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Structural characterization and osteoprotective effects of a novel oligo-glucomannan obtained from the rhizome of *Cibotium barometz* by alkali extraction

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ABSTRACT

Cibotium barometz is mainly distributed in eastern, southern, and southwest China as an important industrial export crop of great economical and medicinal value. The rhizome of *C. barometz* is widely used in Traditional Chinese Medicine clinics to treat conditions such as lumbago, limb-ache, rheumatism, and sciatica. In this study, the results of *in vivo* pharmacological experiments conclusively demonstrated that crude saccharides from *C. barometz* (CBB) exhibited osteoprotective effects in ovariectomized rats, which significantly increased bone mineral content (BMC) and bone mineral density (BMD), and prevented damage of the trabecular bone, consequently improving its biomechanical properties. To investigate the biological active ingredient(s), a novel oligo-glucomannan (denoted CBBP-1) was isolated and purified from CBB via anion-exchange and size-exclusion chromatography. Structural analysis indicated that CBBP-1 consisted of (1 → 4)-linked α-D-glucose, (1 → 6)-linked *β*-D-glucose with (1 → 3, 6)-linked α-D-mannose, and a terminal α-D-glucose. Morphological analysis revealed that CBBP-1 had an irregular sheet structure. Furthermore, osteoblastic MC3T3-E1 cells treated with CBBP-1 had significantly increased mRNA expression of *runt-related transcription factor 2*, *osterix*, *osteopontin*, *osteocalcin*, and *bone sialoprotein*, indicating that CBBP-1 may stimulate osteoblastic differentiation. In conclusion, this study provides evidence that CBBP-1 may have potential as an anti-osteoporosis agent in the pharmaceutical industry.

1. Introduction

Fractures resulting from osteoporosis are a major public health problem and are increasing in prevalence worldwide due to the rapidly aging population. Osteoporosis, characterized by low bone mass and microarchitectural deterioration, is a chronic disease that increases the brittleness of bone and its susceptibility to fracture (Fu et al., 2014). Bisphosphonate (BPH), calcitonin, selective androgen receptor, and hormone replacement therapy (HRT) are the most common and efficacious strategies for reducing the risk of osteoporosis (Thu et al., 2017). However, most of these agents have multiple side effects; for example, the long-term use of BPH has been linked to serious complications, such as osteonecrosis of the jaw and atypical femur fractures (Moro Álvarez et al., 2016). In addition, long-term HRT substantially increases the risk of endometrial cancer and other adverse events, such as thromboembolism and vaginal bleeding (Davison and Davis, 2003; Wiseman, 2004). Thus, the prolonged use of these agents is limited. Consequently, there is a need for natural herbal medicines with relatively few side effects that can be used as alternative therapies for the treatment of osteoporosis.

Cibotium barometz (Linn.) J. Sm. (*Dicksoniaceae* family), known as "Gou-Ji" in Chinese, is mainly distributed in eastern, southern, and southwest China. The total planting area is more than 7000 ha, and its annual production is estimated to be greater than 850 tons, making it one of the most important industrial export plants in China (Yang et al., 2015). As an ornamental plant, *C. barometz* has unique beauty. Due to its large economical and medicinal value, studies have been conducted on techniques for its artificial cultivation (Pu et al., 2015; Praptosuwiryo et al., 2015). According to the theory of Traditional Chinese medicine (TCM), *C. barometz* can strengthen bones and muscles, and its rhizomes are widely used in TCM clinics to treat conditions such as lumbago, limb-ache, rheumatism, and sciatica (Wu and Yang, 2009). Daily oral administration of *C. barometz* extract was shown to significantly contribute to the prevention or treatment of bone loss induced by ovariectomy

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(OVX) in rats (Zhao et al., 2011). To investigate the biological active ingredient(s) of *C. barometz*, a large number of small molecule compounds were isolated, including caffeic acid, protocatechuic acid, palmitic acid and 1-monopalmitin (Cheng et al., 2003), *β*-sitosterol, daucosterol, alternariol, protocatechuic aldehyde, and (24*R*)-stigmast-4-ene-3-one (Wu et al., 2007). However, their effects on osteoporosis are unknown. Consequently, elucidation of its exact anti-osteoporosis active ingredient(s) would be essential for its use in future therapeutic applications and the pharmaceutical industry, which in turn could lead to the development of phytomedicines with industrial feasibility.

There is a general consensus that saccharides (e.g., polysaccharides and oligasaccharides) have various biological activities, but little is known about the saccharide-containing fractions of *C. barometz*. Therefore, the aims of this study were to systematically evaluate the effects of CBB in OVX rats, to isolate and purify CBB to obtain the CBBP-1 oligosaccharide, to determine the detailed structure of this novel oligosaccharide, and to evaluate its effects on the expression levels of five transcription factors, *runt-related transcription factor 2* (*Runx2*), *osterix* (*Osx*), *osteocalcin* (*Ocn*), *osteopontin* (*Opn*), and *bone sialoprotein* (*Bsp*), in MC3T3-E1 cells during osteogenic differentiation.

2. Materials and methods

2.1. Materials

C. barometz was purchased from the Tong Ren Tang medicinal store and identified by Dr. Hongyan Ma of Guangdong Pharmaceutical University (Guangzhou, China). Cellulose DEAE-52 and Sephadex G-75 were purchased from GE Healthcare (Chicago, IL, USA). A total of 32 3-month-old female Sprague–Dawley rats were purchased from the Chinese Medicine Animal Experimentation Center of Guangzhou University (Certificate: SCXK2013-0020). All of the other chemicals and reagents were of analytical grade.

2.2. Extraction and purification of crude saccharide

The rhizome of *C. barometz* (15.0 kg) was ripped into chunks, soaked overnight in deionized water 1: 10 (w/v), and extracted at 80 °C for 3 h; this procedure was repeated three times. The residues of *C. barometz* were extracted with sodium hydroxide ([NaOH] 0.3 mol/L) 1:10 (w/v) at room temperature for 3 h, repeated twice, and pooled. Then the extracts were filtered, neutralized with hydrochloric acid ([HCl] 0.3 mol/L), concentrated (up to 1/30 initial volume), and centrifuged. Next, 95% ethanol was added to the supernatants at a final concentration of 70%, and they were incubated for 24 h at room temperature to obtain crude saccharides, which were deproteinized with Sevag reagent (1-butanol/chloroform, v/v = 1:4). Finally, the supernatants were dialyzed and lyophilized.

2.3. Animals and treatments

The experiments were completed in a specific pathogen-free animal laboratory at a relatively constant temperature of 25 ± 2 °C, 12 h light and 12 h dark cycles, and relative humidity of 40%–50%. Female Sprague–Dawley rats (235 ± 15 g) were adjusted to laboratory conditions for 1 week. Water and food were freely available during the experimental period. After anesthesia by intraperitoneal injection of pentobarbital sodium, rats in the ovariectomized group (OVX, n = 21) were treated with bilateral ovariectomy, and those in the sham-operated group (sham, n = 7) were only given laparotomy to cut off fat but not the ovaries. After a 1-week recovery from surgery, the OVX rats were randomly divided into three sub-groups: OVX group, 17*β*-estradiol group (25 μg/kg E2 body weight/day), and CBB group (400 mg/kg) (Liu et al., 2009). Distilled water, E2, and CBB were all administered orally for 12 weeks, and the rats were weighed every week to adjust the doses of E2 and CBB. After 12 weeks, the rats were secured in the supine position after anesthesia, and blood was obtained from the abdominal aorta. After the rats were sacrificed, the heart, liver, spleen, kidney, brain, and uterus of each rat were weighed and used to calculate the coefficient of the organ and uterus. The femurs were obtained and kept in 70% alcohol at −20 °C for the measurements of bone mineral content (BMC), bone mineral density (BMD), and biomechanical properties. All of the procedures for animal care and use were in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committee of Guangdong Pharmaceutical University (License No: SYXK [Yue] 2017-0125).

2.4. BMC and BMD analyses

The BMC of the entire left femur, distal left femur (2 cm), and proximal left femur (1 cm) was measured by dual-energy X-ray absorptiometry (WI 85003, Hologic Discovery, Marlborough, MA, USA) according to a previously reported method (Li et al., 2011). BMD was determined using the BMC of the measured area, and all values were automatically calculated using a software package (Encore 2006, GE Healthcare, Madison, WI, USA).

2.5. Three-point bending test

After the femora thawed at room temperature for 5 h, each left femur was placed at a distance of two points on a 20-mm fixing device, after which the femoral diaphysis was loaded to the breaking point using a MTS Mini Bionix Tabletop Test System (MTS 858, MTS Systems, Eden Prairie, NJ, USA) at a speed of 6 mm/min. All force and displacement data were recorded for later evaluation. Calculations of biomechanical parameters were based on previously published formulas (Zhang et al., 2009).

2.6. Micro-computed tomography analysis

The left femur of rats was scanned using the Explore Locus SP Pre-Clinical Specimen Micro-Computed Tomography (MicroCT) Scanner (GE Healthcare) to estimate the effects of CBB on trabecular microarchitecture. The distal part of the femur, which is richer in trabecular bone than the proximal and middle regions, was scanned from the proximal growth plate at an isotropic voxel resolution of 22 μm^3. The volume of interest, which included the relative bone volume (BV/TV), trabecula number (Tb.N), trabecula separation (Tb.Sp), trabecula thickness (Tb.Th), connectivity density (Conn.D), and structure model index (SMI), were chosen for subsequent architectural parameter analyses (Cai and Zhang, 2016). All specimens were scanned in the dark.

2.7. Isolation and purification of the CBBP-1 saccharide

CBB has shown significant osteoprotective effects *in vivo* in pharmacological studies. Therefore, it was selected for further isolation and purification by dissolution in deionized water and centrifugation, after which the supernatants were separated and purified on a DEAE-52 cellulose chromatography column (Ø 2.6 × 40 cm). A constant NaCl gradient from 0 to 1 M (400 mL each step) was used as the elution solvent, and the eluents were determined with the phenol-sulfuric acid colorimetric method. Eluents with 0 M NaCl were collected, concentrated, and lyophilized to obtain the CBB-1 saccharide. CBB-1 was further purified using a Sephadex G-75 gel permeation chromatography column (Ø 1.6 × 100 cm) and eluted with distilled water at a flow rate of 0.3 mL/min to obtain the novel purified saccharide denoted CBBP-1.

2.8. Homogeneity and average molecular weight determination of CBBP-1

CBBP-1 was analyzed using an ultraviolet-visible spectrophotometer to detect the presence of nucleic acids and proteins. High-performance