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Structural Characterization and Immunomodulatory Activity of a Novel Polysaccharide from *Lepidium meyenii*

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ABSTRACT: A novel polysaccharide named as MC-1 was isolated from the roots of *Lepidium meyenii* using a water extraction method. Structural characterization revealed that MC-1 had an average molecular weight of 11.3 kDa and consisted of arabinose (26.21%), mannose (11.81%), glucose (53.66%), and galactose (8.32%). The main linkage types of MC-1 were proven to be (1→5)-α-L-Ara, (1→3)-α-L-Man, (1→2,6)-α-L-Man, (1→)-α-D-Glc, (1→4)-α-D-Glc, (1→6)-α-D-Glc and (1→6)-β-D-Gal by methylation analysis, periodate oxidation-Smith degradation and NMR analysis. The immunostimulating assay indicated that MC-1 could significantly enhance the pinocytic and phagocytic capacity and promote the NO, TNF-α and IL-6 secretion of RAW 264.7 cells, involving toll-like receptor 2, complement receptor 3 and mannose receptor mainly. These results suggested the potential utilization of MC-1 as an attractive functional food supplement candidate for hypoimmunity population.

INTRODUCTION

Maca (*Lepidium meyenii*) is a food source in the Andes region and belongs to the Brassicaceae. It grows in altitudes varying between 3700 and 4450 m.1 In the Andes, people have used maca to enhance fertility for centuries.2 Recently, maca has attracted interests as a dietary supplement due to its pharmacological activity, such as enhancing fertility, anti-fatigue, anti-depression, anti-osteoporosis, anti-oxidative, anti-inflammation, and so on.3-12 And many effective compounds in maca were identified, including macaenes, macamides, glucosinolates, alkaloid, and flavonolignans.3, 13-16 However, little attention was devoted to the maca polysaccharide and its activity. Especially, there was little research on the structural information of maca polysaccharides. Polysaccharides obtained from natural sources are known as kinds of biological activities, for example, immunomodulatory activity. But only the antioxidant activity of maca polysaccharides was reported.1 More activities need further exploration.

Macrophages play a unique role in the immune system. They can not only initiate innate immune responses, but also contribute to fight against infection and inflammation. Macrophages can kill pathogens directly by phagocytosis and indirectly by releasing cytotoxic molecules such as NO and secreting cytokines including TNF-α and IL-6.17-19 Thus, macrophages are usually used as an ideal cell model to evaluate the immunomodulatory activity of bioactive compounds.

In the present study, a new polysaccharide, named as MC-1, was separated from the roots of *L. meyenii*. The primary chemical structure of MC-1 was characterized.
Also, the current experiments were designed to investigate the immunomodulatory activity of MC-1 on the murine macrophage cell line, RAW 264.7 cells, by determining the effect on the pinocytic and phagocytic capacity, production of NO, TNF-α and IL-6, and explore the membrane receptors of MC-1 on RAW 264.7 cells. The results from this study might supply useful information to further study on polysaccharides in maca.

**MATERIALS AND METHODS**

**Materials and Chemicals**

The roots of *L. meyenii* were collected from Peru. Myoinositol and standard monosaccharides (xylose, rhamnose, arabinose, fucose, mannose, glucose, galactose) were purchased from Sigma Company (St. Louis, MO, USA). Diethylaminoethyl (DEAE)-Sepharose Fast Flow were obtained from Shanghai Yuanye Biotechnology Company Limited (Shanghai, China). Sephadex G-100 was acquired from GE Healthcare Life Science (Piscataway, NJ). RAW 264.7 cells were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Dulbecco’s modified eagle’s medium (DMEM), fetal bovine serum (FBS), penicillin, and streptomycin were purchased from Gibco Life Technologies (Grand Island, NY). lipopolysaccharide (LPS), neutral red were purchased from Sigma Company (St.Louis, MO, USA). Vybrant Phagocytosis Assay Kit was purchased from Molecular Probes (Carlsbad, CA, USA). Griess reagent was purchased from Sigma-Aldrich (NSW, Australia). Mouse TNF-α and IL-6 detecting ELISA kits were from R&D Systems. Trizol was purchased from Invitrogen, USA; Transcriptor First
Strand cDNA Synthesis Kit, FastStart Universal SYBR Green Master (ROX) was purchased from Roche. Anti-scavenger receptor I antibody (anti-SR), anti-mannose receptor antibody (anti-MR), anti-beta glucan receptor antibody (anti-GR), anti-toll-like 2 antibody (anti-TLR2), anti-complement receptor 3 antibody (anti-CR3), and anti-toll-like 4 receptor antibody (anti-TLR4) were obtained from Abcam (Cambridge, MA). All of the other chemical reagents used in this study were analytical grade.

**Extraction and Purification of Polysaccharides from the Roots of *L. meyenii***

The dried roots of *L. meyenii* were crushed into powder using a tissue triturator. The powder was extracted with boiling water at a ratio of 1:30 (w/v) for 2 h, and the obtained extract was centrifuged at 4000×g for 15 min. The supernatant was then collected and concentrated at 60 °C. After that, the protein in concentrated solution was removed by the Sevag method. The deproteinated process was repeated 30 times. The resulting solution was precipitated with four volumes of 100% ethanol at 4 °C for overnight. The supernatant and precipitate were then separated by centrifugation at 4000×g for 15 min. Finally, the precipitates was centrifuged and redissolved in distilled water before lyophilization to obtain crude polysaccharides.

A total of 50 mg of crude polysaccharides was dissolved in 10 mL of ultrapure water and loaded onto a pre-equilibrated DEAE-Sepharose Fast Flow chromatography column (1.6×35 cm) at a flow rate of 1 mL/min, then sequentially eluted with distilled water and 0.05, 0.1, 0.2, 0.3, 0.5 M NaCl solution, respectively. The eluent fractions were collected and analyzed by the phenol-sulfuric acid method. The fraction eluted
by ultrapure water were concentrated at 60 °C by rotary vacuum evaporator, then
dialyzed (MW cut off 5.0 kDa) at 4 °C for 48 h and freeze-dried. Sephadex G-100
chromatography column (1.6×60 cm) was used to purify further the fraction. The
fraction (20 mg) was dissolved in distilled water (10 mL) at 25 °C. The column was
washed with 0.3 L of distilled water at a flow rate of 1 mL/min for 300 min. The
eluent was detected with phenol-sulfuric acid method, then collected and concentrated
the polysaccharide solution. Three peaks were gotten. In this study, we mainly
focused on the research of MC-1. The resultant solution was dialyzed and
freeze-dried.

**Molecular Weight Determination of MC-1**

The weight-average molecular weight of MC-1 was conducted on
high-performance gel permeation chromatography (HPGPC) using a Waters HPLC
system including two serially linked columns a TSK-GEL G-5000 PWXL column
(300 mm×7.8 mm inner diameter, 10µm) and a TSK-GEL G-3000 PWXL column
(300 mm×7.8 mm inner diameter, 6µm), a Waters 2410 differential refractive index
detector, eluted with 0.02 mol/L KH₂PO₄ at a flow rate of 0.6 mL/min. MC-1 (2.5 mg)
was dissolved in 1 mL mobile phase. The polysaccharide solution was filtered through
a 0.22 µm microporous filtering film.

**Infrared Spectrum Analysis**

The MC-1 samples (2-3 mg) were ground to a fine powder and analyzed through
the potassium bromite pellet method with a Fourier transform infrared (FTIR)
spectrophotometer (Bruker, Ettlingen, Germany) in the 400-4000 cm⁻¹ vibrations region.²⁰

**Determination of Triple-helix Structure**

The conformational structure of MC-1 was determined following the Congo red method.²¹

**Monosaccharide Composition**

A total of 10 mg MC-1 sample was hydrolyzed in 4 mL of 2 mol/L trifluoroacetic acid (TFA) for 8 h at 110 °C. Polysaccharides hydrolysis transformed into alditol acetates. The alditol acetates product of MC-1 was analysed by gas chromatography (GC) (Agilent, US) fitted with a HP-5 capillary column (30 nm×0.32 mm×0.25 µm, 160~210 °C at 2 °C/min, and then 210~250 °C at 10 °C/min) equipped with a flame ionization detector (FID). Glucose, galactose, fucose, rhamnose, mannose, xylose, and arabinose were used as the monosaccharide standards. Myoinositol was used as the interior reference.

**Periodate Oxidation-Smith Degradation**

25 mg of MC-1 sample was dissolved in 12.5 mL of ultrapure water and incubated with 12.5 mL of NaIO₄ (30 mmol/L) in the dark at room temperature. During the incubation period, 0.1 mL of the reaction liquid was taken out from the reaction system at different time intervals (0, 6, 12, 24, 36, 48 and 60 h) until the absorbance value becoming stable under an ultraviolet visible spectrophotometer (model UV-18000, Shimadzu, Japan) in the 233 nm. The periodate product (2 mL) was used to quantify the production of formic acid by titration with 0.098 mol/L sodium
hydroxide solution (screened phenolphthalein as indicator) after addition of glycol. The rest solution was dialyzed in distilled water for 3 days at 4 °C. The dialyzed was concentrated and added with 70 mg sodium borohydride reacting for 12 h in the dark to destroy the furfural. The solution was neutralized to pH 6.0-7.0 with 50% acetic acid and dialyzed for another 3 days at 4 °C. The dialyzed was concentrated and freeze-dried. A total of 10 mg of the residues was hydrolyzed by 4 mL of 2 mol/L TFA at 105 °C for 6 h. The lysate was acetylated with 1 mL of pyridine and 10 mg of hydroxylamine hydrochloride at 90 °C for 30 min. Then, 1 mL of acetic anhydride was added to the reaction system and continuously heating at 90 °C for another 30 min. The production of acetate derivative was analyzed by a GC (Aglient, USA) with a DB-1701 capillary column (30m×0.25mm×0.25µm, J&W Scientific, Fulsom, CA) and a flame ionization detector. The linearily heating program is from 80 to 220 °C at a speed of 2 °C/min, from 220 to 250 °C at a speed of 5 °C/min, and kept at 250 °C for 5 min. The temperature of the detector was set at 300 °C. Phycite, glycol, glycerol, rhamnose, xylose, arabinose, mannose, glucose, and galactose were used as standards.

Methylation Analysis

Methylation analysis of polysaccharide was performed according to the modified method reported by Nie et al. The dried MC-1 (10 mg) was dissolved in 6 mL anhydrous DMSO at 60 °C for 2 h and sonicated for 1 h to ensure a completed solution. Sodium hydroxide (240 mg) was added to the solution at 60 °C throughout the night. The mixture was added 3.6 mL methyl iodide with stirring for 8 min. This procedure was conducted at three times and stopped by the addition of 6 mL distilled
water. The obtained solution was dialyzed against distilled water for 48 h at 4 °C. The methylated polysaccharide was extracted with dichloromethane three times. The dichloromethane extract was then dried over sodium sulfate, and evaporated to dryness. The dried methylated polysaccharide was hydrolyzed as describe above. The hydrolysate was reduced by sodium borodeuteride (70 mg) and acetylated with acetic anhydride (0.5 mL). Finally, the resultant was analysed by gas chromatography (GC) coupled with mass spectrometry (MS) (Agilent, USA) using a TR-b5MS capillary column (30 m × 0.25 mm × 0.25 µm, 150~180 °C at 10 °C/min, and then 180~260 °C at 15 °C/min).

**NMR Spectroscopy**

About 30 mg of MC-1 was dissolved with 0.55 mL of D$_2$O in a NMR tube and then the $^{13}$C NMR and $^1$H NMR spectra were recorded on a Bruker 600 MHz NMR apparatus (Bruker Corp, Fallanden, Switzerland) at 60 °C.

**Determination of Pinocytic and Phagocytic Capacity**

*Pinocytic Capacity* RAW 264.7 cells were incubated at 37 °C in a humidified atmosphere with 5% CO$_2$. DMEM medium with 10% FBS, 100 µg/mL streptomycin, and 100 units/mL, penicillin was used as the culture medium. Cells were adjusted to a concentration of $1\times10^6$ cells/mL in the exponential phase, loaded onto the 96-well or 6-well plate, and continuously incubated for 24 h. Then, cells were treated with MC-1 at different concentrations (62.5, 125, 250, 500, 1000 µg/mL) or LPS (20 µg/mL) were added into each well. After 24 h incubation, the medium was removed, and 100 µL of 0.1% neutral red dissolved in PBS was added to each well and incubated for 1 h.
The cells were washed with PBS three times, and then 100 µL of 1% acetic acid solution (v/v) in 50% ethanol (v/v) was added to each well. Cell culture plate was statically placed overnight. The absorbance at 540 nm was measured using a microplate reader (Biotek, USA).

**Phagocytic Capacity** RAW 264.7 cells were seeded on 6-well plates and stimulated with MC-1 at different concentrations (62.5, 250, 1000 µg/mL) or LPS (20 µg/mL) for 6 h. A solution of FITC-labeled E. coli provided by Vybrant Phagocytosis Assay Kit was added to the cells, and incubated for 2 h. The Bio Particle loading suspension was removed and 100 µL of the prepared trypan blue suspension was added. After 1 minute at room temperature, the excess trypan blue suspension was removed immediately. Images of the cells were captured using a fluorescence microscope (ECCIPSE 50, Nicon, Japan). The fluorescence intensity was measured by flow cytometry using a FC500 flow cytometer (Beckman Coulter Ltd, USA).

**Measurement of NO and cytokines** Cells were treated with the different concentrations of MC-1 (62.5, 125, 250, 500, 1000 µg/mL) or LPS (20 µg/mL) and incubated for 24 h. After that, the supernatants of cells were collected and the levels of NO, TNF-α and IL-6 were measured using a Griess reagent and ELISA kits, respectively.

**QPCR Analysis** RAW 264.7 cells were seeded on 6-well plates at a concentration of 1×10⁶ cells/mL. After 24 h, cells were treated with the different concentrations of MC-1 (62.5, 125, 250, 500, 1000 µg/mL) or LPS (20 µg/mL). After 12 h, cells were lysed to
isolate total RNA.

Total RNA was isolated using TRIzol reagent according to the manufacturer’s protocol, and the RNA was used for cDNA synthesis using reverse transcriptase. The cDNA encoding iNOs, TNF-α, IL-6 genes was quantified by quantitative real-time PCR assay (QPCR). GAPDH was used as the internal reference. The specific primers were used (Table 1). Gene amplification was carried out with the ABI 7500 sequence detection system (Applied Biosystems, Foster, USA).

Investigation of Membrane Receptors

The cells were pretreated with antibodies (5 µg/mL) of membrane receptors (SR, MR, GR, CR3, TLR2 and TLR4) or the mixed antibodies of MR, CR3 and TLR2 for 2 h prior to stimulation with MC-1 (125 µg/mL). The group treated with only MC-1 (125 µg/mL) was used as a control. The untreated cells were used as the negative control group. LPS was used as the positive control. The levels of NO, TNF-α, and IL-6 were measured after 24 h.

Statistical Analysis

Data are expressed as means± standard deviation (SD) for three replicates. One-way ANOVA was used to analyze the significant differences between the groups by SPSS 21.0. p <0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Extraction and Purification of MC-1 Polysaccharide from L. meyenii

Crude polysaccharides were isolated from the roots of L. meyenii with a yield of 6.36%. The extracts were first purified by a DEAE-Sepharose Fast Flow
chromatography (Figure 1A). Two independent fractions, eluted by ultrapuren water and 0.05 mol/L NaCl, respectively, were obtained. The fraction eluted by ultrapuren water which was at much higher content was further purified by a Sephadex G-100 column. As shown in Figure 1B, three peaks, MC-1, MC-2 and MC-3, were observed. In the present study, we mainly focused on MC-1, and the property of MC-2 and MC-3 will be studied in our future work. Finally, MC-1 was detected using the phenol-sulfuric acid method for the determination of 97.5% content.

**Ultraviolet Rays (UV) Spectrum**

UV absorption of MC-1 spectra did not show any absorption peaks in the wavelength 260 and 280 nm (Figure 1C), indicating MC-1 didn’t contain any impurity of nucleotides or protein. 

**Characterization of MC-1**

**Molecular Weight of MC-1**

The average molecular weight of MC-1 was measured by HPGPC as illustrated in Figure 2A. A single peak was observed. It indicated that MC-1 was a homogeneous polysaccharide. The average molecular weight of MC-1 was determined to be 11.3 kDa. Generally, the molecular weight of polysaccharides could reach to hundreds of thousands of Da. So MC-1 was a low molecular weight polysaccharide. This might give MC-1 some characters different from the high molecular weight polysaccharides.

**FT-IR Spectrum**

In the FT-IR spectrum of MC-1 polysaccharides (Figure 2B), most of the absorption bands could be assigned according to previous literature. The vibrations region
3406 cm\(^{-1}\) corresponds to the O-H stretching vibration and that at 2927 cm\(^{-1}\) corresponds to the C-H stretching vibration. The band at 1637 cm\(^{-1}\) may be due to the C=O stretching vibration. The absorption peaks at 1422 cm\(^{-1}\) represented the C=C stretching vibration and the strong peak at 1043 cm\(^{-1}\) was ascribed to the stretching vibrations of pyranose ring. A characteristic absorption at 897 cm\(^{-1}\) was also observed, indicating the β-pyranoside linkage. Finally, an absorption peak at 573 cm\(^{-1}\) indicated the existence of α-configurations. The spectra indicated that MC-1 had the typical groups of sugars.

The Conformational Structure of MC-1

The Congo red method was applied at different concentrations of NaOH (0.05 to 0.5 N) solution to identify the triple helix conformation of MC-1. As shown in Figure 2C, an obvious shift of maximum absorption wavelength from 496 to 506 nm was induced by the presence of the polysaccharides in Congo red solution, indicating that polysaccharide-Congo red complexes had formed. Along with the increase of NaOH concentrations, the maximum absorption wavelength decreased slightly. With the increase of concentrations of NaOH, the triple-helix conformation will translate into single coil conformation, and the maximum absorption wavelength will decrease dramatically. So the result indicated that MC-1 didn’t exhibit a triple helical conformation. But a bathochromic shift was observed after mixing MC-1 and Congo red. The reason might be that MC-1 had another order conformation which could form a complex with Congo red, and the conformation was stable in the NaOH. So the precise conformation of MC-1 needs to study further.
**Monosaccharide Composition**

The monosaccharide composition of MC-1 was analyzed using gas chromatography. The result was shown in Figure 3A (monosaccharide standard) and Figure 3B (MC-1). The polysaccharide MC-1 was found to mainly consist of arabinose, mannose, glucose and galactose in an approximate molar percentage of 26.21%, 11.81%, 53.66% and 8.32%, respectively. It indicated that MC-1 was a kind of heteropolysaccharide. The monosaccharide composition of MC-1 was different from the previous study. For example, the mannose in MC-1 wasn’t reported, and there was not rhamnose in MC-1. The reason might be that the extraction methods were different, or the rhamnose existed in MC-2 or MC-3.

**Periodate Oxidation-Smith Degradation**

The result of periodate oxidation analysis showed that 1 mol of hexose residue consumed 1.13 mol of periodate and produced 0.467 mol of formic acid. The mole number of periodate was more than two times of formic acid. It indicated that one or more of (1→6), (1→2) or (1→4)-linked glycosidic bonds might exist. Then MC-1 was further analyzed by Smith degradation. Glycerol, phycytose, arabinose, glucose were observed after Smith degradation (Figure 3C). Glycerol corresponded to the existence of (1→2) or (1→6)-linked glycosidic bonds. The appearance of erythritol indicated that some of the linkages were (1→4) or (1→6)-linked glycosidic bonds. The presence of monosaccharides revealed the appearance of (1→3)-linked glycosidic bonds. More precise glycosidic bonds were confirmed in the methylation analysis.

**Methylation Analysis**
Methylation analysis followed by GC-MS was employed to obtain more structural information on MC-1. According to the result in Table 2, seven homogeneous peaks were observed. According to the retention time, the peaks were identified as 2,3-Me₂-Araf, 2,4,6-Me₃-Manp, 3,4-Me₂-Manp, 2,3,4,6-Me₄-Glcp, 2,3,6-Me₃-Glcp, 2,3,4-Me₃-Glcp and 2,3,4-Me₃-Galp with a molar ratio of 27.13:3.03:1.83:8.75:43.37:14.01:1.88. The result suggested that MC-1 contained seven linkage forms: (1→5) -linked arabinose, (1→3) -linked mannose, (1→2, 6) -linked mannose, (1→)-linked glucose, (1→4)-linked glucose, (1→6)-linked glucose, and (1→6)-linked galactose. This inference also agreed with the result from the periodate oxidation-Smith degradation. The ratio between terminal units (T-Glcp) and the branching points (1, 2, 6-Manp) was 4.78, indicating that number of terminal units was much more than that of branching points. These results indicated that MC-1 contained the linear and branched polysaccharides. And, the linear polysaccharide was approximately two times more than the branched polysaccharide. Additionally, the degree of branching (DB) value of MC-1 was calculated as 10.58% following the equation DB = (NT + NB)/ (NT + NB + NL), where NT, NB, and NL are the numbers of the terminal residues (T-Glcp), branch residues (1,2,6-Manp), and linear residues (1,5-Araf, 1,3-Manp, 1,4-Glcp, 1,6-Glcp, 1,6-Galp), respectively.²⁷

NMR Analysis of MC-1

The spectra of ¹³C NMR and ¹H NMR of MC-1 was shown in Figure 4. Signals of MC-1 in ¹³C NMR and ¹H NMR spectra were analyzed through referring to the previous research.²⁸-³⁰ The resonances in the region of δ 95.0-107.0 in ¹³C NMR were
assigned to the anomeric carbon atoms of D-galactose, D-glucose, L-mannose, and L-arabinose. The anomeric proton signals (δ 5.28, 5.12, 5.05, 4.96, 4.53, 5.03 and 4.51) and the anomeric carbon signals (δ 106.36, 95.72, 106.85, 99.48, 99.56, 99.69 and 103.62) corresponded with H-1 and C-1 of seven anomeric residues, including (1→5)-α-L-Ara, (1→3)-α-L-Man, (1→2,6)-α-L-Man, (1→)-α-D-Glc, (1→4)-α-D-Glc, (1→6)-α-D-Glc, and (1→6)-β-D-Gal, respectively. The entire assignment of the $^{13}$C and $^1$H chemical shifts was shown in Table 3.

**Immunomodulatory Activity of MC-1**

**Effects of MC-1 on RAW 264.7 cells Viability**

To investigate the toxic effect of MC-1 on the RAW 264.7 cells, after being treated with MC-1 (62.5, 125, 250, 500, 1000 µg/mL) for 24 h, the viability of cells were detected by MTT assay. As shown in Figure 5A, MC-1 didn’t affect the viability of RAW 264.7 cells, which indicated that MC-1 under 1000 µg/mL was nontoxic to RAW 264.7 cells. So the concentration of MC-1 used in the following study was below 1000 µg/mL.

**Effects of MC-1 on the Pinocytic and Phagocytic Capacity of RAW 264.7 cells**

The pinocytic capacity was examined by the uptake of neutral red. As shown in Figure 5B, compared to the control group, 62.5 µg/mL MC-1 significantly enhanced the uptake of neutral red (p<0.05). With the increase of concentration, the enhancement was stronger. Although the effect of 1000 µg/mL was lower than 500 µg/mL, there was no significant difference between two groups. The result indicated that MC-1 could enhance the pinocytic capacity of RAW 264.7 cells.
Meanwhile, we also determined the effect of MC-1 on phagocytic capacity to E.coli (FITC-labeled) of RAW 264.7 cells. The photos of RAW 264.7 cells taken by fluorescence microscope were shown in Figure 5C. The photo of the control group showed that the untreated cells could phagocytose a little of E.coli. The fluorescence intensity of cells treated with MC-1 or LPS was obviously stronger than the control group. Then, the flow cytometer was used to further confirm the result (Figure 5D). The cell percentage in gating area of the control group was 12.00%. The percentages of groups treated with 62.5, 250, 1000 µg/mL MC-1 or 20µg/mL LPS were 38.00%, 54.98%, 62.82%, 65.46%, respectively. From the results, it was known that MC-1 could promote the phagocytic capacity of RAW 264.7 cells.

When the concentration of MC-1 was below 250 µg/mL, the enhancement on pinocytic and phagocytic capacity increased rapidly. It increased slowly or indistinctively as the concentration was higher than 250 µg/mL. This might suggest that the enhancement effect of MC-1 was limited. In fact, previous studies showed that macrophages over-activated would lead inflammatory damage.\textsuperscript{31, 32} Thus, it might hint that MC-1 exhibited remarkable immunomodulatory activity by enhancing the pinocytic and phagocytic capacity of RAW 264.7 cells moderately.

**Effects of MC-1 on NO, TNF-α and IL-6 Production of RAW 264.7 cells**

Macrophage activated can induce NO expression and enhance production of cytokines(TNF-α, IL-6 etc).\textsuperscript{33} Thus, the effects of MC-1 on levels of NO, TNF-α, IL-6 in RAW 264.7 cells were analyzed. The results were shown in Figure 6A, Figure 6B and Figure 6C. Cells of the control group secreted a basal level of TNF-α, IL-6 and
NO. The addition of MC-1 resulted in remarked increase in TNF-α, IL-6 and NO levels. The concentration of TNF-α, IL-6 and NO after treatment by 62.5 µg/mL MC-1 improved 1017 pg/mL, 153 pg/mL, 9.31 µM more than the control group, respectively. Like the result shown in Figure 5B-5D, the production cytokines or NO increased rapidly at the low concentrations of MC-1. The increase was slow, or inapparent at the high concentrations. To further confirm the result, we examined the effect of MC-1 on mRNA expression of nitric oxide synthase (iNOs), TNF-α and IL-6 using QPCR. As shown in Figure 6D, Figure 6E and Figure 6F, compared to the control group, the mRNA expression of TNF-α, IL-6 and iNOs were increased significantly. The mRNA expression of TNF-α, IL-6 and NO after treatment by 62.5 µg/mL MC-1 were 2.21, 9.12, 12.9 times more than the control group, respectively. But the mRNA levels of cytokines or iNOs still increased rapidly at the high concentrations of MC-1. It might result from that the sampling time for RNA extraction and collecting supernatants was different. These results suggested that MC-1 could promote the expression and secretion of TNF-α, IL-6 and NO by activating RAW 264.7 cells.

Receptors of MC-1 on RAW 264.7 cells

Macrophage activation is mediated primarily through the stimulation of pattern recognition receptors (PRRs). The PRRs binding polysaccharides mainly include scavenger receptor I (SR), mannose receptor (MR), beta glucan receptor (GR), complement receptor 3 (CR3), toll-like receptors (TLR2 and TLR4). In the present study, the roles of SR, MR, GR, CR3, TLR2 and TLR4 on immunemodulatory
activity of MC-1 were investigated. As shown in Figure 7A, Figure 7B and Figure 7C, after treatment of anti-CR3, anti-MR, anti-TLR2, the levels of NO, TNF-α and IL-6 were all visibly decreased in comparison to the group treated with MC-1 only. The decrement was the most, after the treatment of anti-TLR2, especially. Indistinctive decrease was found in the groups treated by anti-SR, anti-GR, and anti-TLR4. It indicated that TLR2, CR3 and MR were receptors of MC-1. The levels of TNF-α and NO in the group treated with the mixed antibodies of TLR2, CR3 and MR didn’t show significant difference with the control group. Interestingly, there was a significant difference between the two groups on the level of IL-6 (p<0.05). The result hinted that TLR2, CR3 and MR were the major receptors of MC-1, but it could not be excluded that the other receptors existed. The six receptors used in the study just were major receptors of polysaccharides. There are more than the six receptors on the cytomembrane of macrophages. So the receptors of MC-1 need further confirmed and investigated in the future.

TLR2 ligation leads to the activation of IL-1R-associated kinase (IRAK) via an adaptor myeloid differentiation protein 88 (MyD88), with subsequent activation of TNF receptor-associated factor 6 (TRAF-6), Jun N-terminal kinase (JNK) and NF-κB. CR3 has been proven to be involved in the activation of phosphoinositide -3-kinase (PI3K), activation of the mitogen-activated protein kinase (MAPK), and NF-κB signal transduction pathways. MR activation leads to activation of macrophage phagocytosis, endocytosis and NF-κB. Activation of these transcription pathways induces expression of cytokines and iNOs. Therefore, we speculated that the
possible mechanism of immunomodulatory activity of MC-1 was mainly through the activation of the pathways induced by the three receptor types cooperating with each other (Figure 7D). These pathways will be characterized in our future study.

The immunomodulatory activity of polysaccharides has close contact with the structure, such as monosaccharide composition and glycosidic bonds.\footnote{Previous studies have proven that polysaccharides containing mannose or glucose are most likely recognized by TLR2, CR3 or MR.\textsuperscript{38,41}} Therefore, the mannose and glucose composition of MC-1 may contribute to the immunomodulatory activity. The glycosidic bonds in polysaccharides, such as the $\alpha$-(1→3)-Man, $\alpha$-(1→4)-Glc and $\alpha$-(1→6)-Glc, always contact with the immunomodulatory activity.\footnote{Also, MC-1 possessed these glycosidic bonds.}

In this study, a new polysaccharide, MC-1 of 11.3 kDa, was purified from the roots of L. meyenii. MC-1 consisted of arabinose (26.21%), mannose (11.81%), and glucose (53.66%), and galactose (8.32%). The main linkage type of MC-1 were proven to be (1→5)-$\alpha$-L-Ara, (1→3)-$\alpha$-L-Man, (1→2,6)-$\alpha$-L-Man, (1→)$\alpha$-D-Glc, (1→4)-$\alpha$-D-Glc, (1→6)-$\alpha$-D-Glc and (1→6)-$\beta$-D-Gal. MC-1 possessed significant immunomodulatory activity by enhancing the pinocytic and phagocytic capacity of RAW 264.7 cells, and increasing the levels of NO, TNF-$\alpha$, and IL-6. TLR2, CR3 and MR were confirmed to be the major membrane receptors of MC-1 on RAW 264.7. From the above results, MC-1 could be explored as a functional food supplement candidate for hypoimmunity population.

\textbf{FUNDING}
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20. Seedevi, P.; Sudharsan, S.; Kumar, S. V.; Srivivasan, A.; Vairamani, S.; Shanmugam, A., Isolation and characterization of sulphated polysaccharides from Codium tomentosum (J. Stackhouse, 1797) collected from southeast coast of India.


FIGURE CAPTIONS

**Figure 1.** Chromatography of the polysaccharides from *Lepidium meyenii* by DEAE-Sepharose Fast Flow chromatography (A) and Sephadex G-100 (B); UV spectrum (C) of MC-1.

**Figure 2.** HPGPC (A), FTIR spectrum (B), and triple helical conformation analysis (C) of MC-1.

**Figure 3.** Ion-exchange chromatography of the monosaccharide mixture (A) and MC-1 sample (B). GC of MC-1 after Smith degradation (C). The production of MC-1 after Smith degradation: 1.693 (glycol), 3.361 (glycerol), 8.260 (phycite), 11.320 (arabinose), 17.620 (glucose), 19.555 (inositol).

**Figure 4.** $^{13}$C NMR spectrum (A) and $^1$H NMR spectrum (B) of MC-1.

**Figure 5.** (A) Effect of MC-1 on the viability of RAW 264.7 cells. (B) Effect of MC-1 on taking neutral red of RAW 264.7 cells. Fluorescence microscopic images (C) and the fluorescence intensity (D) of RAW 264.7 cells phagocytosing FITC-labeled E.coli. The group without MC-1 was used as the negative control, and LPS (20µg/mL) was used as the positive control group. *, $p < 0.05$ and **, $p < 0.01$, versus the negative control group. The data shown are means ± SD.

**Figure 6.** Effects of MC-1 on secretion levels of TNF-α(A), IL-6(B), NO(C) and mRNA levels of TNF-α(D), IL-6(E), iNOS (F) in RAW 264.7 cells. The group without MC-1 was used as the negative control, and LPS (20µg/mL) was used as the positive control group. *, $p < 0.05$ and **, $p < 0.01$, versus the negative control group. The data shown are means ± SD.
**Figure 7.** Roles of SR, MR, GR, CR3, TLR2, TLR4 on MC-1 enhancing TNF-α (A), IL-6 (B) and NO (C) secretion in RAW 264.7 cells. The cells were incubated with antibodies of the receptors for 2h and then washed with PBS three times before stimulating with MC-1 (125µg/mL). The anti-mix was the group combined treatment of anti-TLR2, anti-CR3, and anti-MR. (D) Possible mechanism of MC-1 activating RAW 264.7 cells. *, *p* <0.05 and **, *p* < 0.01, versus the negative control group; Δ *p* <0.05 and ΔΔ *p* < 0.01, the groups treated with antibodies versus the group treated with only MC-1 (125 µg/mL). The data shown are means ± SD.
Table 1. Primers used in QPCR.

<table>
<thead>
<tr>
<th>genes</th>
<th>primer sequences</th>
</tr>
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<tbody>
<tr>
<td>iNOs</td>
<td>Forward: 5'-CGGCAAAACATGACTTCAGGC-3'</td>
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<tr>
<td></td>
<td>Reverse: 5'-GCACATCAAAGCGGCCATAG-3'</td>
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<tr>
<td>TNF-α</td>
<td>Forward: 5'-GGGGATTATGGGCTCAGGGTC-3'</td>
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<td></td>
<td>Reverse: 5'-CGAGGCTCCAGTGAATTCCG-3'</td>
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<td>IL-6</td>
<td>Forward: 5'-TACTCGGCAAACCTAGTGCG-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-GTGTCCCAAACATTCTATTGTTCAGT-3'</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward: 5'-TTTGTCAGCTCATTCCCTGTATG-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-TGGGATAGGGCCTCCTTG-3'</td>
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Table 2. Glycosidic linkage composition of methylated MC-1

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<tr>
<th>retention time (min)</th>
<th>methylated sugar</th>
<th>linkage</th>
<th>molar ratio</th>
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<tbody>
<tr>
<td>21.083</td>
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<td>1,5-</td>
<td>27.13</td>
</tr>
<tr>
<td>23.064</td>
<td>2,4,6-Me₁-Manp</td>
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<td>23.311</td>
<td>3,4-Me₂-Manp</td>
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<td>22.232</td>
<td>2,3,4,6-Me₄-Glc</td>
<td>T-</td>
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<tr>
<td>24.240</td>
<td>2,3,6-Me₃-Glc</td>
<td>1,4-</td>
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<td>2,3,4-Me₃-Glc</td>
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</tr>
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<td>24.981</td>
<td>2,3,4-Me₃-Galp</td>
<td>1,6-</td>
<td>1.88</td>
</tr>
</tbody>
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Table 3. Chemical shifts of resonances in the ¹H and ¹³C NMR spectra of MC-1

<table>
<thead>
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<th>sugar residue</th>
<th>chemical shift (ppm)</th>
</tr>
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<tr>
<td>(1→5)-α-L-Ara</td>
<td>C1/H1 106.36/5.28</td>
</tr>
<tr>
<td>(1→3)-α-L-Man</td>
<td>C2/H2 80.93/4.09</td>
</tr>
<tr>
<td>(1→2,6)-α-L-Man</td>
<td>C3/H3 76.64/3.92</td>
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<tr>
<td>α-D-Glc(1→4)</td>
<td>C4/H4 84.00/3.64</td>
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<tr>
<td>α-D-Glc(1→6)</td>
<td>C5/H5 69.14/4.00</td>
</tr>
<tr>
<td>β-D-Gal(1→6)</td>
<td>C6/H6 +/-</td>
</tr>
<tr>
<td>→4)-α-D-Glc(1→6)</td>
<td>C1/H1 99.56/4.53</td>
</tr>
<tr>
<td>→6)-α-D-Glc(1→6)</td>
<td>C2/H2 76.64/3.68</td>
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<tr>
<td>→6)-β-D-Gal(1→6)</td>
<td>C3/H3 73.93/4.09</td>
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<tr>
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<td>C4/H4 72.81/3.83</td>
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<td>→6)-α-D-Glc(1→6)</td>
<td>C5/H5 84.49/3.54</td>
</tr>
<tr>
<td>→6)-β-D-Gal(1→6)</td>
<td>C6/H6 76.15/3.50</td>
</tr>
</tbody>
</table>
Figure 1

A

Absorbance (400 nm)

Concentration of NaCl (mol/L)

Tube number

B

Absorbance (400 nm)

MC-1

MC-2

MC-3

Tube number

C

Absorbance

260nm

280nm

Wavenumber (cm⁻¹)

ACS Paragon Plus Environment
Figure 2
Figure 3
Figure 5

**Figure Description:**

A. Cell survival rate % vs. MCb1 concentration (µg/mL) graph.

B. OD540nm vs. MCb1 concentration (µg/mL) graph.

C. Magnification ×200 images showing control and treated samples.

D. Flow cytometry histograms comparing control, LPS, and treated samples.

**Legend:**
- **Control**
- **LPS**
- **MCb1:** 62.5 µg/mL, 250 µg/mL, 1000 µg/mL
Figure 6
Figure 7
Graphic for table of contents