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Evaluation of an Antioxidative Polysaccharide Fraction from *Russula pseudocyanoxantha*, a Novel Mushroom, as a Strategy to Enhance Innate Immunity

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Abstract: With a long history of appreciation as a health-beneficial food, our team has recently discovered *Russula pseudocyanoxantha*, which displays the presence of water-soluble bioactive polysaccharides. As part of our continuing interest in searching compounds from traditional yet unexplored food, the present research was conducted to harness the residual components by subjecting them to a hot alkali solution, yielding a bioactive rich fraction (RP-HAP). Chemical analysis unveiled that this β-glucan-enriched extract consisted of a homogeneous polymer with M*w* of ~111.25 kDa. RP-HAP exhibited significant antioxidant properties, manifesting through multiple mechanisms such as inhibition of radical generation, chelating abilities, and electron donation capacity, where EC_{50} values ranged from 1193 to 1683 µg/mL. Moreover, RP-HAP demonstrated its potential by promoting various immune responses in RAW264.7 macrophage cells within 24 h, including cell proliferation, phagocytosis, pseudopod formation, oxidative burst, and NO synthesis. This heightened cellular activity was supported by increased expressions of key immune-related genes, such as TLR-2, TLR-4, NF-κB, iNOS, TNF-α, COX-2, IFN-γ, and Iκ-Bα underlining robust immune-boosting properties of the fraction mediated through TLR/NF-κB pathway. Thus, further exploration of RP-HAP may hold promise for the development of potent pharmaceuticals that will contribute to a healthier future for humanity.

Keywords: chromatography; β-glucan; free radical scavenging; pro-inflammatory response; RAW264.7; tribal food.

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1. Introduction

West Bengal, a geographically unique state in India, is inhabited by numerous tribal communities that rely on forest products for survival. Among these resources, wild mushrooms play a crucial role in providing food security and offering health benefits to these indigenous populations [1]. One such unique macrofungal species has recently been discovered during recent field expeditions across lateritic regions of the state. An in-depth investigation revealed that the collected specimen was a complete discovery to the world of science, and we named it *Russula pseudocyanoxantha*. Our study also highlighted that this novel Basidiomycetes contained valuable bioactive polysaccharides successfully extracted through the hot water process [2]. However, it was evident that the remaining residues still held a wealth of essential

macromolecules, presenting significant potential for various industrial applications. The re-utilization of these waste materials could lead to the recovery of therapeutic substances, promoting the growth of the circular economy and ensuring sustainability within the nutraceutical sector [3].

In this context, the alkaline extraction technique proves to be highly efficient in maximizing polysaccharide yields from mushrooms. This method effectively disrupts cell walls and protein-glycan bonds, releasing intracellular macromolecules. Moreover, it transforms previously water-insoluble substances into soluble forms, significantly enhancing recovery rates [4]. This straightforward and cost-effective process has demonstrated its potential in isolating polysaccharides rich in β-glucans, as exemplified in studies with *Pleurotus eryngii* [5] and *Fomitopsis betulina* [6]. β-glucans, a vital component in macrofungi, have garnered global attention due to their diverse biological activities. Extensive research has shown their remarkable antioxidant properties, which are capable of protecting cells from damage caused by free radicals (unstable molecules produced during normal metabolism) [7]. Consequently, these antioxidant compounds have the potential to reduce the risk of various disorders, including diabetes, aging, hepatic conditions, cancer, cataracts, cardiovascular diseases, and neurodegenerative ailments [3]. Beyond their radical-quenching abilities, β-glucans are also highly valued as "biological response modifiers" [4]. These biopolymers can directly activate macrophages, essential immune system components responsible for pathogen engulfment, inflammation promotion, and immune response initiation. Ongoing research endeavors continuously uncover new polysaccharides with immune-modulatory properties, offering potential applications in developing functional foods, nutraceuticals, and pharmaceuticals to enhance immune health [8].

Considering this background, the present study was dedicated to repurposing the residue that has undergone a series of extraction methods to isolate therapeutic macromolecules from this traditional yet overlooked food source. These polymers were further scrutinized for their physico-chemical and functional properties, with the aim of expanding practical applications of *R. pseudocyanoxantha*.

2. Materials and Methods

2.1. Mushroom collection and preliminary treatment.

Fruiting bodies of *R. pseudocyanoxantha* (accession no. CUH AM652) were harvested from their natural habitat in West Bengal. To identify these collected basidiomes, we conducted a comprehensive analysis that included morphological examination and DNA barcoding, a methodology detailed in our previous publication [2].

2.2. Polysaccharide extraction.

We employed a series of steps to isolate the polysaccharides from the powdered fruit bodies. Firstly, low molecular weight (M*w*) components were removed by immersing the powder in ethanol overnight. The solution was then filtered, and the remaining residue was soaked in boiled distilled water. After filtration of the suspension, the residue was treated with 10% NaOH solution. Following 24 h incubation at 4°C, the residue was separated and exposed to 10% w/v NaOH solution at 65°C for one h. The resulting remnants were discarded after filtration, and the solution was neutralized using glacial acetic acid. Four volumes of absolute ethanol were added to the solvent to isolate the polysaccharides further and incubated overnight

at low temperatures. The polysaccharides were then separated by centrifugation (11,000 rpm for 10 min at 4°C) and repeatedly dissolved in water. Subsequently, precipitation, centrifugation, and washing steps were carried out to isolate the water-soluble fraction of hot alkali-extracted polysaccharides from *R. pseudocyanoxantha*, designated as RP-HAP [9]. The obtained polysaccharides were stored in amber containers under cold and dry conditions till further analysis.

2.3. Determination of architecture.

To assess the total sugar content, the phenol sulfuric acid method was conducted, where 100 µL of the extract solution (10 mg/mL) was combined with 900 µL of distilled water, 500 µL of 6% phenol, and 2.5 mL of sulfuric acid. After a 30-minute incubation period, the absorbance was measured at 490 nm. Protein content in the fraction was determined using Bradford reagent (750 µL). After 5-min incubation, the absorbance was recorded at 595 nm. Additionally, the Mushroom and Yeast β-Glucan Assay Kit (Megazyme Institute in Wicklow, Ireland) was employed following the provided manual to calculate the quantities of total glucan, β-glucan, and α-glucan. The molecular composition of RP-HAP was assessed using high-performance thin-layer chromatography (HPTLC) and gas chromatography-mass spectrophotometry (GC-MS) methods, as previously described in our publication [10]. To determine the homogeneity of the fraction and M*w* of the existing polymer, gel-permeation chromatography (GPC) was performed using a Seralose 6B column (1.6 cm×60 cm) with water as the eluent and dextrans (110, 70, and 40 kDa) as standards, as detailed in our previous works [8, 11]. Moreover, the polymers and KBr were pressed into pellets and analyzed using Fourier transform infrared (FT-IR) spectrophotometer within the frequency range of 400–4000 cm^{-1}. Lastly, an analysis of the helical structure of the carbohydrate backbone was conducted through characterization of the Congo red-polysaccharide reaction [12].

2.4. Estimation of antioxidant potential.

The ability of RP-HAP to chelate ferrous ions was assessed using 5 µL ferrous chloride (3 nM) and 10 µL ferrozine (0.12 nM) in a 200 µL reaction mixture. After 10 min incubation, absorbance was estimated at 595 nm using a microplate reader (Bio-Rad iMark™ Microplate Reader, USA), and the results were compared with ethylenediaminetetraacetic acid (EDTA). For the estimation of 2,2-diphenyl-1-picryl-hydrazyl-hydrate radical (DPPH•) scavenging activity, 4% DPPH radical solution was mixed with the extract in a microtiter plate, making the reaction mixture up to 200 µL. After 30-min incubation, the final color was measured at 595 nm. To understand the effect of RP-HAP on superoxide radicals ($O_2^{-\bullet}$), methionine, EDTA, nitroblue tetrazolium, and riboflavin were added to the polysaccharides. The test tubes were exposed to light for 10 min, and finally, absorbance was recorded at 595 nm. Furthermore, we evaluated the potential against 2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical using RP-HAP at different levels, and the absorbance was detected at 750 nm. Hydroxyl radical (OH•) quenching ability was determined by adding KH_2PO_4-KOH buffer, RP-HAP, 2-deoxy-D-ribose, vitamin C, $FeCl_3$, EDTA, and H_2O_2. The tubes were then incubated at 37°C for one hour at room temperature. Following this, two mL thiobarbituric acid (TBA)-trichloroacetic acid (TCA) mixed solution was added to each reaction, which was then subjected to 100°C for 15 min, and the absorbance was measured at 535 nm. Different RP-HAP concentrations were mixed in a 96-well plate with 0.2 M Na-phosphate buffer and

$K_3[Fe(CN)_6]$ to assess the reducing power. After 20 min, TCA was added to the mixture, followed by the addition of $FeCl_3$. At the end of the experiment, absorbance at 750 nm was noted. Additionally, total antioxidant capacity was assessed, and the outcome was compared with a standard ascorbic acid [13].

2.5. Determination of immune-stimulatory activity.

RAW264.7 murine macrophages were cultured in our laboratory following established protocols. We investigated the impact of RP-HAP on macrophage viability by co-incubating cells with varying polymer concentrations (50, 100, and 200 µg/mL) for 24 and 48 h. Subsequently, 20 µL of water-soluble tetrazolium (WST) reagent was added to each well, and a microplate reader was used to measure absorbance at 450 nm. Cells were exposed to the polysaccharide for different time intervals to evaluate the effect of RP-HAP on phagocytic activity. After the incubation period, the reaction mixture was discarded, and 100 µL of media containing 0.07% (w/v) neutral red was added. After one h, the cells were washed with phosphate-buffered saline (PBS) twice, and 100 µL of cell lysate solution (comprising ethanol and 0.01% acetic acid at a 1:1 ratio) was added into each well to lyse cells. The plate was then incubated for two hours, and optical density was measured at 575 nm using a microplate reader [14]. For the assessment of nitric oxide (NO) production after one day of treatment, Griess reagent (1% sulfanilamide, 0.1% naphthyl ethylenediamine dihydrochloride, and 5% H_3PO_4) was used. The mixture was left for approximately 10 min, and the absorbance intensity was recorded at 545 nm for treated and untreated monocytes. To observe the cellular morphology of both stimulated and unstimulated macrophages following 24 h of incubation, 4',6-diamidino-2-phenylindole (DAPI) solution (1 µg/mL in PBS) was used, and images were captured using a fluorescent microscope (FLoid Cell Imaging Station, Life Technologies, India) [10, 12]. Further, the effect of RP-HAP on the production of intracellular reactive oxygen species (ROS) was assessed using 2´,7´-dichlorofluorescin diacetate (DCFDA) dye (5 µM) with the assistance of flow cytometry (BD Bioscience, USA). Finally, the impact on gene expression was determined using primers for Toll-like receptor (TLR)-4, TLR-2, nuclear factor (NF)-κB, Iκ-Bα, cyclooxygenase (COX)-2, inducible nitric oxide synthase (iNOS), tumor necrosis factor (TNF)-α, and interferon (IFN)-γ genes, where β-actin served as the control. The polymerase chain reaction (PCR) cycle conditions were as follows: 95ºC for 4 min, followed by 35 cycles of 94ºC for 20 sec, annealing temperature (Tm) specific to each primer for 30 sec, and 72ºC for 45 sec, with a final extension step of 7 min at 72ºC [14].

2.6. Statistical analysis.

The findings established herein are conveyed as mean±SD of three independent experiments. The analysis was procured with Student's *t*-test by $p<0.05$ as the minimal level of significance using IBM SPSS Statistics, v. 23.0. (IBM Corp., Armonk, New York, United States).

3. Results and Discussion

3.1. Physico-chemical characterization.

In this study phase, the challenge was to extract crude polysaccharides from thoroughly processed residues after a series of extraction steps. The technique ultimately led to isolating a

highly water-soluble polysaccharide fraction, denoted as RP-HAP, with a brown color and substantial recovery percentage (Table 1). Notably, the yield was comparatively higher than the alkali-extracted fraction of *Fomitopsis betulina* [6].

Table 1. Extractive yield and chemical characterization of RP-HAP.

Parameters	RP-HAP
Yield (%)	10.42 ± 1.45
Carbohydrate (g per 100 g of dry polysaccharide)	54.76 ± 4.62
Protein (g per 100 g of dry polysaccharide)	11.54 ± 0.31
Total glucan (g per 100 g of dry polysaccharide)	42.46 ± 2.88
α-Glucan (g per 100 g of dry polysaccharide)	0.43 ± 0.07
β-Glucan (g per 100 g of dry polysaccharide)	42.03 ± 2.81
Monosaccharide composition	Xylose: mannose: glucose: galactose = 15.9: 16.6: 53.1: 14.4
Homogeneity and molecular weight	Homogenous, ~111.25 kDa

Several biochemical assays were conducted to discern the molecular composition of the isolated extract. At first, the total carbohydrate content was determined, i.e., 54.76 ± 4.62 g per 100 g of dry polysaccharide, indicating it as the primary component in RP-HAP. A notable amount of protein was also detected. Moreover, the total amount of glucan was quantified as well, i.e., 42.46 ± 2.88 g per 100 g of dry polysaccharide. Among the glucan types, β-glucan content was found to be 42.03 ± 2.81 g per 100 g of dry polysaccharide, suggesting that the carbohydrates in RP-HAP primarily consisted of glucans linked by β-glycosidic bonds. The β-glucan content in RP-HAP was found to be superior to the hot water-extracted fractions from *Agaricus bisporus*, *Pleurotus ostreatus*, and *Coprinus atramentarius* [15].

To verify the spectroscopic data, we employed chromatography-based fingerprinting techniques to elucidate the fine architecture of the polymers. As shown in Figure 1a, the HPTLC chromatogram results corroborated previous findings by displaying a prominent band of glucose, along with bands of three additional monomers: galactose, mannose, and xylose, indicating a hetero-polysaccharide nature. GC-MS was also performed to validate these findings, where the preparation from *R. pseudocyanoxantha* displayed four peaks, with glucose as the predominant element (Figure 1b). The homogeneity of the fraction was further confirmed through GPC. As illustrated in Figure 1c, the elution fingerprint revealed a single, prominent peak at tube number 20, indicating the presence of a single polymer. Utilizing a standard curve, M*w* was calculated to be approximately ~111.25 kDa, slightly lower than the previous observations [3, 11].

Additionally, FT-IR spectroscopy was performed within the wavenumber range of 4000 to 400 cm^{-1} to analyze molecular vibrations of covalent bonds. The spectra of RP-HAP (Figure 1d) exhibited an extremely broadband with maximum absorption (minimum transmittance) at around 3439 cm^{-1}. This could be attributed to the normal vibrational modes of asymmetric and symmetric stretching of hydroxyl groups, which are present in significant numbers in polysaccharides [16]. A weak absorption peak at 2925 cm^{-1} corresponded to the vibrational types of asymmetric and symmetric stretches of C–H groups [17]. The strong signal at 1637 cm^{-1} was due to the stretching of C–N and N–H protein groups, indicating amide linkages consistent with the chemical analysis. The band at 1076 cm^{-1} suggested a pyranose form of glucosyl residue. Finally, a peak at 776 cm^{-1} indicated the presence of β-glycosidic anomeric bonds specific to β-glucan [2]. In conclusion, the FT-IR data indicated that RP-HAP displayed characteristic wavenumbers typical of carbohydrates containing β-glucan and proteins.

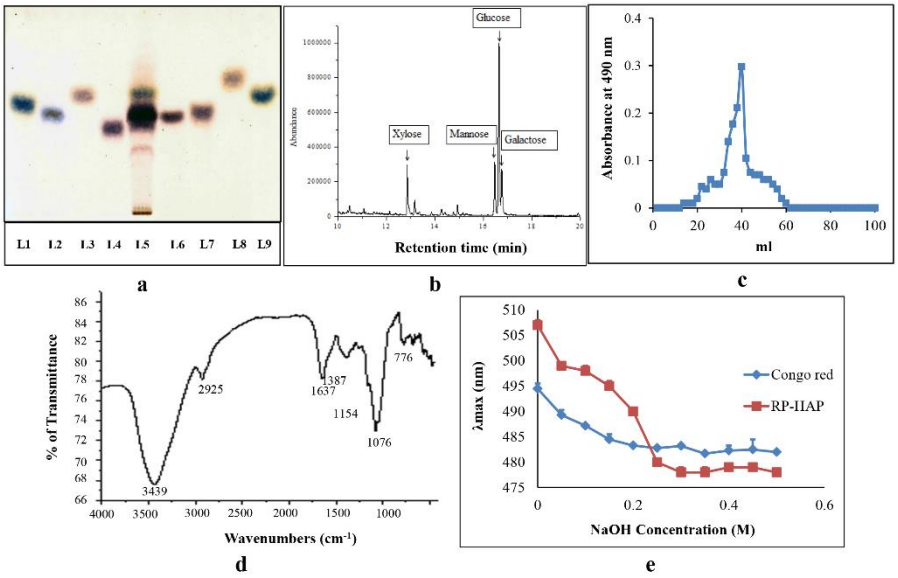

Figure 1. Enumeration of structural features of RP-HAP. (**a**) Identification of monomers by HPTLC (lanes are as follows: 1, L-arabinose; 2, D-fructose; 3, D-fucose; 4, D-galactose; 5, RP-HAP; 6, D-glucose; 7, D-mannose; 8, D-rhamnose; and 9, D-xylose); (**b**) GC-MS; (**c**) Estimation of homogeneity and molecular weight; (**d**) FT-IR chromatogram; (**e**) Changes in λmax of Congo red and RP-HAP complex at various concentrated NaOH solutions.

A well-established Congo red binding assay was performed to determine the helical conformation of the carbohydrate backbone. As shown in Figure 1e, the interaction of RP-HAP with the dye did not exhibit any increase in absorption maxima. Instead, the λmax of the mixtures gradually decreased with the increment of NaOH strength from 0 to 0.5 M. The absence of a red shift phenomenon suggested a lack of a triple helical conformation in the polysaccharides. This could be attributed to using an alkali solution during extraction, known to disrupt hydrogen bonds necessary for the three-dimensional triple helical structure [18]. To date, many mushroom-derived polysaccharides have been found not to contain any ordered structure but have displayed strong antioxidant and immune-enhancing potential [19].

3.2. Estimation of antioxidant potential.

Several assays were conducted based on different principles to evaluate the antioxidative properties of RP-HAP. First, the chelating assay was performed to assess the ability of RP-HAP to bind ferrous ions, as Fe^{2+} can lead to the generation of free radicals, and metal chelation is effective in reducing oxidative stress. As depicted in Figure 2a, RP-HAP exhibited strong binding affinity to Fe^{2+} in a dose-dependent manner. At 100, 500, and 1000 µg/mL concentrations, RP-HAP demonstrated iron binding capabilities of 9.97%, 32.3%, and 57.6%, respectively, indicating its moderate potency. Furthermore, a DPPH$^{\bullet}$ scavenging assay was conducted to assess the impact of RP-HAP on free radicals. The results revealed potent antioxidative effects, with the scavenging ability of 21%, 39.2%, and 55.1% in the presence of 100, 1000, and 2000 µg/mL dosages, respectively (Figure 2b). The radical scavenging effect of RP-HAP was more pronounced in the case of $O_2^{\bullet-}$ radicals. As presented in Figure 2c, the fraction exhibited 14.1% and 45.8% inhibition of radical generation at 10 and 50 µg/mL levels, respectively. When the concentration was increased to 100 µg/mL, a 65.2% radical scavenging effect was recorded. Similarly, RP-HAP displayed a strong capacity for $ABTS^{\bullet+}$ radical scavenging. The fraction quenched 38.89%, 86.56%, and 94.21% of radicals at 100, 500, and 1000 µg/mL concentrations, respectively (Figure 2d). However, the antioxidative effect was

less pronounced in the case of OH• scavenging, as shown in Figure 2e, where RP-HAP reduced radical generation by 27.1%, 40.8%, and 64% at the levels of 100, 500, and 1000 µg/mL, respectively. Nevertheless, RP-HAP exhibited significant electron-donation ability, as evidenced by the reducing power assay. The fraction demonstrated a reducing power of 0.19, 0.4, and 0.77 at dosages of 500, 1000, and 2000 µg/mL, respectively (Figure 2f). We also conducted a total antioxidant activity assay, indicating that the polysaccharide possessed moderate potential (Table 2).

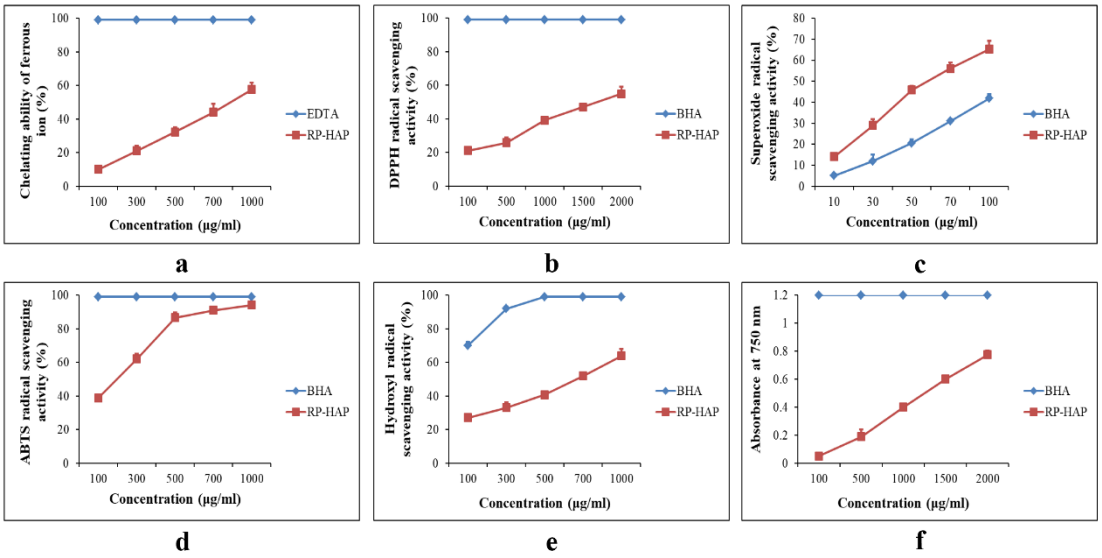

Figure 2. The antioxidant activity of RP-HAP was determined as follows: **(a)** Chelating ability of ferrous ion; **(b)** DPPH; **(c)** Superoxide; **(d)** ABTS; **(e)** Hydroxyl radical scavenging; **(f)** Reducing power activities.

Comparative analysis with previous studies revealed that RP-HAP displayed superior antioxidant activities compared to *A. bisporus*, *P. ostreatus*, *C. atramentarius* [15], and *Russula senecis* [19]. Researchers have suggested that the bioactivity of such polymers depends on a combination of their physico-chemical characteristics. Specific monosaccharide composition, presence of various substituents, and functional groups, such as hydroxyl groups, can influence the antioxidant activity of mushroom-derived macromolecules by donating electrons or hydrogen atoms, scavenging free radicals, or chelating metal ions involved in oxidative reactions [20]. Therefore, it can be inferred that the molecular composition of the polymers present in RP-HAP likely played a significant role in its potent antioxidant activity.

Table 2. Antioxidant activity of RP-HAP. Butylated hydroxyanisole (BHA) was considered a standard in radical scavenging and reducing power assays, while EDTA was used in the chelating ability of the ferrous ion method for reference.

Antioxidant parameters		**RP-HAP**	**Standards**
EC_{50} values (µg/mL)	Chelating ability of ferrous ion	849 ± 7^{a}	2.51 ± 0.43^{b}
	DPPH radical scavenging	1683 ± 29^{a}	2.17 ± 0.3^{b}
	Superoxide radical scavenging	635 ± 23^{a}	262 ± 5^{b}
	ABTS radical scavenging	193 ± 4.6^{a}	3.2 ± 0.08^{b}
	Hydroxyl radical scavenging	698 ± 35^{a}	68 ± 2^{b}
	Reducing power	1250 ± 49^{a}	14.8 ± 0.9^{b}
Total antioxidant activity (µg ascorbic acid equivalent per mg of dry extract)		1.45 ± 0.39	Not applicable

In each row, dissimilar letters designate significant alterations between RP-HAP and standard ($p < 0.05$).

3.3. Estimation of immune-modulatory activity.

As previously mentioned, macrophages are integral to our immune defense, and their increased growth and proliferation serve as markers of immune modulation [21]. To assess the impact of RP-HAP on RAW264.7 cells and determine its cytotoxicity, we incubated the cells with various doses of the preparation. After 24 h of co-treatment, the monocytes did not exhibit significant proliferation. This observation aligns with findings described by Zhang *et al*., where pure polysaccharides extracted from *Tricholoma lobayense* did not alter macrophage viability within the tested concentration range of 25–400 µg/mL over 24 h [22]. However, after 48 h of exposure to RP-HAP, an increase in cell division was observed (Figure 3a). Notably, RP-HAP at 50, 100, and 200 µg/mL concentrations enhanced proliferation by 246.17%, 377.73%, and 352.84%, respectively, compared to the negative control. In fact, at a dose of 100 µg/mL, RP-HAP exhibited the highest potency, surpassing even lipopolysaccharide (LPS).

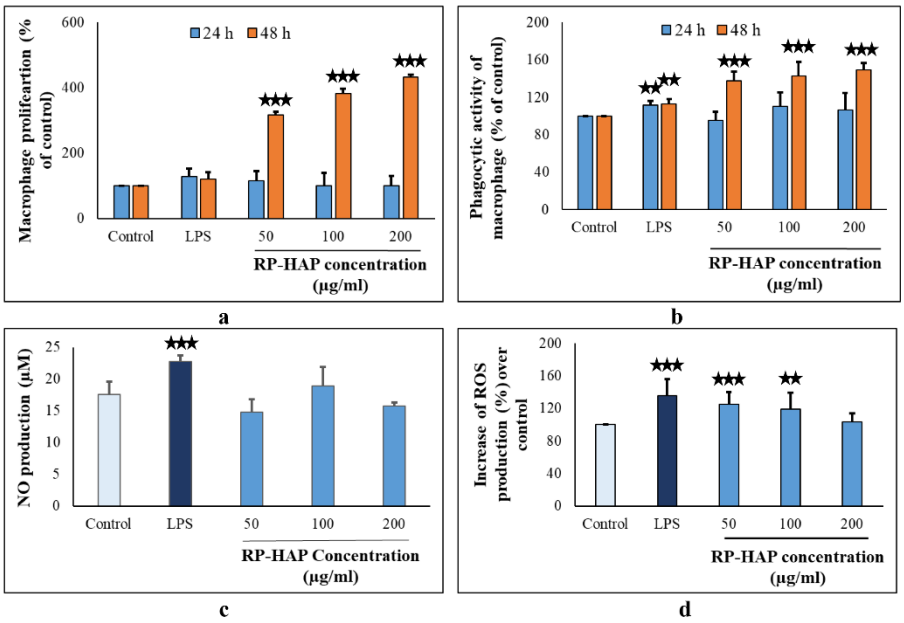

Figure 3. The influence of RP-HAP on (**a**) proliferation; (**b**) phagocytic uptake; (**c**) NO production; and (**d**) intracellular ROS synthesis by RAW264.7 cells was monitored after treatment of polysaccharides at different concentrations. In all assays, 5 µg/mL of LPS was used as a positive control. **p*< 0.05, ***p*< 0.01 and ****p*< 0.001 (unpaired *t* test).

Based on the results, we further explored the impact of RP-HAP on other macrophage functions, such as phagocytosis, the process by which immune cells engulf and digest foreign particles like bacteria or cellular debris. The neutral red method was conducted to assess this, and the results are presented in Figure 3b. Similar to the proliferation assay results, RP-HAP treatment did not significantly enhance the phagocytic capabilities of macrophages compared to untreated cells. However, when the co-incubation period was extended by another 24 h, the phagocytosis index increased in a dose-dependent manner. Specifically, an increase of 137.55%, 142.96%, and 149.24% in activity over the negative control was observed in the presence of 50, 100, and 200 µg/mL of RP-HAP, respectively. Comparative analysis with previous literature suggests that RP-HAP can enhance phagocytosis more effectively than the pure polysaccharides extracted from *Morchella importuna* [23].

Activated macrophages synthesize essential inflammatory mediators such as NO and ROS, contributing to engulfed pathogens' destruction. As depicted in Figure 3c, untreated cells released minimal amounts of NO, while LPS-stimulated cells significantly increased NO

production. In the presence of RP-HAP, there was a moderate enhancement of NO release in the culture medium, with the most significant effect observed at the concentration of 100 µg/mL. As expected, co-incubation of RP-HAP with RAW264.7 cells increased ROS production compared to un-stimulated cells, although the positive control yielded better results (Figure 3d). Ghosh *et al*. illustrated the immune-enhancing effect of *Macrocybe lobayensis*, where the water-soluble polysaccharide fraction exhibited more potential than RP-HAP in terms of NO and ROS production [12].

Previous studies have demonstrated that RAW264.7 cells undergo morphological changes after being stimulated by an activator [14]. To visualize such alterations, treated and untreated monocytes were observed under a microscope after 24 h of incubation. In the negative control set, RAW264.7 cells displayed typical features such as small size, minimal spreading, and few cell extensions from the surface (Figure 4). In contrast, the presence of RP-HAP at all tested concentrations altered the morphology, resulting in visibly larger cells that adhered more tightly to the culture dish and displayed irregular shapes. Additionally, microvilli-like structures were formed, indicating an enhanced cellular capacity to engulf and eliminate pathogens efficiently. A similar trend has also been reported when polysaccharides from *Pleurotus nebrodensis* [24] and *Craterellus cornucopioides* [25] were introduced to RAW264.7 cells.

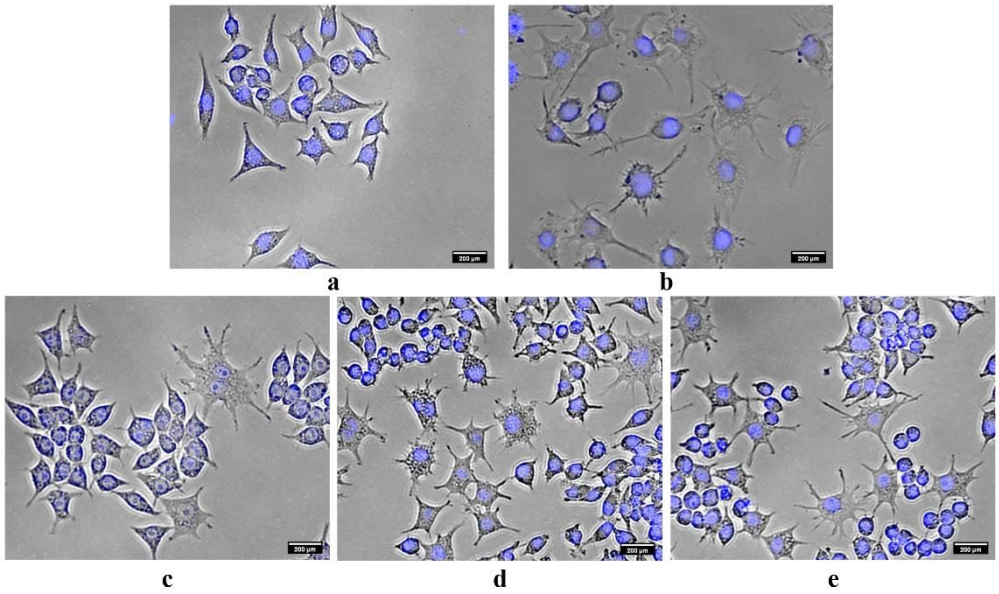

Figure 4. The effect of RP-HAP on the morphology of murine macrophages was visualized under a fluorescence microscope. (**a**) Negative control; (**b**) LPS (5 µg/mL); RP-HAP at the concentrations of (**c**) 50 µg/mL; (**d**) 100 µg/mL; (**e**) 200 µg/mL. Scale 50 µM.

Activated macrophages secrete various immune factors and cytokines that enhance the overall immune response of monocytes [14]. RT-PCR was performed using gene-specific primers to investigate the effect of RP-HAP on the synthesis of these molecules and the underlying mechanism. The results indicated that RP-HAP, at all tested doses, significantly upregulated the mRNA expression of immune-related genes (Figure 5). As expected, the intensities of all examined genes were markedly increased in the presence of 50, 100, and 200 µg/mL of each fraction compared to the negative control. Specifically, RP-HAP at the 100 µg/mL dose induced a 6637% increase in COX-2 expression, a 490% increase in iNOS, a 4941% increase in TNF-α, and a 244% increase in IFN-γ over the control. In most cases, the effect of RP-HAP at the level of 100 µg/mL was found to be better than that of LPS. These findings suggest that TLR4 and TLR2 recognition of the hot alkali-extracted crude

polysaccharide from the studied mushroom stimulated the expression of transcription factors, subsequently promoting the release of cytokines, indicating the potential use of RP-HAP as an immune-stimulatory agent in the future.

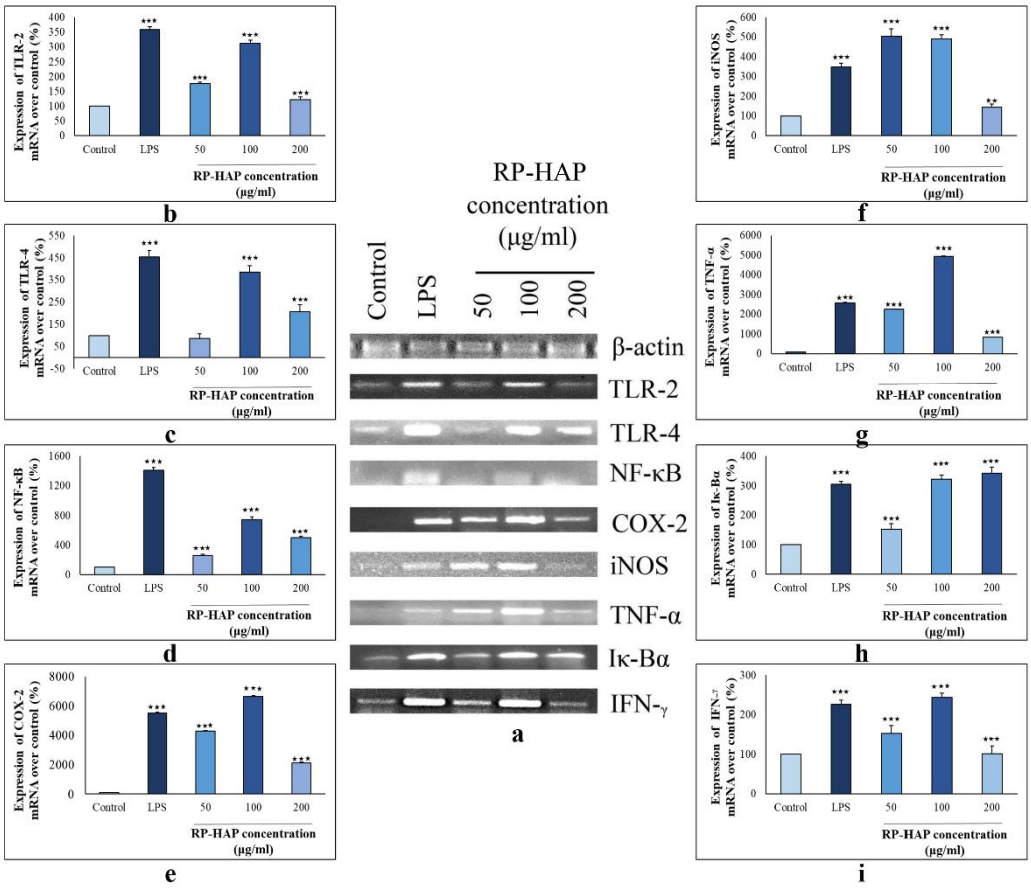

Figure 5. To explore the molecular mechanisms behind the immune-enhancing effects of RP-HAP, the following experimental procedure was employed: (**a**) Macrophage cells were incubated for 24 h with either LPS or RP-HAP, with a set of untreated cells serving as controls. Total RNA was then extracted from these cells, followed by the synthesis of cDNA from the isolated RNA samples. Semi-quantitative reverse transcription PCR was conducted to evaluate the expression levels of eight (**b**–**i**), specific genes: TLR-2, TLR-4, NF-κB, COX-2, iNOS, TNF-α, Iκ-Bα, and IFN-γ, using β-actin as a housekeeping gene. The PCR band intensities were measured using ImageJ software to quantify the relative increase in transcription levels of the target genes, expressed as a percentage relative to the control group. Statistical significance was determined using an unpaired t-test, with significance levels indicated as $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

To date, numerous studies have reported the immune-boosting effects of edible mushrooms. For example, treatment with water-extractable polysaccharides from *Macrolepiota procera* resulted in increased NO and IL-6 production by RAW264.7 cells [26]. Chen *et al*. described that co-incubation of purified polysaccharides from *Russula griseocarnosa* with murine macrophages could trigger the production of NO, IL-6, iNOS, COX-2, and TNF-α mediated through NF-κB and mitogen-activated protein kinase (MAPK) signaling pathways [27]. Shi *et al*. purified a glucan (M*w* 1670 kDa) from fruiting bodies of *Pleurotus citrinopileatus* and reported an activating effect evident by the NF-κB signaling pathway [28]. In another study, a novel water-soluble heteropolysaccharide was purified using *Ganoderma leucocontextum* fruiting bodies. Investigations showed that the polymer possessed significant immunostimulatory effects mediated through MAPK and NF-κB pathways [29]. Such unique bioactivity largely depends on the chemical structure of biopolymers. Chen *et al*. suggested that polysaccharides' immunomodulatory capacity mainly depends on the main-chain structure, branching pattern, M*w*, helical conformation, and orientation of hydrophilic

groups [30]. Most fungal polysaccharides with M*w* ranging from 104 to 106 Da have shown potent immune-modulatory effects [31]. Therefore, it can be inferred that the presence of water-soluble β-glucans with high M*w* in the studied extract likely played a key role in its strong immune-modulatory effect.

4. Conclusions

In summary, the extraction method employed in this study facilitated the isolation of a polysaccharide-rich fraction from *R. pseudocyanoxantha* that is extractable with hot alkali. Our *in vitro* assays revealed that this extract, RP-HAP, exhibits robust antioxidant properties, with EC_{50} values ranking in the following order: DPPH radical scavenging< reducing power< chelating ability of ferrous ions< inhibition of hydroxyl radical< superoxide radical scavenging< ABTS radical quenching. Additionally, RP-HAP demonstrated potent immune-boosting effects, particularly at a concentration of 100 µg/mL, by promoting the proliferation of RAW264.7 cells, enhancing phagocytosis, and increasing the secretion of pro-inflammatory cytokines through activation of TLRs and NF-κB pathway. The research thus suggests that RP-HAP has the potential to enhance the immune system's ability to defend against pathogens and maintain overall immune health. These multifaceted bioactivities are likely attributed to the presence of a homogeneous polymer composed of various monosaccharides, with β-glucan being the predominant component. Altogether, these findings indicate that RP-HAP could be considered for future use as an antioxidative agent or adjuvant in immunological therapies, offering potential health benefits.

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Conflicts of Interest

The authors declare no conflict of interest.

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