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# Extraction, characterization of polysaccharides from lycium barbarum and its effect on bone gene expression in rats

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ABSTRACT

In this study, water-soluble polysaccharides were isolated from dried fruits of lycium barbarum. Gas chromatography–mass spectrophotometer (GC–MS) and Fourier-transform infrared (FT-IR) spectroscopy were used to characterize the compounds in polysaccharides from lycium barbarum (LBP). GC–MS analysis revealed that glucose and mannose were the main monosaccharides of LBP. Xylose, galactose, arabinose and galactose were detected in trace amounts. The Fourier-transform infrared spectra (FT-IR) of LBP revealed typical characteristics of polysaccharides. The pharmacological study indicated that LBP could enhance bone mineral density (BMD) and bone mineral content (BMC) in rats. RT-PCR studies had shown that LBP treatment resulted in increase in the mRNA expression of Col5a2 and Alpl in rat blood. Western blot analyses elicited a decreased Col5a2 and Alpl protein level after 45 days of LBP administration. Taken together, our results provide evidence that LBP increase bone gene expression, bone mineral density (BMD) and bone mineral content (BMC) in ovariectomized rats.



## 1. Introduction

Osteoporosis is defined as a skeletal disorder characterized by compromised bone strength predisposing a person to an increased risk of fracture. It is most common in postmenopausal woman, because estrogen deficiency causes an imbalance of osteoclastic bone resorption and osteoblastic bone formation, resulting in deterioration of bone tissue and low bone mass (Eichner, Lloyd, & Timpe, 2003; Lane, Haupt, Kimmel, Modin, & Kinney, 1999). The ovariectomized (OVX) animal model mimics bone loss in the estrogen deficiency condition of postmenopausal woman (Giavaresi et al., 2001). The estrogen-deficient ovariectomized (OVX) osteoporosis model in animals is useful for evaluation of osteoporotic drugs.

Lycium barbarum fruit (goji) has become more popular for the last few years due to its public acceptance as a "super food" with highly advantageous nutritive and antioxidant properties. It has been widely used in these countries for medicinal purposes and as a functional food for more than 2500 years (Amagase & Nance, 2008; Chang & So, 2008). The ancient herbalist classics recorded that lycium barbarum nourishes the liver and kidney and brightens the eye. In support of such traditional properties, modern studies indicate that extracts from lycium barbarum possess a range of biologic activities, including antioxidant properties (Amagase & Nance, 2008; Chang & So, 2008). The extracts also exhibit antiaging effects, neuroprotection, promotion of endurance, increased metabolism, improved control of glucose and other diabetic symptoms, antiglaucoma effects, immunomodulation, antitumor activity, and cytoprotection (Lin, Wang, Chang, Stephen Inbaraj, & Chen, 2009; Zhu, Zhao, Zhao, & Chen, 2007). The effect of polysacharides extract on the bone loss, immunity and antioxidant activities have already been investigated in ovariectomized animals (Zhu, Wang, Zhang, Pei, & Fen, 2008).

In this model, the effects of the polysaccharides from lycium barbarum on bone mineral density (BMD) and bone mineral content (BMC) were investigated. Then, we investigated bone gene expression.

## 2. Materials and methods

### 2.1. Materials

Fresh lycium barbarum specimens were collected from a herb shop, Zhejiang province of China, in March, 2009.

### 2.2. Isolation and purification of polysaccharides

Dried lycium barbarum fruits (43 g) was extracted successively with refluxing $CHCl_3$–MeOH (2:1 v/v) and 80% aqueous MeOH, in

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order to extract low-molecular-mass components according to Yin and Dang's method (2008). The residual material was then extracted three times with distilled water (500 ml) at 100 °C for 2 h and deproteinated by the Sevage procedure. The combined extracts were concentrated, and the retained solution was freeze–thawed to give a precipitate (crude polysaccharides).

### 2.3. Analysis of monosaccharide composition

LBP (10 mg) were hydrolysed by 10 ml of 2 M trifluoroacetic acid at 100 °C for 4 h (Erbing, Jansson, Widmalm, & Nimmich, 1995). Derivatization of the released monosaccharides was then carried out by the trimethylsilylation reagent according to the method of Guentas et al. (2001). The trimethylsilylated derivatives were loaded onto a GC-2010 gas chromatography system (Shimadzu, Shanghai, China) equipped with a RTX-5 capillary column and a flame ionization detector. The following program was adopted for gas chromatography analysis: injection temperature: 230 °C; detector temperature: 230 °C; column temperature programmed from 130 to 180 °C at 2 °C/min, holding for 3 min at 180 °C, then increasing to 220 °C at 10 °C/min and finally holding for 3 min at 220 °C. Nitrogen was used as the carrier gas and maintained at 40.0 ml/min. The speed of air and hydrogen gas were 400 and 40 ml/min, respectively. The split ratio was set as 10:1. Six monosaccharides were used as the external standard to quantify the monosaccharide content.

### 2.4. IR spectral analysis

The structural characteristics of the LBP were determined using a Fourier-transform IR spectrophotometer (Bruker, Karlsruhe, Germany) equipped with OPUS 3.1 software. The purified polysaccharides were ground with KBr powder and then pressed into pellets for transform IR spectral measurement in the frequency range of 4000–500 $cm^{-1}$ (Kumar, Joo, Choi, Koo, & Chang, 2004).

### 2.5. Animal and treatment

Thirty-two adult albino female Wistar rats weighing 220–255 g were used in this study. The rats were divided into four groups: I, control ($n = 8$); II, ovariectomized ($n = 8$); III, ovariectomized + LBP ($n = 8$) and IV, ovariectomized + LBP ($n = 8$). The rats in the control group were neither operated on nor treated with LBP. The animals were acclimatized for 1 week to our laboratory conditions prior to experimental manipulation and were exposed to a 12-h light and 12-h dark cycle at a room temperature of 22 °C. The animals had free access to standard laboratory chow and water ad libitum. All animal procedures used were in strict accordance with the National Institutes of Health Guidelines on the Care and Use of Laboratory Animals, and the approval of the ethic committee of our university was obtained prior to the study.

The rats in groups III and IV were anaesthetized with ketamine (Ketalar, Eczacibasi Pharmaceutical Co.) and underwent a bilateral ovariectomy via ventral incision. Ovaries were excised and oviducts replaced, with minimum disruption to surrounding soft tissues, and the incisions were closed with clips.

The control rats (group I) orally received an equal volume of physiological saline. The model rats (group II) also orally received an equal volume of physiological saline. The group III rats orally received LBP (50 mg/kg b.w.). The group IV rats orally received LBP (100 mg/kg b.w.).

At the end of the experiment (45 days), the rats were anesthetized using diethyl ether. Blood samples were taken from the left atrium of the heart. Plasma was prepared and used for laboratory tests. The weight gain of the rats was recorded. Both femurs were removed for examination.

### 2.6. Bone mass measurement

The femur was used for dual-energy X-ray absorptiometry (DEXA) assessment of the bone mineral content (BMC) and bone mineral density (BMD) using an Hologic QDR 1000 and Hologic's small animal program. The measurements were repeated on the femur by a blinded investigator at a different center (analysis center of our institute), also using an Hologic QDR 1000. This femur was subsequently ashed to burn off organic material and the remaining mineral structure was weighed and the length measured. Thereafter, the ash was dissolved in dilute hydrochloric acid and calcium, and magnesium and inorganic phosphate content determined by standard colorimetric assays performed by blinded investigators. Accuracy of the DEXA measurements was demonstrated by the close correlation between ash weight and BMC, and precision by multicenter comparison.

### 2.7. Reverse-transcriptase polymerase chain reaction (RT-PCR)

Total RNA (5 μg) from blood cells was used for cDNA synthesis with oligo (dT) 12–18 primers and Superscript II (RNaseH-) (Life Technologies) in reverse transcription (RT) reactions. About 2 μL of RT product was used as a template for polymerase chain reaction (PCR) amplification of Col5a2 mRNA, Alpl mRNA and GAPDH gene products. PCR primers were commercially synthesized (Sigma-Genosys) for Col5a2 mRNA and Alpl mRNA. Col5a2 mRNA sequences that were employed were 5′-GGCAGACACTGGATGGTTATG-3′ for forward sequence and 5′-AGTTGAGCACTTCTTGGCAC-3′ for reverse sequence. Alpl mRNA sequences that were employed were 5′-GGTACTCGGACAATGAGATGC-3′ for forward sequence and 5′-TTCAGTGCGGTTCCAGACAT-3′ for reverse sequence. Non-regulated GAPDH sequences 5′-CACCATGGAGAAGGCCGGGG-3′ for forward primer sequence and 5′-GACGGACACATTGGGGGTAG-3′ for reverse primer sequence. RT-PCR products were separated and analyzed by gel electrophoresis. Resulting images were captured using a Gel-Doc (Bio-Rad) imaging system equipped with UV light and a gel scanner. PCR results were quantifies using Bio-Rad's phosphoimager system and Quantity One software to assess relative differences. RT-PCR products were assessed by subcloning RT-PCR products into pGEM-T (Promega) and DNA sequencing to determine authenticity.

### 2.8. Western blot analysis

The cellular lysates were prepared as described previously (Tang, Chiu, Tan, Yang, & Fu, 2007). Proteins were resolved on SDS–PAGE and transferred to Immobilon polyvinyldifluoride (PVDF) membranes. The blots were blocked with 4% BSA for 1 h at room temperature and then probed with rabbit anti-mouse antibodies against p-Akt, Akt or BMP-2 (1:1000) for 1 h at room temperature. After three washes, the blots were subsequently incubated with a donkey anti-rabbit peroxidase-conjugated secondary antibody (1:1000) for 1 h at room temperature. The blots were visualized by enhanced chemiluminescence using Kodak X-OMAT LS film (Eastman Kodak, Rochester, NY). Quantitative data were obtained using a computing densitometer and ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

### 2.9. Statistical analysis

Unless otherwise indicated, the results were expressed as the means ± SD of data obtained from triplicate experiments. Statistical analysis was performed by a paired $t$-test. $p$ values <0.05 were considered statistically significant.

## 3. Results and discussion

### 3.1. GC–MS analysis of LBP

LBP was hydrolysed by trifluoroacetic acid into individual monosaccharides that were further trimethylsilylated for gas chromatography analysis (Fig. 1). On the basis of comparison of GC–MS and retention times of sample peaks with those of standards, monosaccharide composition of LBP polysaccharides was identificated (Fig. 1 and Table 1). Table 1 shows the monosaccharide composition of LBP. The water-soluble polysaccharide mainly contained glucose and mannose. Xylose (0.16%), galactose (0.5%), arabinose (1.08%), and ribose (0.14%) were detected in trace amounts.

### 3.2. FT-IR of LBP

The FT-IR spectra of carbohydrates are used for determination of their structural features. The LBP had IR bands at 500–900 $cm^{-1}$, 1000–1100 $cm^{-1}$, 1400–1530 $cm^{-1}$, 2800–2900 $cm^{-1}$, and 3100–3500 $cm^{-1}$, which were distinctive absorptions of polysaccharides (Fig. 2). Since monosaccharide analysis revealed that LBP was mainly composed of glucose, FT-IR spectra of LBP were compared against the commercial glucose standards (Fig. 2). It was found that the FT-IR spectra of LBP exhibited similarities in absorption pattern to glucose, confirming the preliminary conclusion derived from chemical composition analysis (Table 1) that the polysaccharide is a glucan. The polysaccharides was examined by IR which showed strong absorption at 3500–3300 $cm^{-1}$ due to –OH, 3100 $cm^{-1}$, and 1600–1400 $cm^{-1}$ absorptions. The band at 1630 $cm^{-1}$ reflects the absorption of aromatic C=C bonds and of the C=O group that is part of glycosides. The wave number between 950 and 1200 $cm^{-1}$ is often called the fingerprint of molecules because it allows the identification of major chemical groups in polysaccharides: the position and intensity of the bands that are specific for each polysaccharide (Fellah, Anjukandi, Waterland, & Williams, 2009; Kawabata et al., 1998). In addition, in the IR spectrum of LBP, the characteristic band at 898 $cm^{-1}$ belonged to the β-anomeric configuration, the band at l029 $cm^{-1}$, 864 $cm^{-1}$, 817 $cm^{-1}$, 778 $cm^{-1}$ (Fig. 2) can be attributed to the pyranose ring (Fellah et al., 2009; Teli & Waghmare, 2009).

### 3.3. Effect of LBP on rats' body weight, BMD and BMC

As shown in Table2, there was no significant difference in body weight between groups. As previously reported, ovariectomy induced a significant ($P < 0.01$) decrease in femur BMD (group II). The administration of LBP dose-dependently significantly enhanced BMD in groups III and IV rats. The same pattern was observed for femur BMC.

**Table 1**
Chemical composition of LBP.

| Components | Retention time | Percentage (%) |
|---|---|---|
| Ribose | 5.29 | 0.14% |
| Arabinose | 5.48 | 1.08% |
| Xylose | 5.61 | 0.16% |
| Mannose | 7.75 | 7.3% |
| Glucose | 7.95 | 90.8% |
| Galactose | 8.11 | 0.5% |

Body weight (lean and/or fat mass) changes have been used as predictors of bone density in OVX rodents (Lorden & Caudle, 1986). However, no significant or meaningful changes in body weights were detected in the present study. Although it is generally accepted that the changes of bone weight are not a critical index in detecting the efficacy of antiosteoporotic agents (except for bone ash weight) (Yamamoto et al., 1998), a trend toward slightly increased relative bone weights was detected at all dosage levels tested in this study. Therefore, this trend toward increased bone weight may be considered to be directly related to the potency of the antiosteoporotic effect of LBP. Generally, bone mineral contents are significantly decreased in osteoporotic status. Among bone mineral contents, Ca and P contents are the most dramatically decreased in osteoporotic bone, but their ratio, Ca/P, generally does not change (Tanaka et al., 2001; Tarvainen et al., 1994). LBP significantly increased the tibiae Ca and P contents that were decreased by ovariectomy. These increases in bone mineral content by LBP subsequently cause gains in bone strength and quality.

### 3.4. Effect of LBP on mRNA expression of the Col5a2 and Alpl in the blood of control and LBP-treated rats

The COL5a2 gene encoded Collagen alpha-2(V) chain, a protein that in humans. Activation mutations in this gene lead to increased bone formation in the crania of both human and mice (Välkkilä et al., 2001). ALPL gene exists in single copy in the haploid genome and is composed of 12 exons distributed over more than 50 kb. Damaged or diseased tissue releases enzymes into the blood, so serum ALP measurements can be abnormal in many conditions, including bone disease and liver disease.

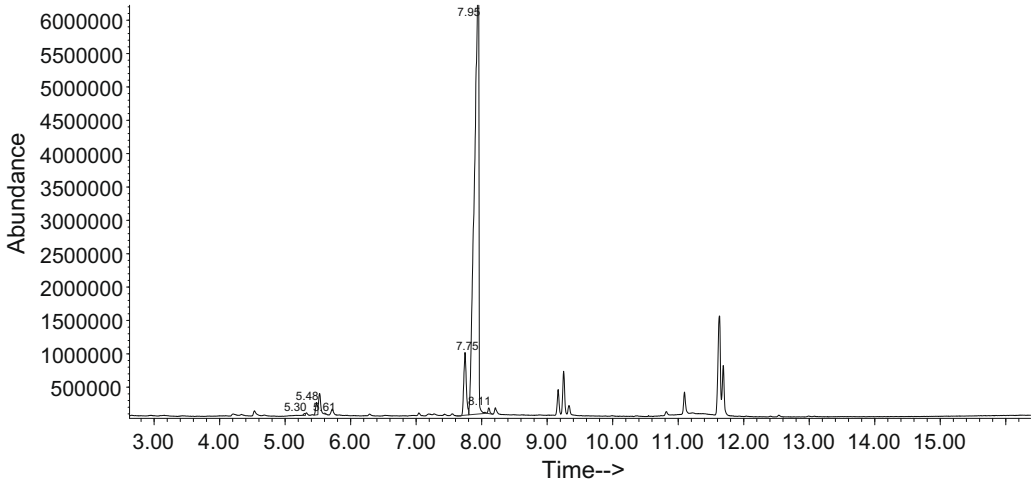


**Fig. 1.** GC–MS analysis of LBP.

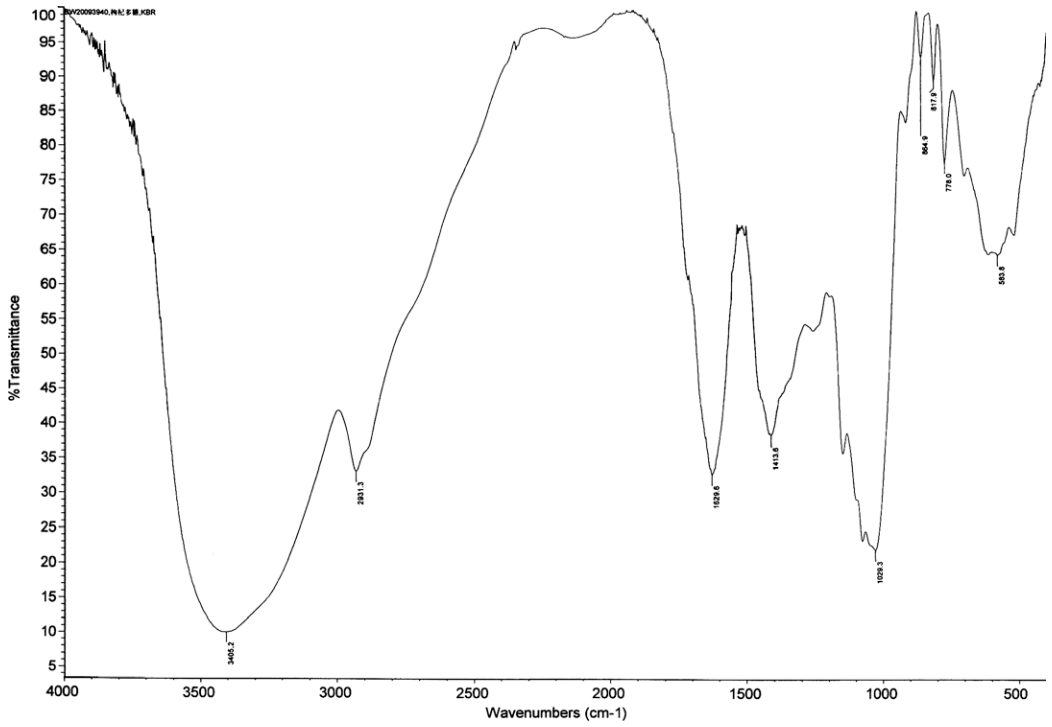


**Fig. 2.** FT-IR analysis of LBP.

**Table 2**
Effect of LBP on body weight, BMD and BMC in ovariectomized rats.

| Group | Body weight | BMD(g/cm$^2$) | BMC(g) |
|---|---|---|---|
| I | 346.1 ± 16.5 | 0.241 ± 0.009 | 0.573 ± 0.024 |
| II | 350.2 ± 22.1 | 0.297 ± 0.013[b] | 0.602 ± 0.017[b] |
| III | 347.8 ± 19.3 | 0.328 ± 0.011[d] | 0.643 ± 0.021[d] |
| IV | 343.8 ± 25.1 | 0.352 ± 0.013[d] | 0.692 ± 0.030[d] |

[b] $P < 0.01$, compared with group I.
[d] $P < 0.01$, compared with group II.

To investigate the tissue expression profile of Col5a2 mRNA and Alpl mRNA, RT-PCR was used to analyze its transcript. As shown in Fig. 3, the lower Col5a2 and Alpl mRNA levels were detected in blood of group II rats. The higher Col5a2 and Alpl mRNA levels were detected in blood of group III and IV rats, indicating that LBP could stimulate bone gene expression and growth.

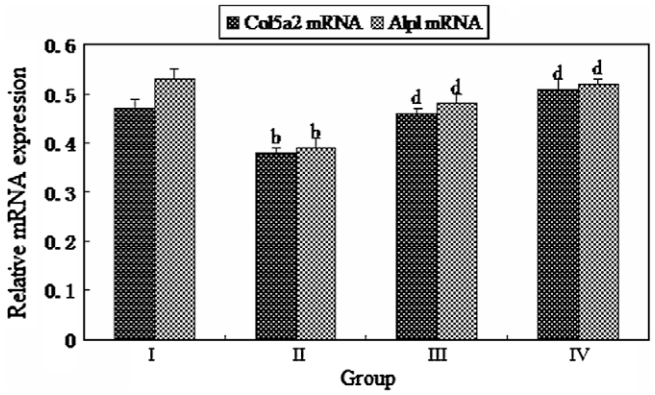


**Fig. 3.** Effect of LBP on mRNA expression of the Col5a2 and Alpl in the blood of control and LBP-treated rats. [b]$P < 0.01$, compared with group I; [d]$P < 0.01$, compared with group II.

Our data support the importance of Col5a2 and Alpl expression in the female rat femur, and we have now demonstrated their regulation during ovariectomy-induced bone formation. Because LBP clustered tightly with several known bone formation activity genes (Col5a2 and Alpl) there is great interest in pursuing a deeper understanding of their relevance to bone formation in the ovariectomized rat.

### *3.5. Effects of LBP on Col5a2 and Alpl protein levels in the blood of control and LBP-treated rats*

Various animal and in vitro cell culture studies have demonstrated the efficacy of polysaccharides isolated from natural plants on bone growth (Liao, Chen, & Yang, 2005). It has been well-known for decades that LBPs are biologically active components of lycium barbarum with potential pharmacological and biological functions (Luo et al., 2006; Ma, Liu, Yu, Chen, & Zhang, 2009). As shown in Fig. 4, the ovariectomy operation caused down-regulation of Col5a2 and Alpl proteins ($P < 0.01$) in group II rats. The treatment with LBP induced a strong over-expression of Col5a2 and Alpl protein ($P < 0.01$) of group III and IV rats in a dose-dependent manner. Collectively, these observations further lead to the possibility that the increased production of Col5a2 and Alpl proteins may account for the improvement of bone quality after LBP administration.

## 4. Conclusion

Polysaccharides were extracted from lycium barbarum fruits in this work. Six monosaccharides, namely glucose, mannose, xylose, galactose, arabinose, and ribose, were identified for LBP. Glucose was confirmed to be the dominant monosaccharide in the polysaccharides with relative molar percentages of >90%. It constructed the backbone in combination with mannose. The infrared spectra of LBP showed typical characteristics of polysaccharides. Our results provide evidence that LBP increase bone gene expression, bone mineral density (BMD) and bone mineral content (BMC) in

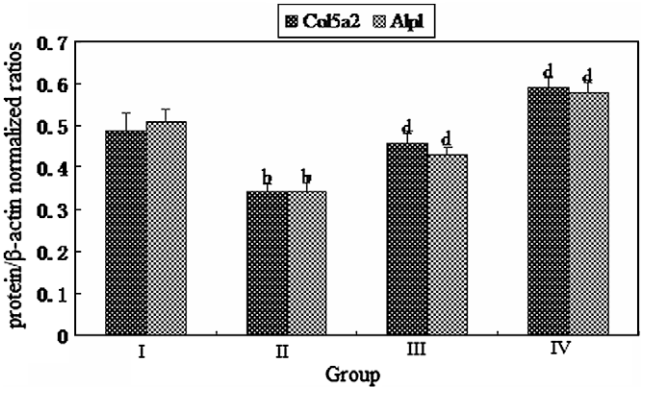


**Fig. 4.** Effects of LBP on Col5a2 and Alpl protein levels in the blood of control and LBP-treated rats. $^{b}P < 0.01$, compared with group I; $^{d}P < 0.01$, compared with group II.

ovariectomized rats. Further bioactivity investigation of LBP is worthy to be done in the future work for better exploiting this substance.

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