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Water-soluble polysaccharide from dried *Lycium barbarum fruits*: Isolation, structural features and antioxidant activity

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ABSTRACT

The purpose of this study was to investigate the protective effect of purified *Lycium barbarum* polysaccharides (PLBP) against tissue oxidative injury. *Lycium barbarum polysaccharides* were isolated from dried *Lycium barbarum* fruit fruits by boiling water. Crude polysaccharides were divided into seven small fractions and one major fraction using DEAE–cellulose column. Major fraction, namely purified *Lycium barbarum* polysaccharides (PLBP), was analyzed. Results indicated that the average molecular weight of PLBP was 1.21 × 105 Da. The carbohydrate-related absorbances appeared at 3400.38, 2930.49, 1629.66, 1411.40, 1151.44, 1078.24, 1032.50, 920.72, 864.33, 817.08, and 777.04 cm^{-1}. Then, strong DPPH radical scavenging activity of PLBP was detected. In addition, after the oral administration of LPBP to animals for 30 days, levels of malondialdehyde (MDA) and antioxidant enzymes activities in skins markedly reduced or increased in aged animals. The results clearly demonstrated that PLBP possessed an antioxidant activity and protective effect against skin oxidative injury.

1. Introduction

The skin is the barrier which protects organisms from an environment that is becoming increasingly hostile. Endogenous antioxidants protect tissue from the effects of oxidative stress; however, the levels of antioxidants in the tissue decrease with age (Venditti, Scirè, Tanfani, Greci, & Damiani, 2008), thus resulting in less protection and a greater potential for tissue damage (Lau, Bagchi, Zafra-Stone, & Bagchi, 2009). With advancing age and thus total exposure, oxidative stress begins to overwhelm the tissue's capacity to respond. This causes subtle changes that accumulate, accelerating the aging process and resulting in a state of chronic inflammation (Jenkins, 2002). Furthermore, ROS are known to be involved in many skin disorders such as skin cancer, cutaneous autoimmune diseases, xeroderma pigmentosum, and skin aging (Giacomoni & D'Alessio, 1996).

Much attention has recently been focused on naturally occurring antioxidants, in particular botanical polysaccharides, that provide effective protection from age-induced oxidative damage (Angerhofer, Maes, & Giacomoni, 2009; Lin, Wang, Chang, Stephen Inbaraj, & Chen, 2009; Subathra, Shila, Devi, & Panneerselvam, 2005). *Lycium barbarum* L. is perennial foliage plants endemic to Korea, Japan, and China and is widely used in medicine (Park, Kim, Lee, Sung, & Lim, 1996). It has been widely used in these countries for medicinal purposes and as a functional food for more than 2500 years (Shi, Jia, & Dong, 1997; Wen, Chung, Chou, Lin, & Hsieh, 2006). In support of such traditional properties, modern studies indicate that polysaccharides from *Lycium barbarum* possess a range of biologic activities, including antioxidant properties (Amagase & Nance, 2008; Chang & So, 2008).

There are, however, few reports about age-related changes in oxidative damage to skin throughout the aging process. In order to clarify how oxidative damage in skin accumulates during aging, we examined age-related changes and antioxidant activity of PLBP.

2. Materials and methods

2.1. Materials

Dried fruits of *Lycium barbarum* L. in the family Solanaceae, commonly called Gouji, were produced from NingXia province, China. The materials were air-dried at room temperature. Other reagents were analytical grade as commercially available.

2.2. Polysaccharides extraction

Dried fruits of *Lycium barbarum* (200 g) was defatted in a Soxhlet apparatus with petroleum ether (boiling point: 60–90 °C) and pre-

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treated with 80% ethanol twice to remove some coloured materials, monosaccharides, oligosaccharides, and small molecule materials. The organic solvent was volatilized and the pretreated dry powder was obtained and immersed in distilled water for 2 h. The suspensions were extracted with water under reflux for 4 h. The water phase was filtered and freeze-dried. All the polysaccharides were stored at −20 °C.

2.3. Purification of polysaccharide

The crude polysaccharide was further purified by column chromatography. Fifty milligrams of crude polysaccharide dissolved in 10 ml of dH_2O was applied to a DEAE–cellulose column (3 cm × 45 cm) pre-equilibrated with water and eluted in NaCl gradient (0–3 M) until no carbohydrate is detected. Each fraction was assayed for carbohydrates content by phenol–sulfuric acid method (Taylor, 1995). The carbohydrate-positive fractions were pooled together and dialyzed (MWCO 14,000) for 24 h against dH_2O and lyophilized. Seven small fractions and one major fraction were observed. The major fraction was taken as purified *Lycium barbarum* polysaccharides and used in animal test.

2.4. Molecular weight detection

Molecular weights of PLBP were determined by high performance size exclusion chromatography. Superdex column (30 cm × 0.32 cm i.d., Pharmacia PC 3.2/30, Uppsala, Sweden) and a Waters Model 590 pump were used. The column was maintained at 35 °C, and the mobile phase was 0.7% (w/v) Na_2SO_4 buffer with flow rate of 0.5 ml min^{-1}. Samples were filtered through 0.45 μm filter membrane before analysis. The samples were injected with an injector Perkin-Elmer ISS 100 and the injection volume was 25 μl. The detection was carried out at 35 °C with a refractive index detector (Agilent 1100 Series). Column calibration was performed with standard dextran, and calculations of molecular weights were carried out using Turbochrom software (Perkin-Elmer, Norwalk, CT, USA).

2.5. FT-IR

The spectra were collected on a Nicolet Magna 750 FT-IR spectrometer with a DTGS detector. The samples were pressed into KBr pellets (2 mg of sample per 200 mg of KBr). 256 scans at a resolution of 4 cm^{-1} were averaged. A blank KBr disk was used as background.

2.6. DPPH radical scavenging activity

The free radical scavenging activity of the extracts, based on the scavenging activity of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical, was determined by the method described by Braca et al. (2001). Plant extract (0.1 ml) was added to 3 ml of a 0.004% MeOH solution of DPPH. Absorbance at 517 nm was determined after 30 min, and the percentage inhibition activity was calculated from $[(A_0 - A_1)/A_0] \times 100$, where A_0 is the absorbance of the control, and A_1 is the absorbance of the extract/standard.

2.7. Animals groups and treatments

Male rats 360 ± 20 g (more than 24 months old) or 145 ± 23 g (2 months old) were purchased from Small Animal Breeding Centre, Shenyang, China and were kept for a week under environmentally controlled conditions with free access to standard food and water ad libitum. Animals were handled according to the rules and regulations of Institutional Animal Ethics Committee (IAEC), China Medical University, China.

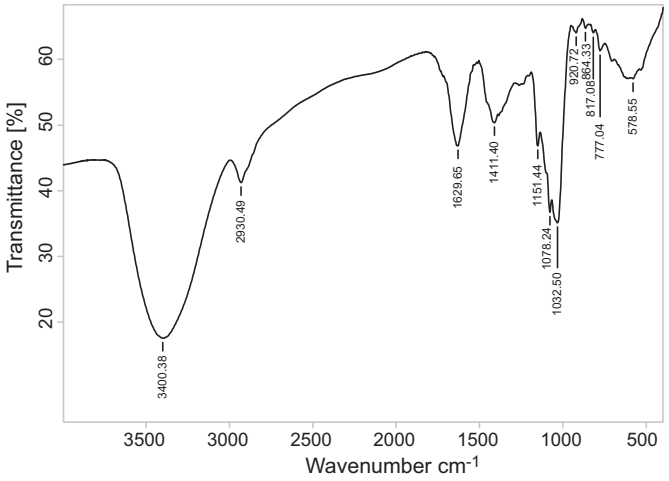

Fig. 1. FT-IR spectra of PLBP.

Animals weighing approximately 145 ± 23 g (nearly 2 months) were taken as normal young control (Group I). Animals weighing approximately 360 ± 20 g (more than 24 months old) considered as aged (Group II) and were divided into three groups: untreated model control; low (200 and 400 mg/kg B.W.); high doses of polysaccharides groups (Groups III and IV) of six animals each. Two groups of aged animals received polysaccharides. The polysaccharides were administered orally once daily for 30 days. Similarly animals in the young and aged control groups were administered with distilled water. Twenty-four hours after the last dose, animals were sacrificed by decapitation. Blood was collected from abdominal aorta into dried tubes and centrifuged at 4 °C, 1000 × *g* for 15 min. Serum was separated to determine serum antioxidant enzyme activities.

The tissues MDA concentration was determined using the method described by Jain, McVie, Duett, and Herbst (1989), based on TBA reactivity. The superoxide dismutase (E.C.1.15.1.1) level was determined using a method of Asada, Takahashi, and Nagate (1974). The enzyme catalase (CAT; EC 1.11.1.6) converts H_2O_2 into water. Catalase activity in tissue supernatant was measured spectrophotometrically at 240 nm by calculating the rate of degradation of H_2O_2, the substrate of the enzyme (Xu, Yuan, & Lang, 1997). GSH-Px activity was determined with a GSH-Px Assay Kit A005 (Institute of Biological Engineering of Nanjing Jianchen, Nanjing, China).

2.8. Statistical analysis

The results are expressed as mean and standard error. The means were compared for differences among completely random treatments using ANOVA. Duncans New Multiple Range Test was used for comparison. The differences were considered to be statistically significant when $P < 0.05$.

3. Result

3.1. Chemical analysis of PLBP

The average molecular weight of PLBP was calculated to be 1.21 × 105 cmDa. The characteristic absorptions of PLBP were identified in the FT-IR spectra (Fig. 1). The carbohydrate-related absorbances appeared at 3400.38, 2930.49, 1629.66, 1411.40, 1151.44, 1078.24, 1032.50, 920.72, 864.33, 817.08, and 777.04 cm^{-1}. The signal at 3400.38–2930.49 cm^{-1} might correspond to the bending vibration of C–H, C=C bonds. The absorbance at 1629.66 cm^{-1} can be assigned to CH_3–, CH_2– groups. The small

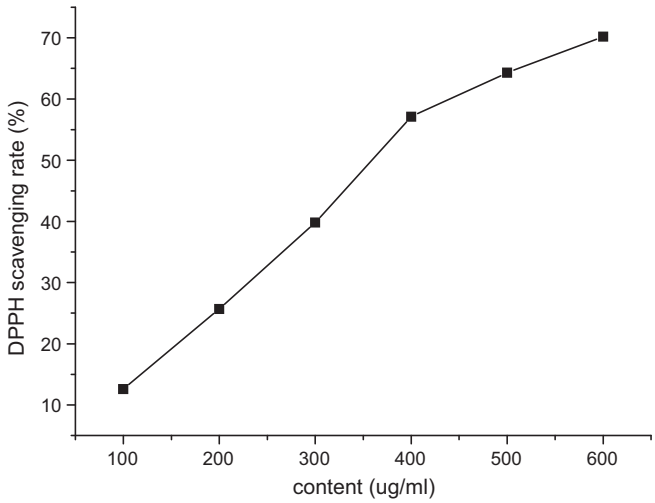

Fig. 2. Scavenging activity of PLBP against 1,1-diphenyl-2-picrylhydrazyl radical.

sharp band at 920 cm^{-1} is characteristic of β-glycosidic linkages between the sugars, indicating a β-glycosidic linked polysaccharides.

3.2. DPPH radical scavenging activity

DPPH is usually used as a reagent to evaluate free radical scavenging activity of antioxidants (Oyaizu, 1986). DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule (Soares, Dins, Cunha, & Ameida, 1997). The reduction capability of DPPH radical is determined by the decrease in absorbance at 517 nm induced by antioxidants. The extracts are able to reduce the stable radical DPPH to the yellow-coloured diphenylpicrylhydrazine. From Fig. 2 we observe that a dose–response relationship is found in the DPPH radical scavenging activity; the activity increased as the concentration increased for PLBP. The involvement of free radicals, especially their increased production, appears to be a feature of most, if not all human diseases, including cardiovascular disease and cancer (Deighton, Brennan, Finn, & Davies, 2000). It has been found that cysteine, glutathione, ascorbic acid, tocopherol, flavonoids, tannins, and aromatic amines (p-phenylene diamine, p-aminophenol, etc.), reduce and decolourise DPPH by their hydrogen donating ability (Blois, 1958; Yokozawa et al., 1998). We supposed that phenolic compounds of the PLBP are probably involved in their antiradical activity.

3.3. In vivo antioxidant activity

Effect of PLBP on MDA and GSH in tissue of animals is shown in Fig. 3. When compared to control (healthy animals), MDA and GSH levels increased markedly or reduced in untreated aged control animals. After the treatments of PLBP for 30 consecutive days, there was a significant decrease or decrease in MDA levels in PLBP-treated animals' tissue ($P < 0.01$).

In untreated aged control group, SOD, CAT and GSH-Px activities in tissue were significantly reduced when compared with normal control group. Administration of PLBP provided a marked suppression on oxidative injury caused by aging, and high dose of PLBP illustrated a significant effect in the increase of antioxidant enzymes activities (Fig. 4).

4. Discussion

$DPPH^-$ is a free radical compound and has been widely used to test the free radical-scavenging ability of various samples (Hatano, Takagi, Ito, & Yoshida, 1997; Sakanaka, Tachibana, & Okada, 2005; Shimoji et al., 2002). It is a stable free radical with a characteristic absorption at 517 nm, was used to study the radical-scavenging effects of extracts. As antioxidants donate protons to this radical, the absorption decreases. DPPH, which shows absorption at 515 nm, is reduced to the corresponding hydrazine when it reacts with hydrogen donors and this can be detected as a decrease of absorption (Brand-Williams, Cuvelier, & Bersec, 1995). To evaluate the scavenging effects of $DPPH^-$ of PLBP, $DPPH^-$ inhibition was investigated. Results indicated that PLBP could significantly scavenge DPPH radicals in a dose-dependent manner.

A free radical is any atom or molecule that is missing an electron in its outer shell. This free radical will attack and destroy other necessarily healthy atoms to get its missing electron (Santiard, Ribière,

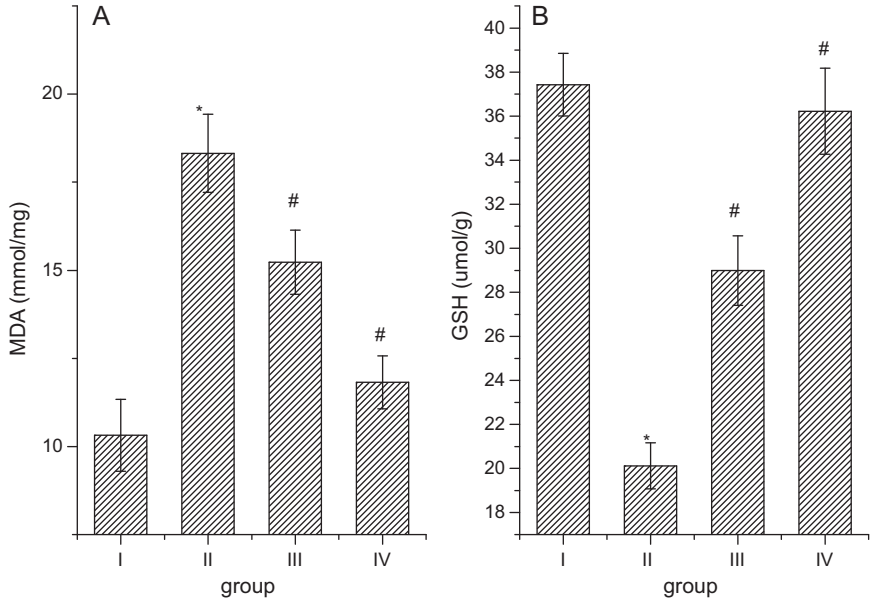

Fig. 3. (A) Blood MDA levels in skin of untreated and PLBP-treated animals and (B) blood GSH levels in skin of untreated and PLBP-treated animals. Values are expressed as mean ± S.D. for six rats in each group. $^*P < 0.01$, comparisons are made between Group I and Group II. $^\#P < 0.01$, comparisons are made between Groups III, IV and II.

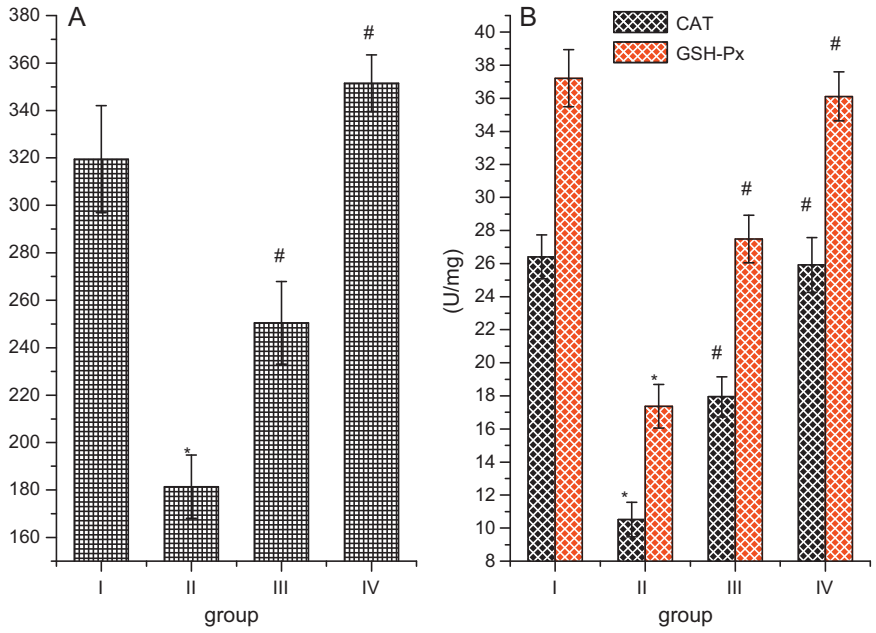

Fig. 4. (A) Activities of SOD in skin of untreated and PLBP-treated animals and (B) Activities of CAT and GSH-Px in skin of untreated and PLBP-treated animals. Values are expressed as mean ± S.D. for six rats in each group. Units: SOD: units/mg; CAT: micromoles of H_2O_2 consumed/min/mg protein; GSH-Px: micromoles of GSH oxidized/min/mg protein. $^{*}P < 0.01$, comparisons are made between Group I and Group II. $^{\#}P < 0.01$, comparisons are made between Groups III, IV and II.

Nordmann, & Holiee-Levin, 1995). These free radicals can accumulate and cause oxidative damage. This oxidative damage causes biological organisms to age. In other words, free radicals turn the oils of our skin rancid which in turn damages the collagen in the skin (Takeshita, Chi, Hirata, Ono, & Ozawa, 2006). Collagen is the protein in fibers that serve as the building blocks of our skin. In fact, free radicals have even been associated with skin cancer and premature skin-aging due to sun exposure (which speeds up this free radical damage) (Nguyen, Maquart, & Monboisse, 2005; Kerkvliet, Jansen, Schoenmaker, Beertsen, & Everts, 2003; Adesina, Japhet, Donbraye, Kumapayi, & Kudoro, 2009). Scientists are now focusing their efforts on understanding how free-radicals work what effects they have on the body. For those interested in how free-radicals affect the skin, let it be said that free-radicals are definitely harmful to the skin. Free-radicals initiate the deterioration of the skin's structural support and decrease the elasticity, resilience, and suppleness of skin. They are often tagged as the culprit in the case of wrinkles, loss of skin elasticity and suppleness (Komsa-Penkova, Koynova, Kostov, & Tenchov, 2000).

Human skin, which is directly exposed to environmental events that may give rise to the formation of ROS, is equipped with enzymatic and non-enzymatic defense mechanisms to modulate ROS levels. There have been many reports demonstrating the increase in oxidative damage during aging (Perchellet & Perchellet, 1989; Mamic, Holman, Roberts-Thomson & Monteith, 2000; Keramat, Kalantari, & Arvin, 2009). In this study, we observed that the contents of MDA increased linearly in animals during aging. Antioxidants helped to remove the free radicals and their damage from these cells. That is, they slowed down and could even stop this oxidative damage that caused the skin to age. Our results showed that PLBP treatment reduced skin MDA level.

Although no data concerning the age-related changes in the antioxidant enzyme activities in animal skin have been reported, there are many reports about changes in these activities in other organs. For example, it has been observed that the activities of SOD, catalase, and GSH-Px decrease with age in brain, hepatocytes, and kidneys from male animals (Santiago, Osato, Liu, & Mori, 1993). As for the effect of age on antioxidant enzyme activities in the skin, GSH-Px activity has been found to decrease with age in mouse tissue (Lopez-Torres, Shindo, & Packer, 1994). In this study, the activities of SOD, catalase and GSH-Px in skin underwent much change during aging. Since significant decreases in the antioxidant enzyme activities were observed, the repair system that protects against oxidative damage to biological molecules may become weak in aged animals.

Chinese herbs have been used in clinical applications for many centuries. Driven by rising consumer interest and demand for natural products, there has been great interest and attention in the application of Chinese herbs in the development of tissue care cosmetics (Aburjai & Natsheh, 2003). Currently, various Chinese herbs are used in tissue care cosmetics and claimed that they can improve the physical appearance of aged tissue. Individual polysaccharides isolated from *Lycium barbarum* (LBP) (Huang, Tian, Wang, Dong, & Wu, 2001; Peng & Tian, 2001) have shown various effects including antioxidant activities. The bioactive components of *L. barbarum* fruit have been mainly attributed to its polysaccharide–protein complex (LBP), which contains several fractions separated by ion exchange chromatography and size exclusion chromatography (Bensky & Gamble, 1993; Huang, Lu, Shen, & Lu, 1999; Li, Yang, Ren, & Wang, 2002; Wang et al., 2010; Xin et al., 2007; Zou, Zhang, Yao, Niu, & Gao, 2010). LBP have been also reported to increase the destruction of free radicals (Ni, Qing, Kaisa, & Lu, 2004; Rhee, Park, & Cho, 2009). The results of the other study indicated that LBP protect the structure of the murine seminiferous epithelium (Wang et al., 2002). A study on testicular degeneration in vitro showed that, although oxygen radicals significantly damaged the shape of the murine seminiferous epithelium, the addition of LBP prevented this damage (Huang et al., 2001; Yu et al., 2005). Thus, one of the mechanisms of action of LBP may be direct protection of membranes from oxygen radical damage. The results of the present study demonstrated, for the first time, that treatment of animal with PLBP effectively protected the animals against age-induced oxidative damage, as evidenced by decreased tissue MDA elevated the antioxidants levels. Moreover, PLBP treatment still markedly reduced skin antioxidant enzyme activities.

In conclusion, ageing generates ROS, including H_2O_2 and O_2^-, by increasing lipid peroxidation level and decreasing antioxidant enzymes activities in aged animals' skin. Because free radicals are implicated in many diseases and age-related conditions, the antioxidant dependent actions of *Lycium barbarum* may have a wide range of beneficial effects. The PLBP may support health by increasing endogenous factors, such as SOD and GSH-Px, reducing the MDA level and protecting skins from oxygen radical-mediated damage.

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