·实验研究·

苦瓜多糖的纯化、结构解析及其免疫调节和抗肿瘤活性研究

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摘要：目的 提取并纯化苦瓜多糖，对其结构特征进行解析并研究其免疫调节和抗肿瘤活性。**方法** 热水提取苦瓜多糖，透析后纤维素阴离子交换色谱和凝胶过滤层析纯化得到多糖产物。利用高效凝胶渗透色谱法测定多糖分子量，并采用 FT-IR 和 NMR 法对其局部结构特征进行解析。利用细胞分子生物学技术和药理学等手段测定苦瓜多糖的免疫调节作用和抗肿瘤活性。**结果** 分离纯化获得分子量为 745 kDa 的杂多糖 MCP-2，测定其单糖组成为鼠李糖、半乳糖醛酸、半乳糖、木糖和阿拉伯糖，对应的摩尔比为 1.19∶15.97∶3.40∶0.73∶0.94。此外，还发现 MCP-2 能够促进正常小鼠淋巴细胞增殖，提高巨噬细胞 RAW264.7 中炎症因子 NO、IL-1β 和 TNF-α 的表达量。MCP-2 还具有体内抑制 S180 肿瘤细胞的活性。**结论** 本研究表明 MCP-2 具有显著的免疫增强和肿瘤抑制的活性。

关键词：苦瓜多糖；纯化；结构特征；免疫调节活性；抗肿瘤
中图号：R285.5 **文献标志码：**A **文章编号：**1672－0482(2017)01－0033－07
DOI：10.14148/j.issn.1672-0482.2017.0033

Purification, Structural Characterization and Immunomodulatory and Antitumor Activities of a Polysaccharide Isolated from *Momordica Charantia L.*

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ABSTRACT: OBJECTIVE To isolate and purify an active polysaccharide of *Momordica charantia* L. and investigate its structural characterization and immunomodulatory and antitumor activities. **METHODS** A polysaccharides was isolated from *Momordica charantia* L. in hot water extraction followed by dialysis and purification through anion exchange cellulose chromatography and gel filtration chromatography, and its molecular weight was measured by high performance gel permeation chromatography. Additionally, Fourier transform infrared spectroscopy (FT-IR) and nuclear magnetic resonance (NMR) were used to determine its partial structural characterization, and its immunomodulatory and antitumor activities were measured by cell and molecular biological techniques and pharmacological methods. **RESULTS** A heteropolysaccharide named MCP-2 with molecular weight of 745 kDa was isolated and purified, which was shown to be consisted of rhamnose, galacturonic acid, galactose, xylose, and arabinose with corresponding molar ratio being 1.19∶15.97∶3.40∶0.73∶0.94. Furthermore, MCP-2 was found to promote the lymphocyte proliferation of normal mice and to raise the levels of NO, IL-1β and TNF-α produced by macrophage RAW264.7. Intriguingly, MCP-2 presented the antitumor activity against S180 cells *in vivo* as well. **CONCLUSION** All experimental data showed that MCP-2 had apparent immunologic enhancement and antitumor activities.
KEY WORDS: polysaccharide from *Momordica charantia* L.; purification; structural characterization; immunomodulatory activity; antitumor

Momordica charantia (MC), a climber and bitter melon belonging to family Cucurbitaceae, widely grows in Asia, Africa and other developing areas. MC has been used primarily as a vegetable and traditional Chinese medicine as well. With the application of modern experimental methodology, the function of MC as a medicine can be explored. Many studies have been performed to document the pharmacological properties of MC and its extractions in antidiabetic, anti-

收稿日期：2016-10-28；**修稿日期：**2016-12-28
基金项目：江苏省自然科学基金(BK20161041)；江苏省自然科学基金普通高校项目(16KJB310009)；江苏高技术研究重点实验室开放项目(FJGJS-2015-03)；江苏省卫生计生委面上科研项目(H201625)；南通市科技计划项目(GY12016047)
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bacterial, antiviral, anticancer, and immunomodulatory activities, and so on[1-3]. Since infection and cancer diseases present significant challenges to healthcare providers today, researches on the immunomodulatory and antitumor effects of MC are of particular importance.

Many studies have shown that MC components exert dual effects on immune system as immunostimulatory and immunosuppressive agents. Both *in vivo* and *in vitro* data showed that *Momordica charantia* inhibitory protein could ameliorate the immune response to concanavalin A. In contrast, Cunnick et al also reported that MC resulted in interferon production and natural killer cell activity[4]. Because MC is a living organism with complicated mixtures of various components, it is not hard to understand these contradictory results. With multiple and varied hypotheses and claims being put forward, identification of the specific components in MC which may have medicinal properties become an urgent need for the scientific community.

In the current paper, we reported the immunostimulatory and antitumor effects of a specific polysaccharide isolated from MC. We believe that the further evaluation of MCP-2 will provide a unique opportunity for the discovery of novel therapeutic agents and adjuvants that exhibit beneficial immunomodulatory and antitumor properties.

1 Materials and methods

1.1 Materials and reagents

The dried pieces of *Momordica charantia* were purchased from the ZhenJiang LvJian Natural Health Products Co., Ltd. (Jiangsu, China). Mannose, rhamnose, glucose, galactose, arabinose, xylose, glucuronic acid, galacturonic acid, ribose, fucose, trifluoroacetic acid (TFA), 1-Phenyl-3-methyl-5-pyrazolone (PMP), lipopolysacccharide (LPS), concanavalin A(ConA), tumor necrosis factor-α(TNF-α), actinomycin D and MTT (Thiazolyl blue) were purchased from Sigma (St. Louis, MO, USA). Dextran standards (MW 2 000 000, 133 800, 84 400, 10 000, 4 600, 180) were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). RPMI 1640 medium, DMEM HG medium and fetal calf serum (FCS) were the products of Gibco (Grand Island, NY, USA). Penicillin and streptomycin were purchased from Invitrogen (Carlsbad, CA, USA). All other reagents were of analytical grade and obtained locally.

1.2 Animals and cell cultures

Male ICR mice between 6 and 8 weeks old (weight: 20.0±1.0 g) were purchased from the Experimental Animal Center of Qinglong Mountain (Jiangning, Nanjing, China. Quality Certificate Number: SCXK, 2007-0001). The mice were housed under normal laboratory conditions, i.e. room temperature, 12/12 h light-dark cycle with free access to standard rodent chow and water.

RAW 264.7 mouse macrophage-like cells (ATCC TIB-71) were generously donated by Prof. Xiangdong Gao of China pharmaceutical University and cultured in DMEM HG medium (Gibco, USA) supplemented with 10% fetal calf serum (Gibco, USA), 100 U/mL of penicillin, and 100 μg/mL of streptomycin in a humidified 5% CO_2 atmosphere at 37 ℃.

1.3 Extraction, isolation, purification of MCP

The dried pieces of *Momordica charantia* were pulverized by agitation. After passing through a 40-mesh stainless steel sieve, the collected bitter melon powder was degreased by 80% ethanol at 80 ℃ under reflux, then extracted with distilled water (g/mL＝1∶50, pH＝8) for 5 h at 95 ℃, then the water extract was collected and concentrated to 20% of the original volume under a reduced pressure. A four times dilution with 95% ethanol was made by adding 95% ethanol slowly and stirring to precipitate the polysaccharide, and then the mixture was stored overnight at 4 ℃ and finally the polysaccharide pellet was obtained by centrifugation at 4 000 r/min for 15 min. The polysaccharide pellet was completely dissolved in an appropriate volume of distilled water and exhaustively dialyzed for 2 days against distilled water using dialytic membranes (cut off MW 14 000 Da), and the nondialysable phase was concentrated and freeze-dried to supply crude polysaccharide. The crude polysaccharide (1.5 g) was redissolved in distilled water and applied to DEAE Cellulose-32 anion exchangechromatography column(3.5 cm×30 cm), followed by stepwise elution with 0.15, 0.2, 0.25, and 0.3 mol/L sodium chloride solutions at a flow rate of 60 mL/h. The eluates (1.5 mL/tube) were collected automatically and the carbohydrates were determined by the phenol-sulfuric acid method using glucose as standard. The main peak eluated by 0.2 mol/L sodium chloride solutions was fractionated on a Sephacryl S-400 column(1.6 cm×100 cm) eluted with distilled water at a flow rate of 6 mL/h, and two completely separated fractions were obtained. The main fraction (MCP-2) was collected and lyophilized for further study.

1.4 Molecular weight measurement

The molecular weight of MCP-2 was determined by gel chromatographic technique[5] in combination with an Agilent 1100 HPLC system equipped with Shodex OHpak SB802 and SB804 columns (8 mm×300 mm) and a refractive index (RI) detector. An 80 μL of 1% sample solution was injected in the run followed by pre-calibration with T-series Dextran standards (molecular weight 180, 4 600, 21 400, 41 100, 133 800, 2 000 000) and eluted with distilled water at the mobile phase at 0.5 mL/min. The GPC software was used to draw the standard curve and calculate the molecular weight.

1.5 Analysis of monosaccharides

Monosaccharide composition was analyzed according to the PMP labeling procedure[6]. In brief, 1 mL of MCP-2 (2.5 mg/mL) mixed with 1 mL TFA (2 mol/L) was hydrolyzed at 120 ℃ for 2 h. The resulting solution was concentrated in vacuum and the excess acid was removed by repeated co-distillations with distilled water. The solution was directly labeled with PMP by adding 25 μL NaOH (0.6 mol/L) and 50 μL PMP (0.4 mol/L in methanol), vortexing and incubating at 70 ℃ for 120 min. 50 μL HCl (0.3 mol/L) was added to neutralize the mixture, together with 850 μ L of water and 1 mL of chloroform being added and mixed thorough-

ly by vortexing for 3 min. The organic phase (upper layer) was carefully removed and discarded. After the extraction was repeated for two more times, the resulting aqueous phase was ready for HPLC analysis. An Agilent Eclipse XDB-C_{18} (4.6 mm×250 mm) column with the mobile phase (acetonitrile: 0.1 mol/L phosphate buffer=17∶83) at the detection wavelength of 254 nm was utilized. Ten kinds of monosaccharides were labeled by the same procedure.

1.6　UV spectrum analysis of MCP-2

One milligram of MCP-2 was dissolved in 10 mL of distilled water. The sample solution was scanned from 200 to 400 nm via using an ELISA reader (Thermo, USA) when the spectra were recorded.

1.7　Infrared spectrum analysis of MCP-2

The infrared spectrum of MCP-2 was determined through using a Fourier transform infrared spectrophotometer (Bruker, Karlsruhe, Germany) equipped with OPUS 3.1 software. The chromatographically purified MCP-2 was grounded with KBr powder and then pressed into pellet for Fourier transform infrared spectrum measurement in a frequency range of 4 000～500 cm^{-1} [7].

1.8　Assay of mitogenic and comitogenic activities

The mitogenic and comitogenic activities were tested according to a slightly modified method described previously[8]. Spleens collected from sacrificed ICR mice under aseptic conditions were minced using a pair of scissors and passed through a 200-mesh Nylons sieve to obtain a homogeneous cell suspension, and the erythrocytes were lysed with sterile distilled water and 1.8% sodium chloride solution. After centrifugation (1 000×g for 5 min), the pelleted cells were washed three times in PBS and resuspended in RPMI 1640 complete medium. Cell numbers were counted with a haemocytometer by trypan blue dye exclusion technique, and cell viability exceeded 95%. Splenocytes, seeded into a 96-well flat-bottom microtiter plate used at a concentration of 5×10^5 cells per 0.1 mL per well, were stimulated by MCP-2 at final concentrations from 0 to 500 μg/mL. In the comitogenic test, MCP-2 was added at the same concentrations in the presence of ConA (5 μg/mL). The cells were cultured for 48 h at 37 ℃ in a humidified 5% CO_2 atmosphere, and further incubated for 4 h with 20 μL of MTT (5 mg/mL) per well. The plates were centrifuged (2 000×g for 5 min) and the untransformed MTT was carefully removed by pipetting. To each well, 100 μL DMSO was added and the absorbance was evaluated in an ELISA reader (Thermo, USA) at 570 nm after 15 min. The direct mitogenic effect of the tested compounds was expressed as SImit = mean $A570_{\text{test compound}}$/mean $A570_{\text{control}}$, and the comitogenic effect was expressed as SIcomit = mean $A570_{\text{(ConA+test compound)}}$/mean $A570_{\text{ConA}}$.

1.9　Measurement of nitrite production as an assay of NO release

NO production was determined indirectly by assaying the culture supernatant for accumulated nitrite, the stable end product of NO reacted with molecular oxygen as previously described[9]. Briefly, RAW264.7 cells were plated into a 96-well flat-bottom microtiter plate used at a concentration of 1×10^5 cells per 0.1 mL per well and adhered RAW264.7 macrophages were cultured with various concentrations of MCP-2 from 0 to 500 μg/mL or LPS (2 μg/mL) for 24 h at 37 ℃ in a humidified 5% CO_2 atmosphere. After treatment, 100 μL of isolated supernatants were allowed to react with Griess reagent (1% sulfanilamide, 0.1% N-1-naphthylethylenediamine dihydrochloride and 2.5% phosphoric acid) at room temperature for 10 min. Nitrite products were determined by measuring absorbance at 540 nm versus a $NaNO_2$ standard curve using an ELISA reader at 540 nm and the results were shown in μmol/L.

1.10　Assay for TNF-α activity

Briefly, RAW264.7 cells were plated into a 96-well flat-bottom microtiter plate used at a concentration of 1×10^5 cells per 0.1 mL per well, and adhered RAW264.7 macrophages were cultured with various concentrations of MCP-2 from 0 to 500 μg/mL for 4 h at 37 ℃ in a humidified 5% CO_2 atmosphere. After incubation, conditioned supernatants were collected for assaying TNF-α release by determining cytotoxicity using a bioassay of TNF-α sensitive L929 cells[10]. L929 cells (5×10^4 cells, 100 μL) were cultured with diluted supernatants (100 μL) or TNF-α standard preparation in the presence of actinomycin D(1 μg/mL) for 24 h in 96-well flat-bottom microtiter plate. The cells were washed once with PBS and stained with 0.1% crystal violet for 1 h at 37 ℃. The plates were extensively washed with water, and the dye was extracted with methanol. The percentage of L929 cells inhibition was calculated from the absorbance values at 570 nm as follows: $(A_{\text{untreated control}} - A_{\text{treated}})/A_{\text{untreated control}}\times100\%$.

1.11　Assay for IL-1 activity

RAW264.7 cells were plated into a 96-well flat-bottom microtiter plate used at a concentration of 1×10^5 cells per 0.1 mL per well and adhered RAW264.7 macrophages were cultured with various concentrations of MCP-2 from 0 to 500 μg/mL or LPS (2 μg/mL) for 24 h at 37 ℃ in a humidified 5% CO_2 atmosphere. After incubation, conditioned supernatants were collected for assaying IL-1 activity by modified thymocyte proliferation assay[11]. Thymocytes (2×10^6 cells, 100 μL) of ICR mice were plated into a 96-well flat-bottom microtiter plate in RPMI 1640 medium with diluted supernatants (100 μL) in the presence of ConA (final concentration 2 μg/mL). The cells were cultured for 68 h at 37 ℃ in a humidified 5% CO_2 atmosphere, and further incubated for 4 h with 20 μL of MTT (5 mg/mL) per well. The plates were centrifuged (2 000×g for 5 min) and the untransformed MTT was carefully removed by pipetting. To each well, 100 μL DMSO was added and the absorbance was evaluated in an ELISA reader (Thermo, USA) at 570 nm after 15 min.

1.12　NMR analysis

Ten milligrams of MCP-2 was dissolved in 0.5 mL of D_2O at 333 K in 5 mm internal-diameter tubes. ^1H and ^{13}C NMR spectra were recorded (operating frequency of 500.00 MHz) by a Bruker AVANCE AV-500 spectrometer (Bruker, Switzerland) at 25 ℃. The chemical shifts were expressed in ppm relative to the resonance of the internal stand-

ard tetramethylsilane.

1.13　MCP-2 Treatment of S180 Tumor *in vivo*

To examine the therapeutic effects of MCP-2 on the growth of S180 Tumors *in vivo*, each kind of tumor cell was injected subcutaneously into mice at a dose of 1×10^7 cells/mouse separately. Sixty mice were randomized into six groups (each group containing 10 mice): control group, model group, positive control group, and MCP-2-treated groups (of low, middle and high doses). Mice in polysaccharide-treated groups (low, middle and high doses) were orally fed with MCP-2 (5, 10, 20 mg/kg B.W.) once every day, mice in control group and model group were treated with vehicle alone and mice in positive control group were injected with cyclophosphamide once a day (20 mg/kg). The experiment lasted for 2 weeks. At the end of the experiment, mice were sacrificed and spleen and thymus gland of each mouse were taken and weighed. The indexes of spleen and thymus gland were calculated as follows, Inhibitory rate of tumor (%) = 1− (Average tumor weight of treated mice)/(Average tumor weight of model group mice), Spleen index = Spleen weight (mg) /Whole body weight (g), Thymus gland index = Thymus gland weight (mg) /Whole body wight (g).

1.14　Statistical analysis

All experiments were performed with each assay in triplicate. Data were presented as $\bar{x}\pm s$. Statistical differences among different groups were assessed by the Student t-test in Excel 2007. The level of significance was at a P value less than 0.05 or 0.01.

2　Results

2.1　Isolation and purification of MCP-2

The crude polysaccharide was obtained from *Momordica charantia* by hot water extraction followed by ethanol precipitation with yields being 17.2%. After fractionation on DEAE-Cellulose 32 and Sephacryl S-400 gel-permeation chromatography, MCP-2 (52 mg) was obtained. The homogeneity of MCP-2 was elucidated as a single and homogeneous peak by high performance liquid chromatography (HPLC). Profile of MCP-2 in Shodex sugar KS 804 column appeared a single elution peak (Fig. 1).

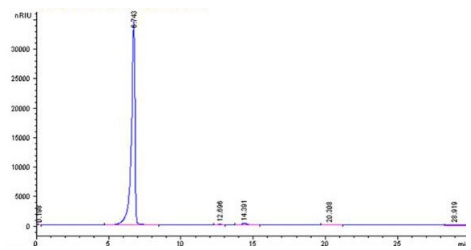


Fig. 1　Elution profile of MCP-2 with HPLC using a refractive index detector

2.2　Monose compositions and MW of purified MCP-2

High performance gel permeation chromatography is often employed to determine the MW of a polysaccharide. Based on the equation of the standard curve made by different dextran standards and the retention time of MCP-2, the MW of the purified MCP-2 was estimated to be 7.45×10^5 Da.

According to PMP labeling procedure, MCP-2 was composed of rhamnose, galacturonic acid, galactose, xylose, and arabinose with molar ratio being 1.34∶15.49∶3.88∶1∶1.29. It was worth mentioning that the polysaccharide consisted mainly of galacturonic acid. PMP precolumn derivation profiles of the mixture solution of standard monosaccharides and MCP-2 were shown in Fig. 2.

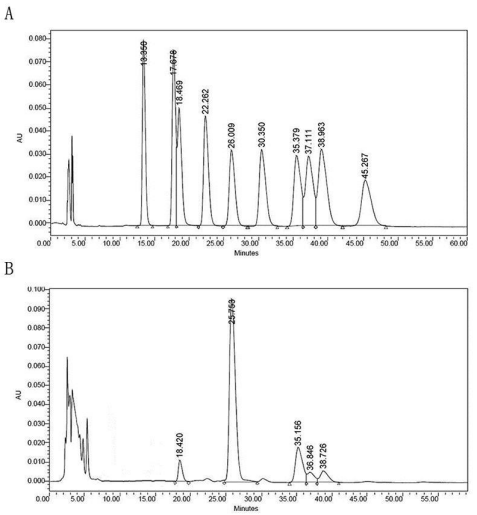


Fig. 2　PMP precolumn derivation profiles of monosaccharide composition of MCP-2

The upper profile (A) represents the sugar standards (From left to right: Man, Rib, Rham, GlcUA, GalUA, Glc, Gal, Xyl, Ara, Fuc). The bottom profile (B) represents the MCP-2 tested.

2.3　Structural determination of MCP-2

As shown in Fig. 3A, the UV absorption spectra of MCP-2 showed no absorbance at 280 nm and 260 nm, implying that protein and nucleic acid were absent in this polysaccharide.

As shown in Fig. 3B, the IR spectrum of MCP-2 displayed a broad stretching intense characteristic peak at around 3 442.59 cm^{-1} for the hydroxyl group, and a weak C−H stretching band was observed at 2 935.54 cm^{-1} [12]. Uronic acids were characterized by the carboxylic group which could lead to two absorbance peaks. The band at 1 744.36 cm^{-1} was attributed to the stretching vibration of C=O in protonated carboxylic acid. Two peaks at 1 628.99 and 1 425.13 cm^{-1} were attributed to the absorbance of COO^- deprotonated carboxylic group[13]. Two distinct-absorbance peaks at 1 744.36 and 1 628.99 cm^{-1} in the IR spectra of MCP-2 resulted from the presence of uronic acids. The absorbance of polysaccharide in the range of 950～1 200 cm^{-1} was C−O−C and C−O−H link band positions. Three stretching peaks at 1 145.48, 1 102.39, and 1 016.29 cm^{-1} in the IR spectrum of MCP-2 suggested the presence of C−O bonds. A stretching peak at 916.16 cm^{-1} in the IR spectrum suggested the presence of D-galactose. The peak at 639.71 cm^{-1} was due to O−H out-of-plane vibration[14].

The ^1H and ^{13}C NMR spectra of MCP-2 were shown in Fig. 4. The ^1H NMR spectrum (Fig. 4A) showed reso-

nances corresponding to C-methyl proton at 1.137 and anomeric proton at d 5.08 due to the a-Rhap residues[15]. The intensity signals at d 2.016 were characteristic of acetyl groups with chemical shifts from 3.435 to 4.425 ppm, showing the overlapping peaks assigned to protons of carbons C−2 to C−5 (or C−6) of the glycosidic ring. The protons (1.93 ppm) were related through two bonds to a carbonyl group that resonances at 173.47 ppm[16]. The ^{1}H NMR spectrum showed the characteristic protons of β-Xylp residues (4.66 ppm), and the relative ^{13}C spectrum signal of C−1 of β-Xylp was shown at 102.8 ppm. The ^{13}C signal at 55.592 ppm confirmed the existence of $-O-CH_3$[16].

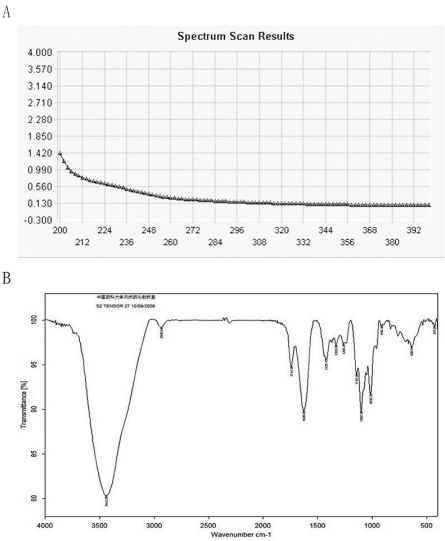


Fig.3 (A) UV spectra of MCP-2 from 200 to 400 nm (B) Infrared spectrum of MCP-2

From the profile of ^{13}C NMR spectrum (Fig. 4B), the signals at 103.080, 70.739, 81.404, 71.351, 76.037, and 173.460 ppm in the ^{13}C spectrum of MCP-2 were related to C−1, C−2, C−3, C−4, C−5 and C−6 of β-D-GalA. The chemical shift at 173.460 ppm showed the existence of uronic acid[17]. The galactan side-chains were characterized by the minor signals at 102.825, 72.013, 74.147 and 75.595 assigned to C−1, C−2, C−3 and C−5, respectively[18]. The chemical shift at 5.045 ppm of anomeric H−1 indicated a form of L-arabinofuranosyl unit[17]. The anomeric region showed signals due to the anomeric carbon of the reducing sugar residues (94.915 ppm).

2.4　Effects of MCP-2 on splenocyte proliferation *in vitro*

Splenocyte proliferation has been shown to be a good index of measurement when investigating the immunomodulatory activity of drug, as shown in Fig. 5A, the polysaccharide MCP-2 was found to significantly increase the proliferation of splenocytes compared with the control in a dose-dependent manner at the concentrations from 100 to 500 μg/mL ($P<0.01$). Consequently, MCP-2 is directly mitogenic for mouse splenocytes. Meanwhile, we detected comitogenic effects of MCP-2 on splenocytes with mitogen such as ConA (5 μg/mL). As shown in Fig. 5B, MCP-2 has no comitogenic activity in low concentrations, but showed a comitogenic activity on ConA-stimulated lymphocytes in a dose-dependent manner at concentrations from 100 to 500 μg/mL ($P<0.05$).

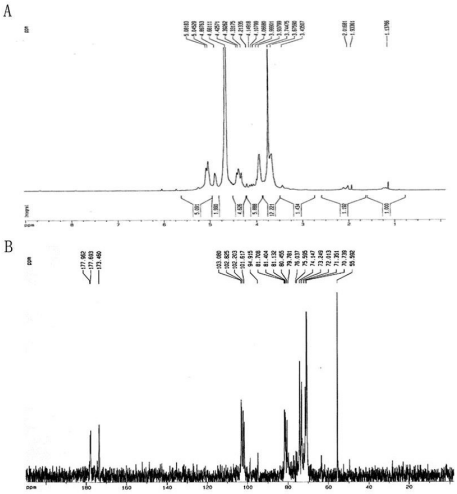
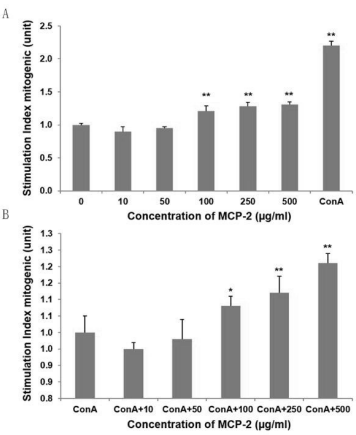


Fig. 4　^{1}H (A) and ^{13}C (B) NMR spectra of MCP-2



(A) Effects of MCP-2 on proliferation of mouse splenocytes *in vitro*. (B) Comitogenic effects of MCP-2 on ConA-stimulated mouse splenocytes *in vitro*. $n=5$. $*P<0.05$ vs. control, $**P<0.01$ vs. control.

Fig. 5　Effects of MCP-2 on mouse splenocytes

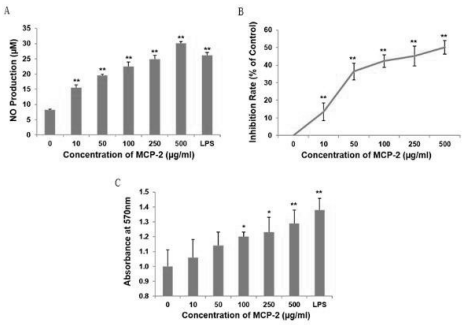
2.5　Effects of MCP-2 on NO production in mononuclear macrophage RAW264.7 cell line

For further estimating the potential of enhancing the innate immune response to MCP-2 stimulation, the concentration dependence of MCP-2 on NO production from mononuclear macrophage RAW264.7 cell line was investigated. As shown in Fig. 6A, MCP-2 was found to significantly increase NO production by RAW264.7 compared with control in a dose-dependent manner at the concentrations from 0 to 500 μg/mL ($P<0.01$) and went through a peak at a concentration of 500 μg/mL. In this study, MCP-2 was proved to be potent activator of mononuclear macrophage RAW264.7 cell line.

2.6　Effects of MCP-2 on TNF-α secretion in mononuclear macrophage RAW264.7 cell line

To further confirm the MCP-2 mediated activation of macrophages, we also assessed the effect of MCP-2 on TNF-α secretion by macrophages. As shown in Fig. 6B, MCP-2 was found to significantly increase TNF-α secretion by RAW264.7 resulting in the augmentation of TNF-α cytotox-

icity against L929 cells compared with control in a dose-dependent manner at the concentrations from 0 to 500 μg/mL ($P<0.01$), this result was also in agreement with that MCP-2 was proved to be potent activator of mononuclear macrophage RAW264.7 cell line.



(A) Concentration dependence of MCP-2 on NO production by mononuclear macrophage RAW264.7 cell line. (B) Inhibition rate of L929 cells treated with the supernatants from MCP-2 stimulated RAW264.7 cells. (C) The proliferation of thymocytes treated with the supernatants from MCP-2 stimulated RAW264.7 cells in the presence of suboptimal concentration of ConA (2 μg/mL). $n=5$. $*P<0.05$ vs. control, $**P<0.01$ vs. control.

Fig.6　Effect of MCP-2 on macrophage cells

2.7　Effects of MCP-2 on IL-1 secretion in mononuclear macrophage RAW264.7 cell line

The presence of IL-1 can stimulate the thymocytes proliferation associate with suboptimal concentration of ConA (2 μg/mL). In this study, we used this bioassay method to investigate the effects of MCP-2 on IL-1 secretion in RAW264.7 cells. As shown in Fig. 6C, MCP-2 was found to increase the IL-1 secretion by RAW264.7 cells in a dose-dependent manner at concentrations from 100 to 500 μg/mL ($P<0.05$ ~0.01).

2.8　MCP-2 treatment of S180 tumor *in vivo*

As shown in Table 1, it was found that, compared to the model group, MCP-2 could inhibit the growth of S180 tumor *in vivo* in a dose-dependent manner at concentrations from 5 to 20 mg/kg. Although it seemed that cyclophosphamide was more effective, the spleen and thymus gland indexs of the cyclophosphamide-treated group were much lower than those of the polysaccharide-treated groups, which meant that cyclophosphamide could potentially destroy the immune system of mice whereas MCP-2 could enhance the immune system of mice especially in its high-dose group.

Table 1　Antitumor activity of MCP on S180 tumor($\bar{x}\pm s, n=10$)

Group	Tumor weight/g	TI	SI	Inhibitory rate of tumor/%
Negative control	1.84±0.27	2.17±0.62	8.32±2.59	0
Positive control(CTX 20 mg)	0.88±0.26**	1.20±0.37	6.23±0.77	52.17
MCP(5 mg)	1.32±0.38**	2.11±0.35##	9.65±1.83 ##	28.26
MCP(10 mg)	1.17±0.194**	2.28±0.51##	10.19±1.39##	36.41
MCP(20 mg)	1.08±0.292**	2.86±0.56##	10.74±1.35##	41.30

Significant differences from negative control group (weight of tumor) evaluated using Student's t-test: $**P<0.01$. Significant differences from positive control group (spleen and thymus gland indexes): $##P<0.01$. TI means thymus gland index; SI means spleen index; CTX means cyclophosphamide.

3　Discussion

Our major finding in this work is the successful isolation and purification of a specific polysaccharide (MCP-2) from MC. We identify the primary structure of MCP-2 and document that this component can be used as an immunostimulatory and antitumor agent. To the best of our knowledge, this is the first scientific report that the immunostimulatory activities of polysaccharide from MC. Polysaccharides exhibit a number of beneficial therapeutic properties and their mechanisms involved in producing these effects are due to the modulation of innate immunity and more specifically, macrophage function. Compared to other components from the extractions such as proteins and hormones, the components from polysaccharides could be provided for further analysis and application. In addition, polysaccharide has less possibility to induce the host-immunorejection and is convenient in storage and delivery[19].

Here we have obtained direct evidence that MCP-2 contributes to the mitosis of splenocytes and shows an additive effect with the presence of concanavalin A, the strongest known mitotic promoter of splenocytes. This part supports that MCP-2 is a potential activator of the humoral immunity.

The macrophage is a cornerstone component of the immune system. Activation of the macrophage is a key event in many pathogenic disorders. The corresponding effects of post-activation of macrophages include the secretion of inflammatory factors and the enhancement of clearance: endocytosis and phagocytosis. Here we observed that a low concentration of MCP-2 could dramatically increase the production of NO. By using two different cell lines, we documented that a similar pattern of TNF activation was achieved by MCP-2. Thus we concluded that MCP-2 could trigger the cellular immunity by activation of macrophages[20].

Polysaccharide compounds commonly have no direct cytotoxicity on tumors, but they play an important role as immunomodulators, and they can raise the body-specific and non-specific immune function. The antitumor effect of MCP-2 may be derived from its immunostimulatory function, and the increase of the thymus gland and spleen indexes in polysaccharides-treated groups correlates well with our hypothesis[21-23].

4　Conclusions

Overall, the primary effect of polysaccharides is to enhance and/or activate macrophage immune responses, resul-

ting in immunomodulation, anti-tumor activity, wound-healing and other therapeutic effects. Based on our experimental data, MCP-2 exerts significant immunomodulatory and anti-tumor effects. We propose that further investigation on therapeutic potentials and toxicity of MCP-2 will shed a light on the ultimate utilization of bitter melon.

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(编辑:董宇)