promising antithrombotic agent.

1. Introduction

Thrombosis diseases are primary causes of death, especially cardiovascular disease and stroke, and their incidences have been increasing annually around the world (Juliana, Elaine, Philip, & Marcello, 2005; Li et al., 2010). Thrombosis, associated with blood coagulation and endothelial lesions, is the main source of thromboembolic complications (Fan et al., 2010; Jin et al., 2004; Juliana et al., 2005; Martinichen, Carbonero, Sassaki, Gorin, & Iacomini, 2005). Thrombus formation is the key factor, which is widely accepted that it determines the pathogenesis of cardiovascular or cerebrovascular disorders (Kang, Lim, &Yuk, 1999; Li et al., 2010). The search for antithrombotic agents is urgent.

Thrombus is mainly composed of platelet aggregate, which is a primary pathogenic mechanism of thrombosis. Platelets play a key role at the site of damaged blood vessels. Adhesive ligands will generated at the injury site, and promote platelets activation and coagulation cascades (Han et al., 2012; Li, Ji, Cheng, Li, & Ng, 2002). This thrombus is the source of thromboembolic material, so regulation of it may be an approach for the disease, such as antithrombotic therapy.

A number of drugs with antithrombotic effects, have been developed for preventing thrombosis. Some platelet inhibitors, such as aspirin, heparin and ticlopidine, have been proven to be effective. Heparin has been widely used in the therapy (Li et al., 2011; Rana, Zeynep, Nurcan, Ali, & Yusuf, 2011; Sa, Kim, & Choi, 2011). In fact, these inhibitors such as aspirin are only partially effective in the prevention of thrombus formation. They also have side effects including internal bleeding, prolonged bleeding time, and palpitation gastrointestinal symptoms and hemorrhage, so alternative drugs for these are in high demand (Li et al., 2011; Rana et al., 2011; Sa et al., 2011).

As an alternative source, antithrombotic agent isolated from traditional Chinese medicine or food with much safety has attracted attentions. Lichens are symbiotic microorganisms of algae and fungi, with 13,500 distinct species. Among them, only about 100 lichens have been researched (Bargagli, Sanchez, & Monaci, 1999; Branislav, Marijana, & Slobodan, 2008; Carbonero et al., 2006a, 2006b; Moosung & Kyungae, 2006), and *Umbilicaria esculenta* has been used as a traditional food in China. *U. esculenta* has been used to treat inflammation and bleeding, and it proved to have inhibitory activities of cholesterol synthesis, antitumor and glycosidase (Lee & Kim, 2000; Muller, 2001; Tomas, Jitka, & Valery, 2003). Polysaccharides isolated from *U. esculenta* (UEP, *U. esculenta* polysaccharide) have several components. Among them, three main polysaccharides have been characterized. Two of them were (1/3)-and (1/6)-linked β-glucans, namely laminaran and pustulan, respectively. The former consisted of a main chain of (1/6)-linked α-mannopyranosyl residues, part of them being substituted at O-2, O-4, and O-2,4 by complex, branched side chains

* Corresponding author. Tel.: +86 25 84396583; fax: +86 25 84396583.
E-mail addresses: joywangyu@sina.com, yufengwang@sohu.com (Y. Wang).

0144-8617/$ – see front matter © 2014 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.carbpol.2014.01.082

containing α-mannopyranosyl and β-galactofuranosyl units. The other was a galactofuranomannan, and mainly a D-galacto-D-gluco-D-mannoglycan (-4Manβ1-4Glcβ1-4(Galα1-6)Manβ1-4Manβ1-), which was analyzed as well as a galactose-free polysaccharide formed on partial hydrolysis (Carbonero et al., 2006b; Karunaratne, 2012). UEP has antimicrobial activities against many microorganisms. Moreover, UEP can boost human immunitys, eliminate oxygen free radicals and inhibite lipid peroxidation. Furthermore, it can also reduce the occurrence of cardiovascular event, which was closely related with the incidence of thrombosis (Lee & Kim, 2000; Muller, 2001; Tomas et al., 2003).

Up to now, its active constituents and antithrombotic activity have not been investigated in depth. In our previous study, a new method for separating its UEP was developed with AB-8 macroporous resins. Based on our preliminary study of UEP, this objective was to investigate its antithrombotic activity and provide evidences for clinical applications. Therefore, we tested its effects on rat thrombus formation in vivo and in vitro. In addition, its in vivo antithrombotic effects on arteriovenous shunt thrombosis model in rats were also examined. This work would contribute to evaluate whether *U. esculenta* could be used as a functional food or medicine ingredient.

2. Materials and methods

2.1. Chemicals and reagents

The AB-8 resin was supplied by Anhui Sanxin Resin Technology Co., Ltd. (Anhui Province, China). Deionized water was used throughout and purified by a Mill-Q water-purification system from Milipore (Bedford, MA, USA). All other chemicals and reagents were of analytical grade and obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China), unless otherwise stated.

2.2. Collection of umbilicaria esculent

U. esculenta were obtained at July 2010 from Huangshan Mountain (30° N, 1864 m altitude) in Anhui Province, China. They were identified according to their morphological characteristics (Lee & Kim, 2000; Muller, 2001; Tomas et al., 2003).

2.3. Preparation of UEP

U. esculenta (100 g) were cleaned and lyophilized (a Model 2K-XL Lyophilizer, Virtis corporation, American), and ground with a Model ZN-100 Grinder (Zhongnan Pharmaceutical Machinery Factory, Shanghai, China) to obtain their powders. The powders were successively extracted with deionized water (800 ml) at 100 °C for 3 h, and the residue was isolated. Each aqueous extract was added to excess absolute ethyl alcohol (ratio of 3:1, v/v) to form a precipitate. The precipitate was isolated by a Model 5804R centrifugation (4680 × *g* for 20 min, at 25 °C, Eppendorf corporation, Germany) and dried or lyophilized.

Then it was further purified at room temperature in a glass column (Φ 26 mm × 300 mm) wet-packed with the AB-8 resin. For adsorption, the concentration, processing volume and flow rate of the sample were 2.0 mg/ml, 3.2 BV and 1.5 BV/h, respectively; the pH of 7 and temperature of 25 °C were also suitable. For desorption, the AB-8 resin column was firstly washed by deionized water with a flow rate of 1 BV/h and then ethanol solution (ethanol: water of 50:50, v/v) of 4.4 BV with a flow rate of 2.0 BV/h; the pH and the temperature was the same as that of the adsorption process. A peristaltic pump (HL-2, Nanpu Huxi Equipment Factory, Shanghai, China) was used to pump the crude UEP solution through the packed column, and the effluent or eluate was collected in an auto-partial collector (DBS-100, Nanpu Huxi Equipment Factory, Shanghai, China) with 6 min per test tube. After treatment with the AB-8 resin, the pure UEP (99.8%) was obtained and lyophilized.

2.4. Animals

Experiments were carried out with male Sprague–Dawley rats weighing 250–300 g (Nanjing University of Traditional Chinese Medicine, China). The rats were maintained in a temperature-controlled room (24 ± 1 °C). The rats had free access to diets before experiments and were acclimatized for at least one week. All experiments were performed in accordance to internationally accepted guidelines on laboratory animal use.

2.5. In vitro anticoagulant activity assay

The rats were anesthetized intramuscularly with ketamine (100 mg/kg) and xylasine (16 mg/kg). Blood was collected from the abdominal aorta, then anticoagulated with citrate (3.8%; 1:9, v/v). The blood was centrifuged (1000 rpm, 10 min) to obtain its PRP (platelet-rich plasma). The residual blood was centrifuged (3000 rpm, 15 min) to obtain its PPP (platelet-poor plasma). Its platelets were centrifuged (2500 rpm, 10 min) to obtain its pellets and washed thrice. They were then suspended in a Tyrode-Hepes solution (1 mM Ca^{2+}, 0.35% BSA; heparin 50 units/ml; pH 7.4). In the solution, heparin at the concentration was used to prevent plasma from coagulating and has no effect on aggregation induced by ADP in PRP (Antonio & Marchien, 2001; Gulzar, Nurbiya, Kerimjan, & Abdulla, 2011; Lee, Park, Jung, Lee, & Oak, 2010). The platelet concentration was adjusted to 4.5×10^8 platelets/ml. They were stored at 4 °C.

Experiments were carried out using UEP or heparin (as a positive control) dissolved in saline at various concentrations, respectively. As controls, saline was added to plasma in a ratio of 1:10. The plasma (90 μL) was mixed with a 10 μL solution of UEP (0–50 μg), or heparin (0–10 μg) and incubated at 37 °C for 7 min. Prothrombin time (PT), activated partial thromboplastin (APTT) and thrombin time (TT) were measured using kits from Nanjing Jiancheng Bioengineering Institute (Nanjing, China) and an automated coagulometer (Medtronic Inc, USA). In brief, 1 μL of the incubated plasma was mixed with 50 μL of cephalin in the process plate, and the coagulation was started by addition of $CaCl_2$ (1 mM), 100 μL of thromboplastin and 100 μL of bovine thrombin into the 100 μL of incubated plasma for APTT, PT and TT assay, respectively.

The decreasing percentage of platelet aggregation rate was also calculated and compared, as well as IC_{50} was calculated, according to the Born method (Lu et al., 2011; Wu et al., 2009; Xie et al., 2007).

2.6. Ex vivo determination of APTT

The rats were anesthetized with ketamine (100 mg/kg intramuscularly) and xylasine (16 mg/kg intramuscularly). The carotid artery was exposed and dissected free from surrounding tissue. Heparin (0–500 μg/kg), or UEP (0–1500 μg/kg) was administered into it. After 5, 15, 30 and 60 min samples of blood was collected (0.5 ml in 3.8% trisodium citrate, 9:1, v/v). Each was centrifuged (2000 × g, 10 min), and plasma was stored at 4 °C. APTT was evaluated using the kit and the automated coagulometer.

2.7. In vivo arteriovenous shunt thrombosis

Thrombus formation was promoted with a combination of stasis and hypercoagulability. The rats were anesthetized by intraperitoneal injection of chloral hydrate (350 mg/kg) and an arteriovenous shunt tube was placed between the right carotid