

Research Article

Structural Characterization and Immunomodulatory Activities of a Novel Polysaccharide From Sacha Inchi (*Plukenetia volubilis* L.) Shell

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Received 25 January 2024; Revised 27 September 2024; Accepted 3 October 2024

Academic Editor: Seyed Mohammad Taghi Gharibzahedi

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The novel bioactive polysaccharide, Sacha inchi shell polysaccharide-3 (SISP-3), was isolated from the Sacha inchi (*Plukenetia volubilis* L.) shell using anion exchange and gel filtration chromatography. This neutral polysaccharide fraction, characterized by a triple-helix structure and a molecular weight of 17,684 Da, was successfully isolated. The composition analysis of SISP-3 revealed the presence of Rha, Ara, Gal, Glc, and Fru in molar ratios of 7.2%:41.7%:40.3%:4.1%:6.7%. The dominant glycosidic linkage in SISP-3 was identified as $\rightarrow 4$ -Galp-(1 $\rightarrow$), accompanied by a complex branching pattern. The results from RAW264.7 cell experiments showed that SISP-3 significantly enhanced cell viability, phagocytosis, and NO production. Additionally, it significantly stimulated the production of cytokines, including TNF- α , IL-1 β , and IL-6, in RAW264.7 cells. These findings suggest that SISP-3 serves as an immune active agent with immunomodulatory properties, indicating its potential as a natural immunomodulatory agent for various applications.

Keywords: immunoregulatory activities; polysaccharide; Sacha inchi shell; structure characterization

1. Introduction

The immune system prevents pathogen invasion and participates in immune surveillance, defense, and regulation. Macrophages are the first line of the body immune system. They are an important cellular bridge between innate and adaptive immunity [1]. Upon encountering pathological triggers or injury, macrophages respond by generating numerous biologically active compounds, including nitric oxide (NO), reactive oxygen species, and various cytokines, which collectively fend off invading pathogens [2]. Given their critical role in immune responses, macrophages have become a target for developing immune-enhancing agents.

In recent years, with the growing concern for health in contemporary society, both consumers and industries have shown increasing interest in finding natural and safe immunomodulatory substances. This demand has driven research toward identifying bioactive compounds with immune-enhancing properties, particularly from natural sources [3]. Among these natural substances, polysaccharides have gained significant attention due to their potential immunomodulatory, antioxidant, antibacterial, and other biological activities [4–6].

Sacha inchi (*Plukenetia volubilis* L.), also known as Inca peanut or Sacha peanut, is a climbing plant native to the Amazon rainforest. This plant has gained prominence due to

its seeds' high content of omega-3 fatty acids and complete protein profile, making it a valuable nutritional supplement [7]. While the seeds have been extensively studied, the shells, which account for 35.6% of the plant's biomass, have been largely overlooked despite their bioactive potential [8]. Recent studies have started to reveal the potential value of this by-product. Previous research has indicated that Sacha inchi shells contain 43.12% protein, 9.90% carbohydrates, and 7.45% total phenols. These bioactive compounds have been linked to significant health benefits [9, 10]. Moreover, polysaccharides isolated from various sources, including the shells of plants, have demonstrated immunomodulatory effects, yet the polysaccharides in Sacha inchi shells remain underexplored.

To investigate the immunomodulatory properties of Sacha inchi polysaccharides, the RAW264.7 macrophage cell line is often used as a model for studying immune responses. Previous research has shown that Sacha inchi seeds enhance the phagocytic capacity of RAW264.7 cells and increase the production of NO and inflammatory factors, demonstrating their immunomodulatory potential [11]. Polysaccharides, particularly those derived from traditional medicinal herbs such as *Dendrobium officinale* and *Polygonatum* spp., have long been recognized for their ability to interact with macrophages and influence immune function [12, 13]. However, research into the polysaccharides present in plant by-products, such as the shells of Sacha inchi, remains limited. Therefore, in this study, we aimed to isolate, purify, and characterize polysaccharides from the shells of Sacha inchi and evaluate their immunomodulatory effects on macrophages. By focusing on this underutilized plant by-product, we seek to contribute to the discovery of new natural immunomodulatory agents with potential health benefits.

2. Materials and Methods

2.1. Materials and Reagents. Sacha inchi's shell was provided by Pu'er Lianzhong Biological Resources Development Co., Ltd., Yunnan Province, China, in 2021. All the other reagents were of analytical reagent grade and made in China.

2.2. Isolation and Purification of Polysaccharides. Isolation: Dry the Sacha inchi shells in an oven at 60°C (DGX-9143B-2, Shanghai Formosa Experimental Equipment Co., Ltd, China) until a constant weight is achieved. Next, crush the dried Sacha inchi shells using a traditional Chinese medicine grinder and sieve it through an 80-mesh screen. Subsequently, mix the Sacha inchi shells with distilled water at a ratio of 1:10. Ultrasonic extraction was carried out for 60 min at 85°C and 500 W using an ultrasonic extractor (FRQ-101OHT, Hangzhou Falante Co., Ltd., China). The filtrate was concentrated approximately fourfold using a rotary evaporator (RE-5298, Shanghai Yarong Biochemical Instrument Factory, China) to obtain a solution at 60°C. The solution was then precipitated for 12 h at 4°C by adding four times its volume of anhydrous ethanol (v/v). The precipitate was collected by centrifugation and dissolved in water to preliminarily obtain crude polysaccharides. The

crude polysaccharides were treated with the Sevage method (chloroform:n-butanol = 4:1) to remove proteins, continuing until no protein layer appeared in the solution after centrifugation. The resulting solutions were dialyzed and freeze-dehydrated to obtain crude Sacha inchi shell polysaccharide (SISP).

Purification: Subsequently, crude SISP was prepared at a concentration of 10 mg/mL for purification using a DEAE-Sephacrose Fast Flow cellulose column (2.5 × 50 cm²) (17070901, Cytiva, Sweden). Elution was performed with ultrapure water and NaCl solutions of 0.1, 0.2, and 0.3 mol/L at a flow rate of 2 mL/min. An automatic collector was used to collect 5 mL per tube. The eluted fractions were collected and analyzed by the phenol-sulfuric acid method, with absorbance measured at 490 nm. The fractions with the highest content were collected, concentrated, dialyzed against a 1000 Da membrane, and lyophilized for further purification. The preliminarily purified polysaccharides obtained from the above steps were prepared at a concentration of 10 mg/mL and further purified through a Sephadex G-100 dextran gel (17-0060-01, GE Healthcare, Sweden) column (1.6 × 60 cm²) at a flow rate of 1 mL/min. Polysaccharide fractions were collected using an automatic collector, with each tube collecting 5 mL. Their absorbance at 490 nm was determined using the phenol-sulfuric acid method. Fractions with an absorbance greater than 0.5 were collected and subsequently lyophilized to obtain the pure polysaccharide sample SISP-3 (Figure 1).

2.3. UV-Vis. An ultraviolet spectrophotometer (Evolution 300, Thermo Fisher Scientific, China) was used to measure the protein and nucleic acid content of SISP-3 by scanning in the 200–800 nm range.

2.4. Evaluation of Polysaccharide Structural Characteristics

2.4.1. Molecular Weight Determination. High-performance gel permeation chromatography (HPGPC) was performed using an LC-20A high-performance liquid chromatography (HPLC) system (1525, Waters, USA) equipped with a 7.8 × 300 mm column (TSK-GEL G5000SWxl, Tosoh, Japan), and a RID detector (RI-101SHODEX, Tosoh, Japan) was used to assess the homogeneity and molecular weight of SISP-3 [14].

Standards were prepared using dextran-T series polysaccharides (922,000, 543,200, 311,400, 175,900, 108,900, 39,600, 3400, 1000), which were dissolved in 20 mM phosphate buffer solution (pH 7.4) to create a 1 mg/mL standard solution. The solution was filtered through a 0.45-μm membrane filter for later use. The standards were analyzed by HPGPC, with an elution mode of GPC water at a flow rate of 1.0 mL/min, a column temperature of 35°C, an injection volume of 20 μL, and a detector of a differential refractometer, with a total detection time of 35 min. A standard curve was created by plotting the weight-average molecular weight (Mw) of standard dextran on the vertical axis against the relative retention volume on the horizontal axis, facilitating regression fitting.

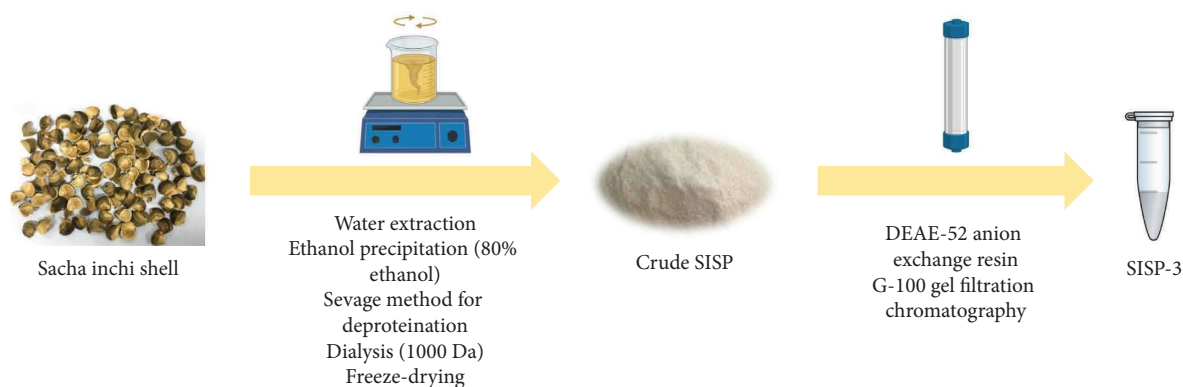


FIGURE 1: Extraction and isolation scheme.

Sample solutions were prepared by weighing 4 mg of the polysaccharide and adding 2 mL of the mobile phase to achieve a concentration of 2 mg/mL. The mixture was fully dissolved, filtered through a 0.45- μ m membrane filter, and set aside for later use.

The prepared polysaccharide sample solution was analyzed under the conditions established for the standard curve. The molecular weight of the sample is calculated based on its retention time in the chromatographic column, using the equation of the dextran standard curve.

2.4.2. Monosaccharide Composition Analysis.

Monosaccharide standards, including fucose, rhamnose, arabinose, galactose, glucose, xylose, mannose, fructose, ribose, galacturonic acid, and glucuronic acid, were precisely weighed to 5 mg each and placed into individual vials. Each standard was treated with 2 mL of 3 M trifluoroacetic acid (TFA) and hydrolyzed at 80°C for 3 h. After hydrolysis, the solutions were transferred into tubes and the solvent was evaporated under a nitrogen stream. Each residue was reconstituted in 5 mL of deionized water and vortexed to ensure thorough mixing, forming the stock standard solutions. These solutions were used to prepare dilutions for calibration, facilitating the creation of a mixed standard for subsequent analysis.

Samples were prepared by accurately weighing 5 mg and placing each sample into a vial, followed by the addition of 2 mL of 3 M TFA. The samples underwent hydrolysis at 80°C for 3 h, following the same conditions applied to the standards. After hydrolysis, the liquid was transferred to a tube and dried under nitrogen. The dried samples were redissolved in 5 mL of water, vortexed for uniformity, and then diluted by transferring 100 μ L of the solution to 900 μ L of deionized water. The resulting solution was centrifuged at 12,000 rpm for 5 min, and the supernatant was collected for ion chromatography analysis.

Chromatographic analyses were performed on an ion chromatography system (ICS5000, Thermo Fisher, China) using a column (4 mm $\times$ 250 mm) (Dionex Carbpac™ PA10, Thermo Fisher, China). The mobile phase consisted of A: H₂O, B: 500 mM NaOH with 50 mM NaOAc, and C: 20 mM NaOH, flowing at 1.0 mL/min. The injection volume was 25 μ L, and the column temperature was maintained at

30°C. Detection was carried out using an electrochemical detector, providing sensitive measurement of the monosaccharides present in the samples.

2.4.3. Fourier Transform Infrared (FT-IR) Spectroscopy Analysis.

A 100 mg quantity of potassium bromide was weighed, ground, pressed, and scanned with infrared light in the full-wavelength mode, with the results used as a reference. Subsequently, 1.5 mg of the SISP-3 sample was weighed and added to 100 mg of potassium bromide, after which the samples were ground and pressed. Infrared full-wavelength mode spectral scanning was then performed using a Bruker Vertex 70 FT-IR spectrometer, in the frequency range of 4000 to 400 cm⁻¹ [6, 15].

2.4.4. I₂-IK Analysis.

The sample solution of 1 mg/mL polysaccharide with 2 mL concentration was admixed with 1.5 mL I₂-KI reagent (0.2% KI solution containing 0.02% I₂) and scanned by using a UV-vis spectrophotometer (Evolution 300, Thermo Fisher Scientific, China) at 300–700 nm.

2.4.5. Methylation Analysis.

The polysaccharide sample was dissolved in dimethyl sulfoxide (DMSO), and the solution was methylated in NaOH and CH₃I. After complete methylation, the permethylated products were hydrolyzed with 2 mol/L TFA at 120°C for 1.5 h, reduced by NaBH₄, and acetylated with acetic anhydride for 2.5 h at 100°C.

The acetates were dissolved in chloroform and analyzed with gas chromatography–mass spectrometry (GC-MS) (6890-5973, Agilent, American) equipped with a chromatographic column (30 m $\times$ 0.25 mm $\times$ 0.25 μ m) (RXI-5 SIL MS, Shimadzu, Japan). The temperature program was set at 120°C for 2 min, subsequently increased to 250°C at 3°C/min, and held at 250°C for 5 min. The temperatures of the injection port were set at 250°C. The flow rate was 1 mL/min, and the carrier gas was nitrogen. The retention time and fragmentation pattern were used to identify and quantify each methylated glycosidic linkage [15].

2.4.6. Congo Red Test. Triple-helix structure of samples in vitro was measured by the Congo red method [16].

2.5. Immunoregulatory Activity

2.5.1. Cell Culture. RAW264.7 cells were obtained from the Medical College of Sun Yat-sen University (Guangzhou, China). The cells were cultured with Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY) and added with 10% fetal bovine serum (Gibco, Grand Island, NY), 100 $\mu\text{g}/\text{mL}$ streptomycin (HyClone, Logan, UT), and 100 units/mL penicillin at 37°C in a humidified incubator with 5% CO_2 .

2.5.2. Viability Assay. The cytotoxicity of SISP-3 on RAW264.7 cells was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (St. Louis, MO) [17]. RAW264.7 cells were cultured in 96-well plates at a density of 1×10^4 cells/well. After 24 h, SISP-3 was added to the cells at concentrations of 25, 50, 100, 200, and 400 $\mu\text{g}/\text{mL}$, followed by incubation at 37°C for another 24 h. Subsequently, the MTT solution was subjoined to each well and incubated for 4 h at 37°C. After incubation, the medium was discarded, and DMSO was added to dissolve the formed formazan crystals. Absorbance was measured at 570 nm on a microplate reader. Cell viability was expressed as the percentage of untreated controls [18] and was assessed in triplicate for each group.

2.5.3. NO, Interleukin (IL)-1 β , IL-6, Tumor Necrosis Factor (TNF)- α , and Phagocytic Activity. RAW264.7 cells were treated with 25, 50, 100, 200, and 400 $\mu\text{g}/\text{mL}$ SISP-3, cell supernatants were collected, and NO levels were determined using nitrate reductase and NO assay kits [19]. The phagocytic capacity of macrophages was evaluated according to neutral red uptake. The determination of IL-1 β , IL-6, and TNF- α in RAW264.7 cell homogenates was conducted using an enzyme-linked immunosorbent assay kit (Jiangsu Meimian Industrial Co., Ltd) and assessed in triplicate for each group.

2.6. Statistical Analysis. Experimental data were reported as mean values with standard deviation (SD). The statistical program SPSS 26.0 and GraphPad Prism 9.4 were used to analyze the data. Probabilities of $p < 0.05$ were used to indicate significance carried out with the LSD test, and values sharing a common letter (A, B, C, D, and E) in each row did not show significant differences.

3. Results

3.1. Isolation and Purification of Polysaccharides. The crude polysaccharide was extracted from Sacha inchi shell with a yield of $(2.80 \pm 0.34)\%$ of the dry material. Following preliminary purification by the Seville method for eliminating nucleic acids and proteins, the crude polysaccharide was separated through the DEAE-52 cellulose column. The elution with distilled water and various concentrations of NaCl resulted in the isolation of three peaks representing three fractions: S-1, S-2, and S-3 (Figure 2(a)). The area under the curve analysis method in GraphPad Prism 9.4 to calculate the area under the curve for absorbance ≥ 0.1 , the

areas under the curves for S-1, S-2, and S-3 were found to account for 25.21%, 16.24%, and 58.55%, respectively (Figure 2(a)). Consequently, S-3 underwent further purification via Sephadex G-100 chromatography and designated as SISP-3 (Figure 2(b)). A UV-vis scan of SISP-3 between 200 and 800 nm (Figure 2(c)) revealed no significant absorption peaks at 260 and 280 nm, indicating that nucleic acids and proteins were nearly completely removed from the polysaccharide [20]. Therefore, the purification of SISP-3 was effectively achieved.

3.2. Molecular Weight and Monosaccharide Analysis. The molecular weight of sample SISP-3 was determined to be 17,684 Da using HPGPC (Figure 3(a)). The polydispersity index (PDI) was measured at 1.16, indicating a relatively uniform molecular weight distribution [21], suggesting a higher purity of SISP-3.

Analysis by HPIC (Figure 3(b)) showed that SISP-3 primarily consisted of arabinose and galactose, with molar ratios of 41.7% and 40.3% of the total composition, respectively. Additionally, minor components such as rhamnose (7.2%), glucose (4.1%), and fructose (6.7%) were also present in SISP-3.

3.3. FT-IR Analysis. The FT-IR spectrum of SISP-3 is shown in Figure 4, which displays the typical features of polysaccharides with a strong and broad absorption band at 3387 cm^{-1} , indicating $-\text{OH}$ stretching vibrations [5]. This absorption may also arise from $\text{N}-\text{H}$ stretching or intramolecular hydrogen bonding. Additionally, a relatively weak peak between 2700 cm^{-1} and 3000 cm^{-1} is attributed to $-\text{CH}$ stretching vibrations in methylene groups ($-\text{CH}_2-$) [5]. Notably, the spectrum reveals the presence of a free carboxylic carbonyl group, evidenced by absorption peaks at 1655 cm^{-1} and 1541 cm^{-1} , alongside a peak at around 1400 cm^{-1} due to $\text{C}-\text{H}$ bending vibrations. The characteristic absorption peaks at $1,078$, $1,041$, and 815 cm^{-1} in the FT-IR analysis further define the polysaccharide structure. The peak at 1078 cm^{-1} is typically associated with the $\text{C}-\text{O}-\text{C}$ ether linkages of sugar rings, and the peak at 1041 cm^{-1} indicates the $\text{C}-\text{O}$ stretching vibrations representing glycosidic linkages between sugar units [22]. Additionally, the region around 1035 cm^{-1} is suggested to display characteristic absorption bands for arabinose, suggesting that SISP-3 may contain a significant proportion of this monosaccharide. Consistent with these findings, galactose has been identified around 1065 and 807 cm^{-1} [5], which aligns with the monosaccharide composition analysis. Furthermore, there was a peak at 815 cm^{-1} , indicating the existence of α -glycosidic bonds from the glucosyl unit [23–25].

3.4. I_2 -IK Analysis. As shown in Figure 5, the maximum absorption of SISP-3 polysaccharides with I_2 -KI was noted at 367 nm and no maximum absorption at 565 nm, which manifested that SISPs may feature more branches and a longer side chain. This is consistent with the results obtained in previous studies conducted by our research group on Sacha inchi seeds [18].

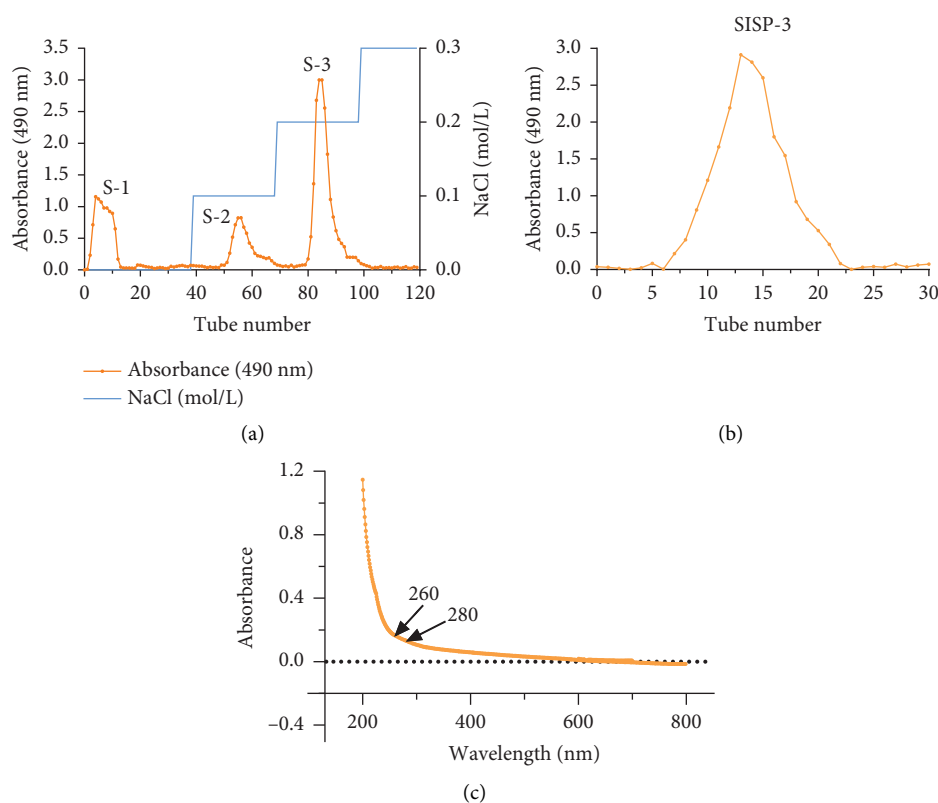


FIGURE 2: DEAE-Sepharose Fast Flow chromatography of the SISP (a), Sephadex G-100 gel chromatography column of SISP-3 (b) and full-wavelength UV scan of SISP-3 (c).

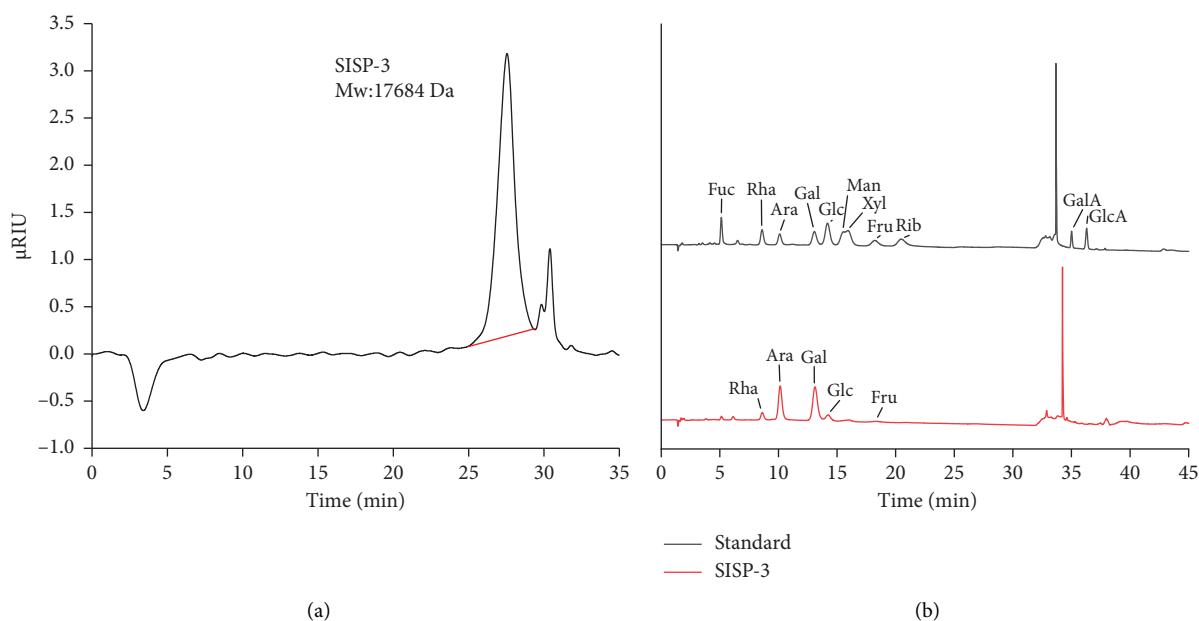


FIGURE 3: HPGPC chromatograms (a) and monosaccharide composition analysis by ion chromatogram of SISP-3 (b).

3.5. Methylation Analysis. Binding ion signals were analyzed using an MS database to determine the types and molar ratios of the methyl glycosides, inferring their linking modes. The results, along with GC-MS data (Figure S1) and mass spectra

(Figure S2), are summarized in Table S1. Combining the results of the monosaccharide composition, SISP-3 contains a portion of fructose. Due to fructose's characteristics as a keto-sugar, mannitol or glucosyl acetate may be produced during the

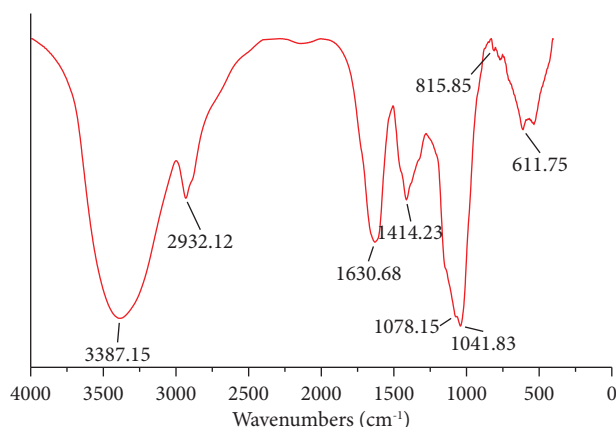
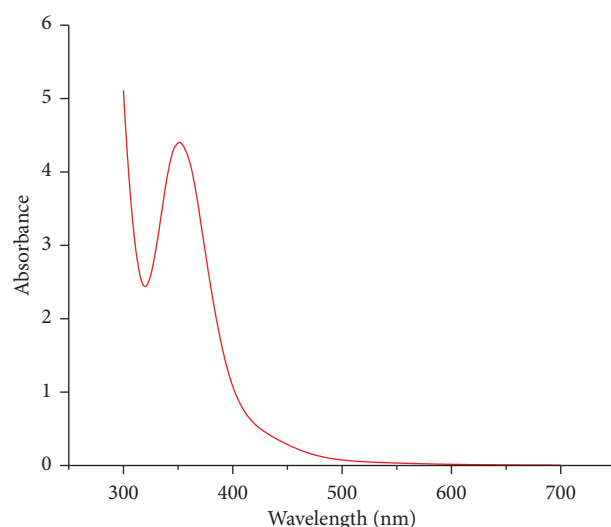


FIGURE 4: FT-IR spectrum of SISP-3.

FIGURE 5: I₂-IK result of SISP-3.

methylation process [15, 26–28]. Therefore, the final results are compiled in Table 1. Due to the absence of standards in the methylation experiment, the analysis focused on qualitative rather than quantitative results, complicating the derivation of standards for each monosaccharide [29]. This issue may explain the inconsistency in the molar ratios of fructose and glucose found in the methylation results compared to the monosaccharide composition analysis. However, combining the monosaccharide composition and methylation data, it can be inferred that the main chain of SISP-3 is likely composed of $\rightarrow 4$ -Galp-(1 $\rightarrow$). The structure of the polysaccharide isolated from Sacha inchi shells is complex and significantly different from the polysaccharides found in Sacha inchi seeds [30]. This suggests that the polysaccharide structures in the shell and seeds of Sacha inchi are not consistent. The branching degree (DB) of SISP-3 was calculated using the formula $DB = (\text{terminal residues} + \text{branch residues}) / (\text{terminal residues} + \text{branch residues} + \text{linear residues})$, yielding a value of 58.2%. This indicates the presence of a complex branching structure, which is consistent with the results from the I₂-IK.

3.6. Triple-Helix Structure Analysis. The triple-helix structure and side-chain structure profoundly affect various physiological activities of polysaccharides [31]. SISPs were dissolved in NaOH solutions of varying concentrations and reacted with Congo red reagent to determine the presence of a triple-helix structure. Congo red interacts with triple-helix polysaccharides, which shifts the maximum absorption toward the long wavelength in solution and does not react with other polysaccharides [32]. The transition from triple-helix conformation to single coil conformation decreases the maximum absorption in Congo red-polysaccharide solution. Laminarin is a typical triple-helix conformational polysaccharide [33]. Maximum absorption wavelength indicated that the laminarin solutions were 502 nm (Figure 6). A shift in the maximum absorption wavelength from 498 to 502 nm occurred in distilled water, indicating that triple-helix polysaccharide-Congo red complexes had formed. Additionally, SISP-3 exhibited a red shift in the concentration range of 0–0.1 mol/L, indicating the presence of a triple-helix structure.

TABLE 1: Methylation results of polysaccharides from SISP-3.

RT (min)	Methylated sugar	Mass fragments (m/z)	Molar ratio	Type of linkage
10.333	2,3,5-Me ₃ -Araf	45, 71, 87, 101, 117, 129, 145, 161	0.058	Araf-(1→
13.764	2-Me ₁ -Rhap	45, 87, 99, 113, 117, 129, 141, 159, 173	0.013	→3,4)-Rhap-(1→
15.002	1,3,4,5-Me ₄ -Manf/Glcf	87, 101, 129, 145, 161	0.106	Fruf-(2→
15.598	2,3-Me ₂ -Araf	45, 71, 87, 99, 101, 117, 129, 161, 189	0.020	→5)-Araf-(1→
16.766	2,3,4,6-Me ₄ -GlcP	45, 71, 87, 101, 117, 129, 145, 161, 205	0.054	GlcP-(1→
17.683	2,3,4,6-Me ₄ -GalP	45, 71, 87, 101, 117, 129, 145, 161, 205	0.027	GalP-(1→
18.304	2-Me ₁ -Araf	45, 58, 85, 99, 117, 127, 159, 201	0.021	→3,5)-Araf-(1→
18.883	3-Me ₁ -Araf	45, 57, 85, 99, 117, 127, 129, 159, 189	0.047	→2,5)-Araf-(1→
20.668	3,4,5-Me ₃ -Manf/Glcf	45, 71, 87, 99, 101, 129, 145, 161, 189	0.110	→1)-Fruf-(2→
20.789	2,4,6-Me ₃ -GlcP	45, 87, 99, 101, 117, 129, 161, 173, 233	0.030	→3)-GlcP-(1→
21.205	2,3,6-Me ₃ -GalP	45, 87, 99, 101, 113, 117, 129, 131, 161, 173, 233	0.142	→4)-GalP-(1→
21.597	2,4,6-Me ₃ -GalP	45, 87, 99, 101, 117, 129, 161, 173, 233	0.066	→3)-GalP-(1→
21.968	2,3,4-Me ₃ -GlcP	45, 87, 99, 101, 117, 129, 161, 189, 233	0.027	→6)-GlcP-(1→
23.494	2,3,4-Me ₃ -GalP	45, 87, 99, 101, 117, 129, 161, 189, 233	0.023	→6)-GalP-(1→
24.211	2,6-Me ₂ -GalP	45, 87, 99, 117, 129, 145, 159	0.102	→3,4)-GalP-(1→
26.442	2,3-Me ₂ -GlcP	45, 71, 85, 87, 99, 101, 117, 127, 159, 161, 201, 261	0.033	→4,6)-GlcP-(1→
26.738	2,3-Me ₂ -GalP	45, 71, 85, 87, 99, 101, 117, 127, 159, 161, 201, 261	0.042	→4,6)-GalP-(1→
28.33	2,4-Me ₂ -GalP	45, 87, 117, 129, 159, 189, 233	0.071	→3,6)-GalP-(1→
29.973	2-Me ₁ -GalP	45, 87, 97, 117, 139, 159, 173, 233	0.008	→3,4,6)-GalP-(1→

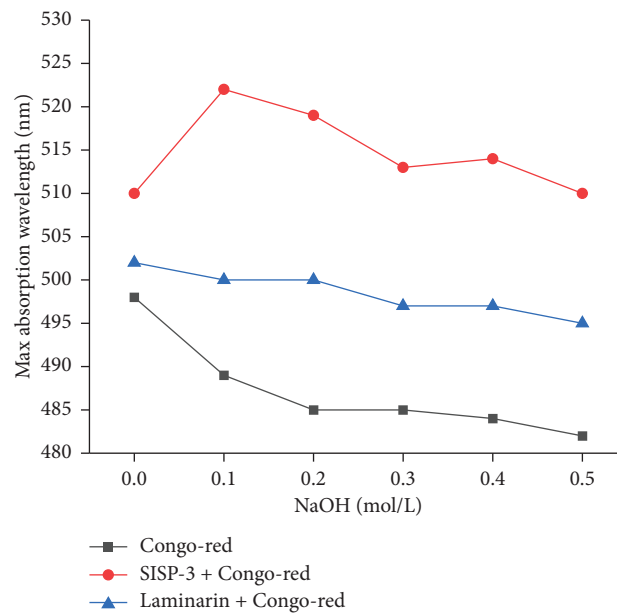


FIGURE 6: Helix-coil transition analysis of SISP-3 and triple-helix conformational glucans at different concentrations of NaOH.

3.7. Immunoregulatory Activity of SISP-3

3.7.1. Cell Viability. In order to investigate the SISP-3 on the survival rate of RAW 264.7 cells, cells with different concentrations of SISP-3 were treated with MTT reagent for 24 h to determine the cell viability. The results are shown in Figure 7(a). Compared with the control group and LPS group, SISP-3 significantly promoted the growth of RAW 264.7 cells ($p < 0.05$) and the cell viability reached the highest at 400 $\mu\text{g/mL}$, 100 $\mu\text{g/mL}$, and 200 $\mu\text{g/mL}$ of SISP-3

($p < 0.05$). The results indicate that there was no cytotoxicity observed in the cells at this experimental concentration.

3.7.2. Phagocytic Activity. The internalization of extracellular materials in the form of phagocytosis is a key indicator of macrophage effects or activities [34]. SISP-3 significantly stimulated the phagocytic activity of RAW264.7 cells in a dose-dependent manner ($p < 0.05$) compared with the control group in the concentration range of 25–400 $\mu\text{g/mL}$,

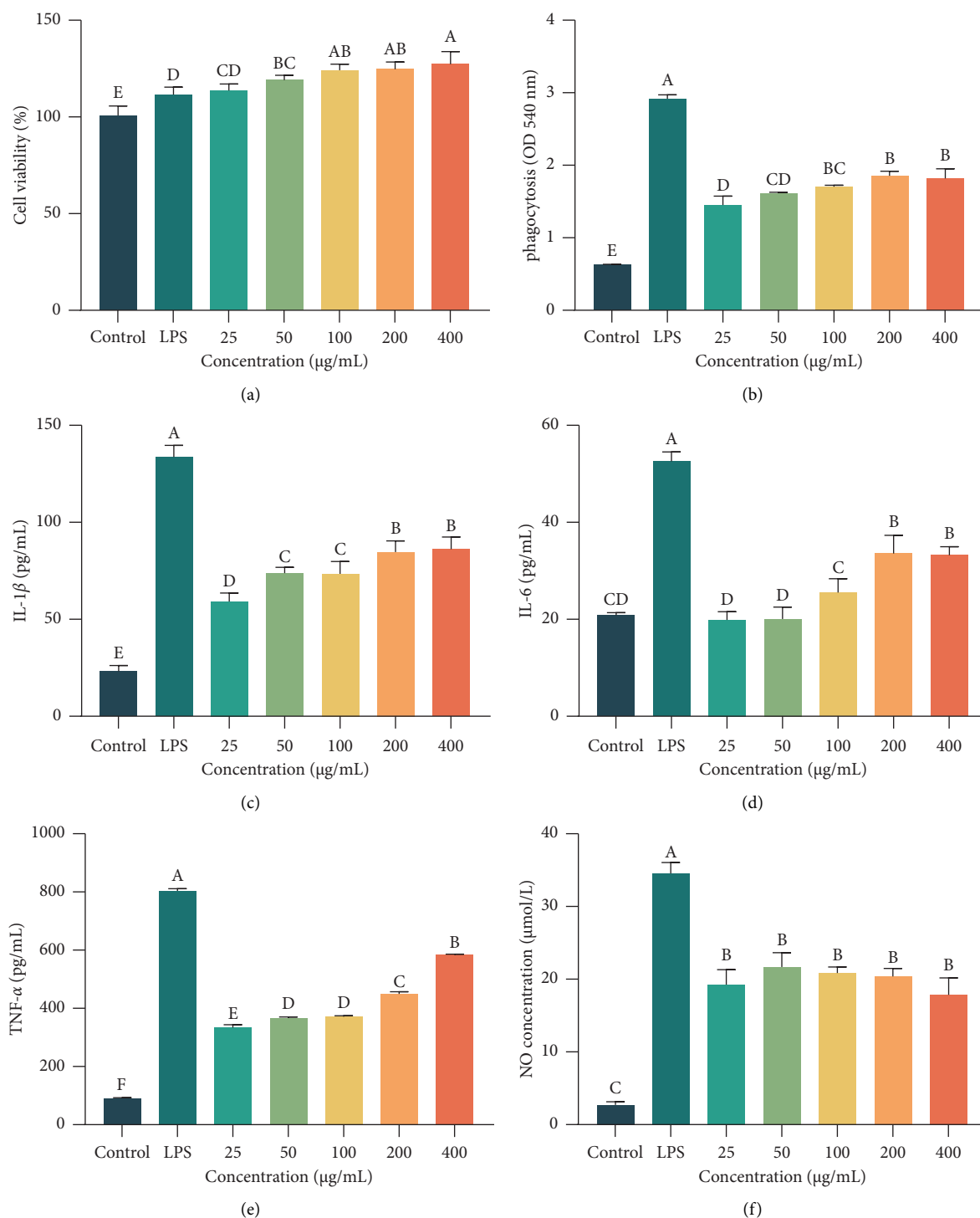


FIGURE 7: Effect of the SISP-3 on the viability of RAW264.7 cells. RAW264.7 cells were treated with various concentrations of SISP-3 or LPS (10 $\mu\text{g/mL}$) for 24 h (a). Effect of the various concentrations of SISP-3 or LPS (10 $\mu\text{g/mL}$) on the phagocytosis (b), and production of IL-1 β (c), IL-6 (d), and TNF- α (e) of RAW264.7 cells for 24 h. The effect of the various concentrations of LPS (10 $\mu\text{g/mL}$) for 24 h (f). The values are presented as mean $\pm$ SD ($n = 6$).

while uptake increased with a higher concentration of the SISP-3. However, the stimulatory effects of SISP-3 on the phagocytic activity of RAW264.7 cells were significantly lower than that of LPS ($p < 0.05$).

3.7.3. Cytokines Production. TNF- α , IL-1 β , and IL-6 are crucial cytokines for regulating immune responses and play significant roles in cell proliferation, differentiation, regulating immune responses, and activating other immune cells

[35]. The effects of SISP-3 on the secretion of IL-1 β , IL-6, and TNF- α by RAW264.7 cells are depicted in Figure 7. Compared to the control group, treatment with SISP-3 significantly increased IL-1 β secretion, which progressively rose with increasing concentrations and plateaued at 200 μ g/mL. Similarly, IL-6 and TNF- α secretion followed a comparable trend, but the maximum secretion of TNF- α by RAW264.7 cells occurred at 400 μ g/mL with SISP-3, significantly higher than other concentrations ($p < 0.05$). However, the stimulation levels of these cytokines by LPS remained significantly higher than those achieved with any dose of SISP-3. Compared to previous research by our team on Sacha inchi seed polysaccharides (PVLP-1) [18], despite SISP-3's lower yield than PVLP-1, SISP-3 exhibited a stronger stimulatory effect on the secretion of IL-1 β , IL-6, and TNF- α in RAW264.7 cells. Specifically, under the same stimulation of 200 μ g/mL, compared to their respective control groups, the secretion levels of the three cytokines enhancing by SISP-3 were approximately 2–6 times that of PVLP-1.

3.7.4. NO Production. NO is a reactive nitrogen species produced by inducible nitric oxide synthase (iNOS) within cells. It not only has oxidative effects that can kill pathogens or inhibit tumor cell proliferation but also serves as a signaling molecule to mediate other immune responses [36]. The impact of SISP-3 on the secretion of NO by RAW264.7 cells is depicted in Figure 7(f). Compared to the control group, SISP-3 significantly enhances the secretion levels of NO in RAW264.7 cells. However, as the concentration increases, there is no significant variation in NO secretion levels, and the promoting effect on NO secretion is consistently weaker than that of LPS. These findings suggest that the stimulatory effect of SISP-3 on NO secretion by cells is limited. However, this limited stimulatory effect may not lead to an excessive immune response or inflammation [37]. Therefore, SISP-3 can be considered a relatively safe immune modulator.

4. Discussion

The SISP-3 polysaccharide exhibits significant immunomodulatory activities, closely linked to its unique structural characteristics. Prior studies have demonstrated that the immunomodulatory functions of polysaccharides are directly correlated with their structural feature [30, 38]. Different structures of polysaccharides show distinct effects on NO secretion and inflammatory cytokine stimulation in RAW264.7 cells [37].

Analysis of SISP-3 reveals complex branching and specific glycosidic linkages that are likely crucial for its effective macrophage activation. Methylation analysis results show that the main structural features of these polysaccharides include various types of sugar units and their linkages, such as $\rightarrow 4$ -Galp-(1 $\rightarrow$, $\rightarrow 3$)-GlcP-(1 $\rightarrow$ Araf-(1 $\rightarrow$, $\rightarrow 3,4$)-Rhap-(1 $\rightarrow$ and $\rightarrow 1$)-Fruf-(2 $\rightarrow$). These structural characteristics affect the spatial conformation, molecular weight, and degree of branching of the polysaccharides, thereby influencing their binding ability to receptors on the surface of

immune cells. For example, galactans and arabinogalactans interact with the immune system through their unique molecular structures, thereby triggering immune responses [39]. Specifically, structures such as $\rightarrow 4$ -Galp-(1 $\rightarrow$ and $\rightarrow 3$)-GlcP-(1 $\rightarrow$ play critical roles in the polysaccharide chains by interacting with pattern recognition receptors (PRRs) such as TLRs and dectin-1 on immune cells [40]. This interaction triggers signaling pathways, ultimately leading to the secretion of cytokines and activation of immune cells. These structural features of polysaccharides have shown significant immunomodulatory effects in both in vitro and in vivo experiments, such as enhancing macrophage phagocytic activity and promoting the secretion of pro-inflammatory cytokines (e.g., TNF- α , IL-6) [41].

Additionally, SISP-3's rich monosaccharide composition, including rhamnose, arabinose, galactose, glucose, and fructose, has been shown to interact with various PRRs such as Toll-like receptors (TLRs), mannose receptors (MRs), scavenger receptors (SRs), and glucan receptors (GRs) on macrophages [37]. The diverse monosaccharide profile of SISP-3 may enhance its ability to be recognized by multiple receptors, thereby amplifying its immunomodulatory effects. This broad receptor interaction is particularly important in activating different signaling pathways, which can lead to a synergistic enhancement of the immune response. The diverse monosaccharide profile of SISP-3 may enhance its ability to be recognized by multiple receptors, thereby amplifying its immunomodulatory effects. This broad receptor interaction is particularly important in activating different signaling pathways, which can lead to a synergistic enhancement of the immune response.

Research has also indicated that the molecular weight of polysaccharides plays a crucial role in regulating their immunoactivity. The molecular weight of SISP-3 is 17,684 Da, which falls within the low molecular weight range. Low molecular weight polysaccharides below 20 kDa generally exhibit enhanced immune-stimulating properties due to their low viscosity, allowing for better interaction with immune cells [42]. The molecular weight of SISP-3 provides these advantages, potentially resulting in significant effects in immune cell activation and pro-inflammatory cytokine secretion.

Based on the aforementioned conclusions, the hypothesized relationship between the structure and activity is illustrated in Figure 8. These characteristics position SISP-3 as a potent candidate for developing novel functional foods and pharmaceuticals, particularly in strategies aimed at enhancing health through immune response modulation. The potential applications of SISP-3 extend beyond basic immunomodulation; it may be explored for its therapeutic effects in inflammatory diseases, infection control, and even cancer therapy, where the modulation of the immune system plays a pivotal role.

Although this study provides a preliminary assessment of the structure of SISP-3 and conducted an analysis of its immunomodulatory activity on RAW264.7 cells, along with an initial exploration of the structure–activity relationship, the specific mechanisms by which the polysaccharide structure influences immunomodulatory activity still

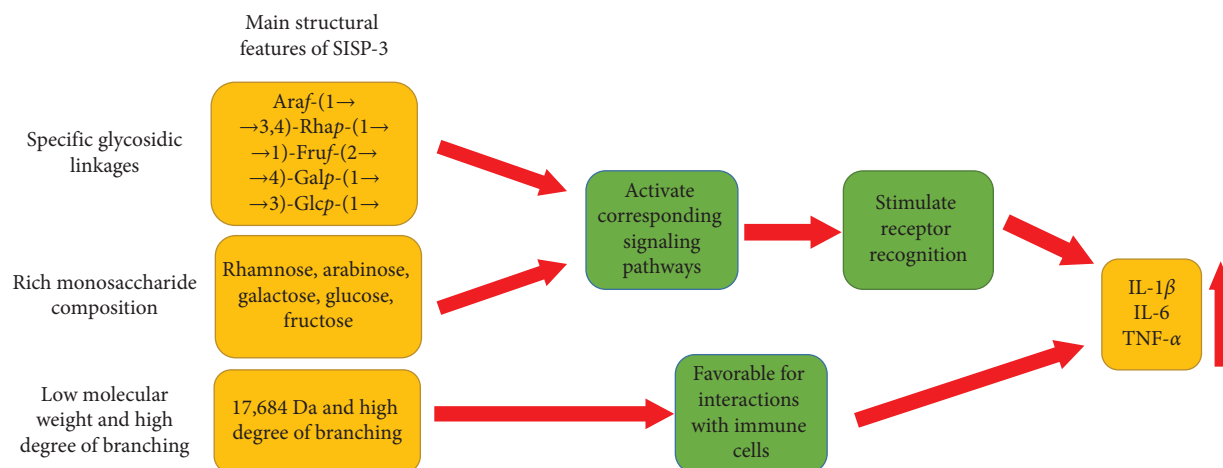


FIGURE 8: The hypothesized relationship between the structure and activity.

require further investigation. Future studies should focus on detailed mechanistic insights, including the identification of key structural motifs responsible for receptor binding and activation, along with *in vivo* studies to confirm the immunomodulatory effects observed *in vitro*. A deeper understanding of these mechanisms in greater detail will not only elucidate the biological functions of SISP-3 but also aid in the rational design of polysaccharide-based therapeutics with optimized efficacy and safety profiles.

5. Conclusion

This was the new report in which chemical characterization and immunoregulatory activities of polysaccharides from the Sacha inchi shell. A neutral polysaccharide fraction SISP-3 with a triple-helix structure was isolated with an Mw of 17,684 Da. The results indicate that SISP-3 contains Rha, Ara, Gal, Glc, and Fru in a molar ratio of 7.2%:41.7%:40.3%:4.1%:6.7%. The main glycosidic linkage in SISP-3 is suggested to be $\rightarrow 4$ -Galp-(1 $\rightarrow$), and it exhibits a complex branching pattern. Notably, SISP-3 demonstrates significant immunomodulatory activities in RAW264.7 cells with promoting cell viability, enhancing phagocytic capacity, elevating NO levels, and stimulating the secretion of IL-6, IL-1 β , and TNF- α . This study provides valuable insights into the relationship between polysaccharides and immunomodulatory activity and offers a theoretical foundation for the development of novel immunomodulators.

Data Availability Statement

Data will be made available on request.

Conflicts of Interest

The authors declare no conflicts of interest.

Author Contributions

Junyuan Huang: conceptualization, data analysis, and drafting of the manuscript. Yanlan Chen: conceptualization,

drafting of the manuscript, and supervision. Kun Wang: methodology and data curation. Changan Chen: methodology. Yang Yang: methodology. Zebin Zou: methodology. Yanli Li: methodology. Bing Du: investigation and conceptualization. Danfeng Tang: reviewing and editing, and funding acquisition. Pan Li: supervision, validation, conceptualization, reviewing and editing, and funding acquisition.

Funding

This work was financially supported by the project of the National Natural Science Foundation of China (Grant No. 32202058) and Independent Research Project of Guangxi Key Laboratory of Medicinal Resources Protection and Genetic Improvement (KL2023KF03).

Acknowledgments

This work was financially supported by the project of the National Natural Science Foundation of China (Grant No. 32202058) and the Independent Research Project of Guangxi Key Laboratory of Medicinal Resources Protection and Genetic Improvement (KL2023KF03).

Supporting Information

Additional supporting information can be found online in the Supporting Information section. (*Supporting Information*)

The methylation analysis of the polysaccharide from the Sacha inchi (*P. volubilis* L.) shell, along with the MS data, is described in the supporting information. Figure S1: GC chromatogram of alditol acetate derivatives from the methylated product of SISP-3. Figure S2: Mass spectra of methylated derivatives of the SISP-3. Table S1: The methylation preliminary results of SISP-3.

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