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journal homepage: <https://www.sciopen.com/journal/2097-0765>Structural analysis of a neutral polysaccharide ATP-1 from the red seaweed *Asparagopsis taxiformis* and its immunomodulatory effects on RAW264.7 macrophagesKun Yang^a, Baojun Xu^c, Wei Hu^a, Yifan Tang^a, Amy Hui-Mei Lin^d, Saiyi Zhong^{a,b,*}^a College of Food Science and Technology, Guangdong Ocean University, Guangdong Provincial Key Laboratory of Aquatic Product Processing and Safety, Guangdong Province Engineering Laboratory for Marine Biological Products, Guangdong Provincial Engineering Technology Research Center of Seafood, Guangdong Provincial Engineering Technology Research Center of Prefabricated Seafood Processing and Quality Control, Zhanjiang 524088, China^b Shenzhen Research Institute, Guangdong Ocean University, Shenzhen 518108, China^c Food Science and Technology Program, Department of Life Sciences, BNU-HKBU United International College, Zhuhai 519087, China^d Singapore Institute of Food and Biotechnology Innovation (SIFBI), Agency for Science, Technology and Research (A*STAR), Nanos 138669, Singapore

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ABSTRACT

As a seaweed supplement, the powdered, entire herb of *Asparagopsis taxiformis* can effectively promote immune response in animals. However, the active factors responsible for this effect are currently unknown. A novel polysaccharide, ATP-1, was isolated and purified from the red alga *A. taxiformis*, and its structural and immunomodulatory properties were determined. ATP-1 is a neutral polysaccharide with a molecular weight of 307.978 kDa and is primarily composed of galactose (94.07%), glucose (1.27%), and xylose (4.66%). Scanning electron microscopy and atomic force microscopy revealed that ATP1 had an amorphous structure and was composed of molecular chain entanglement and intramolecular hydrogen bonding interactions. The microstructure of ATP-1 exhibited asymmetric sheet-like cracks and pores. Results from immunomodulatory activity showed that ATP-1 promoted inducible nitric oxide (NO) synthase and cyclooxygenase (COX)-2 protein expression in RAW264.7 macrophages, improved macrophage phagocytic ability, and stimulated cytokine production (NO, tumor necrosis factor (TNF)- α , and interleukin (IL)-6). Quantitative proteome analysis identified 178 differentially expressed proteins (70 upregulated and 108 downregulated) that were involved in various molecular pathways, among which, TNF was one of the 20 most common and significant signaling pathways in inflammation and immunoregulation. Western blotting indicated that ATP-1 significantly upregulated the expression of IKK α , IKK β , COX-2, and TNF- α proteins in the TNF pathway. In contrast, ATP-1 downregulated the expression of I κ B α , Fas, and AIP1. Results from molecular docking suggested that hydrogen bonding predominantly contributed to the affinity between TNF- α and galactose, glucose, and xylose. These findings indicated that ATP-1 may play an immunomodulatory role by activating the TNF signaling pathway. ATP-1 enhanced macrophage phagocytic capacity and stimulated the production of cytokines NO, TNF- α , and IL-6, thereby exerting immunoregulatory effects. Our findings highlight the potential of ATP-1 as a natural immune modulator with applications as a functional food.

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1. Introduction

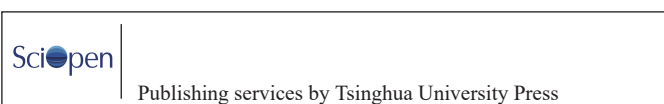
Macroalgae or seaweed has emerged as a promising source of nutraceuticals and pharmaceutical applications owing to the upsurge

of global interest in natural products^[1]. Marine algae, commonly known as seaweed, are widely distributed in the marine world. The main types that are currently being developed and utilized include green algae, blue-green algae, brown algae, and red algae^[2]. Felix et al.^[3] reported that the *Asparagopsis* genus, classified within the Rhodophyta division, produces an array of bioactive compounds, polysaccharides, lipids, proteins, sterols, and bromoform. These compounds exhibit several functional properties such as antioxidant, prebiotic, antimicrobial,

* Corresponding author at: College of Food Science and Technology, Guangdong Ocean University, Zhanjiang 524088, China.

E-mail address: zhongsy@gdou.edu.cn. (S.Y. Zhong)

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anti-inflammatory, and antitumor effects^[4-5]. *Asparagopsis taxiformis* is a temperate, economic, red alga. *A. taxiformis* grows on reefs or corals below the low tide line and is distributed along the coasts of mainland China in the South China Sea^[6].

Recent studies have focused on *A. taxiformis* as a potential feed additive to reduce enteric methane production in ruminants, thereby mitigating greenhouse gas emissions^[7]. Brooke et al.^[8] showed that supplementing ruminant feed with 5% organic matter of *A. taxiformis* reduced enteric methanogenesis by 99%. This supplementation did not negatively affect volatile fatty acid profiles or organic matter digestibility. Rodríguez Sánchez et al.^[9] studied sulfated galactans from *A. taxiformis* gametophytes collected in Venezuela and suggested their potential in preventing abnormal blood clotting. Thépot et al.^[10] have reported that the addition of *A. taxiformis* and its extract to the feed of Atlantic salmon led to a 33% increase in its growth rate and a 13% increase in feed intake. Additionally, the feed conversion ratios improved, and the innate immune response was enhanced by 58% over 4 weeks compared with the controls. These findings indicate the potential of *A. taxiformis* in the areas of drug discovery, functional foods, and various other industrial applications. However, knowledge on the bioactive compounds of *A. taxiformis* is significantly low, which considerably hinders their exploitation and utilization.

Studies have shown that the polysaccharides of red algae, which constitute the primary component of cell walls, exert several physiological and biological effects^[11]. Nonetheless, the beneficial biological effects of polysaccharides are dependent on their chemical composition and structure. Zhao et al.^[12] demonstrated that a relatively high proportion of arabinose, xylose, and galactose could remarkably ($P < 0.05$) increase the immunoregulatory effect of a polysaccharide isolated from the rhizomes of *Dryopteris crassirhizoma* Nakai. Meng et al.^[13] indicated that polysaccharides having a triple helix conformation typically exert a better immunostimulatory effect. Liu et al.^[14] have revealed that the immunomodulatory activity of the polysaccharide IHP-1aa could be attributed to its 3-O-CH₃ group and granular conformation. Molecular weight has an indirect impact on immunomodulatory activity. *Astragalus* polysaccharides (APSs) have been separated into the following 3 fragments based on their molecular weights: > 2 000 kDa (APS-I), approximately 10 kDa (APS-II), and approximately 300 Da (APS-III). Immunity studies have demonstrated the potent immunoenhancing effect of APS-II^[15]. Therefore, elucidating the structure of the polysaccharides from *A. taxiformis* is crucial to determine their biological effects.

Low immunity is currently a prevalent health issue due to rapidly changing modern lifestyles. The development of plant-based formulations having immunoregulatory effects is a research hotspot^[16]. Previous studies have reported that diets supplemented with the red seaweed *A. taxiformis* lead to the most prominent enhancements in humoral and cellular innate immune defenses. Hemolytic activity was increased four-fold in fish receiving *A. taxiformis* compared with control fish^[17]. However, the active compounds that exert an immunoenhancing effect are yet to be identified. Accordingly, water-extracted polysaccharides were used as a starting point to explore the possible material basis of this effect. In this study, the polysaccharide ATP-1 was isolated and purified from the red alga *A. taxiformis* using hot water extraction. The primary structure and chain conformation of the resultant polysaccharide were characterized

using gel chromatography, high-performance liquid chromatography (HPLC), Fourier-transform infrared spectroscopy (FT-IR), ultraviolet (UV) absorption spectroscopy, thermogravimetry (TG)-differential scanning calorimetry (DSC), scanning electron microscopy (SEM), and atomic force microscopy (AFM). The immunomodulatory activity of ATP-1 was preliminarily evaluated *in vitro* by measuring cell viability, phagocytic ability, NO secretion, and the levels of cytokines and other indicators in RAW264.7 cells. The potential mechanism of ATP-1 on RAW264.7 macrophages was determined using proteomic analysis, western blotting, and molecular docking.

2. Materials and methods

2.1 Materials and reagents

A. taxiformis was collected from Naozhou Island in Zhanjiang, China. RAW264.7 cells were maintained in high-glucose Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum, along with penicillin (100 U/mL) and streptomycin (100 µg/mL). The cells were cultured at 37 °C in a humidified environment in an incubator, which was flushed with 5% CO₂. Cell counting kit-8 (CCK-8), lipopolysaccharide (LPS) assay kit, nitric oxide (NO) assay kit, and mouse interleukin (IL)-6 and tumor necrosis factor (TNF)-α enzyme-linked immunosorbent assay (ELISA) kits were purchased from SolarBio (Beijing, China), Beyotime Biotechnology (Shanghai, China), and Enzyme-linked Biotechnology Co., Ltd. (Shanghai, China), respectively. Polyclonal antibodies against inducible NO synthase (iNOS), Ikka, Ikkβ, cyclooxygenase (COX)-2 (Ptgs2), Fas, AIP1, TNF-α, IkBa, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were purchased from Wuhan Sanying Biotechnology Co., Ltd. (Wuhan, China).

2.2 Extraction and purification of ATP-1

A sample of 20 g dry weight of the red alga *A. taxiformis* powder was combined with ultrapure water in a 1:15 ratio (*m/V*), stirred evenly, placed in a water bath (60 °C) for 3 h, and ultrasonicated (200 W) for 50 min. Next, the sample was centrifuged at 4 000 r/min for 20 min to concentrate the resulting mixture. Next, 3 volumes of absolute ethanol (4 °C) were added to the concentrated mixture, allowed to precipitate for 24 h, and centrifuged at 4 000 r/min for 15 min. The residue was collected and dissolved in a small volume of water, and the protein was removed using the Sevage method^[18]. The sample was dialyzed for 24 h and freeze-dried for a further 48 h to obtain the crude polysaccharide of *A. taxiformis*.

A 10 mg/mL solution of the crude polysaccharide was prepared. After filtration through a 0.22-µm membrane, it was loaded onto a DEAE52 exchange column (Φ1.5 cm × 40.0 cm). The polysaccharide was eluted with distilled water and 0.3, 0.9, and 1.2 mol/L NaCl solutions at a flow rate of 2 mL/min, and the eluate was automatically collected (5.0 mL per tube). Polysaccharide content was assessed using the phenol-sulfuric acid method^[19]. An elution curve was plotted with the number of collection tubes as the horizontal axis and the absorbance at OD_{490 nm} as the vertical axis. Eluates from the same peak were collected, concentrated, dialyzed, and lyophilized to obtain 3 polysaccharide components, namely, ATP-1, ATP-2, and ATP-3.

2.3 Determination of chemical properties

The total carbohydrate content was determined using the method reported by Zhong et al.^[18], which involved measuring the absorbance at a wavelength of 490 nm using the phenol-sulfuric acid method with *D*-galactose as the standard and then plotting a standard curve. The protein content was determined following the instructions in the bicinchoninic acid (BCA) protein quantitation kit (Beyotime Biotechnology, Shanghai, China).

2.4 Structural characterization of ATP-1

2.4.1 Molecular weight distribution of ATP-1 using chromatography

The molecular weight distribution of ATP-1 was determined using a gel chromatography–differential multiangle laser light scattering system (HPSEC-MALS-RI) with slight modifications per the method reported by Xing et al.^[20]. The following analytical equipment was used: U3000 liquid phase system (Thermo, USA), Optilab T-REX differential detector (Wyatt Technology, CA, USA), and DAWN HELEOS II laser light scattering detector (Wyatt Technology, CA, USA). The gel exclusion chromatography columns OHpak SB-805 HQ (300 mm × 8 mm, Shodex Showa Denko K.K., Japan) and OHpak SB-803 HQ (300 mm × 8 mm, Shodex Showa Denko K.K., Japan) were coupled in series. The column temperature was 45 °C and the loop volume was 100 µL. Mobile phase A (0.02% NaN₃ (*m/V*), 0.1 mol/L NaNO₃) was used at a flow rate of 0.6 mL/min and the sample was eluted using an isocratic gradient for 75 min.

2.4.2 Monosaccharide composition

Fuc, Rha, Ara, Gal, Glc, Xyl, Man, Fru, Rib, Gal-UAGul-UA, Glc-UA, Man-UA, and other standard samples were accurately weighed, added to pure water to prepare a mixed standard solution, and diluted appropriately to yield a concentration series. Monosaccharide composition was determined using an electrochemical detector and a Dionex™ CarboPac™ PA20 (150 mm × 3.0 mm, 10 µm) liquid chromatography column following the method reported by Wu et al.^[21].

2.4.3 Thermal characterization of ATP-1

The thermal characterization of ATP-1 was performed using a TGDSC thermal analyzer (STA 449F3). Briefly, a sample of 5 mg dry weight of ATP-1 powder was accurately weighed and placed in a crucible. The temperature program involved ramping up the temperature from 50 to 900 °C at a rate of 10 °C/min^[22].

2.4.4 FT-IR spectroscopy and UV scanning

The functional groups in ATP-1 were determined using FT-IR spectroscopy. An appropriate amount of the polysaccharide powder and potassium bromide (dried at 100 °C for 12 h) were mixed evenly, and the mixture was ground to a uniform, fine powder. The powder was pressed into a thin, transparent sheet, and the infrared spectrum of the sample was obtained by scanning at a wavelength range of 4 000–400 cm⁻¹.

Potassium bromide was used as a blank correction background to measure and record the infrared spectrum of the sample^[23]. The UV absorption spectrum was acquired by preparing a 0.5 mg/mL polysaccharide solution and scanning it at a wavelength range of 200–700 nm^[24].

2.4.5 Morphology determination using SEM and AFM

The micromorphology of the polysaccharide ATP-1 was analyzed using SEM (Zeiss Merlin Compact, Japan), and the structural morphology was determined using AFM (Dimension Icon, Bruker, Germany). The scanning frequency was 1.00 Hz, and the AFM model mode was “tapping”^[25].

2.5 Immunomodulatory effect of ATP-1 on RAW264.7 cells

2.5.1 Cell viability

RAW264.7 cells were seeded into a 96-well plate at a concentration of 1×10^4 cells per well. After 24 h of adherence, 0, 0.125, 0.25, 0.5, and 1 mg/mL ATP-1 was added, with 4 replicates for each group. Subsequently, following a 24-h exposure period at 37 °C, 10 µL of CCK-8 solution was added to each well. The plates were incubated for an additional 30 min at 37 °C before determining the absorbance at 450 nm using a microplate reader. Cell viability was calculated following the instructions provided in the CCK-8 assay kit^[26].

2.5.2 Phagocytic activity

The phagocytic activity of RAW264.7 cells was evaluated using the neutral red method^[27]. Briefly, RAW264.7 cells were incubated in a 96-well plate (1×10^4 cells per well) for 24 h. Different concentrations of ATP-1 (0.125, 0.250, and 0.500 mg/mL) were added, with DMEM serving as the blank control and LPS (1 µg/mL) as the positive control. After 24 h of incubation, an aliquot of 20 µL neutral red solution was added to each well for 2 h. The excess neutral red solution was discarded by rinsing the cells 3 times with phosphatebuffered saline (PBS; pH 7.4). Then, an aliquot of 100 µL of the cell lysate was added to each well and lysed at room temperature (37 °C) for 30 min. The absorbance at 540 nm was measured using a microplate reader. The phagocytic index of RAW264.7 cells was calculated using the following formula:

$$\text{Phagocytosis index rate (\%)} = \frac{A_{\text{test}}}{A_{\text{control}}} \times 100$$

where A_{test} and A_{control} represent the absorbances of the test and control samples, respectively.

2.5.3 Determination of cytokine levels

A previously published method was used to determine the levels of the cytokines NO, TNF-α, and IL-6 in the supernatant using a Griess reagent kit and ELISA kits^[28]. RAW264.7 cells were incubated for 24 h in a 96 well plate (10^5 cells per well) with different concentrations of ATP-1 (0.125, 0.250, and 0.500 mg/mL). DMEM was used as the blank control and LPS (1 µg/mL) as the positive control. NO, TNF-α, and IL-6 levels in the supernatant were determined using the corresponding ELISA kits.

2.6 Label-free proteomics and bioinformatics analysis

Cells were cultured in T25 culture flasks at a density of 4×10^5 cells/mL for 24 h, and further treated with DMEM and 0.5 mg/mL ATP-1 solution for 24 h. An adequate amount of protein lysis buffer was added to the samples for 30 min. After centrifugation, protein levels in the supernatant were determined using a BCA kit and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). The samples were completely dissolved in TEAB, and trypsin was added in a 1:50 ratio (enzyme:protein, *m/m*) and allowed to react overnight at 37 °C for enzymatic hydrolysis. The hydrolyzed peptide segments were collected, lyophilized, and quantified using a peptide segment quantification kit (stock number 23275). The peptide segments were dissolved and analyzed using liquid chromatography (LC)–mass spectrometry (MS)/MS using an EASY-nLC 1200 LC system coupled with a timsTOF Pro2 mass spectrometer (Shanghai Meiji Biotechnology Co., Ltd.). Next, the raw MS data were imported into MaxQuant version 2.0.3.1 for library analysis. Gene Ontology (GO) functional annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of the differentially expressed proteins (DEPs) were performed using the Meiji Cloud Biotechnology platform. The UniProt database was used in this study^[29].

2.7 Western blotting

RAW264.7 cells in the logarithmic phase were seeded at a density of 1×10^5 cells/mL in a 6-well plate and incubated for 24 h. The supernatant was discarded, and the cells were treated with 0.125, 0.250, and 0.500 mg/mL of ATP-1 solution, respectively. DMEM was used as the blank control and LPS (1 µg/mL) as the positive control. After another 24 h of incubation, the supernatant was discarded; the cells were washed twice with precooled PBS and lysed for 30 min using RIPA lysis buffer containing 1 mmol/L phenylmethylsulfonyl fluoride. The supernatant was collected after centrifuging for 20 min at 12 000 r/min and 4 °C. Proteins were quantified using a BCA assay kit. The loading amount of the protein was calculated, and a specific volume of 5× SDS-PAGE protein-loading buffer was added. The denatured proteins were separated by loading them onto 4%–20% SDS-PAGE gels and then transferred to a 0.22-µm polyvinylidene fluoride membrane. The membrane was blocked with 5% BSA for 1 h while shaking at room temperature and then incubated overnight with the primary antibodies (iNOS, Ikka, Ikkβ, COX-2 [Prgs2], Fas, AIP1, TNF-α, IkBa, and GAPDH) at 4 °C. The membrane was washed 5 times with Tris-buffered saline before incubation with the secondary antibodies at room temperature for 60 min. The resultant protein bands were visualized using enhanced chemiluminescence reagent and a gel imaging system (Bio-Rad Laboratories, Hercules, CA)^[30].

2.8 Molecular docking

Molecular docking was used to determine the mechanism underlying the interaction between the monosaccharide fraction and TNF-α. The crystal structure of mouse TNF-α (PDB: 2tnf) protein for docking was downloaded from the PDB database. The 3D structures of the small molecules galactose (ID: 6036), glucose (ID: 5793), and xylose (ID: 135191) were downloaded from the PubChem

database (<https://pubchem.ncbi.nlm.nih.gov/>) and subjected to energy minimization using the MMFF94 force field. Subsequently, the ligands and water molecules of the receptor protein were removed using PyMOL 2.5.5, and the polysaccharides and receptor proteins were docked using AutoDock Vina 1.2.3. PyMOL 2.5.5 was used to visualize the results^[31].

2.9 Statistical analysis

All results are presented as mean ± standard deviation. Statistical analysis was performed using SPSS 21.0, and significant differences between groups were analyzed using analysis of variance and Duncan's method.

3. Results and discussion

3.1 Purification and structural characterization

3.1.1 Purification of ATP-1

Hot water extraction was used to obtain 0.56 g of the crude polysaccharide from 20 g of *A. taxiformis* powder, corresponding to a yield of $(2.8 \pm 0.4)\%$. The crude polysaccharide was further purified using a DEAE-52 chromatography column to obtain 3 components (Fig. 1A). The yields of ATP-1, ATP-2, and ATP-3 were $(17.5 \pm 1.4)\%$, $(12.6 \pm 1.1)\%$, and $(18.3 \pm 0.76)\%$, respectively, whereas the total sugar content was $(60.3 \pm 3.2)\%$, $(57.0 \pm 2.1)\%$, and $(41.2 \pm 1.1)\%$, respectively. No proteins were detected. ATP-1 was selected for subsequent structural characterization owing to its highest yield and sugar content.

3.1.2 Molecular weight distribution

Molecular weight distribution is a crucial determinant of physiological activities, such as the antioxidant and immune activities and the overall functional activity of a polysaccharide^[32]. The molecular weight distribution of ATP-1 was determined using a combination of size exclusion chromatography and a MALS-RI detector (Fig. 1B). The number-average molecular weight (*M_n*) and weight-average molecular weight (*M_w*) of ATP-1 were 56.967 and 307.978 kDa, respectively, with a dispersion index (*M_w/M_n*) of 5.41. The *M_w/M_n* value represents the molecular weight distribution of polysaccharides and reflects the width and degree of polydispersity^[33]. Dispersion index data suggested that ATP1 had a small dispersity in its *M_w* distribution, indicating a relatively uniform molecular structure.

ASTRA 6.1 was used to simulate the slope of the double-logarithmic curve of the root mean square radius and *M_w* to determine the conformation of the polysaccharide molecules in aqueous solutions. Typically, a slope (*k*) of 1, 0.5–0.6, or 0.33 indicates the molecular conformation of the polymer to be rod-shaped, irregularly coiled, or spherical, respectively^[34]. The *k* was determined to be 0.16 (Fig. 1C), indicating the spherical conformation of polysaccharide ATP-1 in aqueous solution as well as its compact, branched nature.

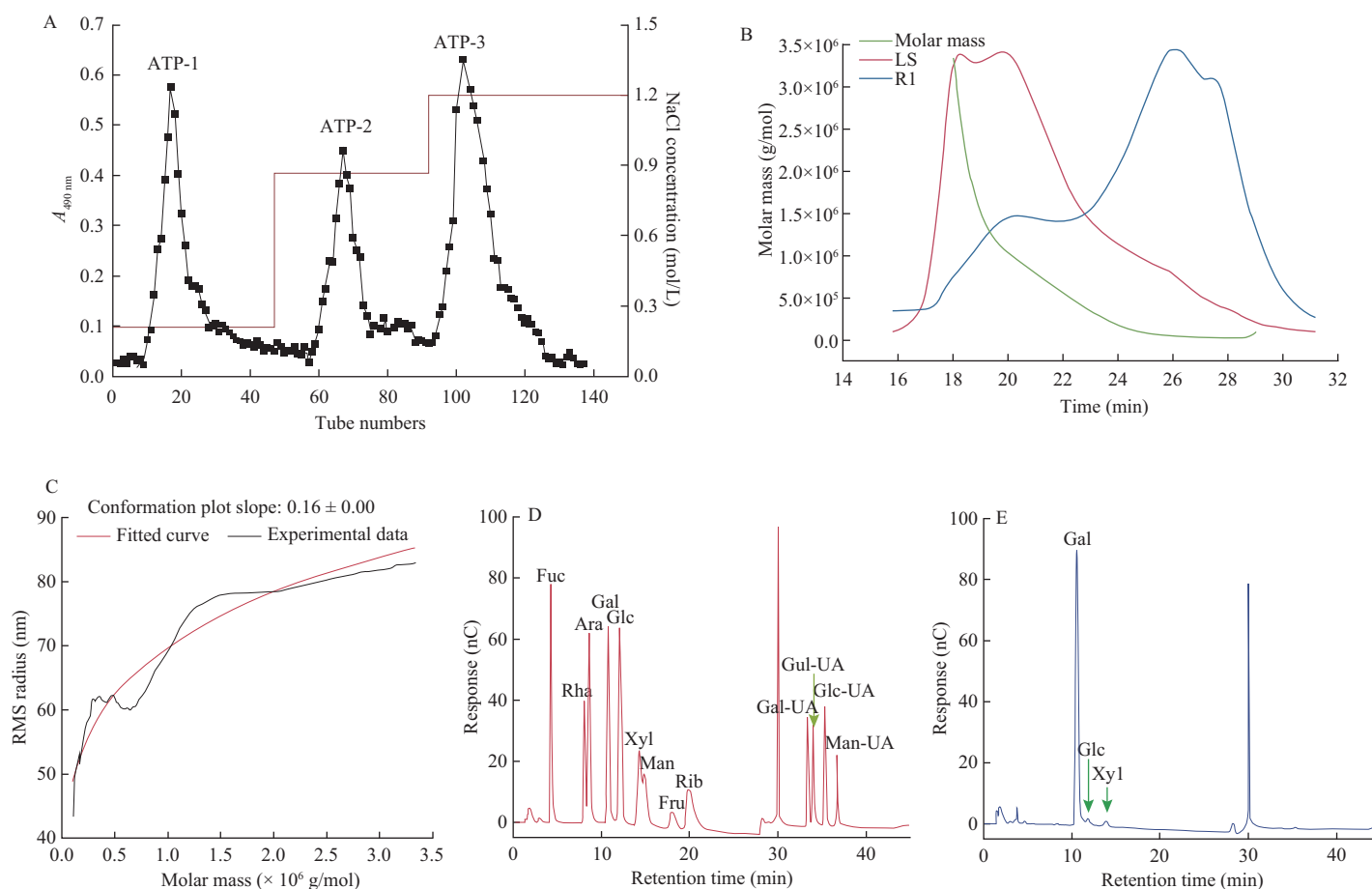


Fig. 1 (A) DEAE chromatogram of the 3 ATP fractions, (B) HPLC-MALS-RI chromatogram demonstrating the molecular weight distribution of purified ATP-1, (C) Conformational chromatogram of the sugar chain, (D) HPLC chromatogram showing the monosaccharide composition of standard monosaccharides, (E) HPLC chromatogram showing the monosaccharide composition of ATP-1.

3.1.3 Monosaccharide composition of ATP-1

The monosaccharide composition of a functional polysaccharide is closely related to its activity^[35]. The monosaccharide composition of ATP1 was analyzed using HPLC (Figs. 1D and E) and was found to be primarily composed of galactose (94.07%), glucose (1.27%), and xylose (4.66%). Wang et al.^[36] have reported that arabinose and xylose mainly affect the prebiotic and immunomodulatory activities of polysaccharides. Lo et al.^[37] found that the monosaccharides arabinose, xylose, mannose, and galactose in polysaccharides played a crucial role in stimulating macrophages. Sun et al.^[38] have reported that increased levels of arabinose, xylose, galactose, and uronic acid play a significant role ($P < 0.05$) in safeguarding SPF3-1 in LPS-stimulated RAW264.7 macrophages. These studies indicate that ATP-1, the polysaccharide from *A. taxiformis*, may have potential immunomodulatory effects.

3.1.4 FT-IR spectroscopy and UV scanning analysis

Infrared spectroscopy is a technique by which the functional groups in molecules can be determined based on their selective absorption of infrared light at certain wavelengths. This absorption leads to transitions in the vibrational and rotational energy levels in molecules. Therefore, the

positions and intensities of the absorption peaks of the functional groups in polysaccharides can be determined, revealing their characteristics^[39]. Shi et al.^[40] used infrared spectroscopy and reported hydroxyl stretching at 3400 cm^{-1} , glycoside bond C–O–C stretching vibrations at 1072 cm^{-1} , strong vibrations of the S=O bonds of the sulfuric acid group at 1260 cm^{-1} , and an absorption peak of *D*-galactose at 870 cm^{-1} . The FT-IR spectrum shows the typical absorption peaks of polysaccharides in the range of $400\text{--}4000\text{ cm}^{-1}$ (Fig. 2A). A strong stretching vibration can be observed at 3448 cm^{-1} , which represents the distinctive absorption peak of O–H; the signal at 2928 cm^{-1} reveals C–H bending vibration. The absorption peaks at 1042 , 1145 , and 1230 cm^{-1} correspond to the stretching vibration of glycoside C–O. The absence of an absorption peak at $1750\text{--}1700\text{ cm}^{-1}$ suggests that ATP-1 lacks a uronic acid moiety, which is consistent with our monosaccharide composition findings. Our results are in agreement with those reported by Liu et al.^[41], wherein infrared spectroscopy was used to determine the structure of *Gracilaria lemaneiformis* polysaccharides. No absorption peaks were noted in their study at $1750\text{--}1700\text{ cm}^{-1}$, indicating the absence of a uronic acid moiety. Furthermore, the spectrum showed characteristic absorption peaks for –OH (3289 cm^{-1}), C–H (2920 cm^{-1}), and C–O (1070 cm^{-1}). The lack of nucleic acids and proteins in ATP-1 was confirmed in our study due to the lack of UV absorption at 260 nm and 280 nm . This finding aligned with the results from protein quantification.

3.1.5 Thermal characterization of ATP-1

TG-DSC is often used to determine the thermal stability of polysaccharides. The stability of raw materials has a significant influence in industrial applications. The DSC curve reflects the endothermic or exothermic rate of samples. However, the DTG curve area represents the first derivative of the TG curve with respect to temperature or time, indicating the relationship between the mass-loss rate and temperature or time. The curve area is proportional to mass loss^[42]. Fig. 2C shows a loss of mass in ATP-1 in 3 stages in the temperature range of 30 to 800 °C. The TG curve at 30.0–250.5 °C showed a slight reduction in the weight of ATP-1, which could be attributed to the dissociation of bound water from the hydrogen bonding of ATP-1 polysaccharide. This finding was consistent with previous research findings^[25]. At 250.5–269.5 °C, the polysaccharide showed a significant trend of mass loss, with the degradation of thermally unstable functional groups being the primary cause of this shift. The mass of the polysaccharide decreased slightly between 269.5 and 799.6 °C. In addition, at 799.6 °C, the residual mass of ATP-1 was 39.41%, which was most likely due to degradation of the polysaccharide branches and oxidative decomposition of organic matter. Furthermore, the DTG curve revealed that polysaccharide ATP-1 had the largest peak area at 261.0 °C, indicating a significant mass loss of the polysaccharide at this temperature. DSC revealed that ATP-1 exhibited an exothermic reaction throughout the process. These results serve as an important reference for the stability and processing applications of ATP-1.

3.1.6 Observation of the microstructure using SEM and AFM

The morphological characteristics and microstructure of ATP-1 were studied using SEM and AFM. Figs. 3A–C show the microstructure of ATP-1 to have asymmetric flaky cracks and holes, suggesting its amorphous nature. The AFM image of ATP-1 shows multiple island-like structures ranging in size from –4.0 to 4.0 nm. ATP-1 has a highly branched structure in aqueous solution, with small rings formed between chains and accompanied by spherical particles. The molecule has a spherical conformation (Figs. 3D–E). These findings are consistent with the results of the conformation chromatogram of the sugar chain (Fig. 1), suggesting that ATP-1 polysaccharide chains have high molecular flexibility and weak intramolecular forces.

Gong et al.^[43] have reported that the roughness of a surface measured using AFM is commonly represented using conventional statistical measurements such as root-mean-square (Rmax), peak-

to-valley ratio (Rq), and average roughness (Ra); for ATP-1, these values were 5.60, 0.692, and 0.483 nm, respectively. The preceding data imply that the structure of ATP-1 may be related to the aggregation of molecular chains caused by the entanglement and interaction of intramolecular hydrogen bonds. Structure influences activity, and structural characteristics such as molecular weight, monosaccharide composition, and the functional groups of polysaccharides are strongly related to their immunological activity^[44]. To elucidate the correlation between the structural characteristics and activity of polysaccharides, RAW264.7 cells were used as an *in vitro* model to determine the immunomodulatory activity of ATP-1.

3.2 Effect of ATP-1 on RAW264.7 cells

3.2.1 Cell viability

Macrophages are important immune cells in the body that are involved in both specific and nonspecific immunity^[45]. To determine the immunomodulatory effect of ATP-1 *in vitro*, the toxicity of ATP-1 on RAW264.7 macrophages was studied using the CCK-8 assay. As shown in Fig. 4A, the viability of treated cells was similar to that of control cells at concentrations ranging from 0.125–0.500 mg/mL. This finding demonstrated that ATP-1 was not toxic to RAW264.7 macrophages in the concentration range used in this study. However, the viability of cells treated with 1 mg/mL of ATP-1 decreased significantly compared with control cells ($P < 0.05$). Therefore, a concentration range of 0.125–0.500 mg/mL was chosen for subsequent experiments to determine the immunomodulatory effect of ATP-1.

3.2.2 ATP-1 facilitated phagocytic activity in RAW264.7 cells

Enhanced phagocytosis is one of the most significant aspects of macrophage activation and is commonly used to assess immunity. Activated macrophages promote the innate immune response through phagocytosis, preventing the invasion of pathogenic microorganisms^[46]. The neutral red technique was used to evaluate the effect of ATP-1 on the phagocytic ability of RAW264.7 cells. Fig. 4B shows that cells treated with varying ATP-1 concentrations exhibited a significant increase in phagocytic ability after 24 h compared with control cells ($P < 0.05$). Moreover, low concentrations of ATP-1 showed higher phagocytic activity than high concentrations of ATP-1. These findings indicated that ATP-1 could significantly improve the phagocytic ability of RAW264.7 cells, thereby exerting an immunomodulatory effect.

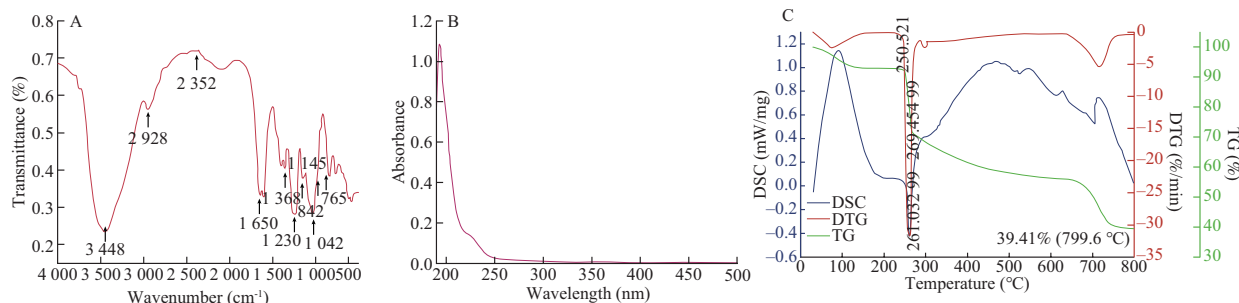


Fig. 2 (A) FT-IR spectra, (B) UV spectra, and (C) TG-DSC thermogram of ATP-1.

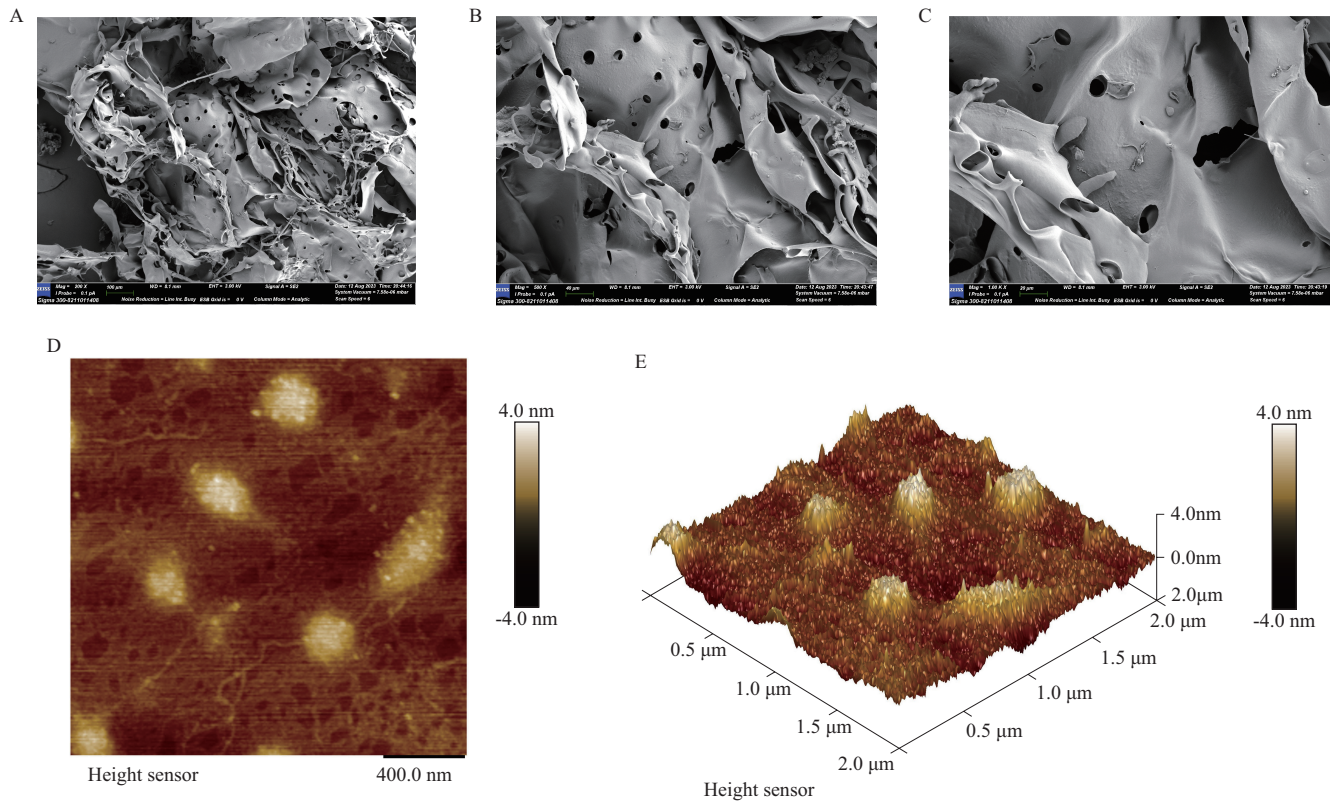


Fig. 3 (A-C) SEM and (D-E) AFM images for ATP-1.

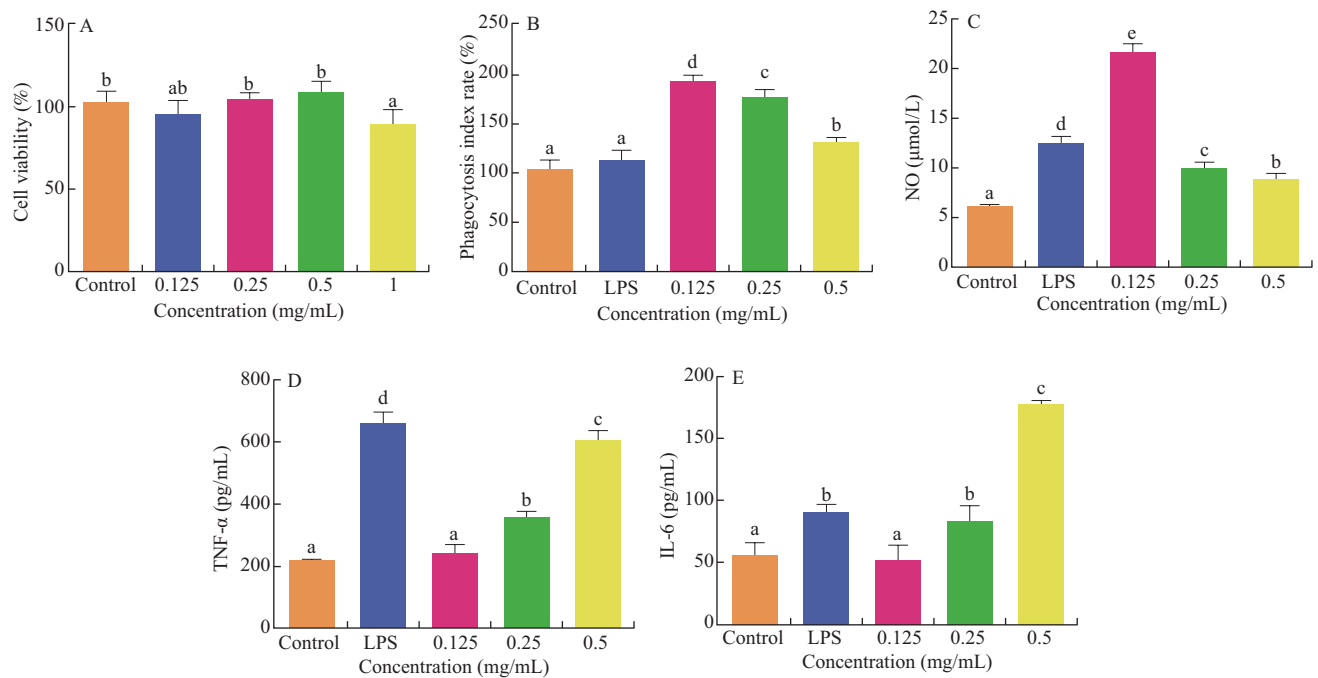


Fig. 4 Effect of ATP-1 on RAW264.7 cells. (A) Cell viability. (B) Phagocytic activity. (C-E) Secretion of cytokines (NO, TNF- α , IL-6). Values are expressed as mean $\pm$ SD ($n = 3$). Different letters indicate a different significance among groups at $P < 0.05$.

3.2.3 ATP-1 promoted the secretion of NO, TNF- α , and IL-6

NO is an effector molecule produced by macrophages. It is an essential host defense effector in the immune system that is capable of eliminating pathogenic bacteria and exerting other effects^[47]. NO is frequently used as an indicator of the immunostimulatory ability of

polysaccharides. The Griess method was used to study the effect of ATP-1 on the release of NO from cells. As shown in Fig. 4C, ATP-1 treatment significantly promoted NO secretion versus the untreated control group ($P < 0.05$). The highest NO secretion was 21.69 μ mol when 0.125 mg/mL of ATP-1 was used. The lowest NO secretion was 8.88 μ mol when 0.5 mg/mL of ATP-1 was used. This outcome

was speculated to be related to the bidirectional immunomodulatory or anti-inflammatory activity of ATP-1. NO is produced by the catalysis of NO synthase, with iNOS being a major enzyme for NO synthesis, which can mediate various immunological responses^[48]. Therefore, Western blotting was used to determine the expression of iNOS protein. The findings revealed that ATP-1, at concentrations ranging 0.125–0.500 mg/mL, could significantly promote iNOS expression in cells (Fig. 6D). This finding was consistent with the results from ELISA, which was used to determine NO content in the cell supernatants.

Furthermore, Fig. 6D shows that the treatment with ATP-1 substantially upregulated COX-2 protein levels compared with those in the control group ($P < 0.05$). Wu et al.^[49] have established that COX-2 is an inducible enzyme that catalyzes the conversion of arachidonic acid into prostaglandins. Prostaglandins play a pivotal role in immune cells and influence their differentiation and activation. Our findings revealed that ATP-1 exerted an immunomodulatory effect by promoting COX-2 release from RAW264.7 cells. Overall, ATP-1 may have stimulated NO secretion in RAW264.7 macrophages by activating iNOS and COX-2 protein expression, making it a safe biological immunomodulator. These findings are similar to the observations reported by Shan et al.^[50]

Immunoregulation involves the activation of macrophages, which not only enhances phagocytic activity but also secretes biological signaling molecules including NO, TNF- α , and IL-6 to protect from pathogens and boost immunity. TNF- α , a pro-inflammatory cytokine predominantly produced by macrophages and monocytes, stimulates the release of the bioactive substances IL-1 β and IL-6^[51]. IL-6 is secreted by monocytes, activated lymphocytes, and other activated cells, and regulates both innate and acquired immunity^[52]. The effect of ATP-1 on the release of TNF- α and IL-6 in RAW264.7 macrophages was determined using ELISA. Fig. 4D and E demonstrate that ATP-1 significantly increased TNF- α and IL-6 secretion in RAW264.7 macrophages compared with that in the control group. TNF- α and IL-6 secretion peaked at 606.04 and 178.31 pg/mL ($P < 0.05$), respectively, at an ATP-1 concentration of 0.5 mg/mL.

The pathways through which polysaccharides stimulate macrophages to exert their immunoregulatory effects mainly include

improving the phagocytic ability of macrophages; secreting cytotoxic molecules such as NO and ROS; and stimulating macrophages to secrete cytokines such as TNF- α , IL-6, and IL-10^[53–54]. These results highlighted the immune-enhancing effect of ATP-1, wherein the phagocytic ability of RAW264.7 macrophages was enhanced, the secretion of cytokines NO, TNF- α , and IL-6 was stimulated, and the innate immune response was regulated. Label-free proteomics and bioinformatics analysis were conducted to better understand the immunoregulatory mechanism of ATP-1 on RAW264.7 cells.

3.3 Label-free proteomics and bioinformatics analysis on RAW264.7 cells

RAW264.7 cell extracts were subjected to label-free quantitative proteomic analysis to determine the impact of ATP-1 on protein expression. A total of 3 986 proteins were identified in RAW264.7 cell samples (Fig. 5A), including 3 860 common proteins, 47 proteins in the treatment group, and 79 proteins in the control group. Cluster analysis of protein expression after data normalization revealed that protein expression in the control groups (D1, D2, and D3) differed significantly from that in the ATP-1 treatment group (C1, C2, and C3) (Fig. 5B). Fig. 5C illustrates the volcano plot of DEPs (x -axis, $\log_2$ fold change). Compared to the untreated RAW264.7 control, 178 proteins (70 upregulated and 108 downregulated) were identified as DEPs (> 2 -fold or < 0.5 -fold; $P < 0.05$) following treatment with 0.5 mg/mL ATP-1.

GO functional annotation of DEPs was analyzed based on the following 3 aspects: biological process (BP), cellular component (CC), and molecular function (MF). Fig. 5D shows the top 20 GO functional annotations. Regarding BPs, in C vs. D upregulated or downregulated differential proteins were mostly enriched in cellular activities, biological regulation, metabolic processes, stimulus responses, and developmental processes, with 27 enriched in immune system functions. In terms of CCs, they were mainly enriched in cellular anatomical entity and protein-containing complex. With respect to MFs, they were mainly enriched in binding, catalytic activity, transporter activity, and molecular function regulator activity.

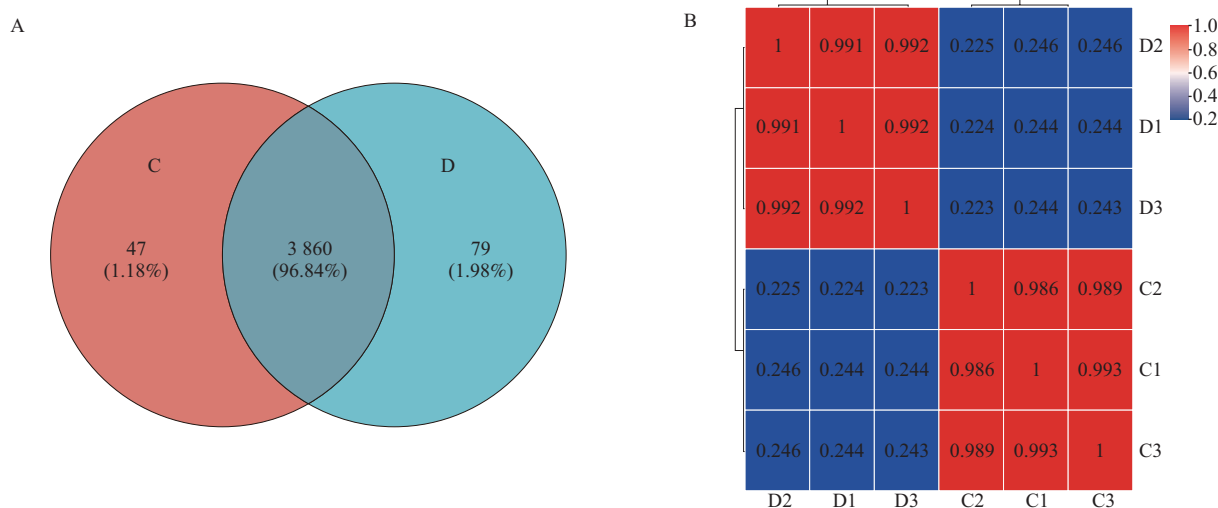


Fig. 5 Changes in differential protein expression in RAW264.7 cells and bioinformatics analysis. (A) Wayne diagram. C and D represent ATP-1–treated samples and the controls, respectively. (B) Cluster analysis of proteins from RAW264.7 cells (treatments: C1, C2, and C3; controls: D1, D2, and D3); (C) Volcano map of differentially expressed proteins in RAW264.7 cells; (D) GO annotation analysis.

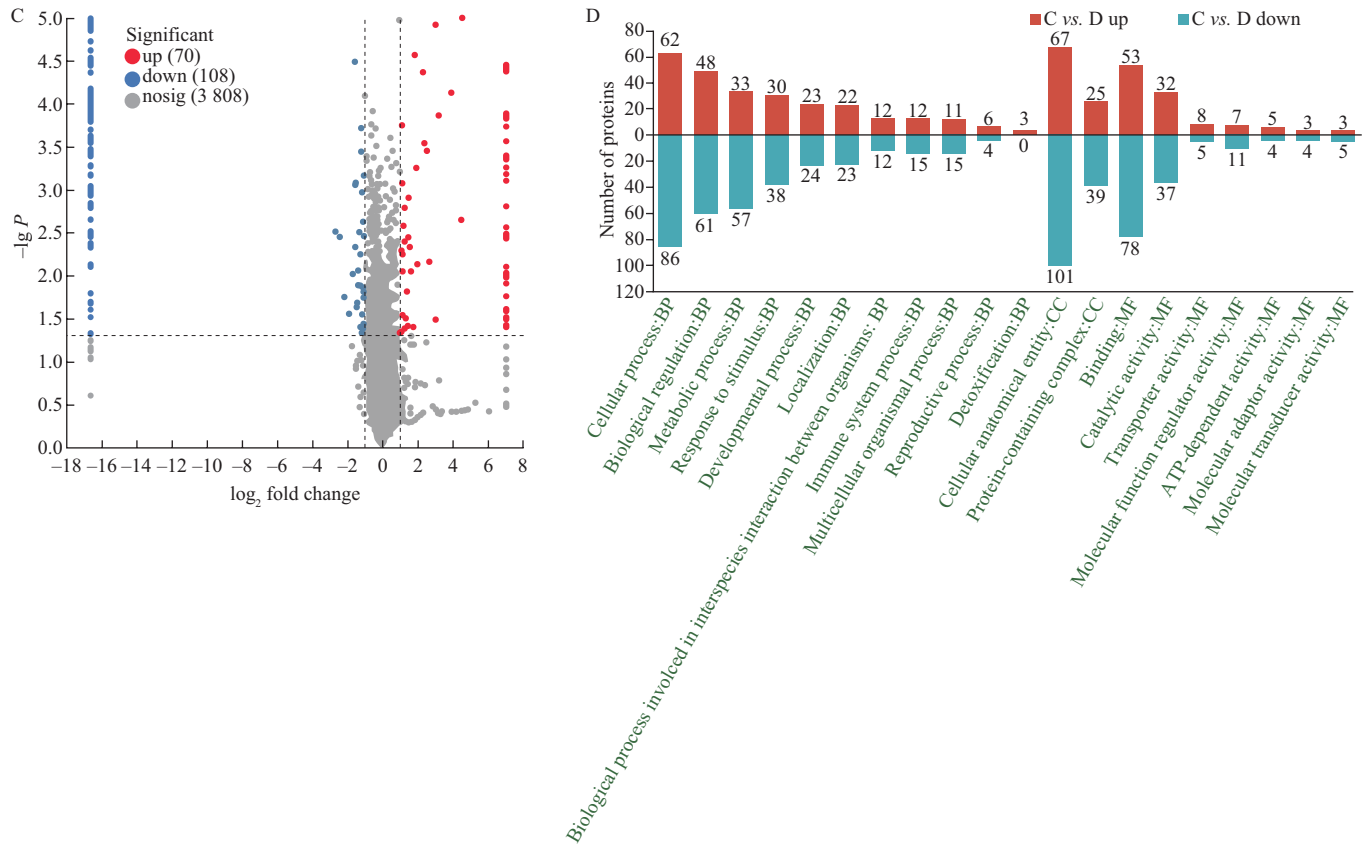


Fig. 5 (Continued)

3.4 ATP-1 stimulated TNF signaling pathways in RAW264.7 cells

KEGG pathway analysis was performed to identify and study the pathways activated by ATP-1 in RAW264.7 cells. Chen et al.^[55] have reported KEGG as a classic and practical database for integrating chemical, genomic, and functional information at the cellular and ecological levels. KEGG analysis in this study revealed that DEPs were implicated in 191 pathways, with 20 pathways being significantly enriched. Several major pathways were represented, including mineral absorption; peroxisome; fatty acid degradation; calcium signaling; cytosolic DNA-sensing; glycine, serine and threonine metabolism; PPAR signaling; and TNF signaling (Fig. 6A). Among them, TNF was determined to be one of the most prevalent and important signaling pathways in inflammation and immunoregulation^[56].

Significant changes in 5 proteins were noted in the TNF signaling pathway after treatment with ATP-1 for 24 h (Figs. 6B-C). Specifically, the proteins Ikk α , Ikk β , and COX-2 (Ptgs2) were significantly upregulated and caused inflammation and cell proliferation ($P < 0.05$). In contrast, Fas and AIP1 were significantly downregulated and resulted in cell apoptosis and inflammation ($P < 0.05$). These findings suggested that ATP-1 may exert its immunomodulatory effects by activating the TNF signaling pathway. Next, the effect of ATP-1 on the TNF signaling pathway in RAW264.7 cells was validated. Western blotting was used to determine the protein expression of Ikk α , Ikk β , COX-2 (Ptgs2), Fas, AIP1, TNF- α , and Ikb α in each group. ATP-1 significantly upregulated the protein expression of IKK α , IKK β , COX-2, and TNF- α ($P < 0.05$) and downregulated that of Ikb α , Fas, and AIP1 ($P < 0.05$). These findings indicated that

the TNF signaling pathway was involved in the ATP-1-induced activation of RAW264.7 cells (Fig. 6D).

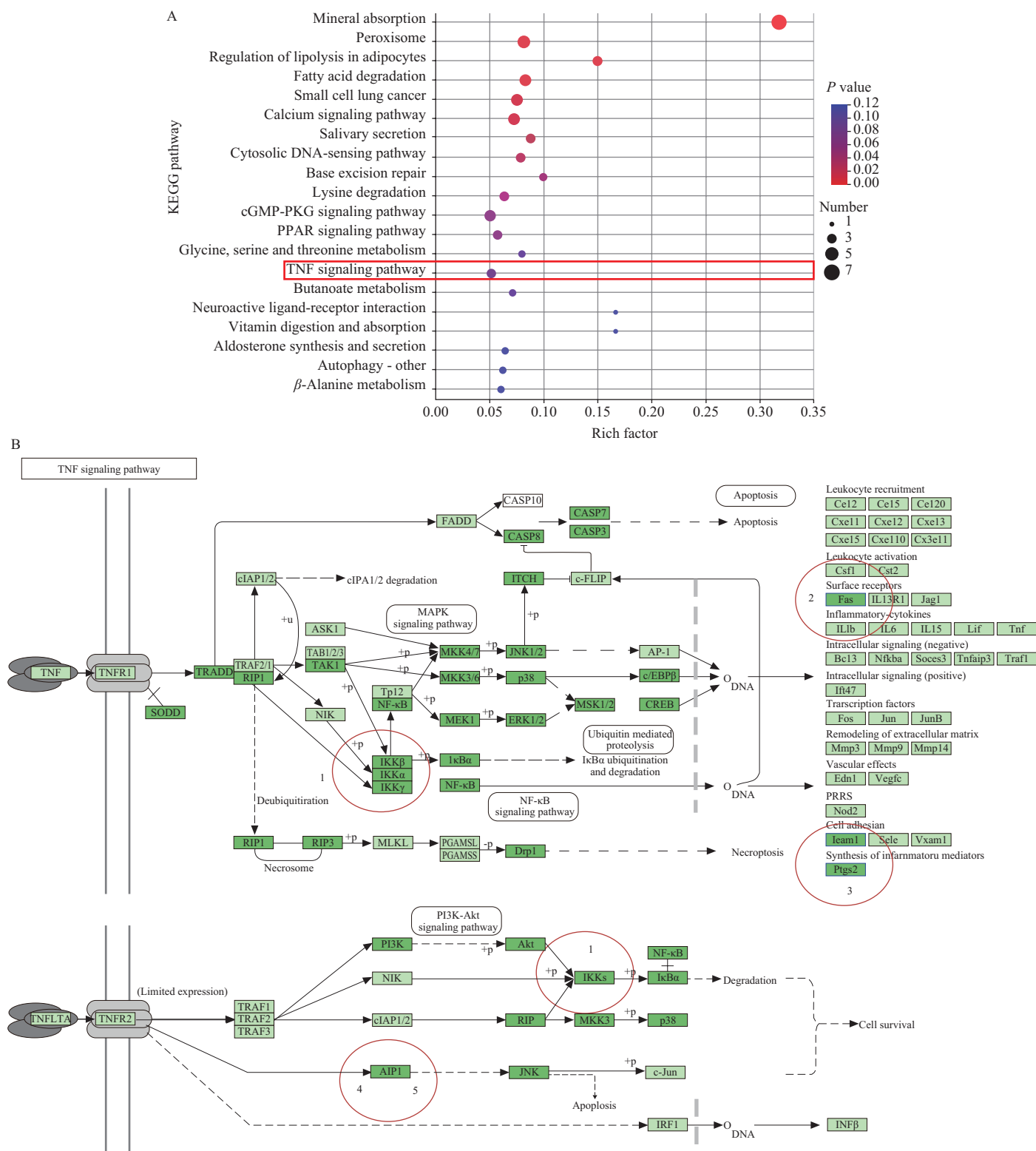
TNF exerts multiple biological effects. It can bind to specific receptors on the cell membrane to promote cell growth, differentiation, apoptosis, and inflammation^[57]. TNF- α is a member of the TNF family and exerts antiviral, immunoregulatory, and apoptotic functions by activating caspase protease, Janus kinase, and nuclear factor-kappa B (NF- κ B) signaling pathways among others^[58]. NF- κ B often exists in an inactive state in cells by binding to the inhibitory factor Ikb. TNF induces Ikb (especially Ikb α) degradation, activating the NF- κ B signaling pathway. IKK α and IKK β are key components of Ikb kinase (IKK), and IKK phosphorylation activates the NF- κ B signaling pathway^[59]. Our results showed that ATP-1 downregulated the protein expression of Ikb α and upregulated that of IKK α , IKK β , and TNF- α . Collectively, these findings suggested that the NF- κ B signaling pathway may potentially play a role in the ATP-1-induced activation of RAW264.7 cells.

Svandova et al. discovered that the Fas/FasL pathway is crucial in regulating apoptosis. Activation of the Fas ligand can induce Fas-dependent apoptosis while also increasing the immune activity of cells, thereby exerting an antitumor effect^[60]. Kang et al.^[61] found that the *Gracilaria lemaneiformis* polysaccharide could inhibit tumor cell proliferation by upregulating the gene and protein levels of Fas and FasL, activating Fas/FasL pathway-induced apoptosis and thereby inhibiting tumor cell proliferation. This finding could mean that ATP-1 augmented the immune activity of RAW264.7 cells by reducing Fas protein expression. Liu et al. have identified TNF- α -induced protein 1 (TNFAIP1) as a protein involved in DNA replication, DNA damage

repair, apoptosis, and disease progression^[62]. Their finding aligns with the conclusions of our study that ATP-1 mediates apoptosis in RAW264.7 cells by downregulating AIP1 protein expression.

We hypothesized that ATP-1 exerts its immunomodulatory effect by activating the TNF/NF- κ B signaling pathway to enhance the

phagocytic ability of macrophages and stimulate the production of cytokines including NO, TNF- α , and IL-6. Next, molecular docking was used to simulate the mechanism of interaction between ATP-1 and TNF- α and elucidate the molecular mechanism of ATP-1 in activating the TNF signaling pathway.



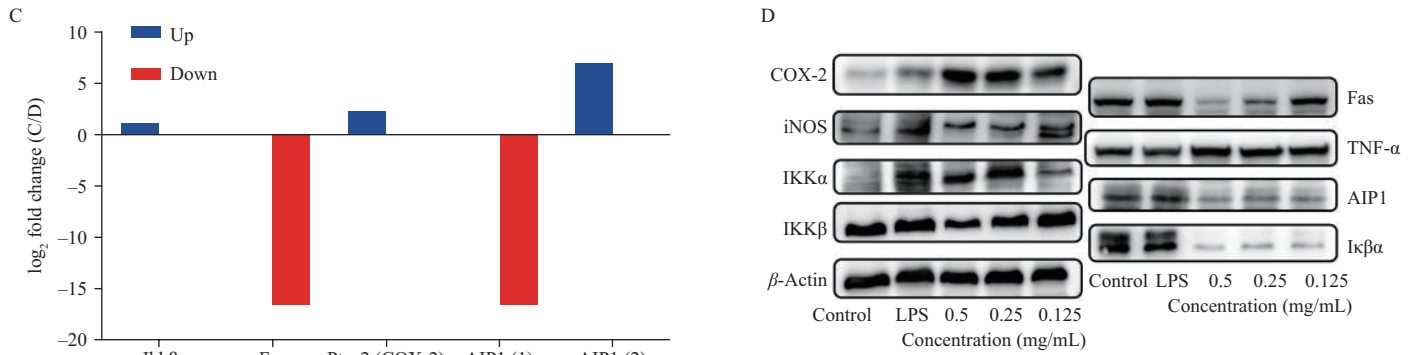


Fig. 6 (Continued)

3.5 Molecular docking

Molecular docking is an important tool in drug discovery and structure-based drug design. It can be used to predict interactions between molecules and compounds based on parameters such as hydrogen bonding, salt bridges, and electrostatic attraction within a specific range^[63]. Wu et al.^[64] discovered that mushroom polysaccharides interact primarily with specific amino acids of dectin-1 protein through hydrogen bonding and CH- π hydrophobicity. Therefore, molecular docking can be considered a rapid and efficient technique to identify target proteins and binding sites.

Molecular docking was used in this study to determine the molecular mechanism underlying the interactions between each monosaccharide fraction and TNF- α (Fig. 7). Galactose formed hydrogen bonds with TNF- α residues including B:LYS-98, B:TYR-119, C:TYR-119, C:PRO-117, and C:LYS-98. The binding energy was calculated to be -6.677 kcal/mol, implying that galactose could effectively interact with the active site of TNF- α . Similarly, docking configurations between glucose and TNF- α (binding energy, -6.12 kcal/mol) as well as between xylose and TNF- α (binding energy, -5.563 kcal/mol) were studied (Table 1). Binding is induced more readily when the negative binding affinity energy is less than -5 kcal/mol^[26]. The docking scores of TNF- α with galactose, glucose, and xylose in our study were all < -5 kcal/mol, indicating that hydrogen bonding largely contributed to the affinity between TNF- α and monosaccharides. Wei et al.^[65] have reported similar findings of the extracellular polysaccharide EPS-1 and α -CAS mainly interacting *via* hydrogen bonding. Our findings indicated that ATP-1 could bind to the active sites of TNF- α , resulting in potent immunological effects in RAW264.7 cells.

Table 1

Summary of the estimated binding energy and chemical interactions for the best poses obtained using molecular docking of TNF- α (PDB: 2tnf) at the galactose (ID: 6036), glucose (ID: 5793), and xylose (ID: 135191) binding sites.

Complexes	Affinity (kcal/mol)	Hydrogen bond binding site
TNF- α -galactose	-6.677	B:LYS-98, B:TYR-119, C:TYR-119, C:PRO-117, C:LYS-98
TNF- α -glucose	-6.120	A:PRO-117, A:TYR-119, A:LYS-98, B:TYR-119, C:TYR-119, C:PRO-117
TNF- α -xylose	-5.563	A:TYR-119, A:PRO-117, B:LYS-98, C:PRO-117, C:TYR-119

The immunological effects of polysaccharides are closely related to their structural characteristics such as molecular weight, monosaccharide composition, and functional groups. Zeng et al.^[66] found that polysaccharides containing galactose, glucose, arabinose, mannose, etc., exhibit immune activity as they are more readily identified by macrophage receptors. Yuan et al.^[67] reported that polysaccharides containing galactose and arabinose as the primary monosaccharides exert potent immunomodulating effects. In this study, the neutral polysaccharide ATP-1 was determined to be primarily composed of galactose, glucose, and xylose and have a molecular weight of 307.978 kDa. SEM and AFM studies indicated that ATP-1 may be formed due to molecular chain entanglement and intramolecular hydrogen bond interaction. These interactions were likely responsible for its spherical structure that showed asymmetric sheet-like cracks and pores in the microstructure. Therefore, it could be postulated that the immunomodulatory activity of ATP-1 is related to its monosaccharide composition and chain conformation.

However, the polysaccharide ATP-1 obtained in this study differed considerably from complex sulfated galactans. Rodríguez Sánchez et al.^[9] isolated a polysaccharide from *A. taxiformis* and reported a high AtC1-1 yield of 3.8%. The major extract was fractionated using cetrinide precipitation. Seven fractions were obtained, which showed similarities in general characteristics such as large amounts of carbohydrates and sulfate. In terms of monosaccharide composition, galactose was present in relatively large amounts, with a distinct predominance of the *D*-configuration. Substantial amounts of glucose and a small amount of xylose were also present. The extracts and fractions of *A. taxiformis* were found to have a moderate anticoagulant effect compared with heparin. The molecular weights of the fractions ranged from 51.0 to 366.4 kDa.

The structure of polysaccharides varies considerably depending on the geographical location, extraction time, extraction temperature, and methods of fractionation. These variations are particularly evident in aspects such as polysaccharide yield, content of sulfate group, molecular weight, monosaccharide composition, and biological activity. For example, *A. taxiformis* in our study was collected from Naozhou Island in Zhanjiang, China. In contrast, nonfertile gametophytes of *A. taxiformis* (Delile) Trevisan were hand-collected in the intertidal zone in the Central Littoral of Venezuela. Therefore, it is necessary to study *A. taxiformis* from different geographical origins and explore the structure-activity relationships.

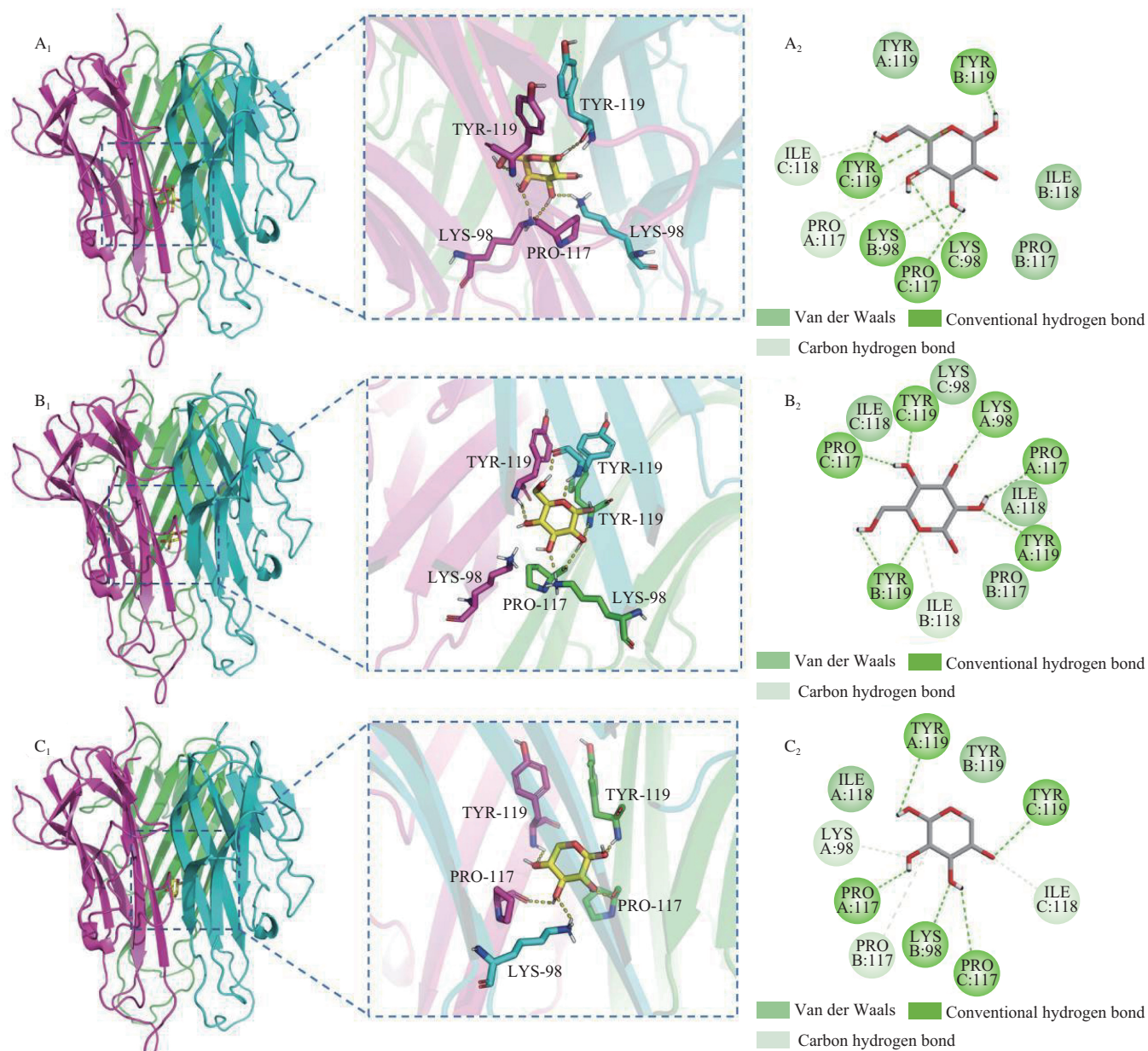


Fig. 7 Molecular docking to determine the binding mode of ATP-1. (A₁) Crystal structure of TNF α (PDB: 2tnf) and its binding site with galactose (ID: 6036); (A₂) 2D diagram of the docking of galactose with TNF- α ; (B₁) Crystal structure of TNF- α and its binding site with glucose (ID: 5793); (B₂) 2D diagram of the docking of glucose with TNF- α . (C₁) Crystal structure of TNF- α and its binding site with xylose (ID: 135191); (C₂) 2D diagram of the docking of xylose with TNF- α .

4. Conclusions

The neutral homogeneous polysaccharide ATP-1 was isolated and purified from the red alga *A. taxiformis* and was determined to have a molecular weight of 307.978 kDa. ATP-1 is composed of galactose (94.07%), glucose (1.27%), and xylose (4.66%). The immunomodulatory effect of ATP-1 was related to its monosaccharide content and chain conformation. Galactose, glucose, and xylose in ATP-1 activated the TNF signaling pathway by binding to the active site of the receptor protein TNF- α *via* hydrogen bonding. This activation significantly upregulated the expression of IKK α , IKK β , COX-2, and TNF- α proteins ($P < 0.05$) and downregulated that of I κ B α , Fas, and AIP1 ($P < 0.05$). These processes also improved the phagocytic ability of macrophages and stimulated the secretion of cytokines NO, TNF- α , and IL-6, thereby exerting

an immunoregulatory effect. Overall, our findings highlight the potential of ATP-1 as a potent immunomodulator. However, the association between the structural characteristics of ATP-1 and immunomodulatory function warrants further investigation.

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

- [1] S. Adarshan, V.S.S. Sree, P. Muthuramalingam, et al., Understanding macroalgae: a comprehensive exploration of nutraceutical, pharmaceutical, and omics dimensions, *Plants* 13 (2023) 113. <https://doi.org/10.3390/plants13010113>.
- [2] K.L. Cheong, K. Liu, W. Chen, et al., Recent progress in *Porphyra haitanensis* polysaccharides: extraction, purification, structural insights, and their impact on gastrointestinal health and oxidative stress management, *Food Chem. X* 22 (2024) 101414. <https://doi.org/10.1016/j.fochx.2024.101414>.
- [3] R. Felix, P. Dias, C. Felix, et al., The biotechnological potential of *Asparagopsis armata*: what is known of its chemical composition, bioactivities and current market? *Algal. Res.* 60 (2021) 102534. <https://doi.org/10.1016/j.algal.2021.102534>.
- [4] M. Zanolli, R. Carmona, L. Mata, et al., Concise review of the genus *Asparagopsis* Montagn, *J. Appl. Phycol.* 34 (2022) 1-17. <https://doi.org/10.1007/s10811-021-02665-z>.
- [5] Z.P. Patwary, M. Zhao, T. Wang, et al., A proteomic analysis for the red seaweed *Asparagopsis taxiformis*, *Biology* 12 (2023) 167. <https://doi.org/10.3390/biology12020167>.
- [6] P. Zhu, D. Li, Q. Yang, et al., Commercial cultivation, industrial application, and potential halocarbon biosynthesis pathway of *Asparagopsis* sp., *Algal. Res.* 56 (2021) 102319. <https://doi.org/10.1016/j.algal.2021.102319>.
- [7] B. Ridoutt, S.A. Lehnert, S. Denman, et al., Potential GHG emission benefits of *Asparagopsis taxiformis* feed supplement in Australian beef cattle feedlots, *J. Clean. Prod.* 337 (2022) 130499. <https://doi.org/10.1016/j.jclepro.2022.130499>.
- [8] C. Brooke, B. Roque, C. Shaw, et al., Methane reduction potential of two Pacific coast macroalgae during *in vitro* ruminant fermentation, *Front. Mar. Sci.* 7 (2020) 561. <https://doi.org/10.3389/fmars.2020.00561>.
- [9] R.A. Rodríguez Sánchez, K. Saluri, R. Tuvikene, et al., Complex sulfated galactans from hot water extracts of red seaweed *Asparagopsis taxiformis* comprise carrageenan and agaran structures, *Carbohydr. Polym.* 322 (2023) 121314. <https://doi.org/10.1016/j.carbpol.2023.121314>.
- [10] V. Thépot, A.H. Campbell, M.A. Rimmer, et al., Dietary inclusion of the red seaweed *Asparagopsis taxiformis* boosts production, stimulates immune response and modulates gut microbiota in Atlantic salmon, *salmo salar*, *Aquaculture* 546 (2021) 737286. <https://doi.org/10.1016/j.aquaculture.2021.737286>.
- [11] S.M. Qiu, J.J. Aweya, X. Liu, et al., Bioactive polysaccharides from red seaweed as potent food supplements: a systematic review of their extraction, purification, and biological activities, *Carbohydr. Polym.* 275 (2022) 118696. <https://doi.org/10.1016/j.carbpol.2021.118696>.
- [12] Y. Zhao, W. Hu, H. Zhang, et al., Antioxidant and immunomodulatory activities of polysaccharides from the rhizome of *Dryopteris crassirhizoma* Nakai, *Int. J. Biol. Macromol.* 130 (2019) 238-244. <https://doi.org/10.1016/j.ijbiomac.2019.02.119>.
- [13] Y. Meng, F. Lyu, X. Xu, et al., Recent advances in chain conformation and bioactivities of triple-Helix polysaccharides, *Biomacromolecules* 21(5) (2020) 1653-1677. <https://doi.org/10.1021/acs.biomac.9b01644>.
- [14] X. Liu, Q. Wang, J. Wang, et al., Structural characterization, chain conformation and immunomodulatory activity of a heteropolysaccharide from *Inonotus hispidus*, *Int. J. Biol. Macromol.* 260 (2024) 129187. <https://doi.org/10.1016/j.ijbiomac.2023.129187>.
- [15] K. Li, Y.X. Cao, S.M. Jiao, et al., Structural characterization and immune activity screening of polysaccharides with different molecular weights from *Astragali radix*, *Front. Pharmacol.* 24(11) (2020) 582091. <https://doi.org/10.3389/fphar.2020.582091>.
- [16] S. Song, H. Peng, Q. Wang, et al., Inhibitory activities of marine sulfated polysaccharides against SARS-CoV-2, *Food Funct.* 11 (2020) 7415-7420. <https://doi.org/10.1039/d0fo02017f>.
- [17] V. Thépot, A.H. Campbell, N.A. Paul, et al., Seaweed dietary supplements enhance the innate immune response of the mottled rabbitfish, *Siganus fuscus*, *Fish Shellfish Immun.* 113 (2021) 176-184. <https://doi.org/10.1016/j.fsi.2021.03.018>.
- [18] C. Zhong, W. Tian, H. Chen, et al., Structural characterization and immunoregulatory activity of polysaccharides from *Dendrobium officinale* leaves, *J. Food Biochem.* 46 (2022) e14023. <https://doi.org/10.1111/jfbc.14023>.
- [19] M. Dubois, K. Gilles, J.K. Hamilton, et al., A colorimetric method for the determination of sugars, *Nature* 168 (1951) 167. <https://doi.org/10.1038/168167a0>.
- [20] H. Xing, M. Lu, L. Xian, et al., Molecular weight determination of a newly synthesized guanidinylated disulfide-containing poly (amido amine) by gel permeation chromatography, *Asian J. Pharm. Sci.* 12 (2017) 292-298. <https://doi.org/10.1016/j.ajps.2016.11.001>.
- [21] R. Wu, P. Li, Y. Wang, et al., Structural analysis and anti-cancer activity of low-molecular-weight chondroitin sulfate from hybrid sturgeon cartilage, *Carbohydr. Polym.* 275 (2022) 118700. <https://doi.org/10.1016/j.carbpol.2021.118700>.
- [22] A. Nawrocka, A. Szymańska-Chargot, M. Monika, et al., Effect of dietary fibre polysaccharides on structure and thermal properties of gluten proteins-a study on gluten dough with application of FT-Raman spectroscopy, TGA and DSC, *Food Hydrocolloid.* 69 (2017) 410-421. <https://doi.org/10.1016/j.foodhyd.2017.03.012>.
- [23] W. Cao, J. Wu, X. Zhao, et al., Structural elucidation of an active polysaccharide from *Radix Puerariae lobatae* and its protection against acute alcoholic liver disease, *Carbohydr. Polym.* 325 (2024) 121565. <https://doi.org/10.1016/j.carbpol.2023.121565>.
- [24] S. Pattanayak, S. Chakraborty, S. Biswas, et al., Degradation of methyl parathion, a common pesticide and fluorescence quenching of rhodamine B, a carcinogen using β -D-glucan stabilized gold nanoparticles, *J. Saudi. Chem. Soc.* 22 (2018) 937-948. <https://doi.org/10.1016/j.jscs.2018.02.004>.
- [25] L. Feng, N. Han, Y.B. Han, et al., Structural analysis of a soluble polysaccharide GSPA-0.3 from the root of *Panax ginseng* C. A. Meyer and its adjuvant activity with mechanism investigation, *Carbohydr. Polym.* 326 (2024) 121591. <https://doi.org/10.1016/j.carbpol.2023.121591>.
- [26] K. Yang, X. Jia, J. Chen, et al., Sulfate glycosaminoglycan from swim bladder exerts immunomodulatory potential on macrophages via toll-like receptor 4 mediated NF- κ B signaling pathways, *Int. J. Biol. Macromol.* 271 (2024) 132439. <https://doi.org/10.1016/j.ijbiomac.2024.132439>.
- [27] Y. Shen, J. Xu, S. Zhi, et al., MIP From *Legionella pneumophila* influences the phagocytosis and chemotaxis of RAW264.7 macrophages by regulating the lncRNA GAS5/miR-21/SOCS6 axis, *Front. Cell. Infect. Mi.* 12 (2022) 810865. <https://doi.org/10.3389/fcimb.2022.810865>.
- [28] Y. Gou, D. Gu, J. Fan, et al., Purification and structural characterization of polysaccharides from *Polygonum multiflorum* Thunb and their immunostimulatory activity in RAW264.7 Cells, *Foods* 13 (2024) 932. <https://doi.org/10.3390/foods13060932>.
- [29] Y. Liu, L. Su, R. Wang, et al., Comparative 4D label-free quantitative proteomic analysis of *Bombus terrestris* provides insights into proteins and processes associated with diapause, *Int. J. Mol. Sci.* 25 (2023) 326. <https://doi.org/10.3390/ijms25010326>.
- [30] R. Fan, C. Zhu, D. Qiu, et al., Activation of RAW264.7 macrophages by an acidic polysaccharide derived from *Citrus grandis* 'Tomentosa', *Int. J. Biol. Macromol.* 156 (2020) 1323-1329. <https://doi.org/10.1016/j.ijbiomac.2019.11.172>.
- [31] H.Q. Chen, H. Zhang, Y.J. Tian, et al., Exopolysaccharide from *Streptococcus thermophilus* as stabilizer in fermented dairy: binding kinetics and interactions with casein of milk, *Int. J. Biol. Macromol.* 140 (2019) 1018-1025. <https://doi.org/10.1016/j.ijbiomac.2019.08.146>.
- [32] T.S. Kalinina, D.V. Zlenko, A.V. Kiselev, et al., Antiviral activity of the high-molecular-weight plant polysaccharides (Panavir®), *Int. J. Biol. Macromol.* 161 (2020) 936-938. <https://doi.org/10.1016/j.ijbiomac.2020.06.031>.
- [33] Y. Wang, Structural analysis and immunological activity study of *Codonopsis pilosula* polysaccharide CPC [D]. Shanxi Medical University, China. 2021.
- [34] J. Wang, W. Yang, Y. Tang, et al., Regioselective sulfation of *Artemisia sphaerocephala* polysaccharide: solution conformation and antioxidant

- activities *in vitro*, Carbohydr. Polym. 136 (2016) 527-536. <https://doi.org/10.1016/j.carbpol.2015.09.073>.
- [35] M. Zhu, R. Huang, P. Wen, et al., Structural characterization and immunological activity of pectin polysaccharide from kiwano (*Cucumis metuliferus*) peels, Carbohydr. Polym. 254 (2021) 117371. <https://doi.org/10.1016/j.carbpol.2020.117371>.
- [36] Z. Wang, Y. Zheng, Z. Lai, et al., Effect of monosaccharide composition and proportion on the bioactivity of polysaccharides: a review, Int. J. Biol. Macromol. 254 (2024) 127955. <https://doi.org/10.1016/j.jbiomac.2023.127955>.
- [37] T.C. Lo, Y.H. Jiang, A.L. Chao, et al., 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. <https://doi.org/10.1016/j.aca.2006.10.051>.
- [38] S. Sun, K. Li, L. Xiao, et al., Characterization of polysaccharide from *Helicteres angustifolia* L. and its immunomodulatory activities on macrophages RAW264.7, Biomed. Pharmacother. 109 (2019) 262-270. <https://doi.org/10.1016/j.biopha.2018.10.039>.
- [39] X. Long, X. Hu, H. Xiang, et al., Structural characterization and hypolipidemic activity of *Gracilaria lemaneiformis* polysaccharide and its degradation products, Food chem. X 14 (2022) 100314. <https://doi.org/10.1016/j.fochx.2022.100314>.
- [40] F. Shi, X. Yan, K.L. Cheong, et al., Extraction, purification, and characterization of polysaccharides from marine algae *Gracilaria lemaneiformis* with anti-tumor activity, Process Biochem. 73 (2018) 197-203. <https://doi.org/10.1016/j.procbio.2018.08.011>.
- [41] Q.M. Liu, Y. Yang, S.J. Maleki, et al., Anti-food allergic activity of sulfated polysaccharide from *Gracilaria lemaneiformis* is dependent on immunosuppression and inhibition of p38 MAPK, J. Agric. Food Chem. 64 (2016) 4536-4544. <https://doi.org/10.1021/acs.jafc.6b01086>.
- [42] M. Bataliotti, F. Costa, F. Minussi, et al., Characterization of tellurium dioxide thin films obtained through the pechini method, J. Sol-Gel Sci. Techn. 103 (2022) 378-385. <https://doi.org/10.1007/s10971-022-05844-7>.
- [43] Y. Gong, S.T. Mixture, P. Gao, et al., Surface roughness measurements using power spectrum density analysis with enhanced spatial correlation length, J. Phys. Chem. C 120 (2016) 22358-22364. <https://doi.org/10.1021/acs.jpcc.6b06635>.
- [44] N. Chen, T. Jiang, J. Xu, et al., The relationship between polysaccharide structure and its antioxidant activity needs to be systematically elucidated, Int. J. Biol. Macromol. 270 (2024) 132391. <https://doi.org/10.1016/j.jbiomac.2024.132391>.
- [45] A.O. Byankin, E.V. Sokolovaa, S.D. Anastuyk, et al. Polysaccharide structure of tetrasporic red seaweed *Tichocarpus crinitus*, Carbohydr. Polym. 98 (2013) 26-35. <https://doi.org/10.1016/j.carbpol.2013.04.063>.
- [46] S. Zhang, Q. Zhang, L. An, et al., A fructan from *Anemarrhena asphodeloides* Bunge showing neuroprotective and immunoregulatory effects, Carbohydr. Polym. 229 (2020) 115477. <https://doi.org/10.1016/j.carbpol.2019.115477>.
- [47] H. Yue, Q. Xu, G. Bian, et al., Structure characterization and immunomodulatory activity of a new neutral polysaccharide SMP-0b from *Solanum muricatum*, Int. J. Biol. Macromol. 155 (2020) 853-860. <https://doi.org/10.1016/j.jbiomac.2019.11.071>.
- [48] F. Aktan, iNOS-mediated nitric oxide production and its regulation, Life Sci. 75 (2004) 639-653. <https://doi.org/10.1016/j.lfs.2003.10.042>.
- [49] D. Wu, S.N. Meydani, Mechanism of age-associated up-regulation in macrophage PGE2 synthesis, Brain Behav. Immun. 18 (2004) 487-494. <https://doi.org/10.1016/j.bbi.2004.05.003>.
- [50] J. Shan, W. Ma, Y. Guo, et al., Unveiling the immunomodulatory mechanism of polysaccharides from *Polygonum cyrtoneura* based on RNA-seq, Food Res. Int. 175 (2024) 113755. <https://doi.org/10.1016/j.foodres.2023.113755>.
- [51] W. Lei, J.D. Browning, C.H. Lu, et al., Immuno-stimulatory activity of a polysaccharide-enriched fraction of *Sutherlandia frutescens* occurs by the toll-like receptor-4 signaling pathway, J. Ethnopharmacol. 172 (2015) 247-253. <https://doi.org/10.1016/j.jep.2015.06.013>.
- [52] C.C. Lin, I.H. Pan, Y.R. Li, et al., The adjuvant effects of high-molecular-weight polysaccharides purified from *Antrrodia cinnamomea* on dendritic cell function and DNA vaccines, PLoS One 10 (2015) e0116191. <https://doi.org/10.1371/journal.pone.0116191>.
- [53] Y. Miao, G. Ren, D. Zhen, et al., Molecular mechanism of *Cordyceps militaris* polysaccharides regulating the immune activity of mouse macrophages RAW264.7, Food Sci. 40 (2019) 188-194. <https://doi.org/10.7506/spkx1002-6630-20180122-294>.
- [54] Y. Hong, M. Shen, L. Huang, et al., *Mesona chinensis* benth polysaccharides alleviates liver injury by beneficial regulation of gut microbiota in cyclophosphamide-induced mice, Food Sci. Hum. Well. 11 (2022) 74-84. <https://doi.org/10.1016/j.fshw.2021.07.009>.
- [55] X. Chen, S. Wu, R. Huang, et al., Immunomodulatory activity and mechanism of chinese yam polysaccharide after sulfated modification, Ind. Crop. Prod. 197 (2023) 116549. <https://doi.org/10.1016/j.indcrop.2023.116549>.
- [56] Y. Liu, X. Chen, J.Y. Yang, et al., RNA-seq analysis reveals an immunomodulatory peptide from highland barley activating RAW264.7 macrophages via TNF/NF- κ B signaling pathway. Funct. Integr. Genomic. 23 (2023) 253. <https://doi.org/10.1007/s10142-023-01180-3>.
- [57] T. Fiedler, R. Fairless, K. Pichi, et al., Co-modulation of TNFR1 and TNFR2 in an animal model of multiple sclerosis, J. Neuroinflamm. 20 (2023) 100. <https://doi.org/10.1186/s12974-023-02784-z>.
- [58] L. Pinton, E. Masetto, M. Vettore, et al., The immune suppressive microenvironment of human gliomas depends on the accumulation of bone marrow-derived macrophages in the center of the lesion, J. Immunother. Cancer. 7 (2019) 58. <https://doi.org/10.1186/s40425-019-0536-x>.
- [59] J. Zhang, R. Zhang, W. Li, et al., I κ B kinase β (IKK β): structure, transduction mechanism, biological function, and discovery of its inhibitors, Int. J. Biol. Sci. 19 (2023) 4181-4203. <https://doi.org/10.7150/ijbs.85158>.
- [60] E. Svandova, B. Vesela, H. Lesot, et al., Expression of Fas, FasL, Caspase-8 and other factors of the extrinsic apoptotic pathway during the onset of interdigital tissue elimination, Histochem. Cell Biol. 147 (2017) 497-510. <https://doi.org/10.1007/s00418-016-1508-6>.
- [61] Y. Kang, Z.J. Wang, D. Xie, et al., Characterization and potential antitumor activity of polysaccharide from *Gracilaria lemaneiformis*, Mar. Drugs. 15 (2017) 100. <https://doi.org/10.3390/md15040100>.
- [62] N. Liu, K. Wei, Y. Xun, et al., Transcription factor cyclic adenosine monophosphate responsive element binding protein negatively regulates tumor necrosis factor alpha-induced protein 1 expression, Mol. Med. Rep. 12 (2015) 7763-7769. <https://doi.org/10.3892/mmr.2015.4336>.
- [63] T.J. Ewing, S. Makino, A.G. Skillman, et al., Dock 4.0: Search strategies for automated molecular docking of flexible molecule databases, J. Comput. Aid. Mol. Des. 15 (2001) 411-428. <https://doi.org/10.1023/A:1011115820450>.
- [64] X. Wu, Z. Zheng, T. Guo, et al., Molecular dynamics simulation of lentinan and its interaction with the innate receptor dectin-1, Int. J. Biol. Macromol. 171 (2021) 527-538. <https://doi.org/10.1016/j.jbiomac.2021.01.032>.
- [65] G. Wei, X. Dai, Bo. Zhao, et al., Structure-activity relationship of exopolysaccharides produced by *Limosilactobacillus fermentum* A51 and the mechanism contributing to the textural properties of yogurt, Food Hydrocolloid. 144 (2023) 108993. <https://doi.org/10.1016/j.foodhyd.2023.108993>.
- [66] Y.J. Zeng, H.R. Yang, X.Y. Ou, et al., Fungal polysaccharide similar with host *Dendrobium officinale* polysaccharide: preparation, structure characteristics and biological activities, Int. J. Biol. Macromol. 141 (2019) 460-470. <https://doi.org/10.1016/j.jbiomac.2019.08.238>.
- [67] L. Yuan, Z.C. Zhong, Y. Liu, et al., Structures and immunomodulatory activity of one galactose- and arabinose-rich polysaccharide from *Sambucus adnata*, Int. J. Biol. Macromol. 207 (2022) 730-740. <https://doi.org/10.1016/j.jbiomac.2022.03.132>.