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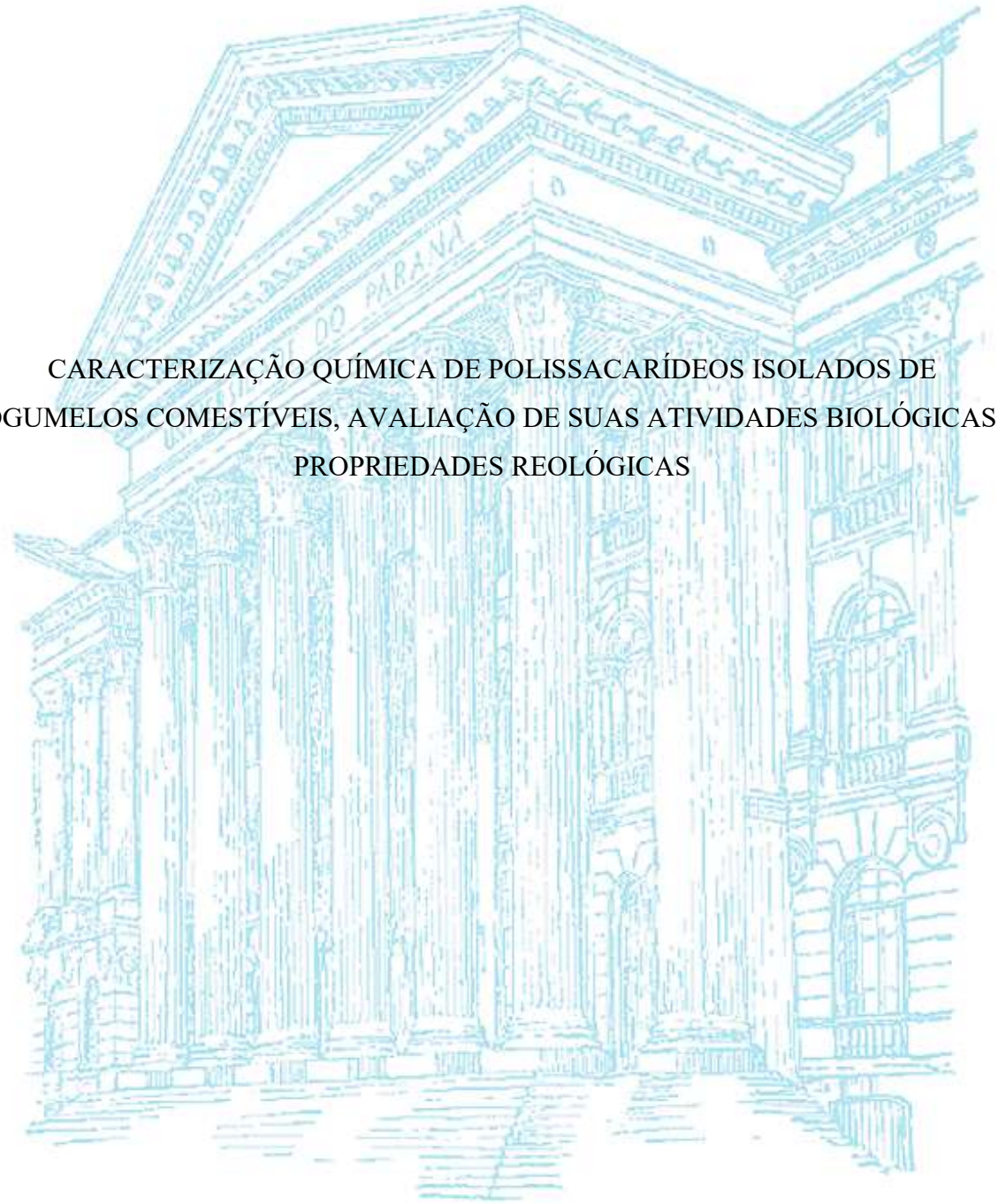

CARACTERIZAÇÃO QUÍMICA DE POLISSACARÍDEOS ISOLADOS DE COGUMELOS COMESTÍVEIS, AVALIAÇÃO DE SUAS ATIVIDADES BIOLÓGICAS E PROPRIEDADES REOLÓGICAS

CURITIBA

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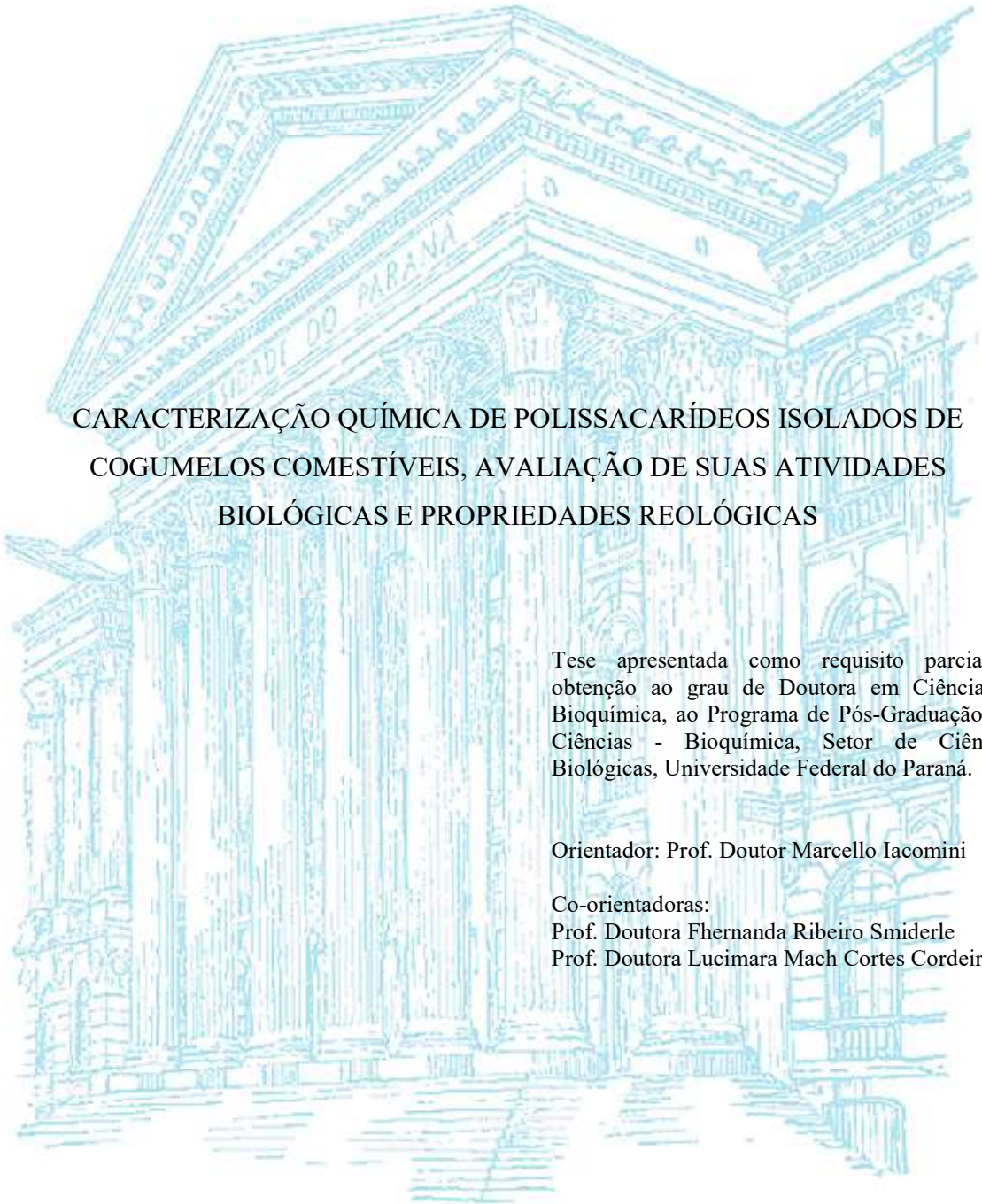

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Tese apresentada como requisito parcial à obtenção ao grau de Doutora em Ciências – Bioquímica, ao Programa de Pós-Graduação em Ciências - Bioquímica, Setor de Ciências Biológicas, Universidade Federal do Paraná.

Orientador: Prof. Doutor Marcello Iacomini

Co-orientadoras:
Prof. Doutora Fhernanda Ribeiro Smiderle
Prof. Doutora Lucimara Mach Cortes Cordeiro

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TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em CIÊNCIAS (BIOQUÍMICA) da Universidade Federal do Paraná foram convocados para realizar a arguição da Tese de Doutorado de **HELLEN ABREU**, intitulada: **CARACTERIZAÇÃO QUÍMICA DE POLISSACARÍDEOS ISOLADOS DE COGUMELOS COMESTÍVEIS, AVALIAÇÃO DE SUAS ATIVIDADES BIOLÓGICAS E PROPRIEDADES REOLÓGICAS**, sob orientação do Prof. Dr. MARCELLO IACOMINI, após terem inquirido a aluna e realizado a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.
A outorga do título de Doutor está sujeita à homologação pelo colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e ao pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

Curitiba, 17 de Fevereiro de 2020.

MARCELLO IACOMINI
Presidente da Banca Examinadora

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Avaliador Interno (UNIVERSIDADE FEDERAL DO PARANÁ)

DEDICATÓRIA

Dedico este trabalho à Deus, que sempre foi meu amparo, refúgio e fonte de força e persistência, pois com fé e muitas orações à Nossa Senhora eu concluí este trabalho e hoje eu sei que até onde não enxergamos Deus é perfeito.

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Acelera que tem muito pela frente!

- *Ayrton Senna* do Brasil

NOTA EXPLICATIVA

Esta tese está estruturada na forma de artigo segundo as normas do Programa de Pós Graduação em Ciências – Bioquímica e do Sistema de Bibliotecas (SiBi) da Universidade Federal do Paraná (UFPR). A tese contém introdução, revisão bibliográfica, justificativa, objetivos, artigos científicos, conclusões, referências e anexo. Os artigos científicos incluem revisão bibliográfica, materiais, metodologias, resultados, discussão de resultados, referências e material suplementar.

RESUMO

A pesquisa desenvolvida com os cogumelos comestíveis *Pholiota nameko* (nameko) e *Pleurotus eryngii* (eryngii) envolveu extração dos polissacarídeos em água, à temperatura ambiente, à temperatura a 100 °C, e em auto-clave (121 °C 1 h 1 atm) e, posteriormente, purificação por técnicas como congelamento e degelo, tratamento por solução de Fehling, diálise por membranas e ultrafiltração. Tanto os extratos quanto os polissacarídeos purificados foram caracterizados quimicamente com técnicas metodológicas como: Cromatografia Gasosa acoplada à Espectrometria de Massa, Ressonância Magnética Nuclear, Cromatografia de exclusão por tamanho de alto desempenho, e as estruturas químicas dos polissacarídeos confirmadas também por análises de metilação. Ao purificar o extrato bruto de *Pholiota nameko,* uma β-D-glucana foi obtida por extração em água quente e purificada por tratamento com amilase. As análises de RMN e de metilação da fração purificada (GHW-PN) indicaram a presença de uma β-D-glucana (1→3), altamente substituída (~ 27%) em *O*-6 por unidades únicas de β-D-Glc*p* ou por fragmentos (1→6)-β-D-Glc*p*. Esta β-D-glucana foi submetida à avaliação de sua propriedade reológica sob diferentes concentrações e temperatura, onde foi constatado que este polissacarídeo apresenta comportamento de gel e que é estável frente à variações térmicas de 90°C - 4 °C. A atividade terapêutica *in vivo* também foi avaliada para a β-D-glucana, indicando que sua aplicação intraperitoneal em animais da raça *Mus musculus* (camundongos) teve efeito antinociceptivo. Em outro estudo, o cogumelo eryngii foi submetido a três processos de extração para polissacarídeos utilizando àgua à temperatura ambiente (CW), à temperatura de 100 °C (HW) e autoclave (AE) gerando, respectivamente, três frações denominadas CW, HW e AE, e quando quimicamente caracterizadas apresentaram, respectivamente, manogalactana, β-D-Glucana(1→6) linear e β-D-Glucana(1→3),(1→6) ramificada como principais polissacarídeos em cada fração, podendo-se concluir que o processo de extração aplicado influencia o tipo de polissacarídeo extraído. As frações CW, HW e AE foram submetidos a testes *in vitro* com células da linhagem THP-1, onde foi possível observar o potencial imunoestimulador, visto que não foram tóxicas e estimularam liberação das citocinas IL-1β e IL-10. Finalmente, ao investigar as estruturas químicas de heteropolímeros presentes em extratos aquosos, obtidos à temperatura ambiente dos cogumelos nameko e eryngii, três heteropolissacarídeos foram purificados. Duas manogalactanas de nameko foram purificadas por ultrafiltração sendo uma retida na membrana de 30 kDa e outra eluída, denominadas respectivamente como R30PN e E30PN. E, uma manogalactana de eryngii foi obtida pelo tratamento com solução de Fehling e denominada FPE. Os heteropolissacarídeos foram quimicamente caracterizados como manogalactanas naturalmente metiladas em *O*-3, com cadeia principal de α-Gal*p*-(1→6), ramificada em *O*-2 por unidades β-D-Man*p*. Porém, há diferenças quanto ao grau de ramificação das estruturas químicas, o percentual de grupos *O*-metil e o peso molecular. Os resultados demonstraram que os métodos de extração utilizados em *P. nameko* e *P. eryngii* são processos simples que proporcionam obter estruturas polissacarídicas diferentes quimicamente, como glucanas e heteropolímeros que possuem propriedades reológicas e biológicas imunoestimuladoras e antinociceptiva com potencial aplicação para a indústria de alimentos e de produtos terapêuticos.

Palavras-chave: Polissacarídeos. Cogumelos comestíveis. Extração e purificação. Caracterização química. Propriedade Reológica. Propriedades biológicas.

ABSTRACT

The research carried out with the edible mushrooms *Pholiota nameko* (nameko) and *Pleurotus eryngii* (eryngii) involved extracting the polysaccharides in water, at room temperature, at a temperature of 100 °C, and in an autoclave (121 °C 1h 1 atm) and, subsequently, purification by techniques such as freezing and thawing, treatment by Fehling's solution, membrane dialysis and ultrafiltration. Both the extracts and the purified polysaccharides were chemically characterized with methodological techniques such as Gas Chromatography coupled to Mass Spectrometry, Nuclear Magnetic Resonance, High-performance size exclusion chromatography, and the chemical structures of the polysaccharides also confirmed by methylation analyzes. When purifying the crude extract of *Pholiota nameko*, a β-D-glucan was obtained by extraction in hot water and purified by treatment with amylase. NMR and methylation analyzes of the purified fraction (GHW-PN) indicated the presence of a β-D-glucan (1→3), highly substituted (~ 27%) in *O*-6 by single units of β-D-Glc*p* or by fragments (1→6)-β-D-Glc*p*. This β-D-glucan was submitted to the evaluation of its rheological property under different concentrations and temperature, where it was found that this polysaccharide has a gel behavior and is stable in the face of thermal variations of 90 °C - 4 °C. In vivo therapeutic activity was also evaluated for β-D-glucan, indicating that its intraperitoneal application in animals of the *Mus musculus* breed (mice) had an antinociceptive effect. In another study, the eryngii mushroom was subjected to three extraction processes for polysaccharides using water at room temperature (CW), at a temperature of 100 °C (HW) and autoclave (AE), generating, respectively, three fractions called CW, HW, and AE, and when chemically characterized they presented, respectively, mannogalactan, linear β-D-Glucana (1→6) and β-D-Glucana (1→3), (1→6) as main polysaccharides in each fraction, it is concluded that the applied extraction process influences the type of polysaccharide extracted. The CW, HW, and AE fractions were subjected to in vitro tests with cells of the THP-1 lineage, where it was possible to observe the immunostimulatory potential since they were not toxic and stimulated the release of cytokines IL-1β and IL-10. Finally, when investigating the chemical structures of heteropolymers present in aqueous extracts, obtained at room temperature from nameko and eryngii mushrooms, three heteropolysaccharides were purified. Two nameko mannogalactans were purified by ultrafiltration, one being retained in the 30 kDa membrane and the other eluted, named respectively R30PN and E30PN. And, an eryngii mannogalactan was obtained by treatment with Fehling's solution and called FPE. The heteropolysaccharides were chemically characterized as mannogalactans naturally methylated in *O*-3, with α-Gal*p*-(1→6) main chain, branched in *O*-2 by β-D-Man*p* units. However, there are differences in the degree of branching of chemical structures, the percentage of *O*-methyl groups, and the molecular weight. The results demonstrated that the extraction methods used in *P. nameko* and *P. eryngii* are simple processes that provide chemically different polysaccharide structures, such as glucans and heteropolymers that have immunological and antinociceptive rheological and biological properties with potential application for the food industry and therapeutic products.

Keywords: Polysaccharides. Edible mushrooms. Extraction and purification. Chemical characterization. Rheological Property. Biological properties.

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Heteropolysaccharides obtained from the edible mushrooms *Pholiota nameko* and *Pleurotus eryngii*

LISTA DE ABREVIATURAS, SIGLAS E SÍMBOLOS

Frações obtidas de *P. nameko*

HW	Fração obtida a partir do extrato aquoso quente de *P. nameko*
PN	Extrato aquoso obtido à temperatura ambiente
SHW	Fração solúvel obtida do processo de congelamento e degelo de HW
IHW	Fração insolúvel obtida do processo de congelamento e degelo de HW
GHW-PN	Fração obtida após tratamento com amilase da fração IHW
R30PN	Fração retida na membrana de 30kDa na ultrafiltração (Manogalactana purificada)
E30PN	Fração eluída na membrana de 30kDa na ultrafiltração (Manogalactana purificada)

Frações obtidas de *P. eryngii*

CW	Extrato aquoso frio de *P. eryngii* (obtido à temperatura ambiente)
PE	Extrato aquoso obtido à temperatura ambiente
ICW	Fração insolúvel obtida do processo de congelamento e degelo de CW
SCW	Fração solúvel obtida do processo de congelamento e degelo de CW
HW	Extrato aquoso quente (100 °C)
SHW	Fração solúvel obtida do processo de congelamento e degelo de HW
IHW	Fração insolúvel obtida do processo de congelamento e degelo de HW
AE	Extrato aquoso em autoclave (121 °C 1atm)
FPE	Fração obtida após tratamento com solução de Fehling (Manogalactana purificada)

Termos associados às estruturas dos polissacarídeos

Δ	Deslocamento químico
σ	Tensão de cisalhamento

T	Taxa de cisalhamento
Γ	Deformação
$\dot{\gamma}_c$	Taxa de cisalhamento crítica
H	Viscosidade
η_{sp}	Viscosidade específica
K	Índice de consistência
N	Índice de comportamento de fluxo
R^2	Coeficiente de Regressão
η_0	Viscosidade de taxa de cisalhamento zero
η_∞	Viscosidade de taxa de cisalhamento infinita
$\dot{\gamma}_c$	Taxa de cisalhamento crítica
K	Coeficiente de Consistência
G’	Módulo elástico
G”	Módulo viscoso
β	Configuração Beta
A	Configuração Alfa
ºC	Graus Celsius
D	Conformação D
C-1	Carbono 1
C-2	Carbono 2
C-3	Carbono 3
C-4	Carbono 4
C-5	Carbono 5
C-6	Carbono 6
2,3,4,6-Me_4-Glc	2,3,4,6-Tetrametil-glucose
2,3,4-Me_3-Glc	2,3,4-Trimetil-glucose
2,4,6-Me_3-Glc	2,4,6-Trimetil-glucose
2,4-Me_2-Glc	2,4-Dimetil-glucose
2,3,4,6-Me_4-Man	2,3,4,6-Tetrametil-manose

2,3,4-Me_3–Gal	2,3,4-Trimetil-galactose
3,4-Me_2–Gal	2,4-Dimetil-galactose
AcOH	Ácido Acético
COSY	Correlation Spectroscopy (Espectroscopia de Correlação)
$CHCl_3$	Clorofórmio
CH_2O_2	Ácido fórmico
DEPT-135	Distortionless enhancement by polarization transfer (Aperfeiçoamento sem distorção por transferência de polarização)
DMSO	Dimetilsulfóxido
ID50	Dose necessária para reduzir a resposta nociceptiva em 50%
DMSO-d_6	Dimetilsulfóxido deuterado
Gal	Galactose
GC-MS	Gas Cromatography-Mass Spectrometry (Cromatografia gasosa acoplada à espectrometria de massas)
Glc	Glucose
Hz	Hertz
HPLC	High Performance Liquid Chromatography
HPSEC	High Pressure Size Exclusion Chromagraphy (Cromatografia de exclusão estérica de alta pressão)
HSQC	Heteronuclear Single Quantum Correlation Spectroscopy (Espectroscopia de Correlação Heteronuclear Quântica Simples)
i.p.	Intraperitoneal
kDa	Quilodalton
KOH	Hidróxido de potássio
Man	Manose
Me	Grupamento metil (-CH_3)
MeOH	Metanol
Mn	Massa molecular numérica média
M_W	Massa molecular ponderal média
$NaBD_4$	Boroidreto de sódio deuterado
$NaBH_4$	Boroidreto de sódio
NaOH	Hidróxido de sódio
p	Piranosídico

Pa	Pascal
Pa.s	Pascal por segundo
ppm	Partes por milhão
per-O-metilado	Parcialmente *O*-metilado
Rg	Raio de giro
rpm	Rotações por minuto
RMN-^{13}C	Ressonância magnética nuclear de carbono 13
RMN-^{1}H	Ressonância magnética nuclear de hidrogênio
SEC	Size Exclusion Chromatography
spp.	Espécies pertencentes ao mesmo gênero
TFA	Ácido trifluoroacético
Tocsy	Total Correlation Spectroscopy (Espectroscopia de Correlação Total)
W	Distribuição de massa (*weight distribution*)

SUMÁRIO

I. Introdução

Os cogumelos comestíveis são excelentes fonte de nutrientes e seus polissacarídeos não digeríveis pelas enzimas digestivas do ser humano são classificados como fibras (β-glucanas, quitinas, manogalactanas), exercendo um importante papel promovendo á saúde do indíviduo, agindo como prebiótico no meio gastrointestinal (Friedman, 2016). O efeito terapêutico dessa classe de moléculas, bem como dos extratos de cogumelos comestíveis vêm sendo reportado por estudos que indicam sua atividade anti-inflamatória, antinociceptiva, hipocolesterolêmica e antitumoral, relatadas em estudos *in vitro* e *in vivo* (Moreno et al., 2016; Ruthes et al., 2013 a,b; Smiderle et al., 2008; Gil-Ramírez et al., 2018).

Para obtenção de extratos de cogumelos foram desenvolvidos diversos processos que impactam no rendimento e qualidade dos produtos obtidos. Tecnologias mais comuns utilizam métodos exaustivos de extração com água à temperatura ambiente, ou à 100 °C, além da extração com soluções contendo substâncias alcalinas (hidróxido de sódio ou potássio) (Ruthes et al., 2015).

Com o desenvolvimento da ciência e a crescente preocupação com a preservação do meio ambiente, novas tecnologias de extração passaram a ser utilizadas e consideradas rentáveis, do ponto de vista de preservação dos nutrientes dos cogumelos comestíveis e, consequentemente, do efeito terapêutico, bem como do menor consumo de recursos e energia e menor geração de resíduos tóxicos ao ambiente (Smiderle et al., 2017). Tais considerações são importantes, principalmente, para os produtores e beneficiadores de cogumelos e subprodutos com fins alimentícios e terapêuticos que buscam obter maior qualidade no produto final, com finalidade de agregar valor e fornecer um produto funcional.

Para a indústria alimentícia interessa também a utilização dos polissacarídeos como aditivo de corpo, ou seja, como um produto capaz de conferir caráter de gel por meio do seu potencial viscoelástico, que é o caso de alguns polímeros encontrados em extratos polissacarídicos de cogumelos (Sovrani et al., 2017).

Os extratos polissacarídicos de cogumelos são geralmente compostos por moléculas de glucose, e/ou galactose, e/ou manose, unidas por ligação glicosídica do tipo alfa (-α) ou beta (-β), que não são hidrolisadas pelas enzimas liberadas no intestino humano. Estes polissacarídeos, quando em solução, podem alterar o caráter viscoelástico

do meio, sendo esta uma propriedade reológica de algumas glucanas de cogumelos. A propriedade reológica de polissacarídeos é conhecida e bastante utilizada pela indústria alimentícia e farmacêutica, as quais a utilizam para alterar/manter textura, sabor, qualidade e conservação de alimentos ou outros produtos. Os polissacarídeos são aplicados como biofilmes ou agentes espessantes e geleificantes, dependendo do seu caráter viscoelástico, que pode também apresentar estabilidade frente a variações térmicas (Rao, 2007; Xu et al., 2016; Sovrani et al., 2017).

O desenvolvimento de tecnologias para aprimorar e aproveitar o potencial dos cogumelos é constante e tem demonstrado ser eficaz para obter biomoléculas com função terapêutica. Tem-se observado que novos métodos de extração estão sendo aplicados a fim de melhorar o rendimento de extração tanto de polissacarídeos, como de outros compostos bioativos presentes em cogumelos (Smiderle et al., 2017; Morales et al., 2017; Morales et al., 2018). Além disso, a evidência de que as diferentes características químicas dos polissacarídeos (grau de ramificação, massa molar e conformação) influenciam diretamente nas propriedades físico-químicas (solubilidade, ponto de congelamento e ebulição), e consequentemente na sua atividade biológica, impulsiona e justifica o estudo químico detalhado destes compostos para possibilitar aplicações como coadjuvantes alimentício e farmacológico (Elisashvili, 2012; Polishchuk e Kovalenko, 2009).

Os estudos referentes aos basidiomicetos do gênero Pleurotus têm identificado e caracterizado heteropolíssacarídeos, como manogalactanas com sua cadeia principal composta por α-D-galactopyranose (1→6) ligada com unidades substituídas em *O*-2 por β-D-manopiranose; e homopolissacarídeos como glucanas que podem ser compostas por diversos tipos de ligação como: β(1→3), β(1→6), α(1→3), α(1→6) (Rosado et al., 2003; Alquini et al., 2004; Carbonero et al., 2005; Smiderle et al., 2008; Carbonero et al., 2008; Smiderle et al., 2013; de Jesus et al., 2018; Castro-Alves & Nascimento, 2018).

Estes hetero e homopolissacarídeos quando submetidos à experimentos biológicos *in vivo* ou *in vitro* alteram padrões de ação celular e molecular, gerando novas hipóteses sobre as atividades anti-inflamatórias, antitumorais, imunomoduladoras. Em relação especificamente ao eryngii e ao nameko, que são espécies comercialmente conhecidas e mundialmente consumidas, os extratos de polissacarídeos são obtidos, no entanto a caracterização química não é realizada, podendo-se observar em diversos estudos apenas afirmações com base na composição de monossacarídeos ou em testes colorimétricos sobre a existência de glucanas e manogalactanas nestes extratos, e em seguida são

realizados experimentos que alegam as mais diversas atividades biológicas, sem poder afirmar que a atividade está de fato relacionada ao conteúdo de polissacarídeos presentes e qual a estrutura polimérica seria a responsável por determinada ação biológica (Li & Shah, 2016; Xu, et al., 2016; Zhang et al., 2017; Ma et al., 2014; Ren et al., 2016; Yang et al., 2013; Synytsya et al., 2009; Li & Shah, 2016b; Zhang et al., 2014; Yan et al., 2019a; Yan et al., 2019b; Li, et al., 2007; Li, et al., 2008; Li, et al., 2010; Ji., et al., 2012; Li, et al., 2012; Chen, et al. 2013; Zheng, et al., 2014; Rodrigues, et al., 2017).

A caracterização química realizada por Carbonero et al. em 2006 e 2008 mostrou a presença de uma β-D-glucana(1→3)(1→6) e de uma galactana 3-*O*-metilada oriundas do eryngii, também presente no eryngii foi encontrada uma manogalactana para a qual foi observado o efeito antimelanoma (Biscaia et al., 2017). Uma β-D-glucana(1→3)(1→6) foi extraída, purificada e caracterizada quimicamente por Sovrani et al. (2017), que avaliou sua propriedade viscoelástica. Porém, até o presente momento não foi relatado atividade biológica para estruturas purificadas oriundas de nameko. Ambas espécies, eryngii e nameko, têm estudos prévios que demonstram ter em sua composição diversos polissacarídeos de interesse e também estruturas a serem elucidadas e testadas, sendo excelentes para a investigação de mecanismos de ação biológica proporcionando embasamento o desenvolvimento de novos produtos terapêuticos. Assim, as espécies *Pholiota nameko* e *Pleurotus eryngii* foram as selecionadas para proceder os estudos de caracterização química de polissacarídeos isolados de cogumelos comestíveis, avaliação de suas atividades biológicas e propriedades reológicas.

II. Revisão de literatura

a. Cogumelos comestíveis e polissacarídeos

Os cogumelos são frutificações macroscópicas de fungos e fazem parte de dois grandes grupos: Ascomicetos e Basidiomicetos (McLaughlin et al., 2009). Das vinte e duas mil espécies registradas, duas mil são de cogumelos comestíveis, e muitos são ingredientes culinários há mais de 2000 anos, sendo usados para prevenção de enfermidades, servindo como suplemento alimentar, de baixo teor calórico e fonte de macro e micronutrientes (Wasser, 2002; Ruthes et al., 2015). Além do mais, desempenham papel ecológico decompondo matéria orgânica e interagindo com plantas, animais e outros organismos (McLaughlin et al., 2009).

O *Pholiota nameko,* é um basidiomiceto conhecido popularmente como nameko, os estudos químicos revelam que ele apresenta manose, glucose e galactose em sua composição de carboidratos, os quais estão relacionados a algum tipo de atividade biológica, tais como antitumoral, anti-inflamatória, hipolipidêmica e prébiótica (Li et al., 2007; Li et al., 2008; Li et al., 2010; Hu et al., 2012; Ji et al., 2012; Chen et al., 2013; Zhen et al., 2014; Sovrani, 2017; Rodrigues et al., 2015).

O *Pleutorus eryngii* é um cogumelo bastante utilizado na culinária oriental e por isso teve seu estudo impulsionado. Atualmente existem diversos estudos com esta espécie, porém a diversidade de moléculas encontradas e também de atividades biológicas relacionadas a estas trazem mais enigmas a serem desvendados pela ciência, o que finda por proporcionar maior profundidade ao seu estudo científico. Dentre as atividades relatadas constam a atividade imunoestimuladora, hepatoprotetora, antitumoral, hipolipidêmica, prébiótica e antimelanoma (Xu et al., 2016; Zhang et al., 2014; Ren et al., 2016; Synytsya et al., 2009; Biscaia et al., 2017).

Os cogumelos comestíveis são fonte de α- e β-glucanas, sendo estas não digeríveis pelas enzimas humanas e, por isso, consideradas fibras dietéticas, que podem atuar auxiliando na motilidade intestinal, aumentando o volume fecal e reduzindo a absorção de substâncias tóxicas pelo organismo e, consequentemente, prevenindo enfermidades (Ruthes et al., 2015; Cheng, 2012). Dessa forma, os cogumelos comestíveis têm importância nutricional e terapêutica, o que agrega valor comercial a estes alimentos.

Cada espécie de cogumelo pode conter diferentes polissacarídeos, em diferentes proporções e, que podem exercer variadas funções para o fungo, tais como

armazenamento de energia ou ainda sendo constituinte da parede celular (McLaughlin et al., 2009). Os polissacarídeos que contém unidades monossacarídicas unidas por ligações glicosídicas, podendo formar uma diversidade de cadeias com diferentes graus de ramificação, tipos de ligação e peso molecular (Ruthes et al., 2015).

Existem os homopolissacarídeos de cogumelos comestíveis que possuem apenas um tipo de monossacarídeo em toda sua estrutura, podendo ser glucanas lineares α-(1→3), α-(1→4) (Ruthes et al., 2013; Smiderle et al., 2010), ou ainda β-(1→3) ou β-(1→6). Estes homopolissacarídeos podem ser ramificados ou *O*-substituídos por terminais não redutores ou por cadeias laterais (Carbonero et al., 2006; Smiderle et al., 2013; Moreno et al., 2016). Um exemplo de homopolissacarídeo abundante é o glicogênio que possui uma cadeia principal de glucose unidas por ligações α-(1→4) podendo conter substituições no carbono 6, por cadeias laterais de glucose com ligações α-(1→4) (Palacios et al., 2012). Outro homopolissacarídeo bastante encontrado em cogumelos comestíveis é a β-D-glucana com ligações (1→3) na cadeia principal e substituições no carbono 6, por unidades de β-glucose ou cadeias laterais ligadas (1→6) (Carbonero et al., 2006; Moreno et al., 2016).

Já os heteropolissacarídeos são compostos por mais de um tipo de monossacarídeo, como por exemplo as heterogalactanas e heteromananas, que podem ter uma cadeia principal de galactose ou manose, respectivamente, com algumas unidades ramificadas por manose, galactose, fucose, xilose em diferentes proporções. As fucogalactanas, manogalactanas, xilomananas e galactomananas são comumente encontradas em cogumelos comestíveis (Ruthes et al., 2016).

O modo de isolamento e purificação influi no tipo de polissacarídeo obtido. A extração exaustiva com água proporciona maior rendimento de polissacarídeos mais solúveis e menos aderidos à parede celular. A matéria não extraída com água, denominada resíduo pode ser submetida na sequência à extrações alcalinas, obtendo polissacarídeos mais firmemente aderidos à parede celular (Ruthes et al., 2015).

Os polissacarídeos mais comumente extraídos dos corpos de frutificação de cogumelos são as glucanas, as quais podem encontrar-se complexadas com outros compostos por meio de pontes de hidrogênio e forças de Van der Waals. Para promover a separação destes compostos, a aplicação de solventes como etanol, dimetilsulfóxido, clorofórmio, metanol entre outros é realizada, pois estes solventes atuam solvatando e extraindo os compostos que com eles possuem afinidade química (Synytsya et al., 2009).

b. *Pholiota nameko*

O nameko é original do Oriente e seus registros de ocorrência relatam mais precisamente o Japão como país de origem, sendo encontrados cultivos também na China. Identificado como espécie *Pholiota nameko* (T. ITO) S. Ito & S. Imai de 1933, sua classificação taxonômica está descrita no Quadro 1.

Quadro 1. TAXONOMIA *Pholiota nameko*	
Reino	Fungi
Filo	Basidiomycota
Classe	Agaricomycotina
Subclasse	Agaricomycetes
Ordem	Agaricales
Família	Strophariceae
Gênero	Pholiota
Espécie	*Pholiota nameko*

Fonte: Genebank, National Institute of Agrobiological Sciences.

Em 1996 um estudo conduzido por Malick et al. (1996) com micélio de *Pholiota nameko* despertou interesse na comunidade científica devido a comprovação da presença de um importante elemento para o metabolismo animal e vegetal, o inositol ou mio-inositol. Essa descoberta não apenas despertou interesse como culminou em estudos posteriores sobre a composição deste basidiomiceto e suas funções (Joh et al., 1998; Joh et al., 2001).

O potencial anti-inflamatório e antitumoral de extratos polissacarídicos de *Pholiota nameko* foram investigados, no entanto os detalhes sobre quais os componentes bioativos e mecanismos de ação não foram esclarecidos (Li et al., 2007; Li et al., 2008). Outros estudos, utilizando extratos, buscaram comprovar a ação antitumoral, hipolipidêmica e hepatoprotetora (Li et al., 2010; Li et al., 2012; Zheng et al., 2014).

Sovrani et al. (2017) em um estudo avançado sobre a caracterização química do extrato aquoso frio dessa espécie concluiu, por meio de análises com ressonância

magnética nuclear ^{13}C e cromatografia gasosa acoplada a espectrometria de massas, que este contém β-glucana com cadeia principal ligada (1→3), com presença de substituições em O-6 em algumas unidades da cadeia principal por terminais não-redutores de β-Glc*p* ou pequenas cadeis de β-Glc*p* ligadas (1→6). Este estudo compõe um importante avanço à caracterização dos polissacarídeos deste fungo, revelando a importância de avaliar também a sua atividade biológica. Até o momento não foram relatados outros estudos de isolamento e caracterização química de polissacarídeos para a espécia *Pholiota nameko*.

A seguir, no Quadro 2, constam os trabalhos desenvolvidos com ênfase em caracterização química e atividade biológica de extratos polissacarídicos de *Pholiota nameko*.

Quadro 2:
Estudos com extratos polissacarídicos de *Pholiota nameko*

Tipo de extrato	Atividade relatada	Monossacarídeo identificado	Referência
[1]HW	Antitumor	[2]Man, Glc, Gal, Ara, Xil	Li et al. (2007)
HW	Anti-inflamatória	Man, Glc, Gal, Ara, Xil	Li et al. (2008)
HW	Hipolipidêmica	Man, Glc, Gal, Ara, Xil	Li et al. (2010)
CW, HW, EtOH	Antioxidante	Glc, Ram, Gal, Xil, Ara	Ji et al. (2012)
HW	Anti-inflamatória		Li et al. (2012)
HW	ND[3]	Xil, Man, Glc, Gal	Chen et al. (2013)
HW	Anti-hiperlipidêmica e hepatoprotetora	Ara, Man, Glc, Gal	Zheng et al. (2014)
CW	ND	Man, Gal, Glc	Sovrani (2017)
HW	Pré biótica e anti-diabética	Glc, Gal, Man, Ara, Xil, Ácidos Urônicos	Rodrigues et al. (2017)

Notas: [1]HW - extrato aquoso quente, CW - extrato aquoso frio, EtOH - extrato etanólico. [2] Man – Manose, Glc – Glucose, Gal – Galactose, Ara – Arabinose, Xil – Xilose, Ram – Ramnose. [3] ND - Nada descrito.

c. *Pleurotus eryngii*

O *Pleurotus eryngii* é um cogumelo apreciado pela culinária internacional, e sua origem é francesa. Identificado como *Pleurotus eryngii* (DC.) Gillet de 1872. Sua classificação taxonômica consta no Quadro 3, a seguir:

Quadro 3. TAXONOMIA *Pleurotus eryngii*	
Reino	Fungi
Filo	Basidiomycota
Classe	Agaricomycotina
Subclasse	Agaricomycetes
Ordem	Agaricales
Família	Pleurotaceae
Gênero	Pleurotus
Espécie	*Pleurotus eryngii*

Fonte: Genebank, National Institute of Agrobiological Sciences.

O eryngii e seus subprodutos são objetos de estudo de diversas pesquisas, devido sua utilização como alimento e como substância terapêutica. Os estudos confirmam que além de serem excelente fonte de fibras dietéticas (polissacarídeos), eles são benéficos ao organismo humano e animal. A caracterização química de extratos e frações polissacarídicas e o estudo das atividades biológicas se difundiu e evolui conforme se obtém mais dados a respeito dos mecanismos biológicos dos quais os polissacarídeos participam. Atividade antinflamatória, antioxidante, hepatoprotetora, inibitória de tumor, antibacteriana e hipolipidêmica foram relatadas por estes estudos (Li et al., 2016a; Li et al., 2016b; Ren et al., 2016; Carbonero et al., 2006, Zhang et al., 2014). Apesar de a caracterização química ser habitual nos estudos em extratos de *Pleurotus eryngii*, é possível observar variações na composição química e no comportamento biológico, justificado pelos diferentes meios de preparo, extração e purificação que influenciam o tipo de polissacarídeo obtido (Li et al., 2016a; Li et al., 2016b; Ren et al., 2016; Carbonero et al., 2006, Zhang et al., 2014).

No Quadro 4 constam os trabalhos desenvolvidos com ênfase em caracterização química e atividade biológica de extratos polissacarídicos de *Pleurotus eryngii*.

Quadro 4:
Estudos com extratos polissacarídicos de *Pleurotus eryngii*

Tipo de extrato [1]	Atividade relatada	Monossacarídeo identificado [2]	Polissacarídeo identificado	Referência
HW	Antioxidante Bifidogênica	Man, Glc, Gal	ND [3]	Li & Shah (2016)
HW	Imunoestimuladora	Man, Glc, Gal	Manogalactana	Xu et al. (2016)
HW	Antioxidante (*in vitro*) Hepatoprotetora (*in vivo*)	Man, Glc, Gal	ND	Zhang et al. (2017)
HW	Antitumor (HepG-2)	Ác. Urônico, Man, Glc, Gal, Xil	ND	Ma et al. (2014)
HW	Antitumor (HepG-2)	Man, Glc, Gal	ND	Ren et al. (2016)
HW	Antitumor renal (*in vivo*)	Ác. Urônico, Ara, Man, Gal	ND	Yang et al. (2013)
HW	ND	Glc	β-Glucana(1,3)(1,6)	Carbonero et al. (2006)
HW	ND	3-*O*-Me Gal, Gal, Glc	Galactana 3-*O*-metilada	Carbonero et al. (2008)
NaOH	Potencial prébiótico	Glc, Gal, Man, GlcN	β-Glucana (1,3)(1,6) β-Glucana (1,3)	Synytsya et al. (2009)
HW	Anti-inflamatória (*in vitro*) Antiproliferativa (Caco2; HepG-2)	ND	ND	Li & Shah (2016b)
CW	Antioxidante (*in vitro*)	ND	ND	Zhang et al. (2014)
CW	Anti-melanoma	Man, Glc, Gal	Manogalactana	Biscaia et al. (2017)
CW	Antioxidante	ND	β-Glucana (1,3)(1,6)	Yan et al. (2019a)
HW	Imunomoduladora	Man, Glc, Gal	3-*O*-metil heterogalactana	Yan et al. (2019b)

Notas: [1]HW - extrato aquoso quente, CW - extrato aquoso frio, EtOH - extrato etanólico, NaOH - extrato com solução alcalina de hidróxido de sódio. [2]Man – Manose, Glc – Glucose, Gal – Galactose, Ara – Arabinose, Xil – Xilose. [3]ND: Nada descrito.11

III. JUSTIFICATIVA

Esse estudo tem como objetivo a caracterização química e a avaliação das atividades biológicas e propriedades reológicas de alguns polissacarídeos presentes nos cogumelos comestíveis *Pholiota nameko* e *Pleurotus eryngii*. Ambas espécies têm comprovações a respeito de sua atividade biológica e propriedades reológicas. No entanto, estes estudos precisam ser avançados, pois muitas vezes a estrutura química relacionada às atividades biológicas relatadas não são elucidadas, e em outros casos a estrutura foi caracterizada quimicamente, porém estudos biológicos não foram realizados, indicando que o estudo dos polissacarídeos destas espécies pode fornecer avanços científicos na área de química de carboidratos e também de sua aplicação terapêutica. A presente pesquisa contribui ao desenvolvimento científico para aproveitamento dos polissacarídeos oriundos de cogumelos comestíveis, desde a extração, purificação e caracterização química, sendo estes resultados importantes para o monitoramento do controle de qualidade de produtos que são ou que possam vir a ser fornecidos comercialmente, como por exemplo, os suplementos alimentares. O comprometimento com a continuidade dos estudos com tais espécies é justificável, visto que os polissacarídeos já são conhecidos por promover à saúde, e são utilizados com fins medicinais e alimentícios.

IV. Objetivos

a. Objetivo geral

Extrair, purificar, e caracterizar quimicamente os polissacarídeos dos cogumelos *Pholiota nameko e Pleurotus eryngii* e investigar e avaliar atividades biológicas e propriedade reológica.

b. Objetivos específicos

- Extrair os polissacarídeos dos cogumelos *Pholiota nameko* e *Pleurotus eryngii*, utilizando água em diferentes condições de temperatura e pressão
- Purificar as frações polissacarídicas
- Caracterizar a estrutura química dos polissacarídeos purificados
- Investigar e avaliar atividades biológicas dos polissacarídeos
- Avaliar a propriedade reológica de polissacarídeos que apresentem caráter viscoelástico

Artigo 1

Gelling functional property, anti-inflammatory and antinociceptive bioactivities of β-D-glucan from the edible mushroom *Pholiota nameko*

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ABSTRACT

A β-D-glucan was obtained from the edible mushroom *Pholiota nameko* by hot aqueous extraction and purification. NMR and methylation analyses of the purified fraction (GHW-PN, 1.46% yield) indicated the presence of a (1→3)-linked β-D-glucan, highly substituted (~27%) at *O*-6 by single units of β-D-Glc*p* or by (1→6)-β-D-Glc*p* fragments. The β-glucan (at 0.5, 1, and 2%) showed shear thinning behavior and when the concentration of the solution increased, there was an increase in apparent viscosity. The β-D-glucan presented gel-like behavior and thermal stability under a simulated pasteurization process, suggesting its potential as a thickening and gelling agent in products submitted to temperature variations. The β-D-glucan at 0.3, 1.0 and 3.0 mg kg^{-1} significantly inhibited the inflammatory pain in 24.8, 56.9 and 82.3%, respectively, in the formalin-induced nociception in mice. The results pointed out that the β-D-glucan (GHW-PN) isolated from *P. nameko* presents potential application for the food industry or for medical purposes.

Keywords: β-D-Glucan; gel-like behavior; pain; *Pholiota nameko*; edible mushroom.

1. Introduction

Edible mushrooms are a source of various nutrients, including fibers such as β-glucans, which contribute to the most diverse forms of application in the food and pharmaceutical industry. Depending on their chemical structure and intermolecular interactions, glucans can act as thickening or gelling agents, which are very useful and important in the food industry [1, 2, 3, 4, 5]. They influence the texture, taste, quality, and shelf life of foods, being therefore used to formulate new food products. Rheological studies have been applied to determine viscosity and gelling properties of glucans, with the aim of using such molecules as food additives. These evaluations have shown that glucans present interesting properties for this field and therefore should be carefully examined about their rheological characteristics [2, 6, 7].

In eastern culture, mushroom extracts have been used for the treatment of several diseases, including pain and inflammatory conditions. The mushroom-extracted polysaccharides, mainly β-D-glucans, represent the major component responsible to its bioactive functions [8, 1, 9, 10]. Nevertheless, the anti-inflammatory and immunomodulatory effects of β-D-glucans are strongly related to their chemical structures and could variate depending on the tertiary structure in solution, molecular weight, branching ratio and solubility in water [11, 12, 3, 13, 14].

Pholiota nameko is a basidiomycete popularly known as Nameko and it contains approximately 60% of carbohydrates [15]. The great amount of carbohydrates grabbed the attention of researchers and a branched β-glucan has recently been extracted and purified from a cold-water extract of this edible mushroom by [7].

Different isolation and purification procedures may result in distinct extracted molecules. For example, cold water extractions are able to isolate more soluble

polysaccharides, while hot water or alkaline extractions are stronger enough to obtain the insoluble ones [10]. This was also observed by Moreno et al. (2016) [11], who extracted β-D-glucans with different molecular weight and branching degrees by distinct extractions procedures from the *Cookeina tricholoma* mushroom. There are other extraction techniques such as pressurized liquid extraction (PLE), supercritical fluids (SFE), as well as microwave extraction (MAE) and ultrasound (UAE), which improve the extraction of extracts rich in polysaccharides or other bioactive compounds [16, 17, 18, 19].

Therefore, this work aimed to develop further scientific knowledge on β-D-glucans from *P. nameko*, through extraction at high temperature followed by detailed chemical and rheological characterization of the purified glucan, as well as its functional property as antinociceptive agent using an inflammatory pain model *in vivo*.

2. Material and Methods

2.1. *Pholiota nameko* mushroom

P. nameko mushroom was kindly provided *in natura* (2.5 kg) by Nayumi Cogumelos Especiais company, located in Mogi das Cruzes, São Paulo, Brazil (Latitude 23º 31 '29 "South; Longitude 46º 11' 14" West).

2.2. Extraction and purification of β-D-glucan

Freeze-dried mushroom was defatted and submitted to cold water extractions following the reported procedures of Sovrani et al. (2017) [7]. The remained residue from this extraction was used to proceed with hot water extractions. Briefly, residue II (Fig. 1) was extracted with water at 100 °C for 6 h (3 x 1 L each). The extracts were separated from residue by centrifugation (8000 rpm, 15 min., 25 ºC). The polysaccharides were

recovered by precipitation with 3 volumes of cold ethanol, followed by centrifugation (8500 rpm, 20 min, 10 ºC), dialysis (12 – 14 kDa, for 24 h), and lyophilization. This was termed hot aqueous extract (HW), which was then solubilized in a small volume of water, frozen and submitted to slowly thaw (3 x) until complete precipitation of the cold water-insoluble polysaccharides, which were recovered by centrifugation (9000 rpm, at 4 ºC, for 20 min) [20]. In order to remove glycogen, the insoluble fraction (IHW) was treated with α-amylase, according to the manufacturer's instructions, to promote lysis of α-(1→4) bonds. The purified β-D-glucan (GHW-PN) was recovered by precipitation with cold ethanol (2 volumes), centrifugation, dialysis and lyophilization (Fig. 1).

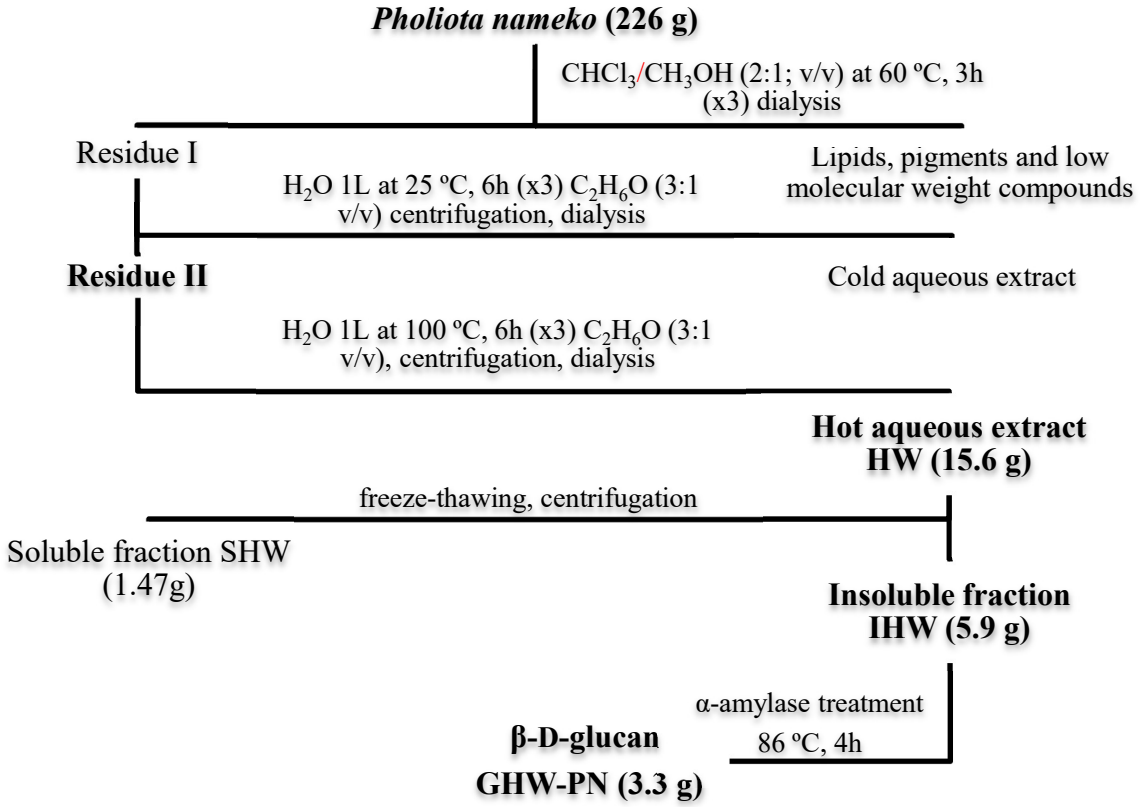


Fig. 1. Scheme of extraction and purification of β-D-glucan from *P. nameko*.

2.3 Analysis of monosaccharide composition by GC-MS

The polysaccharide fractions (1 mg) were hydrolyzed with 2 M TFA at 100 ºC for 8 h, followed by evaporation to dryness. The dried carbohydrate samples were dissolved in distilled water (100 µL) and 1 mg of $NaBH_4$ was added. The solution was held at room temperature overnight to reduce aldoses into alditols [21]. The product was dried and neutralized by the addition of acetic acid. The excess of $NaBH_4$ was removed with an addition of methanol (x 2), and evaporation of methyl tetraborate under a compressed air stream in a fume hood. Acetylation of the alditols was performed in pyridine–Ac_2O (200µL; 1:1, v/v), for 30 min at 100 ºC. The resulting alditol acetates were extract with $CHCl_3$. The pyridine was removed by 5% $CuSO_4$ complexation. The resulting derivatives were analyzed by GC-MS (Varian CP-3800 gas chromatograph coupled to an Ion-Trap 4000 mass spectrometer), using a VF5 column (30 m x 0.25 mm i.d.) programmed from 100 to 280 °C at 10 °C min^{-1}, with He as the carrier gas. Monosaccharides were identified by their typical electron impact breakdown profile and retention time, in comparison with standards, and the results were expressed as mol%.

*2.*4 Static light Scattering

Molar mass of GHW-PN was determined by static light scattering (SLS) in batch, unfractionated, using the static mode of the equipment NanoDLS Brookhaven. The batch quantities determined was the mass-average molar mass (M_w). For SLS measurement the follow equation was used:

$$\frac{Kc}{I_R} = \frac{1}{M_w} \qquad \text{(eq. 1)}$$

where $I_R(q, c)$ is the excess Rayleigh scattering ratio (cm^{-1}) at the scattering vector (q) and concentration (c). K is an optical constant, given vertically polarized incident light by equation 2:

$$K = \frac{4\,\pi^2 n^2 \left(\frac{dn}{dc}\right)^2}{N_A \lambda^4} \qquad \text{(eq. 2)}$$

where *n* is the solvent refraction index as 1.4768 for DMSO, λ is the vacuum wavelength of the incident light (632.8 nm) and *dn/dc* is the differential refractive index for the polysaccharides determined in DMSO using a Viscotek VE3580 refractometer. The *dn/dc* used are 0.05 mL/g for the GHW-PN in DMSO. The *dn/dc* results are compatible to Qin, Kes & Christensen (2013) [22] and Ding et al. (2001) [23] using similar solvents. The concentration used was 1×10^{-3} g mL^{-1} using DMSO as solvent. The sample was stirred during 12 h and heated at 70 ºC during 30 min to solubilize. The scattering vector was obtained by $q = \frac{4\pi}{\lambda}\sin(\frac{\theta}{2})$.

The dynamic light scattering (DLS) measurements were performed in the NanoDLS Brookhaven at 90º detection and it was used for intensity autocorrelation computation and z-average equivalent sphere hydrodynamic radius (R_h) as determined by the Stokes-Einstein equation 3.

$$R_h = \frac{kT}{6\pi\eta D} \qquad \text{(eq. 3)}$$

where k is the Boltzmann constant, t is the temperature in Kelvin, η is the solvent viscosity, and D is the diffusion coefficient.

2.4. Methylation analysis

Per-*O*-methylation of the GHW-PN (5 mg) was carried out using NaOH-Me_2SO-MeI [24]. After isolation of the products by neutralization (HOAc), dialysis, and evaporation, the methylation process was repeated. The per-*O*-methylated derivatives were submitted to methanolysis with MeOH-HCl 3 N (1 mL) for 2 h at 80 °C, followed by evaporation and hydrolysis using sulphuric acid (1 mL, 2 $mol.L^{-1}$) for 16 h at 100 °C. The acid solution was neutralized with $BaCO_3$ and then filtrated, and the sample was

reduced with $NaBD_4$ (sodium borodeuteride) and acetylated as described above, to give a mixture of partially *O*-methylated alditol acetates, which was analyzed by GC-MS using a VF5 capillary column. The derivatives were identified by their typical retention time and electron impact profile, in comparison to standards according to Sassaki et al. (2008) [21].

2.5. Controlled Smith Degradation of the β-D-glucan

An aliquot of GHW-PN (100 mg) was dissolved in 15 mL of 0.05 M $NaIO_4$ in the dark at room temperature. After 72 h under stirring, the solution was dialyzed (2 kDa, 24h) and reduced with $NaBH_4$ at pH 8-9, for 12 h. The solution was then neutralized with HOAc, dialyzed (2 kDa, 24 h), and concentrated to 50 mL [25] The residue was subjected to partial hydrolysis with TFA (2 $mol.L^{-1}$) for 30 min at 100 °C, under reflux, followed by dialysis (2 kDa, 24 h). The resistant material was lyophilized and analyzed by NMR spectroscopy.

2.6. Nuclear Magnetic Resonance (NMR)

^{1}H, ^{13}C, HSQC, HMBC, COSY, TOCSY NMR spectra of polysaccharide fractions were obtained using a 400 MHz Bruker model Avance III spectrometer with a 5 mm inverse probe, at 70 °C in Me_2SO-d_6. Chemical shifts (δ) were expressed in ppm relative to Me_2SO-d_6 ^{13}C (δ 39.5) and ^{1}H (δ 2.40) resonances. The NMR signals were assigned according to 1D and 2D NMR experiments and literature data.

2.7. Rheological measurements

Fraction GHW-PN was solubilized in distilled water at 0.5, 1 and 2% (w/w) and kept under magnetic stirring for 24 h at 25 °C. After that, it was heated at 50 °C with

continuous stirring for 15 min and let stand for 30 min to reach the room temperature. Rheological measurements were carried out using a HAAKE MARS II rheometer (Thermo Fisher Scientific, Karlsruhe, Germany), at 25 °C with a cone-plate (C60/2◦TiL) measurement system. A 1 mm measurement gap was used. The temperature was controlled by a circulating water bath (DC5, Haake) coupled to a Peltier temperature control device (TC 81, Haake). During measurements on temperature variation, the system containing the sample was covered with a sample hood (POM 222-1903) to prevent water evaporation. Before all rheological measurements, samples were maintained on the plate for 300 s, to allow temperature equilibrium.

Viscoelastic behavior of samples was evaluated using frequency sweeps (0.02–10 Hz) with a strain of 1%. On thermostability studies, temperature sweeps were performed by fast heating (25 – 90 °C), where the sample was maintained for 1 minute under 90 ºC, and subsequent cooling (90 – 4 °C), mimicking the pasteurization process, at a rate of 2 °C/ min, at a frequency of 1 Hz and a strain of 1%.

Flow curves were evaluated in the CR mode (controlled shear rate) by applying an increasing shear rate (0.005–1000 s^{-1}) during 300 s. The shear stress (τ) was then measured as a function of shear rate. The data of flow curves were evaluated and fitted according to the rheological models of Power Law [$\sigma = K\dot{\gamma}^n$], - where σ is the shear stress (Pa), K is the consistency index (Pa.s^n), $\dot{\gamma}$ is the shear rate (s^{-1}), n is the flow behavior index (dimensionless); and the Cross model [$\eta_a = \eta_\infty + (\eta_0 - \eta_\infty)/ 1 + (\alpha_c\dot{\gamma})^m$], where η_a is apparent viscosity, η_∞ is infinite shear rate viscosity (Pa.s), η_0 is zero-shear-rate viscosity (Pa.s), and α_c is Cross time constant related to the relaxation times from polymer in solution (Rao 2007 - 7). The $\alpha_c = 1/\dot{\gamma}_c$, where $\dot{\gamma}_c$ is the critical shear rate that marks the end of the zero shear rate Newtonian plateau and m (dimensionless) is (1 - n) [2].

The software RheoWin 4 Data Manager was employed to obtain the rheological parameters. All the analyses were performed in triplicates, and graphs show the mean values and their corresponding standard error of the mean (SEM). Data of frequency sweeps were compared using one-way analysis of variance (ANOVA) and Tukey's tests were applied to verify the differences between mean at the same frequency (0.1, 1, or 10 Hz). Data were considered different at a significant level of $p < 0.05$

2.8. Experimental animals

The experiments were conducted using male Swiss mice (*Mus musculus*), weighing 25-35 g. The animals were kept under a 12 h light/dark cycle, controlled temperature (22 ± 2 °C), air exhaustion, with food (Nuvi-Lab CR-1, Quimtia S/A, Brazil) and water provided *ad libitum*. The animals were supplied by the Biological Sciences Sector of the Federal University of Paraná (UFPR) and all the experimental protocols were in agreement with the "Guide for the Care and Use of Laboratory Animals" (8th edition, National Research Council, 2011) and approved by the local Ethics Committee (CEUA / BIO – UFPR, number 657).

2.9. Nociception induced by formalin

The intraplantar injection of formalin applied in the hind paw allowed us to evaluate distinct types of pain. The first moment (0 to 5 min, phase 1) corresponds to a direct chemical stimulation of nociceptors (neurogenic pain). While the subsequent 15 to 30 min (phase 2) corresponds to a pain caused by the release of inflammatory mediators (inflammatory pain) [26, 27]. Then, the mice were pretreated by intraperitoneal (i.p.) route with vehicle (C: 0.9% sterile saline, 20 μL) and β-D-Glucan (GHW-PN) (0.3, 1.0 and 3.0 mg kg^{-1}). After 30 min, the nociception was induced with 2.5% formalin

intraplantar injection (20 μL, i.pl.). The time that the animal spent licking and/or biting the injected hind paw, in both phases, was considered as an indicative of nociception and measured in seconds (s).

2.10. Statistical analysis

Data were analyzed by the statistical program GraphPad Prism 6®, version 6.01, (GraphPad Software, USA). The data of formalin test were presented as mean ± standard error of the mean (SEM) and was evaluated using one-way ANOVA, followed by Bonferroni multiple comparison test. The ID_{50} value (i.e. the dose of GHW-PN necessary to reduce the formalin nociceptive response by 50% relative to the control value) was determined by nonlinear regression analysis and reported as geometric means accompanied by their respective 95% confidence limits. The significant difference between groups (n= 6-8) was considered when $P < 0.05$.

3. Results and discussion

3.1. Structural characterization of β-D-glucan

The hot water extraction performed on *P. nameko* yielded 15.6 g of HW fraction. Its monosaccharide composition showed mainly glucose (79.6%), as well as small amounts of mannose (6.1%) and galactose (4.9%). Accordingly, NMR spectra (Fig. 2A, B) showed characteristic signals of glucans. Resonances at δ 102.6/4.52 and 102.6/4.22 ppm correspond to glucose in β configuration, while signals at δ 85.9/3.48, 85.9/ 3.30 and 85.9/3.26 ppm are typical of substituted C-3/H-3 from (1→3)-linked β-D-glucans. Furthermore, less intense signals at δ 100.3/5.03 and 79.0/3.38 ppm, from anomeric carbon in α-configuration and substituted C-4/H-4, respectively, were also observed suggesting the presence of an α-D-glucan. Taking in consideration that glycogen is the

energy reserve polymer of fungi [28, 29], it is most probable that this sample is composed of a mixture of β-D-glucans and glycogen. Besides, NMR signals from mannose and galactose were not observed, probably due to their small concentration in HW.

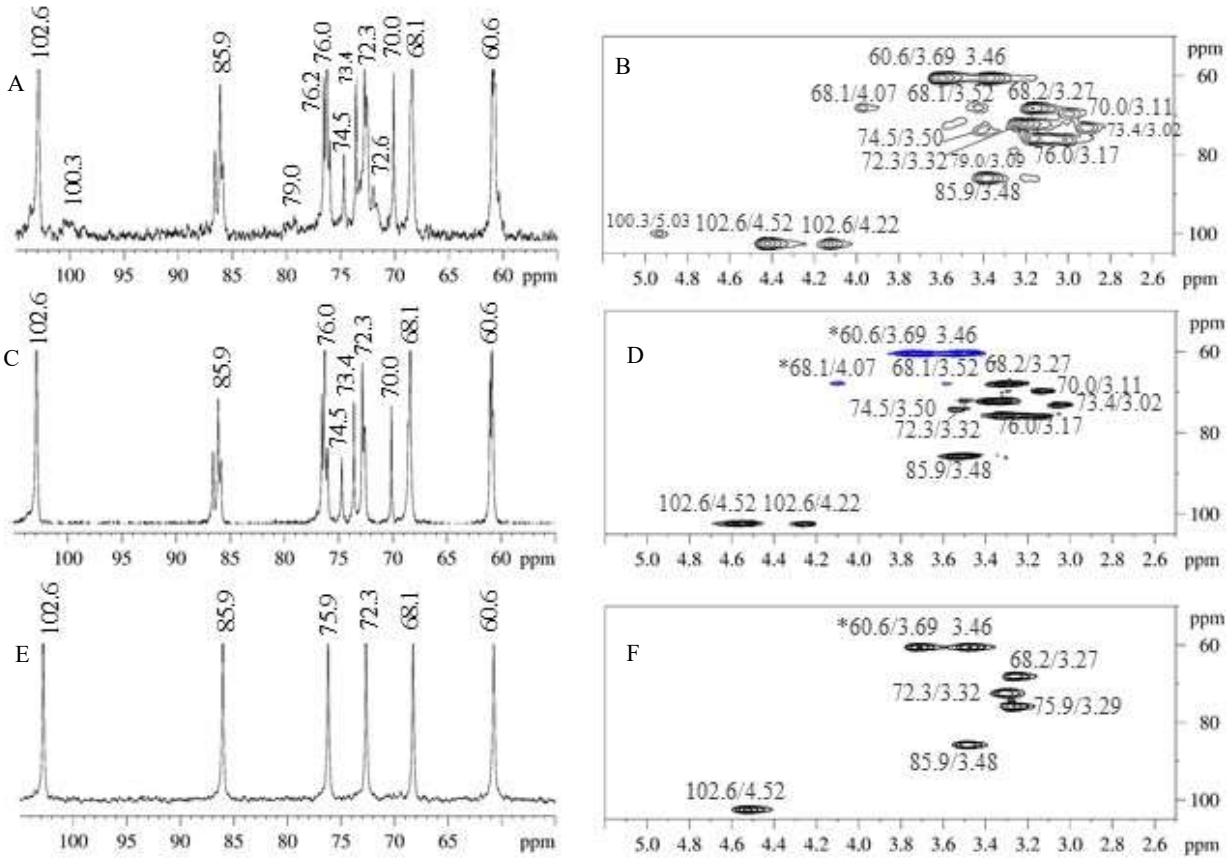


Fig. 2. ^{13}C NMR and HSQC spectra of hot water extract - HW (A, B), purified β-glucan - GHW-PN (C, D) and polymer obtained after controlled Smith degradation of GHW-PN (E, F); Samples in Me_2SO-d_6 at 70 ºC (chemical shifts are expressed in δ ppm)

.

As an attempt to purify this fraction, it was solubilized in water and subjected to freeze and slowly thaw, until obtaining a complete separation of cold water-soluble (SHW) and insoluble (IHW) fractions, which were recovered by centrifugation (Fig. 1). Since IHW presented the higher yield, it was selected for the purification process. Thus,

as it still presented NMR signals of glycogen (data not shown) it was further treated with α-amylase. The freeze-thawing process followed by enzymatic treatment to remove glycogen was successful in generating a purified sample named GHW-PN. Its monosaccharide composition presented only glucose and the NMR spectra (Fig. 2 C, D) showed only signals relative to the β-D-glucan. Signals were observed in ^{13}C and HSQC spectra at δ 102.6/4.52 and 102.6/4.22 ppm corresponding to C-1/H-1 in β configuration; while signal at δ 85.9/3.48 arose from substitution at *O*-3 in β-D-Glc*p* units. Signals from non-substituted and *O*-substituted C-6 were observed at δ 60.6/3.69-3.46 and at δ 68.2/4.07-3.52 ppm, respectively. This result was confirmed by inverted signals in DEPT-^{13}C NMR experiment (data not shown). All the assignments were compared with the literature data [11, 3, 7] and are listed on Table 1. Mass-average molar mass (M_w) obtained from SLS experiments was 1.4 x 10^6 g mol^{-1} in DMSO. Using the Stokes-Einstein equation the R_h was determined in DMSO as 80 ± 19 nm.

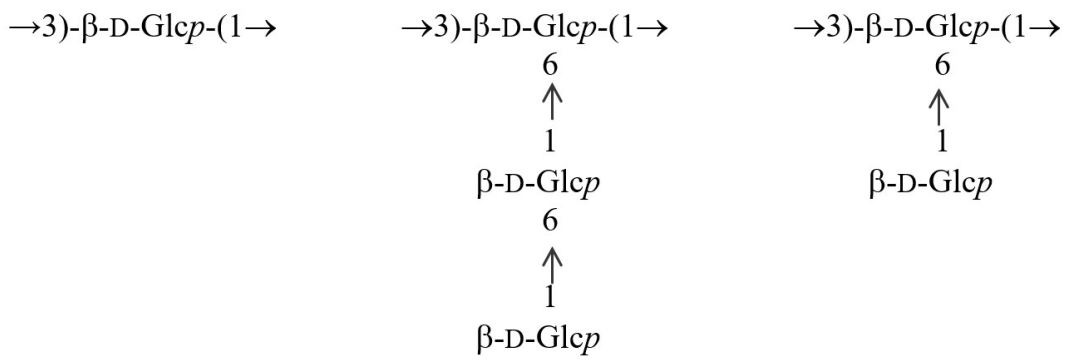
Table 1: ^{1}H and ^{13}C NMR chemical shifts for the branched (1→3)(1→6)-β-D-glucan present in fraction GHW-PN isolated from *Pholiota nameko* mushroom.

Sample	Units/assignments[a]		1	2	3	4	5	6	
								6a	6b
GHW-PN	Glc*p*-(1→	^{13}C	102.6	73.4	na[b]	70.0	76.0	60.6	
		^{1}H	4.22	3.02	na[b]	3.11	3.17	3.69	3.46
	3→)-Glc*p*-(1 →	^{13}C	102.6	72.3	85.9	68.2	75.9	60.6	
		^{1}H	4.52	3.32	3.48	3.27	3.29	3.69	3.46
	6→)-Glc*p*-(1 →	^{13}C	102.6	73.4	na[b]	70.0	74.5	68.2	
		^{1}H	4.22	3.02	na[b]	3.11	3.50	4.07	3.52
	3,6→)-Glc*p*-(1→	^{13}C	102.6	72.3	85.9	68.2	74.5	68.2	
		^{1}H	4.52	3.32	3.48	3.27	3.50	4.07	3.52

[a] Samples in Me_2SO-d_6 at 70 ºC. Assignments based on ^{1}H, ^{13}C, HSQC, HMBC, COSY, TOCSY experiments.

[b] Not assigned due to overlapping.

Methylation analysis of GHW-PN (Table 2) presented the derivatives 2,3,4,6-Me_4-Glc*p* (27.0%), 2,4,6-Me_3-Glc*p* (36.5%), 2,3,4-Me_3-Glc*p* (8.9%), and 2,4-Me_2-Glc*p* (27.0%). These data suggest that the β-D-glucan is composed mainly by (1→3)-linkages, but also in high proportion (~27%) of (1→6) branches. The presence of 2,3,4-Me_3-Glc*p* indicates that side-branches may be composed by two or more (1→6)-linked Glc*p* residues. Thus, the β-D-glucan present in GHW-PN fraction is formed by the following structural fragments:



To confirm if the main chain of this β-D-glucan was (1→3)- or (1→6)-linked, the GHW-PN fraction was subjected to Controlled Smith Degradation (see item 2.5). Based on the ^{13}C and HSQC spectra (Fig. 2E, F), it can be stated that the main chain of this β-D-glucan is (1→3)-linked, which is resistant to the oxidation with $NaIO_4$. Only six signals were observed at δ 102.6/4.52 (C-1/H-1); δ 72.4/3.30 (C-2/H-2); δ 85.9/3.48 (C-3/H-3); δ 68.2/3.26 (C-4/H-4); δ 76.2/3.11 (C-5/H-5); and δ 60.6/3.69 and 3.46 (C-6/H-6).

A (1→3)-(1→6)-branched β-glucan has recently been extracted and purified from a cold-water extract of this edible mushroom by Sovrani et al. (2017) [7]. Although both have the same type of glycosyl linkages, they differ slightly in the amounts of (1→6)-linked Glc*p* units in the side chains. This structural difference was enough to influence the gel strength formed by the glucans, as discussed below in detailed rheological studies. Moreover, a functional property as inhibitor of inflammatory pain induced by formalin was also showed herein for β-D-glucan present in GHW-PN fraction.

Table 2. Partially *O*-methylalditol acetates formed on methylation analysis of *P. nameko* β-D-glucan present in GHW-PN fraction.

Partially *O*-methylated alditol acetates[a]	**Mol %**	**Linkage type**[b]	**Ions**
2,3,4,6 Me_4-Glc	27.0	Glc*p*-(1→	87, 102, 118, 129, 145, 161
2,4,6 Me_3-Glc	36.5	3→)-Glc*p*-(1→	87, 101, 118, 129, 161
2,3,4 Me_3-Glc	8.9	6→)-Glc*p*-(1→	87, 99, 102, 118, 129, 162, 189
2,4 Me_2-Glc	27.0	3,6→)-Glc*p*-(1→	87, 101, 118, 129, 139, 160, 189, 234

[a] GC-MS analysis on a VF5 capillary column.

[b] Based on derived *O*-methylalditol acetates.

3.2. Rheological characterization of β-D-glucan GHW-PN

The flow curves of β-D-glucan (GHW-PN) in all the tested concentrations showed shear-thinning behaviors. These are typical behaviors of polysaccharides in solution, where they tend to align towards the flow, reducing their viscosity [2]. It is also possible to observe that as the concentration of the solution increases (from 0.5% to 2%), there is an increase in apparent viscosity.

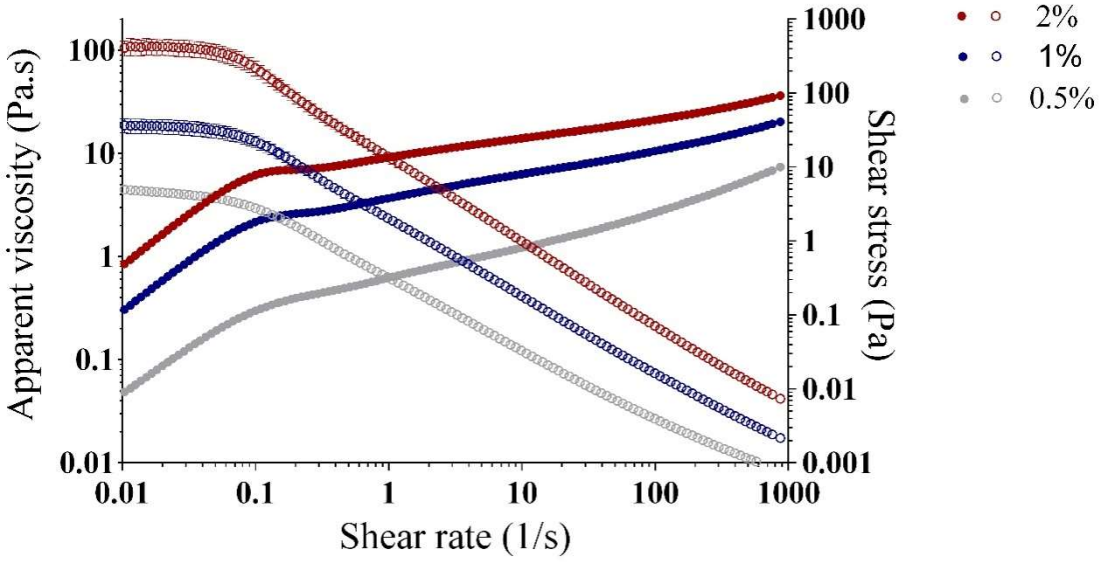


Fig. 3. Flow (full symbols) and viscosity (empty symbols) curves of β-glucan (GHW-PN) at 0.5%, 1% and 2% (w/w).

A similar behavior was observed for the β-glucan (at 2%) purified from cold aqueous extract, but the viscosity values were much lower (3.5 Pa s at 1 s^{-1}) [7] than the ones obtained herein for the hot water extracted β-glucan (GHW-PN), which had a viscosity of 31.6 Pa s at 1 s^{-1} at the same concentration. This may be caused by their differences in side branch sizes, as showed above, which provides different molecular interactions and consequently solutions with different viscosities [30].

The experimental data of all flow curves were well fitted to both Power Law and Cross models with high regression coefficients (R^2) values (≥ 0.99). Each model brings different parameters, Power Law model brings data about the consistency index and the flow behavior index while the Cross model brings data about zero shear rate viscosity, infinite shear rate viscosity, and critical shear rate. All samples showed flow behavior index lower than 1 (Table 3), indicating shear-thinning behaviors [2]. Other studies about mushrooms polysaccharide dispersions demonstrate similar behaviors [6, 31], indicating that the glucans from *Pholiota nameko* have potential technological applications. The

increase in the GHW-PN concentration was accompanied by an increase in the shear thinning behavior, and in the consistency coefficient (*k*), with a decrease in the flow behavior index (*n*). The Cross model was better fitted than the Power Law model. The Cross model revealed the viscosity at zero shear rate (η_0), which gradually increased according to the concentration. The infinite shear rate viscosities of glucan at both 0.5% and 1% were similar, while the glucan at 2% reached η_∞ twice as high as these. The critical shear rate ($\dot{\gamma}_c$) for glucan dispersions at 0.5%, 1%, and 2% were 0.01245 s^{-1}, 0.02675 s^{-1}, and 0,01991 s^{-1}, respectively (Table 3), suggesting that although zero shear rate viscosity of glucan at 2% was almost nine times higher than that found glucan at 1% and nineteen times higher than that for glucan at 0.5%, all three dispersions started their power law region under almost similar shear rate.

Table 3. Rheological parameters based on flow curves of β-glucan (GHW-PN) at 0.5%, 1% and 2% (m/v).

Rheological parameters	**β-D-glucan (%)**		
	0.5	**1.0**	**2.0**
Power Law			
K ($Pa.s^n$)	0.4192	1.82	9.002
N	0.4058	0.2965	0.1976
R^2	0.9949	0.9981	0.9945
Cross			
M	0.7324	0.7589	0.8475
η_0 (Pa.s)	15.54	33.65	282.2
η_∞ (Pa.s)	0.00369	0.003567	0.00885
$\dot{\gamma}_c$ (s^{-1})	0.01245	0.02675	0.01991
R^2	0.9998	0.9998	0.9993

K, consistency index; *n*, flow behavior index; m, (1-*n*); R^2, regression coefficient, η_0, zero shear rate viscosity; η_∞ is infinite shear rate viscosity;$\dot{\gamma}_c$, critical shear rate.

Analyzing the viscoelastic property of β-glucan (GHW-PN) (Fig. 4), gel-like behavior can be observed, since elastic modulus (G') was higher than viscous modulus (G") over an entire analyzed range of frequencies [32]. The gel strength increased with the polysaccharide concentration and all analyzed GHW-PN solutions could be classified as weak-gel since both moduli were frequency-dependent [33]. This viscoelastic behavior was also found for other polysaccharides from edible mushrooms as *Auricularia auricular-judae* and *Flammulina velutipes* [31, 6, 34].

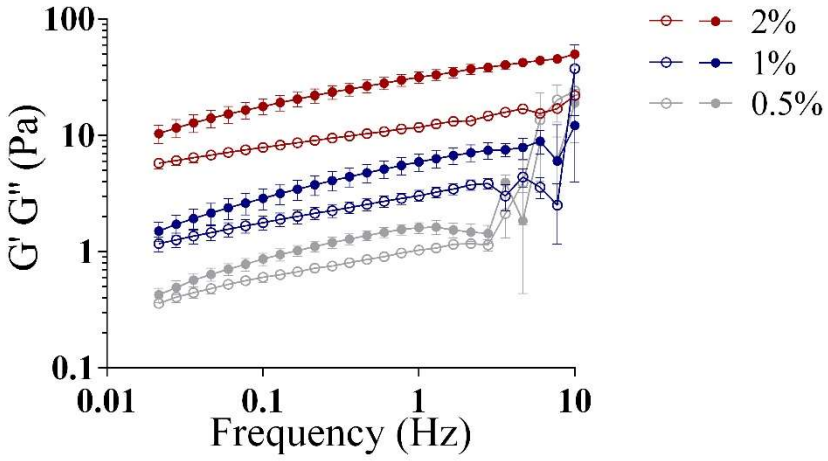


Fig. 4. Frequency sweeps at 25°C of β-glucan (GHW-PN) at 0.5%, 1% and 2% (m/v). Elastic modulus (G') represented by full symbols while viscous modulus (G") by open symbols. The strain was fixed at 1%.

Temperature is a factor that directly affects the characteristics of a solution or dispersion, as well as its quality, and in some cases, the temperature can irreversibly alter its physico-chemical properties. Since GHW-PN can be considered an additive as a thickening or gelling agent, it is important to know its behavior in thermal processes, as pasteurization. In Fig. 5, the viscoelastic behavior of GHW-PN solutions that were subjected to a temperature of 90 °C for one minute, and then underwent cooling (up to 4

°C), simulating the pasteurizing process is showed. This thermal treatment is applied in several food industrial processes [35].

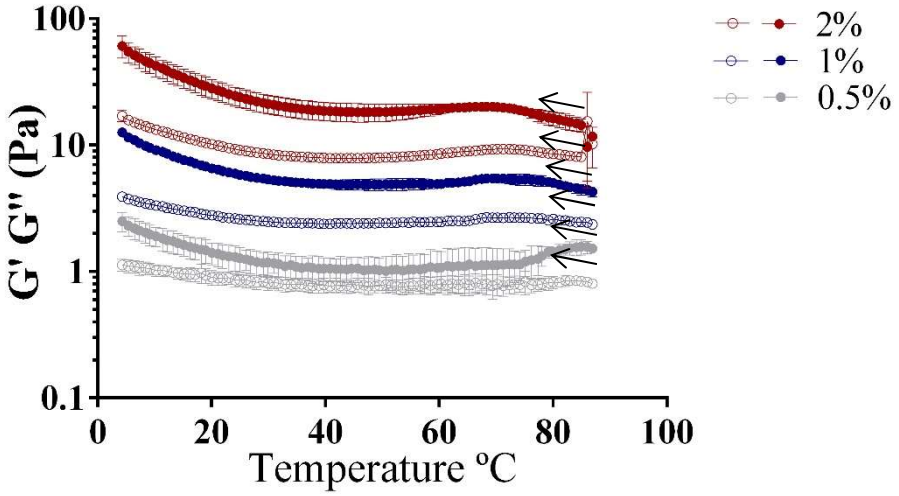


Fig. 5. Elastic moduli (*G'*, full symbols) and viscous moduli (*G''*, open symbols) as a function of temperature cooling of β-glucan (GHW-PN) at 0.5%, 1% and 2%, at 1 Hz and 1 Pa.

The results suggested that high temperature (90 ºC) does not affect the gel-forming capacity of GHW-PN. Thermal stability of β-glucans was also observed by Sovrani et al. (2017) [7] and Bao et al. (2016) [31], in a temperature ramp varying from 5 °C→60 °C→5 °C and 5 °C→80 °C→5 °C, respectively. In contrast, Xu et al. (2016) [6] noted that their sample presented thermal hysteresis between 20 °C and 40 °C, at both heating and cooling process (temperature ramp 10 °C→50 °C→10 °C), suggesting that gel behavior was thermally reversible.

The gel strength of GHW-PN increased at the cooling process, from 90 ºC to 4 ºC, this was more evident at 1% and 2% where G' values were almost five times higher at 20 °C to 4 °C than those at 90° to 20°C, suggesting the formation of new inter- and

intramolecular interactions spots under cooling. In this cooling process any loss of gel formation capacity was observed, even at the lowest concentration (0.5%). This result from pasteurization simulation suggests that glucan GHW-PN can be subjected to this fundamental sterilization industrial process, especially for certain food products [35].

3.3. Effect of GHW-PN (β-D-glucan) on formalin-induced nociception

The antinociceptive effect of polysaccharides has been studied by several research groups due to its interesting potential, and a very efficient and simple model to evaluate this activity is the formalin test.

Following the intraplantar injection of formalin, a first phase of pain response (neurogenic pain) is attributed to the direct stimulation of nociceptors with consequent neuropeptides release, such as substance P and calcitonin gene-related peptide [27]. After a quiescent period, the second phase of pain response appears as a result of the release of prostaglandins, histamine and bradykinin, namely inflammatory pain [36].

Therefore, we investigated the antinociceptive effect of β-D-glucan (GHW-PN) against nociception induced by formalin *in vivo*. The intraperitoneal pre-treatment with GHW-PN at all doses tested was not able to reduce the nociception triggered in the first phase of the formalin test (Fig. 6A). Conversely, several studies demonstrated that polysaccharides can inhibit the nociceptive behavior only in the inflammatory phase of the formalin test [37, 38, 39]. Interestingly, the same pattern was also observed in the present study. β-D-glucan (GHW-PN) at 0.3, 1.0 and 3.0 mg kg^{-1} significantly reduced the inflammatory pain response induced by formalin in 24.8% (136.4 ± 21.0 s), 56.9% (83 ± 13.6 s) and 82.3% (32.75 ± 9.0 s), respectively, compared to the vehicle control group (C: 190 ± 12.9 s). Considering the pharmacological point of view, it is important to mention that the ID_{50} of GHW-PN was 0.7 (0.4 - 1.0) mg kg^{-1}, indicating the dose of

this polysaccharide that can reduce in 50% the second phase of formalin-induced nociception (Fig. 6B).

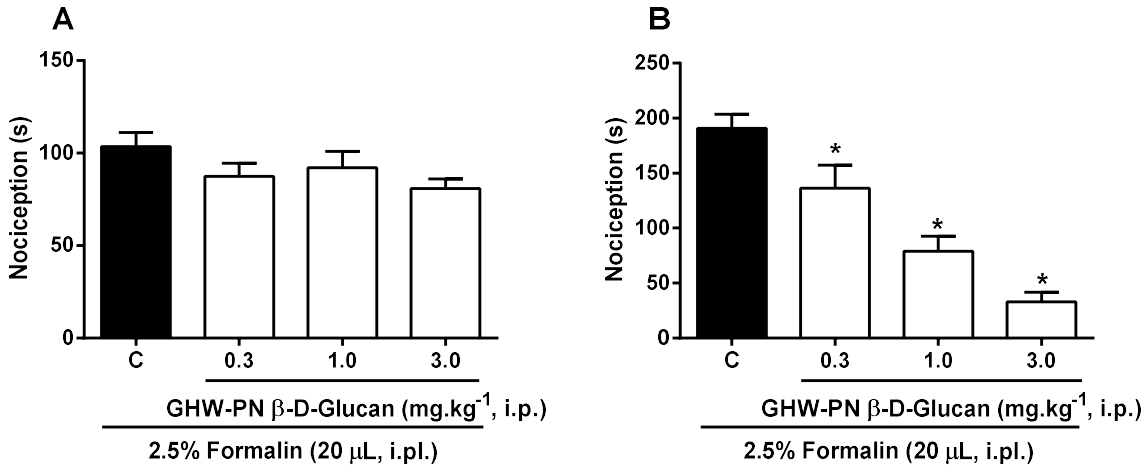


Fig. 6. Effect of intraperitoneal administration of β-D-Glucan (GHW-PN) for the formalin test in mice (Panel A, neurogenic phase; Panel B, inflammatory phase). The animals were pretreated with vehicle (C: 0.9% sterile saline, i.p.) or β-D-Glucan (0.3, 1.0 and 3.0 mg kg^{-1}, i.p.) and after 30 min, nociception was induced by intraplantar 2.5% formalin injection (20 μl/i. pl.). Results are expressed as mean ± standard error of the mean (n=6-8). The comparison between groups was performed by One-way ANOVA followed by the Bonferroni test. *$p \leq 0.05$ in comparison to the control group.

Previous studies using the formalin test showed that β-D-glucans from *Lactarius rufus*, *Amanita muscaria* [12, 40]; and *Cookeina tricholoma* [11], also presented antinociceptive activity. Smiderle et al. (2008) [**3]** demonstrated that a (1→3), (1→6)-linked β-glucan from *Pleurotus pulmonarius* reduced the nociception in both phases of formalin (neurogenic and inflammatory) at 30 mg kg^{-1}. Moreover, Baggio et al. (2010) and Baggio et al. (2011) [41, 42] complemented its mechanism of antinociception showing the involvement of ionotropic glutamate receptors, transient receptor potential channels and interleukin-1β pathways.

The non-specific immunomodulatory property of polysaccharides includes the reduction of pro-inflammatory cytokine release and function enhancement of the host immune system [12, 9]. The effectiveness of these glucans in reducing the inflammatory pain was related to their chemical structure (molecular weight and degree of branching), however, it is worth noting that a glucan from the alkaline extract of *C. tricholoma* had no bioactivity [11]. In comparison to the studies above mentioned in which the ID_{50} of glucans varies between 2.35 to 12.9 mg kg^{-1}, the lower ID_{50} of 0.7 mg kg^{-1} of GHW-PN indicates that the β-D-glucan isolated from *P. nameko* is more potent in promoting antinociceptive effects in the formalin model. Although we did not investigate the mechanisms underlying the antinociceptive effect of GHW-PN, it is possible to suggest that the inhibition of synthesis or release of inflammatory mediators such prostaglandins and/or cytokines could be involved [37, 39]. In addition, a polysaccharidic fraction extracted from *P. nameko* promoted the downregulation of the NF-κB signaling pathway via the TLR2 receptor, suggesting a new mechanism that could also explain the anti-inflammatory effect observed in this study [43].

4. Conclusions

A β-D-glucan (GHW-PN) was obtained and purified from the hot water extract of *P. nameko* fruiting bodies. Chemical analyses showed it has a main chain of (1→3)-linked β-D-Glc*p* units, highly substituted at *O*-6 by single β-D-Glc*p* units or (1→6)-linked β-D-Glc*p* branches. A branched (1→3), (1→6)-β-D-glucan (bG-PN) had already been isolated by cold water extraction from this mushroom. However, slight differences in their chemical structure produced an interesting variation in their rheological behavior demonstrated by the higher viscosity of GHW-PN in comparison to bG-PN. The GHW-PN gel behavior was observed at all tested concentrations; although they were weak-gels

since both modulus were frequency-dependent. Under a pasteurization simulation, GHW-PN showed to be resistant, suggesting that it could be used as a food additive, as a gelling or thickening agent in the food industry, including the processes which involve this thermal process. In addition, in the formalin-induced pain model, GHW-PN significantly reduced the inflammatory pain response, with the lowest ID_{50} value (0.7 mg kg^{-1}) when compared with other β-D-glucans previously tested in the same experimental conditions.

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References

1. Wasser S. Medicinal mushrooms as a source of antitumor and immunomodulating polysaccharides. *Appl Microbiol Biotechnol*. 2002; 60(3):258-274.
2. Rao MA (Department of FS and T. *Rheology Of Fluid And Semisolid Foods - Principles And Applications*. Second ed. (BARBOSA-CÁNOVAS GV (DEPARTAMENT OBSE, ed.). GENEVA; 2007.
3. Smiderle FR, Olsen LM, Carbonero ER, et al. Anti-inflammatory and analgesic properties in a rodent model of a (1→3),(1→6)-linked β-glucan isolated from Pleurotus pulmonarius. *Eur J Pharmacol*. 2008;597(1):86-91.
4. Sabaratnam V, Kah-Hui W, Naidu M, & Rosie David P. Neuronal health – can culinary and medicinal mushrooms help?. *Journal of Traditional and Complementary Medicine*. 2013; *3*(1), 62–68.
5. Elisashvili V. Submerged Cultivation of Medicinal Mushrooms : Bioprocesses and Products (Review). *Int J os Med Mushrooms*. 2012;14(3):211-239.

6. Xu J, Zhang J, Liu Y, Sun H, Wang J. Rheological properties of a polysaccharide from floral mushrooms cultivated in Huangshan Mountain. *Carbohydr Polym*. 2016;139:43-49.
7. Sovrani V, Jesus LI de, Simas-tosin FF, Smiderle FR, Iacomini M. Structural characterization and rheological properties of a gel-like β-D-glucan from *Pholiota nameko*. *Carbohydr Polym*. 2017;169:1-8.
8. Rahar S, Swami G. Preparation , characterization , and biological properties of b -glucans. *J Adv Pharm Technol Res*. 2011;2(2):94-103.
9. Smiderle FR, Ruthes AC, Iacomini M. Natural polysaccharides from mushrooms: Antinociceptive and anti-inflammatory properties. In: *Polysaccharides: Bioactivity and Biotechnology*.; 2015.
10. Ruthes AC, Smiderle FR, Iacomini M. D-Glucans from edible mushrooms: A review on the extraction, purification and chemical characterization approaches. *Carbohydr Polym*. 2015;117:753-761.
11. Moreno RB, Ruthes AC, Baggio CH, Vilaplana F, Komura DL, Iacomini M. Structure and antinociceptive effects of β-d-glucans from *Cookeina tricholoma*. *Carbohydr Polym*. 2016;141:220-228.
12. Ruthes AC, Carbonero ER, Córdova MM, et al. *Lactarius rufus* (1→3),(1→6)-β-D-glucans: Structure, antinociceptive and anti-inflammatory effects. *Carbohydr Polym*. 2013;94(1):129-136.
13. Okasaki M. Structure-Activity Relationship of (1-3)-β-D-Glucans in the Induction of Cytokine Production from Macrophages, *in vitro*. *Biol Pharm Bull*. 1995;18(10):1320-1327.
14. Synytsya A, Novák M. Structural diversity of fungal glucans. *Carbohydr Polym*. 2013;92(1):792-809.
15. Rodrigues DMF, Freitas AC, Rocha-Santos TAP, et al. Chemical composition and nutritive value of *Pleurotus citrinopileatus* var *cornucopiae*, *P. eryngii*, *P. salmoneo stramineus*, *Pholiota nameko* and *Hericium erinaceus*. *J Food Sci Technol*. 2015;52(11):6927-6939.
16. Smiderle FR, Morales D, Gil-Ramírez A, et al. Evaluation of microwave-assisted and pressurized liquid extractions to obtain β-D-glucans from mushrooms. *Carbohydr Polym*. 2017;156:165-174.
17. Morales D, Gil-Ramirez A, Smiderle FR, Piris AJ, Ruiz-Rodriguez A, Soler-Rivas C. Vitamin D-enriched extracts obtained from shiitake mushrooms (*Lentinula edodes*) by supercritical fluid extraction and UV-irradiation. *Innov Food Sci Emerg Technol*. 2017;41(February):330-336.
18. Alzorqi I, Singh A, Manickam S, Al-Qrimli HF. Optimization of ultrasound assisted extraction (UAE) of β-D-glucan polysaccharides from *Ganoderma lucidum* for prospective scale-up. *Resour Technol*. 2017;3(1):46-54.
19. Morales D, Smiderle FR, Piris AJ, Soler-Rivas C, Prodanov M. Production of a β-D-Glucan-rich extract from Shiitake mushrooms (*Lentinula edodes*) by an extraction/microfiltration/reverse osmosis (nanofiltration) process. *Innov Food Sci Emerg Technol*. 2018;51:80-90.
20. Gorin PAJ, Iacomini M. Polysaccharides of the lichens *Cetraria islandica* and *Ramalina usnea*. *Carbohydr Res*. 1984;128(1):119-132.
21. Sassaki GL, Souza LM, Serrato R V., Cipriani TR, Gorin PAJ, Iacomini M. Application of acetate derivatives for gas chromatography–mass

spectrometry: Novel approaches on carbohydrates, lipids and amino acids analysis. *J Chromatogr A*. 2008;1208(1-2):215-222.

22. Qin F, Kes M, Christensen BE. A study of bioactive, branched (1→3)-β-D-glucans in dimethylacetamide/LiCl and dimethyl sulphoxide/LiCl using size-exclusion chromatography with multi-angle light scattering detection. *J Chromatogr A*. 2013;1305:109-113.
23. Ding Q, Zhang L, Xu X, Zhang X, Wu C. Solution properties of pachyman from *Poria cocos* mycelia in dimethyl sulfoxide. *J Macromol Sci - Phys*. 2001;40 B(2):147-156.
24. Ciucanu I, Kerek F. A Simple and Rapid Method For The Permethylation Of Carbohydrates. *Carbohydr Res*. 1984;131(2):209-217.
25. Goldstein J, Hay GW, Lewis BA, Smith F. Controlled degradation of polysaccharides by periodate oxidation, reduction, and hydrolysis. *Methods in Carbohydrate Chemistry.* 1965;5 (1): 361-370.
26. Hunskaar S, Hole K. The formalin test in mice: dissociation between inflammatory and non-inflammatory pain. *Pain*. 1987;30(1):103-114.
27. Tjølsen A, Berge O-G, Hunskaar S, Rosland JH, Hole K. The formalin test: an evaluation of the method. *Pain*. 1992;51(1):5-17.
28. Synytsya A, Novák M. Structural diversity of fungal glucans. *Carbohydr Polym*. 2013;92(1):792-809.
29. Palacios I, García-Lafuente A, Guillamón E, Villares A. Novel isolation of water-soluble polysaccharides from the fruiting bodies of *Pleurotus ostreatus* mushrooms. *Carbohydr Res*. 2012;358:72-77.
30. Yalpani M, Hall LD. Some Chemical and Analytical Aspects of Polysaccharide. *Macromolecules*. 1984;281(16):272-281.
31. Bao H, You S, Cao L, Zhou R, Wang Q, Cui SW. Chemical and rheological properties of polysaccharides from fruit body of *Auricularia auricular - judae*. *Food Hydrocoll*. 2016;57:30-37.
32. Zhong Q, Daubert CR, *Food Rheology*. In: M Kutz (Eds.), Handbook of Farm, Dairy and Food Machinery Engineering 2nd Edition, Elsevier Inc.; 2013, pp. 403-426.
33. Wang W, Li C, Du G, Zhang X, Zhang H. Characteristics and Rheological Properties of Polysaccharide Nanoparticles from Edible Mushrooms (*Flammulina velutipes*). *J Food Sci*. 2017;82(3):687-693.
34. Lewis M, Heppell N. Continuous Thermal Processing of Foods Pasteurization and *UHT* Sterilization. Gaithersburg, Maryland; 2000.
35. Fischer M, Carli G, Raboisson P, & Reeh P. The interphase of the formalin test. *Pain*. 2015,*155*(3), 511–521.
36. Ribeiro NA, Abreu TM, Chaves HV, et al. Sulfated polysaccharides isolated from the green seaweed *Caulerpa racemosa* plays antinociceptive and anti-inflammatory activities in a way dependent on HO-1 pathway activation. *Inflamm Res*. 2014;63(7):569-580.
37. Silveira MLL, Smiderle FR, Agostini F, et al. Exopolysaccharide produced by *Pleurotus sajor-caju*: Its chemical structure and anti-inflammatory activity. *Int J Biol Macromol*. 2015;75:90-96.

38. de Araújo IWF, Rodrigues JAG, Quinderé ALG, et al. Analgesic and anti-inflammatory actions on bradykinin route of a polysulfated fraction from alga *Ulva lactuca*. *Int J Biol Macromol*. 2016;92:820-830.
39. Ruthes AC, Carbonero ER, Córdova MM, et al. Fucomannogalactan and glucan from mushroom *Amanita muscaria*: Structure and inflammatory pain inhibition. *Carbohydr Polym*. 2013;98(1):761-769.
40. Baggio CH, Freitas CS, Martins DF, et al. Antinociceptive Effects of (1→3),(1→6)-Linked β-Glucan Isolated From *Pleurotus pulmonarius* in Models of Acute and Neuropathic Pain in Mice: Evidence for a Role for Glutamatergic Receptors and Cytokine Pathways. *J Pain*. 2010;11(10):965-971.
41. Baggio CH, Freitas CS, Marcon R, et al. Antinociception of β-D-glucan from *Pleurotus pulmonarius* is possibly related to protein kinase C inhibition. *Int J Biol Macromol*. 2012;50(3):872-877.
42. Li H, Liu X, Li Y, Hua Y, Zhi D, Pang G. Effects of the polysaccharide from *Pholiota nameko* on human cytokine network in serum. *Int J Biol Macromol*. 2012;50(1):164-170.

Artigo 2

Pleurotus eryngii: polysaccharides extraction with water, its chemical structure and immunostimulating activity

Pleurotus eryngii: polysaccharides extraction with water, its chemical structure and immunostimulating activity

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ABSTRACT

The mushroom *Pleurotus eryngii* was submitted a three process of the extraction using only cold water (CW), hot water (HW), and under pressure in the autoclave (AE) process. The polysaccharide fractions CW, HW and AE, were characterized by Nuclear Magnetic Resonance Spectroscopy (NMR) studies, Gas-Chromatography coupled to Mass Spectrometry (GC-MS), UV-VIS spectrophotometry and High-Performance Size Exclusion Chromatography (HPSEC) analysis. Different partially purify polysaccharides were extracted in each these fractions with high percentages from a mannogalactan in CW, a linear β-D-Glucan β-(1→6)-linked by the in HW, and a β-D-Glucan-(1→3),(1→6)-linked in AE. The CW and HW fractions showed random coil conformation, while AE fraction presented characteristics of triple helix by Congo red experiment. The immunostimulatory properties of CW, HW, and AE fractions went investigated in human THP-1 monocyte cell line. The fractions are not cytotoxic. The CW and HW fractions were able to stimulate the release of IL-1β and IL-10, indicating that there was an immunostimulatory effect associated with the characteristics of classical macrophages activation designing a defense system to combat invading microorganisms or can be act as a factor for tumor regression.

Keywords: extraction process, polysaccharides, water, β-D-glucans, immunostimulatory properties.

1. Introduction

Pleurotus eryngii (DC.) Quél. is a very appreciated mushroom by international cuisine, and its origin is French. This basidiomycete is one of the most studied worldwide and it is an important source of fiber. A great number of studies were performed by the scientific community to evaluate its chemical composition and the biological activities from the variety of extracted molecules, especially their polysaccharides (Zervakis, Venturella & Papadopoulou, 2001; Zhang et al., 2020).

Polysaccharides are important representatives of the class of biomolecules known as carbohydrates due to their associated biological activities and other applications for both the food and pharmaceutical industries (Carbonero et al., 2006). In this context, it were observed that different authors who studied *P. eryngii* extracts have reported anti-inflammatory, antioxidant, hepatoprotective, tumor inhibitory, antibacterial, and hypolipidemic activities. Different methods of evaluation went used to determine such biological properties. Among the published data about the chemical characterization of *P. eryngii,* it is possible to observe chemical variations, which is justified by the different manners of preparation, extraction and purification procedures (Li et al., 2016a; Li et al., 2016b; Ren et al., 2016; Carbonero et al., 2006; Zhang, et al., 2014; Biscaia et al. 2017; Yan et al., 2019b).

Among antitumoral and antioxidant activities, polysaccharides obtained in aqueous extracts have also reported immunomodulatory properties (Carbonero et al., 2008; Jung et al., 2011; Biscaia et al., 2017; Yan et al., 2019a). The macrophages usually are the cells used to study such properties, because they are involved in various body phenomena, such as immune response to pathogens, homeostasis, and tissue repair and modeling (Wynn et al., 2013; Rendra et al., 2018).

Macrophages expressing inflammatory phenotype are characterized by the release of TNF-α, IL-6, and IL-1β and normally called M1 macrophages (Wynn et al., 2013), while the anti-inflammatory ones are known to present an M2-like phenotype and express the cytokine IL-10 and membrane receptors such as CD-206 and CD-163 (Zhang et al., 2013b). The polarization to M1 or M2 will depend on the stimuli and are usually associated with their function such as to combat pathogenic foreign organisms and tumors (M1 macrophages) or to combat parasitic infection and stimulates angiogenesis (M2 macrophages) (Mantovani et al., 2004; Wynn et al., 2013; Locati, Curtale e Mantovani, 2019). Polarization M2-like are associated with protumor functions, and its byproducts such as ARG-1, PDL-2, IL-1Ra, IL-10, CCL17, 18, 22, 24 are immunosuppressors, favoring the tumor microenvironment, and are therefore macrophages associated with tumor (TAMs) (Mantovani et al., 2004; Kratochvill et al., 2015; Murray, 2017).

Although these classifications are used, that macrophages in their various phenotypes (M1, M2 or M2-like) can coexist in the body and are modulated by various stimuli, showing plasticity in its profile. Therefore, *in vivo* and *in vitro* studies are needed and encouraged to better understand the immune system (Murray et al., 2014; Wynn & Vannela, 2016; Murray, 2017; Locati, Curtale e Mantovani, 2019).

Given the present scientific discoveries, the objective of this work was to develop new scientific knowledge about the polysaccharide extraction process of *P. eryngii* and to detail the chemical characteristics of the polysaccharide fractions to investigate the immunostimulatory effect on THP-1 cell line by the polysaccharide fractions.

2. Material and Methods

2.1. *Pleurotus eryngii* mushroom

P. eryngii was purchased (600 g dried) at Municipal Market of Curitiba-PR, Brazil, lyophilized and ground.

2.2. Extraction of polysaccharides

The dried and ground fruiting bodies (600g) were pre-extract with acetone (1L, 60 °C, for 6 h) to remove low molecular mass compounds such as lipids, phenols, and terpenes. Subsequently, the insoluble residue was submitted to dialysis using a membrane of MWCO 6-8 kDa.

2.2.1 Common water extractions

After dialysis, the retained material was lyophilized (420 g) and subjected to extraction with water at room temperature, under stirring (3 times for 6 h). After the suspension was filtered and the aqueous extract was concentrated under reduced pressure. The polysaccharides were precipitated with 3 volumes of cold ethanol. The ethanolic solution was centrifuged at 10,000 rpm at 10 °C for 20 min and the precipitate material was dialyzed (6–8 kDa Mw cut-off membrane) against tap water for 24 h and freeze-dried. Aliquots of the resultant fraction (CW yield: 6.98 g) were submitted to chemical analyses.

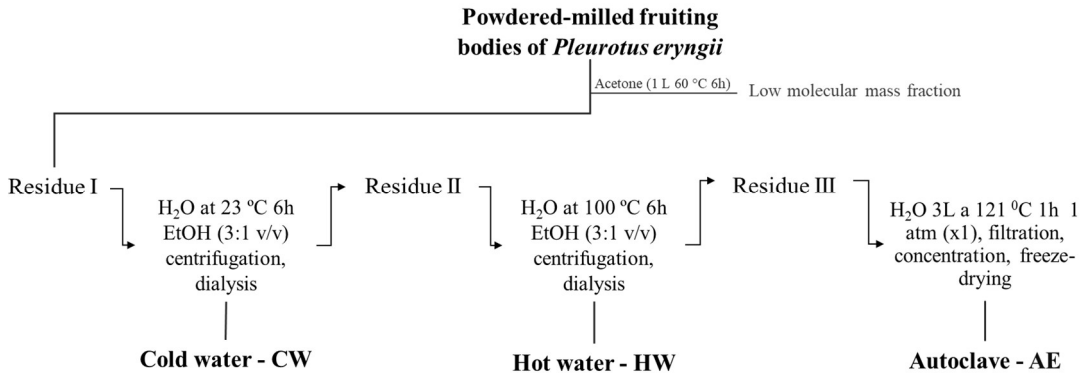

Fig. 1. *P. eryngii* scheme of extraction.

Briefly, residue II (Fig. 1) was extracted with water at 100 °C for 6 h (3 x, 1 L/ cycle). The extracts were separate from residue by centrifugation (8,000 rpm, 15 min., 25 ºC). The polysaccharides were recovered by precipitation with three volumes of cold ethanol, followed by centrifugation (10.000 rpm, 20 min, 10 ºC), dialysis (6 – 8 kDa, for 24 h), and lyophilization. This fraction was termed hot aqueous fraction (HW yield: 38.8g).

2.2.2 Extraction in Autoclave

After extraction with hot water, the residual material was then conditioned in an Erlenmeyer flask with 3 L of distilled water and kept closed, in an autoclave, where it was held for 1 hour at 121 ºC and 1.2 atm. After extraction, the material was filtered and the eluate was concentrated and lyophilized, being named AE (Figure 1). The autoclave fraction yielded 8 g.

2.3 Analysis of monosaccharide composition by GC-MS

Aliquots of the polysaccharide fractions (1 mg) were hydrolyzed with 2M TFA at 100 ºC for 8 h, followed by evaporation to dryness. The dried carbohydrate samples were dissolved in distilled water (100 µL) and 1 mg of $NaBH_4$ was added. The solution was held at room temperature overnight to reduce aldoses into alditols (Sassaki et al., 2008).

The product was dry and neutralized by the addition of acetic acid. The excess of $NaBH_4$ was remove with addition of methanol (x 2), and evaporation of methyl tetraborate under a compressed air stream in a fume hood. Acetylation of the alditols was performed in pyridine–acetic anhydride (200 µL; 1:1, v/v), for 30 min at 100 ºC. The resulting alditol acetates were extract with $CHCl_3$. The pyridine was remove by 5% $CuSO_4$ complexation. The aqueous phase was removed, and the chloroform phase containing the alditol acetate derivatives was evaporated. The samples were res uspended in hexane prior analysis on GC-MS.

The GC–MS analysis was carried out on a Shimadzu system consisting of a GC2010 Plus gas chromatograph coupled to a TQ8040 mass spectrometer (Shimadzu, Kyoto, Japan). The GC was equipped with an AOC5000 auto-injector and a split–splitless injector. Chromatographic separation was achieved on a GC Column SH-RTX-5 ms (30m × 0.25 mm × 0.25 µm) with helium 5.0 as the carrier gas at a flow rate of 1.0 mL min^{-1} and a column head pressure of 20 psi. Sample (1 µL) was injected at a split ratio of 1:10. Both injector and ion source temperatures were set at 250 °C. The interface temperature was 300 °C. The GC oven temperature was kept at 100 °C (2 min); 10 °C/min to 160 °C (2 min); 5 °C/min to 210 °C; 10°C/min to 280°C; 5°C/min to 300 °C (1 min); 10 °C/min to 320 °C (4 min). The total analysis time was 30 min.

The mass spectrometer was operated in the full-scan (SCAN) mode over a mass range of m/z 50–300 before selective ion monitoring mode, both with electron ionization at 70 eV. The software GCMS Solution (Tokyo, Japan) was used for data analysis.

Monosaccharides were identified by their typical electron impact breakdown profile and retention time, in comparison with standards, and the results were expressed as mol%.

2.4. Methylation analysis

Per-*O*-methylation of the samples (5 mg) was carried out using NaOH-Me_2SO-MeI (Ciucanu & Kerek, 1984). After that, the reaction was neutralized with glacial acetic acid, dialyzed, and lyophilized. The methylation process was repeated to guarantee that all the free hydroxyls of the polysaccharide were methylated. The per-*O*-methylated derivatives were submitted to methanolysis with MeOH-HCl 3 N (1 mL) for 2 h at 80 °C, followed by evaporation and hydrolysis using sulphuric acid (1 mL, 2 N) for 27 h at 100 °C. The acid solution was neutralized with $BaCO_3$, filtrated, and the eluate was reduced with $NaBD_4$ (sodium borodeuteride) and acetylated as described above, to give a mixture of partially *O*-methylated alditol acetate, which was analyzed by GC-MS using a VF5 capillary column. The derivatives were identified by their typical retention time and electron impact profile, in comparison to standards according to Sassaki et al. (2008).

2.6. Nuclear Magnetic Resonance (NMR) spectroscopy

The nuclear magnetic resonance experiments (^1H, ^{13}C, HSQC) were obtained using a 400 MHz Bruker spectrometer model Avance III with a 5 mm inverse probe, at 70 °C in Me_2SO-d_6. Chemical shifts (δ) were expressed in ppm relative to Me_2SO-d_6 ^{13}C (δ 39.7) and ^1H (δ 2.40) resonances. The NMR signals were assigned according to 1D and 2D NMR experiments and literature data.

2.7 Determination of Triple Helix with Congo Red Dye

Dextran standards (1 mg), samples (1 mg; CW, HW, and AE) and Congo Red dye (80 μM) were dissolved in 50 nM NaOH (980 μL). After complete solubilization, 20 μL of the Congo Red solution were added to the samples and dextran standards. Congo Red solution were used as a negative pattern. Colorimetric analysis was performed on an

Evolution 600 UV-vis spectrophotometer (ThermoFisher Scientific, Brazil) and the absorbance were read from 400 to 640 nm at 10 nm intervals (Palacios et al., 2012; Smiderle et al., 2014).

2.8 High Performance Size Exclusion Chromatography (HPSEC)

The samples (CW, HW and AE) were injected into a high-performance size-exclusion chromatography system (HPSEC) (Waters, Massachussets, USA) coupled to refractive index detector (Waters, Massachussets, USA). The chromatograph was equipped with four gel-permeation Ultrahydrogel columns disposed in series, each with the following exclusion sizes: $7x10^6$, $4x10^5$, $8x10^4$ and $5x10^3$ Da. The eluent used was 0.1 aqueous $NaNO_2$ containing 200 ppm aqueous NaN_3 at 0.6 mL min^{-1}. Samples were solubilized using that solution (1 mg mL^{-1}), and filtered through a 0.22 μm membrane before injection (100 μL loop). Data were analyzed using Astra software version 4.70.

2.9 Cell culture and macrophage differentiation

The human monocytic cell line THP-1 (Rio de Janeiro cell bank, Rio de Janeiro, Brazil) was grown in RPMI 1640 culture medium (Sigma–Aldrich, cat. R8758) supplemented with 10% heat-inactivated fetal calf serum Sterile A (Gibco, cat. 161010-159), 100 μg mL^{-1} streptomycin and 100 U mL^{-1} penicillin (Sigma–Aldrich), in a humidified incubator at 37 °C in 5% CO_2 atmosphere. The medium was replaced twice a week. Cells were grown to a density of 1–8 × 10^5 cells mL^{-1}, and used in the experiments at the maximum passage of 10. The cells were cultured in 96-well culture plates with 200 μL of cell suspension/well (2 × 10^5 cells mL^{-1}) for cytotoxicity and reactive oxygen species production assessment or in 24-wells culture plates with 1 mL of cell suspension/well (2 × 10^5 cells mL^{-1}) for cytokine measurement. The mature macrophage-

like state was induced by treating THP-1 monocytes to differentiate for 48 h with 62.5 ng mL^{-1} (~100 nM) phorbol 12-myristate 13-acetate (PMA; Sigma–Aldrich). After differentiation, cells adhered to the surface allowing removal of the culture medium with PMA, then the wells were washed twice with sterile PBS (phosphate buffered saline) and fresh medium, free of PMA, was added. Subsequently, the THP-1 macrophages (differentiated cells) were incubated for 24 h at 5% CO_2, at 37 °C, for resting, before initiating the treatments.

3.0 Cytotoxicity assessment

To measure CW, HW or AE cytotoxic activity in THP-1 macrophages, the MTT [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was performed. This experiment is based on the reduction of MTT salt by viable mitochondrial dehydrogenase to purple formazan crystals (Reilly et al., 1998).

After cell differentiation, the medium was removed and replaced with fresh medium containing CW, HW or AE treatment (50, 250 and 500 μg mL^{-1}) or PBS as control and then incubated for 24 h, 37°C and 5% CO_2. The assay was performed in quadruplicate. Following the incubation period, 20 μL of MTT solution (5 mg mL^{-1}) was added to each well and incubated at the same conditions described before. After 3 h of incubation, the media was removed, and the formazan crystals were solubilized in DMSO:Ethanol (100 μL $well^{-1}$). The absorbance was measured at 595 nm.

3.1 Cytokines IL-1β and IL-10 secretion by THP-1 macrophages

After differentiation and resting, THP-1 macrophages were exposed to the treatments: fresh medium containing CW, HW or AE treatment (at 50, 250, and 500 μg mL^{-1}) or sterile PBS (negative control, 50 μL) or lipopolysaccharide (LPS; at 500 ng mL^{-}

[1] from *Escherichia coli* O111:B4 – Sigma, pro-inflammatory control). The cells were subsequently incubated in humidified 5% CO_2 atmosphere at 37 °C for 18 h. This exposure time was chosen according to Chanput et al. (2010), who observed that the maximal cytokine secretion upon LPS-induced inflammation occurred at this time point. At the end of the incubation period, the cell-free supernatants were collected and stored at −80 °C for measurements of cytokine secretion. The concentration of pro-inflammatory (IL-1β) and anti-inflammatory (IL-10) cytokines was quantified by Elisa Ready-Set-Go kits specific for human cytokines (Invitrogen kits, cat. nos. 88-7261-88 and 88-7106-88, respectively). The experiments were performed according to the manufacturer's instructions.

3.2 Reactive Oxygen Species Determination

After cell cultivation and differentiation like described on item 2.9, the experiments to determination of reactive oxygen species released under treatment containing CW, HW or AE (50, 250 and 500 μg mL^{-1}) or PBS or LPS as control.

3.2.1. Production of Nitric Oxide

To measure nitric oxide production, the nitrite curve was performed (Concentration between 0 - 100μM) in quintuplicate. After cell cultivation, 50 μl of the supernatant was collected and 50 μl of 1:1 Griess reagente (1: 1 mixture of 0.1% sodium chloride stock solutions in water and 1% sulfanylamide in 5% H_3PO_4, prepared at the time of use) added, which was incubated for 10 minutes at room temperature, protected from light (Stuehr & Marletta, 1985). The absorbance reading will performed in microplate reader at 550 nm.

3.2.2. Superoxide Anion Production

The production of super hibernated anions is done by reducing NBT (nitro blue tetrazolium) (Madhavi et al., 1994). THP-1 differentiated cells were incubated and protected from light for 1 hour in presence of 0.2% NBT and 400 nM PMA at 37 °C. Afterward, this plate was centrifuged and the supernatant discarded. The wells are washed by centrifugation with PBS. THP-1 macrophages are fixed with 50% methanol (100 µl) for 10 minutes, the supernatant discarded and plate dried. The KOH 2M solution - 120 µl more 140 µl dimethyl sulfoxide was added. After 30 minutes, the reading will be performed at 550 nm and the results will be expressed as a percentage.

3.2.3. Ability to Adhere

After washing with PBS, 0.15 mL of 50% methanol added to wells and incubated for 10min (at room temperature). The supernatant was discarded, and 0.1 mL of 0.2% Giemsa solution was added. After incubating at room temperature for 40 min, washing with distilled water, and incubating with 0.2 mL of 50% methanol at room temperature the absorbance was measured at 550 nm (Pipe, Coles e Farley, 1995). The mean adhesion absorbance values of each group were used to normalize data for ROS production assays.

3.3. Statistical analysis

The results were expressed as mean ± standard error of the mean (SEM) and were analyzed by one-way analysis of variance (ANOVA). As a post hoc test the Newman-Keuls Multiple Comparison Test was used. The threshold of statistical significance was $p<0.05$. The graphs were drawn and the statistical analyses were performed using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, CA, USA).

4. Results and discussion

4.1. Polysaccharide extraction and chemical characterization

The fruiting bodies obtained from the mushroom *Pleurotus eryngii* after low mass molecules removal with acetone (60 °C, 1h) were submitted to successive cold water extractions (6.98 g - yield 1.7 %), hot water extraction (38.8 g – yield 9.2 %) and autoclave extraction (8 g - yield 1.9 %). A sample (1 mg) of each fraction obtained was used to determine the monosaccharide composition, the cold water fraction (CW) presented galactose (66.5%), mannose (19.8%), 3-*O*-Methyl-galactose (2.4 %) and a lower amount of glucose (11.3%).

The CW HSQC-DEPT spectrum (figure 2) shows characteristic signals and correspondents to mannogalactan, β-glucan and small amounts of glycogen present of this fraction. The anomeric region, C1/H1 signals at δ 98.8/5.02, and 98.6/4.84 corresponding to galactose in α configuration and δ 101.6/4.66 corresponding to mannose in β configuration, these signals are corresponding to the mannogalactan. The signal at δ 102.8/4.35 to glucose in β configuration which is corresponding to a β-glucan polysaccharide and the signal at δ 99.0/5.18 to glucose in α configuration corresponds glycogen. A typical signal of methyl group observed at δ 56.3/3.45 indicate substitution that is observed in several heteropolysaccharides from genus *Pleurotus* described like *O*-me-Gal*p* units (Carbonero et al., 2008; Smiderle et al., 2008; Silveira et al., 2015; Biscaia et al., 2017).

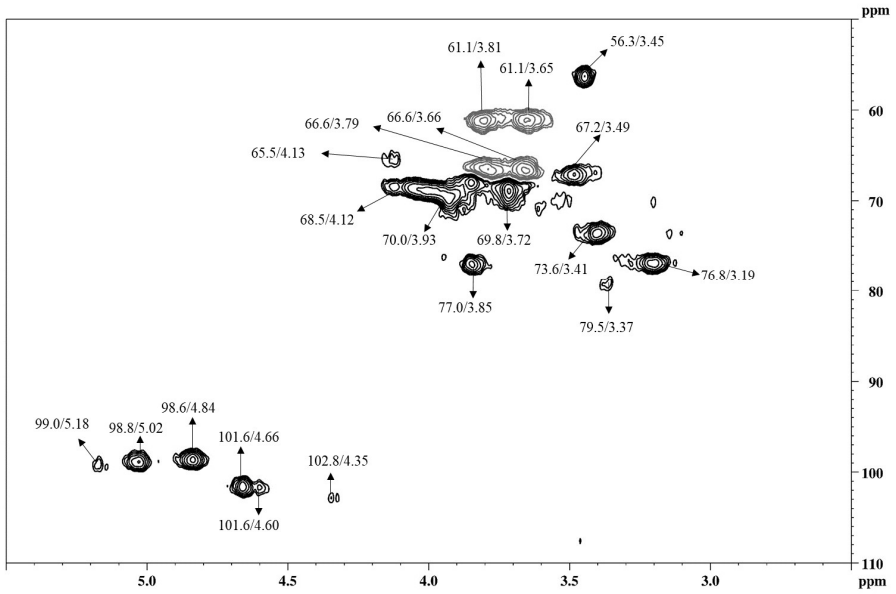

Fig. 2. HSQC-DEPT spectrum of cold water fraction from *P. eryngii* (CW). Sample was solubilized in Me_2SO-d_6 at 70 ºC (chemical shifts are expressed in δ ppm).

Concerning the linkage region of these chemical structures, signals can be observed in C-2 *O*-substituted of α-Gal*p* units at δ 77.0/3.85 furthermore, the main chain 6-*O*-substitution was confirmed by inverted signals at δ 66.6/3.79 and 66.6/3.66 and non-substituted CH_2-OH groups of mannose are also observed by signals at δ 61.1/ 3.81 and 61.1/3.65. All carbon frequencies were assigned according to the literature for the mannogalactan and the main signals observed for CW fraction are in concordance with the typical signals presented for these types of polysaccharides normally extracted from basidiomycetes (Carbonero et al., 2008; Silveira et al., 2015; Biscaia et al., 2017; Zhang et al., 2020). Therefore, cold-water extraction is an important process for obtaining mannogalactan heteropolysaccharides.

The hot water fraction (HW) showed only glucose in its composition determined by GC-MS analysis. HSQC-DEPT spectrum (Fig. 3) of this fraction indicated the

presence of a linear β-glucan (1→6)-linked by the presence of six intense signals at δ 102.9/4.24 (C1/ H1), 73.2/3.02 (C2/ H2), 76.5/3.21 (C3/ H3), 70.0/3.11 (C4/ H4), 75.5/3.33 (C5/ H5), and 68.5/4.00 and 3.61 (C6/ H6). All carbon frequencies were assigned according to the literature (Smiderle et al., 2013).

The less intense anomeric signals at δ 102.9/4.34 and 102.9/4.37 arise also from C1/H1 of β-Glc*p* units, indicating a β-glucan with (1→3)-linkages attributed to the presence of the signal at 87.2/3.42. The non-substituted C-6 signals at δ 61.1/3.69-3.49 were observed by the inversion in the HSQC-DEPT spectrum, according literature Sovrani et al. (2017); Morales et al. (2019). In this spectrum it is also possible to observe the presence of anomeric signals at δ 99.3/5.06 and 82.9/3.64 as linkage region which indicate the presence of small amounts of a (1→3)-linked α-glucan that were previously described for *Lentinula edodes* and *Fomitopsis betulina* (Morales et al., 2020; de Jesus et al., 2018[a]). In this way, by extraction with hot water (HW), it is possible to obtain mainly a linear (1→6)-β-D-glucan, and less (1→3)-β-D-glucan and small contents of a linear (1→3)-α-D-glucan.

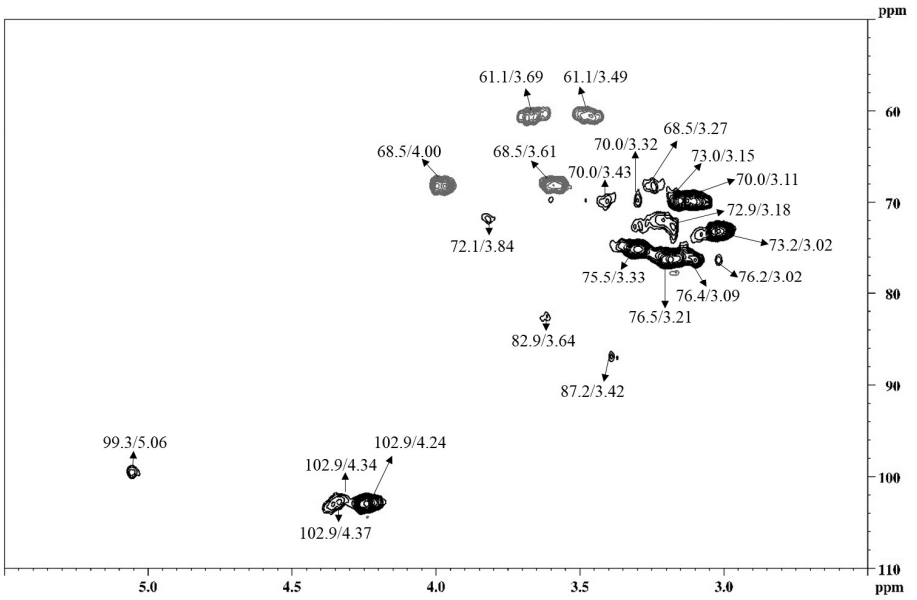

Fig. 3. HSQC-DEPT spectrum of *P. eryngii* obtained from hot water fraction (HW). Sample was solubilized in Me_2SO-d_6 at 70 ºC (chemical shifts are expressed in δ ppm).

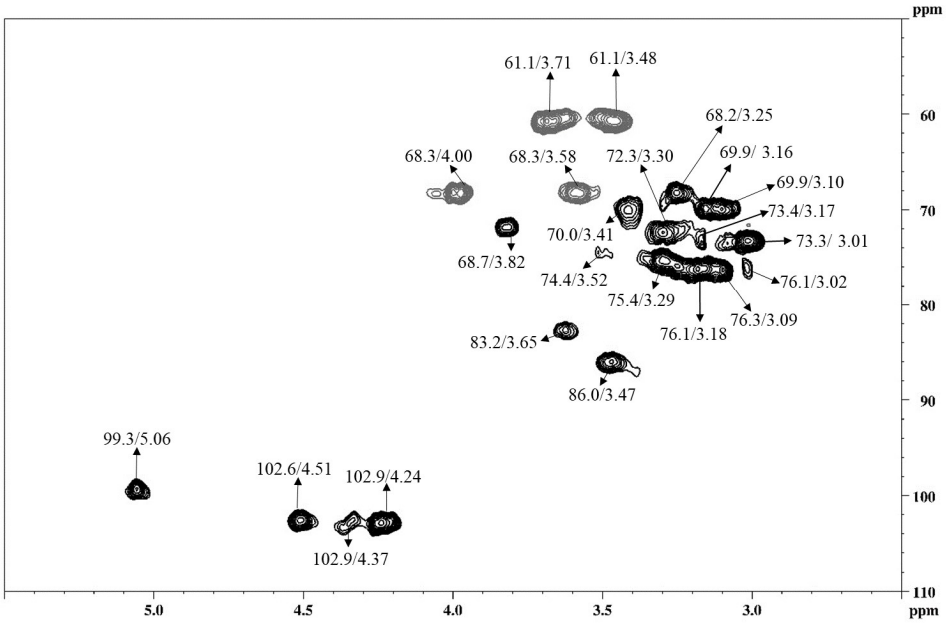

Fig. 4. HSQC-DEPT spectrum of *P. eryngii* obtained from autoclave fraction (AE). Sample was solubilized in Me_2SO-d_6 at 70 ºC (chemical shifts are expressed in δ ppm).

The extraction with autoclave was conducted for 1 hour at 121ºC and 1.2 atm of pressure to extract the residual material from the other extractions. Yielding 8g, a sample of AE fraction after acid hydrolysis, reduction, and acetylation presented 100% of glucose in its monosaccharide analysis determined by GC-MS.

The HSQC-DEPT spectrum of AE (Fig. 4) showed characteristic signals of whose signs show and presence of β-D- glucans (1→3),(1→6)-linked and a linear α-D-glucan (1→3)-linked previously observed by hot water (HW) fraction. The branched β-D-glucan with very intensive anomeric signals were observed at δ 102.6/4.51 and 102.9/4.24 (C1/H1); and the linkage region at δ 86.0/3.47 (3-*O*-substituted, C3/H3); at δ 68.3/4.00 and 68.3/3.58 (6-*O*-substituted, C6/H6) and δ 61.1/3.71 and 61.1/3.48 (6-*O*-non-substituted, C6/H6) (Ruthes et al., 2015; Sovrani et al., 2017; Morales et al., 2020). The main signals of the linear α-D-glucan (1→3)-linked were observed with low intensity on spectrum at δ 99.3/5.06 (C1/ H1) and 83.2/ 3.65 (C3/ H3) as well referenced in de Jesus et al. (2018a), Morales et al. (2019), and Morales et al. (2020). These data demonstrate the pressure of the autoclave extraction is efficient to recover higher amounts of this linear α-D-glucan (1→3)-linked polysaccharide that is more insoluble than the others β-D-glucans.

The NMR analysis were a fundamental tool for elucidating chemical structures of polysaccharides presents on the crude polysaccharide fractions. A table (S1) with the main chemical assignments obtained on NMR analyses and references used were provided in the supplementary material.

Methylation analysis of the CW (Table 1) presented the derivatives 2,3,4-Me_3–Gal*p* (30.8%) suggesting an α-D-Gal*p*-(1→6) – linked as the main chain with branches in *O*-2 represented by 3,4-Me_2–Gal*p* (26.4%) methylated derivative, the total branches are composed of β-D-Man*p* units represented by 2,3,4,6 Me_4-Man*p* (26.6%) methylated

derivative, so characterizing the mannogalactan, like in accordance results of HSQC-NMR signals that are characteristic of a heteropolymers with main chain containing galactose in α-configuration and mannose in β-configuration linked to C2 of galactose units. Thus, as in the NMR experiments, through methylation data it is possible to identify and characterize a β-D-glucan(1→3),(1→6)-linked represented by the partially *O*-methylalditol acetates derivatives with 2,3,4,6-Me_4-Glc*p* (1.8%), 2,4,6-Me_3-Glc*p* (8.2%), 2,3,4-Me_3-Glc*p* (4.8 %), and 2,4-Me_2-Glc*p* (1.5%) – present in smaller proportion.

Table 1. Alditol acetates partially *O*-methylated obtained from CW, HW, AE fractions of the *P.eryngii*.

O-methylated alditol acetates[a]	Fraction / (Mol %)			Linkage type[b]	Ions
	CW	HW	AE		
2,3,4,6 Me_4-Glc	1.8	7.0	14.2	Glc*p*-(1→	87, 102, 118, 129, 145, 161, 207
2,4,6 Me_3-Glc	8.2	10.3	63.0	3→)-Glc*p*-(1→	87, 101, 118, 129, 161
2,3,4 Me_3-Glc	4.8	74.7	3.6	6→)-Glc*p*-(1→	87, 99, 102, 118, 129, 162, 189
2,4 Me_2-Glc	1.5	8.0	19.2	3,6→)-Glc*p*-(1→	87, 101, 118, 129, 139, 160, 189, 234
2,3,4,6 Me_4-Man	26.6	-	-	Man*p*-(1→	87, 102, 118, 129, 145, 161, 207
2,3,4-Me_3–Gal	30.8	-	-	→6)-Gal*p*-(1→	87, 99, 102, 118, 129, 142, 162, 189, 233, 254
3,4-Me_2–Gal	26.4	-	-	→2,6)-Gal*p*-(1→	87, 100, 117, 130, 143, 190, 234

Legend: CW – cold water fraction, HW – hot water fraction, AE – Autoclave fraction – from *P. eryngii*.
[a] GC-MS analysis on a GC Column SH-RTX-5 ms.
Electron ionization at 70 eV.
[b] Based on derived *O*-methylalditol acetates.

The methylation results of the fraction HW presents the derivative 2,3,4-Me_3-Glc*p* (74.7 %) in the high percentage, suggesting the presence of β-D-glucan with linear structure (1→6)-linked, but also have lowest proportion (10.3%) with the linear structure of β-D-glucan(1→3)-linked represented by methylated derivative 2,4,6-Me_3-Glc*p*

(10.3%), and the presence of low concentration of branched β-D-glucan-(1→3),(1→6)-linked indicated by 2,4-Me_2-Glc*p* (8%) methylated derivative, as shown in table 1.

Finally the methylated derivatives obtained in the autoclave fraction (AE) are 2,3,4,6-Me_4-Glc*p* (14.2%), 2,4,6-Me_3-Glc*p* (63%), 2,3,4-Me_3-Glc*p* (3.6%) and 2,4-Me_2-Glc*p* (19.2%). Indicating the presence of a β-D-glucan (1→3)-linked like main chain, by the presence of the 2,4,6-Me_3-Glc*p* derivative in a high percentage - 63%, and β-D-glucan (1→3),(1→6)-linked represented by derivative 2,4-Me_2-Glc*p* (19.2%), and branches of β-D-Glc*p* units with β-(1→6)-linkages represented by 2,3,4-Me_3-Glc*p* (3.6%) and non-reducing terminals represented by 2,3,4,6-Me_4-Glc*p* (14.2%) which is following the NMR results for this fraction.

To observe the three-dimensional conformation of the structures present in *P. eryngii* fractions were conducted using the Congo Red dye, which may complex with polysaccharide structures in triple-helix conformation. Some polysaccharides have a helical conformation in solution, while others are present as random-coil structures, which are stabilized by hydrogen bonds and hydrophobic interactions (Ogawa, Tsurugi, & Watanabe, 1972; Nitschke et al., 2011; Palacios et al., 2012; Synytsya & Novak, 2013; Smiderle et al., 2014; Morales et al., 2020).

The Congo Red experiment showed that among *P. eryngii* fractions, only AE showed a 10 nm bathochromic shift (Fig. 5-A), indicating that the polymers present in this fraction, characterized here as mainly β-D-glucan-(1→3),(1→6) have a triple helix conformation as their tertiary structure. The result of Congo Red experiment converges with that observed for β-D-glucans-(1→3),(1→6)-linked and β-D-glucans-(1→3) studied by Ogawa, Tsurugi, & Watanabe (1972), Nitschke et al. (2011), Smiderle et al. (2014), Semedo, Kamarli e Fonseca (2015) and Morales et al. (2020).

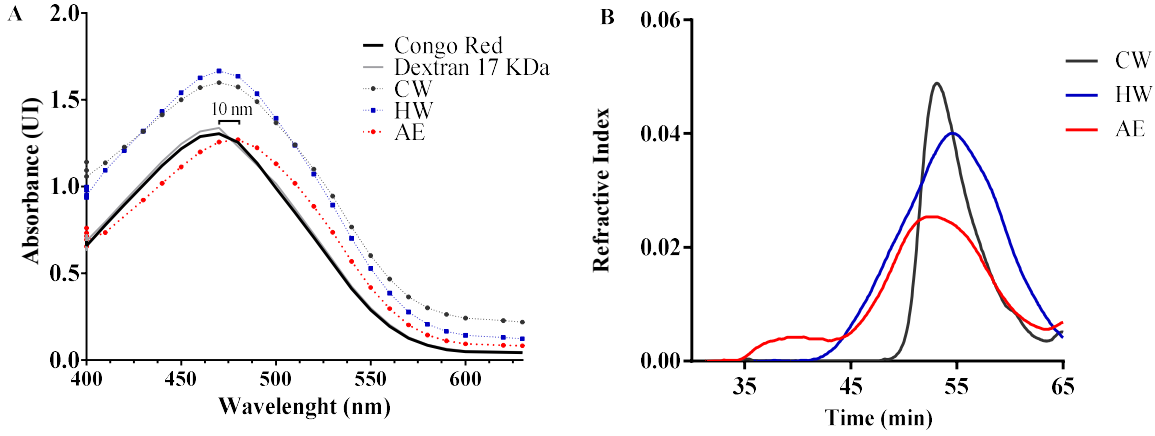

Fig 5. Three-dimensional conformation by Congo red and Elution Profile by HPSEC. **A**: Absorption spectrum of Congo red (control), Congo red with dextran (random coil control), and Congo red with CW, HW or AE fraction**. B:** Elution profiles obtained on HPSEC analysis of CW, HW, and AE fraction from *P. eryngii*.

The CW fraction, represented mainly by mannogalactan structure, show behavior to the random coil conformation as like as dextran standard, which does not form a complex with dye. The HW fraction, chemically characterized by the present study, have by the main component representative the linear β-D-glucan-(1→6), that too demonstrated have random coil conformation, because does not show a bathochromic shift on Congo Red experiment (Zhang et al., 2007; Nitschke et al., 2011).

The high-performance size-exclusion chromatography showed by the refractive index (RI) detectors (Fig. 5-B), indicated that CW presents the most homogeneous population of polysaccharides confirmed by the presence of only one peak (50-58 min). HW and AE presented the wider peaks starting at ~45 min showing a heterogeneous population of polysaccharides. Furthermore, AE also presented a large M_w population by the peak at ~40 min, which was not observed on the other fractions and may be related to the linear, insoluble and α-(1→3)-D-glucan.

4.2 Immunostimulatory properties of *P. eryngii* fractions *in vitro*

The immunostimulatory effects of *P. eryngii* fractions – CW, HW, and AE went investigated in human THP-1 monocyte cell line. The toxicity of the fractions was verified and the MTT test showed that none of the fractions were toxic to the cells up to 500 µg/mL (Fig. 6 A – MTT). The adherence of the cells was also evaluated, and no difference was observed when the cells were treated with the fractions or with vehicle (control), showing that the fractions did not interfere in the macrophage function of the ability to adhere (Fig. 6 B).

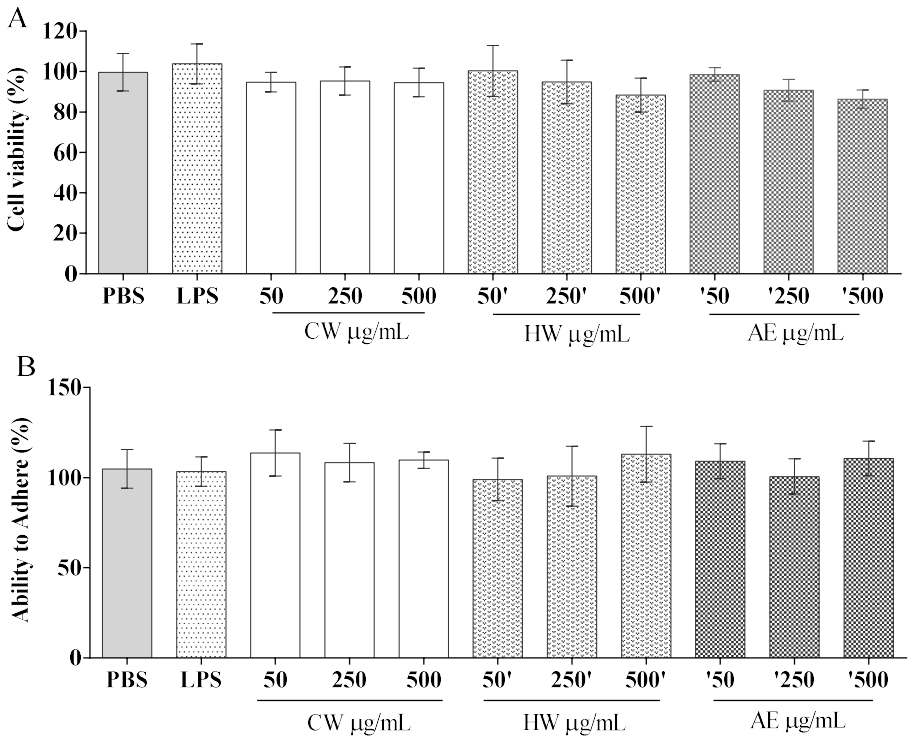

Fig. 6 Evaluation of treatments with fractions CW, HW, and AE from *P. eryngii.* Positive control (LPS 500 ng/mL) and negative control (PBS) on THP-1 cells. **A** Effect of fractions on viability of THP-1 macrophages. **B** Ability to adhere of macrophages treated with fractions**.** The results for three independent experiments in quadruplicate. No statistical differences were found at the ($p < 0.05$).

The CW and HW fraction were able to stimulate the release of the proinflammatory cytokine IL-1β and also stimulated a small production of the anti-inflammatory cytokine IL-10 (Fig. 7 A), however, the autoclave fraction did not alter the pattern of the evaluated cytokines (Fig. 7 A - B). The nature of inflammatory responses depends on a series of factors, including the type of receptor that recognizes the pathogen or antigen molecule and is consequently activated. After activation of such receptors (i.e. TLR-2, TLR-4, dectin-1) the immune response is initiated and the cells release pro or anti-inflammatory cytokines, which will modulate cell polarization and its function. The IL-1β is a pro-inflammatory cytokine that is synthesized by monocytes, macrophages and dendritic cells after an inflammatory stimulus. The release of IL-1β, and TNF-α indicates an inflammatory response after activation of the signaling pathways NF-kB and STAT-1. This reaction may also induce increased production of reactive oxygen species, and it is usually activated as response against tumors and microorganism invasion (Wynn et al., 2013; Murray et al., 2014; Wynn & Vannela, 2016; Murray et al., 2017; Locati, Curtale e Mantovani, 2019).

The release of nitric oxide was observed when the cells were incubated with the three fractions, mainly at the highest concentration tested (500 µg/mL) (Fig. 7 C). Curiously, the superoxide anion production did not alter by the treatments in comparison to the control (Fig. 7 D). The response of the THP-1 cell line by releasing nitric oxide but not altering the superoxide release pattern is interesting, considering that recently immunotherapy-based treatments have proposed that inhibition of superoxide release specifically blocks differentiation of macrophages to Tumor-associated macrophages (TAM), which have a suppressive function of the inflammatory system, allowing tumor growth. Therefore, blocking the superoxide release may provide some therapeutic advantages like tumorigenesis suppressing activity (Zhang et al., 2013b).

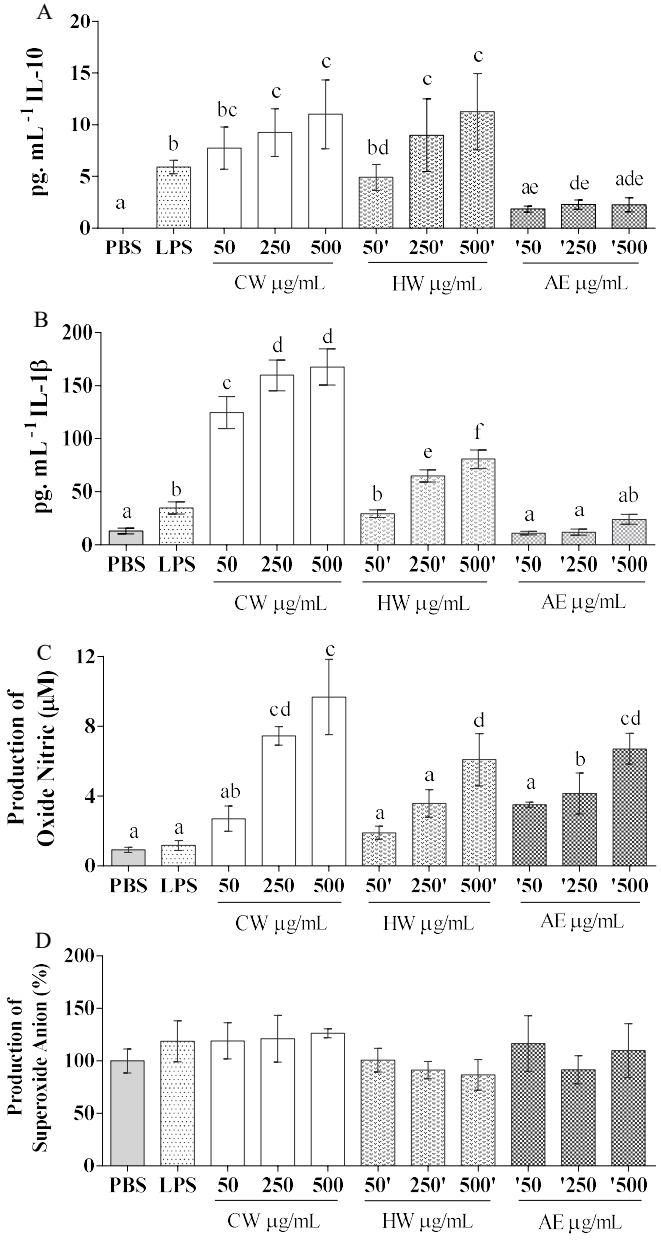

Fig. 7 Cytokines production and release of Reactive Oxygen Species after treatment with CW, HW and AE fractions from *P. eryngii*. **A** Production of IL-10 by THP-1 cells after 18h of incubation with treatments or controls. **B** Production of IL-1β by THP-1 cells after 18h of incubation with treatments or controls. **C** Production of Oxide Nitric (µM). **D** Superoxide anion production. The results are expressed as means of secretion index (SI) ± SEM for two independent experiments in quadruplicate. Different letter represents statistically significant difference between treatments ($p < 0.05$).

The release of NO observed after the treatment with CW, HW, and AE (Fig. 7 C) is characteristic of macrophages with classical type I polarization, accompanied by high production of inflammatory cytokines. (Mantovani et al., 2004; Zhang et al., 2013b; Locati, Curtale e Mantovani, 2019). The results observed in this study suggest that CW and HW can to induce a type I polarization of macrophages and consequently initiate inflammatory responses.

5. Conclusions

This study showed that aqueous extraction process is a simple process that can extract polysaccharides with different chemical structures, the aqueous extraction at room temperature (cold water process) made it possible to obtain a mannogalactan heteropolymer as the main chemical structure. However, by using boiling water (hot aqueous extraction at 100 ºC) it was possible to extract a β-D-glucan (1→6) -linked as the main polysaccharide present in the fraction. The extraction process by autoclave (a stronger extraction process, including the pressure of 1.2 atm and higher temperature - 121 ºC) was extracted a β-D-glucan (1→3),(1→6)-linked and a linear (1→3)-α-D-glucan rarely observed in other extraction procedures. Therefore, interesting extraction processes can be observed that can work selectively to obtain high concentrations of certain polysaccharides. These results indicate that *P. eryngii* mushroom is a rich source of glucans and different methods of extraction can be employed depending on the polysaccharides that are desired. CW and HW fractions showed biological activity, by stimulating the production of cytokines and Oxide nitric reactive specie. Interestingly, they did not alter the production of superoxide. The fractions were not cytotoxic and did not interfere in the adherence of the macrophages. The results observed suggest that CW

and HW may stimulate the release of cytokines (IL-1β and IL-10) of macrophages, designing a defense system that is important to combat pathogen invasion and tumors.

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The authors have declared no conflicts of interest.

Supplementary material

Supplementary material associated with this article can be found, in the online version, at

6. References

1. Alquini G, Carbonero E R, Rosado F R, Consentino C, Iacomini M. **Polysaccharides from the fruit bodies of the basidiomycete *Laetiporus sulphureus* (Bull.: Fr.) Murr.** *FEMSS Microbiology Letters*. 2004; 230: 47-52.
2. Biscaia S M P, Carbonero E R, Bellan D L, et al. **Safe therapeutics of murine melanoma model using a novel antineoplasic, the partially methylated mannogalactan from *Pleurotus eryngii*.** *Carbohydr Polym*. 2017; 178 (August):95-104.
3. Carbonero E R, Cordeiro L M C, Mellinger C G, et al. **Galactomannans with novel structures from the lichen *Roccella decipiens* Darb.** *Carbohydr Res*. 2005; 340(10):1699-1705.
4. Carbonero E R, Gracher A H P, Rosa M C C, et al. **Unusual partially 3-*O*-methylated α-galactan from mushrooms of the genus Pleurotus**. *Phytochemistry*. 2008; 69(1):252-257.
5. Carbonero E R, Gracher A H P, Smiderle F R, Rosado F R, Sassaki G L, Gorin P A J, & Iacomini M. **A β-glucan from the fruit bodies of edible mushrooms *Pleurotus eryngii* and *Pleurotus ostreatoroseus***. *Carbohydrate Polymers*. 2006; *66*(2), 252–257.
6. Chanput W, Mês J, Vreeburg R A M, Savelkoul H F J, Wichers H J. **Transcription profiles of LPS-stimulated THP-1 monocytes and macrophages: a tool to study inflammation modulating effects of food-derived compounds.** *Food Funct.* 2010; 1 , 254 -261.

7. Ciucanu I, Kerek F. **A simple and rapid method for the permethylation of carbohydrates**. *Carbohydr Res*. 1984; 131(2):209-217.
8. de Jesus L I, Smiderle F R, Cordeiro L M C, Freitas R A de, Van Griensven L J L D, Iacomini M. **Simple and effective purification approach to dissociate mixed water-insoluble α- and β-D-glucans and its application on the medicinal mushroom *Fomitopsis betulina***. *Carbohydr Polym*. 2018a; 200: 353-360.
9. de Jesus L I, Smiderle F R, Ruthes A C, et al. **Chemical characterization and wound healing property of a β-D-glucan from edible mushroom *Piptoporus betulinus***. *Int J Biol Macromol*. 2018b; 117:1361-1366.
10. Jung H Y, Bae I Y, Lee S, Lee H G. **Effect of the degree of sulfation on the physicochemical and biological properties of *Pleurotus eryngii* polysaccharides**. *Food Hydrocoll*. 2011; 25: 5, 1291-1295.
11. Kratochvill, F. et al. TNF Counterbalances the Emergence of M2 Tumor Macrophages. **Cell Reports**, 2015; Sep 22;12(11)1902-14
12. Li S, & Shah N P. **Anti-inflammatory and anti-proliferative activities of natural and sulphonated polysaccharides from *Pleurotus eryngii*.** *Journal of Functional Foods*. 2016a; *23*, 80–86.
13. Li S, & Shah N P. **Characterization, antioxidative and bifidogenic effects of polysaccharides from *Pleurotus eryngii* after heat treatments**. *Food Chemistry*. 2016b; *197*, 240–249.
14. Locati M, Curtale G, Mantovani **A. Diversity, Mechanisms, and Significance of Macrophage Plasticity**. 2019:123-147.
15. Madhavi N, Das U N, Prabha P S, Kumar G S, Koratkar R, Sagar P S. **Suppression of human T-cell growth in vitro by cis-unsaturated fatty acids: relationship to free radicals and lipid peroxidation.** Prostaglandins Leukot Essent Fatty Acids. 1994; v. 51, n. 1, p. 33-40.
16. Mantovani A, Sica A, Sozzani S, Allavena P, Vecchi A, Locati M. **The chemokine system in diverse forms of macrophage activation and polarization.** *Trends Immunol*. 2004; 25, pp. 677-686.
17. Morales D, Rutckeviski R, Villalva M, et al. **Isolation and comparison of α- and β-D-glucans from shiitake mushrooms (*Lentinula edodes*) with different biological activities**. *Carbohydr Polym*. 2020; 229 (1 February)115521.
18. Morales D, Smiderle F R, Villalva M, Abreu H, Ricco C, Santoyo S, Iacomini M, Soler-Rivas C. **Testing the effect of combining innovative extraction technologies on the biological activities of obtained β-glucan-enriched fractions from *Lentinula edodes***. *Journal of Functional Foods*. 2019; 60: 103446.
19. Murray P J. **Macrophage Polarization**. *Annual Review of Physiology*. 2017; 79, 541-566.
20. Murray P J, Allen J E, Biswas S K, Fisher E A, Gilroy D W, Goerdt S, et al. **Macrophage activation and polarization: Nomenclature and experimental guidelines**. *Immunity*. 2014; 41 (1), pp. 14-20.
21. Nitschke J, Modick H, Busch E, von Rekowski R W, Altenbach H J. & Molleken H. **A new colorimetric method to quantify beta-1,3-1,6-glucans**

in comparison with total beta-1,3-glucans in edible mushrooms. *Food Chemistry*. 2011; 127(2), 791–796.

22. Ogawa K, Tsurugi J, & Watanabe, T. **Complex of gel-forming β-1, 3-D-glucan with Congo red in alkaline solution**. *Chemical Letters*. 1972; 689-692.
23. Palacios I, Garcia-Lafuente A, Guillamón E, & Villares A. **Novel isolation of water-soluble polysaccharides from the fruiting bodies of *Pleurotus ostreatus* mushrooms**. *Carbohydrate Research*. 2012; 358: 72-77.
24. Pipe R K, Coles J A, Farley, S R. **Assays for measuring immune response in the mussel *Mytilus edulis***. . In: J. S. STOLEN;T. C. FLETCHER, et al (Ed.). Techniques in Fish Immunology - 4. Immunology and Pathology of Aquatic Invertebrates. Fair Haven: SOS Publications. p.93-100, 1995.
25. Ren D, Wang N, Guo, J, Yuan L, Yang X. **Chemical characterization of *Pleurotus eryngii* polysaccharide and its tumor-inhibitory effects against human hepatoblastoma HepG-2 cells**. *Carbohydrate Polymers*. 2016; *138*, 123–133.
26. Rendra E, Riabov, V, Mossel D M, Sevastyanova T, Harmsen M C, Kzhyshkowska, J. **Reactive oxygen species (ROS) in macrophage activation and function in diabetes**. *Immunobiology.* 2018; V. 224(2):242–253.
27. Reilly T P, Bellevue F H, Woster P M, Svensson C K. **Comparison of the *in vitro* cytotoxicity of hydroxylamine metabolites of sulfamethoxazole and dapsone**. *Biochem. Pharmacol*. 1998, 55, pp. 803-810.
28. Ruthes A C, Smiderle F R, Iacomini M. **D-Glucans from edible mushrooms: A review on the extraction, purification and chemical characterization approaches**. *Carbohydr Polym*. 2015; 117:753-761.
29. Sassaki G L, Souza L M, Serrato R V., Cipriani T R, Gorin P A J, Iacomini M. **Application of acetate derivatives for gas chromatography–mass spectrometry: Novel approaches on carbohydrates, lipids and amino acids analysis**. *J Chromatogr A*. 2008; 1208(1-2):215-222.
30. Semedo M C, Karmali A, Fonseca L. **A high throughput colorimetric assay of β-1,3-D-glucans by Congo Red Dye**. *Journal of Microbiological Methods*. 2015; 109: 140-148.
31. Silveira M L L, Smiderle F R, Agostini F, et al. **Exopolysaccharide produced by *Pleurotus sajor-caju*: Its chemical structure and anti-inflammatory activity**. *Int J Biol Macromol*. 2015;75:90-96.
32. Sovrani V, de Jesus L I , Simas-tosin F F, Smiderle F R, Iacomini M. **Structural characterization and rheological properties of a gel-like β-D-glucan from *Pholiota nameko***. *Carbohydr Polym*. 2017; 169:1-8.
33. Smiderle F R, Alquini G, Tadra-Sfeir M Z, Iacomini M, Wichers H J, Van Griensven L J L D. ***Agaricus bisporus* and *Agaricus brasiliensis* (1 → 6)-β-d-glucans show immunostimulatory activity on human THP-1 derived macrophages**. *Carbohydr Polym*. 2013; 94(1):91-99.
34. Smiderle F R, Baggio C H, Borato D G, Santana-Filho A P, Sassaki G L, Iacomini M, & Van Griensven L J L D. **Anti-inflammatory properties of the medicinal mushroom *Cordyceps militaris* might be related to its linear (1→3)-β-D-glucan**. *PLoS ONE*. 2014; 9: e110266.

35. Smiderle FR, Olsen LM, Carbonero ER, et al. **A 3- *O*-methylated mannogalactan from *Pleurotus pulmonarius* : Structure and antinociceptive effect**. *Phytochemistry*. 2008; 69:2731-2736.
36. Synytsya A, Novák M. **Structural diversity of fungal glucans.** *Carbohydr Polym*. 2013; 92(1):792-809.
37. Stuehr D J, Marletta M A. **Mammalian nitrate biosynthesis: mouse macrophages produce nitrite and nitrate in response to *Escherichia coli* lipopolysaccharide**. *Proceedings of the National Academy of Sciences of the United States of America*. 1985; v. 82, n. 22, p. 7738-7742.
38. Whistler, R L. (1973). **Solubility of polysaccharides and their behavior in solution**. In H. S. Isbell (Ed.). Carbohydrates in solution. Washington, DC: *Amer Chemical Society Publications.* Ch. 14, pp. 242-255. Advanced in Chemistry Series 117.
39. Wynn T A, Chawla A, Pollard J W. **Macrophage biology in development, homeostasis and disease**. *Nature Intern. Jour. Of Scien*. 2013; V. 496, pg. 445-455.
40. Wynn T A & Vannella K M. **Macrophages in tissue repair, regeneration, and fibrosis.** *Immunity*. 2016; 44, pp. 450-462.
41. Yan J, Meng Y, Zhang M, et al. **A 3-*O*-methylated heterogalactan from *Pleurotus eryngii* activates macrophages**. *Carbohydr Polym*. 2019a; 206:706-715.
42. Yan J, Zhu L, Qu Y, et al. **Analyses of active antioxidant polysaccharides from four edible mushrooms**. *Int J Biol Macromol.* 2019b; 123, pp. 945-956.
43. Zervakis G I, Venturella G, Papadopoulou K. **Genetic polymorphism and taxonomic infrastructure of the *Pleurotus eryngii* species-complex as determined by RAPD analysis, isozyme profiles and ecomorphological characters.** *Microbiology.* 2001; *147 (11):* 3183–3194.
44. Zhang Y, Choksi, S, Chen K, Pobezinskaya Y, Linnoila I, Zeng-Gang L. **ROS play a critical role in the differentiation of alternatively activated macrophages and the occurrence of tumor-associated macrophages**. Cell Research. 2013; V. 23, 898-914.
45. Zhang A, Li X, Xing C, Yang J, & Sun P. **Antioxidant activity of polysaccharide extracted from *Pleurotus eryngii* using response surface methodology**. *Int J Biol Macromol*. 2014; *65*, 28–32.
46. Zhang M, Cui S W, Cheung P C K, Wang Q. **Antitumor polysaccharides from mushrooms: a review on their isolation process, structural characteristics and antitumor activity.** *Trends Food Sci Technol*. 2007;18(1):4-19.
47. Zhang B, Li Y, Zhang F, Linhardt R J, Zeng G, Zhang A. **Extraction , structure and bioactivities of the polysaccharides from *Pleurotus eryngii*** : A Review. *Int J Biol Macromol*. 2020;1342-1347.

Supplementary Material

Table S1: ^{1}H and ^{13}C NMR chemical shifts for the cold water – CW, hot water - HW, and autoclave fraction – AE from *Pleurotus eryngii* mushroom.
Assignments are based on ^{13}C, ^{1}H, HSQC-DEPT and compare with referenced literature.

Fraction	Units	^{13}C sign[a]	^{1}H sign[a]	References[b]
CW	Mannogalactan			
	C1/H1 of β-D-Man*p*-(1→2) Linked	101.7	4.56	*a b c d*
	C1/H1 of α-D-Gal*p*-(1→6)-2-*O*-Me Linked	98.9	4.93	*a b c d*
	C1/H1 of α-D-Gal*p*-(1→6)-2-*O*-Me Linked	98.7	4.74	*a b c d*
	Glucan			
	C1/H1 of β-D-Glc*p*-(1→6)	102.9	4.24	*e*
HW	Glucans			
	C1/H1 of β-D-Glc*p*-(1→6) Linked	102.9	4.24	*e*
	C6/H6 of β-D-Glc*p*-(1→6) Linked	68.5	4.00 – 3.61	*e*
	C1/H1 of β-D-Glc*p*-(1→3)(1→6)	102.9	4.37 – 4.34	*f g h*
	C3/H3 of β-D-Glc*p*-(1→3)(1→6)	87.2	3.42	*f g h*
	C1/H1 of α-D-Glc*p*-(1→3) Linked	99.3	5.06	*h i*
	C3/H3 of α-D-Glc*p*-(1→3)	82.9	3.64	*h i*
AE	Glucans			
	C1/H1 of β-D-Glc*p*-(1→6)	102.9	4.24	*d*
	C6/H6 of β-D-Glc*p*-(1→6)	68.3	4.00 – 3.58	*d*
	C1/H1 of β-D-Glc*p*-(1→3)(1→6)	102.9	4.37 – 4.34	*e f g h*
	C3/H3 of β-D-Glc*p*-(1→3)(1→6)	86.0	3.47	*e f g h*
	C1/H1 of α-D-Glc*p*-(1→3) Linked	99.3	5.06	*i*
	C3/H3 of α-D-Glc*p*-(1→3)	83.2	3.65	*h i*
	C1/H1 of β-D-Glc*p*-(1→3) Linked	102.6	4.51	*j*
	C3/H3 of β-D-Glc*p*-(1→3) Linked	86.0	3.47	*j*

[a] The chemical shifts are expressed in ppm
[b] References: *a,* Carbonero et al., 2005; *b,* Carbonero et al., 2008; *c* Biscaia, et al., 2017; *d,* Smiderle et al., 2008; *e*, Smiderle et al., 2013; *f,* Sovrani et al., 2017; *g,* Morales et al., 2019; *h,* Morales et al., 2020; *i* de Jesus et al., 2018a; *j* Alquini et al., 2004.

Artigo 3

Mannogalactans from edible mushrooms *Pholiota nameko* and *Pleurotus eryngii*

Mannogalactans from edible mushrooms *Pholiota nameko* and *Pleurotus eryngii*

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ABSTRACT

The heteropolysaccharides from mushrooms are known because of your functional activities, like antitumoral, anti-inflammatory, immunostimulatory. Three heteropolysaccharides from *Pholiota nameko* and *Pleurotus eryngii* were obtained from cold-water extraction, and after purification by freeze and thawing process, and then Fehling solution treatment, the resulting fraction from *Pholiota nameko* was purified by ultrafiltration obtaining two heteropolymers - R30PN and E30PN with a molecular weight 37.9×10^4 g mol^{-1} and 2.4×10^4 g mol^{-1}, respectively. On the other hand, the fraction of *Pleurotus eryngii* was purified only by treatment with Fehling's solution producing a pure heteropolysaccharide with a molecular weight of 1.8×10^4 g mol^{-1}. The structural characterization was determined by monosaccharide composition, methylation analysis (by GC-MS), Nuclear Magnetic Resonance – NMR H^1, C^{13}, HSQC-DEPT, HSQC coupled and TOCSY 1D selective, and HPSEC-RI. The structures of the polysaccharides present a linear main chain of (1→6)- α-D-galactan and with simple *O*-2 ramifications of β-D-Man*p* units. The heteropolysaccharides contain mannose and galactose units, plus natural methylated units of 3-*O*-me-Gal*p.* The methodology used for the purification of these heteropolymers is of low cost with interesting results and can be used by other researchers to purify and clarify the chemical structures of these heteropolymers and the first time two mannogalactans with α-(1→6)-linked main chain were extracted and purified in the same fraction from an edible mushroom with different molecular weight.

Keywords: Heteropolysaccharides, *Pholiota nameko*, *Pleurotus eryngii*, cold water extraction; purification, structural characterization.

1. Introduction

Pleurotus eryngii and *Pholiota nameko* are mushrooms traditionally consumed in culinary preparations, like gourmet products, but scientific studies have revealed other wide potential of applications in the food industry, like functional food, and pharmaceutical industry as a therapeutic product, due to the biological potential observed of their polysaccharide fraction (Ren et al., 2016; Ruthes et al., 2016; Biscaia et al., 2017; Yan et al., 2019).

The heteropolysaccharides from edible mushroom represents the class of polysaccharides with biological benefits proved by scientific literature. Molecules like mannoglucans, galactomannas, heterogalactans, and mannogalactans, haves been applied in a variety of experiments *in vitro* and *in vivo* indicating interesting and successful results as immunomodulatory, antitumor, antinociceptive, gastroprotective molecules (Alquini et al., 2004; Kim et al., 2011; Smiderle et al., 2014; Silveira et al., 2015; Ren et al., 2016; Ruthes et al., 2016; Biscaia et al., 2017; Yan et al., 2019).

Intend to develop by-products containing the above-cited properties, a profound knowledge about the chemical constitution of the extracts and molecules remains needed, considering that the identification and characterization of the active compounds are of great importance (Wu et al., 2017).

The heteropolymers from edible mushrooms are known for their diversity of chemical structures. Several heteropolymers were identified by scientific literature. Structures like 3-*O*-methylated mannogalactan, on edible basidiomycetes from the Pleurotus genus, are identified and characterized, their main chain is composed of (1→6) linked α-D-galactopyranosil with units substituted on *O*-2 with β-D-mannopyranose (Rosado et al., 2003). Similar structures are described to *Pleurotus pulmonarius*,

Pleurotus ostreatus, *Pleurotus sajor-caju, Pleurotus eryngii* by Smiderle et al. (2008), Komura et al. (2014), Silveira et al. (2015), Biscaia et al. (2017) respectively.

This research study was developed to obtain, purify, and characterize the chemical structure of heteropolysaccharides from *Pholiota nameko* and *Pleurotus eryngii* edible mushrooms.

2. Material and Methods

2.1. Mushrooms

P. nameko mushroom was kindly provided *in natura* by Nayumi Cogumelos Especiais Company located in Mogi das Cruzes, São Paulo.

P. eryngii mushroom acquired (dried and ground) in the municipal market of Curitiba, Parana, Brazil.

2.2. Extraction and purification of heteropolysaccharides

Pholiota nameko

To extract the heteropolymers from *P. nameko* (Figure 1A), the samples (2.5 Kg) was freeze-dried, yielding 200 g, that were submitted to treatment with chloroform and methanol (2:1 *v:v*) at 60 °C by 3h (3 times), to remove the lower mass weigh compounds. The residual material was dried and then submitted to aqueous extraction at approximately 25 °C (3 times). The aqueous extract obtained was partially evaporated and precipitated with ethanol (3:1 , *v: v*).

The precipitate was collected after centrifugation (8000 rpm) and dialyzed so the retained (Aqueous Extract) was concentrated and then submitted to freeze-thawing process (Gorin & Iacomini, 1984). The insoluble fraction on cold water was subject to dimethyl sulfoxide (DMSO) treatment at 60 °C and centrifugated (8000 rpm 20 °C). The soluble fraction on DMSO went collected and dialyzed by 12 hours. In sequence was

subjected to the Fehling treatment was apply and the cooper-complexed and precipitated material was separated by centrifugation, neutralized with glacial acetic acid, uncomplexed with cationic resin and then dialyzed on a 2 kDa membrane, resulting a polysaccharide solution (Smiderle et al., 2008; Ruthes et al., 2015). This fraction was submitted to the ultrafiltration using 30 kDa membrane, in this process, two pure heteropolysaccharides were obtained, one on the eluted fraction (**E30PN**) and other on the retained fraction (**R30PN**) in this membrane. These heteropolysaccharides were submitted to structural characterization procedures (Figure 1).

Pleurotus eryngii

The *P. eryngii* mushroom powder (600 g) were mixed with organic solvent acetone at 60 °C by 6 h (3 times), the residual material was dialyzed (6 kDa cut- off) to remove the lower mass weight compounds and dried to submit it to aqueous extraction, at approximately 25 °C. To purify this aqueous extract (PE) was performed the method of Smiderle et al. (2008) and Ruthes et al. (2015) of Fehling treatment, just as previously described in *P. nameko* purification methods. The product obtained of Fehling solution fractionation (**FPE**) was submitted to the structural characterization.

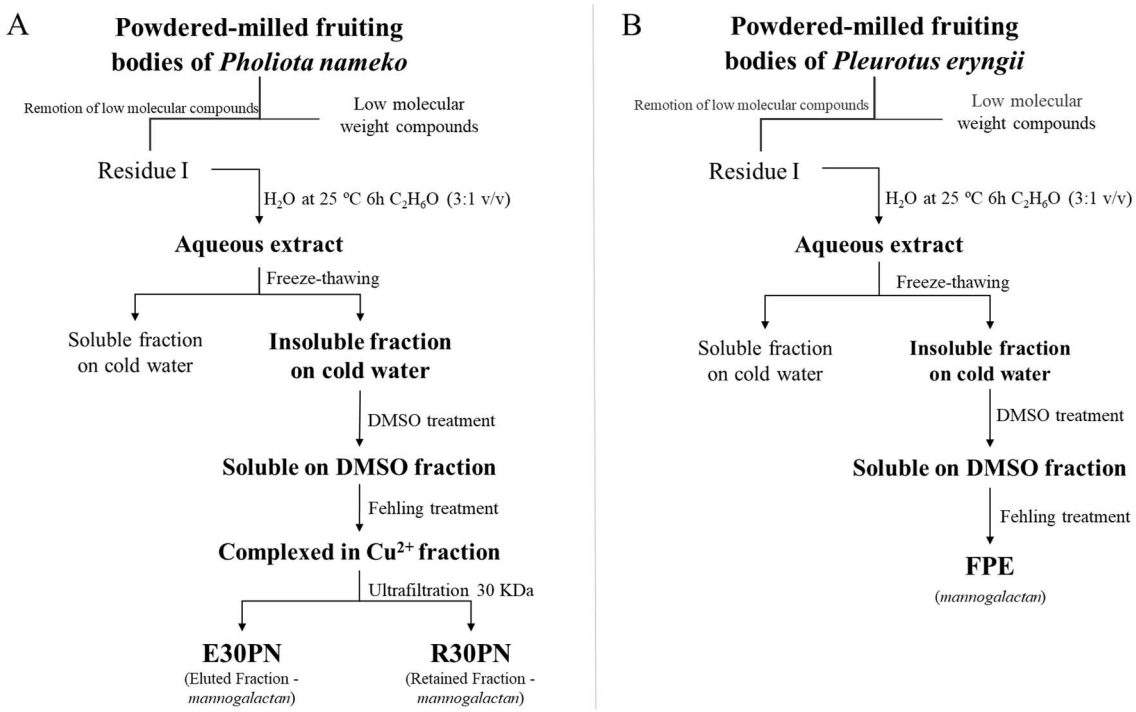

Fig. 1. Scheme of extraction and purification of Heteropolysaccharides from (**A**) *P. nameko* and (**B**) *P. eryngii*.

2.3 Determination of monosaccharide composition by GC-MS

The fractions (1 mg) were hydrolyzed with 2M TFA at 100 ºC for 8 h, followed to dryness. Hydrolyzed carbohydrate samples (1 mL) were reduced with sodium borohydride ($NaBH_4$) at room temperature overnight, after each sample was neutralized with acetic acid and subsequent cationic resin to remove their salts. The alditols was acetylated in pyridine–Ac_2O (200 µL; 1:1, v/v), for 40 min at 100 ºC. The alditol acetates were extracted with $CHCl_3$. The pyridine was removed by complexation with 5% $CuSO_4$.

The resulting derivatives were analyzed by GC-MS (Varian CP-3800 gas chromatograph coupled to an Ion-Trap 4000 mass spectrometer), using a VF5 column (30 m x 0.25 mm i.d.) programmed from 100 to 280 °C at 10 °C min^{-1}, with He as the carrier gas. And others resulting derivatives were analyzed by GC-MS carried out on a Shimadzu system consisting of a GC2010 Plus Gas Chromatograph coupled to a TQ8040 mass spectrometer (Shimadzu, Kyoto, Japan). The GC was equipped with an AOC5000 auto-

injector and a split–splitless injector. Chromatographic separation was achieved on a GC Column SH-RTX-5 ms (30 m × 0.25 mm × 0.25 μm) with helium 5.0 as the carrier gas at a flow rate of 1.0 mL min^{-1} and a column head pressure of 20 psi. Both injector and ion source temperatures were set at 300 °C. The total analysis time was 30 min. The mass spectrometer was operate in the full-scan (SCAN) mode over a mass range of m/z 75–400, both with electron ionization at 70 eV. The GCMS solution software (Tokyo, Japan) was use for data analysis. The monosaccharides have been identified by their typical electron impact breakdown profile and retention time and compared to the standards.

2.4. High Performance Size Exclusion Chromatography coupled to multidetectors (HPSEC-RI)

The fractions polysaccharides were dissolved in the eluent (1 mg mL^{-1}) filtered through a 0.22 μm membrane and then, injected (100 μL loop). The eluent was 0.1 M aqueous $NaNO_2$ containing 200 ppm aqueous NaN3 at flow 0.6 mL min^{-1}. The homogeneity of extracts and purified fractions were evaluated by High Performance Size Exclusion Chromatography (HPSEC), coupled refractive index (RI) detector (Waters 2410, Milford, MA, USA) (HPSEC-RI). Data were analyzed using Astra software version 4.70. The molecular weight of dextran standards (0.49 $_x10^6$ g mol^{-1}, 0.27 $_x10^6$ g mol^{-1}, 0.12 $_x10^6$ g mol^{-1}, 0.072 $_x10^6$ g mol^{-1}, 0.040 $_x10^6$ g mol^{-1}, 0.017 $_x10^6$ g mol^{-1}, and 0.009 $_x10^6$ g mol^{-1} from Sigma) were inject to obtain the calibration curve.

2.5. Methylation analysis

Each sample of the purified polysaccharide fractions (5 mg) from *P.nameko* eluted fraction E30PN and retained fraction R30PN; and from *P. eryngii* FPE were dissolved in DMSO and then methylated using NaOH powder and methyl-iodide (Me_2SO-MeI).

Methylation was interrupted by neutralization with acetic acid and then dialyzed for 24h, in running water. The methylation process was repeated thrice. Partially methylated compounds were extracted with chloroform and brought to dryness. The material was hydrolyzed at 100 °C with formic acid (45%) for 20 hours and lyophilized to remove the acid. The reduction was carried out with $NaBD_4$ (sodium borohydride) overnight and to dryness with methanol. Pyridine and acetic anhydride (1: 1) were used for 40 min to acetylation. The partially *O*-methylated alditol acetates were analyzed by GC-MS like described on topic 2.3. The derivatives were identified by their typical retention time and electron impact profile, in comparison to standards and the literature according to Sassaki et al. (2008).

2.6. Nuclear Magnetic Resonance (NMR)

^{1}H, ^{13}C, HSQC-DEPT, HSQC coupled and TOCSY 1D selective NMR spectra of polysaccharide fractions were obtained using a 400 MHz Bruker model Avance III spectrometer with a 5 mm inverse probe, at 70 °C in Me_2SO-d_6. Chemical shifts (δ) were expressed in ppm relative to Me_2SO-d_6 ^{13}C (δ 39.7) and ^{1}H (δ 2.50) resonances. The NMR signals were assigned according to 1D and 2D NMR experiments and literature data.

3. Results and discussion

The crude aqueous extracted from *P. nameko* (PN), after acid hydrolysis presented the monosaccharide composition of mannose (5.4%), galactose (11.3%), and high quantity of glucose (83.3%), while the aqueous extraction obtained from *P. eryngii* (PE) showed these same monosaccharides but with a low proportions of glucose (11.3%) just opposite of *P. nameko* and higher percentages of mannose (19.8%) and galactose (66.5%), see Table 1.

Table 1. Monosaccharide composition[abc] of *P. nameko* and *P. eryngii* fractions

	PN[b]	*R30PN[b]	*E30PN[b]	PE[c]	*FPE[b]
O-me-Gal*p*	1.6	17.8	23.7	2.4	15.2
Mannose	5.4	33.9	35.3	19.8	49.3
Glucose	83.3	-	-	11.3	-
Galactose	9.7	48.8	41.1	66.5	35.5

PN – aqueous extract from *Pholiota nameko*, R30PN - retained fraction and E30PN – eluated fraction from 30 kDa ultrafiltration; PE – aqueous extract from *Pleurotus eryngii,* FPE – fraction from *P. eryngii* obtained after Fehling treatment. [a]Alditol acetates obtained on successive hydrolysis, $NaBH_4$ reduction, and acetylation, followed by GC-MS analysis. Electron ionization at 70 eV. [b]GC-MS analysis on a Column SH-RTX-5 ms. [c]GC-MS analysis on a VF5 capillary column. * Purified fractions.

The aqueous extracts (crude fractions PN and PE) were subjected to the freezing and thawing process and two fractions, one soluble and other insoluble were produced. Most of the material remained in the insoluble fraction, then was solubilized with hot DMSO and after dialyzed, and then submitted to Fehling solution treatment obtaining a precipitate of the polysaccharides, which is it after uncomplexed with resin then the

material was dialyzed and your volume reduced and then submitted to membrane ultrafiltration (30 kDa) giving a retained R30PN, and an eluted E30PN fraction, both showing to be pure (Fig.1, Fig.3).

The two fractions R30PN and E30PN were composed by galactose 48.8%; 41.1% and mannose 33.9%; 35.3% respectively, but with small differences between fractions (Table 1). While *P. eryngii* mannogalactan (FPE) brings different percentages in these monosaccharides with mannose showed values of 49.3% while the galactose 35.5% just the opposite of the other fractions studied. Interestingly, but all heteropolymers after purification showed also the presence of natural 3-*O*-methyl-Gal*p* but in different percentages 17.8% and 23.0% for R30PN and E30PN respectively, however 15.2% for FPE, with a lower percentage than *P. nameko* (Table 1).

The NMR spectra of crude fractions from *P. nameko* (PN) and *P. eryngii* (PE) (Fig. 2 A and B) showed characteristic signals of whose signs show and presence of glucans and heteropolysaccharides, containing mannose and galactose and glucose. The signals observed on figure 2 (A), in the anomeric region were to α-D-Gal*p* of PN (C1/H1 98.9/5.03 ppm and 98.6/4.84 ppm); and the same region of α-D-Gal*p* of PE (C1/H1 98.8/5.02 ppm and 98.6/4.84 ppm). For both extracts, for units naturally methylated with (-C-O-CH3) was confirmed by the signals at 56.3/3.46 ppm and 56.3/3.45 ppm to PN and PE respectively (Fig. 2, A and B).

The PN spectra (Fig. 2A) presented assignments of β-D-Man*p* (C1/H1 - 101.7/ 4.66 ppm) and β-D-Glc*p* (C1/H1 - 103.3/ 4.51 ppm and 102.8/ 4.34 ppm) the two last signals is typical for β-(1,3) (C3/H3 – 86.9/3.59 ppm) and β-(1,6)-linked glucans (C6/H6 non-substituted – 60.9/3.80 ppm and 60.9/3.58 ppm) (Smiderle et al., 2014; Sovrani et al., 2017; Abreu et al., 2019; Morales et al., 2020). Less intense signals at δ 100.2/5.12 and 79.6/3.44 ppm are observed from anomeric carbon in α-configuration and substituted

C-4/H-4, that indicates an α-D-glucan, normally known as glycogen – that provides energy molecules to fungi (Synytsya et al., 2013; Palacios et al., 2012; Abreu et al., 2019).

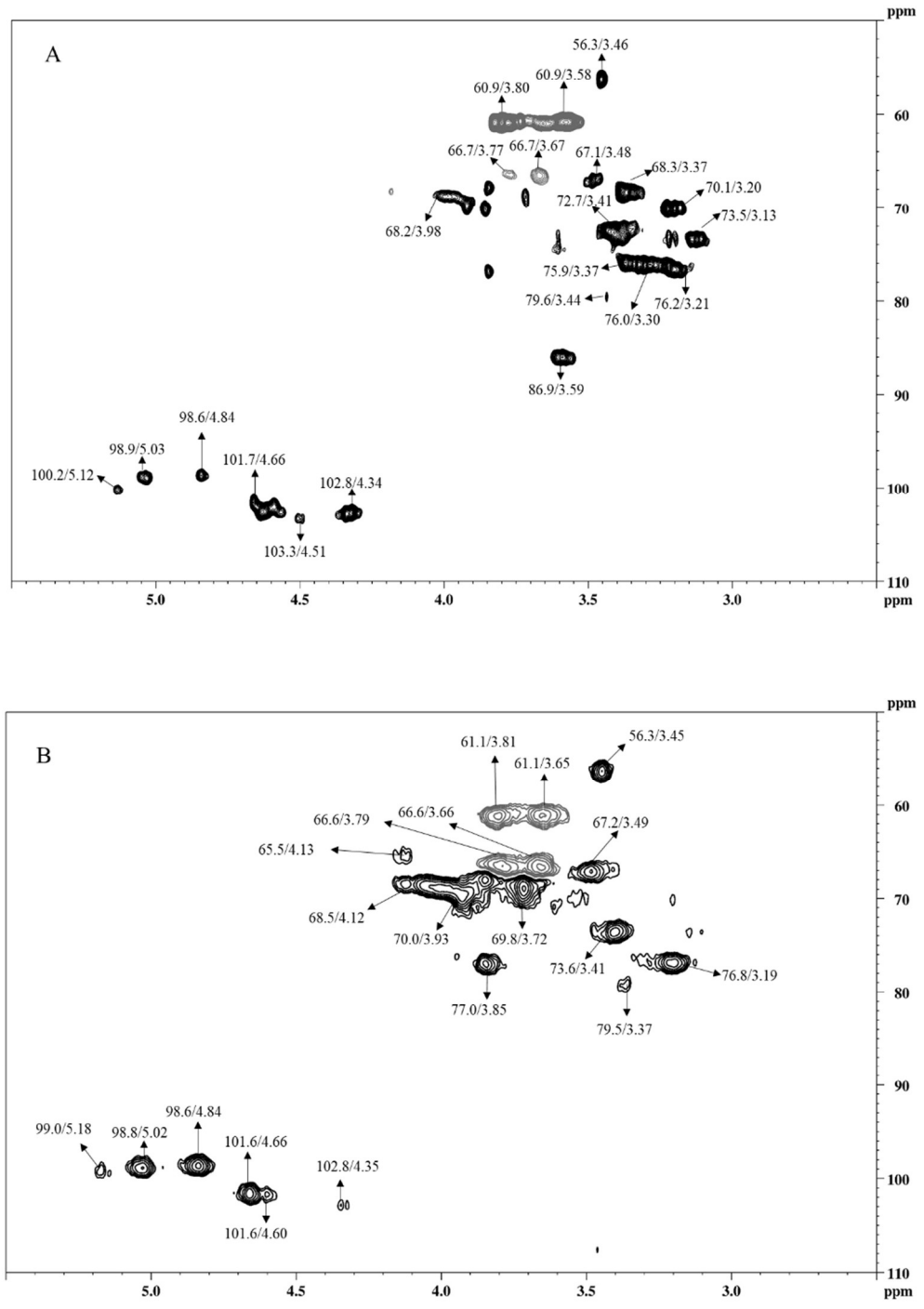

Fig. 2. HSQC-DEPT spectra of crude aqueous extract of *Pholiota nameko* and *Pleurotus eryngii*. (A) Aqueous extract from *Pholiota nameko* – PN and (B) aqueous extract from

Pleurotus eryngii - PE. Samples solubilized Me_2SO-d_6 at 70 ºC (chemical shifts are expressed in δ ppm).

To PE spectra (Fig. 2B) too showed characteristics signals of β-D-Man*p* (C1/H1 - 101.6/ 4.66 ppm and 101.6/4.60 ppm) and β-D-Glc*p* (C1/H1 - 102.8/ 4.35 ppm). Glucans and heteropolysaccharides with different chemical structures are generally present in crude extracts from edible mushrooms and the chemical characterization is determined by several scientific studies (Carbonero et al., 2008; Silveira et al., 2015; Sovrani et al. 2017; Biscaia et al., 2017; Morales et al., 2020).

The purification the aqueous extracts from PN and PE were developed separately by the freeze-thawing process (Gorin & Iacomini, 1984), an easy and important process to develop and bring excellent results in polysaccharide purification because it precisely separates water-soluble and water-insoluble chemical structures. The water-soluble fraction from PN was studied by Sovrani et al. (2017), which founded a β-D-Glucan-(1,3)-(1,6). While the water soluble fraction from PE remains under investigation.

The insoluble fractions of PN in water was solubilized in DMSO and then dialyzed and treated with Fehling solution and subjected to ultrafiltration 30 kDa (Fig 1.), generating the two pure new fractions E30PN (eluted fraction) and R30PN (retained fraction) and can be observed by their elution profile on HPSEC-RI (Fig. 3) as pure fraction. By the analyses of HPSEC-RI, it was possible to observe that the purification procedures efficiently separated two polysaccharides from *P. nameko* with different molecular weights. Ultrafiltration was essential to obtain an elution profile characteristic of a homogeneous solution, with one peak observed to R30PN and E30PN elution profiles, with different molecular weight estimated at 37.9×10^4 g mol^{-1} and 2.4×10^4 g mol^{-1}, respectively. Interesting that by using the ultrafiltration methodology two pure heteropolymers with very different molecular weights were obtained. The purified

heteropolysaccharide from *P. eryngii* (FPE) showed a homogeneous elution profile (Fig. 3), where just only one peak came up on HPSEC-RI profile, being their molecular weight estimated M_w of 1.8×10^4 g mol^{-1}.

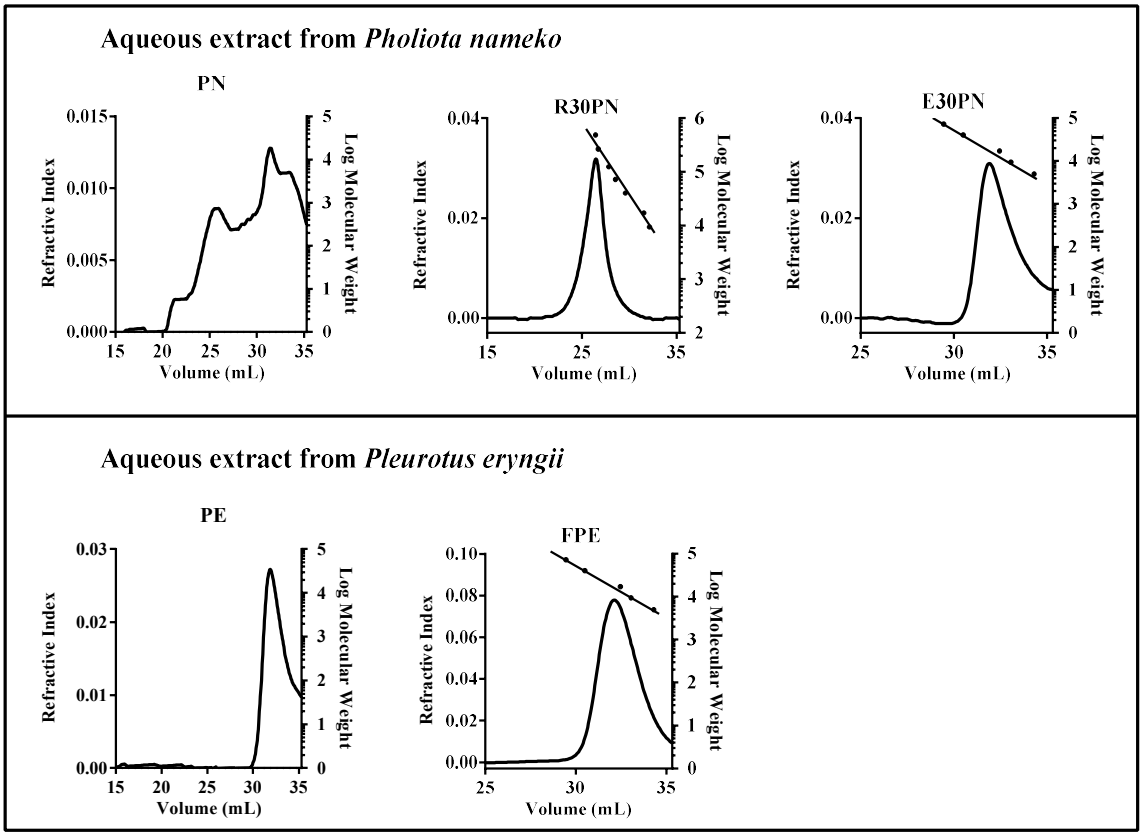

Fig. 3. HPSEC-RI elution profile of *Pholiota nameko* and *Pleurotus eryngii* polysaccharides. **PN** - Aqueous extract from *Pholiota nameko*, **R30PN** - retained fraction and **E30PN** – eluted fraction from 30 kDa ultrafiltration. **PE** - Aqueous extract from *Pleurotus eryngii,* **FPE** – fraction from *P. eryngii* obtained after Fehling treatment. The elution volume of dextran standards (left to right) was employed to construct the calibration curve.

The methylalditol derivatives from R30PN, E30PN and FPE (Table 2) showed great amount of branches for the three polysaccharides confirmed by the presence of 2,3,4,6-Me$_4$-Man*p* and 3,4-Me$_2$–Gal*p* as Gal*p* 2,6 di-*O*-substituted and the derivative 2,3,4-Me$_3$–Gal*p* correspond to 6-*O*-substituted Gal*p* as 2,3,4-Me$_3$–Gal*p* which strongly

indicates that the three polysaccharides have a main chain formed by α-Gal*p*-(1→6)-linked and with ramifications in 2-*O*-Gal*p* by β-D-Man*p* units. These polymers have already been seen in some other mushrooms. However, these mannogalactans have different molecular weights, branches and types of linkages with different percentages of the other heteropolymers described in the scientific literature from *P. ostreatus, P. pulmonarius*, *P. geesteranus*, etc (Rosado et al., 2003; Komura et al., 2014; Smiderle et al., 2008; Zhang et al., 2013a; Maity et al., 2014; Silveira et al., 2015; Biscaia et al., 2017).

Table 2. Partially *O*-methylalditol acetates derivatives of purified heteropolysaccharides fractions R30PN, E30PN, and FPE.

O-methylated alditol acetates[a]	Extract / (Mol %)			Linkage type[b]	Ions
	R30PN	**E30PN**	**FPE**		
2,3,4,6-Me_4-Man	37.4	36.6	36.6	Man*p*-(1→	87, 102, 118, 129, 145, 161, 205
2,3,4-Me_3–Gal	26.7	26.3	35.5	→6)-Gal*p*-(1→	87, 99, 102, 118, 129, 142, 162, 190, 233
3,4-Me_2–Gal	35.7	37.1	27.9	→2,6)-Gal*p*-(1→	87, 100, 130, 160, 190, 234

R30PN - retained fraction and E30PN – eluated fraction – both from *P. nameko*; FPE – fraction from *P. eryngii* obtained after Fehling treatment [a] GC-MS analysis on a Column SH-RTX-5 ms. Electron ionization at 70 eV. [b] Based on derived *O*-methylalditol acetates.

The natural 3-*O*-methylated heteropolymers are highly branched, and the results about their chemical structures indicated heteropolysaccharides from *P. nameko* and *P.eryngii* are similar. These heteropolymers studied have natural 3-*O*-methyl-galactopyranose and this type of monosaccharide is present in the main chain of these polysaccharides studied with varying percentages as 17.8 %. 23.7 % and 15.2 % for R30PN, E30PN and FPE, respectively, the values presents in Table 1 and are represented by the methylated derivatives 2,3,4-Me_3 –Gal*p* and 3,4-Me_2 – Gal*p* (Table 2).

The spectra of NMR (Fig.4.A and B) from R30PN and FPE showed signals in the anomeric region to an α-D-Gal*p* (C1/H1 δ 98.8/5.04) which correspond to a →2,6)- α-D-Gal*p*-(1→ linked; α-D-Gal*p* (C1/H1 δ 98.6/4.84) which correspond to a →6)-3-*O*-Me-α-D-Gal*p*-(1→ linked; β-D-Man*p* (C1/H1 δ 101.6/4.65), and β-D-Man*p* (C1/H1 δ 101.7/4.58) which corresponds to β-D-Man*p*-(1→ as terminal units. This two signals at C-1 / H-1 from Man*p* units are characteristics that these units are in two distinct chemical environments of mannogalactan (Silveira et al., 2015).

The anomeric configuration of each monosaccharide was verified by their respective coupling constants $J_{C\text{-}1,\ H\text{-}1}$ in the coupled HSQC spectrum (data don't show). The HSQC analysis showed also a strong singlet signal at δ 56.2/3.46, referring to a typical *O*-methyl group in C3-linked galactan (O'Rourke et al., 2015), confirming the data found by GC-MS analysis where the natural *O*-methyl group was identified. Just as the methylation data indicates the similarity of composition and structure, the heteropolymers NMR spectra showed high similarity (Fig. 4 A and B), following the description given above. Through the analysis of Selective Total Correlation spectra - TOCSY 1D, the present anomeric hydrogens of heteropolymers were sequentially irradiated, spectra obtained by selective excitation of the H-1 signal of the α-anomers and β-anomers (Fig. S4) is showing just those resonances correlated with the selective peak. By irradiating the H1 (4.84 ppm) of the 2,6-α-D-Gal*p*-linked (C1/H1 98.6/4.84 ppm) unit it is possible to identify the H2 δ 3.72, H3 δ 3.37, H4 δ 3.85, H5 δ 3.97 and H6 δ 3.67; 3.76 correlated to their respective carbons (C2 δ 68.3, C3 δ 79.1, C4 δ 67.9, C5 δ 68.7, C6 δ 66.3). An assignment table (Table 3) was completed after comparing all the spectra (^{1}H, ^{13}C, HSQC-DEPT and TOCSY 1D selective) and with the literature data (Rosado et al., 2003; Komura et al., 2014; Smiderle et al., 2008; Zhang et al., 2013a; Maity et al., 2014; Silveira et al., 2015; Biscaia et al., 2017).

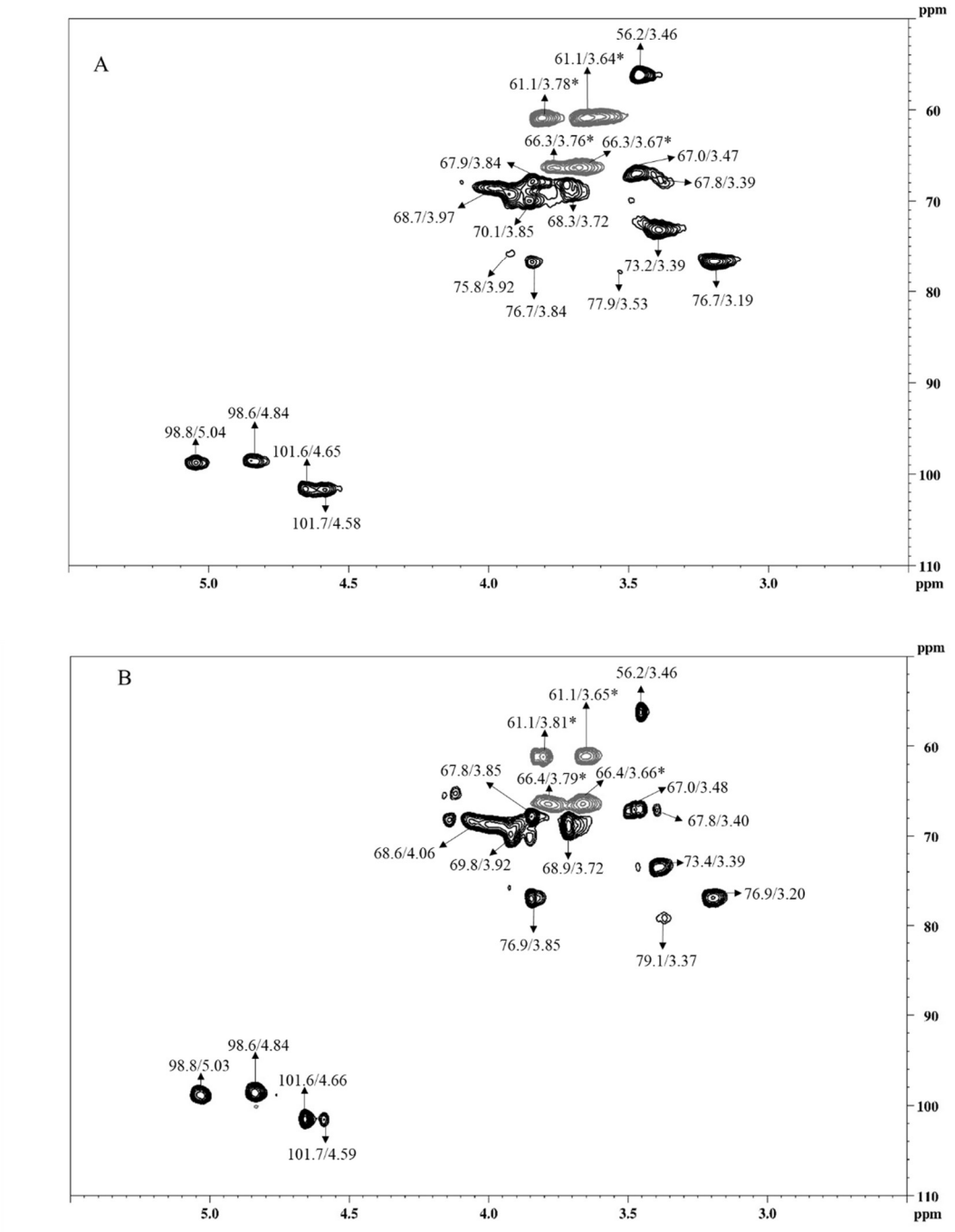

Fig. 4. HSQC-DEPT spectra of R30PN and FPE purified fraction from *Pholiota nameko* and *Pleurotus eryngii*, respectively. (A) R30PN – fraction from *P. nameko* retained in the ultrafiltration of 30 kDa. (B) FPE – fraction from *P. eryngii* obtained after Fehling treatment. Sample solubilized in Me_2SO-d_6 at 70 ºC (chemical shifts are expressed in δ ppm).

Table 3: ^{1}H and ^{13}C NMR chemical shifts for the Heteropolymers from *P. nameko* and *P. eryngii*

Units/assignments[a]	CH	1	2	3	4	5	6	*O*-Me
→6)-3-*O*-Me-α-D-Gal*p*-(1→	^{13}C	98.6	68.7	79.1	67.9	68.7	66.3	56.2
	^{1}H	4.84	3.72	3.37	3.84	3.97	3.67; 3.76	3.43
→2,6)-α-D-Gal*p*-(1→	^{13}C	98.8	76.7	69.3	67.9	68.7	66.3	
	^{1}H	5.04	3.84	3.93	3.84	3.97	3.66; 3.76	
β-D-Man*p*-(1→	^{13}C	101.6; 101.7	70.1	73.2	67.0	76.7	61.1	
	^{1}H	4.65; 4.58	3.85	3.39	3.47	3.19	3.64; 3.78	

[a] Samples in Me_2SO-d_6 at 70 ºC. Assignments based on ^{1}H, ^{13}C, HSQC, TOCSY experiments.

The chemical shifts are characteristic of mannogalactans, revealing their main structure, with their singular properties like anomeric configuration, linkage type present. Besides, of NMR experiments (Fig 4), the methylation data (Table 2) and composition (Table 1) by GC-MS, and the elution profile (Fig. 3) by HPSEC-RI, it is possible identify and characterize the heteropolysaccharides purified in this study.

4. Conclusions

Two different heteropolymers was extracted from the *P. nameko* edible mushroom. These chemical structures are differentiated by molecular weight, monosaccharide composition and branching of their side chains. Another *P. eryngii* mannogalactan also was structurally characterized and differs from the two other polymers mainly in the composition of monosaccharide and side chains present in the structure. All polysaccharides studied are formed by an α-(1→6)-linked the main chain represented by the methylated 2,3,4-tri-*O*-methyl-Gal*p* and 3,4-di-*O*-methyl-Gal*p*

derivatives and single side chains of the Man*p*-β-(1→2)-linked to the main chain α-(1,6)-linked-Gal*p* being represented by the methylated derivative 2,3,4,6-tetra-*O*-methyl-Man*p*. Interestingly, all polysaccharides have a rare and natural monosaccharide represented by 3-*O*-methyl galactopyranose with different percentages ranging from 15.2% to 27.3% for each purified polymer and is present in the main chain α -(1→6)-linked and receives lateral branches of mannose β-(1→2)-linked.

Through their characterized chemical properties, it is possible to compare with other chemical structures presented in the scientific literature and then investigate the probable potential of application in food or pharmaceutical purposes.

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The authors have declared no conflicts of interest.

Supplementary material

Supplementary material associated with this article can be found at page 114.

References

1. Abreu H, Simas F F, Smiderle F R, Sovrani V, Dallazen J L, et al. **Gelling functional property, anti-inflammatory and antinociceptive bioactivities of β-D-glucan from the edible mushroom *Pholiota nameko***. *Int. J. Biol. Macromol*., 122 (2019), pp. 1128-1135.
2. Alquini G, Carbonero E R, Rosado F R, Consentino C, Iacomini M. **Polysaccharides from the fruit bodies of the basidiomycete *Laetiporus sulphureus* (Bull.: Fr.) Murr**. *FEMSS Microbiology Letters*. 2004; 230: 47-52.
3. Biscaia S M P, Carbonero E R, Bellan D L, et al. **Safe therapeutics of murine melanoma model using a novel antineoplasic, the partially methylated mannogalactan from *Pleurotus eryngii***. *Carbohydr Polym*. 2017; 178 (August):95-104.
4. Carbonero E R, Gracher A H P, Komura D L, et al. ***Lentinus edodes* heterogalactan: Antinociceptive and anti-inflammatory effects.** *Food Chem*. 2008;111(3):531-537.
5. Gorin P A J, Iacomini M. **Polysaccharides of the lichens *Cetraria islandica* and *Ramalina usnea***. *Carbohydr Res*. 1984;128(1):119-132.
6. Kim S. P., Kang M. Y., Kim J. H., Nam S. H., and Friedman M. **Composition and Mechanism of Antitumor Effects of *Hericium erinaceus* Mushroom Extracts in Tumor-Bearing Mice**. *Journal of Agricultural and Food Chemistry.* **2011** *59* (18), 9861-9869.
7. Komura D L, Carbonero E R, Gracher A H P, et al. **Structure of *Agaricus spp.* fucogalactans and their anti-inflammatory and antinociceptive properties**. *Bioresour Technol*. 2010;101(15):6192-6199.
8. Komura D L, Ruthes A C, Carbonero E R, Gorin P A J, Iacomini M. **Water-soluble polysaccharides from *Pleurotus ostreatus* var. florida mycelial biomass**. *Int J Biol Macromol*. 2014. 70: 354-359.
9. Maity P, Pattanayak M, Maity S, et al. **A partially methylated mannogalactan from hybrid mushroom pfle 1p: purification , structural characterization , and study of immunoactivation.** *Carbohydr Res*. 2014;395:1-8.
10. Morales D, Rutckeviski R, Villalva M, et al. **Isolation and comparison of α - and β-D-glucans from shiitake mushrooms (*Lentinula edodes*) with different biological activities.** *Carbohydr Polym*. 2020; 229 (1 February)115521.
11. Motoshima R A, Rosa T da F, Mendes L da C, et al. **Inhibition of Leishmania amazonensis arginase by fucogalactan isolated from *Agrocybe aegerita* mushroom.** *Carbohydr Polym*. 2018; 201:532-538.
12. O'Rourke C, Gregson T, Murray L, Sadler IH, Fry S C. **Sugar composition of the pectic polysaccharides of charophytes, the closest algal relatives of land-plants: Presence of 3-*O*-methyl-d-galactose residues**. *Ann Bot*. 2015. 116: 225-236.

13. Palacios I, García-Lafuente A, Guillamón E, Villares A. **Novel isolation of water-soluble polysaccharides from the fruiting bodies of *Pleurotus ostreatus* mushrooms.** *Carbohydr Res*. 2012; 358:72-77.

14. Ren D, Wang N, Guo, J, Yuan L, Yang X. **Chemical characterization of *Pleurotus eryngii* polysaccharide and its tumor-inhibitory effects against human hepatoblastoma HepG-2 cells**. *Carbohydrate Polym.* 2016; *138*, 123–133.

15. Rosado F R, Carbonero E R, Claudino R F, Tischer C A, Kemmelmeier C, Iacomini M. **The presence of partially 3-*O*-methylated mannogalactan from the fruit bodies of edible basidiomycetes *Pleurotus ostreatus* "florida" Berk. and *Pleurotus ostreatoroseus* Sing**. *FEMS Microbiol Lett*. 2003; 221 (1): 119-124.

16. Ruthes A C, Carbonero E R, Córdova M M, et al. **Fucomannogalactan and glucan from mushroom *Amanita muscaria*: Structure and inflammatory pain inhibition**. *Carbohydr Polym*. 2013; 98(1):761-769.

17. Ruthes A C, Smiderle F R, Iacomini M. **D-Glucans from edible mushrooms: A review on the extraction, purification and chemical characterization approaches.** *Carbohydr Polym*. 2015; 117:753-761.

18. Ruthes A C, Smiderle F R, Iacomini M. **Mushroom heteropolysaccharides: A review on their sources, structure and biological effects**. *Carbohydr Polym*. 2016; 136: 358-375.

19. Ruthes A C, Rattmann Y D, Carbonero E R, Gorin P A J, Iacomini M. **Structural characterization and protective effect against murine sepsis of fucogalactans from *Agaricus bisporus* and *Lactarius rufus*.** *Carbohydr Polym*. 2012;87(2):1620-1627.

20. Sabaratnam V, Kah-Hui W, Naidu M, & Rosie D. P. **Neuronal health – can culinary and medicinal mushrooms help?**. *J of Traditional and Complementary Medicine*. 2013; *3*(1), 62–68.

21. Sassaki G L, Souza L M, Serrato R V., Cipriani T R, Gorin P A J, Iacomini M. **Application of acetate derivatives for gas chromatography–mass spectrometry: Novel approaches on carbohydrates, lipids and amino acids analysis**. *J Chromatogr A*. 2008; 1208(1-2):215-222.

22. Silveira MLL, Smiderle FR, Agostini F, et al. **Exopolysaccharide produced by *Pleurotus sajor-caju*: Its chemical structure and anti-inflammatory activity**. *Int J Biol Macromol*. 2015; 75:90-96.

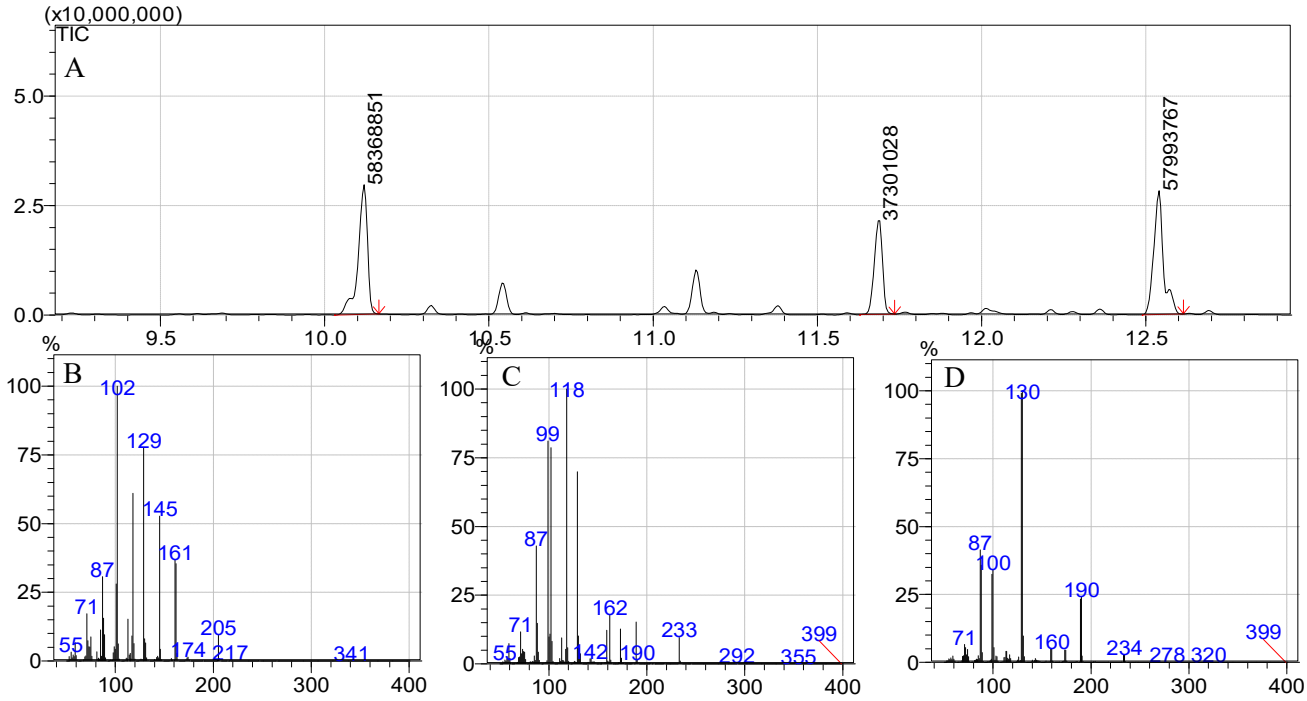
23. Sovrani V, Jesus L de, Simas-tosin FF, Smiderle FR, Iacomini M. **Structural characterization and rheological properties of a gel-like β-D-glucan from *Pholiota nameko***. *Carbohydr Polym*. 2017; 169:1-8.

24. Smiderle F R, Baggio C H, Borato D G, Santana-Filho A P, Sassaki G L, Iacomini M, & Van Griensven L J L D. **Anti-inflammatory properties of the medicinal mushroom *Cordyceps militaris* might be related to its linear (1→3)-β-D-glucan**. PLoS ONE, 9: e110266. 2014.

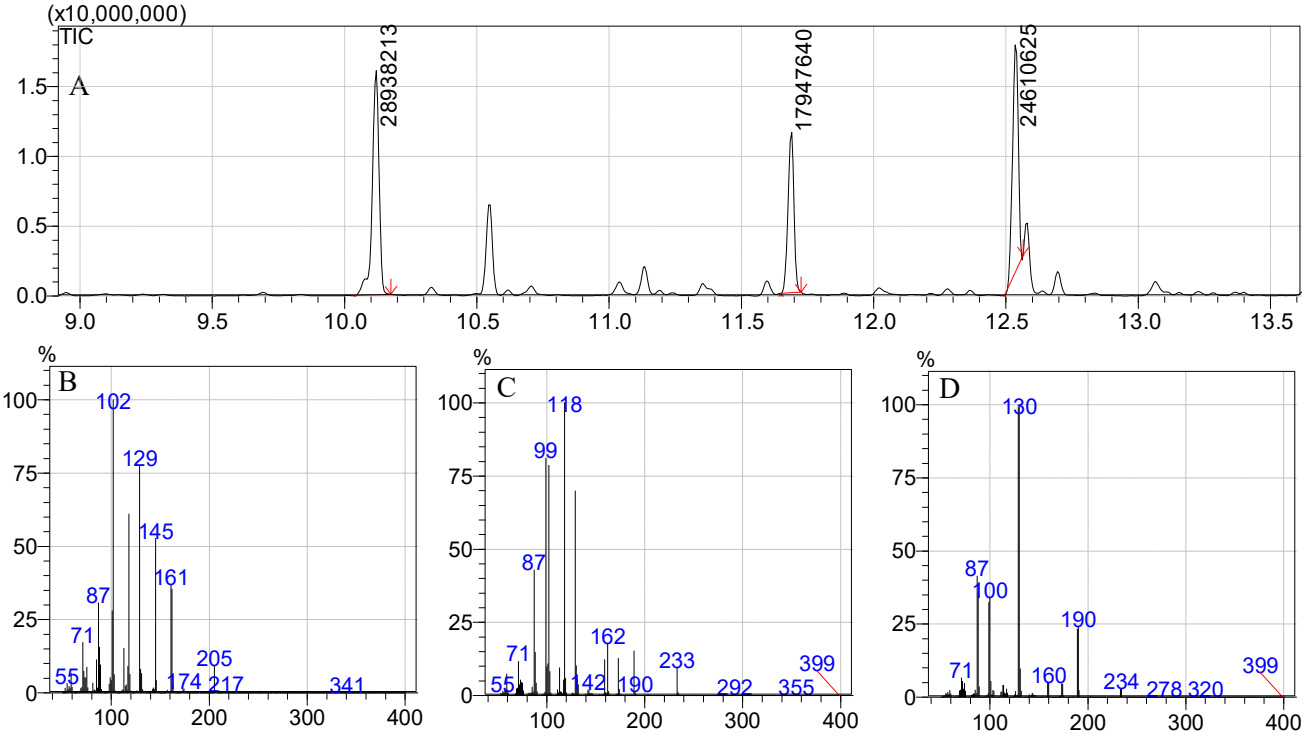
25. Smiderle F R, Olsen L M, Carbonero E R, et al. **A 3-*O*-methylated mannogalactan from *Pleurotus pulmonarius*: Structure and antinociceptive effect**. *Phytochemistry*. 2008; 69(15):2731-2736.

26. Smiderle F R, Ruthes A C, Iacomini M. **Natural polysaccharides from mushrooms: Antinociceptive and anti-inflammatory properties.** In: *Polysaccharides: Bioactivity and Biotechnology*.; 2015.
27. Synytsya A, Novák M. **Structural diversity of fungal glucans.** *Carbohydr Polym*. 2013;92(1):792-809.
28. Wu D.T., Deng Y., Chen L.X., Zhao J., Bzhelyansky A., Li S.P. **Evaluation on quality consistency of *Ganoderma lucidum* dietary supplements collected in the United States**. *Scientific Reports*, 7 (2017), p. 7792.
29. Yan J, Meng Y, Zhang M, et al. **A 3-*O*-methylated heterogalactan from *Pleurotus eryngii* activates macrophages**. *Carbohydr Polym*. 2019; 206: 706-715.
30. Zhang AQ, Xu M, Fu L, Sun PL. **Structural elucidation of a novel mannogalactan isolated from the fruiting bodies of *Pleurotus geesteranus***. *Carbohydr Polym*. 2013; 93 (1): 236-240.

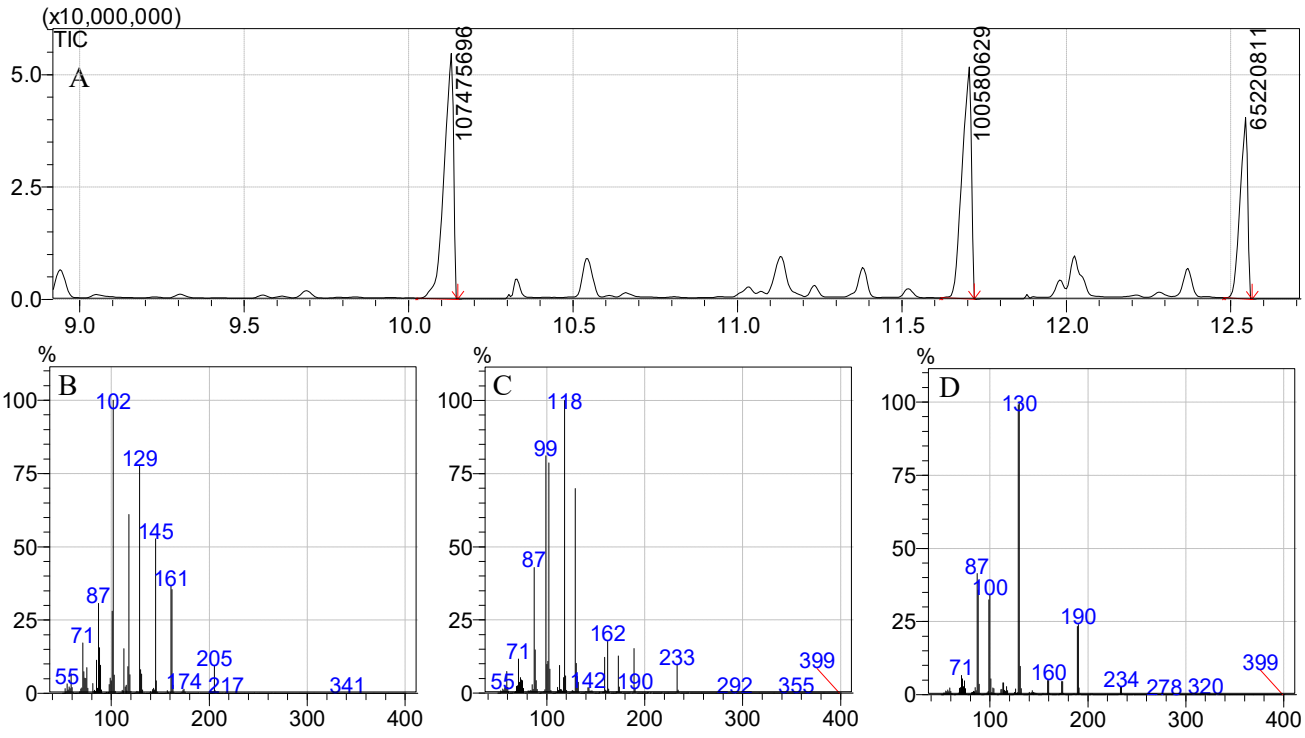
Supplementary material



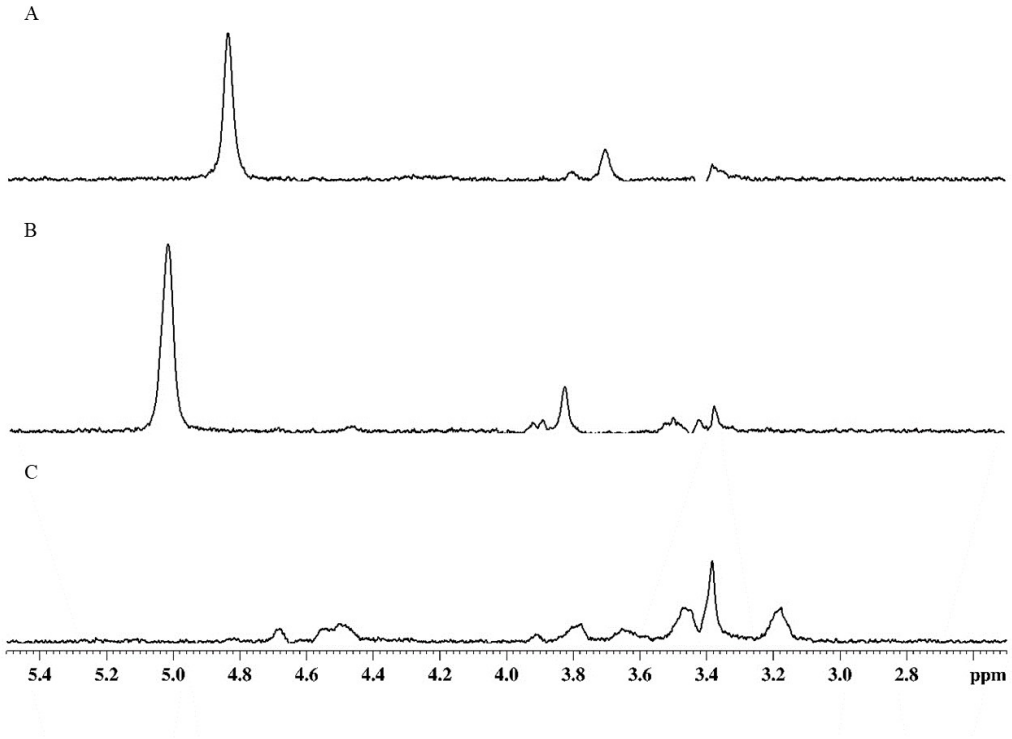
S1: Partially *O*-methylalditol acetates formed on methylation analysis of heteropolymer R30PN. (A) GC-MS chromatogram of Partially *O*-methylalditol acetates formed on methylation analysis of **R30PN**. The retention time of (**B**) 2,3,4,6-Me_4–Man in 10.115 min, (**C**) 2,3,4 Me_3–Gal 11.685 min, (**D**) 3,4 Me_2 –Gal 12.540 min and yours electron impact profile (at *m/z* 75 to 400). Electron ionization at 70 eV.



S2: Partially *O*-methylalditol acetates formed on methylation analysis of heteropolymer E30PN. (A) GC-MS chromatogram of Partially *O*-methylalditol acetates formed on methylation analysis of **E30PN**. The retention time of (**B**) 2,3,4,6-Me_4–Man in 10.115 min, (**C**) 2,3,4 Me_3–Gal 11.685 min, (**D**) 3,4 Me_2 –Gal 12.530 min and yours electron impact profile (at *m/z* 75 to 400). Electron ionization at 70 eV.



S3: Partially *O*-methylalditol acetates formed on methylation analysis of heteropolymer FPE. (A) GC-MS chromatogram of Partially *O*-methylalditol acetates formed on methylation analysis of **FPE**. The retention time of (**B**) 2,3,4,6-Me_4–Man in 10.110 min, (**C**) 2,3,4 Me_3–Gal 11.690 min, (**D**) 3,4 Me_2 –Gal 11.985 min and yours electron impact profile (at *m/z* 75 to 400). Electron ionization at 70 eV.



S4: Selective Total Correlation Spectra (TOCSY) of E30PN. (**A**) signals from the *O*-methyl α-galactose spin system only, spectra obtained by selective excitation of the H-1 signal of the α-anomer at 4.84 ppm. (**B**) signals from the α-galactose spin system only, spectra obtained by selective excitation of the H-1 signal of the α-anomer at 5.03 ppm. (**C**) signals from the β-mannose spin system only, spectra obtained by selective excitation of the H-1 signal of the β-anomer at 4.65 ppm.

I. CONCLUSÕES

- O cogumelo *P. nameko* após processo de extração com água quente e posterior purificação, apresentou uma β-D-Glucana (1→3),(1→6)-ligada que foi caracterizada quimicamente como um polissacarídeo com a cadeia principal de β-D-Glc*p* (1→3) apresentando 27% de suas unidades substituídas em *O*-6 por terminal não-redutor de β-D-Glc*p;* e/ou *O*-substituídas por pequenas cadeias laterais ligadas (1→6) β-D-Glc*p* com unidades substituídas em *O*-6 por terminal não-redutor β-D-Glc*p*, este polímero ainda não havia sido evidenciado para essa espécie. Quando em dispersão aquosa a β-D-glucana apresentou comportamento de gel. A dispersão gerada pela β-D-glucana também apresentou termoestabilidade diante de um experimento que buscou mimetizar o processo de pasteurização. Com este estudo conclui-se que o polissacarídeo extraído de *P. nameko* tem potencial para uso como aditivo alimentar na indústria de alimentos, como geleificante e espessante, podendo ser submetido à processos térmicos que variam de 90 ºC – 4 °C. Quanto ao efeito terapêutico da β-D-glucana à atividade antinociceptiva, concluiu-se que os animais tratados tiveram uma redução significativa da resposta à dor inflamatória (ID 0,7 mg. kg $^{-1}$).

- Métodos de extração aquosa foram aplicados ao cogumelo *P. eryngii*, e as frações obtidas provenientes de processos de extração com água na temperatura ambiente (CW), água quente a 100 °C (HW), e em autoclave (AE) forneceram extratos com diferenciada composição polissacarídica. A fração aquosa à temperatura ambiente foi caracterizada principalmente pelo elevado percentual do heteropolímero manogalactana. Enquanto na fração aquosa quente foi evidente a presença de β-D-glucana (1→6) linear, e na fração obtida de autoclave observou-se a presença de β-D-glucana (1→3) (1→6) em concentração majoritária e também de α-D-glucana (1→3) linear. As frações CW e HW quando testadas em células THP-1 revelaram atividade imunoestimulante aumentando a liberação de IL-1β, IL-10 e óxido nítrico.

- Três heteropolissacarídeos de *Pholiota nameko* e *Pleurotus eryngii* foram obtidos por extração em água à temperatura ambiente e então purificados e caracterizados. Dois heteropolímeros de *P. nameko* foram purificados por ultrafiltração com membrana de 30 kDa, sendo um polímero retido (R30PN) e um eluído (E30PN) nesta membrana, apresentaram pesos moleculares diferentes. O

heteropolímero (FPE) obtido do extrato aquoso à temperatura ambiente de *P. eryngii* foi purificado por tratamento com solução de Fehling e apresentou peso molecular diferente dos heteropolímero de *P. nameko*. Porém, todos os heteropolissacarídeos apresentam uma cadeia principal linear α-D-galactana(1→6) com ramificações simples em *O*-2 por unidades de β-D-Man*p*. Todas as manogalactanas estudadas apresentam grupos metil naturais em *O*-3 das unidades de Gal*p*. A metodologia usada para a purificação desses heteropolímeros é de baixo custo, com resultados interessantes e podem ser usadas por outros pesquisadores para purificar as estruturas químicas de heteropolímeros, principalmente manogalactanas com ligação α-(1→6) na cadeia principal. Interessante observar que pelo processo de ultrafiltração foi possível isolar duas manogalactanas de um mesmo cogumelo comestível, com pesos moleculares diferentes, tal resultado não tinha sido observado até a presente data.

REFERÊNCIAS

ABREU H, SIMAS F F, SMIDERLE F R, SOVRANI V, DALLAZEN J L, et al. Gelling functional property, anti-inflammatory and antinociceptive bioactivities of β-D-glucan from the edible mushroom *Pholiota nameko.* **Int. J. Biol. Macromol.**, 122, pp. 1128-1135, 2019.

ALQUINI, G. et al. Polysaccharides from the fruit bodies of the basidiomycete *Laetiporus sulphureus* (Bull.: Fr.) Murr. **FEMS Microbiology Letters**, v. 230, n. 1, p. 47–52, 2004.

ALZORQI I, SINGH A, MANICKAM S, AL-QRIMLI H F. Optimization of ultrasound assisted extraction (UAE) of β-D-glucan polysaccharides from *Ganoderma lucidum* for prospective scale-up. ***Resour Technol***. 3(1):46-54, 2017.

BAO H, YOU S, CAO L, ZHOU R, WANG Q, CUI S W. Chemical and rheological properties of polysaccharides from fruit body of *Auricularia auricular - judae*. **Food Hydrocoll**.57:30-37, 2016.

BAGGIO C H, FREITAS C S, MARTINS D F, et al. Antinociceptive Effects of (1→3),(1→6)-Linked β-Glucan Isolated From *Pleurotus pulmonarius* in Models of Acute and Neuropathic Pain in Mice: Evidence for a Role for Glutamatergic Receptors and Cytokine Pathways. **J Pain.** 11(10):965-971, 2010.

BAGGIO C H, FREITAS C S, MARCON R, et al. Antinociception of β-D-glucan from *Pleurotus pulmonarius* is possibly related to protein kinase C inhibition. **Int J Biol Macromol**. 50(3):872-877, 2012.

BISCAIA S. M. P., CARBONERO E. R., BELLAN D. L., et al. Safe therapeutics of murine melanoma model using a novel antineoplasic, the partially methylated mannogalactan from *Pleurotus eryngii*. ***Carbohydr Polym***. 178(August):95-104. 2017.

CARBONERO, E. R. Polissacarídeos de fungos liquenizados contendo diferentes fotobiontes. Tese de Doutorado. Departamento de Bioquímica e Biologia Molecular, UFPR, 120p, 2005.

CARBONERO E R, CORDEIRO L M C, MELLINGER C G, et al. Galactomannans with novel structures from the lichen *Roccella decipiens* Darb. **Carbohydr Res**. 340(10):1699-1705, 2005.

CARBONERO E R, GRACHER A H P, ROSA M C C, et al. Unusual partially 3-O-methylated α-galactan from mushrooms of the genus Pleurotus. **Phytochemistry**. 69(1):252-257, 2008.

CARBONERO, E. R., GRACHER, A. H. P., SMIDERLE, F. R., ROSADO, F. R., SASSAKI, G. L., GORIN, P. A. J., & IACOMINI, M. A β-glucan from the fruit bodies of edible mushrooms *Pleurotus eryngii* and *Pleurotus ostreatoroseus*. **Carbohydrate Polymers**, *66*(2), 252–257, 2006.

CASTRO-ALVES, V. C.; NASCIMENTO, J. R. O. DO. α- and β-D-Glucans from the edible mushroom *Pleurotus albidus* differentially regulate lipid-induced inflammation and foam cell formation in human macrophage-like THP-1 cells. **International Journal of Biological Macromolecules**, 2018.

CHANPUT W, MÊS J, VREEBURG R A M, SAVELKOUL H F J, WICHERS H J. Transcription profiles of LPS-stimulated THP-1 monocytes and macrophages: a tool to study inflammation modulating effects of food-derived compounds. **Food Funct**. 1 , 254 -261, 2010.

CHEN J, XIANG Y. Immunological and antitumor activities of the polysaccharides from *Pholiota nameko*. **Mod Food Sci Technol.** 29(8):1800-1804, 2013.

CHENG G Y, LIU J, TAO M X, LU C M, WU G R Activity, thermostability and isozymes of superoxide dismutase in 17 edible mushrooms. **J Food Compos Anal** 26:136–143, 2012.

CIUCANU I, KEREK F. A simple and rapid method for the permethylation of carbohydrates. **Carbohydrates Research**, v. 131, p. 209-217, 1984.

DE ARAÚJO I W F, RODRIGUES J Á G, QUINDERÉ A L G, et al. Analgesic and anti-inflammatory actions on bradykinin route of a polysulfated fraction from alga *Ulva lactuca*. **Int J Biol Macromol.** 92:820-830, 2016.

DE JESUS L I, SMIDERLE F R, CORDEIRO L M C, FREITAS R A DE, VAN GRIENSVEN L J L D, IACOMINI M. Simple and effective purification approach to dissociate mixed water-insoluble α- and β-D-glucans and its application on the medicinal mushroom *Fomitopsis betulina*. **Carbohydr Polym**. 200: 353-360, 2018a.

DE JESUS L I, SMIDERLE F R, RUTHES A C, et al. Chemical characterization and wound healing property of a β-D-glucan from edible mushroom *Piptoporus betulinus*. **Int J Biol Macromol.** 117:1361-1366, 2018b.

DING Q, ZHANG L, XU X, ZHANG X, WU C. Solution properties of pachyman from *Poria cocos* mycelia in dimethyl sulfoxide. **J Macromol Sci - Phys.** 40 B(2):147-156, 2001.

ELISASHVILI, V. Submerged cultivation of medicinal mushrooms: Bioprocess and products (review). **International Journal of Medicinal Mushrooms.** v.14(3), p. 211–239, 2012.

FISCHER M, CARLI G, RABOISSON P, & REEH P. The interphase of the formalin test. ***Pain***. *155*(3), 511–521, 2015.

FRIEDMAN, M. Mushroom Polysaccharides: Chemistry and Antiobesity, Antidiabetes, Anticancer, and Antibiotic Properties in Cells, Rodents, and Humans. **Foods**. 5:80, 2016.

GENEBANK, National Institute of Agrobiological Sciences. National Institute of Genetics. 2016/05/26. Acessado via http://www.gbif.org/occurence/729247832 em 31/05/2016.

GIL-RAMÍREZ A, MORALES D, SOLER-RIVAS C. Molecular actions of hypocholesterolaemic compounds from edible mushrooms. **Food Funct.** 9(1):53-69, 2018.

GOLDSTEIN, J.; HAY, G. W.; LEWIS, B. A.; SMITH, F. Controlled degradation of polysaccharides by periodate oxidation, reduction, and hydrolysis. **Methods in carbohydrate chemistry**. 5, pp. 361-369, 1965.

GORIN, P.A.J., IACOMINI, M. Polysaccharides of the lichens *Cetraria islandica* and *Ramalina usnea.* **Carbohydrate Research.** 128, pp. 119–131, 1984.

HU Q, WANG H, NG TB. Isolation and purification of polysaccharides with anti-tumor activity from *Pholiota adiposa* (Batsch) P. Kumm. (higher Basidiomycetes). **Int J Med Mushrooms.** 14(3):271-284, 2012.

HUNSKAAR, S.; HOLE, K. The formalin test in mice: dissociation between inflammatory and non-inflammatory pain. **Pain**, v. 30, p. 103-114, 1987.

JOH, T., TASAKI, Y., HARA, T., & HAYAKAWA, T. Induction and secretion of RNA-degrading enzymes by phosphate deficiency in *Pholiota nameko*. **Bioscience Biotechnology and Biochemistry**, *65*(11), 2561–2564, 2001.

JOH, T., YAZAKI, J., SUZUKI, K., & HAYAKAWA, T. Isolation and properties of glucose-1-phosphatase from mycelia of Pholiota nameko. **Bioscience biotechnology and biochemistry,** *62*(11), 2251–2253, 1998.

JI H, ZHANG L, ZHANG H, LI G, YANG M. Antioxidant activities of extracts from *Pholiota nameko*. **Adv Mater Res**. 343-344:457-462, 2012.

JUNG H Y, BAE I Y, LEE S, LEE H G. Effect of the degree of sulfation on the physicochemical and biological properties of *Pleurotus eryngii* polysaccharides. **Food Hydrocoll.** 25: 5, 1291-1295, 2011.

KIM S. P., KANG M. Y., KIM J. H., NAM S. H., AND FRIEDMAN M. Composition and Mechanism of Antitumor Effects of *Hericium erinaceus* Mushroom Extracts in Tumor-Bearing Mice. **Journal of Agricultural and Food Chemistry.** *59* (18), 9861-9869, 2011.

KOMURA D L, CARBONERO E R, GRACHER A H P, et al. Structure of Agaricus spp. fucogalactans and their anti-inflammatory and antinociceptive properties. **Bioresour Technol**. 101(15):6192-6199, 2010.

KOMURA D L, RUTHES A C, CARBONERO E R, GORIN P A J, IACOMINI M. Water-soluble polysaccharides from *Pleurotus ostreatus* var. florida mycelial biomass. **Int J Biol Macromol**. 70: 354-359, 2014.

KRATOCHVILL, F. et al. TNF Counterbalances the Emergence of M2 Tumor Macrophages. **Cell Reports**. Sep 22;12(11)1902-14, 2015.

LEWIS M, HEPPELL N. Continuous Thermal Processing of Foods Pasteurization and *UHT* Sterilization. Gaithersburg, Maryland; 2000.

LI, H.; LU, X.; ZHANG, S., LU, M., LIU, H. Anti-inflammatory activity of Polysaccharide from *Pholiota nameko*. **Biochemistry.** *Biokhimiia*, *73*(6), 669–675, 2008.

LI, H.; WANG, S. Kinetics of inhibition of ribonuclease A by *Pholiota Nameko* polysaccharide**. International Journal of Biological Macromolecules,** *40*(2), 134–138, 2007.

LI, H.; ZHANG, M.; MA, G. Hypolipidemic effect of the polysaccharide from *Pholiota nameko*. **Nutrition,** *26*(5), 556–562, 2010.

LI, H., LIU, X., LI, Y., HUA, Y., ZHI, D., & PANG, G. Effects of the polysaccharide from Pholiota nameko on human cytokine network in serum**. International Journal of Biological Macromolecules**, *50*(1), 164–170, 2012.

LI, S., & SHAH, N. P. Anti-inflammatory and anti-proliferative activities of natural and sulphonated polysaccharides from *Pleurotus eryngii*. **Journal of Functional Foods,** *23*, 80–86, 2016a.

LI, S., & SHAH, N. P. Characterization, antioxidative and bifidogenic effects of polysaccharides from Pleurotus eryngii after heat treatments. **Food Chemistry**, *197*, 240–249, 2016b.

LOCATI M, CURTALE G, MANTOVANI A. Diversity, Mechanisms, and Significance of Macrophage Plasticity. **Journal of clinical Investigation.** 123-147, 2019.

MA, G. et al. Purification, characterization and antitumor activity of polysaccharides from *Pleurotus eryngii* residue. **Carbohydrate Polymers**, v. 114, p. 297–305, 19 dez. 2014.

MALICK, D. H., JOH, T., TASAKI, Y., & HAYAKAWA, T. Identification of myo-inositol monophosphates in mycelia of *Pholiota nameko*. **Mycoscience,** *37*(3), 381–383, 1996.

MADHAVI N, DAS U N, PRABHA P S, KUMAR G S, KORATKAR R, SAGAR P S. Suppression of human T-cell growth in vitro by cis-unsaturated fatty acids: relationship to free radicals and lipid peroxidation. **Prostaglandins Leukot Essent Fatty Acids**. v. 51, n. 1, p. 33-40, 1994.

MAITY P, PATTANAYAK M, MAITY S, ET AL. A partially methylated mannogalactan from hybrid mushroom pfle 1p : purification , structural characterization, and study of immunoactivation. **Carbohydr Res**. 395:1-8, 2014.

MANTOVANI A, SICA A, SOZZANI S, ALLAVENA P, VECCHI A, LOCATI M. The chemokine system in diverse forms of macrophage activation and polarization. **Trends Immunol.** 25, pp. 677-686, 2004.

MCLAUGHLIN D J, HIBBETT D S, LUTZONI F, SPATAFORA J W, VILGALYS R. The search for the fungal tree of life. **Trends in Microbiology**. Nov; 17(11):488-97. 2009.

MORALES D, GIL-RAMIREZ A, SMIDERLE F R, PIRIS A J, RUIZ-RODRIGUEZ A, SOLER-RIVAS C. Vitamin D-enriched extracts obtained from shiitake mushrooms (*Lentinula edodes*) by supercritical fluid extraction and UV- irradiation. **Innov Food Sci Emerg Technol.** 41(February):330-336, 2017.

MORALES D, SMIDERLE F R, PIRIS A J, SOLER-RIVAS C, PRODANOV M. Production of a β-D-Glucan-rich extract from Shiitake mushrooms (*Lentinula edodes*) by an extraction/microfiltration/reverse osmosis (nanofiltration) process. **Innov Food Sci Emerg Technol.** 51: 80-90. 2018

MORALES D, SMIDERLE F R, VILLALVA M, ABREU H, RICCO C, SANTOYO S, IACOMINI M, SOLER-RIVAS C. Testing the effect of combining innovative extraction Technologies on the biological activities of obtained β-glucan-enriched fractions from *Lentinula edodes*. **Journal of Functional Foods.** 60: 103446, 2019.

MORALES D, RUTCKEVISKI R, VILLALVA M, ABREU H, et al. Isolation and comparison of α- and β-D-glucans from shiitake mushrooms (*Lentinula edodes*) with different biological activities. **Carbohydr Polym.** 229 (1 February)115521, 2020.

MORENO RB, RUTHES AC, BAGGIO CH, VILAPLANA F, KOMURA DL, IACOMINI M. Structure and antinociceptive effects of β-d-glucans from *Cookeina tricholoma*. **Carbohydr Polym**. 141:220-228, 2016.

MOTOSHIMA R A, ROSA T DA F, MENDES L DA C, et al. Inhibition of Leishmania amazonensis arginase by fucogalactan isolated from *Agrocybe aegerita* mushroom. **Carbohydr Polym.** 201:532-538, 2018.

MURRAY P J. Macrophage Polarization. **Annual Review of Physiology**. 79, 541-566, 2017.

MURRAY P J, ALLEN J E, BISWAS S K, FISHER E A, GILROY D W, GOERDT S, et al. **Macrophage activation and polarization: Nomenclature and experimental guidelines. Immunity**. 41 (1), pp. 14-20, 2014.

NITSCHKE J, MODICK H, BUSCH E, VON REKOWSKI R W, ALTENBACH H J. & MOLLEKEN H. A new colorimetric method to quantify beta-1,3-1,6-glucans in comparison with total beta-1,3-glucans in edible mushrooms. **Food Chemistry**. 127(2), 791–796, 2011.

OGAWA K, TSURUGI J, & WATANABE, T. Complex of gel-forming β-1, 3-D-glucan with Congo red in alkaline solution. **Chemical Letters**. 689-692, 1972.

OKASAKI M. Structure-Activity Relationship of (1-3)-β-D-Glucans in the Induction of Cytokine Production from Macrophages, *in vitro*. **Biol Pharm Bull**. 18(10):1320-1327, 1995.

O'ROURKE C, GREGSON T, MURRAY L, SADLER IH, FRY S C. Sugar composition of the pectic polysaccharides of charophytes, the closest algal relatives of land-plants: Presence of 3-*O*-methyl-d-galactose residues. **Ann Bot.** 116: 225-236, 2015.

PALACIOS I, GARCÍA-LAFUENTE A, GUILLAMÓN E, VILLARES A. Novel isolation of water-soluble polysaccharides from the fruiting bodies of *Pleurotus ostreatus* mushrooms. Carbohydrate Research, v. 358, p. 72–77, 2012.

POLISHCHUK, E. N.; KOVALENKO, A. G. Biological activity of glycopolymers from Basidiomycetes Mushrooms. **Biopolymers and Cell**, v. 25, p. 181-193, 2009.

PIPE R K, COLES J A, FARLEY, S R. **Assays for measuring immune response in the mussel Mytilus edulis**. . In: J. S. STOLEN;T. C. FLETCHER, et al (Ed.). Techniques in Fish Immunology - 4. Immunology and Pathology of Aquatic Invertebrates. Fair Haven: SOS Publications. p.93-100, 1995.

QIN F, KES M, CHRISTENSEN BE. A study of bioactive, branched (1→3)-β-D-glucans in dimethylacetamide/LiCl and dimethyl sulphoxide/LiCl using size-exclusion chromatography with multi-angle light scattering detection. **J Chromatogr A**. 1305:109-113, 2013.

RAO MA (Department of FS and T**. Rheology Of Fluid And Semisolid Foods - Principles And Applications.** Second ed. (BARBOSA-CÁNOVAS GV (DEPARTAMENT OBSE, ed.). GENEVA; 2007.

RAHAR S, SWAMI G. Preparation , characterization , and biological properties of β-glucans. **J Adv Pharm Technol Res**. 2(2):94-103, 2011.

REN, D., WANG, N., GUO, J., YUAN, L., & YANG, X. Chemical characterization of Pleurotus eryngii polysaccharide and its tumor-inhibitory effects against human hepatoblastoma HepG-2 cells. **Carbohydrate Polymers,** *138*, 123–133, 2016.

RENDRA E, RIABOV, V, MOSSEL D M, SEVASTYANOVA T, HARMSEN M C, KZHYSHKOWSKA, J. Reactive oxygen species (ROS) in macrophage activation and function in diabetes**. Immunobiology.** V. 224(2):242–253, 2018.

REILLY T P, BELLEVUE F H, WOSTER P M, SVENSSON C K. Comparison of the in vitro cytotoxicity of hydroxylamine metabolites of sulfamethoxazole and dapsone. **Biochem. Pharmacol.** 55, pp. 803-810, 1998.

RIBEIRO NA, ABREU TM, CHAVES HV, et al. Sulfated polysaccharides isolated from the green seaweed *Caulerpa racemosa* plays antinociceptive and anti-inflammatory activities in a way dependent on HO-1 pathway activation. **Inflamm Res**. 63(7):569-580, 2014.

RODRIGUES DMF, FREITAS AC, ROCHA-SANTOS TAP, VASCONCELOS MW, RORIZ M, et al. Chemical composition and nutritive value of *Pleurotus citrinopileatus* var cornucopiae, *P. eryngii, P. salmoneo stramineus, Pholiota nameko* and *Hericium erinaceus*. **Journal of Food Science and Technology**, *52*(11), 6927–6939, 2015.

RODRIGUES D, FREITAS AC, SOUSA S, AMORIM M, VASCONCELOS MW, et al. Chemical and structural characterization of *Pholiota nameko* extracts with biological properties. **Food Chemistry**, v. 216, p. 176–185, 2017.

ROSADO F R, CARBONERO E R, CLAUDINO R F, TISCHER C A, KEMMELMEIER C, IACOMINI M. The presence of partially 3-*O*-methylated mannogalactan from the fruit bodies of edible basidiomycetes *Pleurotus ostreatus* "florida" Berk. and *Pleurotus ostreatoroseus* Sing. **FEMS Microbiol Lett.** 221 (1): 119-124, 2003.

RUTHES A C, RATTMANN Y D, CARBONERO E R, GORIN P A J, IACOMINI M. Structural characterization and protective effect against murine sepsis of fucogalactans from *Agaricus bisporus* and *Lactarius rufus*. **Carbohydr Polym.** 2012;87(2):1620-1627. 2012.

RUTHES AC, CARBONERO ER, CÓRDOVA MM, ET AL. *Lactarius rufus* (1→3),(1→6)-β-D-glucans: Structure, antinociceptive and anti-inflammatory effects. **Carbohydr Polym**. 94(1):129-136, 2013a.

RUTHES AC, CARBONERO ER, CÓRDOVA MM, et al. Fucomannogalactan and glucan from mushroom *Amanita muscaria*: Structure and inflammatory pain inhibition. **Carbohydr Polym**. 98(1):761-769, 2013b.

RUTHES AC, SMIDERLE FR, IACOMINI M. D-Glucans from edible mushrooms: A review on the extraction, purification and chemical characterization approaches. **Carbohydr Polym.** 117:753-761, 2015.

RUTHES A C, SMIDERLE F R, IACOMINI M. Mushroom heteropolysaccharides: A review on their sources, structure and biological effects. **Carbohydr Polym.** 136: 358-375, 2016.

SABARATNAM V, KAH-HUI W, NAIDU M, & ROSIE DAVID P. Neuronal health – can culinary and medicinal mushrooms help?. **Journal of Traditional and Complementary Medicine.** *3*(1), 62–68, 2013.

SASSAKI GL, SOUZA LM, SERRATO R V., CIPRIANI TR, GORIN PAJ, IACOMINI M. Application of acetate derivatives for gas chromatography–mass spectrometry: Novel approaches on carbohydrates, lipids and amino acids analysis. **J Chromatogr A.**1208(1-2):215-222, 2008.

SEMEDO M C, KARMALI A, FONSECA L. A high throughput colorimetric assay of β-1,3-D-glucans by Congo Red Dye. **Journal of Microbiological Methods**. 109: 140-148, 2015.

SILVEIRA MLL, SMIDERLE FR, AGOSTINI F, ET al. Exopolysaccharide produced by *Pleurotus sajor-caju*: Its chemical structure and anti-inflammatory activity. **Int J Biol Macromol**. 75:90-96, 2015.

SOVRANI V, JESUS LI DE, SIMAS-TOSIN FF, SMIDERLE FR, IACOMINI M. Structural characterization and rheological properties of a gel-like β-D-glucan from *Pholiota nameko*. **Carbohydr Polym**. 169:1-8, 2017.

SMIDERLE, F. R.; OLSEN, L. M.; CARBONERO, E. R.; BAGGIO, C. H.; FREITAS, C. S.; MARCON, R.; SANTOS, A. R. S.; GORIN, P. A. J.; IACOMINI, M. Anti-inflammatory and analgesic properties in a rodent model of a (1→3), (1→6)-linked β-glucan isolated from *Pleurotus pulmonarius*. **Eur J Pharm** 597:86–91, 2008a.

SMIDERLE F R, OLSEN L M, CARBONERO E R, et al. A 3-*O*-methylated mannogalactan from *Pleurotus pulmonarius*: Structure and antinociceptive effect. **Phytochemistry.** 69(15):2731-2736, 2008b.

SMIDERLE FR, RUTHES AC, IACOMINI M. Natural polysaccharides from mushrooms: Antinociceptive and anti-inflammatory properties. In: **Polysaccharides: Bioactivity and Biotechnology.;** 2015.

SMIDERLE F R, ALQUINI G, TADRA-SFEIR M Z, IACOMINI M, WICHERS H J, VAN GRIENSVEN L J L D. *Agaricus bisporus* and *Agaricus brasiliensis* (1 → 6)-β-d-glucans show immunostimulatory activity on human THP-1 derived macrophages. ***Carbohydr Polym***. 94(1):91-99, 2013a.

SMIDERLE F R, BAGGIO C H, BORATO D G, SANTANA-FILHO A P, SASSAKI G L, IACOMINI M, & VAN GRIENSVEN L J L D. Anti-inflammatory properties of the medicinal mushroom *Cordyceps militaris* might be related to its linear (1→3)-β-D-glucan. **PLoS ONE**, 9: e110266, 2014.

SMIDERLE FR, SASSAKI GL, VAN ARKEL J, IACOMINI M, WICHERS HJ, VAN GRIENSVEN LJLD. High molecular weight glucan of the culinary medicinal mushroom *Agaricus bisporus* is an alpha-glucan that forms complexes with low molecular weight galactan. **Molecules**. 5(8):5818-5830, 2010.

SMIDERLE FR, ALQUINI G, TADRA-SFEIR MZ, IACOMINI M, WICHERS HJ, VAN GRIENSVEN LJLD. *Agaricus bisporus* and *Agaricus brasiliensis* (1 → 6)-β-D-glucans show immunostimulatory activity on human THP-1 derived macrophages. **Carbohydr Polym**. 94(1):91-99, 2013b.

SMIDERLE FR, MORALES D, GIL-RAMÍREZ A, et al. Evaluation of microwave-assisted and pressurized liquid extractions to obtain β-D-glucans from mushrooms. **Carbohydr Polym**. 156:165-174, 2017.

STUEHR D J, MARLETTA M A. Mammalian nitrate biosynthesis: mouse macrophages produce nitrite and nitrate in response to *Escherichia coli* lipopolysaccharide. **Proceedings of the National Academy of Sciences of the United States of America.** v. 82, n. 22, p. 7738-7742, 1985.

SYNYTSYA A, NOVÁK M. Structural diversity of fungal glucans. **Carbohydr Polym**. 92(1):792-809, 2013.

SYNYTSYA A, MÍČKOVÁ K, SYNYTSYA A, JABLONSKÝ I, SPĚVÁČEK J, et al. Glucans from fruit bodies of cultivated mushrooms Pleurotus ostreatus and Pleurotus eryngii: Structure and potential prebiotic activity. **Carbohydrate Polymers**, 2009.

TJOLSEN, A.; BERGE, O. G.; HUNSKAAR, S.; ROSLAND, J. H.; HOLE, K. The formalin test: an evaluation of the method. **Pain**, v. 51, p. 5-17, 1992.

WANG W, LI C, DU G, ZHANG X, ZHANG H. Characteristics and Rheological Properties of Polysaccharide Nanoparticles from Edible Mushrooms (*Flammulina velutipes*). **J Food Sci**. 82(3):687-693, 2017.

WASSER, S. P. Medicinal mushrooms as a source of antitumor and immunomodulating polysaccharides. **Applied Microbiology and Biotechnology**, v. 60, p. 258-274, 2002.

WHISTLER, R L. (1973). Solubility of polysaccharides and their behavior in solution. In H. S. Isbell (Ed.). Carbohydrates in solution. Washington, DC: *Amer Chemical Society Publications.* Ch. 14, pp. 242-255. **Advanced in Chemistry Series 117**.

WYNN T A, CHAWLA A, POLLARD J W. Macrophage biology in development, homeostasis and disease. **Nature Intern. Jour. Of Scien**. V. 496, pg. 445-455, 2013.

WYNN T A & VANNELLA K M. Macrophages in tissue repair, regeneration, and fibrosis. **Immunity**. 44, pp. 450-462. 2016.

WU D.T., DENG Y., CHEN L.X., ZHAO J., BZHELYANSKY A., LI S.P. Evaluation on quality consistency of *Ganoderma lucidum* dietary supplements collected in the United States. **Scientific Reports**, 7, p. 7792. 2017.

XU J, ZHANG J, LIU Y, SUN H, WANG J. Rheological properties of a polysaccharide from floral mushrooms cultivated in Huangshan Mountain. **Carbohydr Polym**. 139:43-49, 2016.
YALPANI M, HALL LD. Some Chemical and Analytical Aspects of Polysaccharide. **Macromolecules**. 281(16):272-281, 1984.

YAN J, MENG Y, ZHANG M, ZHOU X, CHENG H, SUN L, ZHOU Y. A 3-*O*-methylated heterogalactan from *Pleurotus eryngii* activates macrophages. ***Carbohydr Polym***. 206: 706-715, 2019a.

YAN J, ZHU L, QU Y, QU X, MU M, ZHANG M, et al. Analyses of active antioxidant polysaccharides from four edible mushrooms. I**nternational Journal of Biological Macromolecules**, v. 123, p. 945–956, 15 fev. 2019b.

YANG Z, XU J, FU Q, FU X, SHU T, BI Y, et al. Antitumor activity of a polysaccharide from Pleurotus eryngii on mice bearing renal cancer. **Carbohydrate Polymers**, 2013.

ZERVAKIS G I, VENTURELLA G, PAPADOPOULOU K. Genetic polymorphism and taxonomic infrastructure of the Pleurotus eryngii species-complex as determined by

RAPD analysis, isozyme profiles and ecomorphological characters. **Microbiology**. *147 (11):* 3183–3194, 2001.

ZHANG A, LI X, XING C, YANG J, & SUN P. Antioxidant activity of polysaccharide extracted from Pleurotus eryngii using response surface methodology. **International Journal of Biological Macromolecules,** *65*, 28–32, 2014.

ZHANG AQ, XU M, FU L, SUN PL. Structural elucidation of a novel mannogalactan isolated from the fruiting bodies of *Pleurotus geesteranus*. **Carbohydr Polym**. 93 (1): 236-240, 2013a.

ZHANG C, LI J, WANG J, SONG X, ZHANG J, WU S, HU C, et al. Antihyperlipidaemic and hepatoprotective activities of acidic and enzymatic hydrolysis exopolysaccharides from *Pleurotus eryngii* SI-04. **BMC complementary and alternative medicine,** 2017.

ZHANG Y, CHOKSI, S, CHEN K, POBEZINSKAYA Y, LINNOILA I, ZENG-GANG L. ROS play a critical role in the differentiation of alternatively activated macrophages and the occurrence of tumor-associated macrophages. **Cell Research**. V. 23, 898-914, 2013b.

ZHANG M, CUI S W, CHEUNG P C K, WANG Q. Antitumor polysaccharides from mushrooms: a review on their isolation process, structural characteristics and antitumor activity. **Trends Food Sci Technol**. 18(1):4-19, 2007.

ZHANG B, LI Y, ZHANG F, LINHARDT R J, ZENG G, ZHANG A. Extraction , structure and bioactivities of the polysaccharides from *Pleurotus eryngii* : A Review. *Int J Biol* **Macromol.** 1342-1347, 2020.

ZHENG L, ZHAI G, ZHANG J, WANG L, MA Z, JIA M, JIA L. Antihyperlipidemic and hepatoprotective activities of mycelia zinc polysaccharide from *Pholiota nameko* SW-02. **International Journal of Biological Macromolecules**, *70*, 523–529, 2014.

ZHONG Q, DAUBERT CR, *Food Rheology*. In: M Kutz (Eds.), Handbook of Farm, **Dairy and Food Machinery Engineering 2nd Edition**, Elsevier Inc.; pp. 403-426, 2013.

ANEXO I