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Anti-inflammation activity of exopolysaccharides produced by a medicinal fungus *Cordyceps sinensis* Cs-HK1 in cell and animal models

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Short running title: **Anti-inflammatory activity of fungal exopolysaccharide**

Abstract

This study was to assess the anti-inflammatory potential of exopolysaccharide (EPS) produced by a medicinal fungus *Cordyceps sinensis* Cs-HK1. The EPS was isolated from the Cs-HK1 mycelial fermentation broth by ethanol precipitation and purified by deproteinization and dialysis. The EPS had a total sugar content of 74.8% and a maximum average molecular weight (MW) over 10^7 Da, and consisted of mainly glucose and mannose, and a small amount of galactose and ribose. In THP-1 and RAW264.7 cell cultures, EPS significantly inhibited lipopolysaccharide (LPS)-induced inflammatory responses of the cells including the release of NF-κB and several pro-inflammatory factors such as NO, TNF-α and IL-1β. In the murine model of LPS-induced acute intestinal injury, the oral administration of EPS to the animals effectively suppressed the expression of major inflammatory cytokines TNF-α, IL-1β, IL-10 and iNOS and alleviated the intestinal injury. The results suggest that the Cs-HK1 EPS has notable anti-inflammatory activity and can be a potential candidate for further development of new anti-septic therapeutics. To the best of our knowledge, this is the first report on the anti-inflammation of an EPS from *C. sinensis* fungal fermentation.

Keywords: *Cordyceps sinensis* fungus; Exopolysaccharide; Anti-inflammation; THP-1 cell; Cytokines; LPS-induced intestinal injury

1. Introduction

Inflammation is a defensive reaction of the body against the harmful stimuli, which is tightly regulated by the inflammatory response system [1, 2]. However, excessive inflammatory responses out of control by the regulation system can lead to a wide range of chronic inflammatory diseases, such as rheumatoid arthritis, chronic hepatitis, and inflammatory bowel disease [3]. Moreover, inflammatory disorders may be linked with other life-threatening diseases such as cancer, and the effective control of the inflammatory reactions is crucial for

prevention and treatment of many diseases including cancer. The overexpression of inflammatory cytokines such as interleukin (IL)-1β and tumour necrosis factor (TNF)-α, is implicated in the pathogenesis of these inflammatory diseases [4]. Therefore, the modulation of cytokine activities is an important strategy for the treatment of chronic inflammatory diseases.

Conventionally, antibiotics are applied for treatment of infections and steroids for control of inflammation [5]. Increasing concerns with their serious side effects and adverse reactions have motivated the considerable effort in recent decades toward the discovery of safer and more effective anti-inflammation drugs [6]. A favorable and effective strategy in the development of new therapeutics is based on molecular targeting on the mediators that can disrupt specific cellular signaling pathways involved in the diseases [7]. Polysaccharides from edible and medicinal fungi are recognized as the biological response modifiers to produce immunomodulatory and anti-inflammatory activities [8], which are promising candidates for new anti-inflammation agents. Another attractive aspect of the fungal polysaccharides is the selective inhibition of pro-inflammatory molecules without significant cytotoxicity [9]. Polysaccharides as complex biomacromolecules have various characteristics such as monosaccharide composition, glycosidic linkage, degree of branching, molecular weight, bonding of proteins or peptides, which can affect the properties and bioactivities [10].

Cordyceps sinensis, generally known as the Chinese caterpillar fungus (or DongchongXiacao in Chinese), is a unique and esteemed medicinal fungus in traditional Chinese medicine and has also been used as a health food or tonic to promote health and alleviate a variety of diseases [11, 12]. Since the natural Chinese caterpillar fungus is very rare and expensive, mycelial fermentation has widely applied for the production of the fungal materials. Cs-HK1 is a fungus species isolated from the fruiting body of a natural *C. sinensis* and the Cs-HK1 mycelial culture has been found to produce significant amount of exopolysaccharide (EPS) in liquid

fermentation [13]. The EPS had a complex composition and a wide molecular weight (MW) distribution, some of the EPS components showed significant antioxidant and moderate immunomodulatory activities [14]. However, the anti-inflammation potential of EPS from the Cs-HK1 mycelial fermentation remains unknown.

This study was to evaluate the potential anti-inflammatory function of Cs-HK1 EPS in both cell culture and animal models and to examine the activity relationship to the EPS composition. EPS was isolated from the Cs-HK1 liquid fermentation broth by ethanol precipitation and purified by deproteinization and dialysis. The EPS was first tested on human THP-1 monocyte cell line as well as RAW264.7 cell as the *in vitro* model and then on lipopolysaccharide (LPS)-induced acute intestinal injury in mice as an *in vivo* model. The major inflammation cytokines and NO were detected to reveal the anti-inflammatory effects and the potential signal pathway.

2. Materials and Methods

2.1. Cs-HK1 mycelial fermentation and EPS extraction

Cs-HK1 is a fungus species isolated from the fruiting of Chinese caterpillar fungus *Cordyceps sinensis* fruit body by Wu's group [15]. The Cs-HK1 mycelial culture was maintained in a liquid medium consisting of 40 g/L glucose, 5 g/L peptone, 1 g/L KH_2PO_4, 0.5 g/L $MgSO_4 \cdot 7H_2O$ and 10 g/L yeast extract. The Cs-HK1 mycelial fermentation and EPS preparation were carried out as reported previously[15, 16]. In the present study, the Cs-HK1 mycelial fermentation was carried out in 1 L conical flasks each filled with 200 ml of liquid medium and placed on a shaking incubator at 200 rpm and 20 °C for 7 days. The fermentation liquid was then centrifuged (12,000 rpm and 20 min) to attain a solid-free medium. Ethanol at a volume ratio of 5:1 (v/v) was added slowly to the fermentation medium with moderate agitation and then maintained at 4°C overnight and the precipitate was recovered by centrifugation (12,000 rpm and 30 min) and lyophilized, yielding the crude EPS fraction, CEPS.

The CEPS was deproteinized with Sevag reagent (chloroform/1-butanol, v/v = 4:1) for several times till free of precipitates, and the aqueous solution was then collected and dialyzed against distilled water through a 3500 MW cut off membrane for 48 h and finally lyophilized to give the purified EPS.

2.2. Analysis of EPS composition and molecular weight

The total carbohydrate content of EPS samples was determined by Anthrone test, involving the acid hydrolysis of samples at 100 °C in the presence of anthrone agents (2 g anthrone dissolved in 80% H_2SO_4) [17]. The absorbance of samples solution was measured at 620 nm with a spectrophotometer using glucose as a reference. The protein content was determined by Lowry method [18] and the absorbance of was measured at 750 nm using bovine serum albumin (BSA) as a reference.

The monosaccharide composition of EPS was analysed by the PMP-HPLC method as reported previously [19] with minor modifications. In brief, the EPS sample was hydrolysed with 2 M TFA at 110 °C for 4 h, followed by evaporation under vacuum to dryness. The residual solid was re-dissolved in DI water and mixed with 0.5 M PMP solution in methanol and 0.3 M NaOH solution at an equal volume and maintained at 70°C for 30 min. The reaction was stopped by adding 0.3 M HCl, followed by washing thrice with chloroform, and the aqueous layer was collected for the HPLC analysis. The analysis was performed with an Agilent Zorbax Eclipse XDB-C18 column (150 mm × 4.6 mm) on an Agilent 1100 instrument at 25 °C. potassium phosphate buffered saline (0.05 M, pH 6.9) containing 15% (solution A) and 40% acetonitrile (solution B) was used as mobile phase.

The MW of EPS samples was determined by HPGPC as reported previously [19]. The HPGPC system consisted of a Waters 1515 isocratic pump, a 2414 refractive index detector, and three

columns in series, Ultrahydrogel 120, 250 and 2000 (7.8 mm × 300 mm) (Waters Co., Milford, MA, USA) at 50 °C. Water was used as the mobile phase. The EPS samples were dissolved in Milli-Q water, centrifuged at 6000 rpm for 15 min and filtered through 0.45 μM membrane before injection. Calibration curve was derived with dextran MW standards of 1.0-670 kDa and computed with the Breeze V3.3 software.

Fourier transform infrared (FT-IR) spectroscopy of CEPS and EPS was performed with a Nexus 670 FT-IR spectrometer (Thermo Nicolet Co., Cambridge, UK) at the wavenumber range of 500-4000 cm^{-1} with KBr pellets and referenced against air in the Spectrum 6.1 software.

2.3 Cell cultures assays

2.3.1 Cell lines and culture conditions

The cell line used in this experiment was THP-1-Dual cell line, obtained from InvivoGen (San Diego, CA, USA). It was derived from the human THP-1 monocyte cell line by stable integration of two inducible reporter constructs (NF-kB-SEAP and IRF-Lucia reporter monocytes). THP-1 cell line is a human monocytic cell line which could be induced by LPS as a prototypical stimulator for cytokine production [20]. Briefly, the THP-1 cell culture was maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), and penicillin (100 U/ml) and streptomycin (100 μg/ml) with extra 25 mM HEPES (Sigma-Aldrich, Shanghai, China) and 100 μg/ml Nornocin (InvivoGen). The culture was incubated at 37°C in a humidified incubator with 5% CO_2 and kept in logarithmic growth at 5-15 × 10^5 cells/ml through routine sub-culturing, according to standard ECACC protocol. During this study, the culture was sub-cultured every 3 days by inoculating 7 x 10^5 cells/ml and the passage number was kept below 20. The newly recovered stock culture was sub-cultured for at least one passage, and then further sub-cultured in the growth medium to which 100 μg/ml of Zeocin™ and 10

μg/ml of blasticidin (InvivoGen) were added every other passage to maintain the selection pressure.

The most commonly used cell line for immuno- and inflammation assays, macrophage RAW264.7 was also used in some of the experiments to confirm the anti-inflammatory activity of EPS. The RAW264.7 cells were maintained in DMEM (glutamine, high glucose) supplemented with 10% FBS, and penicillin (100 U/ml) and streptomycin (100 μg/ml) and were incubated at 37°C under a humidified atmosphere with 5% CO_2. The maximum passage number was 40 in the experiments.

2.3.2 Cell viability assay

For viability assessment, cells were first incubated in 96-well flat-bottom tissue culture plates (10^5 cells/well) for 24 h to give a stable growth, and then stimulated with different CEPS/EPS concentrations for 48 h. After 48 h of incubation, cell viability was evaluated by the MTT (methyl thiazolyl tetrazolium, Sigma) assay according to previously study [21, 22]. The succinate dehydrogenase in mitochondria of living cells reduced exogenous MTT to dirty blue and purple crystals insoluble in water and deposited in cells, but dead cells did not have this function. After 4 h incubated with MTT, Dimethyl sulfoxide (DMSO) was added to dissolve MTT, and the supernatant was shaken well in a plate shaker. Then the optical density (OD) value was determined at 490 nm by a microplate reader. All the experiments and measurements were done in triplicate and arithmetic averages were taken throughout the data analysis and calculations.

2.3.3 NF-kB assay

NF-κB-induced SEAP activity of THP-1 cells was assessed using Quanti-Blue agent (InvivoGen). The cells were first fully recovered and remaining stable growth, then were

incubated in 96-well flat-bottom tissue culture plates (2×10^5 cells/well) for 48 h and then subjected to different treatments, LPS (Sigma-Aldrich, Shanghai, China) at 1 μg/ml, 1 μg/ml LPS + CEPS or EPS at 50 – 500 μg/ml. A control group of cells was included which was not subjected to any treatment. 48 h later, 20 μl culture supernatant was collected from each well and the alkaline phosphatase activity was assayed by adding Quanti-Blue reagent at 1:4 (v/v). The activation of NF-kB was determined by measurement of absorbance at 625 nm relative to that of LPS-stimulated samples.

2.3.4 Nitric oxide (NO) assay

The cells were cultured and subjected to various treatments in the same ways as in section 2.3.3. After 48 h incubation, 100 μl of the culture supernatant was collected from each well and the concentration of nitrite was determined by the Griess assay [23]. All the samples were tested in triplicate by a nitrite detection kit according to the manufacturer's instructions (Beyotime, Shanghai, China).

2.3.5 Analysis of cytokines by ELISA

The cells were inoculated in 24-well plates (5×10^5 cells/well) and pre-incubated for 24 h. At the end of the pre-incubation period, the wells were rinsed with phosphate buffer saline (PBS), and the medium was exchanged to RPMI1640 without FBS. The cells were then stimulated subjected to LPS and CEPS/EPS treatments as in section 2.3.2 for 48 h to induce inflammatory cytokines (IL-1β and TNF-α). The conditioned or treated culture medium was collected and centrifuged, and the concentration of cytokines in the supernatant was measured by DuoSet enzyme-linked immune sorbent assay (ELISA) kits (R&D Systems) (Sigma-Aldrich, Shanghai, China) according to the manufacturer's instructions.

2.4 Animal test with the LPS-induced acute intestinal injury mouse model

The LPS-stimulated mouse model is a well-established animal model for the study of inflammation and *in vivo* assessment of anti-inflammation agents [24]. The LPS-induced pathological process is marked by a range of inflammatory responses such as acute intestinal injury and secretion of inflammatory cytokines by macrophages [25, 26]. In this study, male C57BL/6 mice weighing 20-25 g were used. The animals were housed in temperature-controlled rooms and received food and watered libitum. All experiments were performed in accordance with the China National Accreditation Service for Conformity Assessment (CNAS L3623), which were approved according to the Ethics Committee of Guangdong Medical Experimental Animal Centre. The mice were randomly assigned to five treatment groups (n = 10 per group) as shown in Table 1.

The EPS samples were dissolved in distilled water with low or high dose (EPS-L or EPS-H) and then was administered to the mice via oral gavage for 7 consecutive days before the LPS stimulation. On the last day LPS (an *Escherichia coli* endotoxin O55:B5, Sigma Aldrich Inc., St. Louis, MO, USA) was injected (at 25 mg/kg) to the mice one hour after the oral administration of EPS. Dexamethasone (Dexameth) was used as a positive control drug and 100 mg of the drug was pre-dissolved in 1 ml DMSO, and the solution was diluted to 0.1% (v/v) with normal saline and the diluted solution was intraperitoneally injected to each mouse. The drug was administered to the mice by oral gavage at 0.1 mg/ml immediately after the LPS injection. At 4 h after LPS injection, the jejunum tissue and peripheral blood of each mouse was collected for histography and cytokine analysis. The major inflammatory factors were measured including the pro-inflammatory cytokines IL-1β, TNF-α and the anti-inflammatory cytokine IL-10 in jejunum tissues and the level of iNOS in serum using commercially available ELISA or NO synthase assay kits (Beyotime, Shanghai, China).

For the intestinal histopathology, animals were taken from each group, and their ileum tissues were rinsed with PBS buffer and fixed in 4% paraformaldehyde solution for 24 h, the tissues

were embedded in paraffin and cut into slides, then the sections were dehydrated and stained with hematoxylin and eosin (H&E) for histopathological examination. The histological structures of ileum of experimental animals in each group were observed under the photographed light microscope (Olympus IX73, Japan) to determine the pathological changes such as edema, shedding of intestinal villi and inflammatory cell infiltration.

2.9 Statistical analysis of experiments

All data are presented as the mean ± standard deviation (SD) from at least three independent experiments. The statistical analysis was performed using the SPSS 16.0 statistical software package. Independent-samples T test was performed for comparison the differences among the different treatment groups. The IC_{50} values were calculated according to Beck et al (2017) using the GraphPad Prism 6.02 software package (GraphPad Software Inc., USA).

3. Results and discussion

3.1 Composition and properties of EPS

Table 2 shows the major chemical contents of EPS and its partially hydrolyzed products. The crude EPS (CEPS) had a total sugar content of 45.6% and total protein content of 17.3%. After deproteinization and dialysis of CEPS, the resultant EPS fraction had a much higher total sugar content of 74.8% and much lower protein content of 0.9%. EPS was composed mainly of glucose and mannose, and low contents of ribose and galactose. On GPC, CEPS exhibited three major peaks (Supplemental Fig.1S), two high MW peaks in the order of 10^8 and 10^5, and a very low MW peak of 213, while the purified EPS exhibited two major peaks, a high MW in the order of 10^7 and a very low MW peak of 213 Da. The low MW may be attributed monosaccharide residues. Wang et al. [27] previously reported that the polysaccharide fractions extracted from several natural and cultured *C. sinensis* species had a maximum MW of 950 kDa and about 80% of glucose in the monosaccharide composition. In comparison, the EPS

from the Cs-HK1 mycelial fermentation has a much higher MW and lower content of glucose but higher contents of mannose and galactose.

Fig.1 shows the FT-IR spectrum of EPS. The broad and intense peak around 3400 cm^{-1} is attributed to the axial stretch of -OH group of glycol-chain, and the peak around 1380 cm^{-1} to the OH bending vibration. The peak around 2936 cm^{-1} is ascribed to the weak C-H stretching vibration and that at 1644 cm^{-1}, 1557 cm^{-1} and 1427 cm^{-1} are characteristic of the asymmetric and symmetric vibration of the ring stretching of carboxylate group. The absorption peaks in the region of 1000–1200 cm^{-1} are associated with the stretching vibrations of C-O-H side groups and the C-O-C glycosidic band vibrations [28]. The peak around 1077 cm^{-1} is attributed to the C-O stretching of pyranoside, especially in the glucose residues [29]. The absence of absorption peak at around 1710 - 1740 cm^{-1} confirms the absence of carboxylic acid.

3.2. Effect of EPS on THP-1 cell viability

As shown in Fig.2, the cell viability was reduced by 20-30% in the presence of 50-500 mg/ml of CEPS and EPS. The reduction of cell viability by the EPS fractions was most probably attributed to the increase in the viscosity of the liquid culture medium instead of direct cytotoxicity, increasing the resistance to the transfer of nutrients and oxygen to the cells [30]. This may also explain why CEPS with a higher MW reduced the cell viability more significantly than the EPS. Similarly, in a previous study, EPS caused slight reduction of bifidobacterial growth in liquid culture, which was attributed to the viscosity increase [16]. Nevertheless, the cell viability remained over 70% with EPS even at a concentration up to 500 μg/ml. Therefore, the EPS fractions did not have significant cytotoxicity.

3.3 Effect of EPS on LPS-induced NO production of THP-1 cells

As shown in Fig.3a, LPS (1 μg/ml) effectively stimulated the NO production, the activation of

NF-κB pathway, and the release of two pro-inflammation cytokines TNF-α and IL-1β in the THP-1 cell culture, in comparison with the negative control group. In human or animal systems, LPS may cause alternation of the barrier function of tissue-resident macrophages and mast cells, resulting in the increased permeability or the disrupted tight junction [3, 31]. Microbial LPS leaked into the blood stream through the disrupted tight junction causes inflammation by stimulating granulocytes (neutrophils and eosinophils), monocytes, macrophages, and natural killer (NK) cells in non-specific immune system. The LPS-induced inflammatory responses NO production was suppressed both with CEPS and EPS (Fig. 4a and Fig. 4b). The suppressing effects of CEPS and EPS on the NO production was concentration-dependent and were increasingly more significant as the concentration was increased. Nitric oxide (NO) is widely distributed in various tissues and organs, acting as a key messenger in the pathogenesis of multiple inflammatory diseases as well as the host defense against tumor cells and microorganisms [32].

3.4 Inhibition of inflammatory responses in cell cultures by EPS

As shown in Figs. 4c-d, CEPS and EPS suppressed the LPS-stimulated activation of NF-κB and the release of TNF-α and IL-1β in a concentration-dependent manner, compared with the LPS group. However, the CEPS or EPS fraction did not completely eliminate the LPS-stimulated inflammatory responses compared with the levels of negative control, which is similar to previous studies such as that by Liu et al. [33].

Table 3 presents the IC_{50} values of the CEPS/EPS activities derived from the effect versus concentration data. Compared with CEPS, the purified EPS had a similar or more potent effect on most of the inflammatory factors. As the major difference of EPS from CEPS is its higher sugar content and negligible protein, the result suggests that the polysaccharide constituent of EPS instead of protein was mainly responsible for the anti-inflammatory activity.

Moreover, EPS also showed a significant inhibitory effect on the LPS induced inflammatory responses in RAW264.7 cell culture (Figs. 4a-c) and the inhibitory effect was dose-dependent. At the highest dose, EPS reduced the levels of NO by 11.3%, TNF-α by 46.7% and IL-1β by 68.7%. Similarly, Wang et al. [34] reported that an acidic polysaccharide from *C. sinensis* significantly stimulated the release of several major cytokines, while the EPS had an even more significant effect. These results indicated that the EPS had significant anti-inflammatory activities.

The EPS from Cs-HK1 mycelial fermentation was mainly composed of high molecular weight (MW) polysaccharides and polysaccharide-protein (PSP) complexes [35]. As shown in Table 2, EPS had relative high contents of glucose, and mannose and low contents of galactose and proteins, which may be ascribed to the high MW β-glucan and lower MW galactomannan–protein complex, respectively as reported previously [36,37]. There are specific receptors in the immune cells such as Dectin-1 and Toll-like receptors for the β-glucans [38,39] and mannose receptor (MR) specifically recognizing mannose at polysaccharide terminals [40]. The specific functional groups of polysaccharides can bind to these receptors to mediate the immune response and prevent inflammation [39, 41]. In the present study, the crude EPS (CEPS) attained by ethanol precipitation was deproteinized and dialyzed to give the purified EPS with a much higher total sugar content.

Cytokines are intercellular signaling peptides or proteins with relatively low molecular weight that are released by the cells to alter their own functions (autocrine) or those of adjacent cells (paracrine) [42]. They are most important in modulating the host immune and inflammatory responses, especially during infection and trauma. The NF-κB family of transcription factors is involved in a classic inflammatory pathway. That is crucial for the activation of numerous cytokines, chemokines and adhesion molecules, which regulate the inflammatory response, immune response and cell survival [43, 44]. The NF-κB signaling pathways can be activated

by LPS infection of the granulocytes (neutrophils and eosinophils), monocytes, macrophages, and natural killer (NK) cells, via up-regulation of the DNA-binding activity of NF-κB [4]. For example, several genes related to the inflammatory cytokines and chemokines were up-regulated, as the secretion of the cytokines was promoted [45]. IL-1β and TNF-α are two important pro-inflammatory cytokines that are secreted by inflammatory cells. TNF-α can stimulate the production of genotoxic molecules, such as NO and reactive oxygen species that cause DNA damage and mutations [46]. According to the signal pathway of inflammation responses in the animal cells, the results suggest that the Cs-HK1 EPS inhibited the inflammatory NF-κB pathway by suppressing the secretion of inflammatory cytokines.

An important event of immunity is the activation of macrophages for the initiation and propagation of defense responses against pathogens [47]. The macrophages are activated during inflammation to secrete proper amounts of cytokines (such as TNF-α and IL-1β), NO and other factors contributing to the defense against pathogens. However, over reaction of the immune cells results in inflammatory responses which are harmful to the host health [48,49]. With an immune regulation cell model in which the immune cells such as macrophages are un-activated, the polysaccharide can act a stimulator of immune responses to enhance the secretion of cytokines, proving its immuno-stimulatory activity. On the other hand, in an inflammation cell model as the one used in the present study, the immune cells are stimulated with LPS to secrete excessive inflammatory cytokines. The suppression of the inflammatory responses by the polysaccharide represents an anti-inflammatory effect which is beneficial to the host health. In a previous study by our group [34], a fraction of the EPS has shown both stimulatory effect on the secretion of certain cytokines and suppression of pro-inflammatory cytokines such as TNF-α.

3.5 Anti-inflammatory effects of EPS from *in vivo* experiments

As shown in Fig.5, LPS effectively stimulated the inflammatory responses in mice, including

IL-1β, TNF-α, IL-10 and NO to much higher levels than the control. All these LPS-induced inflammatory responses were significantly suppressed by pre-treatment of the animals with EPS at the higher dose EPS-H, though the levels were still higher than the negative control group. For some of the responses such as IL-1β, the treatment effect of EPS-H was even more remarkable than the positive control drug dexamethasone. The treatment effect of EPS-H on TNF-α was also very significant at $p < 0.01$. IL-10 is a multifunctional negative regulatory cytokine, which is closely related to regulating immune responses to certain infections [50,51]. It has been suggested in a previous study that the extracts of *Cordyceps sinensis* could promote the immune regulation activity and enhance bacteriostatic activity of PA-824 via IL-10 in *Mycobacterium tuberculosis* disease [52]. The results from the present study further proved that the EPS from Cs-HK1 has the anti-inflammatory potency by stimulating IL-10 expression. The LPS-stimulated expression of iNOS in the EPS treatment groups was significantly suppressed ($p < 0.05$).

Fig.6 shows the histological images of colonic mucosa of the test animals in the control and treatment groups. The control group had the normal architecture of the intestinal epithelium and wall (Fig. 6a). Compared with the normal, intact colonic mucosa with smooth and ordered tissue structures in the control animals, the LPS treated group exhibited the typical features of acute tissue injury, including mild dilation of the chylous duct, short and swollen intestinal villi, edema and sloughing of the villus tips, as well as infiltration of inflammatory cells into the mucosa. The degree of LPS-induced tissue injury and alternations was notably reduced in both the EPS-L and EPS-H treatment groups (Fig.6d and Fig.6e) as well as in the positive control group treated with dexamethasone medicine (Fig.6c). Therefore, the animal test results provide further evidence for the anti-inflammatory effect of EPS.

There is a growing concern with gut inflammation due to its prevalence and potential pathogenicity and connection to multiple organ failures. The intestinal injury is an early of the

LPS-induced response of gut inflammation which lead to the gut injury and subsequent systemic organ inflammation, and multiple organ failures [53]. Evidence is accumulating for the secretion of inflammatory cytokines during inflammatory bowel diseases (IBDs) [54, 55]. In the active state of the IBD, macrophages in the intestine are activated to release pro-inflammatory cytokines such as inducible iNOS, IL-1 cytokine family, and TNF-α. The increased production of pro-inflammatory molecules attracts more inflammatory cells to the intestine, amplifying and extending the inflammatory response. On the other hand, the abnormal secretion of these molecules is associated with the survival of intracellular bacteria, inflammatory responses and the onset of IBD [56]. Based on the above *in vitro* and *in vivo* experimental results, we propose that the Cs-HK1 EPS suppressed the expression of inflammatory cytokines and alleviated the inflammation by blocking the activation of NF-κB pathway as illustrated in Fig.7.

4. Conclusions

The extracellular polysaccharide (EPS) produced by the Cs-HK1 fungus had a maximum MW over 10^7 Da and was mainly composed of glucose, mannose and galactose. The Cs-HK1 EPS exhibited significant anti-inflammatory activities in both cell culture and animal models, effectively suppressing the LPS-induced inflammatory cytokine responses and alleviating the intestinal epithelial injury. Therefore, EPS can be a potential candidate for the development of new anti-inflammatory therapy. However, as EPS is a complex of polysaccharides and proteins with a wide MW distribution, further research effort is needed to identify the most active fractions through activity-guided fractionation. Furthermore, purification of the active fractions and characterization of the molecular structures can be performed for establishing the structure and anti-inflammation activity relationship.

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559 **Table 1** Animal test groups and treatments (DW: distilled water; Dexameth: Dexamethasone)

Control	LPS	Positive control	EPS-L	EPS-H
DW oral for 7 days	LPS 25 mg/kg injection on day 7	DW oral for 7 days; Dexameth 100 mg/kg injection on day 7 following LPS injection	EPS 150 mg/kg oral for 7 days; LPS 25 mg/kg injection on day 7	EPS 300 mg/kg oral for 7 days; LPS 25 mg/kg injection on day 7

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562 **Table 2** Molecular weight and chemical composition of Cs-HK1 EPS

	MW (Da)	Area (%)	Total content (wt%)		Monosaccharide molar ratio			
			Sugar	Protein	Man	Rib	Glc	Gal
CEPS	2.768×10^{8}	68.67	45.6 ± 3.5	17.3 ± 0.27	1.983	0.444	6.895	1.000
EPS	1.424×10^{7}	84.57	74.8±0.44	0.9±0.06	2.653	0.755	5.349	1.000

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565 **Table 3** IC_{50} values (μg/ml) of CEPS and EPS on different inflammatory indicators (IC_{50}: the
566 half maximal inhibitory concentration, the drug concentration required for 50% inhibition
567 Yung-Chi, 1973).

568

Pro-inflammation factors	CEPS	EPS
NF-κB	413.6	67.6
NO	41.6	123.3
TNF-α	49.9	51.2
IL-1β	327.0	183.6

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571 **Fig. 1** FT-IR spectrum of EPS.

572 **Fig. 2** Viability of THP-1 cell line treated with EPS after 24 h incubation (error bar: standard
573 deviation SD, $n \geq 3$).

574 **Fig. 3** Effects of CEPS and EPS on LPS-induced inflammatory responses of THP-1 cell: (a)
575 NO production; (b) NF-κB; (c) TNF-α; (d) IL-1β. * and **: statistically significant difference
576 from LPS group at $p < 0.05$ and $p < 0.01$, respectively (error bar: standard deviation $n \geq 3$).

577 **Fig. 4** Effects of EPS on LPS-induced inflammatory responses of RAW264.7 cell: (a) NO
578 production; (b) TNF-α; (c) IL-1β.

579 **Fig. 5** Effects of EPS in LPS-induced inflammatory responses in C57BL/6 mice, on three
580 inflammation cytokines in jejunum tissue: (a) IL-1β, (b) TNF-α; (c) IL-10; and (d) iNOS in
581 serum (EPS-L: 150 mg/kg; EPS-H:300 mg/kg). * and **: statistically significant difference
582 from LPS group at $p < 0.05$ and $p < 0.01$, respectively.

583 **Fig. 6** Effect of EPS on LPS-induced intestinal injury in mice: (a) negative control; (b) LPS-
584 induced intestinal injury group; (c) positive control (LPS + Dexameth); (d) LPS + EPS-L (150
585 mg/kg); (e) LPS + EPS-H (300 mg/kg).

586 **Fig. 7** A proposed signalling pathway for the anti-inflammatory activity of EPS.