Characterization of polysaccharide from *Helicteres angustifolia* L. and its immunomodulatory activities on macrophages RAW264.7

Shuang Sun[a], Kejuan Li[b], Long Xiao[a], Zhongfang Lei[a], Zhenya Zhang[a,*]

[a] *Graduate School of Life and Environmental Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8572, Japan*
[b] *School of Clinical Medicine, Chengdu University of Traditional Chinese Medicine, 37 Twelve Bridge Road, Jinniu District, Chengdu 610075, PR China*

ARTICLE INFO

ABSTRACT

Helicteres angustifolia L. (*H. angustifolia*) has been widely used as a remedy against various types of illness relating to immune response, such as inflammations and fever. In order to characterize the structure and identify the immunomodulatory activity of polysaccharide from *H. angustifolia*, a polysaccharide fraction (SPF3-1) was purified from *H. angustifolia* by using DEAE Sepharose Fast Flow and Sephacry S-400 chromatography, successively. Physicochemical analysis demonstrated that SPF3-1 is an acidic heteropolysaccharide with a molecular weight of about 13.36 kDa; *in vitro* immunomodulatory assay reflects that SPF3-1 could significantly ($p < 0.05$) enhance the proliferation of macrophages, stimulate the macrophages phagocytic capacity, as well as induce NO and immunomodulatory cytokines generation. All the results suggest that SPF3-1 from *H. angustifolia* possesses potent immunomodulatory activity and could be further developed as new products for medicines or functional foods.

1. Introduction

Immunity is defined as the ability of host defence against various infectious diseases. Until now, a number of chemically synthesized compounds have been used as immunomodulators to regulate the functioning of the immune system. However, some adverse effects, such as hepatotoxicity, nephrotoxicity and hypersensitivity limit their clinical usefulness [1]. In recent years, polysaccharides from natural sources have been paid much attention owing to their nontoxic properties and strong immunomodulatory activities [2,3]. Thus, it is demanding for isolation and identification of novel immunomodulatory polysaccharides from natural sources with additional safety and effectiveness.

Helicteres angustifolia L. (*H. angustifolia*) is a traditional medicinal herb widely distributed in southern China, Laos, and other Asian countries. So far, this plant has been used as a folk medicine for the treatment of various diseases such as flu [4], fever, cold, inflammation, and diabetes [5]. Previous phytochemical research of this plant focused on isolation, analysis or determination of quinones, triterpenoids [6,7], lignans, and alkaloids [8]. Biological studies of this plant have demonstrated its antioxidant, antitumor [9], and anti-diabetic activities [10]. In addition, our recent study has indicated that crude polysaccharide (HACP) extracted from *H. angustifolia* inhibited tumor progression and metastasis in 4T1 tumor-bearing mice [11]. However, the

structure features and the activation abilities on macrophages of its major fractions are not clear yet.

In this study, the major bioactive fraction of HACP was purified stepwise with DEAE Sepharose Fast Flow and Sephacry S-400 column chromatography, successfully. The primary structure features and molecular weight of the obtained polysaccharide fraction were determined. Moreover, its *in vitro* immunomodulatory activities on macrophages were also evaluated.

2. Materials and methods

2.1. Plant material and reagents

Fresh *H. angustifolia* roots were collected from Laos in June 2015. The roots were then cleaned and dried at 40 °C, and ground into fine powder for further use.

Dulbecco's modified eagle medium (DMEM), streptomycin, penicillin and fetal bovine serum (FBS) were obtained from GIBCO (Grand Island, NY, USA). Griess reagent, lipopolysaccharide (LPS), {3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide} (MTT) were purchased from Sigma Aldrich, Inc. (Saint Louis, MO, USA). DEAE Sepharose Fast Flow and Sephacry S-400 were obtained from GE Healthcare Bio-sciences (Uppsala, Sweden). Mouse TNF-α, IL-2, IL-4, IL-6, and IL-10 ELISA Kits were purchased from eBioscience (San Diego,

* Corresponding author.
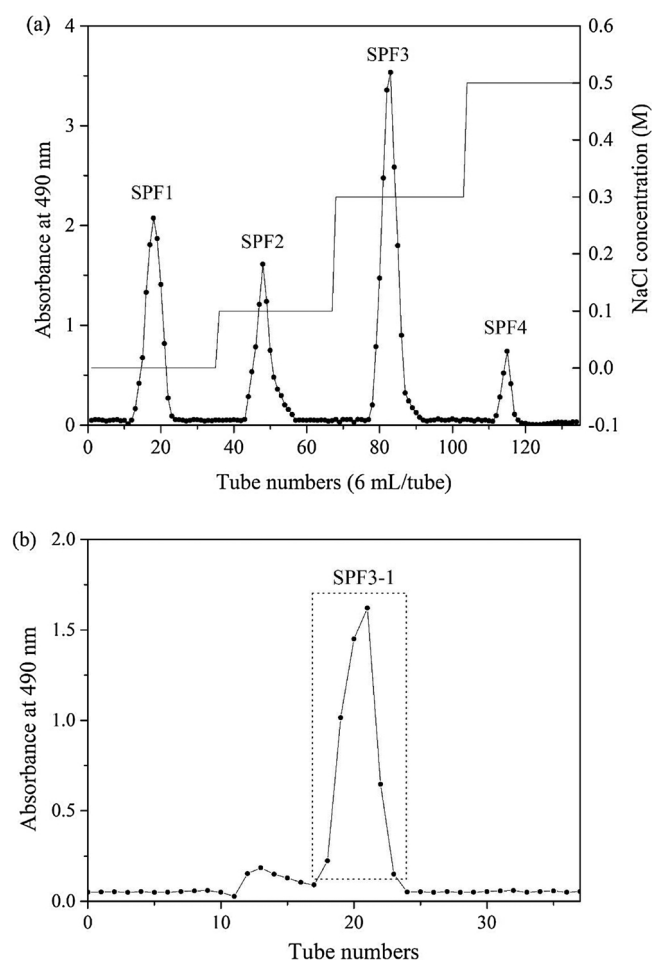*E-mail address:* zhang.zhenya.fu@u.tsukuba.ac.jp (Z. Zhang).

Fig. 1. Separation and purification of HACP: (a) Elution profiles of HACP on the chromatography column DEAE Sepharose Fast Flow; (b) Elution curve of fraction SPF3 on size-exclusion chromatography Sephacry S-400.

CA, USA). Carbazole and D-Galacturonic acid were purchased from Wako (Japan). Protein Quantification Kit-Rapid was obtained from Dojindo Molecular Technologies (Japan). All the reagents used in this study were of analytical grade.

2.2. Extraction and separation of HACP

The crude polysaccharide (HACP) was extracted from *H. angustifolia* according to the method as described previously [11]. The HACP was then purified sequentially by using DEAE Sepharose Fast Flow anion-exchange chromatography and Sephacryl S-400 size exclusion chromatography. Briefly, approximately 300 mg of HACP was dissolved in 10 mL of ultrapure water and applied to DEAE Sepharose Fast Flow column (450 × 40 mm), followed by eluting stepwise with ultrapure water, 0.1, 0.3, and 0.5 M NaCl at a flow rate of 3 mL/min. The elution (6 mL/tube) of each fraction was collected and detected using the phenol-sulfuric acid method [12]. Totally 600 mL of wash solution was used for each fraction. Then, the fractions were concentrated at 50°C by rotary evaporation under reduced pressure, and lyophilized to obtain

SPF1, SPF2, SPF3 and SPF4, respectively. Fraction SPF3 was further fractionated with Sephacry S-400 column (450 × 20 mm) eluted with ultrapure water at a flow rate of 0.5 mL/min. The fractions (3 mL/tube) were collected and analysed by the method mentioned above. Finally, the major fraction named SPF3-1 was then collected, and lyophilized to light yellow powder.

2.3. Cell line and culture

Mouse macrophage cell RAW264.7 and human fibrosarcoma HT1080 cell were obtained from Riken Cell Bank (Tsukuba, Japan). Cells were cultured in DMEM media supplemented with 10% FBS, penicillin (100 U/mL) and streptomycin (100 μg/mL) at 37°C in an incubator with 5% CO_2 atmosphere.

2.4. Preliminary characterization of SPF3-1

2.4.1. Chemical composition and molecular weight (Mw)

The uronic acid content of SPF3-1 was determined by the modified carbazole method according to a previous research [13]. Protein content was determined with the protein quantification kit according to the manufacture procedure.

The neutral monosaccharaides composition of SPF3-1 was determined by using high performance anion exchange chromatography (HPAEC) with pulsed amperometric detection (PAD). SPF3-1 (10 mg) was hydrolysed in a sealed tube with 4 M of trifluoroacetic acid (TFA, 4 mL) at 120°C for 4 h. After cooling to ambient temperature, the excess TFA was removed, then the residue was re-dissolved in 10 mL of ultrapure water, and the resultant solution was diluted for 50 times and filtered through a 0.22 μm filter. The derivatives were then performed on a Dionex HPAEC-PAD system (Dionex, USA) with an advanced gradient pump and eluent degas module. The monosaccharide composition was identified as described previously [14]. The Mw of SPF3-1 was determined by using a high-performance size elusion chromatography instrument equipped with a multi-angle laser light scattering and refractive index (HPSEC-MALLS-RID) system according to the method described by Yang et al. [15] with some modifications. The HPSEC-MALLS-RID system consists of a pump (e2695, Waters, USA), a HPSEC columns (TSKgel SuperMultipore PW-M, TOSOH, Japan), a MALLS detector (DAWN HELEOSII, Wyatt Technology, Santa Barbara, CA, USA), and a RI detector (OPtilab T-rex, Wyatt Technology, Santa Barbara, CA, USA). SPF3-1 (10 mg) was dissolved in 1 mL of distilled water and filtered through a 0.22 μm filter. Twenty μL of SPF3-1 solution was injected into the HPSEC column and eluted with distilled water at a flow rate of 0.5 mL/min.

2.4.2. FTIR and UV spectroscopy

The structure feature of SPF3-1 was analysed by using a Fourier transform infrared spectrophotometer (FT/IR-300, JASCO, Japan). SPF3-1 was ground with potassium bromide (KBr) powder and pressed into a pellet for FTIR measurement at a frequency range of 4000 - 500 cm^{-1}.

For UV spectrum analysis, the aqueous solution of SPF3-1 at 1.0 mg/mL was scanned at the wavelength from 190 to 600 nm on a UV–vis spectrophotometer (UV-3100PC, SHIMADZU, Japan).

Table 1
Chemical composition and molecular weight of SPF3-1.

Carbohydrate	Mw	Uronic acid	Protein	Phenol	Neutral sugar composition (mol%)						
					Rha	Glc	Gal	Ara	Man	Fuc	Xyl
(wt%)	(kD)	(wt%)	(wt%)	(wt%)							
74.8	13.36	58.77 ± 1.64	1.09 ± 0.23	1.01 ± 0.58	22.1	4.5	18.2	37.3	2.0	1.1	14.8

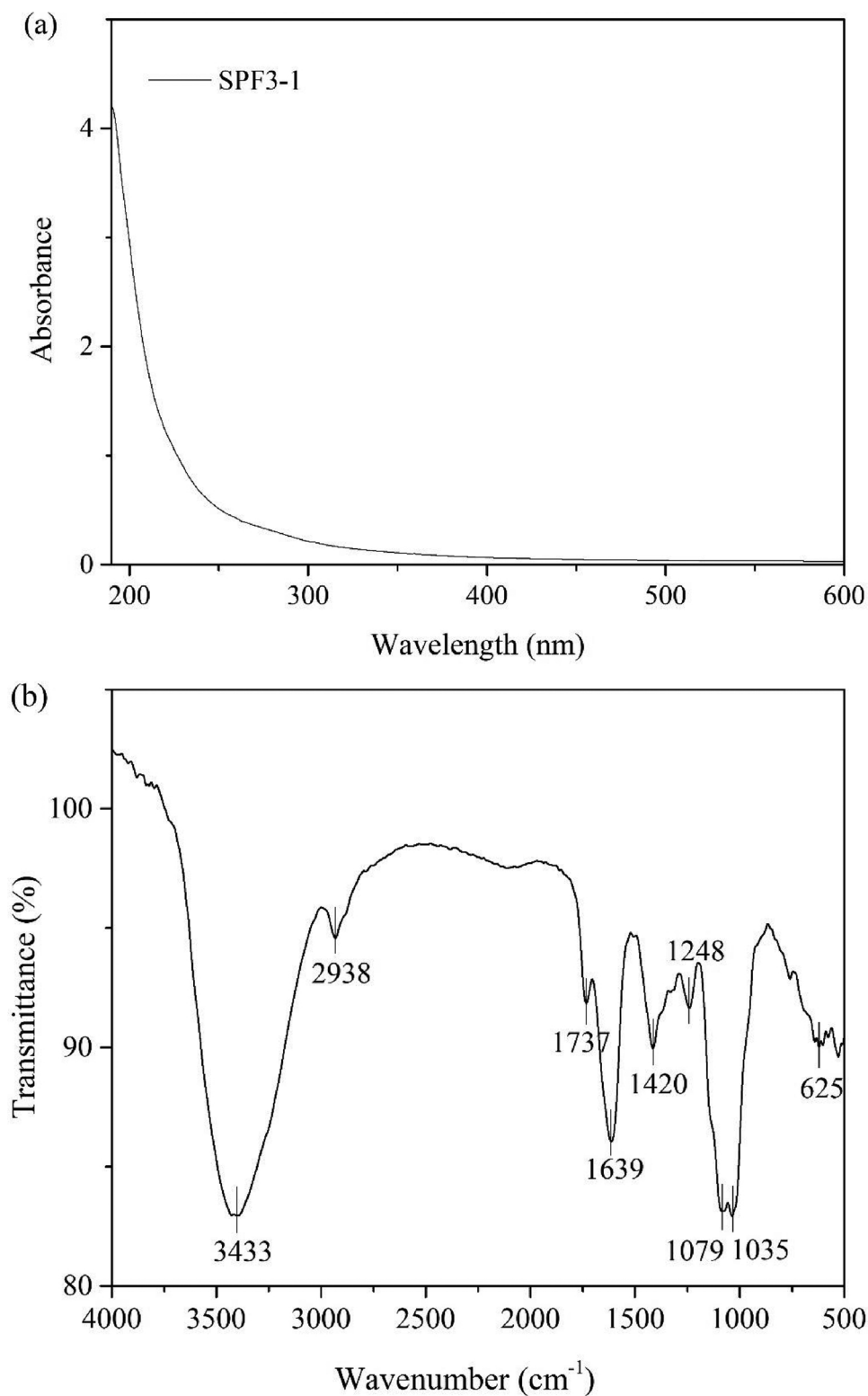Rha- rhamnose, Glc- glucose, Gal- galactose, Ara- arabinose, Man- mannose, Fuc-fucose, Xyl-xylose.

Fig. 2. Structure properties of SPF3-1: (a) UV spectrum of fraction SPF3; (b) FTIR spectrum of SPF3-1; (c) 13C NMR spectrum of SPF3-1; and (d) 1H NMR spectrum of SPF3-1.

2.4.3. Nuclear magnetic resonance (NMR) analysis

SPF3-1 (30 mg) was dissolved in 0.6 mL D_2O in an NMR tube. ^{13}C NMR and ^1H NMR analysis were performed on a Bruker AVANCE-600 NMR Spectrometer.

2.5. In vitro immunomodulatory activity of SPF3-1

2.5.1. Macrophage cell proliferation assay

The proliferation effects of SPF3-1 on RAW 264.7 cell were identified by using MTT assay as described previously [11]. Cells (5000 cell/well) were treated with various concentrations of SPF3-1 (6.25–100 μg/
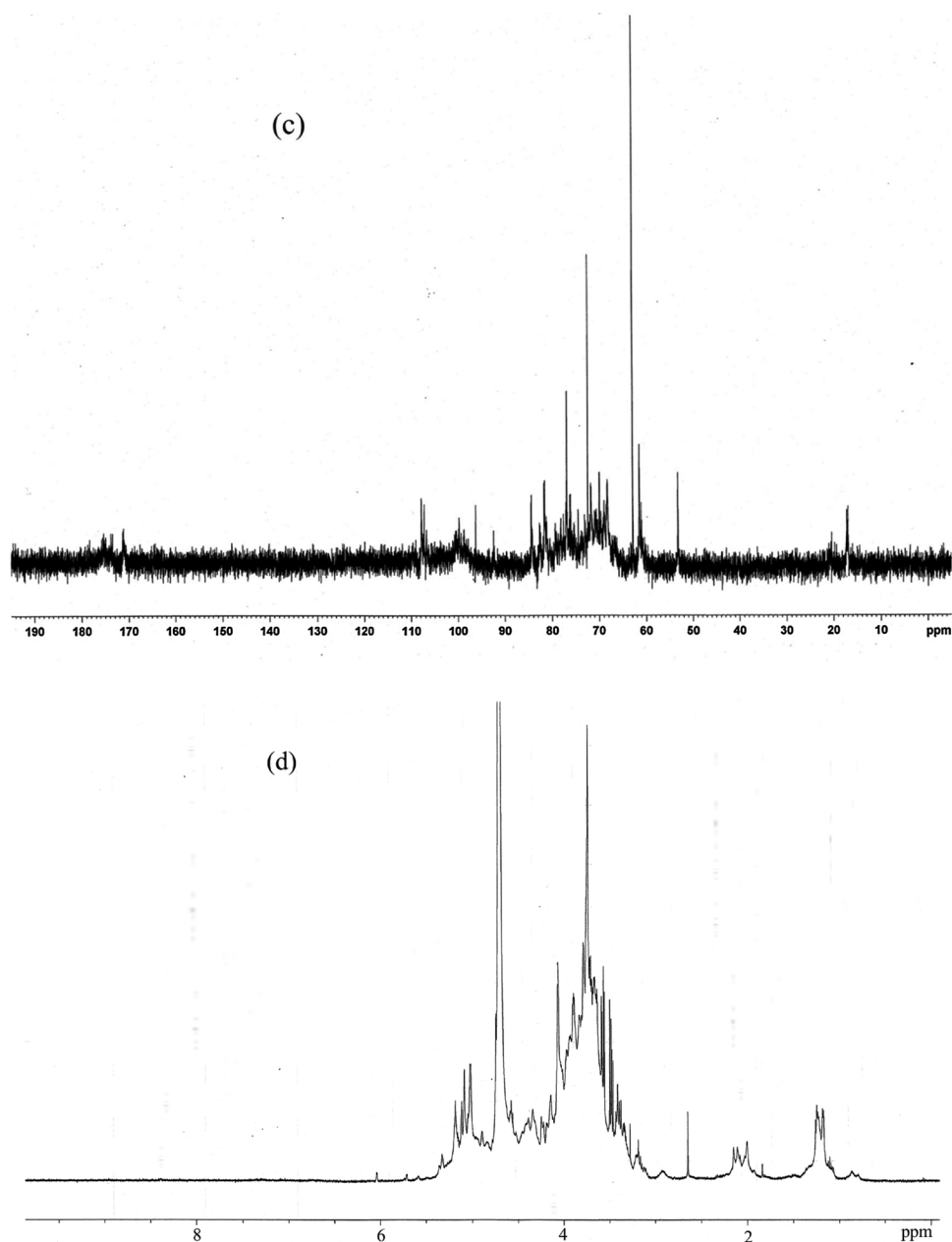
Fig. 2. (continued)

Table 2
Chemical shifts for the resonances in the ^{1}H and ^{13}C NMR spectra of SPF3-1.

Glycosyl residues	Chemical shifts, δ (ppm)					
	C1/H1	C2/H2	C3/H3	C4/H4	C5/H5	C6/H6
→5)-α-Araf-(1→	107.2/5.04	81.3/4.12	76.9/4.01	82.7/4.22	68.3/3.74/3.88	n.d.
→2,4)-α-Rhap-(1→	99.8/4.97	76.3/4.12	71.8/3.87	73.1/3.47	70.6/3.69	16.9/1.15
→1)-α-GalpA-(4→	nd/4.97	68.9/3.85	69.9/3.86	78.1/4.31	72.4/4.73	n.d.
α-Araf-(1→	107.8/5.15	84.4/4.22	79.4/4.03	n.d./4.11	62.8/3.81/3.73	n.d.
α-Rhap-(1→	96.2/4.63	75.4/4.02	69.7/3.89	n.d.	n.d.	17.1/1.31

n.d.: not detected.

mL) for 24 h. The OD values were determined at 490 nm using a Model 550 microplate reader (BIO-RED, Tokyo, Japan). And the macrophage cell viability was then calculated using the following equation: Macrophage cell viability (%) = 100 × (OD$_{SPF3-1}$/OD$_{Control}$), where OD$_{SPF3-1}$ means the optical density of the SPF3-1 treated groups, OD$_{Control}$ is the optical density of control group.

2.5.2. Phagocytic activity determination

The phagocytic uptake ability of macrophages was measured according to the method described previously [16] with some modifications. Macrophages were pre-incubated in 96-well plates for 12 h and then treated without/with SPF3-1 at various doses (6.25–100 μg/ml) for 24 h, followed by addition of 100 μL of neutral red (0.7%, w/v) and
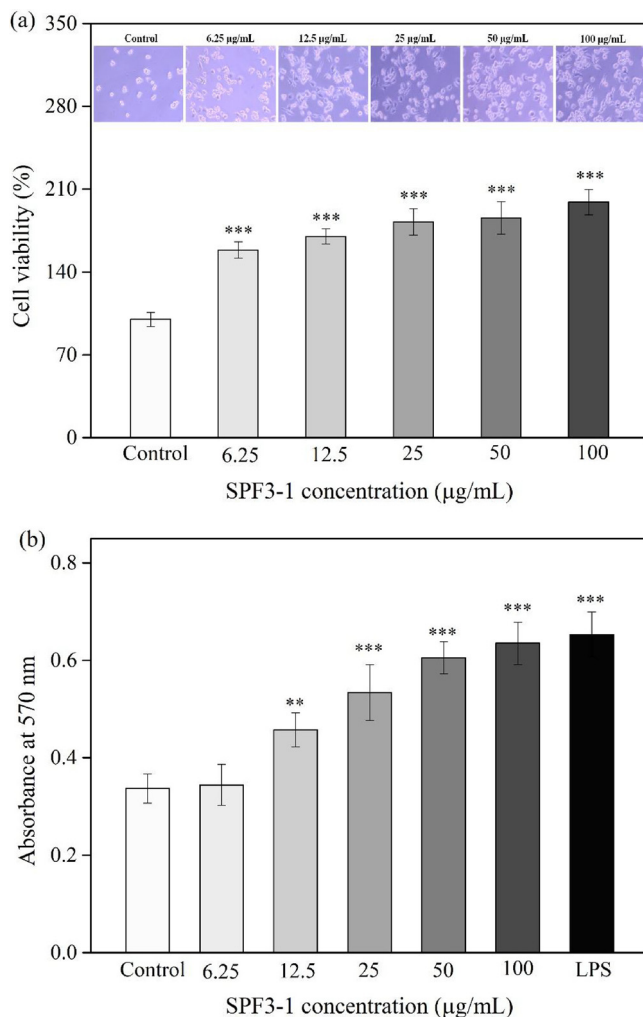
Fig. 3. Effects of SPF3-1 on the viability and phagocytosis of RAW264.7 macrophage cells. (a) Effects on macrophage cell viability; (b) Effects of SPF3-1 on macrophages phagocytosis activity. The cells were treated with various concentrations of SPF3-1 or LPS (10 μg/mL) for 24 h. Results are represented as mean ± SD, n = 5. $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ denote statistically significant difference between the treated and control groups.

Fig. 4. Effect of SPF3-1 on NO, and cytokines production in macrophage cells. (a) NO release in SPF3-1 treated macrophages; (b) Effect of SPF3-1 on TNF-α production; (c) Effect of SPF3-1 on IL-2 generation ; (d) Effect of SPF3-1 on IL-4 generation; (e) Effect of SPF3-1 on IL-6 generation; and (f) Effect of SPF3-1 on IL-10 generation. Results are represented as mean ± SD, n = 5. $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ denote statistically significant difference between the treated and control groups.

cultivation at 37°C for 1 h. Finally, the medium was discarded, and the cells were washed with cold PBS for three times. Afterwards, cell lysis buffer (1% glacial acetic acid: ethanol = 1:1, 100 μL/well) was added and the cells were incubated at room temperature (25 ± 2°C) for another 1 h, and then the optical density was measured at 540 nm by using a microplate reader.

2.5.3. Measurement of nitric oxide (NO) and cytokines production

RAW 264.7 cells (1×10^5 cells/well) were cultured in 48-well plates and stimulated with or without various doses of SPF3-1 (6.25–100 μg/mL). After incubation for 24 h, the culture supernatant was collected, and NO level was measured by using Griess reagent assay [16]. And the cytokines TNF-α, IL-2, IL-4, IL-6 and IL-10 released in the culture supernatant were determined by the ELISA Kits according to the instruction of manufacturers. The cells treated with LPS (10 μg/mL) were used as the positive control, and those cultured in DMEM medium in the absence of SPF3-1 and LPS were used as the negative control.

2.5.4. Cytotoxic effect of SPF3-1 on HT1080 cells

The cytotoxic effect of SPF3-1 on human fibrosarcoma HT1080 cell was identified using MTT assay as described above. Cells were treated with various concentrations of SPF3-1 (6.25–400 μg/mL) for 24 h, cells
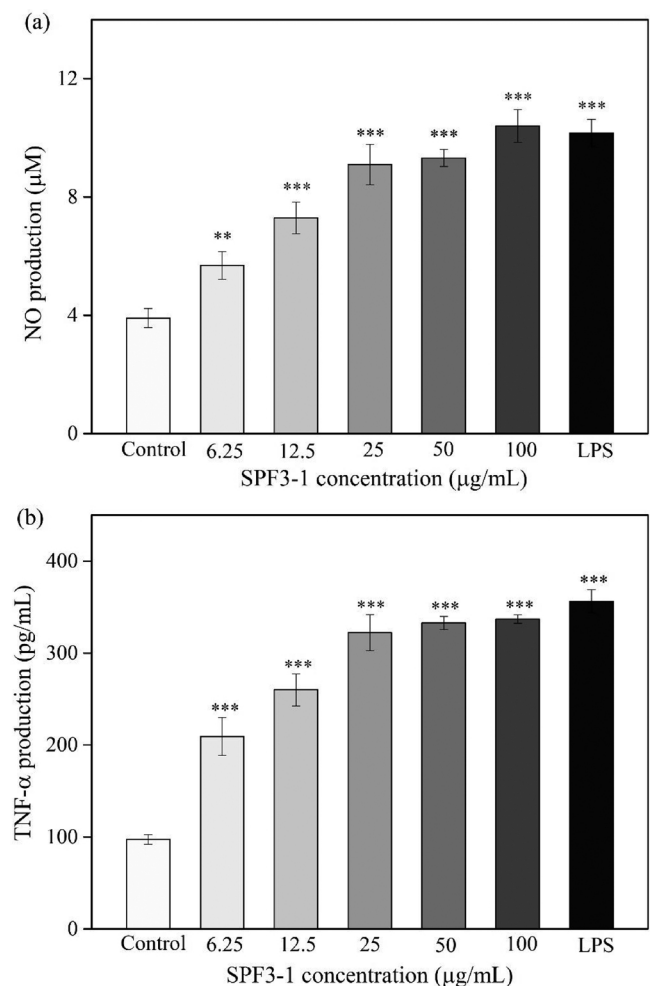
without any treatment were used as control group.

2.5.5. Macrophage-mediated cytotoxicity

The macrophage-mediated cytotoxicity was identified according to Mao et al. [17] and Wang et al. [18] with some modifications. RAW264.7 cells were cultured in 24-well plate (2×10^5 cells/well) overnight for adherent, followed by LPS (10 μg/mL) or various concentrations of SPF3-1 treatment. The cells without any treatment was used as the control group. After 24 h, the supernatant and adherent cells were collected individually. Then, the supernatants were added to a 96-well plate containing adherent HT1080 cells (target cell: 4×10^3 cells/well) and cultured for 48 h. HT1080 cell viability was identified by using MTT assay. Simultaneously, 100 μL of the above SPF3-1 pre-activated macrophage cells (effector cells, 2×10^5 cells/mL) were added to a 96-well plate containing adherent HT1080 cells (target cells, 2×10^3 cells/well) and co-incubated for 48 h. The HT1080 cells co-incubated with macrophage cells which without SPF3-1 treatment was used as negative control group. The cell viability was identified by using MTT assay. And the HT1080 cell viability was calculated by using the following equation: HT1080 cell activity (%) = 100 × (ODT - (ODS - ODE)) / ODT, where ODT means the optical density of target cells control, ODS is the optical density of tested sample, and ODE is the
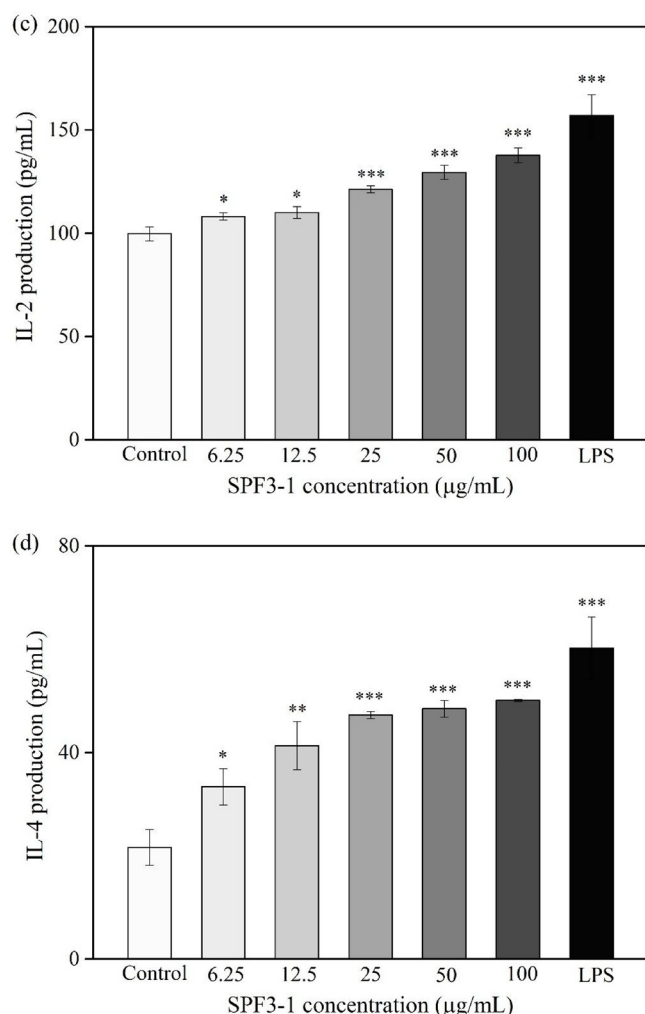
(c)

(e)

(d)

(f)

Fig. 4. (*continued*)

Fig. 4. (*continued*)

optical density of the effector cells control.

2.6. Statistical analysis

All the data were expressed as means ± standard deviation (SD), and the results used in this work were from at least three independent experiments performed in triplicate. In addition, the statistics were analysed using one-way analysis of variance (ANOVA) with the Duncan's multiple-range test. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ were considered as statistically significant.

3. Results and discussion

3.1. Purification and characterization of HACP

3.1.1. Separation and purification of HACP

Since HACP exhibited significant immunomodulatory activity under *in vivo* conditions [11], further separation and structure characterization on its major fractions are necessary. In the present study, the HACP was separated with DEAE Sepharose Fast Flow chromatography as shown in Fig. 1a. Result indicates that the HACP was mainly composed of three fractions (SPF1, SPF2, and SPF3) which were eluted with ultrapure water, 0.1 M, and 0.3 M NaCl, respectively. Due to the highest macrophage cell proliferation effect (data not shown), the fraction SPF3 was selected and its further separation was performed on a Sephacyl S-400 column. Finally, the major fraction SPF3-1 (Fig. 1b) yielding 9.8% of the amount of HACP was collected and lyophilized.
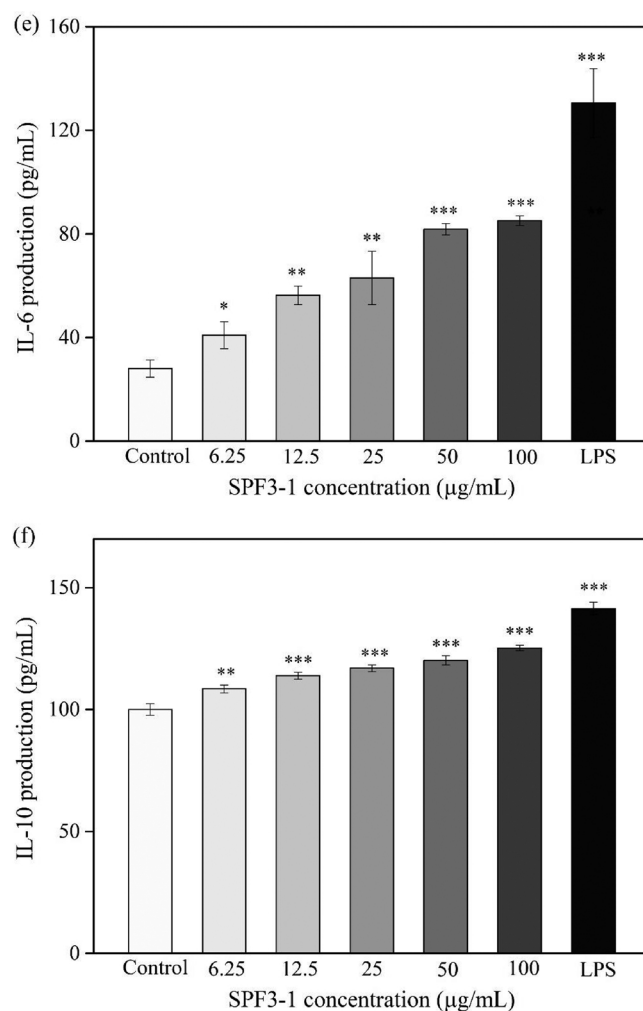
3.1.2. Monosaccharides composition and molecular weight

The chemical composition and molecular weight of SPF3-1 are shown in Table 1. Results reveals that 58.77 ± 1.64% of SPF3-1 is uronic acids. And its main neutral sugars are rhamnose (Rha), arabinose (Ara) and galactose (Gal), indicating that the structure of SPF3-1 possibly consists of a rhamnogalacturonan backbone with arabinan and/or arabinogalactan-rich side chain [19]. Small amounts of xylose (Xyl), glucose (Glc), fucose (Fuc), and mannose (Man) are also detectable. The protein content (1.09 ± 0.23%) is low in SPF3-1, evidenced by a lack of absorbance at 260–280 nm in the UV spectrum (Fig. 2a) as well. In addition, the molecular weight (*Mw*) of SPF3-1 was determined to be 13.36 kDa.

3.1.3. FTIR and NMR spectroscopy

The FTIR spectrum of SPF3-1 (Fig. 2b) reflects that SPF3-1 has the typical characteristic absorption bands of polysaccharide at 4000–500 cm^{-1}. The strong absorption at 3433 cm^{-1} and weak peak at 2938 cm^{-1} are the stretching absorption of O−H and C−H stretching vibrations, respectively [20,21]. The absorptions occurring at around 1737 and 1639 cm^{-1} can be assigned to ester carbonyl (C=O) groups and carboxylate ion stretching band (COO⁻), respectively, reflecting the existence of uronic acids in SPF3-1 [22,23]. This result to a great extent is consistent with the uronic content identified in Table 1. The peak at 1248 cm^{-1} is characteristic absorptions of C=O dilatation vibration [19]. Besides, the peaks at 1035 and 1079 cm^{-1} are attributed to the arabinofuranose units and galactopyanose from the backbone of arabinogalactan. Moreover, the absorption peak at 625 cm^{-1} signals that
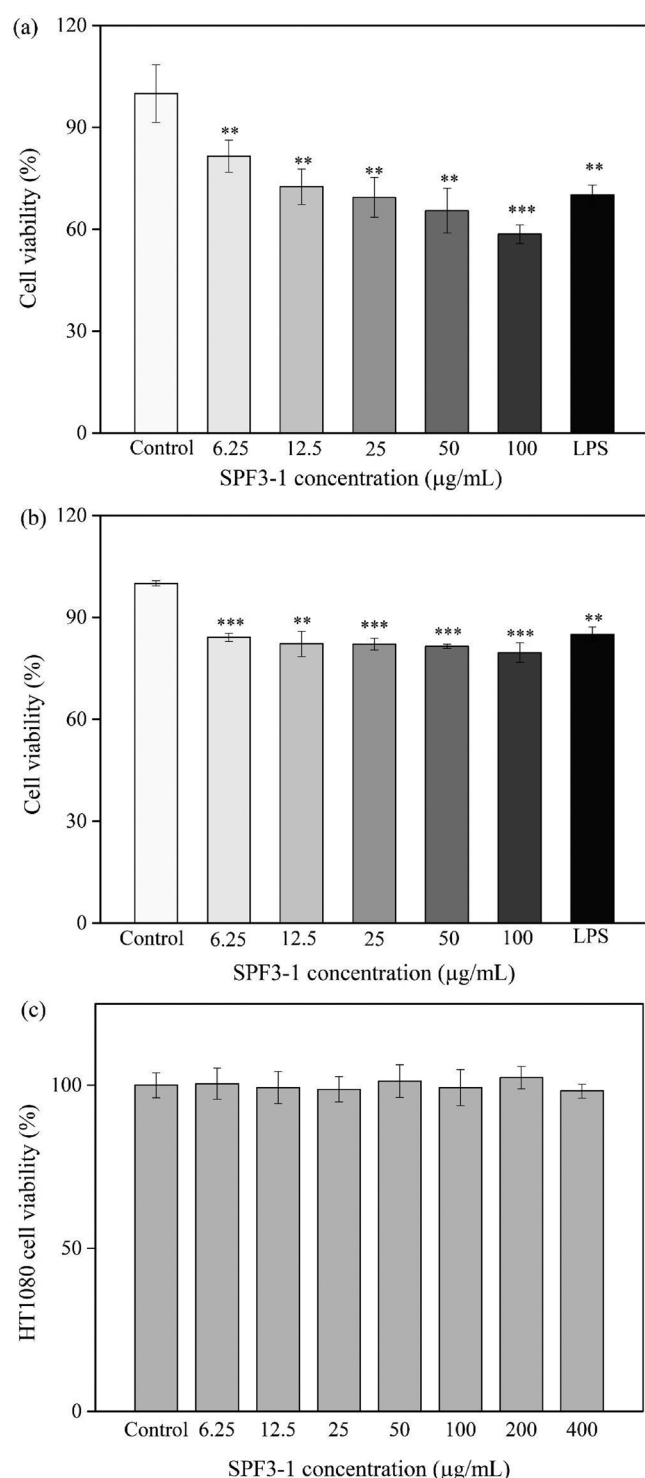
(a)

(b)

(c)

Fig. 5. SPF3-1 induced cytotoxicity of macrophages against HT1080 tumor cells: (a) Cytotoxicity of SPF3-1 activated RAW264.7 towards HT1080 tumor cells; (b) Cytotoxic effect of culture supernatant from SPF3-1 treated macrophages; and (c) Effects of SPF3-1 on HT1080 cell viability. Results are represented as mean $\pm$ SD, n = 5. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ denote statistically significant difference between the treated and control groups.

SPF3-1 contains α-configurations [24].

The structure feature of SPF3-1 was further analysed by the NMR spectra (Fig. 2c and 2d) and the signals of SPF3-1 in ^{1}H NMR and ^{13}C NMR spectra were identified according to previous literatures [25–29]. The region ranging from 3.0 to 5.5 ppm (^{1}H NMR) and 60–110 ppm

(^{13}C NMR) are typical of polysaccharides. As summarized in Table 2, the anomeric carbon signals at δ 107.8, 107.2, 99.8, and 96.2 ppm and those of anomeric protons at δ 5.15, 5.04, 4.97, and 4.63 ppm of SPF3-1 correspond with C-1 and H-1 of anomeric residues α-Araf-(1$\rightarrow$, $\rightarrow$5)-α-Araf-(1$\rightarrow$, $\rightarrow$2,4)-α-Rhap-(1$\rightarrow$, and α-Rhap-(1$\rightarrow$ residues, respectively. In addition, the signals at δ 68.9, 69.9, 78.1 and 72.4 ppm are assigned to C-2, C-3, C-4, and C-5 of 1$\rightarrow$)-α-GalpA-(4$\rightarrow$). The ^{1}H signals at δ 5.14/4.98 ppm and ^{13}C signal at 53.28 ppm of SPF3-1 prove that the α-GalpA is partially methyl esterified. The signal at δ 17.1/1.31 ppm is assigned to the C-6/H-6 of α-L-Rhap.

3.2. In vitro immunomodulatory activity

3.2.1. Effects of SPF3-1 on the viability and phagocytosis of RAW264.7 cells

Macrophage cell, one of the most important immune cells of innate immune system, always act as the first effector cell to aging cells, pathogens and cancer cells [30]. The activation of macrophages has been considered as a primary and necessary step for immune system stimulation. Therefore, in the present study, macrophage cells were used as an *in vitro* cell model to investigate the immunomodulatory effect of SPF3-1. According to Fig. 3a, SPF3-1 exhibits an obvious proliferation effect on macrophage cells, achieving a cell viability of 159.6 $\pm$ 6.9% at 6.25 μg/mL. Besides, the proliferation effect increased with the increase in SPF3-1 concentration, reaching its maximum cell viability of 199 $\pm$ 10.7% at 100 μg/mL.

Phagocytosis is one of the primary features for macrophages response to pathogens and cancer cells [21]. Analysis of the phagocytosis capacity of activated macrophages can reflect the effects of tested sample on immune function. In the present study, SPF3-1 was found to significantly enhance the phagocytosis of macrophages in a dose-dependent manner when its concentrations varied from 12.5 to 100 μg/mL (Fig. 3b). At the highest treatment concentration of 100 μg/mL, the phagocytosis ability increased to 1.9 times of the control group, reflecting that administration of SPF3-1 could efficiently induce macrophages cell immune response.

3.2.2. Increase effects of SPF3-1on NO and cytokines generation in macrophages

As a kind of important molecules produced by macrophages, NO plays a vital role in the regulation of apoptosis and host defence against pathogens and tumor cells [31,32]. At the same time, NO could also enhance the lysis and phagocytosis of macrophages [33]. Thus, the ability to release NO by macrophages reflects the effects of polysaccharides on immune function. In this work, a significant increase in NO release was detected in various SPF3-1 treated macrophages, showing a dose-dependent manner (Fig. 4a). Particularly, the NO production was 10.40 $\pm$ 0.56 μM at 100 μg/mL of SPF3-1 treated group, significantly higher than the control group (3.91 $\pm$ 0.32 μM).

Cytokines are small molecular proteins secreted by activated macrophages. They can mediate and regulate immune response [18]. Among them, TNF-α has been recognized as an important host regulatory molecule with tumor necrosis activity. In the present study, the TNF-α release in the untreated control group was low (97.46 $\pm$ 5.40 pg/mL), while the treatment with SPF3-1 resulted in a significant increase in TNF-α production compared with the control group (Fig. 4b). The highest level of 336.82 $\pm$ 4.63 pg/mL was obtained in the 100 μg/mL SPF3-1 treated group, exhibiting not so much difference with the positive LPS control (356.33 $\pm$ 12.64 pg/mL). As important regulators of host defense responses, IL-2, IL-4, and IL-6 contribute to the differentiation, maturation and activation of cells [34]. As shown in Fig. 4c–e, SPF3-1 exhibited a positive effect on IL-2, IL-4, and IL-6 production in the culture medium of macrophages. In comparison with the untreated cells, SPF3-1 at the concentration of 100 μg/mL caused a 0.38, 1.31, and 3.66-fold increase in the production of IL-2, IL-4, and IL-6, respectively. In addition, with respect to

anti-inflammatory substances, IL-10 has been recognized as a master anti-inflammatory cytokine exhibiting immunosuppressive effects necessary for inflammatory resolution [2]. As shown in Fig. 4f, among the tested dosage, SPF3-1 resulted in an obvious increase of IL-10 production. Taken together, we can conclude that the macrophages activation ability of SPF3-1 is associated with the stimulatory action on the release of NO, and cytokines TNF-α, IL-2, IL-4, IL-6 and IL-10.

3.2.3. Cytotoxic effect of SPF3-1 treated RAW264.7 cells on HT1080 cells

As seen in Fig. 5a, the macrophages induced by SPF3-1 significantly reduced the cell proliferation of HT1080 cells when compared with the control group ($p < 0.01$). The maximum inhibition effect ($41.5 \pm 2.8\%$) was determined at 100 μg/mL of SPF3-1 treated group, greatly in agreement with the above observation on the resultant enhanced macrophages immune response and phagocytosis capacity against cancer cells after pretreatment with SPF3-1. Meanwhile, co-incubation with culture supernatant from SPF3-1 treated macrophages resulted in a significant reduction in cell viability of HT1080 cells (Fig. 5b). The above results demonstrate that after pre-treatment with SPF3-1, the macrophages and culture media possessed a cytotoxic activity against HT1080 cells. No direct cytotoxic effect of SPF3-1 on HT1080 cell was detected in this work (Fig. 5c), indicating that SPF3-1 exhibits an indirect cytotoxicity on HT1080 cancer cells probably by stimulating macrophages immune response.

The immunomodulatory activity of polysaccharides on macrophages is usually correlated with its structure, monosaccharide composition, and molecular weight [35]. Previous results suggest that polysaccharides are mainly composed of Ara, Xyl, Gal, and high content of uronic acid play a positive role in the stimulation of macrophages [30,36,37]. In this study, with relative higher Ara, Gal, Xyl and uronic acid, SPF3-1 was found to strongly stimulate macrophages immune response. In addition, the molecular weight of SPF3-1 was about 13.36 kDa, significantly in accordance with the conclusion that polysaccharides from natural plants with lower molecular weight was proven to possess higher immunomodulatory activities [38].

4. Conclusions

In conclusion, results revealed that the polysaccharide fraction SPF3-1 from HACP displays immunomodulatory activities on macrophages *via* increasing the cell proliferation, phagocytosis ability, and stimulating NO and immunomodulatory cytokines release. This study provides evidence that SPF3-1 from *H. angustifolia* have potent immunomodulatory properties and can be developed as potential immunomodulators for functional foods or drugs.

Funding information

References

[1] I. Jantan, W. Ahmad, S.N.A. Bukhari, Plant-derived immunomodulators: an insight on their preclinical evaluation and clinical trials, Front. Plant Sci. 6 (2015).
[2] Q. Fang, J.F. Wang, X.Q. Zha, S.H. Cui, L. Cao, J.P. Luo, Immunomodulatory activity on macrophage of a purified polysaccharide extracted from Laminaria japonica, Carbohyd. Polym. 134 (2015) 66–73.
[3] C. Nie, P. Zhu, S. Ma, M. Wang, Y. Hu, Purification, characterization and immunomodulatory activity of polysaccharides from stem lettuce, Carbohyd. Polym. 188 (2018) 236–242.
[4] M. Wang, W. Liu, A naphthoquinone from Helicteres angustifolia, Phytochemistry 26 (1987) 578–579.
[5] Y.-S. Chang, Y.-R. Ku, J.-H. Lin, K.-L. Lu, L.-K. Ho, Analysis of three lupane type triterpenoids in *Helicteres angustifolia* by highperformance liquid chromatography, J. Pharmaceut. Biomed. 26 (2001) 849–855.
[6] C.-M. Chen, Z.-T. Chen, Y.-L. Hong, A mansonone from Helicteres angustifolia, Phytochemistry 29 (3) (1990) 980–982.
[7] M.-H. Pan, C.-M. Chen, S.-W. Lee, Z.-T. Chen, Cytotoxic triterpenoids from the root

[8] bark of *Helicteres anguatifolia*, Chem. Biodivers. (2008) 565–574.
[8] G.C. Wang, T. Li, Y.R. Wei, Y.B. Zhang, Y.L. Li, S.C. Sze, W.C. Ye, Two pregnane derivatives and a quinolone alkaloid from Helicteres angustifolia, Fitoterapia 83 (8) (2012) 1643–1647.
[9] K. Li, Z. Lei, X. Hu, S. Sun, S. Li, Z. Zhang, In vitro and in vivo bioactivities of aqueous and ethanol extracts from *Helicteres angustifolia* L. root, J. Ethnopharmacol. 172 (2015) 61–69.
[10] X. Hu, D. Cheng, K. Li, L. Wang, X. Yang, S. Sun, Y. Wang, S. Li, Z. Lei, Z. Zhang, Glucose consumption and α-glucosidase inhibitory activities of aqueous root extract of Helicters angustifolia, Eur. Rev. Med. Pharmacol. 20 (2016) 1423–1429.
[11] S. Sun, K. Li, Z. Lei, L. Xiao, R. Gao, Z. Zhang, Immunomodulatory activity of polysaccharide from Helicteres angustifolia L. on 4T1 tumor-bearing mice. Biomed, Pharmacother 101 (2018) 881–888.
[12] M. DuBois, K. Gilles, J. Hamilton, P. Rebers, F. Smith, Colorimetric method for determination of sugars and related substances, Anal. Chem. 28 (3) (1956) 350–356.
[13] T. Bitter, H.M. Muir, A modified uronic acid carbazole reaction, Anal. Biochem. 4 (4) (1962) 330–334.
[14] W. Cai, H. Xu, L. Xie, J. Sun, T. Sun, X. Wu, Q. Fu, Purification, characterization and in vitro anticoagulant activity of polysaccharides from *Gentiana scabra* Bunge roots, Carbohyd. Polym. 140 (2016) 308–313.
[15] T. Yang, S. Zhang, R. Wang, D. Li, Y. Hu, J. Nie, X. Zhao, Q. Wang, Y. Chen, Y. Zheng, P. Chen, Polysaccharides from Rhizoma Panacis Majoris and its anti-oxidant activity, Int. J. Biol. Macromol. 86 (2016) 756–763.
[16] S. Li, A. Gao, S. Dong, Y. Chen, S. Sun, Z. Lei, Z. Zhang, Purification, antitumor and immunomodulatory activity of polysaccharides from soybean residue fermented with Morchella esculenta, Int. J. Biol. Macromol. 96 (2017) 26–34.
[17] G.H. Mao, Y. Ren, W.W. Feng, Q. Li, H.Y. Wu, D. Jin, T.C.Q. Zhao, L.Q. Xu, X.Y. Wu, Antitumor and immunomodulatory activity of a water-soluble polysaccharide from *Grifola frondosa*, Carbohydr. Polym. 134 (2015) 406–412.
[18] W. Wang, Y. Zou, Q. Li, R. Mao, X. Shao, D. Jin, D. Zheng, T. Zhao, H. Zhu, L. Zhang, L. Yang, X. Wu, Immunomodulatory effects of a polysaccharide purified from Lepidium meyenii Walp. on macrophages, Process Biochem. 51 (4) (2016) 542–553.
[19] X. Wang, Q. Chen, X. Lü, Pectin extracted from apple pomace and citrus peel by subcritical water, Food Hydrocolloid 38 (2014) 129–137.
[20] H. Hu, H. Liang, Y. Wu, Isolation, purification and structural characterization of polysaccharide from Acanthopanax brachypus, Carbohyd. Polym. 127 (2015) 94–100.
[21] W. Liu, H. Wang, J. Yu, Y. Liu, W. Lu, Y. Chai, C. Liu, C. Pan, W. Yao, X. Gao, Structure, chain conformation, and immunomodulatory activity of the polysaccharide purified from Bacillus Calmette Guerin formulation, Carbohyd. Polym. 150 (2016) 149–158.
[22] R. Gnanasambanda, Determination of pectin degree of esterification by diffuse reflectance fourier transform infrared spectroscopy, Food Chem. 68 (3) (2000) 327–332.
[23] S. Zhang, S. Nie, D. Huang, W. Li, M. Xie, Immunomodulatory effect of Ganoderma atrum polysaccharide on CT26 tumor-bearing mice, Food Chem. 136 (3-4) (2013) 1213–1219.
[24] J. Zhao, F. Zhang, X. Liu, K. St Ange, A. Zhang, Q. Li, R.J. Linhardt, Isolation of a lectin binding rhamnogalacturonan-I containing pectic polysaccharide from pumpkin, Carbohyd. Polym. 163 (2017) 330–336.
[25] C. Huang, X.Y. Cao, X.F. Chen, Y.P. Fu, Y.G. Zhu, Z.L. Chen, Q.H. Luo, L.X. Li, X. Song, R.Y. Jia, C.Q. Yin, B. Feng, Y.F. Zou, A pectic polysaccharide from Ligusticum chuanxiong promotes intestine antioxidant defense in aged mice, Carbohyd. Polym. 174 (2017) 915–922.
[26] R.R. Klosterhoff, J.M. Bark, N.M. Glanzel, M. Iacomini, G.R. Martinez, S.M.B. Winnischofer, L.M.C. Cordeiro, Structure and intracellular antioxidant activity of pectic polysaccharide from acerola (Malpighia emarginata), Int. J. Biol. Macromol. 106 (2018) 473–480.
[27] C.Y. Shen, J.G. Jiang, M.Q. Li, C.Y. Zheng, W. Zhu, Structural characterization and immunomodulatory activity of novel polysaccharides from Citrus aurantium Linn. Variant Amara Engl. J. Funct. Foods 35 (2017) 352–362.
[28] C.Y. Wei, P.F. He, L. He, Q.Q. Ye, J.W. Cheng, Y.B. Wang, W.Q. Li, Y. Liu, Structure characterization and biological activities of a pectic polysaccharide from cupule of Castanea henryi, Int. J. Biol. Macromol. 8 (2017) 35–42.
[29] M.M. Zhang, G. Wang, F.R. Lai, H. Wu, Structural characterization and immunomodulatory activity of a novel polysaccharide from *Lepidium meyenii*, J. Agr. Food Chem. 64 (9) (2016) 1921–1931.
[30] H. Sun, J. Zhang, F. Chen, X. Chen, Z. Zhou, H. Wang, Activation of RAW264.7 macrophages by the polysaccharide from the roots of *Actinidia eriantha* and its molecular mechanisms, Carbohyd. Polym. 121 (2015) 388–402.
[31] Q.-M. Liu, S.-S. Xu, L. Li, T.-M. Pan, C.-L. Shi, H. Liu, M.-J. Cao, W.-J. Su, G.-M. Liu, In vitro and in vivo immunomodulatory activity of sulfated polysaccharide from Porphyra haitanensis, Carbohyd. Polym. 165 (2017) 189–196.
[32] Q. Yu, S.P. Nie, W.J. Li, W.Y. Zheng, P.F. Yin, D.M. Gong, M.Y. Xie, Macrophage immunomodulatory activity of a purified polysaccharide isolated from Ganoderma atrum, Phytother. Res. PTR 27 (2) (2013) 186–191.
[33] Z. Ren, T. Qin, F. Qiu, Y. Song, D. Lin, Y. Ma, J. Li, Y. Huang, Immunomodulatory effects of hydroxyethylated Hericium erinaceus polysaccharide on macrophages RAW264.7, Int. J. Biol. Macromol. 105 (Pt 1) (2017) 879–885.
[34] N. Peasura, N. Laohakunjit, O. Kerdchoechuen, P. Vongsawasdi, L.K. Chao, Assessment of biochemical and immunomodulatory activity of sulphated polysaccharides from Ulva intestinalis, Int. J. Biol. Macromol. 91 (2016) 269–277.
[35] Y. Sun, S. Hou, S. Song, B. Zhang, N. Liu, Impact of acidic, water and alkaline extraction on structural features, antioxidant activities of Laminaria japonica

polysaccharides, Int. J. Biol. Macromol. 112 (2018) 985–995.

[36] W. Chen, W. Tang, L. Lou, W. Zhao, Pregnane, coumarin and lupane derivatives and cytotoxic constituents from Helicteres angustifolia, Phytochemistry 67 (10) (2006) 1041–1047.

[37] T.C. Lo, Y.H. Jiang, A.L. Chao, C.A. Chang, Use of statistical methods to find the polysaccharide structural characteristics and the relationships between

monosaccharide composition ratio and macrophage stimulatory activity of regionally different strains of Lentinula edodes, Anal. Chim. Acta 584 (1) (2007) 50–56.

[38] H. Xu, L. Yao, H. Sun, Y. Wu, Chemical composition and antitumor activity of different polysaccharides from the roots of Actinidia eriantha, Carbohyd. Polym. 78 (2) (2009) 316–322.