Structural characterization and immunomodulatory effect of a polysaccharide HCP-2 from *Houttuynia cordata*

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A R T I C L E   I N F O

A B S T R A C T

Immunomodulation of natural polysaccharides has been the hot topic of research in recent years. In order to explore the immunomodulatory effect of *Houttuynia cordata* Thunb., the water extract was studied and a polysaccharide HCP-2 with molecular weight of 60,000 Da was isolated by chromatography using DEAE Sepharose CL-6B and Sephacryl S-400 HR columns. The structure characterization of HCP-2 was performed by Fourier transform infrared spectroscopy (FTIR), acidic hydrolysis, PMP derivation, HPLC analysis and nuclear magnetic resonance spectra (NMR). HCP-2 was elucidated as a pectic polysaccharide with a linear chain of 1,4-linked α-D-galacturonic acid residues in which part of the 6-carboxyl groups were methyl esterified and part of 2-hydroxyl groups were acetylated. The bioactivity assays showed that HCP-2 could increase the secretions of interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), macrophage inhibitory protein-1α (MIP-1α), macrophage inhibitory protein-1β (MIP-1β), and RANTES (regulated on activation, normal T cell expressed and secreted) in human peripheral blood mononuclear cells (PBMCs), which play critical roles in the innate immune system and shape the adaptive immunity. Our results implied that HCP-2 could be an immune enhancer.

1. Introduction

In recent years, polysaccharides from natural resources have attracted extensive attention due to their structural diversity (Caffall & Mohnen, 2009; Maxwell, Belshaw, Waldron, & Morris, 2012) and profound impacts on the immune system (Mazmanian & Kasper, 2006). Some of them have been shown to possess immune-potentiating activities. For example, β-glucans from *Ganoderma lucidum*, and *Astragalus* polysaccharide (APS) induce protective immune responses to prevent microbial invasion and eliminate malignant tumors (Brown & Gordon, 2001; Du et al., 2012). Presumably, these polysaccharides bind to different receptors such as Toll-like receptors (TLR) on macrophages, dendritic cells and other monocytes, and then activate them to release pro-inflammatory

factors, cytokines and chemokines which help the host to constitute an intensive immune response (Shao et al., 2004).

H. cordata is a flowering plant widely grown in Japan, Korea, and southern China. According to the Chinese Pharmacopeia, *H. cordata* is suggested to relieve lung-related symptoms such as lung abscess, phlegm, cough and dyspnea and is effective in treating pneumonia, infectious disease, refractory hemoptysis and malignant pleural effusion (Commision, 2010). In pharmacological studies, *H. cordata* has also been shown to possess anti-inflammatory, anti-allergic (Li, Zhou, Zhang, & He, 2011; Shao et al., 2004), anti-viral (Lau et al., 2008), anti-oxidative (Tian, Zhao, Guo, & Yang, 2011) and anti-cancer (Lai et al., 2010) activities.

Water extract of *H. cordata* has been reported to inhibit the infection of herpes simplex virus (HSV) through inhibition of NF-kB activation (Chen et al., 2011), as well as severe acute respiratory syndrome (SARS) through inhibition of SARS CoV 3C-like protease and RNA-dependent RNA polymerase (Lau et al., 2008). Moreover, production of pro-inflammatory cytokines and PGE_2 in rat macrophages (Kim, Park, Lim, & Kim, 2009), proliferation of mouse splenic lymphocytes and the proportion of $CD4^+$ and $CD8^+$ T cells in

rat (Lau et al., 2008) were all up-regulated by the water extract of *H. cordata*. However, few pharmacological studies on the polysaccharides of *H. cordata* have been performed (Tian et al., 2011).

The present study aimed to isolate and characterize a polysaccharide from the water fraction of *H. cordata* and to evaluate its immunomodulatory activities on human peripheral blood mononuclear cells (PBMCs). The chemical structure of the polysaccharide was elucidated using acid hydrolysis, PMP derivation, infrared (IR) and nuclear magnetic resonance (NMR) analysis. Its biological responses on immune system such as pro-inflammatory factors and cytokines were evaluated.

2. Materials and methods

2.1. Chemicals and materials

Culture medium RPMI-1640, fetal bovine serum (FBS), penicillin, streptomycin, and phosphate-buffered saline (PBS) were purchased from Invitrogen (NY, USA). Ficoll-Paque TM was obtained from GE healthcare (UK). Phytohaemagglutinin (PHA), polymyxin B, 2.3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide (XTT), N-methyl dibenzopyrazine methyl sulfate (PMS), 1-phenyl-3-methyl-5-pyrazolone (PMP), trypan blue, dextrans and standard monosaccharides were purchased from Sigma Chemical Company (MO, USA). Trifluoroacetic acid was purchased from Applied Biosystems (NY, USA). BCA Protein Assay Reagent was purchased from Thermo Scientific (IL, USA). The ELISA kits for IL-1β, TNF-α, and antibodies against CD3, CD4 and CD8 were purchased from BD Biosciences (CA, USA). ELISA kits for MIP-1α, MIP-1β, and RANTES were purchased from R&D Systems (MN, USA). Chloroform, ethanol, methanol and acetonitrile were purchased from Lab-Scan (Thailand). DEAE Sepharose CL-6B, and Sephacryl S-500 were purchased from GE Healthcare Bio-Sciences AB (Uppsala, Sweden). Dialysis tubing (7000 Da cutoff) was purchased from Spectrum Laboratories Inc. (CA, USA).

2.2. Plant material

Dried *H. cordata* were purchased from a herbal shop in Hong Kong. Authentication was performed by morphological characterization and thin layer chromatography in accordance with the Chinese Pharmacopeia (Commision, 2010). Voucher specimen was deposited in the museum of the Institute of Chinese Medicine, The Chinese University of Hong Kong, with voucher specimen number: 2606C.

2.3. Isolation of HCP-2 from water extract of H. cordata

Aerial parts of *H. cordata* (1 kg) were powdered and extracted with boiling water (3 L) for 1 h. The extraction process was repeated for 3 times, and then subsequently the three batches of extract were combined together and centrifuged at 4000 rpm for 20 min. The supernatant was then concentrated and precipitated with 80% ethanol (4 times of volume) overnight at 4 °C. After centrifugation, the pellet was dissolved in double-distilled water and deproteinized with Sevage reagent (CHCl$_3$/BuOH = 4:1, v/v) for 15 min, and the procedure was repeated for seven times. Finally, the extract was centrifuged to remove insoluble materials, and the supernatant was lyophilized to give the crude *H. cordata* polysaccharide (namely HCP, 88.2 g).

A portion of HCP (40 g) was dissolved in water (200 ml) and was loaded onto a DEAE Sepharose CL-6B column (5.0 × 70.0 cm) and eluted with distilled water, 0.1, 0.2, 0.4 M NaCl, and 1.0 M NaCl containing 0.2 M NaOH solution sequentially (each eluant of 3 L). The 0.2 M NaCl fraction was collected for further purification by gel chromatography with Sephacryl S-500 HR eluted with water. A polysaccharide (namely HCP-2) was eventually obtained.

2.4. Characterization of HCP-2

2.4.1. Determination of the purity and molecular weight of HCP-2

The relative molecular weight and the polysaccharide composition in HCP-2 were analyzed according to the method described previously (Han et al., 2012). In brief, after filtration through a 0.45 μm filter, HCP-2 and T-series dextran standards (MW: 2000, 670, 410, 270, 150, 80, 50, 12, 5 and 1 kDa) were subjected to HPLC analysis with a TSK-Gel G3000SWxl column (7.8 mm × 300 mm, 5 μM, Tosoh Bioscience LLC, PA, USA). The column was eluted with water at a flow rate of 0.8 ml/min with evaporative light scattering detector (ELSD). The retention time and the molecular weights of T-series dextran standards were calculated based on a linear equation to determine the molecular weight of HCP-2.

2.4.2. Determination of optical rotation and protein amounts

The optical rotation of HCP-2 (0.35 g/ml) was determined with Perkin Elmer Polarimeter Model 341 at room temperature and of wavelength at 589 nm. The protein percentage of HCP-2 was determined by BCA protein assay.

2.4.3. Determination of the monosaccharide composition of HCP-2

The identification and quantification of monosaccharide composition of HCP-2 were achieved with 1-phenyl-3-methyl-5-pyrazolone (PMP) derivatization method as described previously (Tian et al., 2011). Briefly, HCP-2 was reconstituted with 1 ml distilled water, and hydrolyzed with 2 ml of trifluoroacetic acid (TFA, 2 M) at 100 °C for 8 h. After centrifugation at 10,000 rpm for 5 min, the supernatant (80 μl) was added with distilled water (110 μl), 6 M NaOH (10 μl) and 0.75 M PMP (200 μl), into a 1.5 ml microcentrifuge tube and then vortex for 1 min. For the monosaccharide standards (mannose, rhamnose, glucuronic acid, galacturonic acid, glucose, xylose, galactose, arabinose), standard monosaccharide (0.055 M, 10 μl) was mixed with 6 M NaOH (10 μl), distilled water (180 μl) and 0.75 M PMP solution (200 μl) and then vortex for 1 min. Each mixture was allowed to react for 60 min at 70 °C and subsequently neutralized with 6 M HCl solution (10 μl). The solution was extracted with chloroform and the aqueous layer was filtered through a 0.45 μm membrane for UPLC analysis.

Separation of monosaccharides from HCP-2 was achieved by Waters Acquity UPLC system (Waters, MA, USA) equipped with an Acquity UPLC BEH C8 column (2.1 mm × 100 mm, 1.7 μm) and protected by Acquity UPLC BEH C8 VanGuard Pre-column (2.1 × 5 mm, 1.7 μm). The system was maintained at 50 °C. The mobile phase consisted of (A) 50 mM ammonia formate in 10% ACN and (B) ACN acetonitrile, at a flow rate of 0.35 ml/min with the following gradient: 16–18% B from 0 to 18 min, 18–20% B from 18 to 25 min, 20–16% B from 25 to 27 min. The injection volume was 1 μl and the analytes were monitored with a photodiode array detector (PAD) at the wavelength of 250 nm.

2.4.4. Structural characterization by FT-IR, ^{1}H and ^{13}C NMR spectroscopy

HCP-2 was analyzed by transmittance infrared spectroscopy in the form of KBr disks using a Bruker Equinox 55 FT-IR spectrometer. For the NMR spectroscopy, the polysaccharide samples were exchanged three times in D$_2$O with intermediate freeze-drying. Finally, ^{1}H and ^{13}C NMR spectroscopy was performed on

an AVANCE 600 Superconducting UltraShield[TM] Fourier-Transform NMR spectrometer (CryoProbe[TM]).

2.5. Bioassays of HCP-2

2.5.1. Preparation of human PBMCs

Fresh human buffy coat obtained from the Hong Kong Red Cross Blood Transfusion Service was diluted with phosphate-buffered saline in an equal volume. The diluted sample (20 ml) was put in a 50 ml centrifuge tube together with an equal volume of Ficoll-Plaque Plus solution. The tube was then centrifuged at $800 \times g$ for 20 min at 18 °C. After centrifugation, the supernatant was discarded and PBMCs were re-suspended in 4 ml of RPMI 1640 medium containing 10% v/v fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin. The cell number was counted, and the cell viability was checked by trypan blue exclusion assay.

2.5.2. Cytokine production of PBMCs

The human PBMCs culture was incubated with HCP-2 for 12 h, and the supernatant was subjected to test for the productions of cytokines IL-1β, TNF-α, MIP-1α, MIP-1β, and RANTES. The assays were carried out according to the procedures recommended in the ELISA kit manual.

2.5.3. Involvement of Toll-like receptor-4 (TLR-4) in the activation of IL-1β release by HCP-2 from PBMCs

The PBMCs were pre-incubated with increasing concentrations of TLR-4 inhibitor (LPS-RS) for 15 min, and then treated with HCP-2 for 12 h. The IL-1β levels in the supernatant were determined using an ELISA kit.

2.6. Statistical analysis

All experiments were repeated at least three times and results were presented as mean ± standard deviation (SD). Statistical analysis was performed using one-way ANOVA by Graphpad Prism (v.5.01).

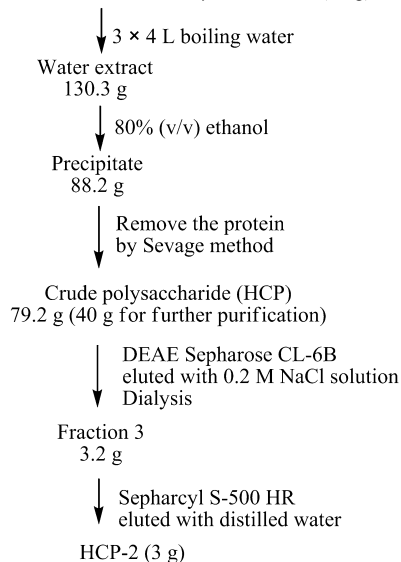Dried vines and leaves of *Houttuynia cordata* (1 kg)

↓ 3 × 4 L boiling water

Water extract
130.3 g

↓ 80% (v/v) ethanol

Precipitate
88.2 g

↓ Remove the protein by Sevage method

Crude polysaccharide (HCP)
79.2 g (40 g for further purification)

↓ DEAE Sepharose CL-6B eluted with 0.2 M NaCl solution Dialysis

Fraction 3
3.2 g

↓ Sepharcyl S-500 HR eluted with distilled water

HCP-2 (3 g)

Fig. 1. Schematic diagram showing the isolation of HCP-2 from *H. cordata*.

3. Results

3.1. Characterization of HCP-2

A pure polysaccharide HCP-2 was isolated with a yield of 4.6% (w/w) from crude water extracts (Fig. 1). Using HPLC analysis, a single and symmetrical peak was shown for HCP-2 (Fig. 2), which indicated HCP-2 is a homogeneously pure polysaccharide based on the distribution of molecular weight. Its molecular weight was determined as 60,000 Da according to the calibration curve based on the T series dextran standards [$\text{Log}(\text{MW}) = 4.06614 + 1.61449t - 0.29322t^2 + 0.01234t^3$, $R^2 = 0.9978$, $t = 7.65$ min]. The optical rotation of HCP-2 was found
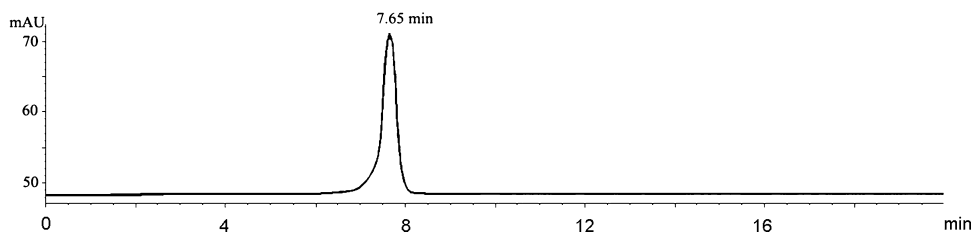

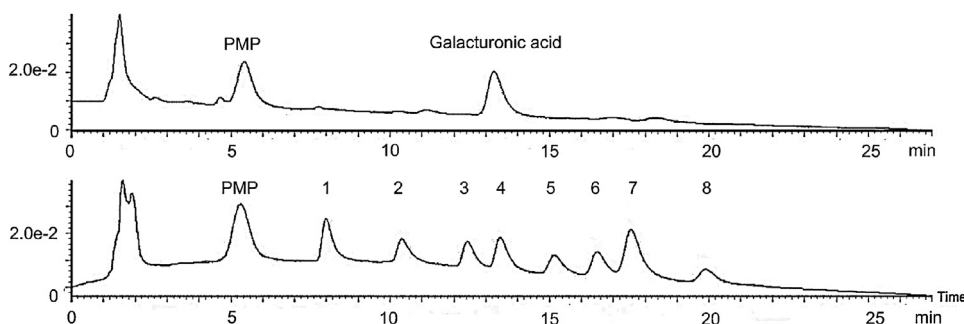**Fig. 2.** HPLC profile of HCP-2.

Fig. 3. UPLC chromatograms of PMP derivatives of constituent monosaccharides from (a) HCP-2 and (b) eight standard monosaccharides. The polysaccharide was hydrolyzed with TFA at 100 °C for 8 h and then labeled with PMP. Peaks in the chromatograms representing the follows: (1) mannose; (2) rhamnose; (3) glucuronic acid; (4) galacturonic acid; (5) glucose; (6) xylose; (7) galactose; (8) arabinose.
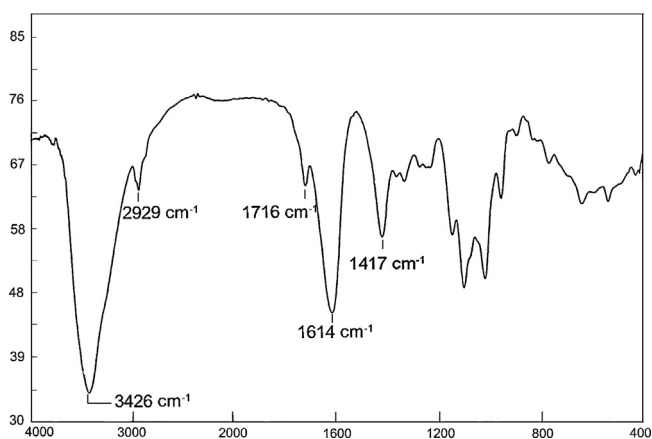
Fig. 4. FT-IR spectrum of HCP-2 in the frequency range of 400–4000 cm^{-1}.

Table 1
^{1}H-NMR (D$_2$O, 600 MHz) and ^{13}C-NMR (D$_2$O, 150 MHz) spectra data of HCP-2.

^{1}H and ^{13}C-NMR data (D_2O) of HCP-2 (δ in ppm)			
Galacturonic acid	δ_C		δ_H
C-1	99.7	H-1	5.06
C-2	68.9	H-2	3.76
C-3	69.6	H-3	3.99
C-4	78.6	H-4	4.40
C-5	72.0	H-5	4.76[a]
C-6	176.2	H-6	
OCOCH$_3$	19.8		2.07
COOCH$_3$	53.6		3.80

[a] Overlapped with H$_2$O.

to be $[\alpha] + 177.7°$ (c = 1.00, H$_2$O) and no protein was detected in BCA assay.

After hydrolysis by 2 M TFA, the monosaccharides of HCP-2 were labeled with PMP for further UPLC analysis. Compared to the eight standard monosaccharides (Fig. 3b), only galacturonic acid could be seen in the monosaccharide composition of HCP-2 (Fig. 3a), which implied that HCP-2 mostly composed of the galacturonic acid residues.

FT-IR spectroscopy (Fig. 4) showed that the IR spectra of HCP-2 displayed a broad stretching intense characteristic peak for the hydroxyl groups at around 3426 cm^{-1}, and one weak C—H stretching bands at 2929 cm^{-1}. The featured signal ester carbonyl groups at 1716 cm^{-1}, and two other strong peaks for free carboxylate groups at 1614 cm^{-1} and 1417 cm^{-1} which suggested that HCP-2 was uronic acid-rich polysaccharide (Tian et al., 2011).

In ^{13}C NMR spectrum (Fig. 5b), the anomeric signal at δ 99.71 was assigned to C-1 of (1 → 4)-linked D-galactopyranosyluronic acid (GalpA), indicating an α-configuration for the GalpA residues, and δ 176.2 was derived from C-6 of α-D GalpA. The signals at δ 68.87, 69.57, 78.62, 72.05 were assigned to C-2, C-3, C-4, C-5 of D-GalpA, respectively (Makarova, Patova, Shakhmatov, Kuznetsov, & Ovodov, 2013; Petersen, Meier, Duus, & Clausen, 2008). The signals at δ 53.61 and δ 19.82 were attributed to the methoxyl groups and acetyl groups, respectively.

In ^{1}H NMR spectrum (Fig. 5a), the signals at δ 5.06 were assigned to the anomeric protons of α-D GalpA. Numerous proton signals at δ 3.76, 3.99, 4.40 and 4.76 ppm were assigned to H-2, H-3, H-4 and H-5 of α-D GalpA. Signals at δ 3.8 and 2.07 were assigned to
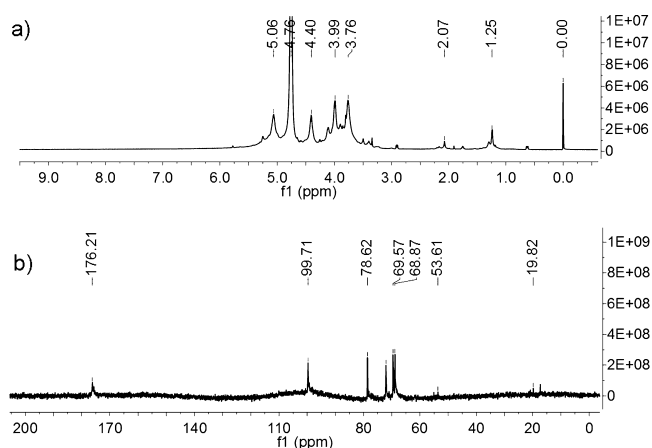
methoxyl groups and O-acetyl groups (data summarized in Table 1). On the whole, HCP-2 is elucidated as a linear poly-(1 → 4)-α-D-galactopyranosyluronic acid with partial methyl esterified carboxyl groups and partial acetylated C-2 hydroxyl groups.

3.2. Bioassay of HCP-2

3.2.1. Cytokines production of PBMCs

After incubating the PBMCs with HCP-2 for 12 h, IL-1β production was significantly stimulated by HCP-2 (0.1–50 μg/ml), while the production of TNF-α was found to be significantly increased by HCP-2 at 10 and 50 μg/ml. The secretion of MIP-1α, MIP-1β and RANTES were all significantly enhanced by HCP-2 at 10 and 50 μg/ml (Fig. 6).

3.2.2. Involvement of TLR-4 in the activation of IL-1β release

To test whether the activation of PBMCs by HCP-2 was through activation of TLR-4, a selective TLR-4 receptor antagonist, lipopolysaccharide from *R. sphaeroides* (LPS-RS) (Lohmann, Vandenplas, Barton, & Moore, 2003), was used. The involvement of TLR-4 was assessed by measuring the levels of IL-1β in the supernatant. Freshly isolated human PBMCs were incubated with mixture of LPS-RS (0, 50 and 200 ng/ml) and HCP-2 (0.1, 1, 5, 10 and 25 μg/ml). After 12 h incubation, the production of the IL-1β induced by HCP-2 was suppressed by LPS-RS in a dose-dependent manner (Fig. 7).

4. Discussion

Following a series of chemical and analytical procedures including acid hydrolysis, PMP derivation, FT-IR, and NMR studies, HCP-2 was finally identified as a pectic polysaccharide with repeating units of (1 → 4)-α-D-galactopyranosyluronic residues. To the best of our knowledge, this is the first report on the isolation of a pure pectic polysaccharide from *H. cordata* with immunostimulating activities. Similar polysaccharides had been isolated from other species, and the differences lie in the molecular weight and optical rotation (Makarova et al., 2013; Shang et al., 2012; Xu, Dong, Qiu, Ma, & Ding, 2011; Zhao et al., 2006). However, the activity study of these polysaccharides focused on mainly the anti-angiogenesis and cell proliferation.

Pectic polysaccharides are the primary components of higher plant cell wall with intriguing structural diversity (Caffall & Mohnen, 2009). Several literatures had reported that pectic polysaccharides exhibit immunomodulatory effects, such as complement fixation (Inngjerdingen et al., 2005), promotion of the ratio of CD4$^+$/CD8$^+$ T cells, and the stimulation of the secretion of cytokines and chemokines (Ye & Lim, 2010). The previous studies on immunomodulation of *H. cordata* concentrated on the water and ethanolic extracts (Lau et al., 2008; Lee et al., 2008), and the study of the *H. cordata* polysaccharide was mainly on the antioxidant activities (Tian et al., 2011). In our study, HCP-2 exhibited

Fig. 5. (a) ^{1}H-NMR (D$_2$O, 600 MHz) and (b) ^{13}C-NMR (D$_2$O, 150 MHz) spectra of HCP-2.
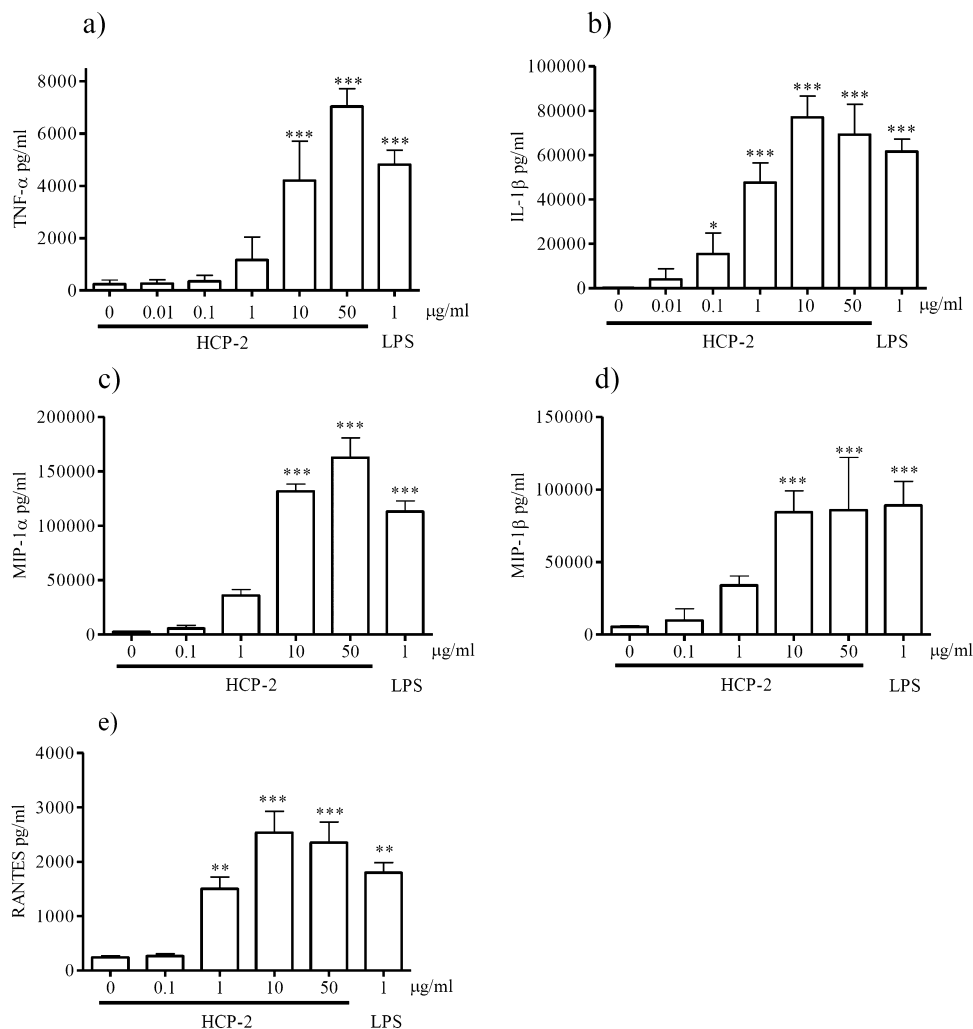
Fig. 6. Effect of HCP-2 on the production of different cytokines in human PBMCs. Freshly isolated human PBMCs were incubated with HCP-2 at concentration of 0.01–50 μg/ml or LPS at 1 μg/ml. (a) TNF-α, (b) IL-1β, (c) MIP-1α, (d) MIP-1β, and (e) RANTES were measured in the supernatant after 12 h of incubation. Data are presented as mean ± S.D. of cells harvested from four independent experiments. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ compared with the control group.

non-cytotoxicity at the range of 0.01–50 μg/ml on human PBMCs *in vitro* (data not shown), and elicited strong responses on IL-1β and TNF-α. As regards to the innate immunity, HCP-2 showed proinflammatory effects by stimulating monocyte functions, *e.g.*, the production of TNF-α and IL-1β, as well as the MIP-1α, MIP-1β, and RANTES. Upon the initiation of pathogen infection, the body requires a few days to develop and expand effector T and B cells. During this critical timeframe, innate immune responses play an important role in controlling the infection. Pathogen recognition receptors (PRR), such as Toll-like receptors of monocytes (especially macrophages and dendritic cells) recognize invasive pathogens, and immediately activate the innate immune to launch the immune and inflammatory responses. Macrophages and dendritic cells secrete pro-inflammatory factors (IL-1β, TNF-α) and chemokines (RANTES, MIP-1α and MIP-1β) to recruit natural killer cells, monocytes and other immune cells for further pathogen elimination, and these cytokines and chemokines also assist in the shape of adaptive immune.

Several studies have reported that natural polysaccharides activate TLR-4 to induce a signaling cascade leading to the activation of NF-κB and the production of proinflammatory cytokines and chemokines (Li & Xu, 2011; Lu, Yeh, & Ohashi, 2008; Yang, Zhao, Wang, & Mei, 2007), which inspired us to explore the underlying mechanism of the stimulation of HCP-2 on innate immunity. After

incubated with LPS-RS, a potent antagonist of TLR-4 for 12 h, the production of IL-1β caused by HCP-2 markedly decreased (Fig. 7), which implied that HCP-2 may launch a series of the immune responses through activation of TLR-4 on the cell membrane of dendritic cells and macrophages.

In this study, the concentrations of IL-1β, TNF-α, MIP-1α, MIP-1β and RANTES were significantly enhanced by HCP-2, which
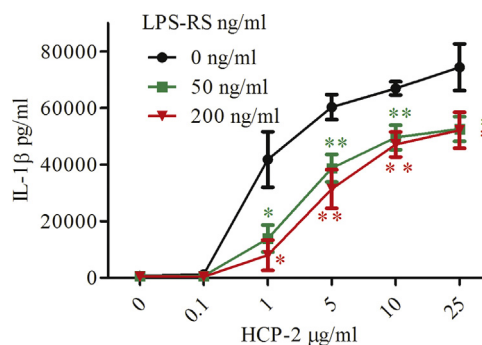
Fig. 7. Antagonistic effect of LPS-RS on HCP-2 induced TLR4. Each point shows the mean ± S.D. of four independent experiments. *$P < 0.05$ and **$P < 0.01$ indicate statistically significant difference from the control group.

imply that the HCP-2 can be used as an immune enhancer, and also provide evidence that this polysaccharide may partly account for the immune enhancement of the water extract of *H. cordata* (Lau et al., 2008). Taken together, HCP-2 induced an enhancement of innate immune responses and these results may benefit the understanding of the immunomodulatory effect of HCP-2 and the underlying molecular mechanisms.

Acknowledgement

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