Carbohydrate Polymers 90 (2012) 1642–1647

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

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

Characterization and anti-allergic effect of a polysaccharide from the flower buds of *Lonicera japonica*

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ARTICLE INFO

Article history:
Received 1 May 2012
Received in revised form 1 July 2012
Accepted 16 July 2012
Available online 22 July 2012

Keywords:
Lonicera japonica
Polysaccharide
Characterization
Anti-allergic effect
Allergic contact dermatitis

ABSTRACT

A water-soluble polysaccharide (LJP-1), with a molecular weight of 1.8×10^5 Da, was isolated from the flower buds of *Lonicera japonica*. Gas chromatography (GC) analysis showed that the LJP-1 was mainly composed of D-glucose and a small amount of D-arabinose. On the basis of methylation analysis, LJP-1 had the backbone chain mainly consisting of 1,6-linked Glc and 1,3,6-linked Glc, which was terminated with 1-linked Ara residues at the O-3 position of 1,3,6-linked Glc in a relative molar ratio of 2.9:1:0.9. The anti-allergic effect of LJP-1 was evaluated on allergic contact dermatitis (ACD) induced by picryl chloride (PC) in mouse ear. Similar to prednisolone, orally administrated LJP-1 (20, 40 and 80 mg/kg) potently inhibited the PC-induced ACD, leading to substantial reductions in ear thickness, serum level of IgE and histamine, as well as tissue TNF-α. These results demonstrate that treatment with LJP-1 may be effective for preventing the development of PC-induced ACD.

1. Introduction

Allergic contact dermatitis (ACD) is a form of contact dermatitis that is the manifestation of an allergic response caused by contact with a specific allergen to which the patient has developed a specific sensitivity, which was characterized by varying degrees of erythema, edema, and vesiculation in the skin. ACD is accepted to be the most prevalent form of immunotoxicity found in humans (Kimber, Basketter, Gerberick, & Dearman, 2002). The mechanisms by which these reactions occur are complex, with many levels of fine control. The main treatment to cure ACD is avoiding the responsible allergen. In the case of drugs, the patient should be treated with other active drugs without potential cross-reactions. While cross-reactions are not always evident, if the disorder is diagnosed early and the allergen avoided, the prognosis is often good (Belsito, 2000). A wide range of anti-inflammatory substances is available for the treatment in the case of contact dermatitis. However, most of them (e.g. corticosteroids) have a high level of collateral and considerable side effect (Thiers, 1989). The interest for new potential active agents against contact dermatitis remains high, and natural products provide an important source for a wide range of potential new agents (Heinemann, Schliemann-Willers, Oberthür, Hamburger, & Elsner, 2004; Itoh, Masuda, Naruto, Murata, & Matsuda, 2009; Lee et al., 2007; Sun et al., 2007).

The flowers and buds of *Lonicera japonica* has been well known as a traditional Chinese medicine and is typically used in the treatment of a wide range of ailments, including fever, headache, upper respiratory tract infections, urinary disorders, rheumatoid arthritis, and diabetes mellitus (Ko, Wei, & Chiou, 2006; Kwak et al., 2003; Ubukata et al., 1992) Pharmacological studies have indicated that the extract of these flower buds have a broad spectrum of biological activities, including antibacterial, anti-inflammatory, antioxidant, antipyretic, antiviral and hepato-protective effects (Oku, Ogawa, Iwaoka, & Ishiguro, 2011). Since 1995, *L. japonica* has been listed in the Pharmacopoeia of the People's Republic of China and more than 500 prescriptions containing *L. japonica* have been used to treat various diseases in China (Shang, Pan, Li, Miao, & Ding, 2011). More than 140 compounds such as alkaloids, cerebrosides, flavonoids, iridoids, polyphenols, and triterpenoid saponins have been isolated and identified from various parts of this plant so far (Lin et al., 2008; Shang et al., 2011). Recently, more and more experiments showed different extracts and constituents of *L. japonica* can inhibit various inflammatory reactions, and suppress various inflammatory factors (Jin, Lim, Kwon, Son, & Kim, 2010; Kang, Choi, Lee, & Kwon, 2010; Lee, Kim, Kim, Lee, & Kang, 2010; Oku et al., 2011). All of these reports supported the traditional use of *L. japonica*, and suggested it be a safe and mild anti-inflammatory agent for treating various inflammatory disorders. The anti-inflammatory activities of *L. japonica* are mainly limited to its small molecules or crude extract, and mainly evaluated in an in vitro study. There are not

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http://dx.doi.org/10.1016/j.carbpol.2012.07.044

sufficient evidences to support its efficacy and action mechanism. Since inflammation is a partial manifestation of the allergic response, we investigated the possible anti-allergic effects of *L. japonica* constituents. However, the anti-allergic effects of polysaccharide from this plant are not yet known. To expand a new anti-allergic medicinal resource, here we are reporting the pharmacological properties of polysaccharide the flower buds of *L. japonica* relating to anti-allergic effects using in vivo animal models.

2. Materials and methods

2.1. Plant materials and chemicals

The flower bud of *L. japonica* was purchased from Anguo Chinese Medicine Market (Hebei, China), and was identified by one of the authors (T.J.), according to the identification standard of Pharmacopeia of the People's Republic of China. Picryl chloride (PC), prednisolone, DEAE-cellulose, the monosaccharide standards, trifluoroacetic acid (TFA) and bovine serum albumin (BSA) were purchased from Sigma Co. (St Louis, MO, USA). Fetal bovine serum and RPMI-1640 medium were purchased from Gibco Invitrogen Co (Grand Island, NY, USA). Sephacryl S-300 was purchased from General Electric Co. All others chemicals and reagents used were of analytical reagent grade.

2.2. Preparation of the polysaccharide fraction from L. japonica

The dry flower buds of *L. japonica* (0.5 kg) were firstly refluxed with 95% EtOH at 70 °C to remove lipophilic compounds, and then successively extracted 4× with 10 vol. of distilled water at 100 °C for 3 h. After centrifugation (1700 × *g* for 10 min, at 20 °C), the supernatant was concentrated in a rotary evaporator, and was deproteinated with the sevag method (Alam & Gupta, 1986), followed by exhaustive dialysis with water for 48 h. Then the crude polysaccharide fraction (CLJP, 23.5 g) was obtained through precipitation with 3 vol. of ethanol and desiccation in vacuo. The CLJP was redissolved in distilled water, centrifuged, and then the supernatant and applied to a DEAE-cellulose column (40 cm × 2.6 cm). The column was first eluted with distilled water followed by 0.3 M and then 0.5 M NaCl. The fractions obtained were combined according to the total carbohydrate content quantified by the phenol–sulfuric acid method. The distilled water eluted fraction was further fractionated on a Sephacryl S-300 column (100 cm × 2.6 cm) eluted with 0.1 M NaCl to yield one completely separated fractions. This main fraction was collected, dialyzed and lyophilized to get a white purified polysaccharide (LJP-1, 578 mg, 2.5% of the crude polysaccharide).

2.3. General methods

The protein concentration of polysaccharide was measured by the Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951), with BSA as the standard. The total carbohydrate content was determined by the phenol–H_2SO_4 method, with glucose as the standard (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). Uronic acid contents were determined by measuring the absorbance at 525 nm using the *m*-hydroxybiphenyl colorimetric procedure and with D-glucuronic acid as the standard (Filisetti-Cozzi & Carpita, 1991). The specific rotation was determined at 20 ± 1 °C with an automatic polarimeter (Model WZZ-2B, China). UV–vis absorption spectra were recorded with a Shimadzu MPS-2000 spectrophotometer between 190 and 290 nm. Gas chromatography (GC), used for identification and quantification, was performed on a Vavian 3400 instrument (Hewlett–Packard Component, USA) equipped with DM-2330 capillary column (30 m × 0.32 mm × 0.2 μm) and flame-ionization detector (FID). The column temperature was kept at 120 °C for 2 min and then increased to 250 °C for 3 min at a rate of 8 °C/min The injector and detector heater temperature were 250 and 300 °C, respectively. The rate of N_2 carrier gas was 1.2 mL/min (Wang, Luo, & Liang, 2004). Gas chromatography–mass spectrometry (GC-MS) was done on a Shimadzu QP-2010 instrument (Shimadzu, Japan) with an HP-5MS quartz capillary column (30 m × 0.25 mm × 0.25 μm).

2.4. Carbohydrate content and monosaccharide composition of polysaccharide

GC was used for identification and quantification of the monosaccharides. Polysaccharide (10 mg) was hydrolyzed with 2 M TFA at 100 °C for 2 h (Sun et al., 2008). The monosaccharides were conventionally converted into the alditol acetates and were analyzed by GC.

2.5. Determination of molecular weight

The molecular weight of the polysaccharide was determined by a high-resolution size-exclusion chromatography (HRSEC) system with a refractive index detector (RID). An aliquot of 20 μL the sample solution (0.5%) was performed on a SHIMADZU HPLC system fitted with one TSK-G3000 PW_{XL} column (7.8 mm ID × 30.0 cm), eluted with 0.1 mol/L Na_2SO_4 solution at a flow rate of 0.5 mL/min and detected by a SHIMADZU refractive index detector (RAD-10A). A standard calibration curve was prepared to determine the molecular weight of the polysaccharide using T-series Dextran (T-130 80, 50, 25, 10) as standards.

2.6. Fourier-transform infrared (FT-IR) spectroscopy

IR spectroscopy was used to investigate the vibrations of molecules and polar bonds between the different atoms. Structures of polysaccharides, such as monosaccharide types, glucosidic bonds and functional groups, could be analyzed using FT-IR spectroscopy. The purified polysaccharides were ground with KBr powder and then pressed into a polymer film for FT-IR measurement (4000–400 cm^{-1}) in a Nicolet 5700 IR spectrometer (Nicolet Instrument Corp., Madison, WI).

2.7. Methylation analysis

The sample (20 mg) was methylated 3 times, according to Needs and Selvendran (1993). Complete methylation was confirmed by the disappearance of the OH band (3200–3700 cm^{-1}) in the IR spectrum. The methylated products were hydrolyzed, then reduced and acetylated as described by Sweet, Shapiro, and Albersheim (1975). The partially methylated alditol acetates were analyzed by GC–MS under the same chromatographic conditions described above.

2.8. Animals

Female ICR strain mice (25–30 g) were provided by the Experimental Animal Center of Harbin Medical University and maintained under SPF (specific pathogen free) conditions at 23 ± 3 °C with 50 ± 10% humidity. The mice were fed a laboratory rodent diet and water ad libitum with a 12 h/12 h light/dark cycle. All animal experiments were approved by the Institutional Animal Care and Use Committee of Harbin Medical University.

2.9. Picryl chloride (PC)-induced contact dermatitis and treatment with poilysaccharide in experimental animals

Contact dermatitis was induced in mouse ear according to the previous method of Asherson and Ptak (1968), with some

modification. Groups of animals ($n = 8$) were sensitized by topical application of 0.1 mL of 7% picryl chloride solution in EtOH to the shaved abdomen. Three days after sensitization, they were challenged on the right ear with 30 μL of 3% picryl chloride in olive oil (the first PC challenge). Polysaccharide (20, 40, 80 mg/kg body weight) or prednisolone (positive control, 3 mg/kg body weight) suspended in normal saline was administered orally twice immediately before and 15 h after the first PC challenge in ear. The control group was treated with 0.2 mL of normal saline for the same way. Ear thickness was measured immediately before and 24 h after the first PC challenge and the difference in the thickness was calculated and expressed in percent value compared to the control group. After the last measurement of ear thickness (24 h after the first PC challenge), the mice were killed by cervical vertebrae dislocation, and three organs (thymus, spleen, and adrenal gland) were excised. The weights of the organs were measured and expressed in a ratio of organ weight to 10 g body weight of mouse.

2.10. Determination of serum total IgE and histamine levels

After blood was collected from the sacrificed mice, serum samples were obtained by centrifugation (1700 × *g*, 10 min) and were stored at −70 °C until use. Serum concentrations of IgE and histamine were determined with an IgE ELISA kit (R&D, USA) and histamine ELISA kit (BD Biosciences Pharmigen, USA), respectively, according to the manufacturers' instructions.

2.11. Measurement of ear tissue TNF-α level

Amounts of cytokines in skin lesions were determined by ELISA. Skin biopsies, cut into small pieces, were homogenized vigorously with a 30-fold volume of potassium phosphate buffer containing 0.1% Tween-20, 100 μg/mL, and aprotinin 2 μg/mL. The homogenates were frozen in nitrogen, thawed in a 37 °C water bath, sonicated for 10 s, and centrifuged for 10 min at 12,000 × *g*. After centrifugation, TNF-α in the supernatant was determined by ELISA in accordance with the protocol provided by the manufacturer. Briefly, 100 μL homogenized supernatants for TNF-α was incubated with the monoclonal capture antibody for 2 h at room temperature and then washed five times. Polyclonal conjugated antibody was added again and incubated for 2 h. It was then washed to remove all unbound material and the substrate solution was added, which was acted upon by the bound enzyme to produce color. The optical density at 450 nm was measured in an ELISA microplate reader.

2.12. Statistical analysis

All the data from the in vivo experiment were expressed as mean ± SE, and the statistical significance was determined using Student's *t*-test. A *P* value less than 0.05 was considered statistically significant.

3. Results

3.1. Isolation, purification and characterization of polysaccharide

The yield of the crude water-soluble polysaccharide from the flower buds of *L. japonica* was 4.7% of the dried material. The crude polysaccharide was separated and sequentially purified through DEAE-cellulose anion-exchange and Sephacryl S-300 gel filtration chromatography, each giving a single elution peak, as detected by the phenol–sulfuric acid assay. The main fraction (LJP-1), with a yield of 2.5% of the crude polysaccharide, was collected for subsequent analyses chemical and anti-allergic effect. LJP-1 appeared as a white powder, which had a negative response to the Bradford test and no absorption at 280 and 260 nm in the UV spectrum,

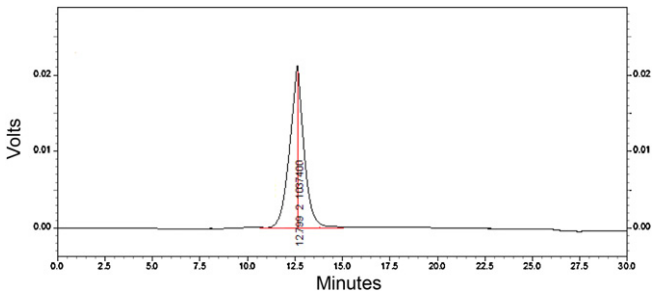

Fig. 1. HRSEC profile of LJP-1.

indicating the absence of protein and nucleic acid. The HRSEC profile (Fig. 1) showed a single and symmetrically sharp peak, indicating that LJP-1 was a homogeneous polysaccharide, with a weight-average molecular weight of ~1.8×10^5 Da. As determined by *m*-hydroxydiphenyl colorimetric method and GC, the polysaccharide did not contain uronic acid. The total sugar content of LJP-1 was determined to be 96.60% and LJP-1 was composed of predominant D-glucose monomers and a small amount of D-arabinose, as detected by GC of the alditol acetate derivatives of the components of the LJP-1 hydrolysate. The relatively high positive value of optical rotation $[\alpha]_D^{20} + 190$ (*c* 0.15, H_2O)] suggested the dominating presence of α-form glycosidic linkages in LJP-1 (Zhao, Kan, Li, & Chen, 2005).

The LJP-1 had IR bands at 1020–1180 cm^{-1}, 1370–1610 cm^{-1}, 2800–2900 cm^{-1}, and 3100–3700 cm^{-1}, which were distinctive absorptions of polysaccharides. The absorption band at 840 cm^{-1} confirmed the existence of α-glycosidic bond (Fig. 2).

The fully methylated product of LJP-1 was hydrolyzed, converted, and analyzed by GC/MS. As shown in Table 1, the GC–MS results revealed two types of glucose (Residue A: 1,6-linked Glc and Residue B: 1,3,6-linked Glc) and one type of arabinose derivatives (Residue C: 1-linked Ara) in a relative molar ratio of 2.9:1:0.9. We can conclude that the main backbone chain of LJP-1 was predominantly composed of Residue A and Residue B, which was branched at O-3 position of Residue B with Residue C.

3.2. The effect of polysaccharide on ear swelling

The immune reaction induced by using this procedure is characterized by swelling at the site of challenge and by an infiltration of monocytes, macrophages and lymphocytes into the epidermis and dermis (Malorny, Goebeler, Gutwald, Roth, & Sorg, 1990; Turk, 1980; Vadas, Miller, Gamble, & Whitelaw, 1975). Since a positive correlation between the intensity of the immune reaction and an increase in ear thickness has been reported (Kimber & Dearman, 1993; Phanuphak, Moorhead, & Claman, 1974), the ear thickness was measured as an index of skin inflammation. This model for skin delayed-type hypersensitivity reaction has been widely used to monitor cell-mediated immune responses in vivo (Thorne, Hawk, Kaliszweski, & Guiney, 1991; Turk, 1980). As shown in Table 2, application of PC to the ear of sensitized mice induced immediate type dermatitis with ear thickness increasing, with a relatively swelling increase of 82.5% in ear, compared to the former before the first PC challenge. When administered orally twice immediately before and 15 h after the first PC challenge in ear, LJP-1

Table 1
GC–MS data of alditol acetate derivatives from the fully methylated product of LJP-1.

Peak no.	Acetates of sugar	Molar ratio	Mode of linkage
Residue A	2,3,4,-O-Me_3-Glc*p*	2.9	1,6-linked Glc
Residue B	2, 4,-O-Me_3-Glc*p*	1.0	1,3,6-linked Glc
Residue C	2,3,5,-O-Me_3-Ara*f*	0.9	1-linked Ara

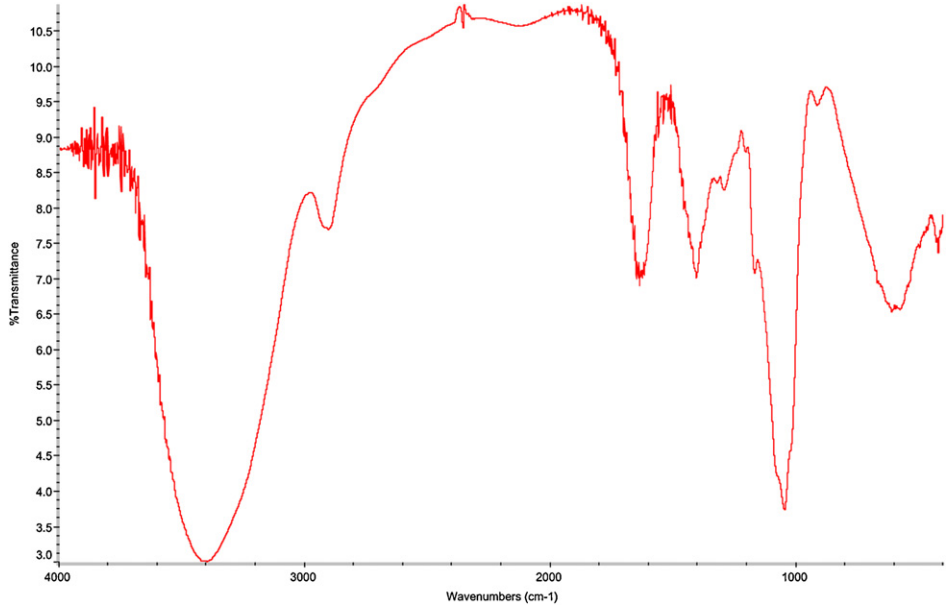

Fig. 2. The FT-IR spectra of LJP-1.

significantly inhibited ear swelling by 32.4, 42.9% and 49.6%, at the dose of 20, 40, and 80 mg/kg, respectively. Furthermore the positive drug, prednisolone, had a maximal inhibitory effect on the ear thickness.

3.3. The effect of polysaccharide on the body weight and the relative weight of thymus, spleen and adrenal gland

The body weights of the treated animals were recorded and no significant difference was found between the durg-treated groups and the control groups. In addition, LJP-1 and prednisolone did not affect the weights of thymus, spleen and adrenal gland (data not shown).

3.4. The effect of polysaccharide on serum IgE level

It has been known that mast cell activation and their histamine release are tightly modulated by IgE from B cells (Dvorak et al., 1985). Therefore, we measured the serum IgE levels in PC-treated mice. As shown in Fig. 3, serum IgE levels in the LJP-1 treated mice (309.2 ng/mL, even at the dose of 20 mg/kg) decrease significantly compared to the mice in control group (650.5 ng/mL) ($P < 0.01$). Especially at the dose of 80 mg/kg, LJP-1 displayed a more potent reduction in the level of serum IgE, which was comparable to the prednisolone treatment.

Table 2
Effect of polysaccharide and prednisolone on the ear thickness 24 h after the first PC challenge.

Groups	Dose (mg/kg)	Administration route	Swelling (%)	Inhibition (%)
Negative control	–	Oral	82.5 ± 3.6	–
Prednisolone	3	Oral	34.2 ± 1.9	58.5
LJP-1	20	Oral	55.8 ± 2.4	32.4*
	40	Oral	47.1 ± 2.1	42.9*
	80	Oral	41.6 ± 1.8	49.6*

Each value represents the mean ± SE ($n = 8$).
* $P \leq 0.01$, significantly different from the negative control group.

3.5. The effect of polysaccharide on serum histamine level

Histamine, a representative content of mast cell granules, exerts many effects related to the immediate phase of allergic inflammation, including vasodilation, increase vascular permeability, tissue erythema, bronchial and gastrointestinal contraction, and increased mucus production (Guo, Mochizuki, Morii, Kitamura, & Maeyama, 1997; White, 1999). To explore its anti-allergic mechanism, we test whether LJP-1 was capable of suppressing the release of this molecule (Hammerberg, Olivry, & Orton, 2001). Interestingly, this fraction remarkably inhibited the enhanced level of histamine induced by PC in a dose-dependent fashion (Fig. 4).

3.6. The effect of polysaccharide on the ear tissue TNF-α

TNF-α, a pro-inflammatory cytokine, plays a crucial role in both acute and chronic inflammation (Holtmann, Schuchmann, Zeller, Galle, & Neurath, 2002). In the early phase of contact hypersensitivity elicitation, the release of serotonin and TNF-α from mast

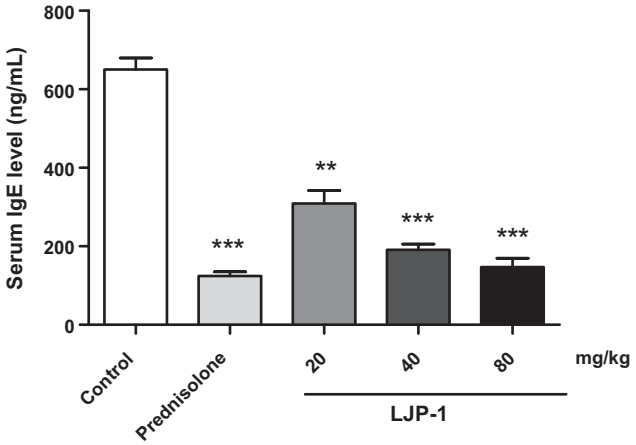

Fig. 3. Serum IgE concentrations in the indicated groups measured 24 h after the first PC challenge. Values are means ± SE. ($n = 8$). $^{**}P \leq 0.01$; $^{***}P \leq 0.001$ vs. model control.

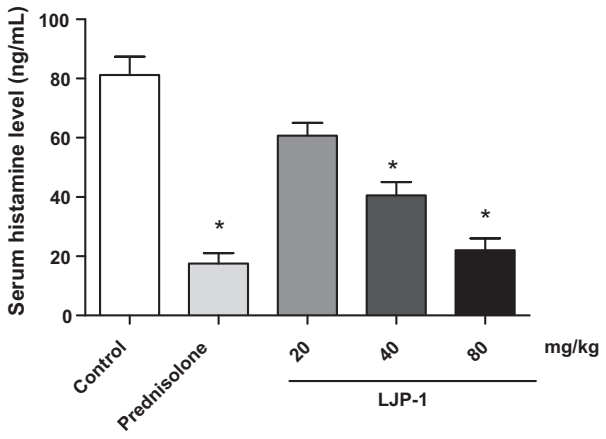

Fig. 4. Serum histamine levels in the indicated groups measured 24 h after the first PC challenge. Values are means ± SE ($n = 8$). $^{*}P \leq 0.05$, vs. model control.

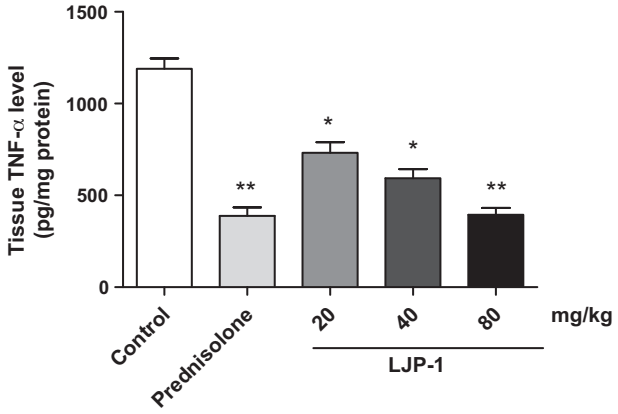

Fig. 5. The TNF-α level in ear tissue homogenates measured 24 h after the first PC challenge. Values are means ± SE ($n = 8$). $^{*}P \leq 0.05$; $^{**}P \leq 0.01$ vs. model control.

cells and platelets led to increased vascular permeability and tissue swelling (Askenase, Bursztajn, Gershon, & Gershon, 1980; Askenase et al., 1995; Van Loveren, Meade, & Askenase, 1983). When TNF-α is specifically blocked, the severity of inflammation is reduced. As shown in Fig. 5, LJP-1 administration significantly decreased tissue TNF-α level compared with the control group, but no difference between prednisolone and LJP-1 (80 mg/kg)-treated group was found. These results suggested that the anti-allergic effect of LJP-1 resulted from its reduction of TNF-α, thus reducing the permeability of endothelial cell monolayers to macromolecules and lower molecular weight.

4. Conclusion

Recently, herbal therapy has become increasingly popular due to the high acceptance of herbal drugs among the population and plant-based preparations with demonstrated clinical efficacy could be a suitable alternative in the treatment of chronic diseases, including ACD, although the mechanism by which these herbal medicines function is not yet fully established (Pinto et al., 2010; Prieto et al., 2008; Ríos, Bas, & Recio, 2005; Sun et al., 2007).

In the present study, we successfully purified and partially characterized a polysaccharide from the flower buds of *L. japonica*. The protective effect of LJP-1 against PC-induced ACD was examined in animal model. Our results indicated that LJP-1 suppressed the ear thickness of PC-induced mice, probably by controlling various Th1- and Th2-associated factors, namely the down-regulation of IgE and TNF-α. Of particular interest was a decrease in serum histamine in PC-induced mice. In addition, there was no toxic indication observed in the weights of body, thymus, spleen and adrenal gland after LJP-1 treatment. Whatever the actual mechanism(s) of LJP-1 was, the results from experiment suggested that LJP-1 had great potential as a safe and effective reagent to prevent the development of dermatitis in ICR mice. Given the fact that the prevalence of ACD is steadily increasing in all major developed countries and virtually no reagent is available for the fundamental treatment of ACD, further investigation into LJP-1 is warranted at the detailed molecular level and will be reported elsewhere.

Acknowledgments

The authors gratefully thank postgraduate Li-Juan He and Laboratory Technician Long Yu (Department of Dermatology, First Affiliated Hospital of Harbin Medical University) for technical assistance in the animal experiment.

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