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Pharmacological Properties of the Polysaccharides Isolated from the Medicinal Mushroom *Phellinus rimosus* (Berk) Pilat

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Abstract

Objectives: *Phellinus rimosus* is a less-explored wood-decaying macrofungus growing exclusively on jackfruit tree trunks in Kerala, India. This investigation focused on the antioxidant, anti-inflammatory, and antidiabetic potential of polysaccharides from this mushroom. **Methods:** Polysaccharides were isolated from the mushroom by hot water extraction and ethanol precipitation. TLC, HPLC, and Sephadex G-100 chromatography were employed for characterization and determination of molecular weight. Total Antioxidant Capacity and Total Reducing Power assays evaluated the antioxidant potential. Inhibition of inflammatory mediators such as Cyclooxygenase, Lipoxygenase, and Inducible Nitric Oxide Synthase was used to study anti-inflammatory potential. Antidiabetic activity was measured through yeast glucose uptake and alpha-amylase inhibition assays. **Findings:** We isolated and characterized a high molecular weight (120,000 Da) Polysaccharide-protein complex (PPC-*Pr*) from *P. rimosus*. TLC analysis showed glucose as the only monomeric unit, indicating it to be a glucan. HPLC analysis revealed aspartic acid, glutamic acid, glycine, alanine, and serine as the major amino acids in PPC-*Pr*. In both antioxidant assays, the compound showed significant dose-dependent activity. Previous reports have revealed the profound ability of PPC-*Pr* to act as free radical scavengers. This study showed the ability of PPC-*Pr* as a reducing agent to neutralize the free radicals in the in vitro system. In murine macrophage RAW 264.7 cells, it significantly inhibited Cyclooxygenase (51.82%), Lipoxygenase (52.30%), and Inducible Nitric Oxide Synthase (48.78%) production at a dose of 100 μg/ml. PPC-*Pr* has been reported to possess significant anti-inflammatory activity in animal models. This investigation supports the previous research through the in vitro anti-inflammatory activity of the compound. While PPC-*Pr* did not enhance glucose uptake, it exhibited 61.57% alpha-amylase inhibition at 1000 μg/ml. **Novelty:** This study revealed that PPC-*Pr* possesses significant antioxidant, anti-inflammatory, and antidiabetic potential in a dose-related manner. The antioxidant activity of PPC-*Pr* might be responsible for its profound anti-inflammatory and antidiabetic effects.

Keywords: *Phellinus*; Mushrooms; Polysaccharides; Anti-oxidant; Anti-inflammatory

1 Introduction

Medicinal mushrooms have been used for centuries in traditional Asian therapies. Historically, mushroom hot water decoctions and essences were commonly used as medicines in the Far East, where mushroom-based treatments originated. Since ancient times, the anticancer properties of medicinal mushrooms have been recognized and incorporated into therapies in countries like Korea, China, Japan, Russia, the USA, and Canada. Macrofungi such as *Ganoderma lucidum*, *Lentinus edodes*, *Fomes fomentarius*, and *Fomitopsis officinalis* have been employed around the world to treat various ailments**.** Mushrooms are highly valued for their nutritional and pharmaceutical potential, being rich in proteins, amino acids, polyunsaturated fats, polysaccharides, dietary fibers, vitamins, minerals, and bioactive compounds such as terpenoids, flavonoids, and polyphenols. Mushrooms are cholesterol-free, low in calories, and offer numerous health benefits, including antioxidant, antimicrobial, antiparasitic, anti-inflammatory, antitumor, anticancer, cytotoxic, antiproliferative, anticoagulant, anti-HIV, hypocholesterolemic, antidiabetic, and hepatoprotective properties[(1),(2)]. Mushroom metabolites are a safe and effective treatment for various disease conditions and are often used without the typical Phase I/II/III clinical trials used for conventional medicines.

Mushroom polysaccharides (PS) are well-researched bioactive compounds with diverse therapeutic effects. Purified PSs from various mushrooms have been clinically used in Japan, China, and the US without significant adverse effects, and are known to possess enhanced efficacy if bound to proteins. Though the mushroom PSs are primarily known for immunomodulatory and anticancer properties, they also exhibit antioxidant, anti-hypertensive, cholesterol-lowering, liver-protective, anti-fibrotic, anti-inflammatory, antidiabetic, antiviral, and antimicrobial effects. Recent studies have highlighted their role in gut microbiota regulation. They support homeostasis through health promotion by short-chain fatty acid production, intestinal mucosal barrier enhancement, lipid metabolism regulation, and activation of key signaling pathways[(3)]. Mushroom polysaccharides are broadly classified into two types: heteropolysaccharides, composed of various types of monomers, and homopolysaccharides, consisting of a single type of monomer**.** The Cell walls of fungi are mainly composed of cellulose or chitin embedded in the matrix of α or β-glucans and glycoproteins[(4)].

The major polysaccharides found in mushrooms belong to the glucans. β-glucans of mushrooms consist of D-glucose monomers linked by 1,3 and 1,6 glucosidic bonds at two distinct positions. The linear backbone is usually composed of 1,3 linkages, while 1,6 linkages are involved in primary branching. α-glucans with α-type linkages are less common in mushrooms[(5)]. Though the glycosidic bonds connecting the glucose monomers are the same in all β-glucans, there exists considerable variation in their structural characteristics, which are influenced by factors like the position of the bond, length of the chain, degree of branching, and three-dimensional structures. The structural and physical properties of β-glucans from mushrooms are also influenced by the species, growth conditions, processing methods, and isolation techniques used. All these factors, in turn, significantly affect the strength and nature of their biological activities[(6)].

Free radicals, inflammation, and diabetes are closely linked, with excess oxidants causing oxidative stress that damages proteins, lipids, DNA, and cell membranes. This occurs when the production of reactive oxygen (ROS) and nitrogen species (RNS) exceeds the neutralization capacity of the host system. Oxidative stress plays a key role in chronic diseases like cancer, arthritis, aging, autoimmune, cardiovascular, and neurodegenerative disorders. If unmanaged, it accelerates aging and contributes to conditions like trauma and stroke. Macrophages and monocytes release inflammatory mediators, including interleukins (IL-1β, IL-6, IL-8), tumor necrosis factor-α (TNF-α), nuclear factor-κB (NF-κB), intercellular adhesion molecule-1 (ICAM-1), inducible cyclooxygenase-2 (COX-2), prostaglandin E2 (PGE2), 5-lipoxygenase (5-LOX), and inducible nitric oxide synthase (iNOS) in response to free radical stimuli. Free radicals play a key role in acute and chronic inflammation. Activation of LOX, COX, and iNOS during inflammatory responses in turn induces oxidative stress, which compromises the antioxidant defense system[(7)].

Diabetes mellitus (DM) is a chronic, non-communicable metabolic disorder characterized by insufficient insulin production or impaired insulin secretion, leading to hyperglycemia. It includes type 1 (T1DM), type 2 (T2DM), gestational, and other specific forms linked to genetics, drugs, or diseases. Free radicals and inflammation play key roles in DM, particularly in insulin resistance. Chronic low-grade inflammation is strongly linked to T2DM and cardiovascular complications, especially in obesity**.** Studies indicate oxidative stress is central to DM progression, disrupting insulin signaling and promoting insulin resistance. Polysaccharides from edible and medicinal mushrooms exhibit hypoglycemic, hypolipidemic, antioxidant, anti-inflammatory, and prebiotic effects, contributing to anti-diabetic benefits. Key mushrooms studied include *Cordyceps militaris, Hericium erinaceus, Phellinus linteus, Inonotus obliquus, Cunninghamella ventricosum, Ganoderma lucidum,* and *Grifola frondosa.* Recent researches highlight novel polysaccharides from *Auricularia auricula, A. polytricha,* and *Dictyophora indusiata* with anti-diabetic properties, improving lipid metabolism, enhancing hepatic glycogen synthesis, reducing insulin resistance, and mitigating inflammation[(8)].

The mushroom genus *Phellinus* is renowned for its pharmacological efficacies due to the presence of a wide array of secondary metabolites, including polysaccharides, steroids, terpenes, coumarins, flavonoids, and styrylpyranones. Polysaccharides are the most effective components derived from mushrooms. *Phellinus* species are a parasitic group of macro fungi with a tough, woody appearance. They are widely distributed in temperate and tropical forests and are primarily involved in hardwood decomposition, contributing to nutrient cycling. Many *Phellinus species,* like *Phellinus linteus, P. igniarius, P. hartigii, P. gilvus, P. pini,* and *Phellinus baummi, P. tuberculosus, P. robustus, P. torulosus,* etc., have been explored extensively for their medicinal properties. They are reported to have anti-cancer, anti-tumor, immunomodulatory, antioxidant, anti-viral, antidiabetic, anti-inflammatory, and hepatoprotective activities [9], [10], [11].

Phellinus rimosus, a wood-decaying medicinal fungus found growing on jackfruit tree trunks in Kerala, India, has traditionally been used by local tribes to treat mumps. Although several *Phellinus* species have been extensively researched for their pharmacological efficacies, studies on the biological potential of *Phellinus rimosus* are very limited. Moreover, existing studies on *P.rimosus* have been carried out mainly using crude ethyl acetate, methanol, and aqueous extracts [9]. Isolation and characterization of active principles from these crude extracts have not yet been attempted, which could shed light on the exact mechanisms underlying the medicinal properties of *P. rimosus.* To accurately understand the active principles present in the aqueous extract of this mushroom, we isolated polysaccharides from the hot water extract in the present study and conducted a preliminary characterization. The use of isolated and purified polysaccharides provides a more accurate understanding of the structure-activity relationships than the crude extracts.

Our prior studies have demonstrated in vitro and in vivo antioxidant activities, as well as anti-inflammatory, antitumor, and anti-arthritic properties of these polysaccharides in animal models**,** along with their anticancer activity against HCT-116 human colon cancer cells through apoptosis**.** The objective of the present investigation was to analyze the ability of these polysaccharides from *P. rimosus* to act as reducing agents in free radical management, along with their in vitro anti-inflammatory and antidiabetic potential.

In previous studies, the antioxidant potential of polysaccharides from *P. rimosus* was assessed using superoxide, hydroxyl, nitric oxide, and 1,1-diphenyl 2-picryl hydrazyl (DPPH) radical scavenging assays, inhibition of lipid peroxidation, and ferric reducing antioxidant power (FRAP). These antioxidant assays are specific against individual free radical species and oxidation systems. In this investigation, we employed antioxidant assays to quantify the overall electron-donating and redox potential of polysaccharides from *P. rimosus*.

There are no existing reports on the in vitro anti-inflammatory and anti-diabetic activities of polysaccharides from *P. rimosus.* In our previous studies, we have reported their profound anti-inflammatory and anti-arthritic potential in animal models. Though useful and essential in studying the efficacy of drugs, in vivo methods reveal only the overall effect of the compound on inflammation. In this study, we employed well-established in vitro techniques that are target-specific and helpful in understanding the mode of action of the polysaccharides at the molecular level.

Free radicals, inflammation, and diabetes are closely interrelated, and the polysaccharides from *P. rimosus* have not yet been explored for their antidiabetic potential either in vivo or in vitro. Here, we report their promising antidiabetic potential through in vitro analysis. This research forms the first report on the in vitro anti-inflammatory and anti-diabetic potential of polysaccharides from *P. rimosus.* The present investigation suggests that polysaccharides from *P. rimosus* can be a suitable candidate for developing safe, multifunctional pharmacological agents from natural sources. The use of isolated polysaccharides and the integration of mechanism-based assays in this research provide valuable insights into the active principles and bioactivity of *P. rimosus.*

2 Methodology

In our previous research, we have reported the isolation and characterization procedure for the Polysaccharide-Protein Complex (PPC-*Pr*) from the basidiocarps of *P. rimosus*. The procedure is briefly summarized below.

2.1 Isolation and characterization of Polysaccharide Protein Complex from the Basidiocarps of *P. rimosus* (PPC-*Pr*)

Sporocarps of *Phellinus rimosus* occurring on the trunks of *Artocarpus heterophyllus* (jackfruit tree) were harvested from regions of Thrissur, Kerala, India. A reference specimen was archived in the Herbarium of the Centre for Advanced Studies in Botany, University of Madras, Chennai, India (HERB.MUBL-3171). The fruiting bodies of the mushroom were made into small pieces, dried at 40–50 °C for 3-4 days, and the polysaccharides were isolated from the hot water extract of *P. rimosus* (Figure 1). The dried mushrooms were powdered, and lipid content was removed by treating with petroleum ether in a Soxhlet apparatus. It was followed by hot water extraction at 95°C for 8-9 hrs and repeated thrice. After filtration and combining extracts, precipitation

was carried out using X5 volume pre-cooled ethanol and maintained for 48 hours at 4 °C. Following ethanol precipitation, centrifugation was carried out at 10,000rpm for 20 minutes. The Sevag method was used to remove the unbound proteins from the precipitate, and the precipitate was dialyzed against distilled water for 3 days and lyophilized. Qualitative and quantitative estimation of carbohydrate content of PPC-*Pr* was done by the Phenol Sulfuric acid method using glucose as a standard. The monosaccharide profile was analyzed by Paper Chromatography and Thin Layer Chromatography (TLC) after hydrolysis of PPC-*Pr* with trifluoroacetic acid (TFA). Bradford method was used to analyze the total protein content, and Bovine Serum Albumin was used as a standard. The analysis of amino acid profile was conducted using Shimadzu HPLC-LC10As amino acid analyzer, with PPC-*Pr* hydrolyzed in 6 N HCl at 110°C for 24 hrs. Sephadex G-100 gel filtration chromatography was performed to determine the molecular weight of PPC-*Pr*.

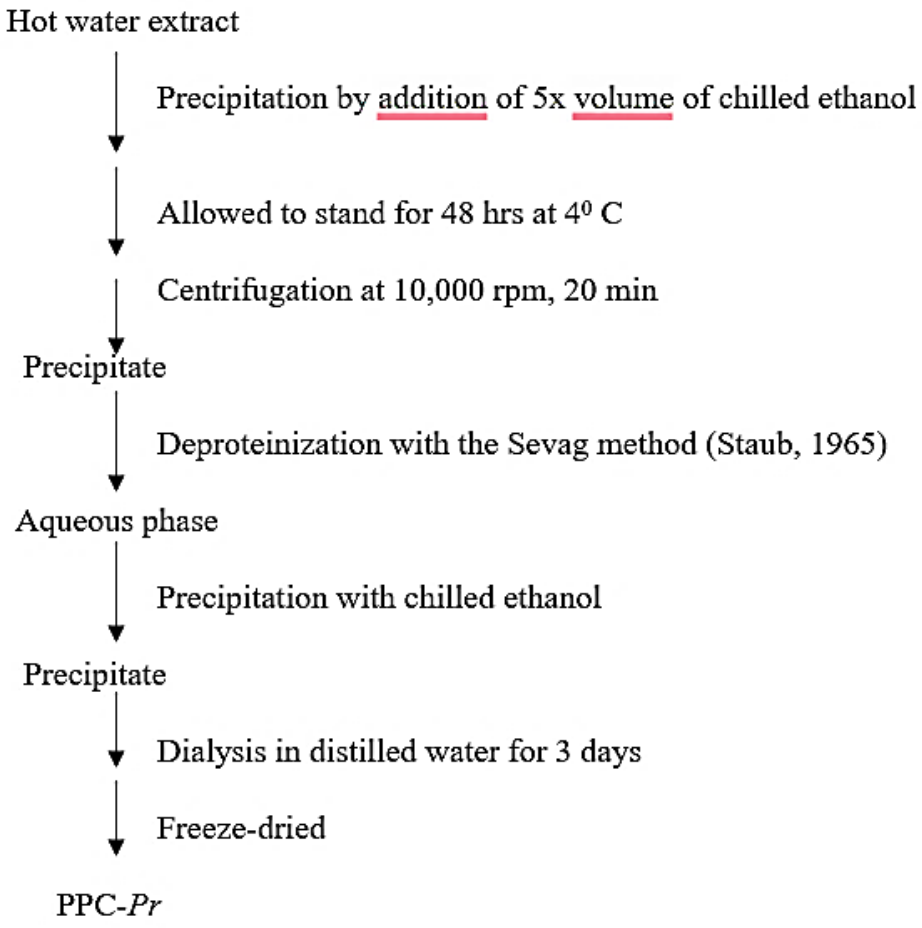

Fig 1. Procedure for isolation of PPC-*Pr* from the fruiting body of *P. rimosus*

2.2 Anti-oxidant potential of PPC-*Pr*

In our previous studies, PPC-*Pr* was reported to possess a significant effect in in vitro antioxidant assays, including Superoxide radical, Hydroxyl radical, DPPH free radical, and Nitric oxide scavenging activities, lipid peroxidation inhibition, and Ferric reducing antioxidant power (FRAP). Here, we evaluated the potential of PPC-*Pr* to act as a reducing agent using the Total Antioxidant Capacity Assay (TACA) and Total Reducing Power Assay (TRPA).

2.2.1. Total Antioxidant Capacity Assay (TACA)

Different concentrations of the drug in water were mixed with 1 mL TACA reagent containing 0.6 M Conc. H_2SO_4, 4 mM Ammonium molybdate, and 2.8 mM Sodium phosphate. After incubating at 95°C for 90 minutes, absorbance was measured at 695 nm. Vitamin C served as the standard reference. The following formula was used to calculate the drug activity.

% Inhibition = (T-C)/ T×100; where T= Absorbance of Treated and C= Absorbance of Control.

2.2.2. Total Reducing Power Assay (TRPA)

Various concentrations of PPC-*Pr* were mixed with 5 mL of TRPA reagent containing 200 mM Phosphate buffer (pH 6.6) and 1% Potassium Ferricyanide. The reaction mixture was incubated at 50°C for 20 min. 10% Trichloroacetic acid (2.5 mL) was added to the reaction mixture after incubation and centrifuged for 10 min. 5 mL was taken from the upper layer of the mixture and mixed with 5 mL of distilled water and 1 mL of 0.1 % ferric chloride solution. Vitamin C served as the standard. O D was measured at 700 nm, and drug activity was calculated as follows:

% Inhibition = (T-C)/ T×100; where T= Absorbance of Treated and C= Absorbance of Control.

2.3 Anti-inflammatory activity of PPC-*Pr*

Our previous studies have reported the ability of PPC-*Pr* to prevent acute and chronic inflammation, as well as Freund's complete adjuvant-induced arthritis in the in vivo animal models. Here, we analyzed the anti-inflammatory potential of PPC-*Pr* in vitro using the RAW 264.7 murine macrophage cell line.

2.3.1. Cell line used

RAW 264.7 murine macrophage cell line was purchased from the National Centre for Cell Sciences (NCCS), Pune, India. Dulbecco's modified Eagle's medium (DMEM) (Sigma Aldrich, USA) was used for cell line maintenance. Cell culture was carried out in tissue culture flasks of 25 cm^2 containing DMEM with sodium bicarbonate, L-glutamine, 10% FBS (Merck, Germany), and an antibiotic solution consisting of 100 μg/mL Streptomycin, 100 U/mL Penicillin, and 2.5 μg/mL Amphotericin B. The cells were incubated in a humidified 5% CO_2 incubator (NBS Eppendorf, Germany) at 37°C until the culture reached a confluency of 60%. The cultured cells were then stimulated using lipopolysaccharide (LPS:1μg/mL) and exposed to various concentrations of PPC-*Pr* drug solution (25, 50, 100 μg/mL), followed by 24 hours of incubation. Cell lysates from the incubated cultures were used for anti-inflammatory experiments, and diclofenac served as the standard reference drug in all assays conducted.

2.3.2. Inhibition of Cyclooxygenase (COX) activity

The cell lysate (100μL) was mixed with glutathione (5 mM/L), Tris-HCl buffer (pH 8), and hemoglobin (5 mM/L) and incubated at 25°C for 1 minute. Arachidonic acid (200 mM/L) was added to initiate the reaction, and after 20 min of incubation, the process for terminated by adding 10% trichloroacetic acid in 1 N hydrochloric acid (200μL). The samples were allowed to cool and were then centrifuged for 3 minutes. The absorbance was measured at 632 nm and COX activity was calculated as follows:

% inhibition = ((C-T)/ C × 100), where C = Absorbance of Control and T = Absorbance of Treated.

2.3.3. Inhibition of Lipoxygenase (LOX) activity

In this assay, 50 μL of cell lysate was mixed with 200 μL sodium linoleate and Tris-HCl buffer (pH 7.4) to obtain a final volume of 2mL. LOX activity, indicated by the increase in absorbance due to the formation of 5-hydroxyeicosatetraenoic acid, was measured at 234 nm (Agilent Cary 60). The inhibition of LOX activity was calculated as in the COX assay.

2.3.4. Inhibition of Inducible Nitric Oxide Synthase (iNOS)

The assay reagent consisted of 0.1mL manganese chloride (4 μmol/L), 0.1mL L-Arginine (2 μmol/L), 0.1mL dithiothreitol (10 mmol/L), 0.1 mL tetrahydropterin (4 μmol/L), 0.1 mL NADPH (1mmol/L), and 0.1 mL oxygenated hemoglobin (10μmol/L). The cell lysate was homogenized in 2mL of HEPES buffer, and 0.1mL of this homogenate was added to the above reagent. The reaction mixture was mixed well, and an increase in absorbance was measured at 401nm. The inhibition of enzyme activity was calculated as in the case of the COX assay.

2.4 Anti-diabetic Potential of PPC-*Pr*

2.4.1. Yeast sugar uptake assay

A 1% suspension of Commercial baker's yeast was prepared in distilled water and left at room temperature overnight. The yeast cell suspension was centrifuged for 5 min at 4200 rpm, and the pellet was repeatedly washed with distilled water and centrifuged

to get a clear supernatant. A 10% v/v suspension of the yeast cells was prepared in distilled water. Different concentrations of PPC-*Pr* were added to 1mL of 25 mM glucose solution and incubated at 37°C for 10 minutes. To this glucose solution containing drug concentrations, 100 μL of the yeast cell suspension was added and further incubated at 37°C for 60 minutes. The tubes were then centrifuged at 2500 rpm for 5 minutes. Supernatant was collected, and the presence of glucose was estimated by Benedict's test, and OD was measured at 520 nm. Metformin was used as the standard reference drug. Increase in glucose uptake was calculated as follows:

% increase in glucose uptake = ((C-T)/ C $\times$ $\times$ 100; where C= Absorbance of Control and T= Absorbance of Treated.

2.4.2. Alpha-Amylase Inhibitory Assay

The reagent consisted of 0.5 mL of 0.5 mg/mL α-amylase solution in 0.002 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl). Various concentrations of PPC-*Pr* were added to the reagent, mixed well, and kept for 10 minutes at room temperature. 0.5 mL of 1% starch solution prepared in 0.002 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl) was added to the reaction mixture and further incubated at room temperature for 10 minutes. 1-2 drops of iodine solution were added to the mixture to terminate of the reaction. 10mL of distilled water was added to each tube to dilute the reaction mixture and the OD was measured at 540nm. The standard reference drug used was Acarbose. The inhibition of enzyme activity was calculated as follows:

% inhibition of α-amylase = (T-C)/ T$\times$100; where T= Absorbance of Treated and C= Absorbance of Control.

2.5 Statistical analysis

All analysis data are presented as mean $\pm$ standard deviation. The statistical significance between different groups was analyzed by Student's *t-test*. < 0.001 was considered significant.

3 Results and Discussion

3.1 Isolation and characterization of Polysaccharide- Protein complex from *P. rimosus*

PPC-*Pr* was separated from the hot water extract of *P. rimosus* through successive ethanol precipitation, deproteinization, centrifugation, dialysis, and freeze drying. The yield of protein-bound polysaccharide (PBP) from the sporocarps was 8%. PPC-*Pr* exhibited typical colour reactions with Phenol Sulfuric acid and Bradford's reagent, revealing the presence of both polysaccharide (54.8%) and protein (28.6%) components. The retention of protein fraction after the Sevag deproteinization confirmed PPC-*Pr* as a protein-bound polysaccharide.

Protein-bound polysaccharides from mushrooms, including Polysaccharide Krestin (PSK) and Polysaccharide Peptide (PSP) from *Trametes versicolor*, have shown enhanced immunostimulatory and anticancer activities compared to free polysaccharides [12]. This highlights the biological relevance of protein-polysaccharide association, suggesting that the protein-bound nature of PPC-*Pr* may similarly contribute to its notable biological properties.

Paper chromatography and TLC after acid hydrolysis of PPC-*Pr* with TFA, identified glucose as the sole monosaccharide, confirming that it is a homopolysaccharide glucan. The amino acid analysis using HPLC LC10A amino acid analyzer revealed that the protein part of PPC-*Pr* mainly consisted of aspartic acid, glutamic acid, glycine, alanine, and serine in molar ratios of 14.1%, 12.4%, 11.6%, 11.6%, and 11.2% respectively. Sephadex G 100 column chromatography estimated the molecular weight of PPC-*Pr* as 1,200,000 (1.2×10^6) Da, confirming PPC-*Pr*, derived from the sporocarps of *P. rimosus*, as a high-molecular-weight glucan-protein complex.

Polysaccharides isolated from *Phellinus* species are generally highly complex, with their molecular weights ranging between 10 to 10^6 Da. Polysaccharides from the *Phellinus* fruiting bodies are heterogeneous, mainly consisting of glucose and other carbohydrates such as xylose, rhamnose, galactose, mannose, and arabinose. For instance, PIPF, polysaccharides from *P. igniarius*, consist of glucose, mannose, galactose, and methyl hexoses. From the sporocarps of *P. linteus*, a proteoglycan containing glucose, galactose, mannose, xylose, and arabinose, with β-(1,3)-D-glucose as the backbone, has been isolated. Additionally, two neutral heteropolysaccharides, PL-A and PL-B, have been identified from *P. linteus*. PL-A is mainly composed of glucose with low mannose content, and PL-B is a rhamnose-containing proteoglycan.

In contrast, the present study revealed the presence of a novel type of protein-bound glucan, distinguished by its homopolysaccharide nature, composed solely of glucose monomers and its high molecular weight of 1.2×10^6 Da. While all reported *Phellinus species* typically yield heteropolysaccharides, we report *P. rimosus* as a source of a unique, water soluble, structurally simpler, high molecular weight glucan-protein complex, which may impart significant biological functions. To our

knowledge, this is the first study on the characterization of a functionally active glucan-protein complex from the fruiting bodies of *P. rimosus*.

3.2 Anti-oxidant potential of PPC-*Pr*

3.2.1. Total Antioxidant Capacity Assay (TACA)

PPC-*Pr* showed significant TACA activity in proportion to dose, which was on par with the activity of the standard reference Vitamin C [Figure 2]. The assay measures the reduction of Molybdate VI to Molybdate V by the drug, which is indicated by the development of green coloured Phosphate-Molybdate complex in the acidic medium. PPC-*Pr* showed 98.81 % activity at the tested dose of 1000 μg/ml. IC_{50} values were 104.10 and 50.43 μg/ml, respectively, for PPC-*Pr* and the standard Vitamin C.

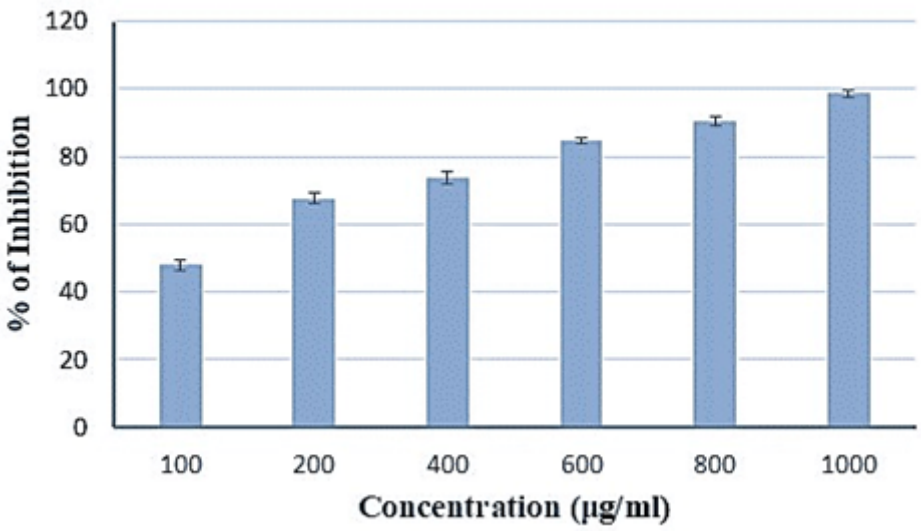

Fig 2. Evaluation of the Total Anti-oxidant Capacity Assay (TACA) of PPC-*Pr*

3.2.2. Total Reducing Power Assay (TRPA) of PPC-Pr

In the TRPA assay, also, remarkable reducing power was demonstrated by PPC-*Pr* with an increase in drug concentration [Figure 3]. This assay relies on the principle that compounds with electron-donating potential cause the reduction of potassium ferricyanide (Fe^{3+}) to potassium ferrocyanide (Fe^{2+}). The resulting Fe^{2+} then reacts with ferric chloride and forms a ferric-ferrous complex, which has maximum absorption at 700nm. The isolated polysaccharide showed 91.69% activity at a dose of 1000μg/ml. IC_{50} values of PPC-*Pr* and Vitamin C were 241.83 and 60.78 μg/ml, respectively.

The antioxidant potential of mushroom polysaccharides has been widely investigated, and it is well documented that antioxidant activity contributes to their anti-cancer, anti-inflammatory, immunomodulatory, hepato-protective, and neuroprotective properties. Polysaccharides from mushrooms like *Inonotus obliquus*, *Cordyceps militaris*, *Entoloma lividoalbum, Hericium erinaceus*, *Agaricus brasiliensis, Pleorotus squarrosulus, Phellinus linteus,* and many other mushrooms have shown high antioxidant activity[(4)],[(13)]. Levels of glucans showed direct association with the antioxidant activity of polysaccharides of *Lentinus edodes, Trametes versicolor, Ganoderma applanatum,* and *Ganoderma lucidum*. PPC-*Pr* isolated from *P. rimosus* contained 54.8% polysaccharides, and this high levels of polysaccharides may be responsible for its profound anti-oxidant activities.

Mushroom polysaccharides exhibit antioxidant activity by scavenging reactive species, reducing oxidative compounds, chelating Fe^{2+}, and inhibiting lipid peroxidation and erythrocyte hemolysis. They also enhance antioxidative enzymes like SOD, CAT, and GPx[(13)]. Our previous studies have revealed PPC-*Pr*'s ability to scavenge superoxide, hydroxyl, nitric oxide radicals as well as DPPH free radicals. The drug also showed the ability to inhibit lipid peroxidation and caused a reduction of ferric compounds[(12)]. Additionally, PPC-*Pr* promoted production of the antioxidant enzymes SOD, CAT, and GPx in the in

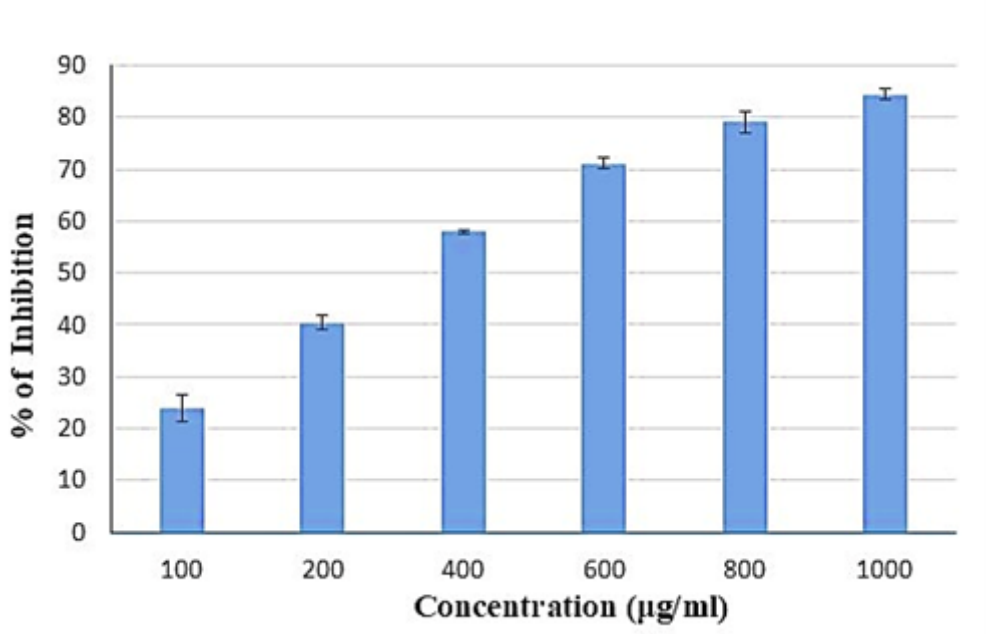

Fig 3. Evaluation of the Total Reducing Power Assay (TRPA) of PPC-*Pr*

vivo models. The in-vitro antioxidant assays carried out yet with the polysaccharides of *P. rimosus* were targeted against either Reactive Oxygen Species (ROS) or Reactive Nitrogen Species (RNS) and against particular oxidation systems. This investigation reveals the ability of polysaccharides from *P.rimosus* to act as a reducing agent by donating electrons to neutralize the free radicals generated. Also, these findings highlight the capacity of PPC-*Pr* to neutralize free radicals through multiple mechanisms.

Literature indicates that the in vitro antioxidant properties of mushroom polysaccharides depend on their structure, solubility, protein moieties, phenolic compounds, and molecular weight[4]. PPC-*Pr* used in this study is a completely water-soluble glucan containing 28.6% protein. Its high-water solubility and protein-bound nature may be the factors contributing to its strong redox potential.

3.3 Anti-inflammatory potential of PPC-*Pr*

The results of the anti-inflammatory assays for COX, LOX, and iNOS are summarized in Figure 4. PPC-*Pr* exhibited a significant dose-dependent inhibition of pro-inflammatory enzymes, showing 51.82%, 52.30%, and 48.78% inhibition of COX, LOX, and iNOS enzyme production, respectively, at the maximum tested dose of 100 μg/ mL. The inhibition was more pronounced against COX and LOX enzyme production.

Polysaccharides derived from mushrooms trigger anti-inflammatory effects by downregulating the production of enzymes like Cyclooxygenase (COX-2), inducible nitric oxide synthase (iNOS), etc., and by enhancing the signaling pathways of NF-κB, which in turn results in the increased production of anti-inflammatory cytokines like IL-10 and decreased production of inflammatory cytokines such as TNF-α and IL-1β[14]. Studies have shown that the polysaccharides isolated from the fruiting bodies and mycelia of *P. linteus* and *P. igniarius* possess significant anti-inflammatory properties. Polysaccharides from the mycelia of *P. linteus* exert anti-inflammatory activity by suppressing the expression of inflammatory cytokines through the prevention of mitogen-activated protein kinase (MAPK) activation, whereas polysaccharides from the fruiting bodies of the same mushroom reduce the inflammatory response by inhibiting nuclear factor (NF)-κB translocation. Fruiting body polysaccharides from *P. igniarius* reduce the expression of the pro-inflammatory cytokine tumor necrosis factor α (TNF-α) and restore the IL-6/IL-10 ratio, thereby ameliorating the inflammatory response[15]. Mushroom polysaccharides also improve intestinal health and inhibit ROS production, further reducing inflammation. Our findings suggest that PPC-*Pr*'s anti-inflammatory action may be due to its antioxidant activity and to its ability to inhibit proinflammatory mediators like COX, LOX, and iNOS.

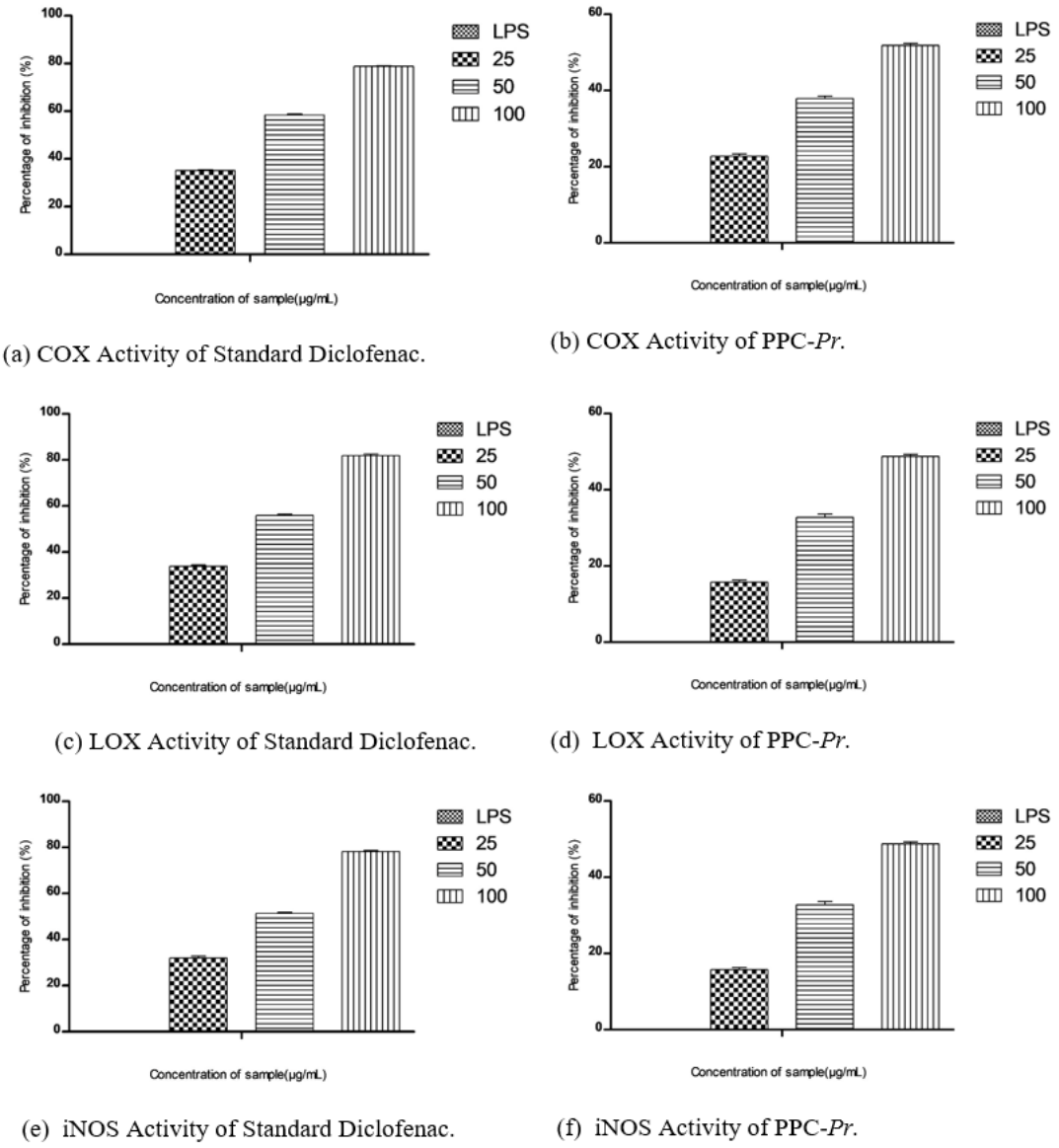

Fig 4. In-vitro Anti-inflammatory activity of PPC-*Pr* and the Standard Reference Diclofenac. Along the Y-axis is the percentage of inhibition (%). Along the X-axis, concentration of the sample (μg/mL)

3.4 Anti-diabetic Potential of PPC-*Pr*

The anti-diabetic activity of PPC-*Pr* was studied using Yeast glucose uptake and Alpha-amylase inhibition assays. The yeast glucose uptake assay showed that PPC-*Pr* did not enhance the absorption of glucose by yeast cells relative to the control. Therefore, PPC-*Pr*'s anti-diabetic action is not through promoting glucose uptake.

Alpha-amylase breaks down dietary starch into absorbable monosaccharides, making its inhibition a strategy for managing diabetes. The polysaccharide from *P. rimosus* showed a dose-dependent inhibitory effect, with 61.57% activity at 1000 μg/mL [Figure 5]. The IC_{50} values for PPC-*Pr* and the standard Acarbose were 819.6 μg/mL and 84.58 μg/mL, respectively. Thus, PPC-*Pr*'s anti-diabetic effect can be due to its inhibition of alpha-amylase and thereby preventing starch breakdown into absorbable sugars.

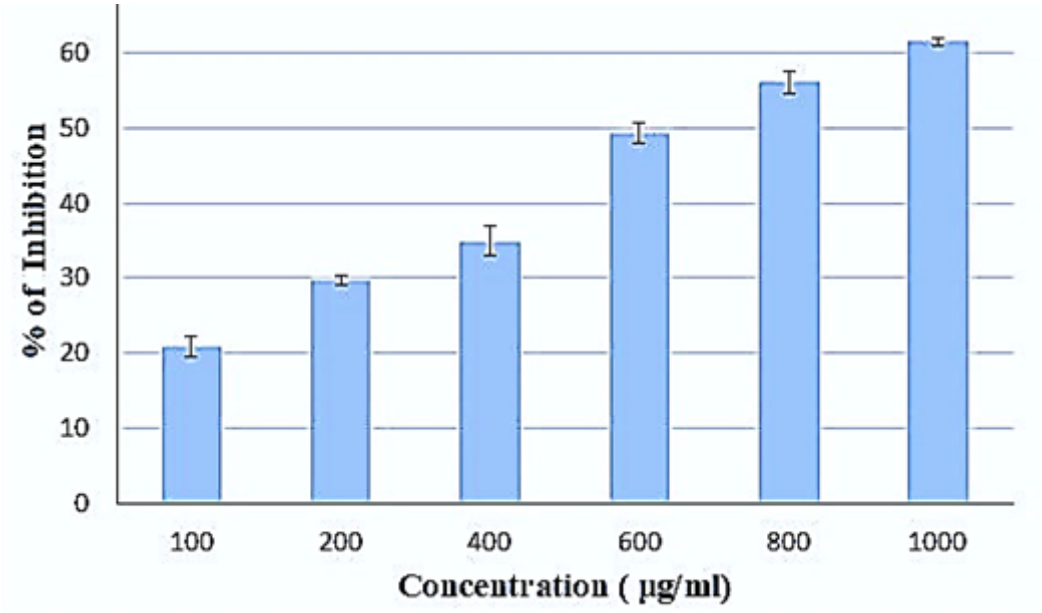

Fig 5. Evaluation of anti-diabetic potential of PPC-*Pr* by Alpha-amylase inhibition assay

An increased activity of alpha-amylase is reported to be associated with type 2 diabetes. In the studies of Zheng *et al.*[16], *Phellinus baumii* extract possessed considerable alpha-glucosidase inhibition. A heteropolysaccharide from *P. linteus* reported to reduce the lipopolysaccharide content in the blood, which in turn helps to reduce inflammation and reverse insulin resistance. A homopolysaccharide of molecular weight 153kDa from *P. linteus* (PLP) significantly brought down the fasting blood glucose (FBG) levels and improved the glucose intolerance through rescuing insulin signal transduction and altering the hepatic phospholipid metabolism. PLP also inhibited the development and progression of inflammation through the downregulation of inflammatory markers like interleukins, macrophages, TNF-α, and IFN-γ[17].

Several studies with diabetes induced animals demonstrated the occurrence of increased oxidative stress and loss of balance in the redox potential, leading to the uncontrolled progression of the disease[18]. Oxidative stress and inflammation have been proven to be implicated in the pathogenesis of type 2 diabetes[19]. Diabetes Mellitus, characterized by hyperglycemia, is a chronic metabolic disorder. Excess glucose levels cause oxidative stress via impaired mitochondrial function and biomolecular glycation, which ends up in chronic inflammatory conditions[20]. Free radicals, inflammation, and diabetes are closely related and interconnected. Free radicals play a crucial role in the pathophysiology of inflammatory diseases and diabetes. Oxidative stress triggers inflammation, which leads to insulin resistance and diabetes; further, diabetes intensifies oxidative stress, worsening inflammation and metabolic dysfunction. *Auricularia* species of mushrooms have been reported to prevent diabetes through the enhancement of the antioxidant system and the inhibition of nuclear factor NF-κB. Polysaccharides from *A. auricularia* induce antidiabetic action through PDX1/GLUT2 modulation, whereas *A. polytricha* decreases the digestion of polysaccharides by acting as an α-amylase inhibitor[21]. The present study showed the antioxidant, anti-inflammatory, as well as anti-diabetic properties of the polysaccharide isolated from *P. rimosus*. As these properties are interrelated to each other, it can be concluded that the free radical scavenging potential seems to be responsible for the anti-inflammatory and anti-diabetic activities of *P. rimosus*. Both the antioxidant and anti-inflammatory activity of *P. rimosus* may also contribute towards the antidiabetic activity of *P. rimosus*. Also, Ajith *et al.*,[22] had reported the absence of in vivo toxicity of *P. rimosus*, supporting its suitability as a natural therapeutic agent.

4 Conclusion

Many mushrooms remain as untapped sources for natural drug development, and *P. rimosus* is one among them. *P. rimosus* have been used by some tribal communities in Kerala, India, for treating mumps and is a less extensively studied mushroom with a repertoire of several biologically active components. The present investigation shows that the isolated protein-bound polysaccharides from *P. rimosus* possess significant antioxidant, anti-inflammatory, and anti-diabetic activities. The protein-bound polysaccharides (PPC-*Pr*) were isolated from the hot water extract of *P. rimosus,* and preliminary characterization was

done. The isolated compound showed the presence of 54.8% polysaccharides and 28.6% proteins. Paper chromatography and TLC analysis after acid hydrolysis showed that the carbohydrate part consisted only of glucose monomers. HPLC analysis revealed aspartic acid, glutamic acid, glycine, alanine, and serine in molar ratios of 14.1%, 12.4%, 11.6%, 11.6%, and 11.2% respectively.

In the Total Antioxidant Capacity Assay, the activity of PPC-*Pr* was comparable to the standard reference and showed 98.81 % activity at the maximum tested dose of 1000 μg/ml. Also, in the Total Reducing Power Assay, PPC-*Pr* showed promising activity, and at the maximum tested dose, the drug showed 91.69% activity. In the anti-inflammatory studies, PPC-*Pr* repressed the pro-inflammatory enzymes in a dose-dependent manner. The inhibition in the production of the enzymes COX, LOX, and iNOS was 51.82%, 52.30%, and 48.78% respectively. The anti-oxidant potential, as well as the inhibition of pro-inflammatory enzymes, might be responsible for the significant anti-inflammatory potential of *P. rimosus*.

Action on Alpha-amylase enzyme, which prevents the breakdown of starch into absorbable sugars, and enhanced glucose uptake, the two key mechanisms behind diabetes, were evaluated. The yeast glucose uptake assay showed that PPC-*Pr* did not enhance glucose uptake by yeast cells compared to the control. However, in the Alpha-amylase inhibition assay, PPC-*Pr* showed 61.57% activity at the maximum tested dose. As the free radicals, inflammation, and diabetes are interrelated physiological phenomena, it can be concluded that the antioxidant, anti-inflammatory as well as Alpha-amylase inhibition property of PPC-*Pr* might be behind its remarkable anti-diabetic activity.

The present study demonstrated the significant in vitro antioxidant, anti-inflammatory, and antidiabetic potential of polysaccharide-protein complex PPC-*Pr* isolated from the fruiting bodies of the macrofungus *Phellinus rimosus*. By incorporating mechanism-oriented assays that target key inflammatory and diabetic pathways, this work addresses the existing gap in the functional characterization of *P. rimosus* polysaccharides. The isolation of PPC-*Pr* from *P. rimosus* is easy to perform, simple and cost effective. Mushroom-derived polysaccharides are least toxic and are safe for long-term use. These properties and results underscore the possibility of *P. rimosus* as a suitable candidate for developing safe, biologically active, multipurpose natural drugs with least adverse impact. Further studies on the complete structural analysis of isolated polysaccharides, molecular studies, and in vivo studies may pave the way to a better understanding of the mode of action of these compounds and help to validate their nutraceutical and pharmaceutical applications.

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