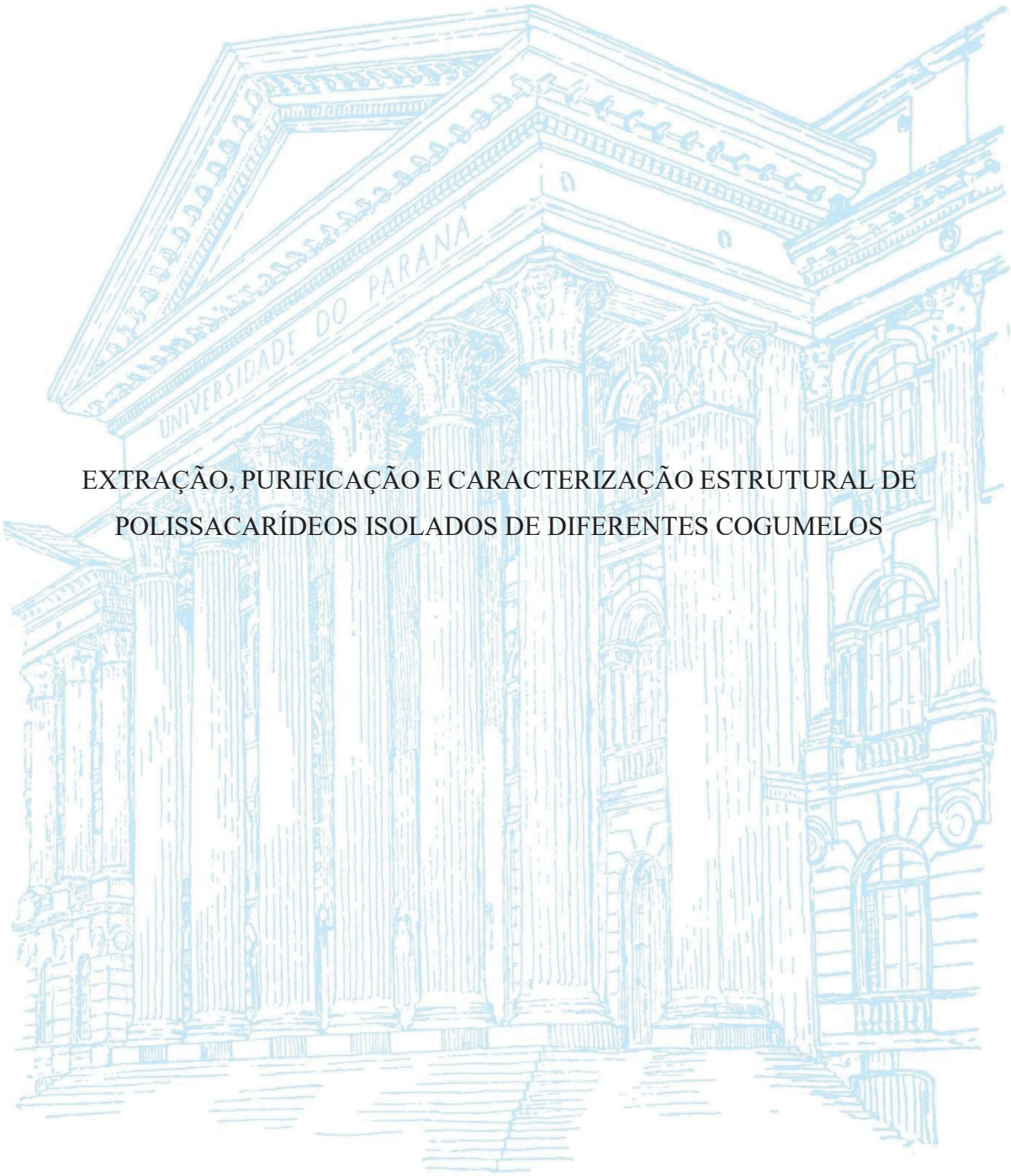
UNIVERSIDADE FEDERAL DO PARANÁ

MATHEUS ZAVADINACK



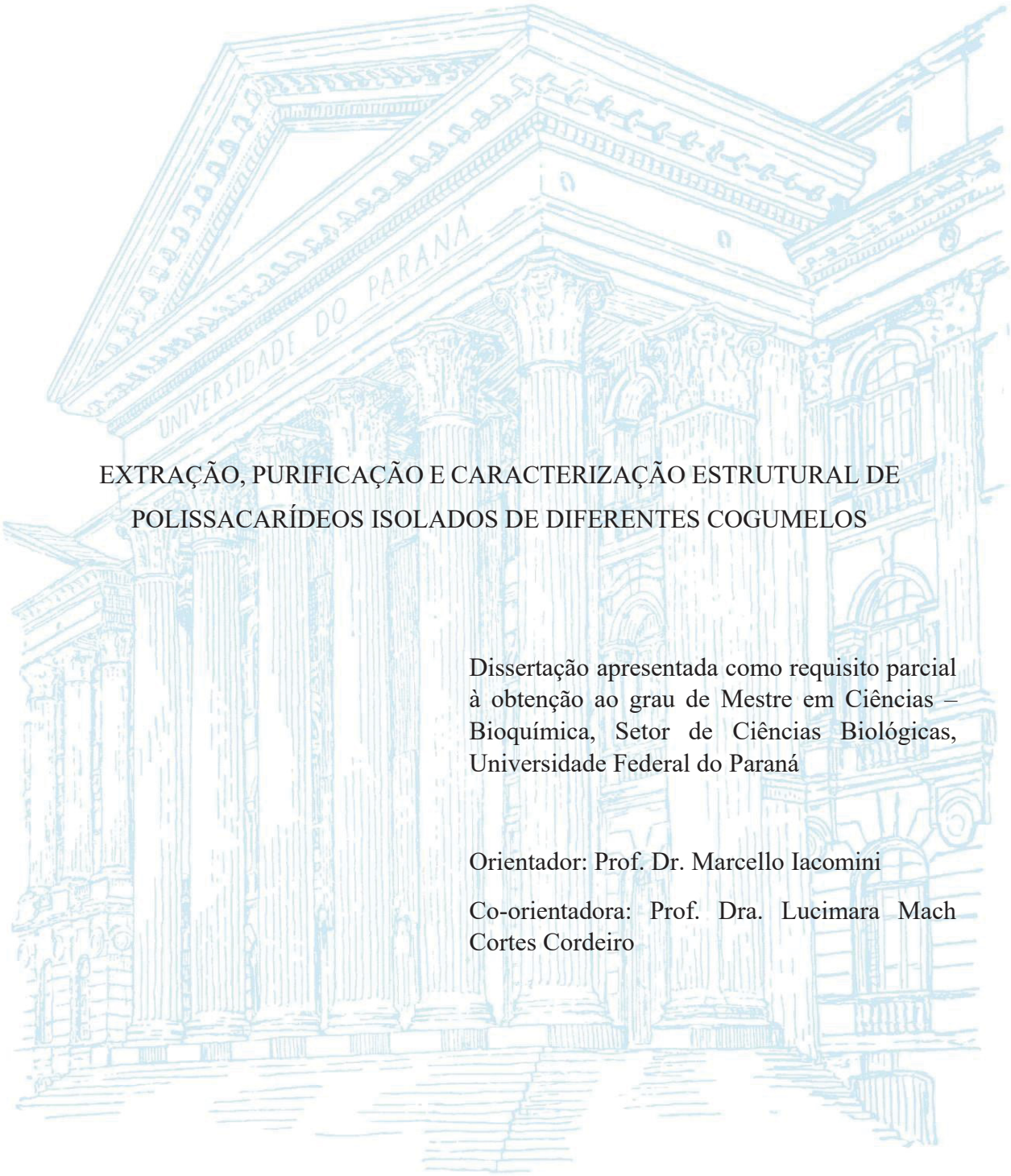
EXTRAÇÃO, PURIFICAÇÃO E CARACTERIZAÇÃO ESTRUTURAL DE POLISSACARÍDEOS ISOLADOS DE DIFERENTES COGUMELOS

CURITIBA

2020

UNIVERSIDADE FEDERAL DO PARANÁ

MATHEUS ZAVADINACK



EXTRAÇÃO, PURIFICAÇÃO E CARACTERIZAÇÃO ESTRUTURAL DE POLISSACARÍDEOS ISOLADOS DE DIFERENTES COGUMELOS

Dissertação apresentada como requisito parcial à obtenção ao grau de Mestre em Ciências – Bioquímica, Setor de Ciências Biológicas, Universidade Federal do Paraná

Orientador: Prof. Dr. Marcello Iacomini

Co-orientadora: Prof. Dra. Lucimara Mach Cortes Cordeiro

CURITIBA

2020

DADOS INTERNACIONAIS DE CATALOGAÇÃO NA PUBLICAÇÃO (CIP)
UNIVERSIDADE FEDERAL DO PARANÁ
SISTEMA DE BIBLIOTECAS – BIBLIOTECA DE CIÊNCIAS BIOLÓGICAS

Zavadinack, Matheus.
Extração, purificação e caracterização estrutural de polissacarídeos isolados de diferentes cogumelos. / Matheus Zavadinack. – Curitiba, 2020.
1 recurso on-line : PDF.

Dissertação (Mestrado) – Universidade Federal do Paraná, Setor de Ciências Biológicas. Programa de Pós-Graduação em Ciências – Bioquímica.
Orientador: Prof. Dr. Marcello Iacomini.
Coorientadora: Prof.ª Dra. Lucimara Mach Cortes Cordeiro.

1. Polissacarídeos. 2. Glucanas. 3. Galactanas. 4. Cogumelos. 5. Análise físico-química. 6. Pleurotus. 7. Pholiota. 8. Agaricus muscarius. 9. Estrutura química. I. Cordeiro, Lucimara Mach C. II. Universidade Federal do Paraná. Setor de Ciências Biológicas. Programa de Pós-Graduação em Ciências - Bioquímica. III. Título.

Bibliotecária: Rosilei Vilas Boas CRB-9/939

ATA Nº495

ATA DE SESSÃO PÚBLICA DE DEFESA DE MESTRADO PARA A OBTENÇÃO DO GRAU DE MESTRE EM CIÊNCIAS (BIOQUÍMICA)

No dia cinco de novembro de dois mil e vinte às 09:00 horas, na sala Virtual do Microsoft Teams, Virtual do Microsoft Teams, foram instaladas as atividades pertinentes ao rito de defesa de dissertação do mestrando **MATHEUS ZAVADINACK**, intitulada**: Extração, purificação e caracterização estrutural de polissacarídeos isolados de diferentes cogumelos**, sob orientação do Prof. Dr. MARCELLO IACOMINI. A Banca Examinadora, designada pelo Colegiado do Programa de Pós-Graduação em CIÊNCIAS (BIOQUÍMICA) da Universidade Federal do Paraná, foi constituída pelos seguintes Membros: MARCELLO IACOMINI (UNIVERSIDADE FEDERAL DO PARANÁ), CAROLINE MELLINGER SILVA (EMPRESA BRASILEIRA DE PESQUISA AGROPECUÁRIA), THALES RICARDO CIPRIANI (UNIVERSIDADE FEDERAL DO PARANÁ). A presidência iniciou os ritos

definidos pelo Colegiado do Programa e, após exarados os pareceres dos membros do comitê examinador e da respectiva contra argumentação, ocorreu a leitura do parecer final da banca examinadora, que decidiu pela APROVAÇÃO. Este resultado deverá ser homologado pelo Colegiado do programa, mediante o atendimento de todas as indicações e correções solicitadas pela banca dentro dos prazos regimentais definidos pelo programa. A outorga de título de mestre está condicionada ao atendimento de todos os requisitos e prazos determinados no regimento do Programa de Pós-Graduação. Nada mais havendo a tratar a presidência deu por encerrada a sessão, da qual eu, MARCELLO IACOMINI, lavrei a presente ata, que vai assinada por mim e pelos demais membros da Comissão Examinadora.

CURITIBA, 05 de Novembro de 2020.

Assinatura Eletrônica
09/11/2020 17:50:29.0
MARCELLO IACOMINI
Presidente da Banca Examinadora (UNIVERSIDADE FEDERAL DO PARANÁ)

Assinatura Eletrônica
19/11/2020 15:41:30.0
CAROLINE MELLINGER SILVA
Avaliador Externo (EMPRESA BRASILEIRA DE PESQUISA AGROPECUÁRIA)

Assinatura Eletrônica
07/11/2020 17:00:19.0
THALES RICARDO CIPRIANI
Avaliador Interno (UNIVERSIDADE FEDERAL DO PARANÁ)

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 Dissertação de Mestrado de **MATHEUS ZAVADINACK** intitulada: **Extração, purificação e caracterização estrutural de polissacarídeos isolados de diferentes cogumelos**, sob orientação do Prof. Dr. MARCELLO IACOMINI, que após terem inquirido o aluno e realizada a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.

A outorga do título de mestre 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, 05 de Novembro de 2020.

Assinatura Eletrônica
09/11/2020 17:50:29.0
MARCELLO IACOMINI
Presidente da Banca Examinadora (UNIVERSIDADE FEDERAL DO PARANÁ)

Assinatura Eletrônica
19/11/2020 15:41:30.0
CAROLINE MELLINGER SILVA
Avaliador Externo (EMPRESA BRASILEIRA DE PESQUISA AGROPECUÁRIA)

Assinatura Eletrônica
07/11/2020 17:00:19.0
THALES RICARDO CIPRIANI
Avaliador Interno (UNIVERSIDADE FEDERAL DO PARANÁ)

DEDICATÓRIA

Dedico este trabalho a todos aqueles que me apoiaram e me acompanharam durante toda esta jornada.

A Deus, que esteve comigo em todos os momentos, me dando força e persistência mesmo diante dos momentos mais difíceis.

A toda minha família, Lenira T. Zavadinack, Celso Zavadinack, Gabriela Zavadinack, Murilo Zavadinack, Daniela Carneiro dos Santos, Lineia Roberta Zen e Guilherme H. G. Cassi, que me apoiou e incentivou durante esses dois anos.

Em especial à minha Mãe e ao meu Pai, Lenira T. Zavadinack e Celso Zavadinack, que sempre me incentivaram a estudar e me deram todo o suporte necessário.

Aos meus irmãos, Gabriela Zavadinack e Murilo Zavadinack, por toda a amizade, carinho e confiança.

À minha namorada, Daniela, por todo o carinho, amor e apoio, mesmo nos meus momentos de ausência – inclusive nos finais de semana –, sempre contribuindo positivamente para que eu alcance meus objetivos.

A todos os meus amigos que de alguma forma contribuíram para que eu chegasse até aqui.

A todos os professores que se empenham e se dedicam à arte de ensinar. Saibam que vocês fazem parte de tudo isso.

Muito obrigado por tudo!

AGRADECIMENTOS

Agradeço a todos aqueles que participaram, direta ou indiretamente, durante esses dois anos de estudo e contribuíram de alguma forma para meu crescimento pessoal e profissional. A todos, muito obrigado!

Em especial, agradeço a Deus pela saúde e por me fornecer todas as condições necessárias para que eu vivenciasse todos esses incríveis momentos de aprendizado.

Ao meu orientador, Professor Dr. Marcello Iacomini, pela oportunidade e pela confiança depositada em mim durante esse período como seu aluno. Pelas conversas matinais e pela paciência para ensinar, algo raro atualmente! Sem dúvida, toda essa bagagem será de grande importância durante toda minha vida. Muito obrigado!

A minha co-orientadora, Professora Drª. Lucimara Mach Cortes Cordeiro, por todos os ensinamentos, oportunidades, contribuições e todo auxílio nos trabalhos desenvolvidos, e por apoiar e dar todo o suporte possível nas ideias que ainda serão desenvolvidas. Muito obrigado!

A todos os amigos e colegas de laboratório, em especial a Hellen Abreu, por ter me ensinado pacientemente toda a prática laboratorial e por produzir ciência junto comigo.

Aos Professores Doutores Thales Cipriani, Guilherme Lanzi Sassaki, Marcelo Muller e Rodrigo Vassoler Serrato.

Aos Técnicos e Técnicas, em especial ao Arquimedes Paixão de Santana Filho, Thiago J. dos Santos, Rosane Bagatin Tambosi, Keylla Lencone Mischiatti e Valter Antonio de Baura.

Às Agências Financiadoras CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior); e CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico).

À toda equipe do Departamento de Bioquímica e do Programa de Pós-Graduação em Ciências – Bioquímica da Universidade Federal do Paraná.

Muito obrigado!

´´Creio firmemente em uma lei de compensação. As verdadeiras recompensas são sempre proporcionais ao esforço e aos sacrifícios feitos. ``

- *Nikola Tesla*

NOTA EXPLICATIVA

Esta dissertação está estruturada no formato de artigo de acordo com as normas do Programa de Pós-Graduação em Ciências – Bioquímica e do Sistema de Bibliotecas (SiBi) da Universidade Federal do Paraná (UFPR). O conteúdo da tese é: introdução, revisão bibliográfica, justificativa, objetivos, artigos científicos, conclusões e referências. Os artigos científicos possuem revisão bibliográfica, materiais, metodologias, resultados, discussão, agradecimentos e referências.

RESUMO

Diversas propriedades biológicas estão relacionadas à estrutura química dos polissacarídeos encontrados nos corpos de frutificação de cogumelos. No presente trabalho, quatro polissacarídeos foram extraídos, isolados e caracterizados a partir dos cogumelos *Pleurotus pulmonarius*, *Pholiota nameko* e *Amanita muscaria* (L.:Fr) utilizando diferentes metodologias, e apresentaram características distintas, como composição monossacarídica, peso molecular, grau de ramificação e solubilidade. As amostras passaram por caracterização química por meio de análises de Ressonância Magnética Nuclear (^{13}C, ^{1}H, HSQC-DEPT, TOCSY, COSY), Cromatografia gasosa acoplada à espectrometria de massa (CG-EM), Cromatografia de exclusão por tamanho de alto desempenho (HPSEC) e Dispersão de luz estática (SLS). Através de extração em autoclave (121 °C, 1,2 atm) em materiais residuais dos cogumelos *P. pulmonarius* e *P. nameko*, os quais já haviam sido submetidos a extrações prévias sequenciais, duas β-D-glucanas com diferentes graus de solubilidade e pesos moleculares foram obtidas. Estas foram purificadas por técnicas de congelamento e degelo e ultrafiltração, respectivamente. Além disso, uma D-galactana e uma D-glucana solúveis em água foram obtidas após extração aquosa fria no cogumelo *A. muscaria* (L.:Fr) e purificadas separadamente por técnicas como diálise por membranas, tratamento por solução de Fehling, congelamento e degelo e ultrafiltração. Os sinais observados nos espectros de RMN e as análises de metilação referentes às D-glucanas indicam a presença de β-D-glucanas com cadeia principal (1→3)-ligada, o que se confirma pela degradação controlada de Smith, substituídas em *O*-6 por cadeias laterais (1→6)-ligadas de β-D-Glc*p* e por terminais não redutores de β-D-Glc*p*. Já a D-galactana apresenta cadeia principal (1→6)-ligada na configuração α substituída em *O*-2 por terminais não redutores de α-D-Gal*p*. Compreender a relação estrutura-função para glicanos pode ser mais difícil do que outras classes de biopolímeros. Portanto, o esclarecimento das estruturas químicas dos diferentes polissacarídeos encontrados em cogumelos é fundamental, pois facilitará a compreensão dos mecanismos de ação e propriedades biológicas para essas macromoléculas.

Palavras-chave: Polissacarídeos; β-D-glucana; α-D-galactana; cogumelos; caracterização química.

ABSTRACT

Several biological properties are related to the chemical structure of the polysaccharides found in the mushrooms fruiting bodies. In the present work, four polysaccharides were extracted, isolated and characterized from the mushrooms *Pleurotus pulmonarius*, *Pholiota nameko* and *Amanita muscaria* (L.:Fr) using different methodologies and presented distinct characteristics, such as monosaccharide composition, molecular weight, degree of branching and solubility. The samples underwent chemical chacarterization by Nuclear Magnetic Ressonance (^{13}C, ^{1}H, HSQC-DEPT, TOCSY, COSY), Gas Cromatography-Mass Spectrometry (GC-MS), High Pressure Size Exclusion Chromagraphy (HPSEC) and Static Light Scattering (SLS). Through autoclave extraction (121 °C, 1,2 atm) in residual fractions from mushrooms *P. pulmonarius* and *P.nameko* who had already been submited to previously sequencial extractions, two β-D-glucans with different solubility degree and molecular weights were obtained. They were purified by freezing and thawing and ultrafiltration process. In addition to these, a water-soluble D-galactan and D-glucan were obtained after cold water extraction from the mushroom *Amanita muscaria* (L.:Fr) and purified separately by techniques such as dialysis, treatment with Fehling solution, freezing and thawing and ultrafiltration. The signs observed at NMR studies and methylation analysis for D-glucans indicate the presence of β-D-glucans with main chain (1→3)-linked, confirmed by contolled Smith degradation, substituted at *O*-6 by side chains of (1→6)-linked β-D-Glc*p* and non-reducing end units of β-D-Glc*p*. The D-galactan has a main chain (1→6)-linked in the α configuration substituted at *O*-2 by non-reducing end units of α-D-Gal*p*. Understanding the structure-function relationship for glycans can be more difficult than other classes of biopolymers. Therefore, clarifying the chemical structure from different polysaccharides found in mushrooms is essential, as it will facilitate the comprehension of the mechanisms of action and the biological properties from these macromolecules.

Keywords: Polysaccharides; β-D-glucan; α-D-galactan; mushrooms; chemical characterization.

LISTA DE QUADROS E FIGURAS

Revisão de literatura

LISTA DE FIGURAS

ARTIGO 1

Structural characterization of an unmethylated α-D-galactan and a β-D-glucan isolated from the mushroom *Amanita muscaria*

ARTIGO 2

β-D-glucans (1→3),(1→6) obtained from residual material by extraction at high temperature and pressure from different mushrooms

LISTA DE TABELAS

ARTIGO 1

Structural characterization of an unmethylated α-D-galactan and a β-D-glucan isolated from the mushroom *Amanita muscaria*

ARTIGO 2

β-D-glucans (1→3),(1→6) obtained from residual material by extraction at high temperature and pressure from different mushrooms

LISTA DE ABREVIATURAS, SIGLAS E SÍMBOLOS

Frações obtidas de *Pleurotus pulmonarius*

Pp-1	Fração obtida após extração aquosa em alta temperatura e pressão
Pp-KOH	Fração obtida após extração aquosa em alta temperatura e pressão em meio alcalino
Pp-GLC	Fração insolúvel obtida após processo de gelo e degelo
Pp-Sm	Polissacarídeo residual obtido após degradação controlada de Smith

Frações obtidas de *Pholiota nameko*

Pn-1	Fração obtida após extração aquosa em alta temperatura e pressão
Pn-2	Fração obtida após precipitação em etanol
Pn-GLC	Fração retida após ultrafiltração em membrana de 3 kDa
Pp-KOH	Fração obtida após extração aquosa em alta temperatura e pressão em meio alcalino
Pn-Sm	Polissacarídeo residual obtido após degradação controlada de Smith

Frações obtidas de *Amanita muscaria*

CW	Extrato obtido após extração aquosa fria
P-Am	Fração obtida após precipitação em etanol
F-1	Fração precipitada na diálise obtida após centrifugação
F-2	Fração sobrenadante obtida após centrifugação
GAL-Am	Fração precipitada do tratamento com solução de Fehling em F-1
S-2	Sobrenadante do tratamento com solução de Fehling realizada em F-1
S-3	Fração solúvel após gelo e degelo em F-2
S-4	Sobrenadante do tratamento com solução de Fehling realizada em S-3
GLC-Am	Fração retida no processo de ultrafiltração em S-4
GLC-Sm	Polissacarídeo residual obtido após degradação controlada de Smith

Compostos químicos e termos associados

AcOH	Ácido Acético
$CHCl_3$	Clorofórmio
CH_2O_2	Ácido fórmico
DMSO	Dimetilsulfóxido
Me_2SO-d_6	Dimetilsulfóxido deuterado
Gal	Galactose
Glc	Glucose
KOH	Hidróxido de potássio
Kc	Constante óptica
Man	Manose
Me	Grupamento metil (-CH_3)
MeOH	Metanol
M*w*	Massa molecular ponderal média
$NaBD_4$	Boroidreto de sódio deuterado
$NaBH_4$	Boroidreto de sódio
NaOH	Hidróxido de sódio
per-*O*-metilado	Parcialmente *O*-metilado
spp.	Espécies pertencentes ao mesmo gênero
TFA	Ácido trifluoroacético

Técnicas de análise e termos associados

COSY	Correlation Spectroscopy (Espectroscopia de Correlação)
GC-MS	Gas Cromatography-Mass Spectrometry (Cromatografia gasosa acoplada à espectrometria de massas)
HPSEC	High Pressure Size Exclusion Chromagraphy (Cromatografia de exclusão estérica de alto desempenho)
HSQC	Heteronuclear Single Quantum Correlation Spectroscopy (Espectroscopia de Correlação Heteronuclear Quântica Simples)
I_R	Índice de refração

RMN-^{13}C	Ressonância magnética nuclear de carbono 13
TOCSY	Total Correlation Spectroscopy (Espectroscopia de Correlação Total)
SLS	Espalhamento de luz estática

SUMÁRIO

1. INTRODUÇÃO

A produção e o consumo de cogumelos vêm crescendo muito devido à conscientização da população sobre suas propriedades benéficas e seu alto valor nutricional. Além de possuírem alto teor de carboidratos não digeríveis, proteínas e baixo teor lipídico, os cogumelos também possuem metabólitos secundários como flavonoides, compostos fenólicos, terpenóides, entre outros, que apresentam atividades biológicas importantes (Sánchez, 2017). Segundo Hardy (2008), o aumento do consumo desses cogumelos também pode ser aliado ao tratamento de cânceres como suplemento dietético alternativo, aliado ao processo de cura.

O número de espécies de cogumelos já conhecidas pode chegar a 12.000, sendoomente 10% identificadas (Aida et al., 2009). Estima-se que o Brasil produza pouco mais de 12.000 toneladas "in natura" de cogumelos comestíveis, sendo 2.000 toneladas de *Pleurotus spp* e, liderando esse cenário, estão os estados do Paraná e São Paulo. Comparada com a produção e o consumo de países como a China, Itália e Estados Unidos, o Brasil ainda apresenta números consideravelmente pequenos. A China lidera esse mercado com uma produção aproximada de 5.000.000 de toneladas e consumo médio de 4 kg/ano por pessoa, enquanto o brasileiro consome aproximadamente 200 g/ano (Amazonas, 2013).

A parede celular dos fungos é composta, principalmente, por quitina, hemicelulose, mananas e β-glucanas, que são polissacarídeos não digeríveis, compondo a classe das fibras dietéticas. Quando consumidas, podem contribuir com a motilidade do trato gastrointestinal e, também, com o aumento do volume das fezes, devido ao conteúdo de material não digerível, além de diminuir a absorção de substâncias tóxicas e carcinogênicas prejudiciais (Manzi & Pizzoferrato, 2000). Cantu-Jungles et al. (2018) mostraram que β-glucanas insolúveis podem modular a composição da microbiota intestinal, aumentando o número de bactérias butirogênicas -o que pode prevenir o desenvolvimento de diabetes, a resistência à insulina, à hipercolesterolemia, as doenças metabólicas e o câncer colorretal-, bem como reduzindo a formação de gases em um modelo de fermentação fecal humano *in* vitro. Segundo Khatun et al. (2015), esta e outras propriedades fazem dos cogumelos comestíveis um alimento nutracêutico, uma vez que seu consumo pode previnir e contribuir no tratamento de doenças.

Os polissacarídeos extraídos dos corpos frutíferos dos cogumelos têm sido foco de atenção na área biomédica devido ao potencial terapêutico e à relativa baixa toxicidade (Schepetkin & Quinn, 2006). Dentre as diversas propriedades biológicas já observadas, pode-se citar: atividade anti-inflamatória, antitumoral, imunomoduladora, analgésica, antibacteriana, antiesclerótica, antiviral e antinociceptiva, sendo a maioria desses mecanismos ainda não compreendidos em sua integridade (Smiderle et al., 2014; Wasser, 2003; Ruthes et al., 2015; Ruthes et al., 2016).

Sabe-se que existe uma íntima relação entre a estrutura e função dos polissacarídeos e que eles são classicamente reconhecidos como ativadores do sistema imunológico (Elisashvili, 2012; Mantovani et al., 2008; Silveira et al., 2015b). Segundo Tong et al. (2009), diferentes espécies de fungos podem produzir polissacarídeos com tipos de ligação, grau de ramificação, posição de ramificação e unidades monossacarídicas distintos. Além disso, o uso de diferentes técnicas de cultivo (Smiderle et al., 2012) e extração (Morales et al., 2016) também podem resultar na obtenção de polissacarídeos distintos, podendo apresentar diferentes propriedades biológicas (Synytsya et al., 2009).

Técnicas alternativas de extração -como extrações em alta temperatura e pressão ou extração assistida por micro-ondas para obter extratos ricos em bioativos de subprodutos industriais- vêm ganhando grande destaque nos últimos anos (Ballard et al., 2010; Machado et al., 2015). A primeira, por exemplo, é um método que leva a água a condições que permitem a dissolução de compostos menos polares (Morales et al., 2016; Plaza & Turner, 2017). A temperatura afeta a taxa de transferência de massa e sua elevação associada à pressão -que mantém a água no estado liquido- favorece a extração devido a ruptura da ultraestrutura celular, causando a liberação de diferentes compostos (Morales et al., 2016). Essas técnicas proporcionam elevados rendimentos com uma redução significativa dos tempos de extração e volumes de solvente, quando comparadas aos métodos de extração convencionais (Ruiz-Aceituno et al., 2016).

Para purificação e caracterização química de polissacarídeos, diversas metodologias são utilizadas. Entre as mais frequentes encontradas na literatura estão: precipitação etanólica (Abreu et al., 2019), congelamento e degelo (Gorin & Iacomini, 1984), tratamento com solução de Fehling (Ruthes et al., 2013b), separação por diálise em membranas semipermeáveis (de Jesus et al., 2018b), ultrafiltração (Smiderle et al., 2008b), purificação por colunas (Yan et al., 2019), degradação controlada de Smith (Sovrani et al., 2017), Ressonância Magnética Nuclear (RMN) (Abreu et al., 2021; Brito

et al., 2018; Carbonero et al., 2012; Rosado et al., 2003), Cromatografia gasosa acoplada à espectrometria de massas (CG-EM) (Pramanik et al., 2005; Synytsya et al 2009) e Cromatografia de exclusão por tamanho de alta pressão (HPSEC) (Brito et al., 2018; Smiderle et al., 2013).

Diferentes técnicas de extração estão relacionadas com a obtenção de diferentes polissacarídeos. Estratégias de purificação e caracterização química destas macromoléculas são essenciais para a compreensão de suas propriedades físico-químicas e biológicas, o que pode favorecer no desenvolvimento de novos fármacos bem como em novas abordagens terapêuticas.

2. REVISÃO DE LITERATURA

2.1 FUNGOS BASIDIOMICETOS

Capazes de degradar lignina e crescerem em troncos de árvores vivas ou mortas principalmente em regiões de clima temperado (Gunde-Cimerman, 1999), os fungos basidiomicetos pertencem ao filo Basidiomycota e são considerados seres heterotróficos pluricelulares, devido à sua capacidade de absorver nutrientes por degradação da matéria orgânica, geralmente complexa e insolúvel, utilizando tais compostos como fontes de nitrogênio, carbono e energia (Putzke & Putzke, 1998) Diversas enzimas hidrolíticas corroboram para gerar substâncias simples que possam ser absorvidas por células especializadas chamadas haustórios. Entre elas, pode-se citar enzimas como celulases, lignina peroxidase, lacase, manganês peroxidase, fenoloxidases, entre outras, que compõem o chamado complexo lignocelulítico (Buswell & Odier, 1987).

O basidiocarpo, formado a partir do micélio dicariótico - também conhecido como corpo de frutificação dos cogumelos - é dividido em píleo, lamelas, anel, estipe e bulbo, como representado na figura 1 (Silveira, 2015a). Essas estruturas apresentam diferenças em sua morfologia e cores bastante variáveis (Urben & Oliveira, 1998)

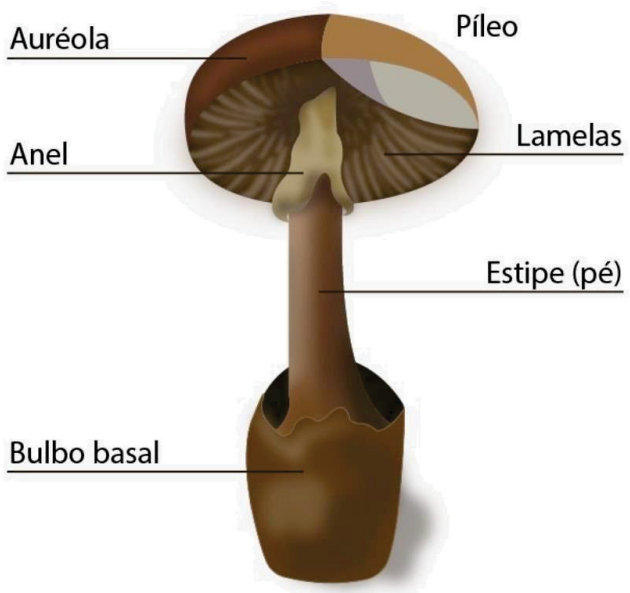


Figura 1. Desenho indicando as principais estruturas de um Basidiomiceto.
Fonte: adaptado de Silveira, 2015a.

Diversas substâncias já foram identificadas a partir de basidiomicetos. Entre os compostos bioativos encontrados, estão as glicoproteínas (Cui et al., 2013), as proteínas (Zhang et al., 2014) e os polissacarídeos de alto valor biológico (Zhang et al., 2011). Além destes, podem-se encontrar vitaminas, minerais como cálcio, potássio, iodo e fósforo (Bonatti et al., 2004) e aminoácidos essenciais (Esposito & Azevedo., 2004). Os cogumelos também apresentam lectinas, compostos fenólicos e compostos orgânicos voláteis (Elsayed et al., 2014). Essas substâncias são utilizadas em diversas áreas, como na produção de ácidos orgânicos, aromas, corantes, enzimas, vitaminas, entre outros (Dufossé et al., 2014).

Os cogumelos estudados no presente trabalho pertencem ao filo Basidiomycota e estão representados no quadro baixo (Quadro 1).

Quadro 1. Taxonomia referente aos cogumelos *Pleurotus pulmonarius*, *Pholiota nameko* e *Amanita muscaria* (L.:Fr).

	P. pulmonarius	***P. nameko***	***A. muscaria***
REINO	Fungi	Fungi	Fungi
FILO	Basidiomycota	Basidiomycota	Basidiomycota
CLASSE	Homobasidiomycetos	Agaricomycotina	Agaricomycetes
ORDEM	Agaricales	Agaricales	Agaricales
FAMÍLIA	Pleurotaceae	Strophariceae	Amanitaceae
GÊNERO	*Pleurotus*	*Pholiota*	*Amanita*
ESPÉCIE	*Pleurotus pulmonarius*	*Pholiota nameko*	*Amanita muscaria*

Fontes: (Holmes, 1983); Genebank, National Institute of Agrobiological Sciences.

2.1.2 *Pleurotus pulmonarius*

Pertencente à ordem Agaricales, os cogumelos do gênero *Pleurotus* podem ser encontrados em tronco de árvores e apresentam basidiocarpo em forma de conchas de ostras crescendo em camadas (Smiderle, 2008a). Muitas das espécies desse gênero são comestíveis e podem ser encontradas facilmente no meio ambiente (Alexopoulos et al., 1996).

O *Pleurotus* tem um histórico de cultivo recente, pois foi somente no início deste século que os alemães, à procura de uma fonte opcional de proteína para o período de guerra, desenvolveram o seu cultivo em troncos de árvores, simulando o *habitat* natural (Laborde & Delmas, 1974). Atualmente, os cogumelos comestíveis do gênero *Pleurotus* são, provavelmente, os mais conhecidos no mundo devido ao seu valor gastronômico e às suas propriedades nutricionais benéficas (Barbosa et al., 2020).

Segundo Stamets (2000), o cogumelo *Pleurotus pulmonarius* foi publicado pela primeira vez como *Agaricus pulmonarius* em 1821. Considerado um fungo comestível bastante versátil, o *P. pulmonarius* cresce sobre uma ampla variedade de resíduos agrícolas de diferentes composições (Rajarathnam & Bano, 1989). Seu crescimento

rápido, mesmo utilizando formas simples de cultivo, fornece uma grande produção com bom valor nutricional (Miles & Chang, 1997).

Os polissacarídeos que compõem sua estrutura são, principalmente, D-glucanas com diferentes tipos de ligação e conformação (Barbosa et al., 2020) e, entre as mais estudadas, estão as β-D-glucanas (Smiderle et al., 2008b).

2.1.3 *Pholiota nameko*

Diferente do *Pleurotus*, o gênero *Pholiota* não é conhecido por sua abundância de espécies de cogumelos comestíveis apetitosos, visto que diversas delas apresentam em sua superfície um véu viscoso, o que torna seu consumo desagradável (Stamets, 2000). Descoberto no Japão, o cogumelo *Pholiota nameko* apresenta excelentes características de sabor e textura e pode ser facilmente cultivado em toras de madeira em regiões úmidas (Stamets., 2000). Atualmente, sua produção nos países orientais, como China e Japão, é uma das mais populares entre os cogumelos comestíveis, sendo utilizado como alimento e para fins medicinais (Abreu, 2020).

O interesse pela comunidade científica pelo *P. nameko* iniciou-se em 1996, após a comprovação na presença de inositol - um importante elemento para o metabolismo animal e vegetal - em sua estrutura, em pesquisa conduzida por Malick. (1996). Segundo Li et al. (2019), diversas propriedades benéficas já foram associadas aos polissacarídeos encontrados nessa espécie, a exemplo da inibição da ribonuclease A, efeitos imunomoduladores, atividade anti-inflamatória, efeito hipolipidêmico, entre outras.

No entanto, ainda há pouca informação sobre polissacarídeos purificados dessa espécie e suas respectivas atividades biológicas (Abreu et al., 2019; Sovrani et al., 2017), uma vez que grande parte dos estudos é conduzida com frações brutas, contendo diferentes polissacarídeos (Li et al., 2019; Li & Wang, 2007; Zhang et al., 2014; Zheng et al., 2014).

2.1.4 *Amanita muscaria*

Conhecido popularmente como cogumelo agárico voador, o cogumelo *Amanita muscaria* é uma espécie nativa de regiões temperadas e boreais do hemisfério norte que foi introduzida no hemisfério sul devido à sua capacidade de crescer em simbiose com árvores Pinus (Ruthes et al., 2013b).

Conhecida por sua toxicidade, o cogumelo *Amanita muscaria* contém muscarina, muscimol, ácido ibotênico e muscazone. Tais compostos estão relacionados com os efeitos psicoativos que deram ao receptor sináptico metabotrópico estimulado por acetilcolina – o receptor muscarínico – seu nome, uma vez que atuam como agonistas nesses receptores após seu consumo (Broadley, 2017). Apesar de tóxico, alguns locais da Europa, norte da América e Japão fazem seu consumo após repetidos processos de fervura (Coville, 2014; Kiho et al., 1992).

Pouco se sabe sobre os polissacarídeos presentes no cogumelo *Amanita muscaria* (Kiho et al., 1992; Ruthes et al., 2013b). A grande maioria dos estudos envolvendo os compostos encontrados em sua composição é direcionada àqueles que apresentam efeitos psicoativos (Maciejczyk & Kafarski, 2013; Michelot & Melendez-Howell, 2003; Størmer et al., 2004)

No presente trabalho o cogumelo *Amanita muscaria* utilizado é uma variedade, chamada de formosa (L.:Fr), apresentando coloração amarelada.

2.2 POLÍSSACARÍDEOS DE BASIDIOMICETOS

Com ampla ocorrência na natureza, os polissacarídeos são compostos por unidades monossacarídicas unidas por ligações glicosídicas, sendo capazes de carregar informações biológicas devido a sua grande variabilidade estrutural (Ooi & Liu, 2012). Diferem entre si em sua composição monossacarídica, no tipo de ligação, no comprimento de suas cadeias e no grau de ramificação (Nelson & Cox, 2014).

As ligações glicosídicas ocorrem entre as unidades monoméricas, entre um carbono anomérico de um hemiacetal (C-1) ou de um hemicetal (C-2) com um grupo hidroxila qualquer de outro monossacarídeo. A ligação poderá ser α ou β, dependendo da configuração do carbono anomérico em ligação (Nelson & Cox, 2014).

Os polissacarídeos desempenham importantes funções e compõem a parede celular dos cogumelos, sendo responsáveis por vários processos biológicos essenciais às células, tais como: fornecer suporte osmótico, proteção física, determinar o formato das células, além de estarem relacionados a eventos de sinalização celular, adesão e reprodução (Fukuda et al., 2009; Pérez & Ribas, 2004). Eles também podem ser encontrados dispersos externamente às células ou associados à membrana plasmática (Figura 2), podendo variar entre as espécies (Adams, 2004). Além dos polissacarídeos,

existem também proteínas que podem estar associadas diretamente a membrana plasmática ou aos polissacarídeos em sua superfície (Silveira, 2015a).

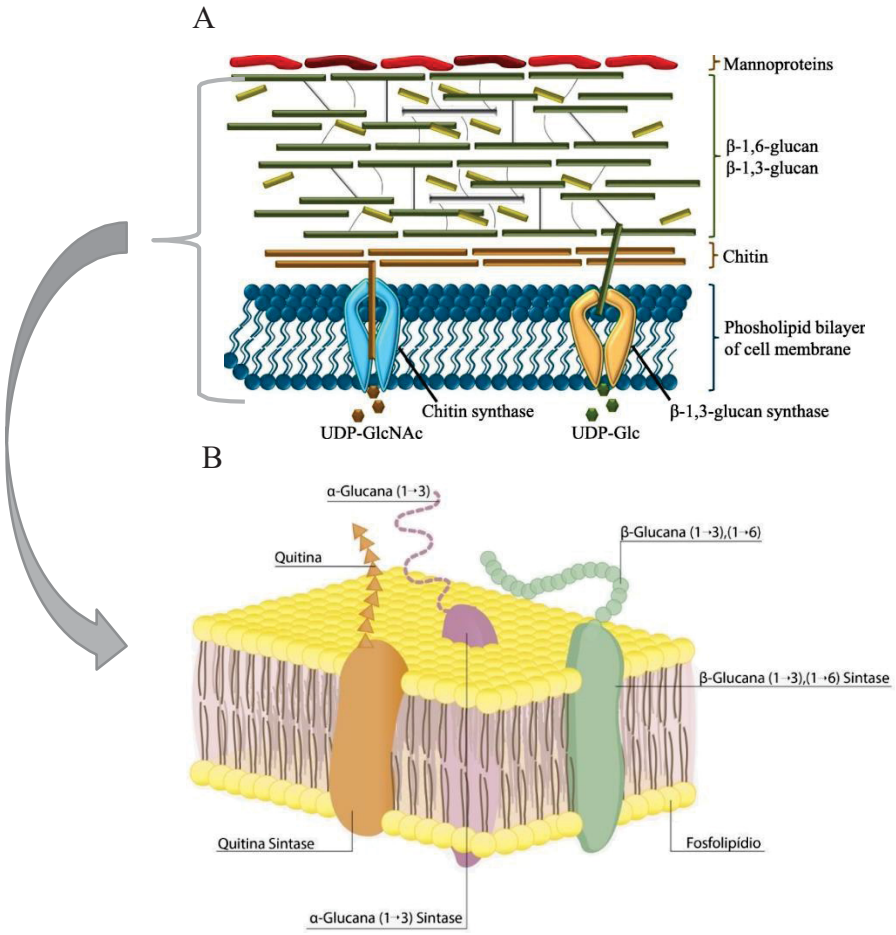


Figura 2. Representação esquemática da estrutura da parede celular (A) e da síntese de α e β glucanas e quitina na parede celular de basidiomicetos (B). Fontes: Fesel et al., 2016; o Autor., 2020.

Os cogumelos comestíveis são fontes de α e β glucanas. Tais estruturas são consideradas homopolissacarídeos, por possuírem apenas glucose em sua composição (Morales et al., 2019; Ruthes et al., 2013a), podendo ser solúveis ou insolúveis em água (de Jesus et al., 2018a). Entre elas, estão as glucanas lineares unidas por ligações do tipo α-(1→3), α-glucanas (1→4), β-glucanas (1→3) e β-glucanas (1→6) (Baggio et al., 2010; Smiderle et al., 2006). Estas unidades podem, ainda, ser *O*-substituídas por terminais não redutores ou por cadeias laterais (Abreu et al., 2019; Moreno et al., 2016), resultando na formação de polissacarídeos, como por exemplo, β-glucanas com ligações (1→3),(1→6) ou glicogênio, com ligações α-(1→4) substituídas no carbono 6 por ligações α-(1→6) (Palacios et al., 2012).

Obtido através de extração aquosa quente, uma β-D-glucana (1→3),(1→6) foi isolada, por Smiderle et al. (2008c), do cogumelo *Pleurotus pulmonarius* e passou por estudos para avaliação do efeito antinociceptivo em ratos (Baggio et al., 2010). Polissacarídeos semelhantes foram obtidos por Ruthes et al. (2013b) através de extração aquosa quente no cogumelo *A. muscaria* e por Abreu et al. (2019) no extrato aquoso frio de *Pholiota nameko*.

Apesar de incomuns, homopolissacarídeos de galactose também já foram extraídos de diferentes cogumelos. Carbonero et al. (2008) isolou uma α-galactana linear parcialmente 3-*O*-metilada, a partir dos cogumelos *Pleurotus eryngii* e *Pleurotus ostreatoroseus*. Outras duas galactanas lineares, unidas por ligações α-(1→6) parcialmente 3-*O*-metiladas na razão 2:1 e 1:1, foram extraídas por Brito et al. (2018) de *Pleurotus citrinopileatus* com massa molecular de 37.6 x 10^3 g.mol^{-1} e 28.5 x 10^3 g.mol^{-1}, respectivamente.

Além destes, também é possível encontrar polissacarídeos com um maior grau de complexidade em sua estrutura, os heteropolissacarídeos (Ruthes et al., 2016). Tais estruturas apresentam composição monossacarídica, grau de ramificação, tipo de ligação e configuração anomérica variados, além de poderem apresentar naturalmente monossacarídeos metilados (Rosado et al., 2003). Smiderle et al. (2008b) obtiveram, através de extração aquosa fria realizada no cogumelo *Pleurotus pulmonarius*, uma molécula pura composta de manose, galactose e *O*-metil-galactose constituída de cadeia principal de 3-*O*-Me-α-D-Gal*p* e α-D-Gal*p* (1→6)-ligadas, sendo que ambas as unidades podem estar substituídas em *O*-2 por terminais não redutores de β-D-Man*p*. Ruthes et al. (2013b) obtiveram uma fucomanogalactana a partir do extrato aquoso quente do cogumelo *A. muscaria*, com massa molecular 25,5 x 10^3 g/mol, composta de uma cadeia principal α-D-Gal*p* (1→6)-ligada, parcialmente substituída em *O*-2 por terminais não redutores de L-Fuc*p* e, em menores proporções, por β-D-Man*p*.

Entender esses aspectos é importante durante os processos de extração, bem como é relevante para a compreensão da relação estrutura-função de tais estruturas, visto que diversas respostas biológicas já foram relatadas a partir de diferentes polissacarídeos, a exemplo de atividades imonumoduladora (Smiderle et al., 2013; Zhang et al., 2013), antinociceptiva (Ruthes et al., 2013a) antitumoral (Jeff et al., 2013; Zhang et al., 2007), prebiótica (Synytsya et al., 2009) antioxidante (Carvajal et al., 2012), anticoagulante e antitrombótica (Barddal et al., 2015; Maas et al., 2012).

No presente trabalho, os polissacarídeos estudados foram obtidos a partir dos cogumelos *Pleurotus pulmonarius*, *Pholiota nameko* e *Amanita muscaria* (L.:Fr) (Quadro 1). No quadro 2, estão representadas as estruturas de polissacarídeos citadas no texto acima, além de outras isoladas de corpos de frutíficação de diferentes cogumelos.

Quadro 2. Polissacarídeos isolados a partir de corpos de frutificação de diferentes cogumelos.

Cadeia Principal	**Ramificação**	**Espécie**	**Métodos de extração e caracterização** [1]	**Massa molecular**	**Referências**
β-D-Glc*p*-(1→3)	*O*-6 β-D-Glc*p*-(1→6)	*Pleurotus Pulmonarius*	HW, EtOH, GD, Sm, RMN, GC-MS, HPSEC	N/I [2]	Smiderle et al. (2008c)
		Pleurotus sajor-caju	HW, EtOH, GD, Sm, RMN, GC-MS, HPSEC	$9,7 \times 10^5$ g/ mol	Carbonero et al. (2012)
		Pholiota nameko	CW, EtOH, Feh, GD, Sm, RMN, GC-MS, HPSEC	N/I [2]	Sovrani et al. (2017)
		Pholiota nameko	HW, EtOH, GD, α-am, Sm, RMN, GC-MS, SLS	1,4 x 10^6 g/mol	Abreu et al. (2019)
		Amanita muscaria	KHW, GD, Feh, Sm, RMN, GC-MS, HPSEC	1,6 x 10^4 g/mol	Ruthes et al. (2013b)
		Fomitopsis betulina	KW, GD, Lm, RMN, GC-MS, Sm, SLS	7,9±1,2 x 10^5 g/mol	de Jesus et al. (2018a)
		Lactarius rufus	HW, EtOH, GD, Feh, Sm, RMN, GC-MS, HPSEC	1,1 x 10^5 g/mol	Ruthes et al. (2013a)
		Piptoporus betulinus	CW, EtOH, GD, Dial(1000), RMN, GC-MS, HPSEC	2,5 x 10^5 g/mol	de Jesus et al. (2018b)
β-D-Glc*p*-(1→6)	-	*Agaricus bisporus*	HW, EtOH, GD, DMSO, RMN, GC-MS, HPSEC	2,9 x 10^4 g/mol	Smiderle et al. (2013)
		Agaricus brasiliensis	HW, EtOH, GD, DMSO, RMN, GC-MS, HPSEC	4,5 x 10^4 g/mol	Smiderle et al. (2013)
α-D-Gal*p*-(1→3)	-	*Fomitopsis betulina*	KW, GD, Lm, RMN, GC-MS, Sm, SLS	3,9±0,8 x 10^5 g/mol	de Jesus et al. (2018a)
		Pleurotus ostreatus	EtOH, KW, α-am, Dial, GPC, GC-MS, FT-IR	2,9 x 10^5 g/mol	Synytsya et al. (2009)

β-D-Glc*p*-(1→3)	-	*Pleurotus sajor-caju*	HW, EtOH, GD, DMSO, RMN, GC-MS, HPSEC	6,01 x 10^4 g/mol	Silveira et al. (2014)
3-*O*-Me-α-D-Gal*p*-(1→6)	*O*-2 β-D-Man*p*	*Pleurotus pulmonarius*	CW, EtOH, GD, Feh, UF(500), UF(30), RMN, GC-MS, HPSEC	2,39 × 10^4 g/mol	Smiderle et al. (2008b)
		Pleurotus eryngii	HW, EtOH, DEAEc, HPSEC, GC-MS, RMN	2,1 x 10^4 g/mol	Yan et al. (2019)
→6)-α-D-Gal*p*-(1→4)-α-D-Glc*p*(1→	*O*-2 β-D-Man*p*	*Pleurotus sajor-caju*	HW, GD, EtOH, Dial, Seph6B, RMN, GC-MS	2,4 x 10^5 Da	Pramanik et al. (2005)
α-D-Gal*p*-(1→6)	*O*-2 α-D-Fuc*p* e β-D-Man*p*	*Amanita muscaria*	HW, GD, Feh, Dial(100), RMN, GC-MS, HPSEC	25,5 x 10^3 g/mol	Ruthes et al. (2013b)
3-*O*-Me-α-D-Gal*p*-(1→6)	-	*Pleurotus eryngii*	HW, EtOH, GD, Feh, UF(300), RMN, GC-MS, HPSEC	17,9 x 10^4 g/mol	Carbonero et al. (2008)
		Pleurotus ostreatoroseus	HW, EtOH, GD, Feh, UF(300), RMN, GC-MS, HPSEC	16,5 x 10^4 g/mol	Carbonero et al. (2008)
		Pleurotus citrinopileatus	CW, EtOH, GD, Feh, RMN, GC-MS, HPSEC	37,6 x 10^3 g/mol 28,5 x 10^3 g/mol	Brito et al. (2018)
α-D-Man*p*-(1→6)	*O*-2 α-D-Man*p*-(1→2) e α-D-Man*p*-(1→3)	*Pleurotus ostreatoroseus*	HW, GD, Feh, DMSO, Sm, RMN, GC-MS, HPSEC	2,8 x 10^4 g/mol 1,7 x 10^4 g/mol	Rosado et al. (2003)

Notas:

[1] HW: extração aquosa quente; CW: extração aquosa fria; KW: extração aquosa alcalina; EtOH: Precipitação etanólica; GD: congelamento e degelo; Feh: tratamento com solução de Fehling; DMSO: tratamento com dimetilsulfóxido; Lm: purificação pelo método proposto por de Jesus, et al (2018); Dial(1000): purificação por diálise (1000 kDa); Dial(100): purificação por diálise (100 kDa); UF(300): ultrafiltração (300 kDa); UF(500): ultrafiltração (500 kDa); ultrafiltração (30 kDa); α-am: tratamento com α-amilase; DEAEc: purificação em coluna de DEAE celulose; Seph6B: purificação em coluna de Sepharose 6B; Sm: degradação controlada de Smith; RMN: análise de ressonância magnética nuclear; GC-MS: análise de cromatografia gasosa acoplada à espectrometria de massas; HPSEC: cromatografia de exclusão por tamanho de alta pressão; SLS: dispersão de luz estática, GPC: cromatografia de permeação em gel.

[2] Não informado.

3. JUSTIFICATIVA

Em razão de possuírem diversas propriedades benéficas à saúde e ainda existirem

muitas dúvidas em relação aos mecanismos de ação dos polissacarídeos obtidos a partir de cogumelos, a sua estrutura química deve ser minunciosamente estudada. O objetivo do presente trabalho foi extrair, purificar e caracterizar diferentes polissacarídeos obtidos dos corpos de frutificação dos cogumelos *Pleurotus pulmonarius*, *Pholiota nameko* e *Amanita muscaria* (L.:Fr), com ênfase na avaliação estrutural através de técnicas de espectroscopia e cromatografia (RMN, GC-MS, HPSEC). Diversas extrações foram realizadas nos três cogumelos, entre elas: extração aquosa fria, extração aquosa quente, extração alcalina e extração em altas temperatura e sob pressão a partir de resíduos obtidos após sucessivas extrações prévias, visando obter e caracterizar polissacarídeos com diferentes características químicas (como composição monossacarídica, grau de ramificação e massa molecular). A presente pesquisa é importante e necessária, pois estudos relativos à identificação e caracterização de polissacarídeos presentes nos cogumelos serão de grande contribuição para o desenvolvimento em ciência e tecnologia voltadas a área da saúde.

4. OBJETIVOS

4.1 Objetivo geral

-Isolar, purificar e caracterizar quimicamente os polissacarídeos obtidos por diferentes formas de extrações desenvolvidas de basidiomas de *Pleurotus pulmonarius, Pholiota nameko e Amanita muscaria* (L.:Fr).

4.2 Objetivos específicos

- Extrair polissacarídeos a partir dos cogumelos *Pleurotus pulmonarius*, *Pholiota nameko* e *Amanita muscaria* (L.:Fr) utilizando técnicas de extração variadas;
-Avaliar os rendimentos e os polissacarídeos das frações brutas obtidas pelos processos de extração em autoclave;
- Purificar as frações de polissacarídeos obtidas;
- Caracterizar quimicamente cada polissacarídeo purificado;

Artigo I

Structural characterization of an unmethylated α-D-galactan and a β-D-glucan isolated from the mushroom *Amanita muscaria*

Structural characterization of an unmethylated α-D-galactan and a β-D-glucan isolated from the mushroom *Amanita muscaria*

Matheus Zavadinack [a], Jessica Bertage [a], Shayane da Silva Milhorini[a], Lucimara M. C. Cordeiro[a] and Marcello Iacomini[a*]

[a] Department of Biochemistry and Molecular Biology, Federal University of Paraná, Curitiba- PR, CEP 81531-980, Brazil.

*Corresponding author: Department of Biochemistry and Molecular Biology, Federal University of Paraná, Mailbox (Caixa Postal) 19046, Curitiba- PR, CEP 81531-980, Brazil. Tel.: +55 (41) 336-1655; Fax: +55 (41) 3266-2042; e-mail: iacomini@ufpr.br

Abstract

Polysaccharides α-D-galactan and β-D-glucan were obtained from fruiting bodies of *Amanita muscaria* (L.:Fr) by aqueous extraction at room temperature. After purification, they were chemically characterized by mono and two-dimensional NMR studies, methylation analysis and controlled Smith degradation. The α-D-galactan showed signals at NMR analysis (HSQC, TOCSY and COSY) which indicates the presence of an unmethylated galactan that has not been previously described, composed of a (1→6)-linked main chain branched at *O*-2 by non-reducing end units, confirmed by the methylated derivatives. The β-D-glucan is composed of a (1→3)-linked main chain, confirmed by controlled Smith degradation, substituted at *O*-6 by β-Glc*p*-(1→6)-linked units. Both polysaccharides are soluble in water and have molecular weight, 9.05 x 10^3 g. $moL^{-1}$and 1.3 x 10^5 g. moL^{-1}, respectively.

Keywords: *Amanita muscaria*; polysaccharide; α-galactan; β-glucan; chemical structure.

1. Introduction

Commonly known as fly agaric mushroom, *Amanita muscaria* (L.:Fr) has toxic and hallucinogenic molecules in its composition. As the main compounds with these properties, muscimol, muscarina, ibotenic acid and muscazone can be found, besides heterocyclic alkaloids in small amounts (Kondeva-Burdina et al., 2019; Ruthes et al., 2013b). Despite this, its consumption is made in regions of Europe, North America and Japan after a process that involve boiling in salt water and steeping in vinegar (Kiho et al., 1992; Coville., 1898)

The body of fungal fruiting, popularly known as mushroom, is an important source of nutrients and fibers, such as β-glucans (Abreu et al., 2019), being considered a delicacy for many cultures and countries (White et al., 2019). In addition, edible mushrooms are used in capsule form as a supporting in patients in the treatment of cancer (Hardy, 2008). Such use is justified by the biological properties found in mushrooms, including: antitumoral, immunomodulatory, hypolipidemic, antibacterial, anti-inflammatory and hepatoprotective effects (Morales et al., 2020).

Many properties are known to be related to the polysaccharides present in fungal cell wall (Ruthes et al., 2013a). These, mainly β-D-glucans are primarily responsible for their biological responses (Abreu et al., 2019), often the (1→3)-linkage type (Willment et al., 2001). D-glucans are the most commonly isolated mushroom glucans and may vary according to their anomericity in α or β configurations, in addition to mixed α/β-D-glucans (Morales et al., 2020). For D-glucans, their immunomodulatory properties and anti-inflammatory effect are strongly related to their structure and may vary depending on the tertiary structure in solution, water solubility, degree of branching and molecular weight (Abreu et al., 2019). The insolubility characteristic is generally related to the presence of branched (1→3),(1→6)-linked β-D-Glc*p*, whereas water-soluble polysaccharides generally present D-glucose, D-galactose, D-mannose and proteins (Rosado et al., 2003).

(1→3),(1→6)-linked β-D-glucans had already been isolated from the mushrooms *Pleurotus pulmonarius* (Smiderle et al., 2008), *Pholiota nameko* (Abreu et al., 2019; Sovrani et al., 2017), *Fomitopsis betulina* (de Jesus et al., 2018a), *Lactarius rufus* (Ruthes et al., 2013a), *Piptoporus betulinus* (de Jesus et al., 2018b), *Amanita muscaria* (Ruthes et al., 2013b) and others. These polysaccharides were obtained from different extraction processes – cold aqueous extraction, hot aqueous extraction and alkaline extraction - and presented molecular weight between 16.2 x 10^3 g/mol and 1.4 x 10^6 g/mol.

Most of them methylated at *O*-3, α-galactans have been already characterized from mushrooms of the genus *Pleurotus*. Carbonero et al. (2008) and Brito et al. (2018) isolated water-soluble (1→6)-linked α-D-galactans partially 3-*O*-methylated via successive cold aqueous extraction and Rosado et al. (2003) isolated a water-soluble 3-*O*-methylated (1→4)-linked α-D-galactan via hot aqueous extraction. In addition, different heteropolysaccharides with galactose in its composition were also reported (Ruthes et al., 2016; Zhang et al., 2013). These polymers usually present a backbone of (1→6)-linked-α-D-galactopyranosyl which can be partially *O*-methylated at 3-*O*-Me-α-D-Gal*p* and can have ramifications by D-mannopyranosyl, D-galactopyranosyl, L-fucopyranosyl or 3-*O*-α-D-mannopyranosyl-α-L-fucopyranosyl residues as side chains generally substituted at C-2 (Ruthes et al., 2016).

A heteropolysaccharide and β-D-glucans were already been isolated from *Amanita muscaria* (L.:Fr). Ruthes et al. (2013b) isolated a fucomannogalactan formed by a (1→6)-linked α-D-galactopyranosyl main chain partially substituted for non-reducing end units at *O*-2 mainly by α-L-fucopyranose and β-D-mannopyranose and a (1→3),(1→6) β-D-glucan that proved efficient in controlling inflammatory pain. In addition, antitumor activity of (1→3),(1→6) β-D-glucan from *Amanita muscaria* (L.:Fr) in mice has already been described (Kiho et al., 1992).

It is known that the biological properties of polysaccharides are intrinsically linked to their composition and chemical structure (Ruthes et al., 2013a; Vetvicka & Yvin, 2004) and there is not much information in the literature of polysaccharides found in *Amanita muscaria* (L.:Fr). Therefore, elucidate the chemical structure of the α-D- galactan unmethylated not yet described from mushrooms as well as the β-D-glucan obtained from *Amanita muscaria* (L.:Fr) described in this study is essential to better understand the structure-activity relationship of these substances.

2. Materials and Methods

2.1 Biological material

The fruiting bodies of *Amanita muscaria* (L.:Fr), were collected in the gardens of the Biological Sciences Sector of the Federal University of Paraná in Curitiba, Paraná - Brazil. The respective mushrooms were cleaned and then ground to a powder.

2.2 General extractions and purification processes

The general extraction and purification processes performed on the *Amanita muscaria* (L.:Fr) are represented in the flowchart (Fig 1). The extraction processes were carried out using 270.86 g dehydrated basidioma. The material was submitted by defatted process with chloroform-methanol (2:1; v/v) at 65 °C in the Soxhlet during the period of 3 hours to remove small molecule compounds including lipids and pigments. Then, the residue was submitted to aqueous extraction at room temperature (25 °C) for 6 h (5x, 4000 mL each) under stirring. The aqueous extract was concentrated in a small volume and subjected to ethanolic precipitation by addition to excess ethanol (3:1, v/v) and centrifuged for 20 min (8000 rpm, 25 °C). The precipitate fraction (P-Am) was dialyzed (6-8 kDa membrane) against tap water for 24 h and showed insoluble and soluble compounds after the dialysis process. The sample was centrifuged for 20 min (10000 rpm, 25 °C) generating the soluble, named F-2, and the insoluble, named F-1, fractions. The sample F-1 was submitted to treatment with Fehling solution using 120 mL of potassium tartarate ($C_4H_4K_2O_6$) and copper sulfate ($CuSO_4$), in the same amount, followed by centrifugation for 20 min (10000 rpm, 25 °C) giving rise the insoluble Cu^{2+} complex and the soluble fractions. Both underwent neutralization with HOAc, dialysis (2 kDa, cut of membrane) and deionized with mixed ion exchange, giving rise the precipitate named GAL-Am and a supernatant fraction.

F-2 was subjected to solubilization in a small volume of water followed by freezing and slowly thaw (2x) until complete precipitation of the cold water insoluble polysaccharides, which were recovered by centrifugation (10000 rpm, 20 min, 4 °C). The soluble fraction (S-3) was subjected to treatment with Fehling solution, using 100 mL of both reagents, as described above. The soluble fraction (S-4) was submitted to ultrafiltration (Millipore®; polyethersulfone; 3 kDa membrane) on a filter holder (Sartorius – Model 16249) with compressed air at 10 psi carrier gas, generating the retained (GLC-Am) and the eluted polysaccharide.

2.3 Monosaccharide composition

An aliquot of the pure polysaccharides (5 mg) was hydrolyzed with 2 M TFA at 100 °C for 8 h and the excess of TFA of each sample was removed with a rotatory evaporator under vacuum. Distilled water (100 µL) was then added to each sample followed by reduction with $NaBH_4$ to reduce aldoses into alditols and acetylation with anhydride-pyridine (200 µL; 1:1; v/v) for 30 min at 100 °C. The extraction of alditol acetates was carried out by adding chloroform followed by the removal of pyridine by

complexing with $CuSO_4$. The analysis of the derivatives obtained were performed by GC-MS using a Varian (model CP-3800) gas chromatograph coupled to an Ion-Trap 4000 mass spectrometer, with He as the carrier gas. The monosaccharides obtained was identified by their typical retention times and electron impact profiles in comparison with standards (Wolfrom & Thompson., 1963; Sassaki et al., 2008)

2.4 Methylation analysis by GC-MS

GAL-Am and GLC-Am were subjected to the methylation process by dissolving the sample in dimethyl sulfoxide and methyl iodide (methylating agent) and kept under stirring for 30 min at 25 °C followed the adding of powdered NaOH as described by Ruthes et al. (2010) and maintained overnight. After, the reaction product was neutralized with acetic acid and the volume was concentrated and lyophilized. The methylation process was repeated three times for each sample and the methylated polysaccharides were separated by addition of chloroform (1 mL). Each sample dissolved in chloroform was transferred to a vial and evaporated and the per-*O*-methylated derivatives were hydrolyzed with 45 % formic acid (1 mL) at 100 °C for 15 h (Carbonero et al., 2012). The excess of acid was removed by lyophilization process followed by reduction with $NaBD_4$ and acetylation as described above (section 2.3) to give a mixture of partially *O*-methylated alditol acetates derivatives which were analyzed by GC-MS using a Varian model 4000 gas chromatograph equipped with VF5 capillary columns. The injector temperature was maintained at 210 ºC and the oven temperature increased from 50 ºC (maintained 2 min) to 90 ºC (20 ºC min^{-1}, then maintained for 1 min), 180 ºC (5 ºC min^{-1}, then maintained for 2 min) and to 210 ºC (3 ºC min^{-1}, then maintained for 5 min). Helium was used as the carrier gas at a flow rate of 1.0 mL min^{-1}. Partially *O*-methylated alditol acetates were identified by the ion *m/z* by comparing their positive ions with standards. The results are expressed as a relative percentage of each component (Sassaki et al., 2005).

2.5 Controlled Smith degradation of the β-glucan

The polysaccharide (GLC-Am, 50 mg) was solubilized in 25 mL of distilled water and oxidized with 25 mL of sodium periodate ($NaIO_4$) 0.1 M at room temperature in the dark under stirring for 72 hours (Delgobo et al., 1998). After the material was dialyzed (2 kDa, cut of membrane) for 24 hours against tap water. Subsequently the material was reduced with $NaBH_4$ to pH 8~9 and kept at room temperature for 20 hours, neutralized

with acetic acid and dialyzed (2 kDa) for 24 hours, hydrolyzed with TFA pH 2.0 (5.0 mL) at 100 °C for 30 minutes and then dialyzed (2 kDa), evaporated to small volume and subsequently precipitated by ethanol (3:1, v/v) and lyophilized. The residual polysaccharide was analyzed by NMR spectroscopy (Ruthes et al., 2015).

2.6 Determination of homogeneity of polysaccharides

The homogeneity of the fractions GAL-Am and GLC-Am were determined by high performance steric exclusion chromatography (HPSEC) using a refractive index (RI) detector and a Wyatt Technology Dawn-F Multi-Angle Laser Light Scattering detector (MALLS). Using 0.1 M $NaNO_3$, containing 0.5 g. L^{-1} NaN_3 as eluent, 4 columns Waters Ultrahydrogel – 120, 250, 500 and 2000 were coupled in series. Each sample was dissolved in the same solvent (1 mg/ mL) and filtered through a membrane, with pores of 0.22 μm diameter (Millipore). The data obtained by HPSEC are analyzed by the Wyatt Technology ASTRA program.

2.7- Nuclear Magnetic Resonance (NMR) spectroscopy

Nuclear magnetic resonance analyzes were performed on a Bruker Avance DRX400 spectrometer (Bruker), at base frequencies of 400 MHz (^{1}H), and 100 MHz (^{13}C), and on a Bruker Avance III 600 spectrometer (Bruker Germany). The samples were solubilized in Me_2SO-d_6 or D_2O, at a concentration of 40 mg/ 500 μL. The analyzes were carried out at temperature of 70 °C. The chemical shift of the samples dissolved Me_2SO-d_6 are expressed in δ (ppm) at δ 39.7 (^{13}C) and 2.5 (^{1}H), while for water-soluble samples it is relative to acetone at δ 30.20 and 2.22 for ^{13}C and ^{1}H.

3.0 Results and Discussion

3.1 Extraction and purification of polysaccharides from *A. muscaria*

The *Amanita muscaria* (L.:Fr) mushroom (powder, 270,86 g) was defatted with chloroform/methanol solution (2:1 v/v) as represented in the flowchart (Figure 1). After, it was subjected to aqueous extraction at room temperature giving rise to the CW fraction (4.54%, 12.4 g) which went through ethanolic precipitation. The precipitate (P-Am, 2.41%, 6.55 g) was subjected to dialysis (6-8 kDa) and centrifuged, generating the fractions insoluble (F-1, 0.69%, 1.86 g) and soluble (F-2, 1.05 %, 2.84 g). The first was

submitted to a Fehling treatment, gave rise the precipitated (GAL-Am, 0.47%, 1.28 g), corresponding to a α-D-galactan and the supernatant fraction S-2. The fraction F-2 was subjected to a freezing and slowly thaw process (2x) and the supernatant fraction recovered by centrifugation was submitted to Fehling treatment. In order to eliminate the residual α-D-galactan, it was performed an ultrafiltration (3 kDa) in the soluble fraction (S-4). The process gave rise a retained (GLC-Am) corresponding to a pure β-D-glucan and an eluted fraction.

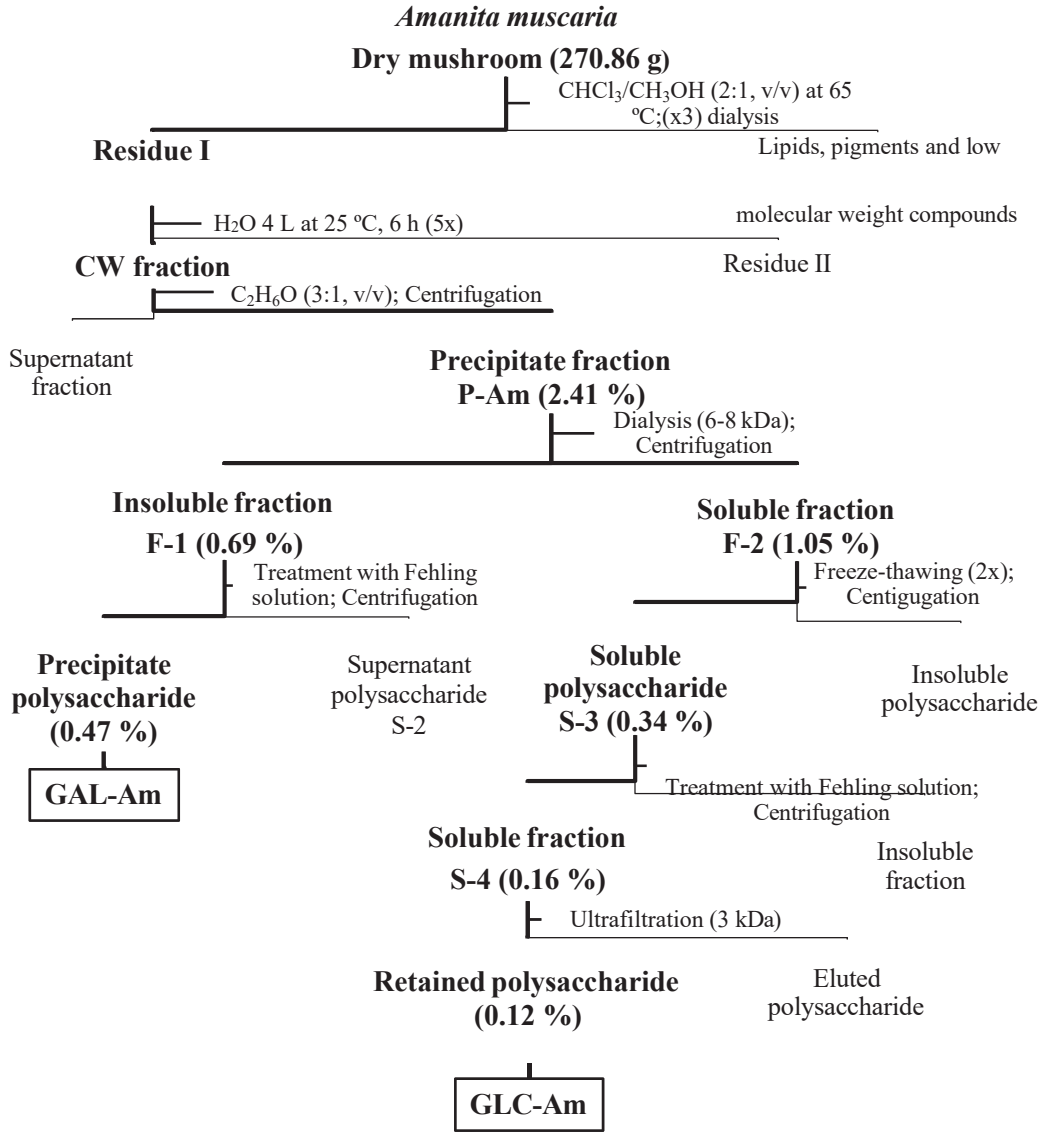


Figure 1. Scheme of extraction and purification of the β-D-glucan (GLC-Am) and the α-D-galactan (GAL-Am) from *A. muscaria* (L.:Fr).

3.2 Polysaccharides presents in the mixture from CW extraction

The fraction P-Am (2.41%, 6.55 g) presented a mixture of polysaccharides, as observed in the HSQC spetrum (Figure 2), which shows the presence of distinct polysaccharides, mainly indicating the presence of a D-galactan and a β-D-glucan-(1→3),(1→6)-linked which can be confirmed by the anomeric region with signs at δ

102.9/4.27, 102.9/4.50 and at 102.5/4.35 indicating the presence of β-Glc*p* and signs in δ

98.4/4.80 and 98.4/4.74 referring to α-Gal*p* (Carbonero et al., 2008; Oliveira et al., 2015). At the linkages region signals were also observed at δ 68.5/3.90, 68.5/3.79 and 66.2/3.69, 66.2/3.54 from β-D-glucan and α-D-galactan, respectively, indicating the (1→6)-linked by *O*-6 substitution. The non-reducing end units are represented at δ 60.5/3.69 and 60.5/3.49 corresponding to free *O*-6, while at δ 86.1/3.48 indicate the presence of molecules (1→3)-linked (Carbonero et al., 2008).

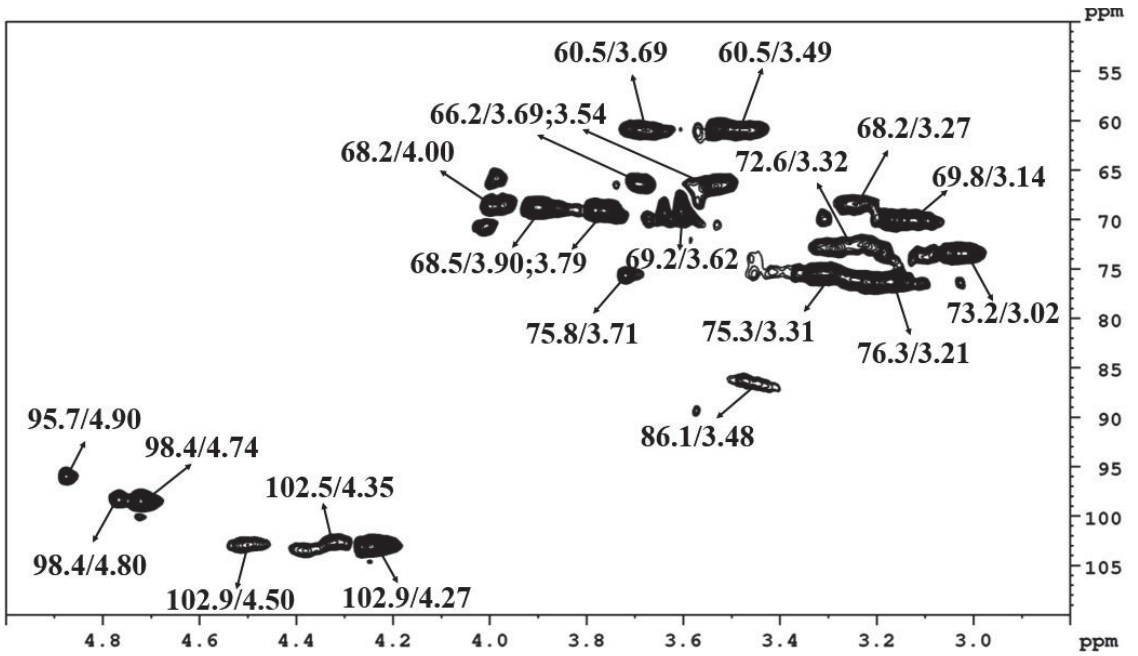


Fig 2. HSQC spectrum of the mixture of polysaccharides (P-Am). The sample was analyzed in Me_2SO-d_6 at 70 °C (chemical shifts are expressed in δ ppm).

3.3 Purification process of the α-D-galactan from *Amanita muscaria* (L.:Fr)

P-am was subjected to dialysis followed by centrifugation process. The insoluble fraction (F-1; 0.69%, 1.86 g) was analyzed by ^{13}C NMR and ^{13}C HSQC and showed similar signs with the P-Am fraction, but with different intensities (Fig 3A and B). The absence of a methyl group ($-CH_3$) is evident as there are no signs between δ 50.0 and 59.0 ppm. The sample F-1 was submitted to a treatment with Fehling Solution (Fig. 1) and the fraction GAL-Am obtained will be discussed in the item 3.4.

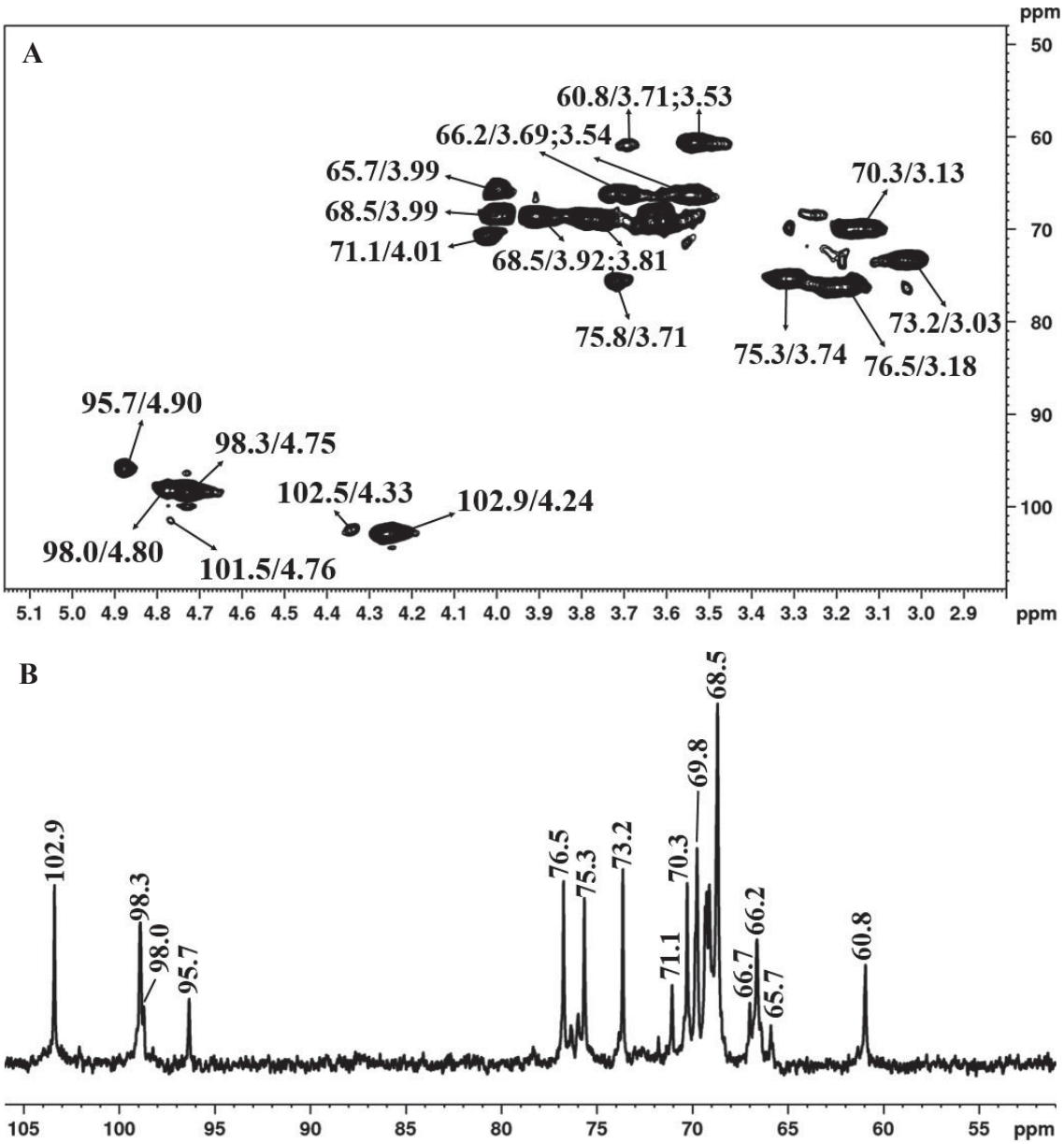


Fig 3. HSQC and ^{13}C NMR spectra of the mixture of polysaccharides (F-1). HSQC (A) and ^{13}C NMR (B). The sample was analyzed in Me_2SO-d_6 at 70 °C (chemical shifts are expressed in δ ppm).

3.4 Chemical structure of the pure α-D-galactan from *Amanita muscaria* (L.:Fr)

The supernatant fraction (S-2) has not been studied but the precipitate fraction GAL-Am (0.47 %, 1.28 g) resulted in a homogeneous elution profile in HPSEC spectrum with *Mw* 9.08 x 10^3 $g.mol^{-1}$. NMR studies [HSQC, COSY and HSQC-TOCSY spectroscopy, (Fig 4, 5 and 6)] contributed to elucidate the structure of the α-D-galactan (Table 1).

The monosaccharide composition showed only galactose in its composition, indicating the presence of a D-galactan. Sign at δ 98.1/4.98 and 98.0/5.02 in the HSQC spectra (Fig. 4) at anomeric region correspond to a galactose (1→6)-linked in α configuration confirmed by the coupling constant $J_{C\text{-}1,H\text{-}1}$ = 173 Hz observed in HSQC spectrum (Perlin and Casu, 1969) branched in C-2 without the presence of methyl group (-CH_3), indicating a structure not yet reported in the literature. The signal at δ 95.7/5.15 corresponding to C-1 of the side chain linked at C-2. In the glycosidic linkages region no signal is observed between δ 85.0 to 79.0 indicating that the main chain of this polymer consists of (1→6)-linked α-Gal*p* units, with characteristic signals for this type at δ 66.7/3.91 and 66.7/3.71, representing C-6 connected, and at δ 61.2/3.76 corresponding to free *O*-6 non-reducing end units (Brito et al., 2018; Carbonero et al., 2008). The signal at δ 75.1/3.97 corresponds to the C-2 of the main chain linked, while δ 68.4/3.85 indicates C-2 of the main chain.

Table 1. ^{1}H and ^{13}C NMR chemical shifts[a] of α-D-galactan fraction (GAL-Am) from *A.muscaria* (L.:Fr).

Units		**1**	**2**	**3**	**4**	**5**	**6 a**	**b**
6→)α-Gal*p*(1→	^{13}C	98.1	68.4	69.6	69.5	68.8	66.7	66.7
	^{1}H	4.98	3.85	3.88	4.03	4.17	3.91	3.71
α-Gal*p*(1→	^{13}C	95.7	69.6	68.4	69.5	68.8	61.2	61.2
	^{1}H	5.15	3.88	3.85	3.94	4.17	3.76	3.76
2,6→)α-Gal*p*(1→	^{13}C	98.0	75.1	66.2	66.9	71.0	66.7	66.7
	^{1}H	5.02	3.97	4.25	3.98	4.18	3.91	3.71

[a] Assignments are based on ^{1}H and HSQC analysis and are expressed as ppm.

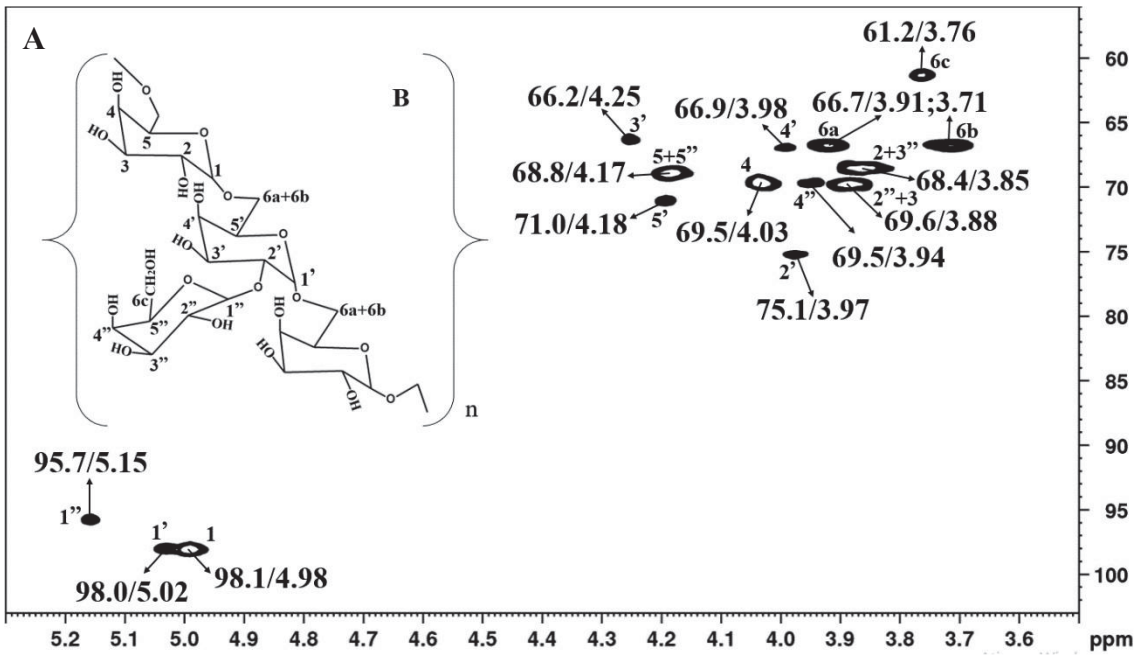


Fig 4. HSQC spectra (A) and the structure of the polysaccharide α-D-galactan (B). The sample was analyzed in D_2O at 70 °C (chemical shifts are expressed in δ ppm).

COSY (Fig. 5) and HSQC-TOCSY analysis (Fig. 6) contributed to elucidate this structure through the correlation between the protons of the adjacent carbons. It is possible to observe in COSY spectrum signals at δ 4.98/3.85, 5.02/3.97 and 5.15/3.88 (1/2, 1′/2′ and 1″/2″, respectively) indicating the correlation between C-1 and C-2 of the three different monosaccharides groups presents in the α-D-galactan (Fig 4B). In HSQC-TOCSY spectrum with superimposed HSQC the correlation between C-5 and C-6 (δ 3.91/68.8, 3.71/68.8, 3.91/68.8, 3.71/68.8, 3.76/71.0 for 5/6a, 5/6b, 5′/6a, 5′/6b, 5″/6c, respectively) is indicated for the three monosaccharides. Besides that, the others correlations are indicated in the Table 2 according to signs already observed by Brito et al. (2018), Zhang et al. (2013), Carbonero et al. (2008) and Rosado et al. (2003).

The analysis of methylated derivatives by GC-MS of the GAL-Am fraction (Table 3) proves the results obtained by NMR studies, demonstrating that the main chain is composed of (1→6)-linked α-D-galactopyranose units based on their high percentage of 2,3,4-tri-*O*-methyl-Gal*p* (71 %) branched at C-2, confirmed by the 3,4-di-*O*-methyl-Gal*p* (14 %), by non-reducing end units, confirmed by 2,3,4,6-tetra-*O*-methyl-Gal*p* (15 %).

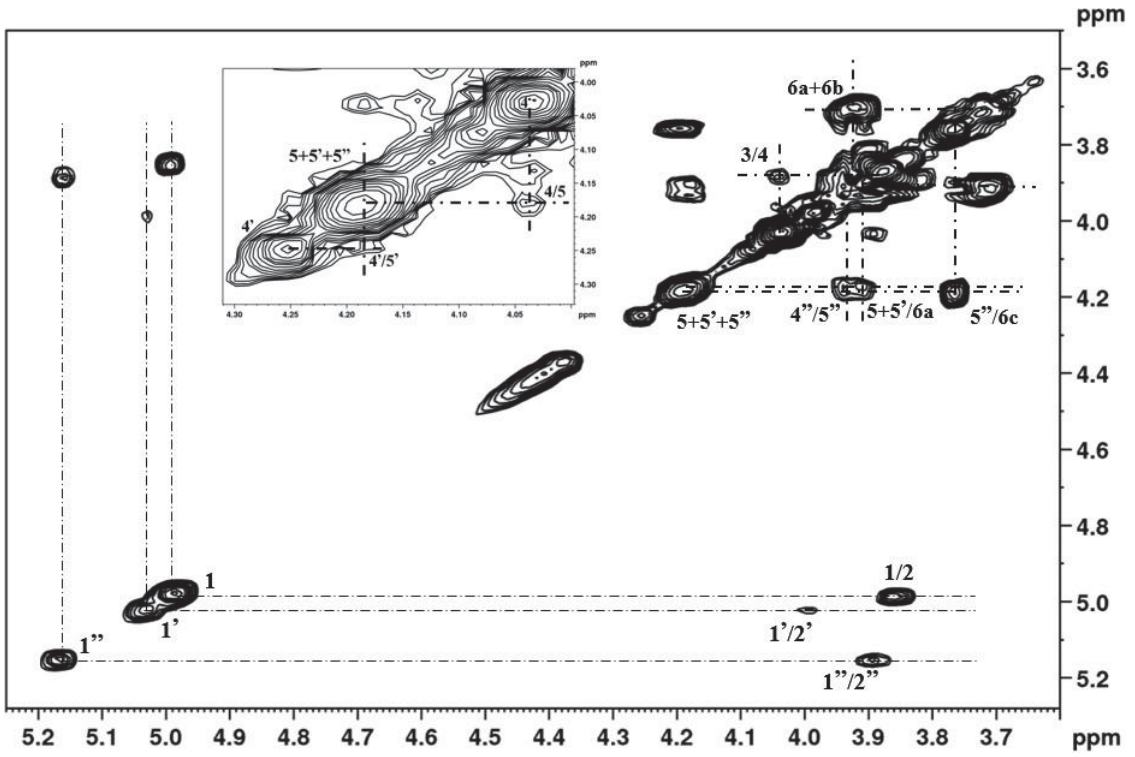


Fig 5. COSY spectrum of α-D-galactan from *Amanita muscaria* (L.:Fr). The sample was analyzed in D_2O at 70 °C (chemical shifts are expressed in δ ppm).

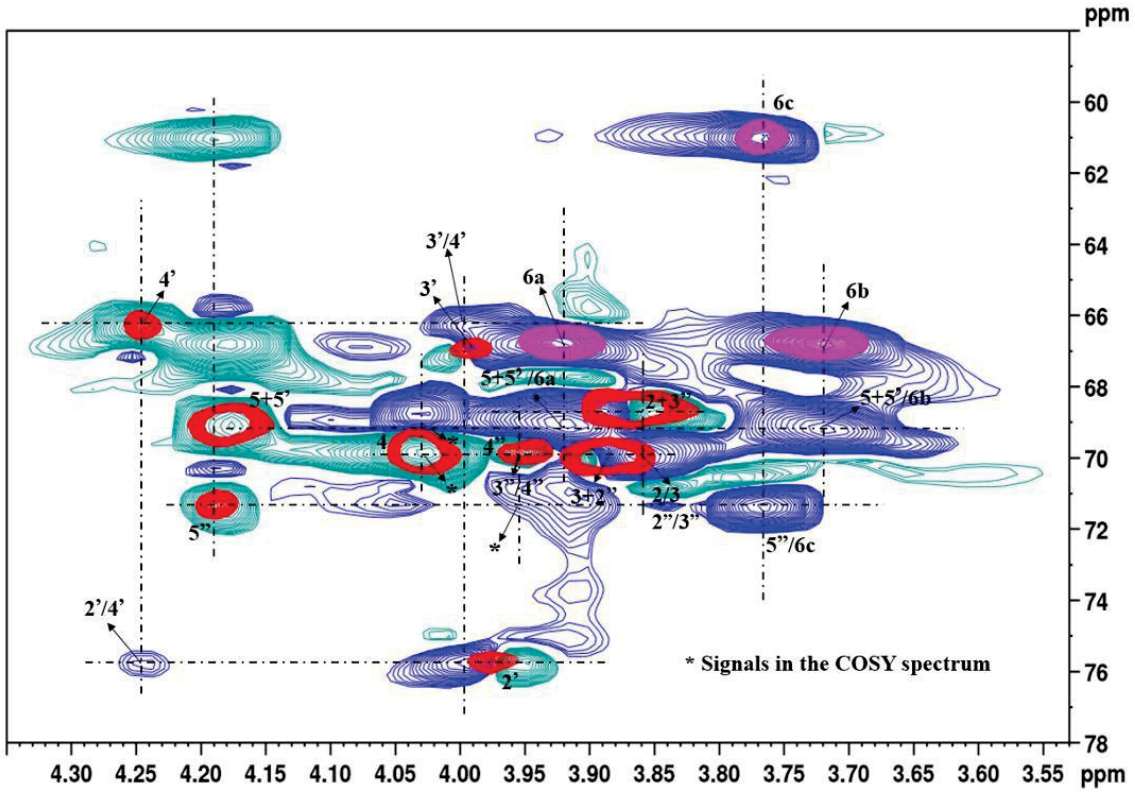


Fig 6. HSQC-TOCSY (in blue and green) spectrum with superimposed HSQC (in red) spectrum of α-D-galactan from *Amanita muscaria* (L.:Fr). The sample was analyzed in D_2O at 70 °C (chemical shifts are expressed in δ ppm).

Table 2. Chemical shift[a] of the correlation between the protons of the adjacent carbons in monosaccharides units of the α-D-galactan.

6→)α-Gal*p*(1→	$^{1}H/^{13}C$	2,6→)α-Gal*p*(1→	$^{1}H/^{13}C$	α-Gal*p*(1→	$^{1}H/^{13}C$
1/2	4.98/3.85[b]	**1'/2'**	5.02/3.97[b]	**1" /2"**	5.15/3.88[b]
2/3	3.85/69.6[c]	**2'/3'**	3.99/75.1[c]	**2" /3"**	3.85/69.6[c]
3/4	3.88/4.03[b]	**3'/4'**	3.98/66.2[c]	**3" /4"**	3.95/68.6[c]
4/5	4.03/4.17[b]	**4'/5'**	4.18/4.25[b]	**4" /5"**	3.94/4.17[b]
5/6a	3.91/68.8[c]	**5'/6a**	3.91/68.8[c]	**5" /6c**	3.76/71.0[c]
5/6b	3.71/68.8[c]	**5'/6b**	3.71/68.8[c]	**-**	**-**

[a]Assignments are based on ^{1}H and HSQC analysis and are expressed as ppm.

[b]Signals in COSY spectrum;

[c]Signals in HSQC-TOCSY/ HSQC spectrum.

In the literature data, no D-galactan has been described from the mushroom *Amanita muscaria*. The D-galactans reported were obtained from mushrooms of the genus *Pleurotus* and have a main chain of α-D-Gal*p*-(1→6)-linked with a methyl group in its structure and none of them showed branches at *O*-2 composed by α-D-Gal*p* non-reducing end units (Brito et al., 2018; Carbonero et al., 2008; Rosado et al., 2003). In addition, heteropolysaccharides have already been reported with the main chain constituted of α-D-Gal*p*-(1→6)-linked partially substituted at *O*-2 by different side chains isolated from the mushrooms *Amanita muscaria* (Ruthes et al., 2013), *Agaricus bisporus* (Komura et al., 2010), *Chroogomphis rutilis* (Sun et al., 2010), *Flammulina velutipes* (Zhang et al., 2012b), *Fomitella fraxinea* (Cho et al., 2011), *Hericium erinaceus* (Zhang et al., 2012a) and *Ganoderma atrum* (Zhang et al., 2014), all of them with distinct chemical characteristics from the α-D-galactan of the present work.

Table 3. Results of partially *O*-methylated alditol acetates of the α-D-galactan (GAL-Am) and β-D-glucan (GLC-Am) polysaccharides obtained from *Amanita muscaria* (L.:Fr).

Partially *O*-methylated alditol acetates[a]	*Rt*[b]	% Area of fragments[c]		Linkage types
		GAL-Am[d]	GLC-Am[e]	
2,3,4,6-Me_4-Gal	14.880	15	-	Gal*p*-(1→
2,3,4,6-Me_4-Glc	15.230	-	19	Glc*p*-(1→
2,3,4-Me_3-Gal	17.160	71	-	6→)-Gal*p*-(1→
2,4,6-Me_3-Glc	16.240	-	45	3→)-Glc*p*-(1→
2,3,4-Me_3-Glc	16.695	-	13	6→)-Glc*p*-(1→
2,4-Me_2-Glc	18.020	-	23	3,6→)-Glc*p*-(1→
3,4-Me_2-Gal	18.425	14	-	2,6→)-Gal*p*-(1→

[a] GC-MS analysis on a Varian 4000 capillary column; [b] Retention time (min); [c] Based on derived *O*-methyalditol acetates; [d] GAL-Am: α-D-galactan isolated from *A.muscaria*; [e]GLC-Am: β-D-glucan isolated from *A.muscaria.*

3.5 Purification process of the β-D-glucan from *Amanita muscaria* (L.:Fr)

After dialysis and centrifugation process in the P-Am fraction, the soluble fraction F-2 obtained (1.05 %, 2.84 g) was analyzed by ^{13}C NMR and HSQC (Fig 7A and B). Signs in the anomeric region at δ 102.9/4.50, 102.6/4.35 and 102.9/4.27 indicate the presence of a β-Glc*p*, while signs at δ 98.4/4.74 and 98.4/4.80 refer to a α-Gal*p*. In the linkage regions it is possible to observe the presence of (1→3)-linked (δ 86.1/3.48), (1→6)-linked by *O*-6 substitution (δ 68.5/3.90;3.79 and 66.2/3.69;3.54) and free *O*-6 non-reducing end units (δ 60.5/3.69 and 60.5/3.49) (Oliveira et al., 2015).

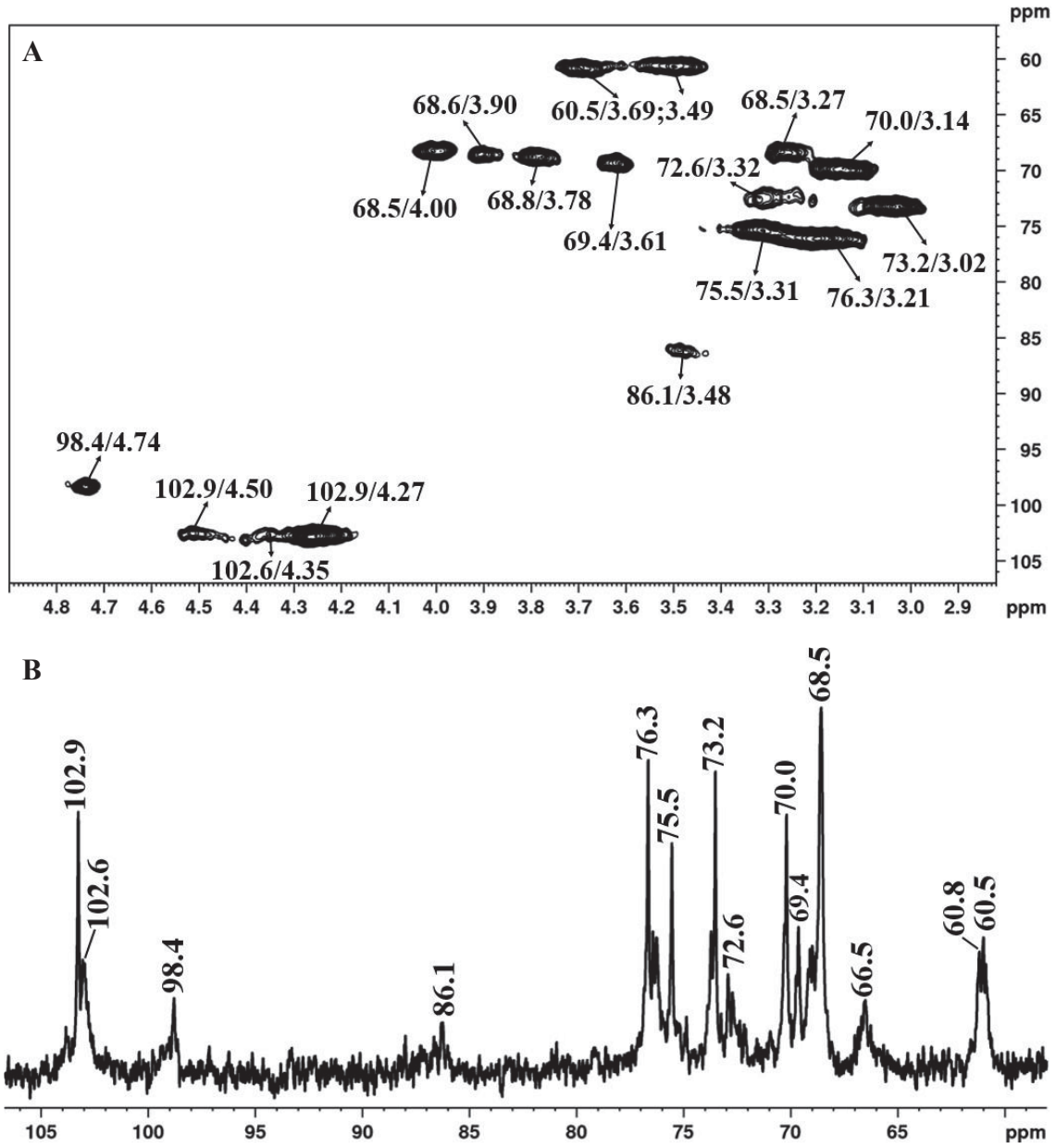


Fig 7. HSQC (A) and ^{13}C NMR (B) and spectra of F-2 fraction from *Amanita muscaria* (L.:Fr). The sample was analyzed in Me_2SO-d_6 at 70 °C (chemical shifts are expressed in δ ppm).

After freezing and thawing process (Gorin & Iacomini, 1984) in the fraction F-2, the soluble polysaccharide S-3 (0.34 %, Fig. 1) was submitted to Fehling treatment and two new fractions were generated: insoluble and soluble fraction (S-4, 0.16 %). The first has not been studied due to the small amount of material, but the soluble fraction (S-4) underwent NMR analisys. ^{13}C NMR and HSQC analysis showed a very similar spectrum to the previous one (Fig 7A and B), however with different intensities (Figure 8A and B).

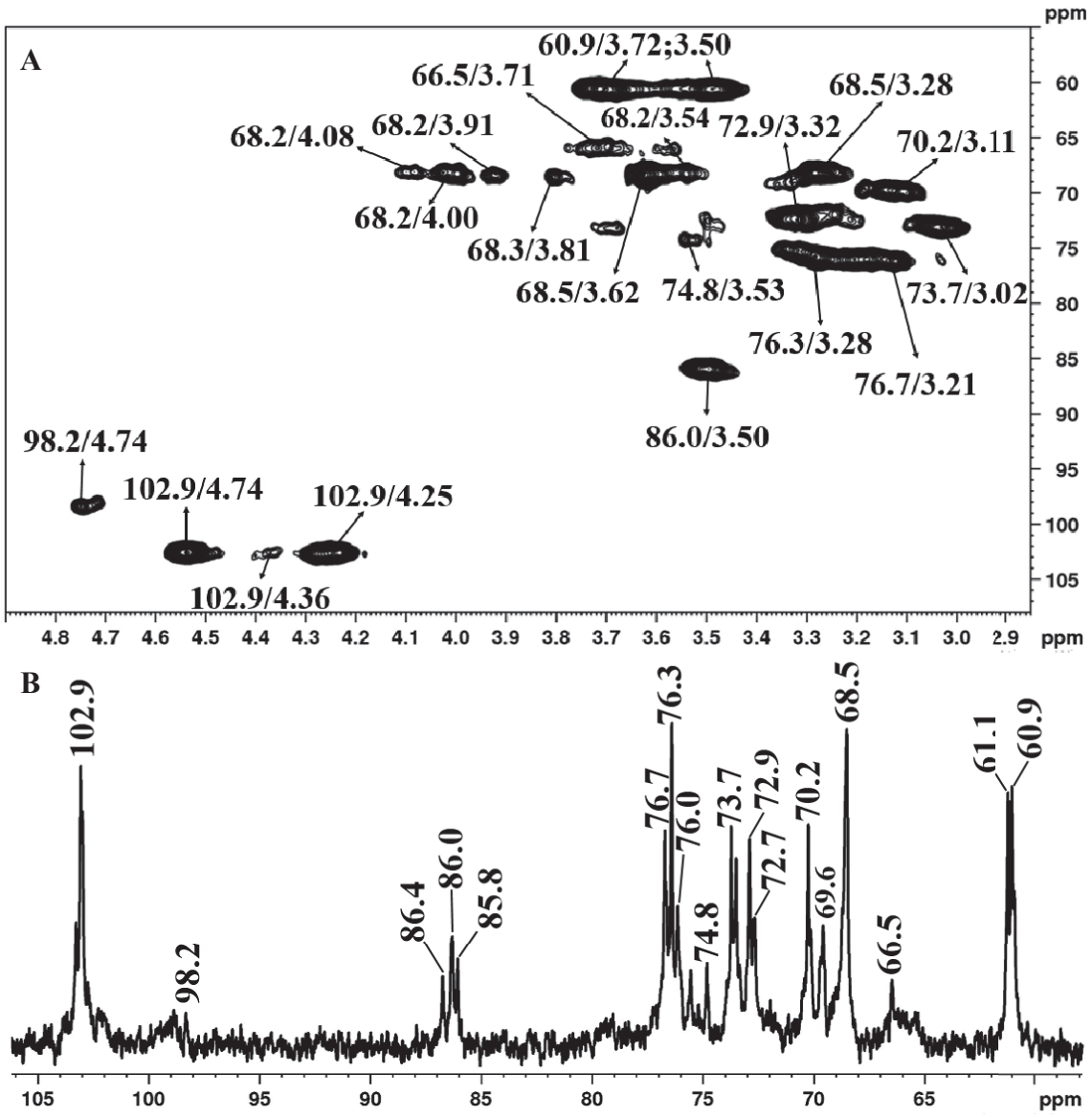


Fig 8. HSQC (A) and ^{13}C NMR (B) spectra of S-4 fraction from *Amanita muscaria* (L.:Fr). The sample was analyzed in Me_2SO-d_6 at 70 °C (chemical shifts are expressed in δ ppm).

In order to separate the mixture of polysaccharides present in the S-4 fraction, an ultrafiltration was performed (3 kDa membrane). The material obtained in the retained fraction (GLC-Am, 0.12 %, 300 mg) showed to be pure and will be discussed in the item 3.6.

3.6 Chemical structure of the (1→3),(1→6)-β-D-glucan from *A. muscaria* (L.:Fr)

GLC-Am fraction showed M*w* 1.3 x 10^5 g.mol^{-1} with a homogeneous elution profile in the HPSEC spectrum and, through the analyzes performed, it proved to be a (1→3),(1→6)-β-D-glucan.

The monosaccharide composition showed only glucose in its composition, confirming the presence of a D-glucan. In the methylation data (Table 2) it is possible to identify and characterize a (1→3),(1→6)-linked β-D-glucan represented by partially *O*-methyl alditol acetates derivatives with 2,3,4,6-tetra-*O*-methyl-Glc*p* (19 %), 2,4,6-tri-*O*-methyl-Glc*p* (45 %), 2,3,4-tri-*O*-methyl-Glc*p* (13 %) and 2,4-di-*O*-methyl-Glc*p* (23 %), which indicate the presence of a (1→3)-linked β-D-glucopyranosyl main chain, partially substituted at *O*-6 by single units of β-D-Glc*p* and by side chains of (1→6)-linked β-D-glucopyranosyl units. These results confirm the signals obtained by NMR studies for the chemical structure of β-D-glucan (Table 3).

The HSQC and ^{13}C NMR spectrum (Fig. 9A and B) showed characteristic signals related to the presence of pure (1→3),(1→6)–linked β-D-glucan, which can be confirmed by the signals anomeric at δ 103.2/4.55 and 103.2/4.27 (C1 / H1). In the region of glycosidic linkages the signal at δ 86.3/3.50 correspond to the substituted 3-*O* units (C3 / H3) and at δ 68.5/4.11 and 68.5/3.58 corresponding to the replaced 6-*O* units (C6 / H6). The non-reducing terminal groups of β-D-Glc*p* units can be observed in δ 60.5/3.69 and 60.5/3.49 (C6 / H6), as seen in Table 4 (Morales et al., 2020; Ruthes et al., 2015; Sovrani et al., 2017).

Table 4. ^{1}H and ^{13}C NMR chemical shift[a] of fractions GLC-Am[b] and GLC-Sm[c] from *Amanita muscaria* (L.:Fr).

	Units		**1**	**2**	**3**	**4**	**5**	**6**	
								a	**b**
GLC-Am	β-D-Glc-(1→	^{13}C	103.2	73.7	76.7	70.2	76.4	60.9	60.9
		^{1}H	4.27	3.09	3.14	3.14	3.29	3.72	3.50
	3→)-β-D-Glc-(1→	^{13}C	103.2	72.8	86.3/86.7	68.7	76.4	60.9	60.9
		^{1}H	4.55	3.35	3.50	3.29	3.29	3.72	3.50
	3,6→)-β-D-Glc-(1→	^{13}C	103.2	72.8	86.1	68.7	74.8	68.5	68.5
		^{1}H	4.55	3.35	3.51	3.29	3.54	4.11	3.58
GLC-Sm	3→)-β-D-Glc-(1→	^{13}C	103.0	72.9	86.2	68.4	76.4	60.9	60.9
		^{1}H	4.53	3.31	3.49	3.25	3.27	3.71	3.48

[a]Assignments are based on ^{13}C NMR, ^{1}H and HSQC analysis and are expressed as ppm; [b](1→3),(1→6)-β-D-Glucan; [c]Smith degration glucan.

The β-D-glucan main chain is a β-D-(1→3)-linked linear structure whose results have been proven by the controlled Smith degradation (Fig. 10A and B). The six signs observed (δ 103.0/4.53, 86.2/3.49, 76.4/3.27, 72.9/3.31, 68.4/3.25, 60.9/3.71;3.48) are relatives to C1/H1, C3/H3, C5/H5, C2/H2, C4/H4, C6/H6a;b, respectively, also referenced in de Jesus et al., 2018b; Morales et al., 2019; Morales et al., 2020.

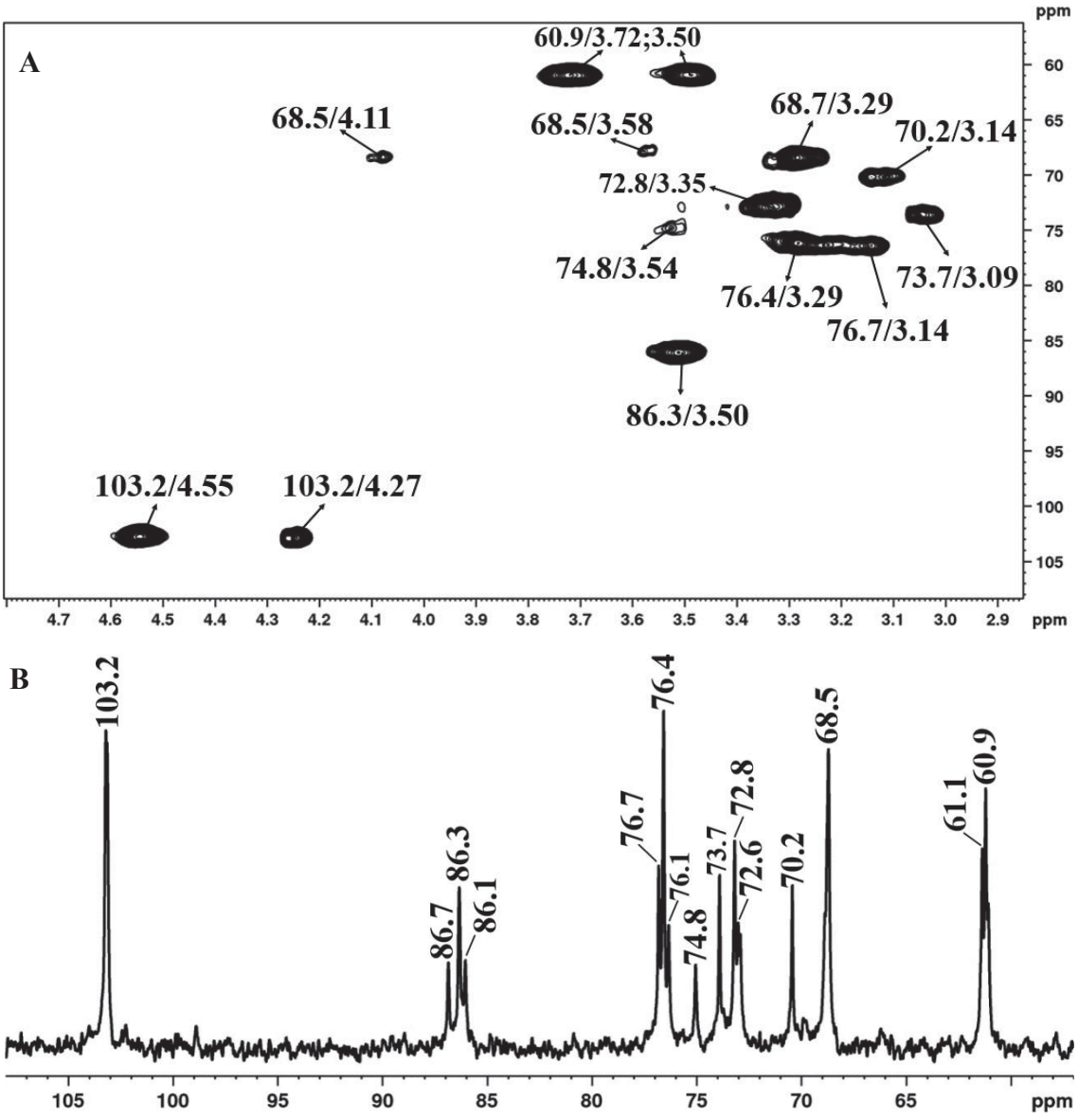


Fig 9. ^{13}C NMR and HSQC spectra of GLC-Am fraction from *Amanita muscaria* (L.:Fr). HSQC (A) and ^{13}C NMR (B) spectra of GLC-Am fraction. The sample was analyzed in Me_2SO-d_6 at 70 °C (chemical shifts are expressed in δ ppm).

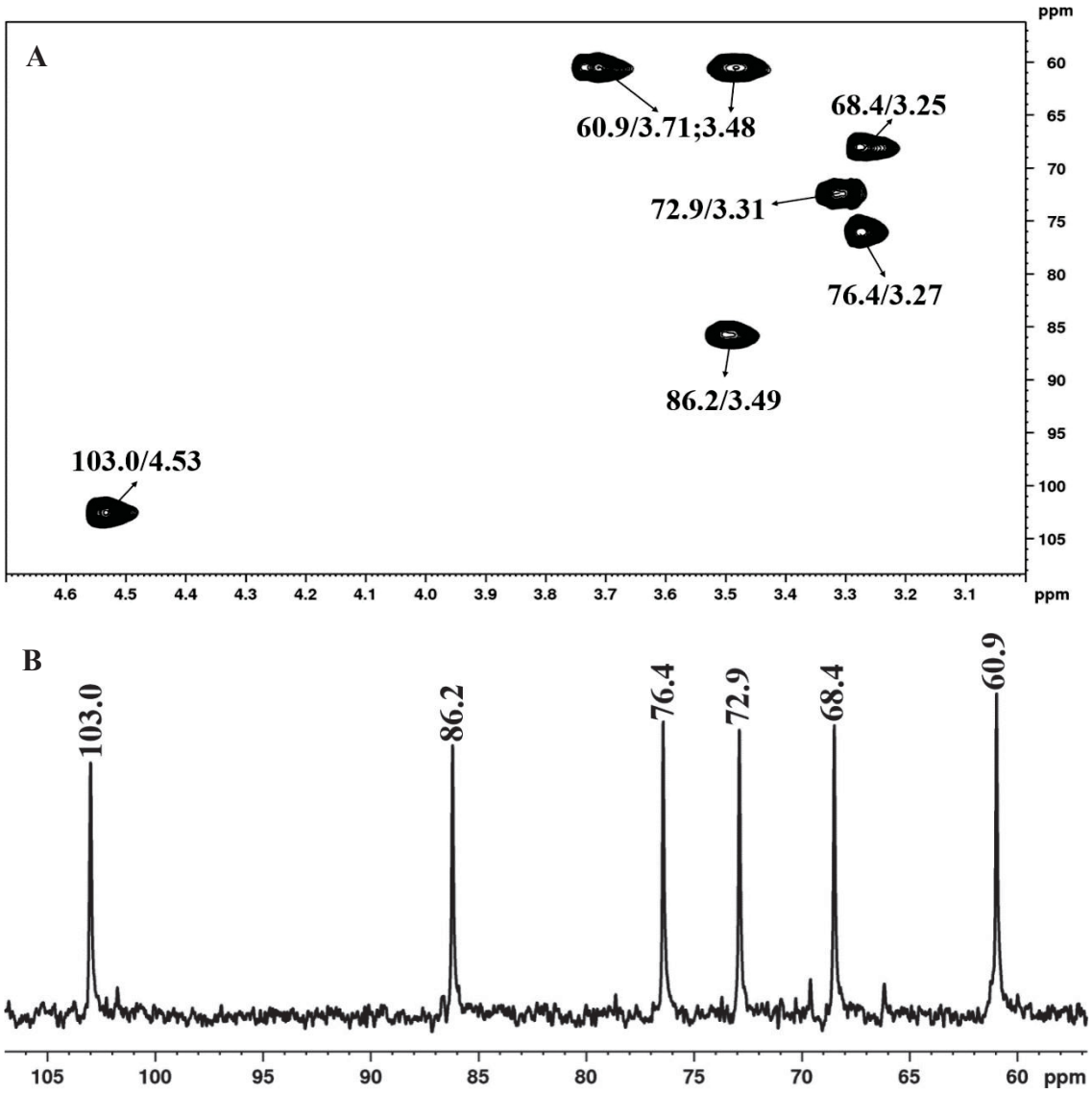


Fig 10. HSQC (A) and ^{13}C NMR (B) spectra of Smith-degraded glucan (GLC-Sm) from *Amanita muscaria* (L.:Fr). The sample was analyzed in Me_2SO-d_6 at 70 °C (chemical shifts are expressed in δ ppm).

GLC-Am (1→3),(1→6) β-D-glucan showed different chemical characteristics - molecular weight, degree of branching and the size of the side chains - to those had already been isolated from the mushroom *Amanita muscaria* by Kiho et al. (1992), from the alkaline extract, with molecular weight estimated 9.5 x 10^3 g/mol^{-1} and main chain (1→3)-linked substituted by two groups of (1→6)-linked D-glucopyranosyl every seven monosaccharide units in the main chain, and by Ruthes et al. (2013b), isolated from the cold water extract, with M*w* 1.6 x 10^4 g/mol^{-1} substituted at *O*-6 mostly by non-reducing end units of β-D-Glc*p* as side chains, and a minor proportion *O*-6 substituted by β-D-glucopyranosyl oligosaccharides.

In addition, GLC-Am appears to have a molecular weight similar to the (1→3),(1→6) β-D-glucan isolated from *Lactarius rufus* (Ruthes et al., 2013a), but differs in degree of branching and in the composition of the side chains.

Conclusions

The α-D-galactan isolated in the present work presented a main chain similar to those extracted by Brito et al. (2018), Carbonero et al. (2008) and Rosado et al. (2003), composed by (1→6)-linked α-D-Gal*p* units. However, its structure does not have a methyl group and has substitutions at *O*-2 by non-reducing end units of α-D-Gal*p*, which makes it a polysaccharide not yet reported in the literature.

Composed by (1→3)-linked β-D-Glc*p* main chain substituted at *O*-6 by single units of β-D-Glc*p* and side chains of (1→6)-linked β-D-Glc*p*, the β-D-glucan isolated showed different chemical characteristics from those already reported for *Amanita muscaria* (Ruthes et al., 2013; Kiho et al., 1992), such as molecular weight, degree of branching and the size of the side chains.

Acknowledgements

The authors would like to thank the Brazilian funding agencies: CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior), CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico), the Chemichal Technician Thiago J. dos Santos and Arquimedes Paixão de Santana Filho for GC-MS and NMR analysis, respectively. The authors have declared no conflicts of interest.

References

Abreu, H., Simas, F. F., Smiderle, F. R., Sovrani, V., Dallazen, J. L., Maria-Ferreira, D., Werner, M. F., Cordeiro, L. M. C., & Iacomini, M. (2019). Gelling functional property, anti-inflammatory and antinociceptive bioactivities of β-D-glucan from the edible mushroom *Pholiota nameko*. *International Journal of Biological Macromolecules*, 122, 1128–1135. https://doi.org/10.1016/j.ijbiomac.2018.09.062

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical*

Biochemistry, 72, 248-254. https://doi.org/10.1016/0003-2697(76)90527-3

Brito, D. R., Carbonero, E. R., Viana, S. R. F., Silva, E. V., Ruthes, A. C., Lião, L. M., & Iacomini, M. (2018). Partially methylated galactans containing different proportions of 3-O-methyl-galactose from *Pleurotus citrinopileatus*. *Carbohydrate Research*, 458-459, 29-34. https://doi.org/10.1016/j.carres.2018.02.002

Carbonero, E. R., Gracher, A. H. P., Rosa, M. C. C., Torri, G., Sassaki, G. L., Gorin, P. A. J., & Iacomini, M. (2008). Unusual partially 3-*O*-methylated α-galactan from mushrooms of the genus *Pleurotus*. *Phytochemistry*, 69, 252-257. https://doi.org/10.1016/j.phytochem.2007.06.018

Carbonero, E. R., Ruthes, A. C., Freitas, C. S., Utrilla, P., Gálvez, J., Silva, E. V. Da, Sassaki, G. L., Gorin, P. A. J., & Iacomini, M. (2012). Chemical and biological properties of a highly branched β-glucan from edible mushroom *Pleurotus sajor-caju*. *Carbohydrate Polymers*, 90, 814-819. https://doi.org/10.1016/j.carbpol.2012.06.005

Cho, S. M., Yun, B. S., Yoo, I. D., & Koshino, H. (2011). Structure of fomitellan A, a mannofucogalactan from the fruiting bodies of *Fomitella fraxinea*. *Bioorganic and Medicinal Chemistry Letters*, 1(1), 204-206. https://doi.org/10.1016/j.bmcl.2010.11.036

Ciucanu, i.; Kerek, f. (1984). A simple and rapid method for the permethylation of carbohydrates. *Carbohydrates Research*, v. 131, p. 209-217. https://doi.org/10.1016/0008-6215(84)85242-8

Coville, F. V. (1898). In Observations on cases of mushroom poisoning in the District of Columbia. https://doi.org/10.5962/bhl.title.162914

de Jesus, L. I., Smiderle, F. R., Cordeiro, L. M. C., de Freitas, R. A., Van Griensven, L. J. L. D., & Iacomini, M. (2018). Simple and effective purification approach to dissociate mixed water-insoluble α- and β-D-glucans and its application on the medicinal mushroom *Fomitopsis betulina*. *Carbohydrate Polymers*, *200*, 353–360. https://doi.org/10.1016/j.carbpol.2018.08.004

de Jesus, L. I., Smiderle, F. R., Ruthes, A. C., Vilaplana, F., Dal'Lin, F. T., Maria-Ferreira, D., Werner, M. F., Van Griensven, L. J. L. D., & Iacomini, M. (2018). Chemical characterization and wound healing property of a β-D-glucan from edible

mushroom *Piptoporus betulinus*. *International Journal of Biological Macromolecules*, 117, 1361-1366. https://doi.org/10.1016/j.ijbiomac.2017.12.107

Delgobo, C. L., Gorin, P. A., Jones, C., & Iacomini, M. (1998). Gum heteropolysaccharide and free reducing mono- and oligosaccharides of *Anadenanthera colubrina*. *Phytochemistry*, 47, 1207-1214. https://doi.org/10.1016/S0031-9422(97)00776-0

Gorin, P. A. J., & Iacomini, M. (1984). Polysaccharides of the lichens *Cetraria islandica* and *Ramalina usnea*. *Carbohydrate Research*, 128, 119-132. https://doi.org/10.1016/0008-6215(84)85090-9

Hardy, M. L. (2008). Dietary Supplement Use in Cancer Care: Help or Harm. *Hematology/Oncology Clinics of North America*, 22, 581–617. https://doi.org/10.1016/j.hoc.2008.04.012

Kiho, T., katsurawaga, M., Nagai, K., Ukai, S., & Haga, M. (1992). Structure and antitumor activity of a branched (1→3)-β-D-glucan from the alkaline extract of *Amanita muscaria*. *Carbohydrate Research*, 224, 237-243. https://doi.org/10.1016/0008-6215(92)84109-6

Komura, D. L., Carbonero, E. R., Gracher, A. H. P., Baggio, C. H., Freitas, C. S., Marcon, R., Santos, A. R. S., Gorin, P. A. J., & Iacomini, M. (2010). Structure of *Agaricus* spp. fucogalactans and their anti-inflammatory and antinociceptive properties. *Bioresource Technology*, 101(15), 6192-6199. https://doi.org/10.1016/j.biortech.2010.01.142

Kondeva-Burdina, M., Voynova, M., Shkondrov, A., Aluani, D., Tzankova, V., & Krasteva, I. (2019). Effects of *Amanita muscaria* extract on different in vitro neurotoxicity models at sub-cellular and cellular levels. *Food and Chemical Toxicology*, 132, 110687. https://doi.org/10.1016/j.fct.2019.110687

Morales, D., Rutckeviski, R., Villalva, M., Abreu, H., Soler-Rivas, C., Santoyo, S., Iacomini, M., & Smiderle, F. R. (2020). Isolation and comparison of α- and β-D-glucans from shiitake mushrooms (*Lentinula edodes*) with different biological activities. *Carbohydrate Polymers*, 229, 115221. https://doi.org/10.1016/j.carbpol.2019.115521

Morales, D., Smiderle, F. R., Piris, A. J., Soler-Rivas, C., & Prodanov, M. (2019).

Production of a β-D-glucan-rich extract from Shiitake mushrooms (*Lentinula edodes*) by an extraction/microfiltration/reverse osmosis (nanofiltration) process. *Innovative Food Science and Emerging Technologies*, 51, 80-90. https://doi.org/10.1016/j.ifset.2018.04.003

Oliveira, K. S. M., Di Bastiani, M., Cordeiro, L. M. C., Costa, M. F., Toledo, K. A., Iacomini, M., Babosa, A. M., Dekker, R. F. H., & Nascimento, V. M. G. (2015). (1→6)- and (1→3)(1→6)-β-glucans from *Lasiodiplodia theobromae* MMBJ: Structural characterization and pro-inflammatory activity. *Carbohydrate Polymers*, 133, 539-546. https://doi.org/10.1016/j.carbpol.2015.07.060

Perlin, A. S.; Casu, B. Carbon-13 and proton magnetic resonance spectra of D-glucose-^{13}C. *Tetrahedron Letters*, 34, 2919-2924, 1969.

Rosado, F. R., Carbonero, E. R., Claudino, R. F., Tischer, C. A., Kemmelmeier, C., & Iacomini, M. (2003). The presence of partially 3-*O*-methylated mannogalactan from the fruit bodies of edible basidiomycetes *Pleurotus ostreatus* "*florida*" Berk. and *Pleurotus ostreatoroseus* Sing. *FEMS Microbiology Letters*, 221, 119-124. https://doi.org/10.1016/S0378-1097(03)00161-7

Ruthes, A. C., Komura, D. L., Carbonero, E. R., Sassaki, G. L., Gorin, P. A. J., & Iacomini, M. (2010). Structural characterization of the uncommon polysaccharides obtained from *Peltigera canina* photobiont *Nostoc muscorum*. *Carbohydrate Polymers*, 81, 29-34. https://doi.org/10.1016/j.carbpol.2010.01.050

Ruthes, A. C., Smiderle, F. R., & Iacomini, M. (2016). Mushroom heteropolysaccharides: A review on their sources, structure and biological effects. *Carbohydrate Polymers*, 136, 358-375. https://doi.org/10.1016/j.carbpol.2015.08.061

Ruthes, A. C., Carbonero, E. R., Córdova, M. M., Baggio, C. H., Santos, A. R. S., Sassaki, G. L., Cipriani, T. R., Gorin, P. A. J., & Iacomini, M. (2013). *Lactarius rufus* (1→3),(1→6)-β-D-glucans: Structure, antinociceptive and anti-inflammatory effects. *Carbohydrate Polymers*, 94, 129-136. https://doi.org/10.1016/j.carbpol.2013.01.026

Ruthes, A. C., Carbonero, E. R., Córdova, M. M., Baggio, C. H., Sassaki, G. L., Gorin, P. A. J., Santos, A. R. S., & Iacomini, M. (2013). Fucomannogalactan and glucan from mushroom *Amanita muscaria*: Structure and inflammatory pain inhibition.

Carbohydrate Polymers, 98, 761-769. https://doi.org/10.1016/j.carbpol.2013.06.061

Ruthes, A.C., Smiderle, F. R., & Iacomini, M. (2015). D-Glucans from edible mushrooms: A review on the extraction, purification and chemical characterization approaches. *Carbohydrate Polymers*, 117, 753-761. https://doi.org/10.1016/j.carbpol.2014.10.051

Sassaki, G. L., Gorin, P. A. J., Souza, L. M., Czelusniak, P. A., & Iacomini, M. (2005). Rapid synthesis of partially O-methylated alditol acetate standards for GC-MS: Some relative activities of hydroxyl groups of methyl glycopyranosides on Purdie methylation. *Carbohydrate Research*, 340, 731-739. https://doi.org/10.1016/j.carres.2005.01.020

Sassaki, G. L., Souza, L. M., Serrato, R. V., Cipriani, T. R., Gorin, P. A. J., & Iacomini, M. (2008). Application of acetate derivatives for gas chromatography-mass spectrometry: Novel approaches on carbohydrates, lipids and amino acids analysis. *Journal of Chromatography A*, 1208(1–2), 215-222. https://doi.org/10.1016/j.chroma.2008.08.083

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. (2008). Anti-inflammatory and analgesic properties in a rodent model of a (1→3),(1→6)-linked β-glucan isolated from *Pleurotus pulmonarius*. *European Journal of Pharmacology*, 597, 86-91. https://doi.org/10.1016/j.ejphar.2008.08.028

Sovrani, V., de Jesus, L. I., Simas-Tosin, F. F., Smiderle, F. R., & Iacomini, M. (2017). Structural characterization and rheological properties of a gel-like β-D-glucan from *Pholiota nameko*. *Carbohydrate Polymers*, 169, 1-8. https://doi.org/10.1016/j.carbpol.2017.03.093

Sun, Y., Li, X., Yang, J., Liu, J., & Kennedy, J. F. (2010). Water-soluble polysaccharide from the fruiting bodies of Chroogomphis rutilus (Schaeff.: Fr.) O. K. Miller: Isolation, structural features and its scavenging effect on hydroxyl radical. *Carbohydrate Polymers*. https://doi.org/10.1016/j.carbpol.2009.12.015

Vetvicka, V., & Yvin, J. C. (2004). Effects of marine β-1,3 glucan on immune reactions. *International Immunopharmacology*, 4, 721-730.

https://doi.org/10.1016/j.intimp.2004.02.007

White, J., Weinstein, S. A., De Haro, L., Bédry, R., Schaper, A., Rumack, B. H., & Zilker, T. (2019). Mushroom poisoning: A proposed new clinical classification. In *Toxicon*, 157, 53–55. https://doi.org/10.1016/j.toxicon.2018.11.007

Willment, J. A., Gordon, S., & Brown, G. D. (2001). Characterization of the Human β-Glucan Receptor and Its Alternatively Spliced Isoforms. *Journal of Biological Chemistry*, 276, 43818-43823. https://doi.org/10.1074/jbc.M107715200

Wolfrom, M. L.; Thompson, A. Acetylation. *Methods in Carbohydrate Chemistry*, 2, 211-215, 1963b.

Zhang, A. Q., Fu, L., Xu, M., Sun, P. L., & Zhang, J. S. (2012). Structure of a water-soluble heteropolysaccharide from fruiting bodies of *Hericium erinaceus*. *Carbohydrate Polymers*, 88(2), 558-561. https://doi.org/10.1016/j.carbpol.2011.12.039

Zhang, A. Q., Xiao, N. N., Deng, Y. L., He, P. F., & Sun, P. L. (2012). Purification and structural investigation of a water-soluble polysaccharide from *Flammulina velutipes*. *Carbohydrate Polymers*, 87(3), 2279-2283. https://doi.org/10.1016/j.carbpol.2011.10.061

Zhang, A. Q., Zhang, Y., Yang, J. H., & Sun, P. L. (2013). Structural elucidation of a novel heteropolysaccharide from the fruiting bodies of *Pleurotus eryngii*. *Carbohydrate Polymers*, 92, 2239-2244. https://doi.org/10.1016/j.carbpol.2012.11.069

Zhang, H., Nie, S. P., Yin, J. Y., Wang, Y. X., & Xie, M. Y. (2014). Structural characterization of a heterogalactan purified from fruiting bodies of *Ganoderma atrum*. *Food Hydrocolloids*, 36, 339-347. https://doi.org/10.1016/j.foodhyd.2013.08.029

Artigo II

β-D-glucans (1→3),(1→6) obtained from residual material by extraction at high temperature and pressure from different mushrooms

β-D-glucans (1→3),(1→6) obtained from residual material by extraction at high temperature and pressure from different mushrooms

Matheus Zavadinack [a], Hellen Abreu [a], Thales Ricardo Cipriani[b], Lucimara M. C. Cordeiro[a], Rilton A. de Freitas[b] and Marcello Iacomini[a*]

[a] Departament of Biochemistry and Molecular Biology, Federal University of Paraná, Curitiba- PR, CEP 81531-980, Brazil.

[b] Department of Chemistry, Federal University of Paraná, Curitiba-PR, Brazil.

*Corresponding author: Department of Biochemistry and Molecular Biology, Federal University of Paraná, Mailbox (Caixa Postal) 19046, Curitiba- PR, CEP 81531-980, Brazil. Tel.: +55 (41) 3361-1655; Fax: +55 (41) 3266-2042; e-mail: iacomini@ufpr.br

Abstract

Two residual mushroom extracts were subjected to autoclave extraction (*Pleurotus pulmonarius* and *Pholiota nameko*) with high temperature and pressure in aqueous and alkaline medium. The aqueous extract from *P. nameko* and the alkaline from *P. pulmonarius* were precipitated with ethanol and the samples were purified by freezing and thawing and ultrafiltration process. After purification, the acid hydrolysis showed only glucose as monosaccharide in its compositions. The D-glucans were characterized by NMR mono and by two-dimensional studies, methylation analysis and contolled Smith degradation. By the spectra of ^{1}H-, ^{13}C- and HSQC-NMR we could conclude that they are β-D-glucans with main chain (1→3)-linked with ramifications replaced by β-D-(1→6)-linked glucopyranose units as non-reducing end units confirmed by the analysis of the methylated derivatives. The main chain of the two polysaccharides were confirmed by Smith degradation sequenced, which gave rise to a linear polymer β-D-Glc*p*-(1→3)-linked. The β-D-glucans demonstrate different water solubilities and molecular weights, 1.5±0.2 x 10^{6} g/mol^{-1} and 13.5±2.4 x 10^{6} g.mol^{-1}, respectively.

Keywords: *Pholiota nameko*, *Pleurotus pulmonarius*, mushroom, β-glucan and polysaccharides

Introduction

Several species of mushroom have already been reported and a wide variety of bioactive compounds, especially polysaccharides, have been identified (Wasser, 2003). Among them are D-glucans, fucomannogalactans, xylomannans and mannogalactans (Smiderle et al., 2008).

Many of the species of the genus *Pleurotus* are edible. Easily found in the environment on tree trunks, it presents basidiocarp in shell-shaped and are commonly called oyster mushroom (Alexopoulos et al., 1996). Belonging to the phylum basidiomycota, *P. nameko* and *P. pulmonarius* are rich in biological compounds (Abreu et al., 2019; Smiderle et al., 2008). In China and Japan, they are extensively cultivated to be used as food and as traditional medicine (Sovrani et al., 2017; Zhang et al., 2014)

Several D-glucans have already been reported in the literature from different mushrooms and showed interesting application in cosmetic, food, agronomic and therapeutic areas. The most commonly isolated glucans from fungi is β-glucans (Morales et al., 2016). They are a basis of fungal cell wall structure and are not found in animals. However, a large variety of beneficial effects on human health was described for them, such antitumoral, hypolipidemic, anti-inflammatory, analgesic, immunomodulatory and others (Chen & Seviour, 2007; Dalonso et al., 2015; Smiderle et al., 2008).

The most common homopolysaccharide present in Basidiomycetes are the branched (1→3),(1→6)-linked β-D-glucans (Ruthes et al., 2013; Santos-Neves et al., 2008; Smiderle et al., 2006). In general, consist mainly of a backbone of β-D-Gl*c*p (1→3)-linked units partially substituted at *O*-6 by side chains of β-D-Glc*p* (1→3) or (1→6)-linked units and/or single β-D-Glc*p* residues as non-reducing end units (Bhanja et al., 2014; Moradali et al., 2007; Moreno et al., 2016; Zhu et al., 2015).

The degree of branching implies variation of their tertiary structure (Zhu et al., 2015). Those with high degrees of (1→6)-glycosidic bonds form a triple helix as their tertiary structure (Laroche & Michaud, 2008; Nitschke et al., 2011) while those with few or no (1→6)-linkages mainly have a single helix structure (Moreno et al., 2016). Such conformations directly imply the type of biological response generated (Moradali et al., 2007). Besides, other chemical characteristics, such is distinct anomeric configurations (α-, β- or mixed α,β), linkage types (1→3; 1→4; 1→6), molecular weight and solubility, are related with their biological properties (Morales et al., 2020; Zhang et al., 2007).

The release of TNF-α by macrophages is only induced by β-D-glucans with high molecular weight and low degree of branching, while tumor activity was more evident in

heteroglucans containing β-D-glucans (1→3)-linked with branches composed of glucuronic acid, arabinoxylose, xylose or mannose (Moradali et al., 2007). The mechanism of action of mushroom polysaccharides capable of generating biological responses are still unknown. Some authors have showed that these molecules can bind immune cells receptors, generating pro- or anti-inflammatory responses through the release of cytokines (Chen & Seviour, 2007; El Enshasy & Hatti-Kaul, 2013).

Different isolation and purification procedures may result in distinct extracted β-D-glucans (Abreu et al., 2019). Such correlation was represented by Moreno et al. (2016) when obtaining, through aqueous and alkaline extraction, β-D-glucans from *Cookeina tricholoma* with different molecular weight and degree of branching.

Baggio et al. (2012) isolated a (1→3),(1→6)-linked β-D-glucan from *P. pulmonarius* by hot aqueous extraction with molecular weight between 1 x 10^5 and 1 x 10^6 g mol^{-1} that showed an antinociceptive effect possibly related to protein kinase C inhibition. A (1→3),(1→6)-linked β-D-glucan with molecular weight 1.4 x 10^6 g mol^{-1} was obtained by Abreu et al. (2019) from *P. nameko* by hot aqueous extraction with significant inhibition of the inflammatory response. In addition, (1→3),(1→6)-linked β-D-glucans were also obtained from *Lentinus edodes* (Morales et al., 2020), *Lactarius rufus* (Ruthes et al., 2013), *Amanita muscaria* (Ruthes et al., 2013), *Fomitopsis betulina* (de Jesus et al., 2018a), *Lasiodiplodia theobromae* (Oliveira et al., 2015) and others.

In this work, two (1→3),(1→6)-linked β-D-glucans were obtained separately by extraction at high temperature and pressure. Both were characterized and showed different chemical characteristics.

2. Materials and Methods

2.1 Biological material

2.1.1 Residual material obtained from *P. pulmonarius*

The residual material from *Pleurotus pulmonarius* (Residue IV, 41.7 g, figure 1) was originated after four sequential extractions (lipid extraction, cold water extraction, hot water extraction and alkaline extraction, respectively) and the purified polysaccharides obtained by these processes were previously published by Baggio et al. (2010), Baggio et al. (2012), Smiderle et al. (2008a) and Smiderle et al. (2008b) and are represented in the flowchart below (Figure 1).

2.1.2 Residual material obtained from *P. nameko*

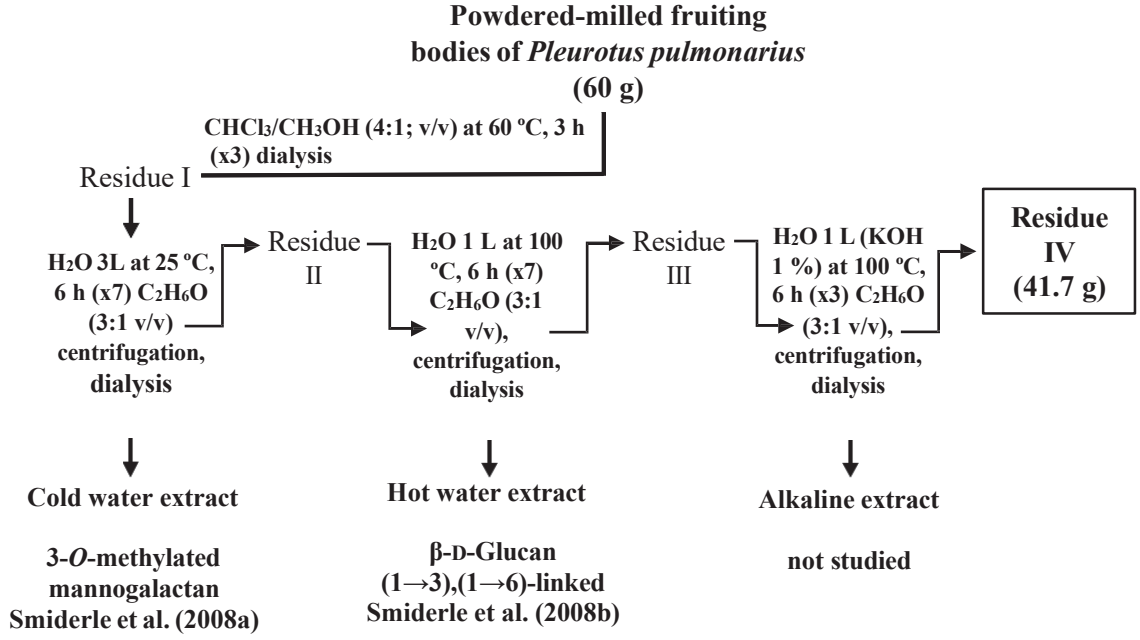


Figure 1. Scheme of extractions previously performed on *Pleurotus pulmonarius* mushroom powdered-milled fruiting bodies.

The residual material from *Pholiota nameko* (Residue IV, 102.4 g, figure 2) was also originated after four sequential extractions (lipid extraction, cold water extraction, hot water extraction and alkaline extraction, respectively) developed by Sovrani et al. (2017) and Abreu et al. (2019). The polysaccharides obtained and their respectively extraction process are shown in the figure 2.

Both residual fractions would possibly be discarded because they have already undergone several extraction processes. The amount of material obtained from these residual fractions was also evaluated and discussed in the present work (section 3.1)

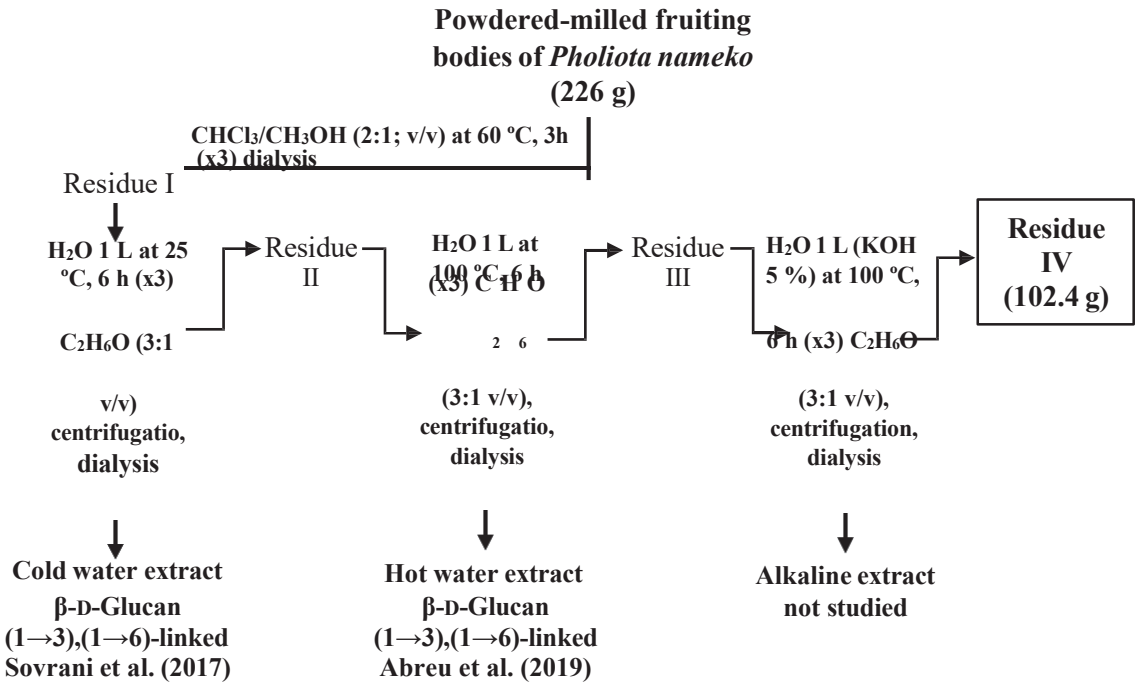


Figure 2. Scheme of extractions previously performed on *Pholiota nameko* mushroom powdered-milled fruiting bodies.

2.2 General extractions and purification processes

The general extraction and purification processes performed on the *Pholiota nameko* and *Pleurotus pulmonarius* are described in the following items (section 2.2.1 and 2.2.2).

2.2.1 *Pholiota nameko* and *Pleurotus pulmonarius*: general extraction and purification processes

The lyophilized residues (102.4 g and 41.7 g, respectively) were dissolved, separately, in distilled water (0.3 L/ 10 g), reduced with a small aliquot of $NaBH_4$ and submitted to high temperature and pressure extraction (121 °C, 1.2 atm, 1 h) using an autoclave. Still hot, the materials were filtered and the eluted fractions were concentrated to a small volume and lyophilized, generating the fractions Pn-1 and Pp-1. Subsequently, Pn-1 was dissolved in a small volume of water and submitted to ethanolic precipitation by addition to excess ethanol (3:1, v/v) and centrifuged for 20 min (10000 rpm, 25 °C), giving rise to the precipitated fraction Pn-2, which was submitted to freezing and thawing process. The soluble fraction was subjected to ultrafiltration process using a 3 kDa cut-off membrane (Millipore®; polyethersulfone membrane) coupled in a cylinder Sartorius (Model 16249), generating the eluted and retained fractions, named Pn-GLC.

The residual fractions obtained after aqueous extraction in autoclave were subjected, separately, to a new autoclave extraction, now in an alkaline medium with 5 %

potassium hydroxide. Both materials were filtered and the eluted fractions were neutralized with acetic acid, concentrated to a small volume and lyophilized, generating the fractions Pp-KOH and Pn-KOH. Then, Pp-KOH was dissolved in a small volume of water and went through ethanolic precipitation by addition to excess ethanol (3:1, v/v) and centrifuged for 20 min (10000 rpm, 25 °C). The precipitate fraction was subjected to solubilization in a small volume of water followed by the freezing and thawing process (3 x), giving rise the soluble and insoluble fraction, named Pp-GLC.

2.3 Monosaccharide composition of the *P. nameko* and *P. pulmonarius*

Each sample (2 mg) was submitted to hydrolysis with 2 M TFA (2 mL) at 100 °C for 8 h. After this time, it was evaporated to dryness and then solubilized in water (1 mL) and reduced with $NaBH_4$ (10 mg). After 12 h the reduction was interrupted with acetic acid and later went through elimination of sodium from the reaction medium with cationic resin. The residual liquid was then evaporated to dryness. Acetylation was developed with acetic anhydride and pyridine (200 µL; 1:1; v/v) for 30 min at 100 °C. The resulting alditol acetates were analyzed by gas liquid chromatography-mass specrometry (GC-MS) using a Varian (model CP-3800) gas chromatograph coupled to an Ion-Trap 4000 mass spectrometer, with He as the carrier gas. The identification of the corresponding alditols was made by comparison with the typical retention times and electron impact profiles with several standards (Sassaki et al., 2008).

2.4 Methylation of the polysaccharides of *P. nameko* and *P. pulmonarius* analysis by GC-MS

Using a method modified from (Ciucanu & Kerek, 1984), per-*O*-methylation of the purified fractions (10 mg) were carried out using NaOH-Me_2SO-MeI, as described by Ruthes et al (2010). The per-*O*-methylated derivatives were hydrolyzed with 1 M TFA (1 mL), at 100 °C, for 20 h, followed by reduction with $NaBD_4$ and acetylation as above (section 2.5). The residues were converted into partially *O*-methylated alditol acetates and analyzed by GC-MS using a Varian (model 4000) gas chromatograph equipped with VF5 capillary columns. Helium was used as the carrier gas at a flow rate of 1.0 mL min^{-1}. Partially *O*-methylated alditol acetates were identified by the ion *m/z* by comparing their positive ions with standards. The results are expressed as a relative percentage of each component (Sassaki et al., 2005).

2.5 Controlled Smith degradation of the β-glucans

An aliquot of each purified β-D-glucan (50 mg) obtained from *P.nameko* and *P. pulmonarius* was submitted to 0.05 M aq. $NaIO_4$ (20 mL) for 72 h at 25 °C in the dark with stirring using magnetic bar (Delgobo et al., 1998). The oxidation process was stopped with ethylene glycol (2 mL, 30 minutes) and each sample was then dialyzed against tap water for 12 h, partially evaporated (5 mL), subsequently reduced with $NaBH_4$ until pH 9~10 and kept overnight at room temperature, followed by neutralization with acetic acid, dialysis (2 kDa) for 24 h and lyophilization. The products of the respective polyalcohols were hydrolyzed with TFA 1 M (4.0 mL) for 30 min at 100 °C and dialyzed (2 kDa) against tap water for 12 h, evaporated to a small volume and lyophilized. The residual polysaccharide was analyzed by NMR spectroscopy (Ruthes et al., 2015).

2.6 Molar Mass determination

The average molar mass (Mw_{app}) was determined by static light scattering (SLS) without any fractioning by size exclusion chromatography. The following equation (Eq. 1) was used to determine the *Mw*.

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

Where I_R (q,c) is the excess Rayeigh scattering in cm^{-1} at specific scattering vector (q), c is the concentration in 0.5 x 10^{-3} g/mL and K*c* is the optical constant obtained for a vertically polarized light, as Eq. 2:

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

The *n* is the DMSO refraction index (1.4768) at 25 °C, λ is the wavelenght (632.8) and the *dn*/*dc* is the differential refractive index for the β-glucan in DMSO as previously published by (de Jesus et al., 2018a).

The samples were dispersed during 48 hours, sonified during 3 min to ensure complete solubilization.

The dynamic light scattering experiments (DLS) were performed in NanoDLS Brookhaven at 90° detection and it was used for intensity autocorrelation computation and z-average equivalent sphere hydrodynamic radius (R_h).

2.7 Nuclear Magnetic Resonance (NMR) spectroscopy

Using a 400-MHz Bruker Avance III spectrometer, the mono- (^{13}C) and bidimensional (HSQC) NMR spectra were obtained. The samples were dissolved in Me_2SO-d_6 and the analyzes were performed at 70 °C. The chemical shifts are expressed in ppm (δ) at δ 39.7 and δ 2.5 for ^{13}C and ^{1}H signals, respectively.

3.0 Results and Discussion

3.1 Crude fractions obtained by autoclave extraction

The residual fractions from polysaccharides *P. nameko* and *P. pulmonarius* were extracted separately by a single autoclave process in aqueous and after in alkaline medium, resulting in four different fractions: Pn-1 (3.8 %, 3.9 g) and Pp-1 (4.8 %, 2.0 g) – from aqueous extract in an autoclave – and Pp-KOH (8.5 g, 21.4 %) and Pn-KOH (17.8 g, 18.3 %) – from alkaline extract in an autoclave. The crude amount obtained in each extraction was significantly (in percentage %) when compared to those previously performed (Figure 3), precisely because the residual samples were submitted to a single extraction process in each condition, while the other extractions (cold water extraction, hot water extraction and alkaline extraction) were performed exhaustively. In addition, it is interesting to note that the autoclave extraction processes follow a pattern in relation to the amount of material obtained, with less amount in the aqueous extract (4.8 % and 3.8 %) and a higher in the alkaline medium (21.4 % and 18.0 %).

The autoclave extracts showed mainly D-glucans in its composition (Figure 4, 5, 8 and 9), whereas in the other crude amounts, the heteropolysaccharide mannogalactan (Smiderle et al., 2008; Sovrani et al., 2017) and β-D-glucans (Sovrani et al., 2017; Abreu et al.,2019; Smiderle et al., 2008) were observed, which suggests that these polysaccharides are easily extracted and that the conditions of high temperature and pressure facilitate the extractions of D-glucans (Morales et al., 2016). In the present work, the alkaline extract from *P. pulmonarius* and the aqueous extract from *P. nameko* were purified and characterized separately and are represented in the following items (section 3.2, 3.3, 3.4 and 3.5).

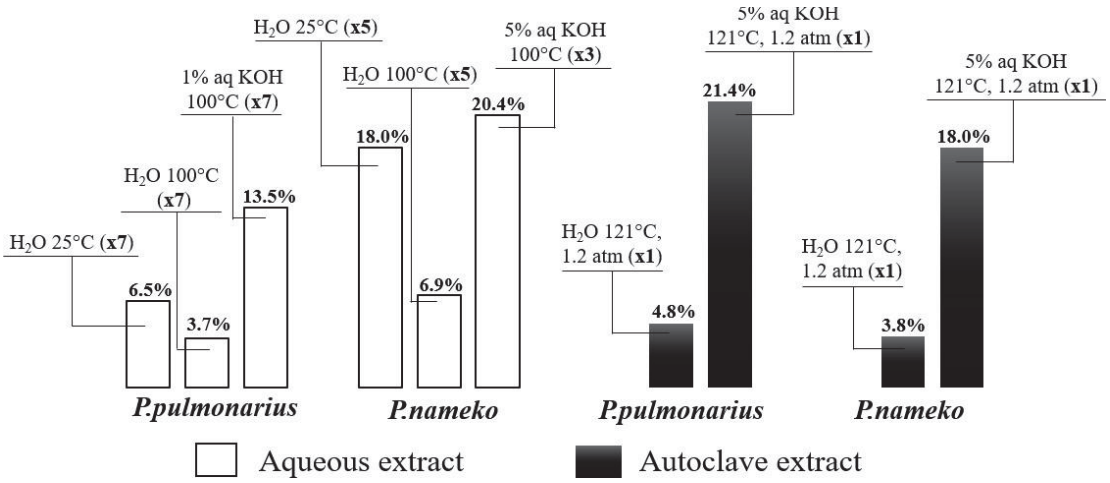


Figure 3. Crude amount obtained in different extractions performed in the mushrooms *P. nameko* and *P. pulmonarius*.

3.2 Purification process from *Pleurotus pulmonarius*

The fraction generated by high temperature and pressure extraction named Pp-1 (2.0 g, 4.8 %) went through NMR analysis (figure 4) and showed a heterogeneous material.

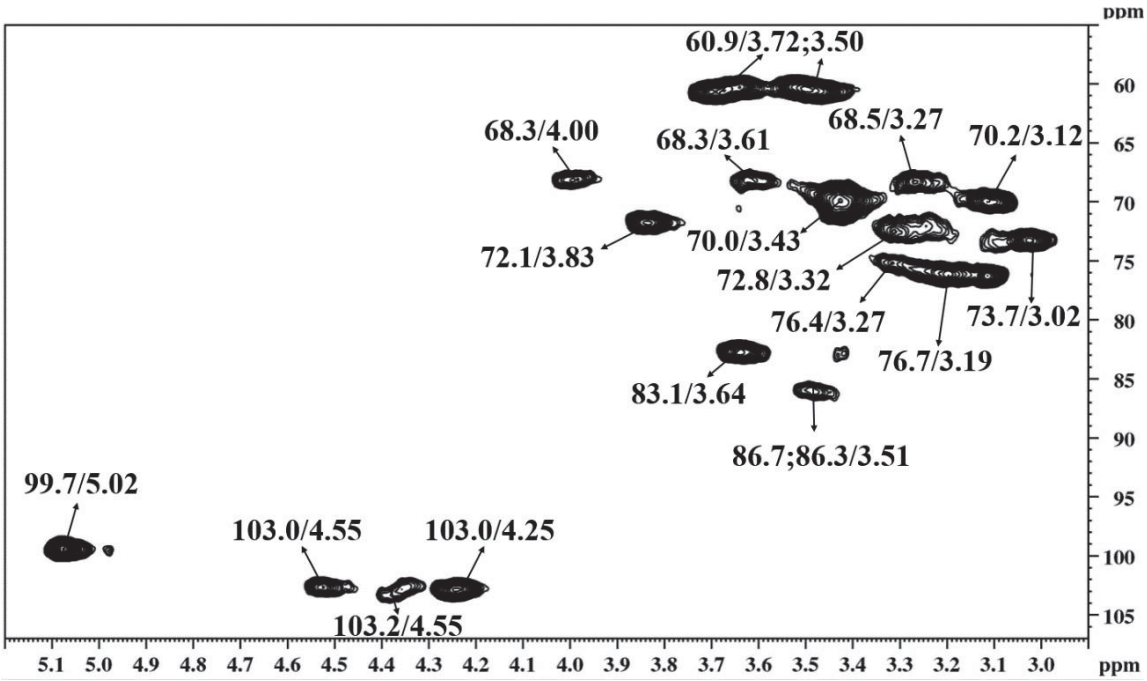


Figure 4. HSQC spectra of the Pp-1 fraction obtained from *Pleurotus pulmonarius*. The sample was analyzed in Me_2SO-d_6 at 70 °C (chemical shifts are expressed in δ ppm).

Three signs can be observed in the anomeric regions compatible with β-D-glucans at δ 103.0/4.55, 103.2/4.37 and 103.0/4.25. In the linkage region the signals at δ 86.7/3.51

and 86.3/3.51 corresponding to β-(1→3)-linked and δ 68.3/4.00 and 68.3/3.61 ppm for (1→6)-linked demonstrating the presence of a ramified polysaccharide β-D-glucan-(1→3),(1→6)–linked. A α-D-glucan can also been observed in the anomeric region at δ 99.7/5.02 and in the linkage region at 83.1/3.64 ppm for α-(1→3)-linked polymer. At δ 60.9/3.72 and 60.9/3.50 ppm signals to the non-reducing terminal units of D-glucans are represented. Then, this spectrum indicates that this fraction (Pp-1) is composed of distinct polysaccharides with a high percentage of α-D-glucan-(1→3)-linked and in low percentage a (1→3),(1→6)-linked β-D-glucan.

The sequential extraction performed in alkaline medium (KOH 5 %) originated the fraction Pp-KOH (8.5 g, 21.4 %), which gave a considerable amount of material extracted when compared to the extractions previously performed (Figure 3). The HSQC spectra showed a predominance of signals in the anomeric region compatible with β-glucans at δ 103.0/4.55, 103.1/4.55 and 103.0/4.25 (Figure 5).

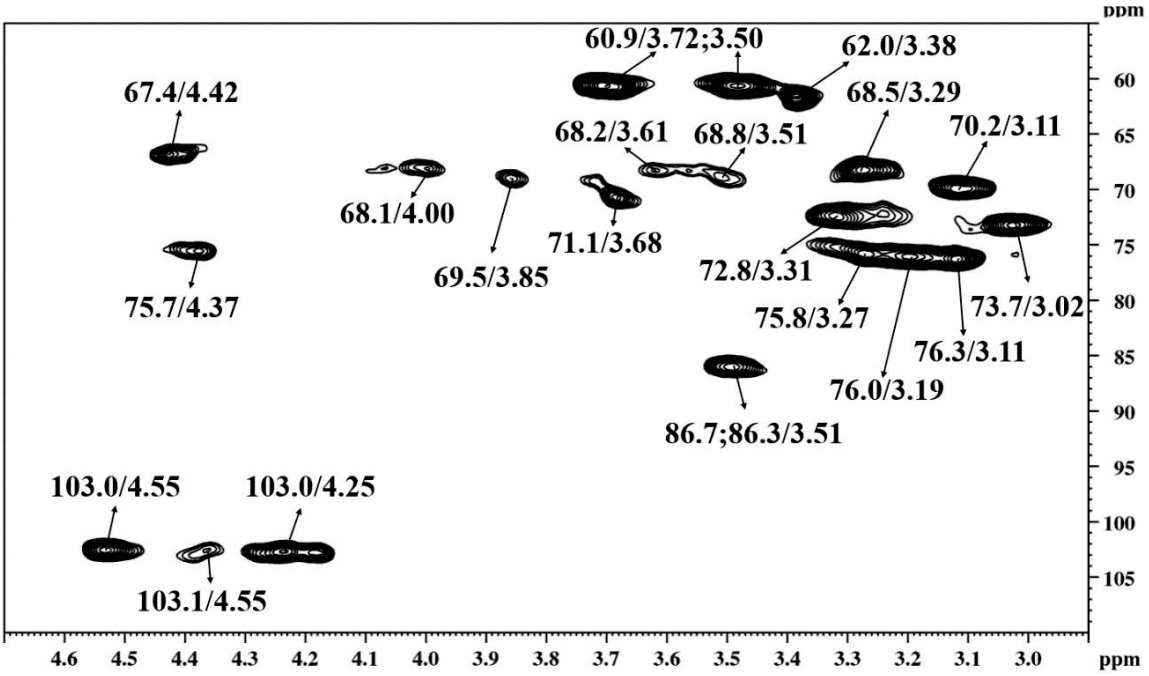
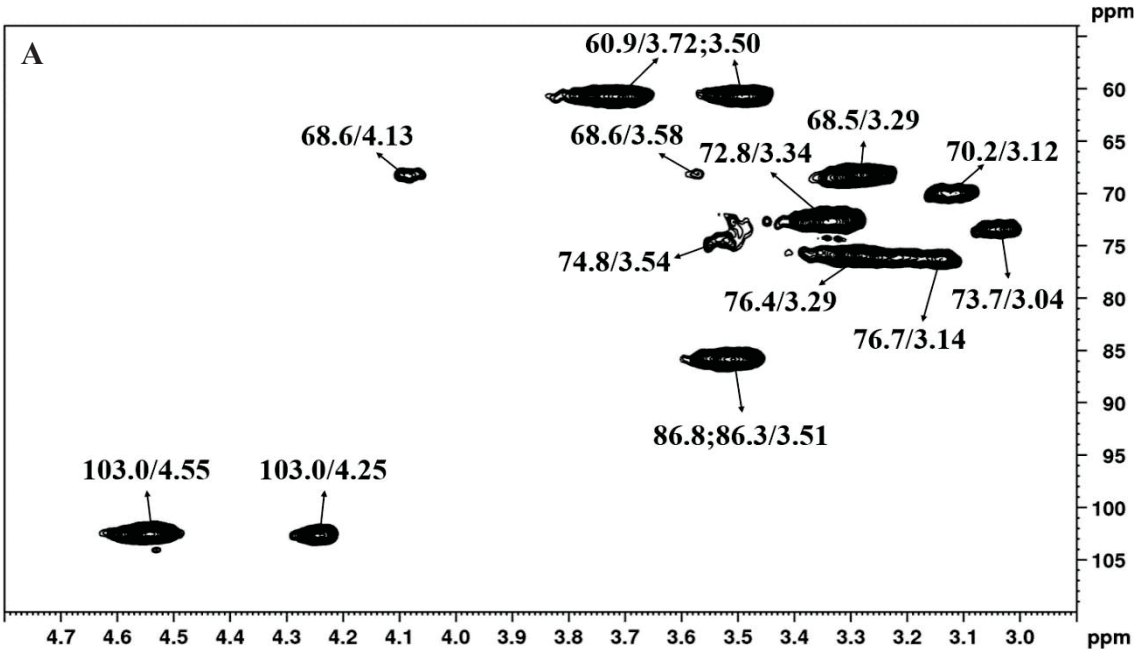


Figure 5. HSQC spectra of the Pp-KOH fraction obtained from *Pleurotus pulmonarius*. The sample was analyzed in Me_2SO-d_6 at 70 °C (chemical shifts are expressed in δ ppm).

The material was dissolved in a small volume of water and went through ethanolic precipitation by addition to excess ethanol (3:1, v/v) and centrifuged for 20 min (10000 rpm, 25 °C). The precipitate fraction (5.5 g, 13.5 %) was subjected to solubilization in a small volume of water followed by freezing and slowly thaw (3 x), giving rise the soluble and insoluble fraction, named Pp-GLC (3.5 g, 8.6 %), discussed in the item 3.3.

3.3 Chemical structure of the (1→3),(1→6) β-D-glucan from *Pleurotus pulmonarius*

After acid hydrolysis it has been shown to be composed exclusively of monosaccharide glucose with *Mw* 13.5±2.4 x 10^6 g.mol^{-1}. As the increase in the molar mass appear to be significant, it is possible that some aggregates are present in solution. Unfortunately, heating of sonification as not significant difference in the results. So, the differences in the molar mass could be associated to an intramolecular interaction of the glucan in DMSO. Using the Stokes-Einstein equation the R_h was determined in DMSO as 126 nm (relative variation of 0.635). The NMR studies proved that the structure is a (1→3),(1→6) β-D-glucan precisely because of the signs obtained by ^{13}C NMR and HSQC analysis (Fig 6 A and B) where the β-configuration at the anomeric region was confirmed by signals in δ 103.0/4.55 and 103.0/4.25 ppms by the units of β-Glc*p*. In the linkage regions, the presence of β-D-Glc*p*-(1→3)-linked was confirmed by signal in δ 86.8/3.51 and 86.3/3.51 ppm and the ramifications β-D-Glc*p*-(1→6)-linked may be observed with corresponded signals of the *O*-6 substitution at 68.6/4.13 and 68.6/3.58, respectively, and free *O*-6 non-reducing end units are indicated at 60.9/3.72 and 60.9/3.50 ppm (Table 1).



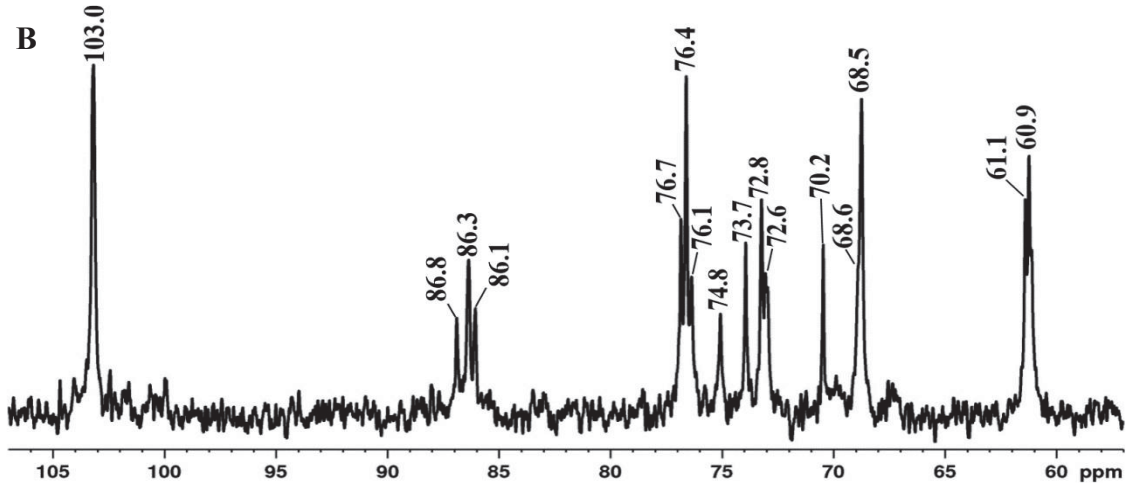


Figure 6. HSQC (A) and ^{13}C NMR (B) of the β-D-glucan obtained from *Pleurotus pulmonarius* (Pp-GLC). The sample was analyzed in Me_2SO-d_6 at 70 °C (chemical shifts are expressed in δ ppm).

To confirm the polysaccharide main chain, a sequenced Smith degradation was developed (Figure 7). Six signals at δ 103.0; 86.7; 76.4; 72.8; 68.5 and 60.9 are present and represent C-1, C-3, C-5, C-2, C-4 and C-6, respectively. Such signs indicate the presence of a linear (1→3)-linked β-D-Glc*p* chain, clarifying the structure of the polysaccharide with a linear main chain β-(1→3)-linked with β-(1→6)-linked branches (Santos-Neves et al., 2008).

The analysis of methylated derivatives by GC-MS of the Pp-GLC fraction (Table 2) proves the results obtained by NMR studies, demonstrating that the main chain is composed of (1→3)-linked β-D-Glc*p* units substituted at *O*-6 by (1→6)-linked β-D-Glc*p* based on their percentage of 2,3,4,6-tetra-*O*-methyl-Glc*p* (21 %), 2,4,6-tri-*O*-methyl-Glc*p*- (52 %), 2,3,4-di-*O*-metil-Glc*p* (8 %) and 2,4-di-*O*-metil-Glc*p* (19 %).

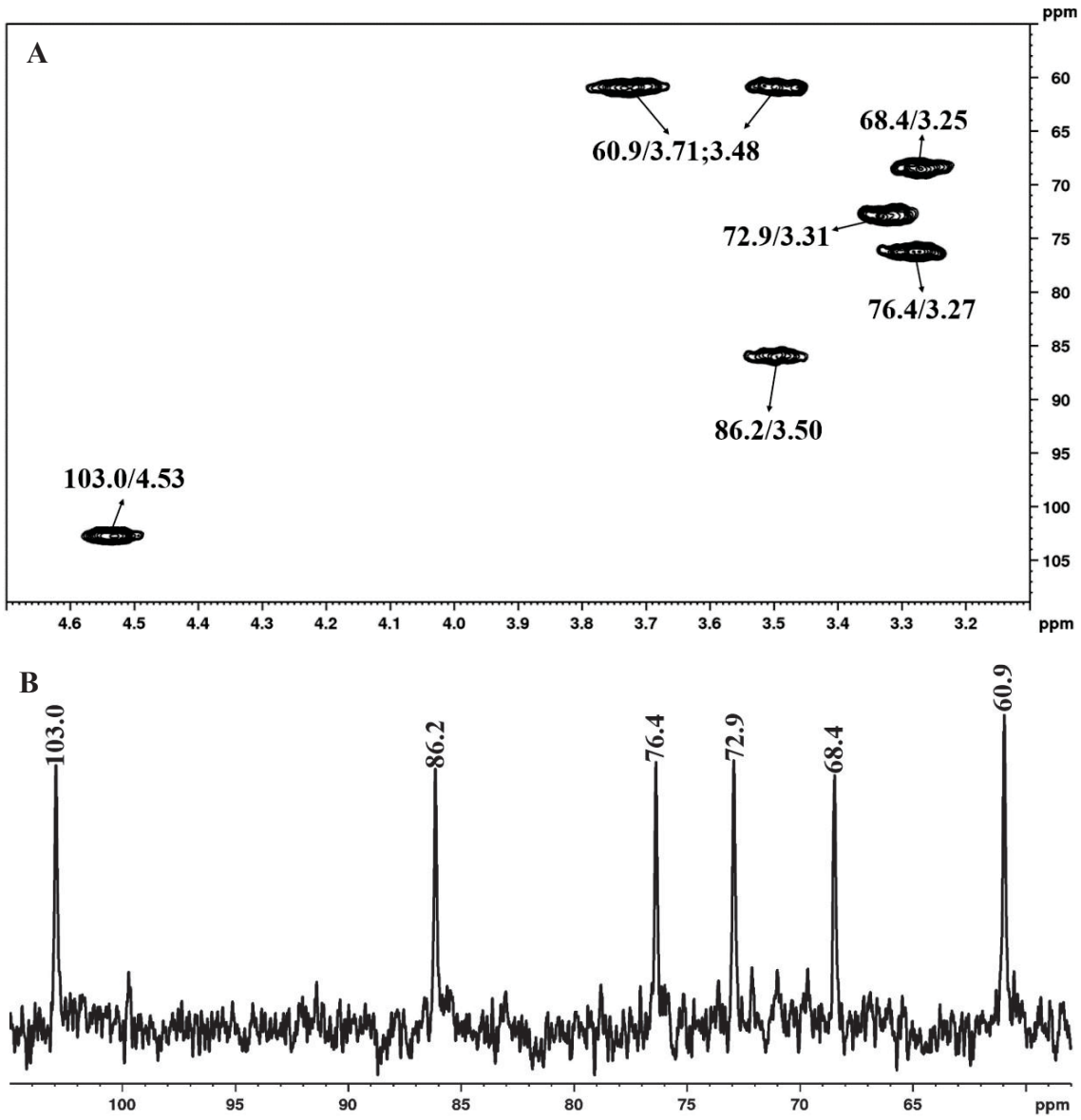


Figure 7. HSQC (A) and ^{13}C NMR spectra (B) of the controlled Smith degradation of the (1→3),(1→6) β-D-glucan obtained from *Pleurotus pulmonarius* (Pp-Sm). The sample was analyzed in Me_2SO-d_6 at 70 °C (chemical shifts are expressed in δ ppm).

Table 1. ^{1}H and ^{13}C NMR chemical shift[a] of the fractions Pn-GLC[b] and Pn-Sm[c] from *Pholiota nameko* and Pp-GLC[d] and Pp-Sm[e] from *Pleurotus pulmonarius*

	Units		1	2	3	4	5	6 a	6 b
Pn-GLC[b]	β-D-Glc-(1→	^{13}C	103.0	73.7	76.7	70.2	76.4	60.9	60.9
		^{1}H	4.25	3.04	3.14	3.12	3.29	3.72	3.50
	3→)-β-D-Glc-(1→	^{13}C	103.0	72.8	86.7/86.3	68.5	76.4	60.9	60.9
		^{1}H	4.55	3.34	3.51	3.29	3.29	3.72	3.50
	3,6→)-β-D-Glc-(1→	^{13}C	103.0	72.8	86.1	68.5	74.8	68.6	68.6
		^{1}H	4.55	3.34	3.51	3.29	3.54	4.13	3.58
Pn-Sm[c]	3→)-β-D-Glc-(1→	^{13}C	103.0	72.9	86.2	68.4	76.4	60.9	60.9
		^{1}H	4.53	3.31	3.49	3.25	3.27	3.71	3.48
Pp-GLC[d]	β-D-Glc-(1→	^{13}C	103.0	73.7	76.7	70.2	76.4	60.9	60.9
		^{1}H	4.25	3.04	3.14	3.12	3.29	3.72	3.50
	3→)-β-D-Glc-(1→	^{13}C	103.0	72.8	86.7/86.3	68.5	76.4	60.9	60.9
		^{1}H	4.55	3.34	3.51	3.29	3.29	3.72	3.50
	β-D-Glc-(1→	^{13}C	103.0	72.8	86.1	68.5	74.8	68.6	68.6
		^{1}H	4.55	3.34	3.51	3.29	3.54	4.13	3.58
Pp-Sm[e]	3→)-β-D-Glc-(1→	^{13}C	103.0	72.9	86.2	68.4	76.4	60.9	60.9
		^{1}H	4.53	3.31	3.49	3.25	3.27	3.71	3.48

[a]Assignments are based on ^{13}C NMR, ^{1}H and HSQC analysis and are expressed as ppm; [b](1→3),(1→6) β-D-glucan from *P. nameko*; [c]Smith degradation glucan from *P. nameko*; [d](1→3),(1→6) β-D-glucan from *P. pulmonarius*; [e] Smith degradation glucan from *P. pulmonarius*.

Table 2

Partially *O*-methylated acetates present on D-glucans (Pp-GLC and Pn-GLC) obtained from *Pholiota nameko* and *Pleurotus pulmonarius,* respectively: linkage types.

Partially *O*-methylated alditol acetates[a]	*Rt*[b]	% Area of fragments[c]		Linkage types
		Pp-GLC[d]	Pn-GLC[e]	
2,3,4,6-Me_4-Glc	14.412	21	16	Glc*p*-(1→
2,4,6-Me_3-Glc	15.427	52	36	3→)-Glc*p*-(1→
2,3,4-Me_3-Glc	15.805	8	30	6→)-Glc*p*-(1→
2,4-Me_2-Glc	17.123	19	18	3,6→)-Glc*p*-(1→

[a]GC-MS analsysis on a Varian model 4000 capillary column; [b]Retention time (min); [c]Based on derived O-methyalditol acetates; [d]Pp-GLC: β-D-glucan-(1→3),(1→6) from *P. pulmonarius*; [e]Pn-GLC: β-D-glucan-(1→3),(1→6) from *P. nameko*.

3.4 Purification process from *Pholiota nameko*

The lyophilized residue (102.4 g) was dissolved in 3 L and submitted to high temperature and pressure extraction (121 °C, 1.2 atm, 1 h) using an autoclave, generating the fraction Pn-1 (3.9 g, 3.8 %). HSQC analysis showed signals compatible with β-glucans at δ 103.0/4.55, 103.2/4.35 and 103.0/4.25 and for α at δ 99.7/ 5.02 in the anomeric region, and signs in the linkage region at δ 86.7;86.3/3.51 and δ 83.1/3.64 corresponding to the β-(1→3)-linked and α-(1→3)-linked, respectively (Fig. 8 A and B), very similar to those obseved in the *P. pulmonarius* mushroom by the same extraction process (Figure 4).

The material went through ethanolic precipitation by addition to excess ethanol (3:1, v/v) and centrifuged. The precipitate fraction Pn-2 (2.4 g, 2.7 %) was subjected to solubilization in a small volume of water followed by freezing and slowly thaw (3 x), giving rise the soluble and insoluble fraction. The soluble fraction went through ultrafiltration (3 kDa), generating the eluted and the retained fraction, named Pn-GLC (1.2 g, 1.2 %), discussed in the item 3.5.

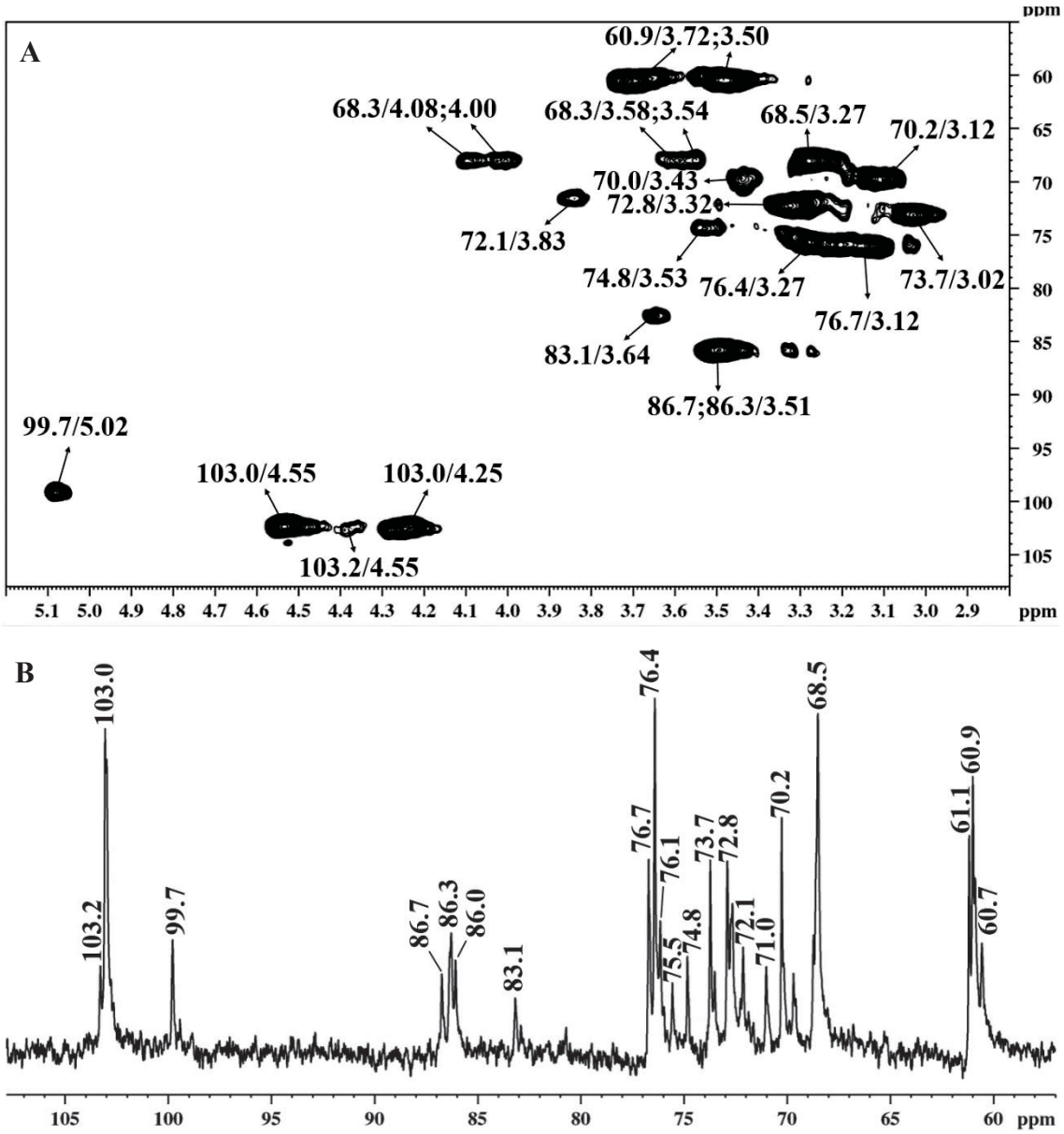


Figure 8. HSQC (A) and ^{13}C NMR (B) spectra of the Pn-1 fraction from *Pholiota nameko*. The sample was analyzed in Me_2SO-d_6 at 70 °C (chemical shifts are expressed in δ ppm).

In order to evaluate the yield and the content of the crude fraction, a new autoclave extraction in alkaline medium (KOH 5 %) was performed in the residue obtained after the extraction previously described, obtaining a significant amount of material (17.8 g, 18 %) with a HSQC spectrum very similar (Figure 9) to that seen previously (Figure 8). It is interesting to note the presence of the signal in the anomeric region in δ 101.7/4.95, which indicate the presence of a β-D-mannose, a polysaccharide not yet obtained from this type of extraction.

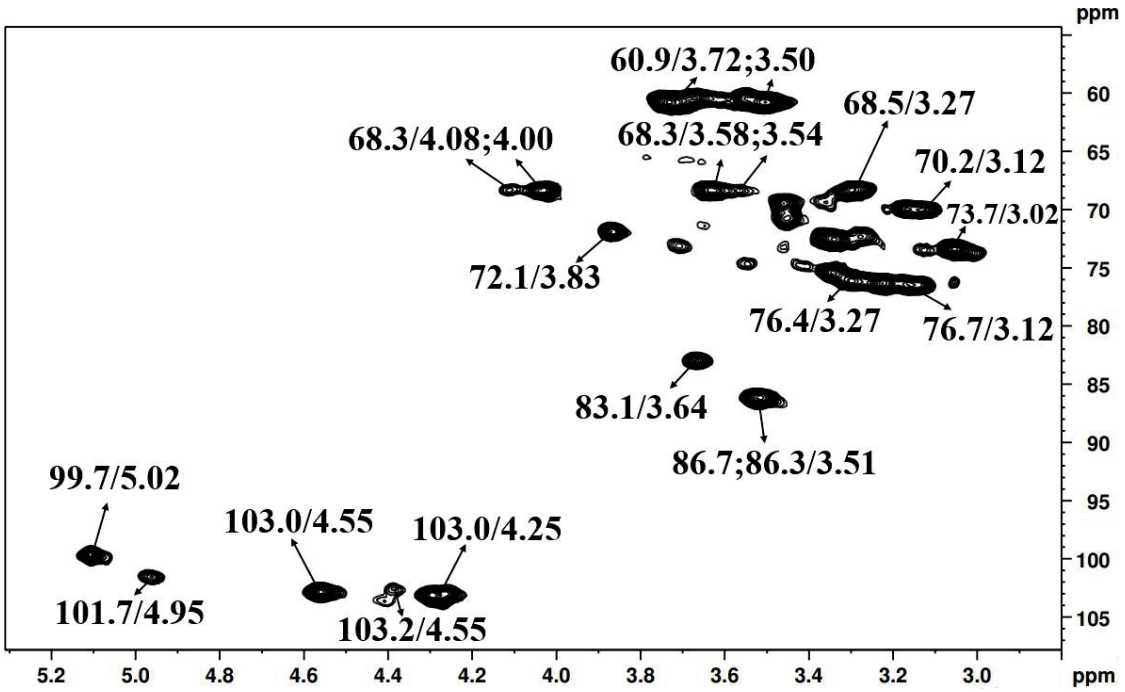


Figure 9. HSQC spectra of the Pn-KOH fraction from *Pholiota nameko*. The sample was analyzed in Me_2SO-d_6 at 70 °C (chemical shifts are expressed in δ ppm).

3.5 Chemical structure of the D-glucan from *Pholiota nameko*

The fraction Pn-GLC presented M*w* 1.5±0.2 x 10^6 g/mol^{-1} with R_h determined in DMSO as 65.4 nm (relative variation of 0.358) using the Stokes-Einstein equation and the monosaccharide composition analysis showed exclusively glucose in its composition. Through NMR studies, the fraction Pn-GLC proved to be a (1→3),(1→6) β-D-glucan precisely because of the signals obtained by HSQC and ^{13}C NMR analysis (Fig 10 A and B) In the anomeric region, at δ 102.7/4.54 and 102.7/4.23 ppms, and in the linkage region, at δ 86.0/3.50, confirming the presence of β-D-Glc*p*-(1→3)-linked, and at 68.1/4.09 and 68.1/3.55, indicating the ramifications at *O*-6 of β-D-Glc*p*-(1→6)-linked, while the free *O*-6 non-reducing end units can be observed in 60.8/3.71 and 60.8/3.48 ppms.

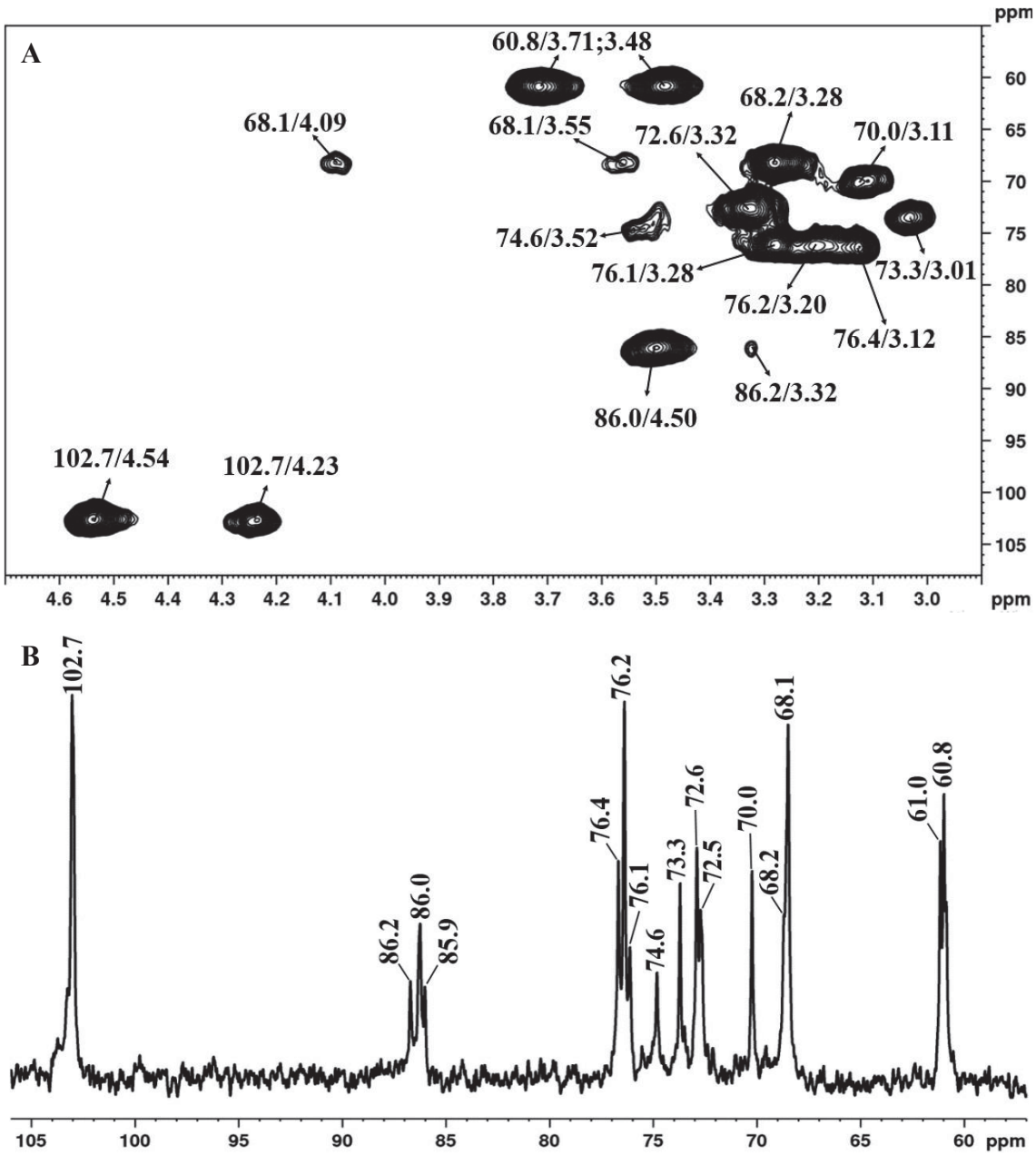


Figure 10. HSQC (A) and ^{13}C NMR spectra (B) of the (1→3),(1→6) β-D-glucan obtained from *Pholiota nameko* (Pn-GLC). The sample was analyzed in Me_2SO-d_6 at 70 °C (chemical shifts are expressed in δ ppm).

A sequenced Smith degradation was developed (Figure 11) and showed six signals at δ 103.0; 86.7; 76.4; 72.8; 68.5 and 60.9 which represent C-1, C-3, C-5, C-2, C-4 and C-6, respectively, indicating that the main chain is composed by a linear β-(1→3)-linked glucan (Santos-Neves et al., 2008) and the methylation analysis confirmed these results with the presence of 2,3,4,6-tetra-*O*-Methyl-Glc*p* (16 %), 2,4,6-tri-*O*-methyl-Glc*p*- (36 %), 2,3,4-tri-*O*-metil-Glc*p* (30 %) and 2,4-di-*O*-metil-Glc*p* (18 %) (Table 2).

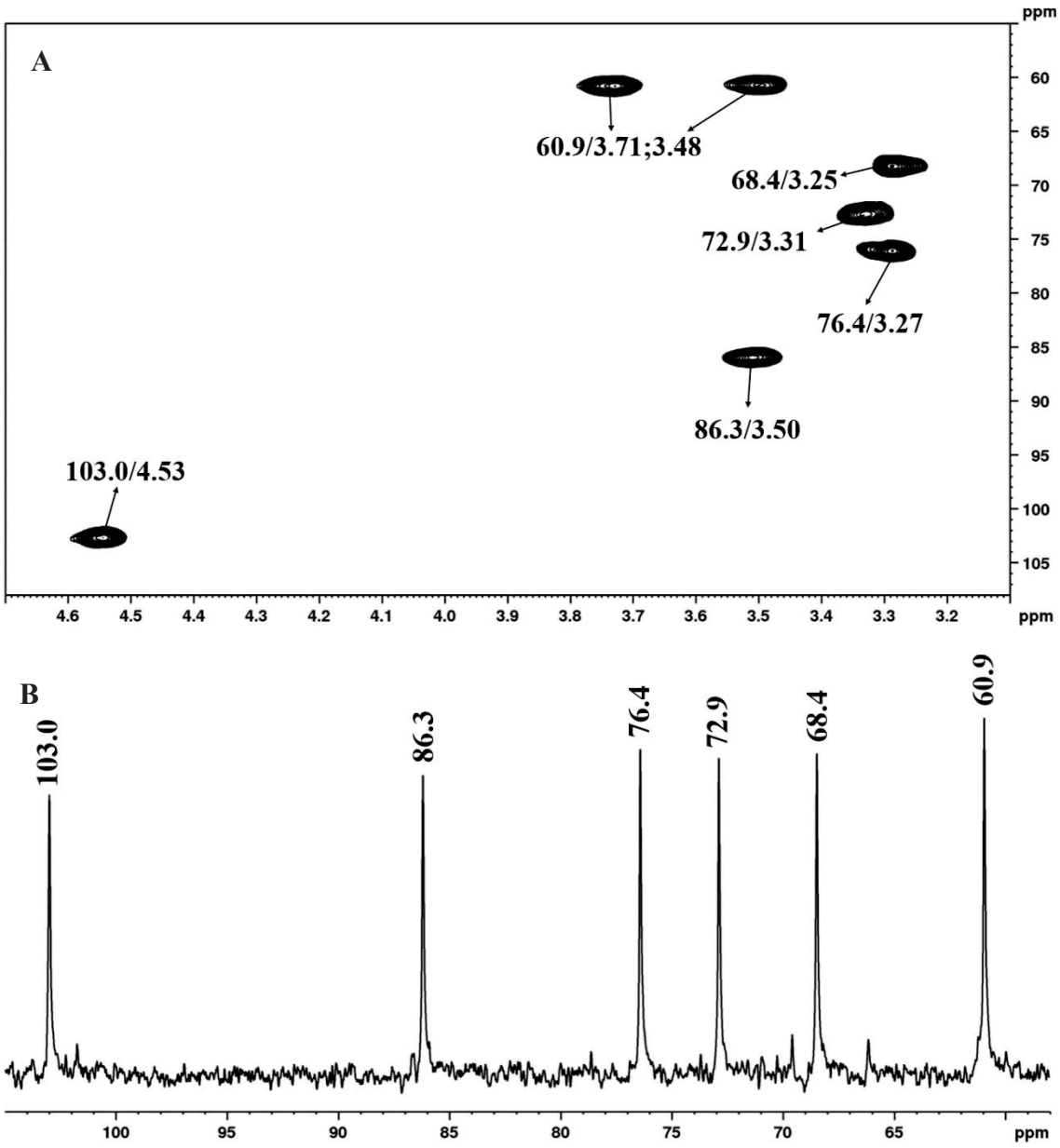


Figure 11. HSQC (A) and ^{13}C NMR spectra (B) of the controlled Smith degradation of the (1→3),(1→6) β-D-glucan obtained from *Pholiota nameko* (Pn-Sm). The sample was analyzed in Me_2SO-d_6 at 70 °C (chemical shifts are expressed in δ ppm).

Even having a similar main chain composed by β-(1→3)-linked glucans substituted at *O*-3, the β-D-glucans obtained in the present work showed significantly differences. *P. pulmonarius* (1→3),(1→6) β-D-glucan presented shorter side chains composed by β-(1→6)-linked, when compared to the (1→3),(1→6) β-D-glucan obtained from *P. nameko*, which can explain the difference observed on solubility among the fractions, showing that the insoluble β-D-glucan is less branched that the soluble one. Besides that, the purified polysaccharides showed different molecular weights.

Conclusions

Autoclave extraction proved to be an effective method to obtaining D-glucans, even when carried out in extraction residues. The literature shows that polysaccharides obtained from mushrooms as (1→3),(1→6) β-D-glucans (Carbonero et al., 2012; Ruthes et al., 2013), (1→6) β-D-glucans (Smiderle et al., 2013), (1→3) α-D-glucans (Synytsya et al., 2009; de Jesus et al., 2018a) and heteropolysaccharides (Yan et al., 2019; Pramanik et al., 2005) were obtained from materials not yet used and/or little used for this purpose, which suggests that the available (discarded) residues obtained after sequentially extractions still have large amounts of important polysaccharides.

The significant amount of D-glucans obtained by autoclave extraction from these materials indicate that the other extractions processes (cold aqueous extraction, hot water extraction and alkaline extraction), usually developed sequentially and exhaustively, are not as effective for the total extraction of these polysaccharides, and the remainder are extracted only with more strongly methods, such as autoclave extraction.

The crude materials obtained in aqueous and alkaline fractions from autoclave process showed different solubilities in water, which resulted in different purification processes. The aqueous extracts from autoclave are soluble in water and have in their composition (1→3),(1→6) β-D-glucans, (1→6) β-D-glucans and (1→3) α-D-glucans, while the alkaline extracts from autoclave are insoluble in water and presented mostly (1→3),(1→6) β-D-glucans with higher molecular weight, which probably explains the large amount of material obtained, in both fractions, in a single autoclave extraction in alkaline medium.

The β-D-glucans of *P. pulmonarius* and *P. nameko* have the same type of main chain - β-(1→3)-linked – but differ in degree of branching at *O*-3 of β-(1→6)-linked and molecular weight.

These results are important and could be developed in future extractions with other residual materials obtained not only from mushrooms, but also with other materials from other sources that have used the same extraction processes. Besides that, the purification of the other D-glucans observed in the autoclave extracts should be evaluated.

Acknowledgements

The authors would like to thank the Brazilian funding agencies: CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) and CNPq (Conselho Nacional de

Desenvolvimento Científico e Tecnológico). The authors have declared no conflicts of interest.

References

Abreu, H., Simas, F. F., Smiderle, F. R., Sovrani, V., Dallazen, J. L., Maria-Ferreira, D., Werner, M. F., Cordeiro, L. M. C., & Iacomini, M. (2019). Gelling functional property, anti-inflammatory and antinociceptive bioactivities of β-D-glucan from the edible mushroom *Pholiota nameko*. *International Journal of Biological Macromolecules*, 122, 1128-1135. https://doi.org/10.1016/j.ijbiomac.2018.09.062

Alexopoulos, C. J., Mims, C. W., & Blackwell, M. (1996). Introductory Mycology. 4th Edition. *Wiley, New York.* https://doi.org/10.2105/AJPH.43.6_Pt_1.781-a

Baggio, C. H., Freitas, C. S., Marcon, R., Werner, M. F. de P., Rae, G. A., Smiderle, F. R., Sassaki, G. L., Iacomini, M., Marques, M. C. A., & Santos, A. R. S. (2012). Antinociception of β-D-glucan from *Pleurotus pulmonarius* is possibly related to protein kinase C inhibition. *International Journal of Biological Macromolecules*, 50(3), 872–877. https://doi.org/10.1016/j.ijbiomac.2011.10.023

Baggio, C. H., Freitas, C. S., Martins, D. F., Mazzardo, L., Smiderle, F. R., Sassaki, G. L., Iacomini, M., Marques, M. C. A., & Santos, A. R. S. (2010). 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. *Journal of Pain*, 11(10), 965-971. https://doi.org/10.1016/j.jpain.2010.01.005

Bhanja, S. K., Rout, D., Patra, P., Sen, I. K., Nandan, C. K., & Islam, S. S. (2014). Water-insoluble glucans from the edible fungus *Ramaria botrytis*. *Bioactive Carbohydrates and Dietary Fibre*, 3(2), 52-58. https://doi.org/10.1016/j.bcdf.2014.01.004

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, 72(1-2), 248-254. https://doi.org/10.1016/0003-2697(76)90527-3

Carbonero, E. R., Ruthes, A. C., Freitas, C. S., Utrilla, P., Gálvez, J., Silva, E. V. Da, Sassaki, G. L., Gorin, P. A. J., & Iacomini, M. (2012). Chemical and biological

properties of a highly branched β-glucan from edible mushroom *Pleurotus sajor-caju*. *Carbohydrate Polymers*, 90, 814-819. https://doi.org/10.1016/j.carbpol.2012.06.005

Chen, J., & Seviour, R. (2007). Medicinal importance of fungal β-(1→3), (1→6)-glucans. *Mycological Research*, 111(6), 635-652. https://doi.org/10.1016/j.mycres.2007.02.011

Ciucanu, I., & Kerek, F. (1984). A simple and rapid method for the permethylation of carbohydrates. *Carbohydrate Research*, 131(2), 209-217 https://doi.org/10.1016/0008-6215(84)85242-8

Dalonso, N., Goldman, G. H., & Gern, R. M. M. (2015). β-(1→3),(1→6)-Glucans: medicinal activities, characterization, biosynthesis and new horizons. *Applied Microbiology and Biotechnology*, 99, 7893-7906. https://doi.org/10.1007/s00253-015-6849-x

de Jesus, L. I., Smiderle, F. R., Cordeiro, L. M. C., de Freitas, R. A., Van Griensven, L. J. L. D., & Iacomini, M. (2018). Simple and effective purification approach to dissociate mixed water-insoluble α- and β-D-glucans and its application on the medicinal mushroom *Fomitopsis betulina*. *Carbohydrate Polymers*, *200*, 353–360. https://doi.org/10.1016/j.carbpol.2018.08.004

de Jesus, L. I., Smiderle, F. R., Ruthes, A. C., Vilaplana, F., Dal'Lin, F. T., Maria-Ferreira, D., Werner, M. F., Van Griensven, L. J. L. D., & Iacomini, M. (2018). Chemical characterization and wound healing property of a β-D-glucan from edible mushroom *Piptoporus betulinus*. *International Journal of Biological Macromolecules*, 177, 1361-1366. https://doi.org/10.1016/j.ijbiomac.2017.12.107

Delgobo, C. L., Gorin, P. A., Jones, C., & Iacomini, M. (1998). Gum heteropolysaccharide and free reducing mono- and oligosaccharides of *Anadenanthera colubrina*. *Phytochemistry*, 47(7), 1207-1214. https://doi.org/10.1016/S0031-9422(97)00776-0

El Enshasy, H. A., & Hatti-Kaul, R. (2013). Mushroom immunomodulators: Unique molecules with unlimited applications. *Trends in Biotechnology*, 31(12), 668-677. https://doi.org/10.1016/j.tibtech.2013.09.003

Laroche, C., & Michaud, P. (2007). New Developments and Prospective Applications for

β-(1,3) Glucans. *Recent Patents on Biotechnology*, 1(1), 59-73. https://doi.org/10.2174/187220807779813938

Moradali, M. F., Mostafavi, H., Ghods, S., & Hedjaroude, G. A. (2007). Immunomodulating and anticancer agents in the realm of macromycetes fungi (macrofungi). *International Immunopharmacology*, 7(6), 701-724. https://doi.org/10.1016/j.intimp.2007.01.008

Morales, D., Iacomini, M., Gilbert-López, B., Smiderle, F. R., de Jesus, L. I., Soler-Rivas, C., & Gil-Ramírez, A. (2016). Evaluation of microwave-assisted and pressurized liquid extractions to obtain β-D-glucans from mushrooms. *Carbohydrate Polymers*, 156, 165–174. https://doi.org/10.1016/j.carbpol.2016.09.029

Morales, D., Rutckeviski, R., Villalva, M., Abreu, H., Soler-Rivas, C., Santoyo, S., Iacomini, M., & Smiderle, F. R. (2020). Isolation and comparison of α- and β-D-glucans from shiitake mushrooms (*Lentinula edodes*) with different biological activities. *Carbohydrate Polymers*, 229, 115521. https://doi.org/10.1016/j.carbpol.2019.115521

Moreno, R. B., Ruthes, A. C., Baggio, C. H., Vilaplana, F., Komura, D. L., & Iacomini, M. (2016). Structure and antinociceptive effects of β-D-glucans from *Cookeina tricholoma*. *Carbohydrate Polymers*, 141, 220-228. https://doi.org/10.1016/j.carbpol.2016.01.001

Nitschke, J., Modick, H., Busch, E., Von Rekowski, R. W., Altenbach, H. J., & Mölleken, H. (2011). A new colorimetric method to quantify β-1,3-1,6-glucans in comparison with total β-1,3-glucans in edible mushrooms. *Food Chemistry*, 127(2), 791-796. https://doi.org/10.1016/j.foodchem.2010.12.149

Oliveira, K. S. M., Di Bastiani, M., Cordeiro, L. M. C., Costa, M. F., Toledo, K. A., Iacomini, M., Babosa, A. M., Dekker, R. F. H., & Nascimento, V. M. G. (2015). (1→6)- and (1→3)(1→6)-β-glucans from *Lasiodiplodia theobromae* MMBJ: Structural characterization and pro-inflammatory activity. *Carbohydrate Polymers*, 133, 539-546. https://doi.org/10.1016/j.carbpol.2015.07.060

Pramanik, M., Mondal, S., Chakraborty, I., Rout, D., & Islam, S. S. (2005). Structural investigation of a polysaccharide (Fr. II) isolated from the aqueous extract of an edible mushroom, *Pleurotus sajor-caju*. *Carbohydrate Research*, 340, 4, 629- 636.

https://doi.org/10.1016/j.carres.2004.12.032

Ruthes, Andrea C., Komura, D. L., Carbonero, E. R., Sassaki, G. L., Gorin, P. A. J., & Iacomini, M. (2010). Structural characterization of the uncommon polysaccharides obtained from *Peltigera canina* photobiont *Nostoc muscorum*. *Carbohydrate Polymers*, 81(1), 29-34. https://doi.org/10.1016/j.carbpol.2010.01.050

Ruthes, Andrea Caroline, Carbonero, E. R., Córdova, M. M., Baggio, C. H., Santos, A. R. S., Sassaki, G. L., Cipriani, T. R., Gorin, P. A. J., & Iacomini, M. (2013). *Lactarius rufus* (1→3),(1→6)-β-D-glucans: Structure, antinociceptive and anti-inflammatory effects. *Carbohydrate Polymers*, 94(1), 129-136. https://doi.org/10.1016/j.carbpol.2013.01.026

Ruthes, Andrea Caroline, Carbonero, E. R., Córdova, M. M., Baggio, C. H., Sassaki, G. L., Gorin, P. A. J., Santos, A. R. S., & Iacomini, M. (2013). Fucomannogalactan and glucan from mushroom *Amanita muscaria*: Structure and inflammatory pain inhibition. *Carbohydrate Polymers*, 98(1), 761-769. https://doi.org/10.1016/j.carbpol.2013.06.061

Ruthes, Andrea Caroline, Smiderle, F. R., & Iacomini, M. (2015). D-Glucans from edible mushrooms: A review on the extraction, purification and chemical characterization approaches. *Carbohydrate Polymers*, 117, 753-761. https://doi.org/10.1016/j.carbpol.2014.10.051

Santos-Neves, J. C., Pereira, M. I., Carbonero, E. R., Gracher, A. H. P., Alquini, G., Gorin, P. A. J., Sassaki, G. L., & Iacomini, M. (2008). A novel branched αβ-glucan isolated from the basidiocarps of the edible mushroom *Pleurotus florida*. *Carbohydrate Polymers*, 73(2), 309-314. https://doi.org/10.1016/j.carbpol.2007.11.030

Sassaki, G. L., Gorin, P. A. J., Souza, L. M., Czelusniak, P. A., & Iacomini, M. (2005). Rapid synthesis of partially O-methylated alditol acetate standards for GC-MS: Some relative activities of hydroxyl groups of methyl glycopyranosides on Purdie methylation. *Carbohydrate Research*, 340(4), 731-739. https://doi.org/10.1016/j.carres.2005.01.020

Sassaki, G. L., Souza, L. M., Serrato, R. V., Cipriani, T. R., Gorin, P. A. J., & Iacomini, M. (2008). Application of acetate derivatives for gas chromatography-mass

spectrometry: Novel approaches on carbohydrates, lipids and amino acids analysis. *Journal of Chromatography A*, 1208(1–2), 215-222. https://doi.org/10.1016/j.chroma.2008.08.083

Smiderle, F. R., Olsen, L. M., Carbonero, E. R., Marcon, R., Baggio, C. H., Freitas, C. S., Santos, A. R. S., Torri, G., Gorin, P. A. J., & Iacomini, M. (2008). A 3-*O*- methylated mannogalactan from *Pleurotus pulmonarius*: Structure and antinociceptive effect. *Phytochemistry*, 69(15), 2731-2736. https://doi.org/10.1016/j.phytochem.2008.08.006

Smiderle, Fhernanda R., Alquini, G., Tadra-sfeir, M. Z., Iacomini, M., Wichers, H. J., & Van Griensven, L. J. L. D. (2013) *Agaricus bisporus* and *Agaricus brasiliensis* (1 → 6)-β-D-glucans show immunostimulatory activity on human THP-1 derived macrophages. *Carbohydrate Polymers*, 94(1), 91–99. https://doi.org/10.1016/j.carbpol.2012.12.073

Smiderle, Fhernanda R., Carbonero, E. R., Mellinger, C. G., Sassaki, G. L., Gorin, P. A. J., & Iacomini, M. (2006). Structural characterization of a polysaccharide and a β-glucan isolated from the edible mushroom *Flammulina velutipes*. *Phytochemistry*, 67(19), 2189-2196. https://doi.org/10.1016/j.phytochem.2006.06.022

Smiderle, Fhernanda 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. (2008). Anti-inflammatory and analgesic properties in a rodent model of a (1→3),(1→6)-linked β-glucan isolated from *Pleurotus pulmonarius*. *European Journal of Pharmacology*, 597(1-3), 86-91. https://doi.org/10.1016/j.ejphar.2008.08.028

Sovrani, V., de Jesus, L. I., Simas-Tosin, F. F., Smiderle, F. R., & Iacomini, M. (2017). Structural characterization and rheological properties of a gel-like β-D-glucan from *Pholiota nameko*. *Carbohydrate Polymers*, 169, 1-8. https://doi.org/10.1016/j.carbpol.2017.03.093

Synytsya, A., Míčková, K., Jablonský, I., Spěváček, J., Erban, V., Kováříková, E., & Čopíková, J. (2009) Glucans from fruit bodies of cultivated mushrooms *Pleurotus ostreatus* and *Pleurotus eryngii*: Structure and potential prebiotic activity. *Carbohydrate Polymers*, 76(4), 548-556. https://doi.org/10.1016/j.carbpol.2008.11.021

Wasser, S. (2003). Medicinal mushrooms as a source of antitumor and immunomodulating polysaccharides. *Applied Microbiology and Biotechnology*, 60, 258–274. https://doi.org/10.1007/s00253-002-1076-7

Yan, J., Meng, Y., Zhang, M., Zhou, X., Cheng, H., Sun, L., & Zhou, Y. (2019). A 3-*O*-methylated heterogalactan from *Pleurotus eryngii* activates macrophages. *Carbohydrate Polymers*, 206, 706-715. https://doi.org/10.1016/j.carbpol.2018.11.063

Zhang, M., Cui, S. W., Cheung, P. C. K., & Wang, Q. (2007). Antitumor polysaccharides from mushrooms: a review on their isolation process, structural characteristics and antitumor activity. *Trends in Food Science and Technology*, 18(1), 4-19. https://doi.org/10.1016/j.tifs.2006.07.013

Zhang, Y., Liu, Z., Ng, T. B., Chen, Z., Qiao, W., & Liu, F. (2014). Purification and characterization of a novel antitumor protein with antioxidant and deoxyribonuclease activity from edible mushroom *Pholiota nameko*. *Biochimie*, 99, 28-37. https://doi.org/10.1016/j.biochi.2013.10.016

Zhu, F., Du, B., Bian, Z., & Xu, B. (2015). β-Glucans from edible and medicinal mushrooms: Characteristics, physicochemical and biological activities. *Journal of Food Composition and Analysis*, 41, 165-173. https://doi.org/10.1016/j.jfca.2015.01.019

CONCLUSÕES

- Dois polissacarídeos distintos, uma α-D-galactana e uma β-D-glucana, foram extraídos a partir do extrato aquoso frio do cogumelo *Amanita muscaria* (L.:Fr), purificados por diferentes metodologias e caracterizados por Ressonância magnética nuclear (RMN), Cromatografia de exclusão estérica de alta pressão (HPSEC) e Cromatografia gasosa acoplada à espectrometria de massas (CG-EM). O primeiro é uma α-D-galactana ainda não reportada na literatura, composta de cadeia principal (1→6)-ligada e substituições em *O*-2 por terminais não redutores de α-D-Gal*p*. A mesma é solúvel em água, não apresenta grupo metil e tem peso molecular 9,08 x 10^3 g.mol^{-1}. O segundo, uma β-D-glucana (1→3),(1→6)-ligada com peso molecular 1,3 x 10^5 g.mol^{-1}, apresenta cadeia principal (1→3)-ligada com substituições em *O*-6 por cadeias laterais (1→6)-ligadas e terminais não redutores de β-D-Glc*p*.
- Métodos de extração em altas temperatura e pressão foram aplicados em resíduos de extração dos cogumelos *Pleurotus pulmonarius* e *Pholiota nameko* e provaram ser eficientes na obtenção de D-glucanas. Além disso, os rendimentos brutos obtidos foram percentualmente significativos quando comparado às extrações realizadas previamente (extração aquosa fria, extração aquosa quente, extração aquosa alcalina).
- Duas β-D-glucanas (1→3),(1→6)-ligadas com diferentes solubilidade e características estruturais foram extraídas, separadamente, em condições de altas temperatura e pressão (autoclave) a partir de materiais residuais finais que já haviam sofrido diversas extrações sequencias dos cogumelos *Pleurotus pulmonarius* e *Pholiota nameko*. Ambas foram purificadas por diferentes metodologias e caracterizadas por Ressonância magnética nuclear (RMN), Cromatografia de exclusão estérica de alta pressão (HPSEC), Cromatografia gasosa acoplada à espectrometria de massa (CG-EM) e por Espalhamento de luz estático (SLS). Suas cadeias principais são similares, compostas de unidades (1→3)-ligadas de β-D-Glc*p*, confirmada pela degradação controlada de Smith, com substituições em *O*-6, e diferem em peso molecular, solubidade em água e no tamanho das cadeiais laterais (1→6)-ligadas.

REFERÊNCIAS

ABREU, H., Caracterização química de polissacarídeos isolados de cogumelos comestíveis, avaliação de suas atividades biológicas e propriedades reológicas. **Tese (Doutorado em Ciências – Bioquímica)**. Universidade Federal do Paraná. Curitiba, p. 25, 2020.

ABREU, H., SIMAS, F. F., SMIDERLE, F. R., SOVRANI, V., DALLAZEN, J. L., MARIA-FERREIRA, D., WERNER, M. F., CORDEIRO, L. M. C., & IACOMINI, M.. Gelling functional property, anti-inflammatory and antinociceptive bioactivities of β-D-glucan from the edible mushroom *Pholiota nameko*. **International Journal of Biological Macromolecules**, v. 122, p. 1128–1135, 2019.

ABREU, H., ZAVADINACK, M., SMIDERLE, F. R., CIPRIANI, T. R., CORDEIRO, L. M. C., & IACOMINI, M. Polysaccharides from *Pleurotus eryngii*: Selective extraction methodologies and their modulatory effects on THP-1 macrophages. **Carbohydrate Polymers**, v. 252, 2021.

ADAMS, D. J.. Fungal cell wall chitinases and glucanases. **Microbiology**, v. 150, p. 2029-2035, 2004.

AIDA, F. M. N. A., SHUHAIMI, M., YAZID, M., & MAARUF, A. G.. Mushroom as a potential source of prebiotics: a review. **Trends in Food Science and Technology,** v. 20, n.11-12, p. 567–575, 2009.

ALEXOPOULOS, C. J., MIMS, C. W., & BLACKWELL, M.. Introductory Mycology. 4ª Edição. **New York; John Wiley & Sons,** 1996.

AMAZONAS, A. O Setor de Cogumelos. **Associação Nacional de Produtores de Cogumelos**, 2013. Disponível em: <https://anpccogumelos.org/cogumelos>. Acesso em: 07 out. 2020.

BAGGIO, C. H., FREITAS, C. S., MARTINS, D. F., MAZZARDO, L., SMIDERLE, F. R., SASSAKI, G. L., IACOMINI, M., MARQUES, M. C. A., & SANTOS, A. R. S. 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. **Journal of Pain**, v. 11, p. 965-971, 2010.

BALLARD, T. S., MALLIKARJUNAN, P., ZHOU, K., & O'KEEFE, S.. Microwave-

assisted extraction of phenolic antioxidant compounds from peanut skins. **Food Chemistry**, v. 120, p. 1185-1192, 2010.

BARDDAL, H. P., GRACHER, A. H. P., SIMAS-TOSIN, F. F., IACOMINI, M., & CIPRIANI, T. R. Anticoagulant activity of native and partially degraded glycoglucuronomannan after chemical sulfation. **International Journal of Biological Macromolecules**, v. 80, p. 328-333, 2015.

BONATTI, M., KARNOPP, P., SOARES, H. M., & FURLAN, S. A. Evaluation of *Pleurotus ostreatus* and *Pleurotus sajor-caju* nutritional characteristics when cultivated in different lignocellulosic wastes. **Food Chemistry**, v. 88, p. 425-428, 2004.

BRITO, D. R., CARBONERO, E. R., VIANA, S. R. F., SILVA, E. V., RUTHES, A. C., LIÃO, L. M., & IACOMINI, M. Partially methylated galactans containing different proportions of 3-*O*-methyl-galactose from *Pleurotus citrinopileatus*. **Carbohydrate Research**, v. 458, p. 29-34, 2018.

BROADLEY, K. J.. Autonomic pharmacology. 1ª Edição. **CRC Press**, 2017.

BUSWELL, J. A., ODIER, E., & KIRK, T. K. Lignin biodegradation. **Critical Reviews in Biotechnology**, v. 6, p. 1-60, 1987.

CANTU-JUNGLES, T. M., RUTHES, A. C., EL-HINDAWY, M., MORENO, R. B., ZHANG, X., CORDEIRO, L. M. C., HAMAKER, B. R., & IACOMINI, M. In vitro fermentation of *Cookeina speciosa* glucans stimulates the growth of the butyrogenic Clostridium cluster XIVa in a targeted way. ***Carbohydrate Polymers***, v. 183, p. 219-229, 2018

CARBONERO, E. R., GRACHER, A. H. P., ROSA, M. C. C., TORRI, G., SASSAKI, G. L., GORIN, P. A. J., & IACOMINI, M. Unusual partially 3-*O*-methylated α-galactan from mushrooms of the genus *Pleurotus*. **Phytochemistry**, v. 69, p. 252-257, 2008.

CARBONERO, E. R., RUTHES, A. C., FREITAS, C. S., UTRILLA, P., GÁLVEZ, J., SILVA, E. V. DA, SASSAKI, G. L., GORIN, P. A. J., & IACOMINI, M. Chemical and biological properties of a highly branched β-glucan from edible mushroom *Pleurotus sajor-caju*. **Carbohydrate Polymers**, v. 90, p. 814-819, 2012.

CARVAJAL, A. E. S. S., KOEHNLEIN, E. A., SOARES, A. A., ELER, G. J., NAKASHIMA, A. T. A., BRACHT, A., & PERALTA, R. M. Bioactives of fruiting

bodies and submerged culture mycelia of *Agaricus brasiliensis* (*A.blazei*) and their antioxidant properties. **LWT - Food Science and Technology**, v. 46, p. 493-499, 2012.

COVILLE, F. V. Observations on recent cases of mushroom poisoning in the District of Columbia. **Forgotten Books**, 2014.

CUI, F., ZAN, X., LI, Y., YANG, Y., SUN, W., ZHOU, Q., YU, S., & DONG, Y. Purification and partial characterization of a novel anti-tumor glycoprotein from cultured mycelia of *Grifola frondosa*. **International Journal of Biological Macromolecules**, v. 62, p. 684-690, 2013.

DE JESUS, L. I., SMIDERLE, F. R., CORDEIRO, L. M. C., DE FREITAS, R. A., 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*. **Carbohydrate Polymers**, v. 200, p. 353–360, 2018.

DE JESUS, L. I., SMIDERLE, F. R., RUTHES, A. C., VILAPLANA, F., DAL'LIN, F. T., MARIA-FERREIRA, D., WERNER, M. F., VAN GRIENSVEN, L. J. L. D., & IACOMINI, M. Chemical characterization and wound healing property of a β-D-glucan from edible mushroom *Piptoporus betulinus*. **International Journal of Biological Macromolecules**, v. 117, p. 1361-1366, 2018.

DUFOSSÉ, L., FOUILLAUD, M., CARO, Y., MAPARI, S. A. S., & SUTTHIWONG, N. Filamentous fungi are large-scale producers of pigments and colorants for the food industry. **Current Opinion in Biotechnology**, v. 26, p. 56-61, 2014.

ELISASHVILI, V. Submerged Cultivation of Medicinal Mushrooms: Bioprocesses and Products (Review). ***International Journal of Medicinal Mushrooms***, v. 14, n. 3, p. 211-239, 2012.

ELSAYED, E. A., EL ENSHASY, H., WADAAN, M. A. M., & AZIZ, R. Mushrooms: A potential natural source of anti-inflammatory compounds for medical applications. **Mediators of Inflammation**, v. 2014, p. 805-841, 2014.

ESPOSITO, E.; AZEVEDO, J. L. Fungos: uma introdução à biologia, bioquímica e biotecnologia. **Caxias do Sul: Educa**, 2004.

FUKUDA, E. K., VASCONCELOS, A. F. D., MATIAS, A. C., DE MELO BARBOSA,

A., DEKKER, R. F. H., & DA SILVA, M. D. L. C. Fungal cell wall polysaccharides: Purification and characterization. **Semina:Ciências Agrarias**, v. 30, p. 117-134, 2009.

GENEBANK, National Institute of Agrobiological Sciences. **National Institute of Genetics**, 2013. Disponível em: <http://www.gbif.org/occurence/729247832>. Acesso em: 03 out. 2020.

GORIN, P. A. J.; IACOMINI, M. Polysaccharides of the lichens *Cetraria islandica* and *Ramalina usnea*. **Carbohydrate Research**. v. 128, p. 119-132, 1984

GUNDE-CIMERMAN, N. Medicinal Value of the Genus *Pleurotus* (Fr.) P.Karst. (Agaricales s.l., Basidiomycetes). **International Journal of Medicinal Mushrooms**, v. 1, n. 1, p. 39-80, 1999.

HARDY, M. L. Dietary Supplement Use in Cancer Care: Help or Harm. **Hematology/Oncology Clinics of North America**, v. 22, n. 4, p. 581-617, 2008.

HOLMES, S. Outline fo plant classification. **London: Longman**, 1983.

JEFF, I. B., LI, S., PENG, X., KASSIM, R. M. R., LIU, B., & ZHOU, Y. Purification, structural elucidation and antitumor activity of a novel mannogalactoglucan from the fruiting bodies of *Lentinus edodes*. **Fitoterapia**, v. 84, p. 338-346, 2013.

KHATUN, S., ISLAM, A., CAKILCIOGLU, U., GULER, P., & CHATTERJEE, N. C. Nutritional qualities and antioxidant activity of three edible oyster mushrooms (*Pleurotus* spp.). **NJAS - Wageningen Journal of Life Sciences**, v. 72–73, p. 1–5, 2015.

KIHO, T., KATSURAWAGA, M., NAGAI, K., UKAI, S., & HAGA, M. Structure and antitumor activity of a branched (1→3)-β-D-glucan from the alkaline extract of *Amanita muscaria*. **Carbohydrate Research**, v. 224, p. 237-243, 1992.

LABORDE, J. e DELMAS, J. Le *Pleurote*: un nouveau champignon comestible cultivé Dissier *Pleurote*. **Sation de Recherches sur les champignons**, p. 39-46, 1974

LI, H., TAO, Y., ZHAO, P., ZHI, D., GAO, X., ZHAO, X., & LI, M. Effect of ultrasound-assisted extraction on physicochemical properties and TLR2-affinity binding of the polysaccharides from *Pholiota nameko*. **International Journal of Biological Macromolecules**, v. 135, p. 1020-1027, 2019.

LI, H., & WANG, S. Kinetics of inhibition of ribonuclease A by *Pholiota Nameko*

polysaccharide. **International Journal of Biological Macromolecules**, v. 40, p. 134-138, 2007.

MAAS, N. C., GRACHER, A. H. P., SASSAKI, G. L., GORIN, P. A. J., IACOMINI, M., & CIPRIANI, T. R. Sulfation pattern of citrus pectin and its carboxy-reduced derivatives: Influence on anticoagulant and antithrombotic effects. **Carbohydrate Polymers**, v. 89, p. 1081-1087, 2012.

MACHADO, A. P. D. F., PASQUEL-REÁTEGUI, J. L., BARBERO, G. F., & MARTÍNEZ, J. Pressurized liquid extraction of bioactive compounds from blackberry (*Rubus fruticosus L.*) residues: A comparison with conventional methods. **Food Research International**, v. 3, p. 675-683, 2015.

MACIEJCZYK, E., & KAFARSKI, P. Mannitol in *Amanita muscaria* - An osmotic blood-brain barrier disruptor enhancing its hallucinogenic action? **Medical Hypotheses**, v. 81, n. 5, p. 766-767, 2013.

MALICK, D. H. Identification of myo-inositol monophosphates in mycelia of *Pholiota nameko*. **Mycoscience**, v. 37, n. 3, p. 381-383, 1996.

MANTOVANI, M. S., BELLINI, M. F., ANGELI, J. P. F., OLIVEIRA, R. J., SILVA, A. F., & RIBEIRO, L. R. β-Glucans in promoting health: Prevention against mutation and cancer. **Mutation Research - Reviews in Mutation Research**, v. 685, n. 3, p. 154-161, 2008.

MANZI, P., & PIZZOFERRATO, L. Beta-glucans in edible mushrooms. **Food Chemistry**, v. 68, p. 315–318, 2000.

MICHELOT, D., & MELENDEZ-HOWELL, L. M. *Amanita muscaria*: Chemistry, biology, toxicology, and ethnomycology. **Mycological Research**, v. 107, n. 2, p. 131-145, 2003.

MILES, P. G., & CHANG, S. T. Mushroom Biology Concise Basics and Current Development. **World Scientific Publishing Company**, 1997.

MORALES, D., IACOMINI, M., GILBERT-LÓPEZ, B., SMIDERLE, F. R., DE JESUS, L. I., SOLER-RIVAS, C., & GIL-RAMÍREZ, A. Evaluation of microwave-assisted and pressurized liquid extractions to obtain β-D-glucans from mushrooms. **Carbohydrate Polymers**, v. 156, p. 165–174, 2016.

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. **Innovative Food Science and Emerging Technologies**, v. 51, p. 80-90, 2019.

MORENO, R. B., RUTHES, A. C., BAGGIO, C. H., VILAPLANA, F., KOMURA, D. L., & IACOMINI, M. Structure and antinociceptive effects of β-D-glucans from *Cookeina tricholoma*. **Carbohydrate Polymers**, v. 141, n. 5, p. 220-228, 2016.

NELSON, D. L., & COX, M. M. Principios de Bioquímica de Lehninger. 6ª Edição. **Artmed**, 2014.

OOI, C. V., & LIU, F.. Immunomodulation and Anti-Cancer Activity of Polysaccharide-Protein Complexes. **Current Medicinal Chemistry**, v. 7, p. 715-729, 2012.

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.

PÉREZ, P., & RIBAS, J. C. Cell wall analysis. **Methods**, v. 33, n1, p. 245-251, 2004.

PLAZA, M., & TURNER, C. Pressurized Hot Water Extraction of Bioactives. **Comprehensive Analytical Chemistry**, v. 71, p. 39-54, 2017.

PRAMANIK, M., MONDAL, S., CHAKRABORTY, I., ROUT, D., & ISLAM, S. S. Structural investigation of a polysaccharide (Fr. II) isolated from the aqueous extract of an edible mushroom, *Pleurotus sajor-caju*. **Carbohydrate Research**, v. 340, n. 4, p. 629-636, 2005.

PUTZKE, J.; PUTZKE, M. T. L. Os Reinos dos Fungos. Santa Cruz do Sul: **EDUNISC**, v. 1, p. 22-23, 2004.

RAJARATHNAM, S., & BANO, Z. *Pleurotus* mushrooms. part III. biotransformations of natural lignocellulosic wastes: Commercial applications and implications. **Critical Reviews in Food Science and Nutrition**, v. 28, n. 1, p. 31-113, 1989.

BARBOSA, R. J., DOS SANTOS FREITAS, M. M., DA SILVA MARTINS, L. H., & DE CARVALHO, R. N. Polysaccharides of mushroom *Pleurotus* spp.: New extraction techniques, biological activities and development of new technologies. **Carbohydrate Polymers**, v. 229, 2020.

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 Microbiology Letters**, v. 221, n. 1, p. 119-224, 2003.

RUIZ-ACEITUNO, L., GARCÍA-SARRIÓ, M. J., ALONSO-RODRIGUEZ, B., RAMOS, L., & SANZ, M. L. Extraction of bioactive carbohydrates from artichoke (*Cynara scolymus* L.) external bracts using microwave assisted extraction and pressurized liquid extraction. **Food Chemistry**, v. 196, p. 1156–1162, 2016.

RUTHES, ANDREA C., SMIDERLE, F. R., & IACOMINI, M. Mushroom heteropolysaccharides: A review on their sources, structure and biological effects. **Carbohydrate Polymers**, v. 136, p. 358-375, 2016.

RUTHES, ANDREA CAROLINE, CARBONERO, E. R., CÓRDOVA, M. M., BAGGIO, C. H., SANTOS, A. R. S., SASSAKI, G. L., CIPRIANI, T. R., GORIN, P. A. J., & IACOMINI, M. *Lactarius rufus* (1→3),(1→6)-β-D-glucans: Structure, antinociceptive and anti-inflammatory effects. **Carbohydrate Polymers**, v. 94, n. 1, p. 129-136, 2013.

RUTHES, ANDREA CAROLINE, CARBONERO, E. R., CÓRDOVA, M. M., BAGGIO, C. H., SASSAKI, G. L., GORIN, P. A. J., SANTOS, A. R. S., & IACOMINI, M. Fucomannogalactan and glucan from mushroom *Amanita muscaria*: Structure and inflammatory pain inhibition. **Carbohydrate Polymers**, v. 98, n. 1, p. 761-769, 2013.

RUTHES, ANDREA CAROLINE, SMIDERLE, F. R., & IACOMINI, M. D-Glucans from edible mushrooms: A review on the extraction, purification and chemical characterization approaches. **Carbohydrate Polymers**, v. 117, p. 753-761, 2015.

SÁNCHEZ, C. Reactive oxygen species and antioxidant properties from mushrooms. **Synthetic and Systems Biotechnology**, v. 2, n. 1, p. 13-22, 2017.

SCHEPETKIN, I. A., & QUINN, M. T. Botanical polysaccharides: Macrophage immunomodulation and therapeutic potential. **International Immunopharmacology**, v. 6, n. 3, p. 317-333, 2006.

SILVEIRA, M. L. L., Caracterização estrutural e ação antinociceptiva e anti-inflamatória de polissacarídeos isolados de *Pleurotus sajor-caju*. **Tese (Doutorado em Ciências – Bioquímica)**. Universidade Federal do Paraná. Curitiba, p. 38. 2015.

SILVEIRA, M. L. L., SMIDERLE, F. R., AGOSTINI, F., PEREIRA, E. M., BONATTI-CHAVES, M., WISBECK, E., RUTHES, A. C., SASSAKI, G. L., CIPRIANI, T. R., FURLAN, S. A., & IACOMINI, M. Exopolysaccharide produced by *Pleurotus sajor-caju*: Its chemical structure and anti-inflammatory activity. **International Journal of Biological Macromolecules**, v. 75, p. 90-96, 2015.

SILVEIRA, M. L. L., SMIDERLE, F. R., MORAES, C. P., BORATO, D. G., BAGGIO, C. H., RUTHES, A. C., WISBECK, E., SASSAKI, G. L., CIPRIANI, T. R., FURLAN, S. A., & IACOMINI, M. Structural characterization and anti-inflammatory activity of a linear β-D-glucan isolated from *Pleurotus sajor-caju*. **Carbohydrate Polymers**, v. 113, p. 588-596, 2014.

SMIDERLE, F.R., Caracterização estrutural de alguns polissacarídeos presentes no basidioma de *Pleurotus pulmonarius* e aplicações. **Dissertação (Mestrado em Ciências – Bioquímica).** Universidade Federal do paraná. Curitiba, p. 15, 2008.

SMIDERLE, F. R., OLSEN, L. M., CARBONERO, E. R., MARCON, R., BAGGIO, C. H., FREITAS, C. S., SANTOS, A. R. S., TORRI, G., GORIN, P. A. J., & IACOMINI, M. A 3-*O*-methylated mannogalactan from *Pleurotus pulmonarius*: Structure and antinociceptive effect. **Phytochemistry**, v. 69, n. 15, p. 2731–2736, 2008.

SMIDERLE, F. R., OLSEN, L. M., RUTHES, A. C., CZELUSNIAK, P. A., SANTANA-FILHO, A. P., SASSAKI, G. L., GORIN, P. A. J., & IACOMINI, M. Exopolysaccharides, proteins and lipids in *Pleurotus pulmonarius* submerged culture using different carbon sources. **Carbohydrate Polymers**, v. 87, p. 368–376, 2012.

SMIDERLE, FHERNANDA 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. **Carbohydrate Polymers**, v. 94, n. 1, p. 91–99, 2013.

SMIDERLE, FHERNANDA 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**, v. 9, n. 10, 2014.

SMIDERLE, FHERNANDA R., CARBONERO, E. R., MELLINGER, C. G., SASSAKI, G. L., GORIN, P. A. J., & IACOMINI, M. Structural characterization of a polysaccharide and a β-glucan isolated from the edible mushroom *Flammulina velutipes*. **Phytochemistry**, v. 67, n. 19, p. 2189-2196, 2006.

SMIDERLE, FHERNANDA 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*. **European Journal of Pharmacology**, v. 597, n. 1-3, p. 86-91, 2008.

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*. **Carbohydrate Polymers**, v. 169, p. 1-8, 2017.

STAMETS, P. Growing gourmet and medicinal mushrooms. **Intern. J. Med. Mushrooms**, 3rd Revise. ed Ten Speed Press, California, US, 2000.

STØRMER, F. C., & JANAK, K. Ibotenic acid in *Amanita muscaria* spores and caps. **Mycologist**, v. 18, n. 3, p. 114-117, 2004.

SYNYTSYA, A., MÍČKOVÁ, K., SYNYTSYA, A., JABLONSKÝ, I., SPĚVÁČEK, J., ERBAN, V., KOVÁŘÍKOVÁ, E., & ČOPÍKOVÁ, J. Glucans from fruit bodies of cultivated mushrooms *Pleurotus ostreatus* and *Pleurotus eryngii*: Structure and potential prebiotic activity. **Carbohydrate Polymers**, v. 76, n. 4, p. 548-556, 2009.

TONG, H., XIA, F., FENG, K., SUN, G., GAO, X., SUN, L., JIANG, R., TIAN, D., & SUN, X. Structural characterization and in vitro antitumor activity of a novel polysaccharide isolated from the fruiting bodies of *Pleurotus ostreatus*. **Bioresource Technology**, v. 100, n. 4, p. 1682-1686, 2009.

URBEN, A. F.; OLIVEIRA, C. Cogumelos comestíveis: utilização e fontes energéticas. **Revisão Anual de Patologia de Plantas**, v. 6, p. 173-196. 1998.

WASSER, S. Medicinal mushrooms as a source of antitumor and immunomodulating polysaccharides. **Applied Microbiology and Biotechnology**, v. 60, p. 258–274, 2003.

YAN, J., MENG, Y., ZHANG, M., ZHOU, X., CHENG, H., SUN, L., & ZHOU, Y. A 3-

O-methylated heterogalactan from *Pleurotus eryngii* activates macrophages. **Carbohydrate Polymers**, v. 206, p. 706-715, 2019.

ZHANG, H., WANG, Z. Y., YANG, L., YANG, X., WANG, X., & ZHANG, Z. In vitro antioxidant activities of sulfated derivatives of polysaccharides extracted from *Auricularia auricular*. **International Journal of Molecular Sciences**, v. 12, n. 5, p. 3288-3302, 2011.

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 in Food Science and Technology**, v. 18, n. 1, p. 4-19, 2007.

ZHANG, S., NIE, S., HUANG, D., LI, W., & XIE, M. Immunomodulatory effect of *Ganoderma atrum* polysaccharide on CT26 tumor-bearing mice. **Food Chemistry**, v. 136, n. 3-4, p. 1213-1219, 2013.

ZHANG, Y., LIU, Z., NG, T. B., CHEN, Z., QIAO, W., & LIU, F. Purification and characterization of a novel antitumor protein with antioxidant and deoxyribonuclease activity from edible mushroom *Pholiota nameko*. **Biochimie**, v. 99, p. 28-37, 2014.

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**, v. 70, p. 523-529, 2014.