

UNIVERSIDADE FEDERAL DO PARANÁ

SHAYANE DA SILVA MILHORINI

CARACTERIZAÇÃO ESTRUTURAL, ATIVIDADE BIOLÓGICA E  
PROPRIEDADES REOLÓGICAS DE POLISSACARÍDEOS ISOLADOS DE  
AGARICOMICETOS

CURITIBA

2022

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PROPRIEDADES REOLÓGICAS DE POLISSACARÍDEOS ISOLADOS DE  
AGARICOMICETOS

Tese apresentada ao Programa de Pós-graduação  
em Ciências – Bioquímica, Departamento de  
Bioquímica e Biologia molecular, Setor de Ciências  
Biológicas, Universidade Federal do Paraná, como  
requisito parcial para a obtenção do título de  
Doutora em Ciências – Bioquímica.

Orientador: Professor Dr. Marcello Iacomini

Coorientadora: Professora Dra. Fhernanda Ribeiro  
Smiderle

CURITIBA

2022

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

Milhorini, Shayane da Silva

Caracterização estrutural, atividade biológica e propriedades reológicas de polissacarídeos isolados de agaricomycetos /

Shayane da Silva Milhorini. – Curitiba, 2022.

1 recurso on-line : PDF.

Tese (Doutorado) – Universidade Federal do Paraná, Setor de Ciências Biológicas, Programa de Pós-Graduação em Ciências (Bioquímica).

Orientador: Professor Dr. Marcello Iacomini.

Coorientadora: : Professora Dra. Fhernanda Ribeiro Smiderle.

1. Polissacarídeos. 2. Reologia (Biologia). 3. Reishi. 4. Melanoma. 5. Mamas – Câncer. I. Iacomini, Marcello, 1947-. II. Smiderle, Fhernanda Ribeiro. III. Universidade Federal do Paraná. Setor de Ciências Biológicas. Programa de Pós-Graduação em Ciências (Bioquímica). IV. Título.

Bibliotecária: Giana Mara Seniski Silva. CRB-9/1406



MINISTÉRIO DA EDUCAÇÃO  
SETOR DE CIÊNCIAS BIOLÓGICAS  
UNIVERSIDADE FEDERAL DO PARANÁ  
PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO  
PROGRAMA DE PÓS-GRADUAÇÃO CIÊNCIAS  
(BIOQUÍMICA) - 40001016003P2

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CURITIBA, 21 de Fevereiro de 2022.

Assinatura Eletrônica  
23/02/2022 17:03:51.0  
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21/02/2022 16:21:33.0  
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25/02/2022 11:47:17.0  
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Assinatura Eletrônica  
21/02/2022 16:11:34.0  
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Dedico este trabalho ao meu avô: Antônio Pedro de Oliveira (*In memoriam*)

*"I'll seek you in heaven*

*Between angels and cherubim*

*I know I'll feel in heaven*

*When I hear you say: Amen!*

*Dear, I'll ask Jesus*

*If I can hug you once again*

*I know I'll find you in heaven*

*While the angels sing: Amen!"*

## AGRADECIMENTOS

Começo meus agradecimentos com uma frase que usei no meu trabalho de conclusão de curso da graduação, na minha dissertação, e usarei agora, pois quanto mais a vida acontece, mais tenho certeza de que estas palavras são verdadeiras: “Agradecer é admitir que houve um momento em que se precisou de alguém; é reconhecer que o homem, jamais poderá lograr para si o dom de ser autossuficiente. Ninguém nem nada cresce sozinho: sempre é preciso um olhar de apoio, uma palavra de incentivo, um gesto de compreensão, uma atitude de amor” (AUTOR DESCONHECIDO). Portanto, não economizarei nos meus agradecimentos.

Ao Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) pela bolsa de estudo que me permitiu ter dedicação integral a esta pesquisa.

Ao meu orientador, professor Dr. Marcello Iacomini, pela orientação detalhada ao longo de todos esses anos e pela sua dedicação à ciência.

À minha coorientadora, professora Dra. Fhernanda Ribeiro Smiderle, pela orientação na química de carboidratos, nas atividades biológicas e pelas conversas.

À Universidade Federal do Paraná e ao Programa de Pós Graduação em Ciências – Bioquímica.

Aos professores Dr. Thales Ricardo Cipriani e Dra. Lucimara Mach Cortes Cordeiro, pela correção do meu projeto, relatórios e qualificação.

Ao professor Dr. Fábio Rogério Rosado e aos integrantes do Núcleo Experimental de Micologia Aplicada (NEMA) da UFPR-Palotina, pela colaboração que foi o coração de boa parte desta pesquisa. Há sete anos fizemos nossa primeira coleta do *Macrocybe titans*, o qual me foi doado para a realização das pesquisas em meu mestrado e doutorado. Agradeço também à professora Dra. Adriana Fiorini Rosado e à Mrs. Fernanda C. B. N. Pereira, pela identificação molecular desse cogumelo. A vocês três, agradeço ainda pela amizade, pela família que me acolheu e que me acompanha.

À empresa Juncao Brazil, de Taboão da Serra, SP, Brasil, pelo fornecimento do cogumelo *Ganoderma lucidum*.

Aos colaboradores que foram essenciais para o desenvolvimento deste trabalho: Dra. Arianne Centa, Dr. Arquimedes Paixão de Santana Filho, Dr. Daniel de Lima Bellan, Eduardo Luis Longoria, Dra. Liana Inara de Jesus, Msc. Matheus

Zavadinack, Msc. Renata Rutckeviski, professora Dra. Fernanda Fagagnoli Simas e professor Dr. Guilherme Lanzi Sassaki.

Aos técnicos Keylla, Arquimedes, Thiago e Rosane, bem como ao pós-doutorando Leociley e ao professor Dr. Lauro Mera de Souza, pelas análises de HPSEC, GC-MS e RMN.

A todos os meus colegas e amigos do grupo de química de carboidratos (laboratórios 252, 250, 247 e E1), pelos auxílios laboratoriais e por todo conhecimento compartilhado. De forma especial, agradeço pela convivência bem-humorada, pela vida que pude compartilhar com vocês e pela vida que vocês compartilharam comigo.

Um agradecimento especial para as cinco pessoas que provavelmente mais me toleraram no âmbito pessoal: Giuliana, Maria e Vanessa, uma amizade baseada em bioquímica, fotos de gato e áudios cantando, tende a durar uma vida toda! Ao Philippe, pelo tempo de compartilhamento de laboratório e de desafios. E à Jéssica, por todo o convívio, crescimento mútuo e aprendizado.

À doutoranda Juliana Danna Kulik, amizade que nasceu por meio dos cogumelos e que me permitiu estender meus conhecimentos fúngicos e iniciar parcerias em diferentes pesquisas. Espero que ainda chova muito em Curitiba e que nossa amizade se fortaleça a cada chuva!

À minha família: Meu marido, William P. Milhorini, pelo time que formamos, pelo amor, parceria, apoio, pelas aventuras e pelo cuidado nos detalhes, te amo! Ao meu felino, Josh (sim, irei agradecer-lo), essa preciosidade que trouxe muita vida para nossa casa, especialmente no trabalho *home-office* durante a pandemia de COVID-19.

Ao meu triozinho mais precioso: Minha mãe/pai Maria Madalena, minha irmã Keila e meu irmão Ederson. Sei que não importa onde eu esteja, eu sempre terei um lugar para voltar, alguém que irá me encontrar. Este amor incondicional que transborda e cada palavra de ânimo e orgulho, me motivaram mais do que na caminhada acadêmica, na caminhada da vida. À minha mãe em especial, que chegou a ter quatro empregos simultaneamente para que pudéssemos não só sobreviver, mas também estudar, sonhar e realizar. Independente da nossa condição social e intempéries da vida, ela decidiu que poderíamos, doou-se, superou-se e, então, pudemos.

À minha família Oliveira, em especial ao meu avô, Antônio Pedro de Oliveira, do qual tive que me despedir durante esta jornada acadêmica, e à minha segunda

mãe, minha avó Rosária. A todos os tios, tias, primos, primas e agregados. Tenho muita felicidade em dizer que tenho uma família gigante que me apoia, com força e com orgulho, desde os meus primeiros passos na vida, incluindo a acadêmica.

À minha segunda família, a família Milhorini, por terem me acolhido, ajudado e estarem, ao longo dos anos, fornecendo amor e cuidado.

Às minhas amigas da graduação, da UFPR para a vida, Dagna, Fernanda, Jéssica, Leidi e Vanessa. Sinto-me muito abençoada por ter vocês ainda acompanhando cada passo da minha jornada, aconselhando-me para o melhor e vibrando a cada conquista.

Agradeço a Deus, minha força motriz, pela força e saúde a mim direcionadas. Por cada dificuldade profissional e, especialmente, por cada dificuldade pessoal que tive que enfrentar durante este caminho, onde, ao me perder de mim, me descobri meramente humana. A cada momento de ansiedade ou dificuldade de clareza mental, a cada fadiga e a cada cansaço, a cada perda e a cada dor, eu agradeço. Que, através dessas experiências, eu nunca me esqueça da minha própria fragilidade humana e da beleza de reconhecer que não possuo minha própria redenção, que preciso do Divino, Jesus, pois Nele eu encontro e reencontro, quantas vezes forem necessárias, a minha única e eterna identidade: a minha verdadeira fé, a minha verdadeira motivação, os meus verdadeiros sonhos e objetivos: "Porque quando sou fraca, então, sou forte" (II Cor. 2:10).



Look up child! (LD)  
=)

## RESUMO

Os cogumelos são largamente consumidos na culinária e na medicina tradicional devido ao seu valor nutricional e propriedades medicinais. Essas propriedades são decorrentes da presença de compostos bioativos. Dentre esses, os polissacarídeos são moléculas muito conhecidas no meio científico, principalmente pelas suas atividades antitumoral e imunomoduladora. Além da importância medicinal, alguns polissacarídeos apresentam características físico-químicas que os tornam interessantes para a aplicação na indústria de alimentos e cosmética, pois possuem propriedades reológicas interessantes, como capacidade de geleificação. Baseado nisso, diferentes polissacarídeos foram isolados dos cogumelos *Macrocybe titans* e *Ganoderma lucidum*, através de extrações aquosas (25 °C e 100 °C), bem como extrações alcalinas (NaOH 5 % ou KOH 10 %). A partir do *M. titans* foram obtidas duas fucogalactanas, uma previamente reportada (F-1) e uma inédita (F-2), cujas massas moleculares ( $M_w$ ) se diferem em 22 vezes. Ambas foram testadas em duas linhagens de células de câncer de mama (MCF7 e MDA-MB-231). Foi observado que a diferença de  $M_w$  afeta os efeitos biológicos resultantes, uma vez que apenas a molécula com maior  $M_w$  foi capaz de causar apoptose e necrose nas células MDA-MB-231, apesar de ambas as frações terem apresentado citotoxicidade sobre as duas linhagens celulares. Além disso, duas frações de glucanas também foram obtidas do *M. titans*: GFI (mistura de  $\beta$ -glucanas e glicogênio) e  $\beta$ -GLC (mistura de  $\beta$ -glucanas de diferentes  $M_w$ ). Ambas foram investigadas quanto as suas propriedades reológicas e apresentaram comportamento não-Newtoniano pseudoplástico e comportamento de gel em baixas temperaturas (<11 °C para GFI, e <20 °C para  $\beta$ -GLC). Todavia, a fração  $\beta$ -GLC produziu um gel mais forte do que a mistura com glicogênio. Adicionalmente, uma fração purificada de  $\beta$ -glucana (GLC;  $M_w$   $1,1 \times 10^4$  g/mol) com ligações (1→3) e (1→6), foi obtida desse mesmo cogumelo. A partir do *G. lucidum* foi purificada uma fucoxilomanana (FXM;  $M_w$   $3.59 \times 10^4$  g/mol), a qual ocasionou a redução da proliferação e formação de colônias de células de melanoma murino B16F10, além de alterar o ciclo celular das mesmas. Desse cogumelo também foram isoladas duas frações de  $\beta$ -glucanas ramificadas (R12 e E12) de diferentes  $M_w$  ( $1.3 \times 10^4$  e  $5 \times 10^3$  g/mol, respectivamente), as quais possivelmente são formadas por ligações (1→3), (1→4) e (1→6). Por fim, diferentes tipos de glucanas lineares foram obtidas do *G. lucidum*, sendo: duas frações contendo  $\beta$ -glucana com ligação (1→3) (GLC-1 e GLC-2), uma fração apresentando glucana com as mesmas ligações, porém em configuração alfa (GLC-3), e uma mistura de três glucanas lineares:  $\beta$ -glucana-(1→3),  $\alpha$ -glucana-(1→3) e  $\alpha$ -glucana-(1→4) (GLC-4). Em suma, heteropolissacarídeos e homopolissacarídeos com diferentes estruturas químicas foram obtidos dos cogumelos *M. titans* e *G. lucidum*. Algumas dessas moléculas apresentaram potencial para serem aplicadas na indústria médica, farmacêutica ou alimentícia.

Palavras-chave: *Macrocybe titans*; *Ganoderma lucidum*;  $\beta$ -glucanas; Fucogalactanas; Fucoxilomanana; Reologia; Bioatividade; Melanoma; Câncer de mama.

## ABSTRACT

Mushrooms are widely consumed in culinary and traditional medicine due to their nutritional value and medicinal effects. Such properties are related to the presence of bioactive compounds. Among these, polysaccharides are well known molecules in the scientific community, mainly due to their antitumor and immunomodulatory activities. In addition to their medicinal importance, some polysaccharides have physicochemical features that allow their application in the food and cosmetic industries, since they present interesting rheological properties, such as gelling capacity. Based on this, different polysaccharides were isolated from the mushrooms *Macrocybe titans* and *Ganoderma lucidum*, through aqueous extractions (25 °C or 100 °C) as well as alkaline extractions (5 % NaOH or 10 % KOH). Two fucogalactans were obtained from *M. titans*, one previously reported (F-1) and an unpublished one (F-2), whose molecular masses (Mw) differ by 22 times. Both were tested in two breast cancer cell lines (MCF7 and MDA-MB-231). It was observed that the difference in Mw affects the resulting biological effects since only the molecule with the highest Mw (F-2) was able to cause apoptosis and necrosis in MDA-MB-231 cells, even though both fractions showed cytotoxicity on the two cell lines. Furthermore, two glucan fractions were obtained from *M. titans*: GFI (mixture of  $\beta$ -glucans and glycogen) and  $\beta$ -GLC (mixture of  $\beta$ -glucans of different Mw). Both were investigated about their rheological properties and exhibited non-Newtonian pseudoplastic behavior and gel-like behavior at low temperatures (<11 °C for GFI, and <20 °C for  $\beta$ -GLC). However, the  $\beta$ -GLC fraction produced a stronger gel than the mixture with glycogen. Additionally, a purified fraction of  $\beta$ -glucan (GLC; Mw  $1.1 \times 10^4$  g/mol) with (1 $\rightarrow$ 3) and (1 $\rightarrow$ 6) linkages was obtained from the same mushroom. On the other hand, a fucoxylomannan (FXM; Mw  $3.59 \times 10^4$  g/mol) was purified from *G. lucidum*. This heteropolysaccharide led to a reduction in proliferation and colony formation of B16F10 murine melanoma cells, apart from altering their cell cycle. From such mushroom, two fractions of branched  $\beta$ -glucans (R12 and E12) of different Mw ( $1.3 \times 10^4$  and  $5 \times 10^3$  g/mol, respectively) were also isolated, which are possibly formed by (1 $\rightarrow$ 3), (1 $\rightarrow$ 4), and (1 $\rightarrow$ 6) linkages. Finally, different types of linear glucans were obtained from *G. lucidum*, as follows: two fractions containing  $\beta$ -(1 $\rightarrow$ 3)-glucan (GLC-1 and GLC-2), one fraction containing a glucan with the same linkage but with alpha-configuration (GLC-3), and a mixture of three linear glucans:  $\beta$ -(1 $\rightarrow$ 3)-glucan,  $\alpha$ -(1 $\rightarrow$ 3)-glucan, and  $\alpha$ -(1 $\rightarrow$ 4)-glucan (GLC-4). In summary, heteropolysaccharides and homopolysaccharides with different chemical structures were obtained from the mushrooms *M. titans* and *G. lucidum*. Some of such molecules showed potential to be applied in the medical, pharmaceutical, or food industries.

**Keywords:** *Macrocybe titans*; *Ganoderma lucidum*;  $\beta$ -glucans; Fucogalactans; Fucoxylomannan; Rheology; Bioactivity; Melanoma; Breast cancer.

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## 1- INTRODUÇÃO

Os cogumelos são apreciados e consumidos há décadas devido ao seu valor nutricional e suas propriedades medicinais (SANTOS-NEVES, 2017). Esses fungos são ricos em moléculas que atuam como modificadores da resposta biológica, como os compostos secundários (BABY et al., 2015), proteínas (TEHRANI et al., 2012) e polissacarídeos (MILHORINI et al., 2018).

Dentre esses compostos bioativos, os polissacarídeos recebem atenção no meio científico e tem sido alvo de diversas pesquisas que indicam seus potenciais efeitos biológicos (SILVEIRA, 2015; ZHANG et al., 2016; ROMÁN et al., 2016; MILHORINI et al., 2018; ABREU et al., 2021). Dentre os polímeros obtidos de cogumelos, esses são os mais conhecidos e mais potentes quanto às atividades antitumoral e imunomoduladora (SMIDERLE; RUTHES; IACOMINI, 2014). Diversos trabalhos relataram a efetividade dos polissacarídeos sobre distintas linhagens de células tumorais, incluindo células de melanoma (BISCAIA et al., 2017; MILHORINI et al., 2018) e células de câncer de mama (SHI et al., 2013).

Os efeitos biológicos reportados para os polissacarídeos estão relacionados com a estrutura química dos mesmos, portanto, polissacarídeos com diferentes características estruturais, como ligações glicosídicas, composição monossacarídica e massa molecular, possivelmente apresentarão distintas atividades biológicas (ELISASHVILI, 2012; RUTHES; SMIDERLE; IACOMINI, 2015). Devido a isso, se faz necessário realizar a caracterização química dessas moléculas em conjunto com os estudos de suas propriedades biológicas, visando investigar quais características químicas estão relacionadas com os efeitos biológicos obtidos (FERREIRA et al., 2015).

Polissacarídeos com diferentes características estruturais já foram isolados de diversas espécies de cogumelos (SOVRANI et al., 2017; MILHORINI et al., 2018; OLIVEIRA et al., 2019; TEL-ÇAYAN et al., 2020), no entanto, muitos organismos fúngicos da classe Agaricomycetes não tiveram seus compostos explorados e podem conter polímeros bioativos relevantes, mas que ainda não foram avaliados.

Dentre as espécies de cogumelos pouco explorados, encontra-se o cogumelo *Macrocybe titans*. Quanto a seus polissacarídeos, até o momento foi isolada uma fucogalactana capaz de reduzir a migração de células de melanoma murino B16F10,

sem alterar a viabilidade das mesmas (MILHORINI *et al.*, 2018). Todavia, outros polissacarídeos de interesse médico, farmacêutico e alimentício, possivelmente podem ainda serem obtidos desse fungo.

Por outro lado, o cogumelo *Ganoderma lucidum* é uma espécie utilizada por milênios na medicina oriental com o intuito aumentar a longevidade e promover a saúde (LESKOSEK-CUKALOVIC *et al.*, 2010; LIANG *et al.*, 2019; AHMAD *et al.*, 2020). Segundo Nie *et al.* (2013), a principal substância bioativa encontrada nos cogumelos da espécie *Ganoderma*, são os polissacarídeos. Por ser muito consumido e conhecido na medicina tradicional, o *G. lucidum* é uma espécie em potencial para ser utilizada em pesquisas de polissacarídeos bioativos.

Além do potencial medicinal, os polissacarídeos também podem possuir características físico-químicas que possibilitam sua utilização na indústria alimentícia e cosmética. Portanto, extratos, frações polissacarídicas ou polissacarídeos isolados, podem ser avaliados através de estudos reológicos, investigando a possível aplicação industrial dos mesmos como agentes espessantes, emulsificantes ou geleificantes (XU *et al.*, 2016; SOVRANI *et al.*, 2017; ABREU *et al.*, 2019; WANG *et al.*, 2020a).

Considerando a ampla aplicação médica e industrial dos polissacarídeos, aliado a pouca exploração do cogumelo *Macrocybe titans* e a utilização do *Ganoderma lucidum* na medicina tradicional, ambas as espécies foram selecionadas para a realização deste trabalho. A partir dessas, visa-se realizar a obtenção, purificação e caracterização dos polissacarídeos. Adicionalmente, alguns desses polímeros serão avaliados quanto a seus efeitos sobre células de melanoma ou células de câncer de mama, além de suas propriedades reológicas.

## **2- REVISÃO DE LITERATURA**

### **2.1 FILO BASIDIOMYCOTA**

Os fungos pertencentes ao filo basidiomycota possuem hifas septadas e são denominados basidiomicetos. Esses organismos produzem esporos fúngicos sexuais, chamados de basidiósporos, os quais são formados externamente em um pedestal, denominado basídio. Alguns basidiomicetos produzem esporos assexuais, chamados conidiósporos, os quais são abrigados em uma hifa aérea denominada conidióforo (TORTORA; FUNKE; CASE, 2017).

No filo Basidiomycota, três subfilos são existentes: Agaricomycotina, Pucciniomycotina e Ustilaginomycotina (MAO; WANG, 2019). Dentre esses, os membros do subfilo Agaricomycotina são as principais espécies envolvidas na desconstrução de biomassa lignocelulósica (SANTOS, 2018). Além disso, o Agaricomycotina contém cerca de um terço das espécies de fungos já descritas, incluindo cogumelos, fungos gelatinosos e leveduras (HIBBETT, 2006).

O subfilo Agaricomycotina, por sua vez, possui três classes: Tremelomicetes, Dacrymycetes e Agaricomycetes (HIBBETT, 2006). Os representantes da classe Agaricomycetes possuem como característica o desenvolvimento de basidiomas (cogumelos), onde são produzidos os basídios e basidiosporos. Grande parte dos representantes desse grupo degradam componentes da madeira, sendo assim chamados de lignolíticos ou lignocelulolíticos. Além disso, podem agir como simbiontes ou como parasitas obrigatórios ou facultativos (LIRA, 2012).

#### **2.1.1 Classe Agaricomycetes**

Os cogumelos, fungos pertencentes a classe Agaricomycetes, são definidos como macrofungos cujo corpo de frutificação pode ser epígeo (acima do solo) ou hipógeo (sob o solo) (figura 1) (CHANG; MILES, 1992; RATHORE et al., 2019).

FIGURA 1 – EXEMPLOS DE BASIDIOMAS (CORPO DE FRUTIFICAÇÃO OU COGUMELO). TODOS OS EXEMPLARES FORAM ENCONTRADOS NO CENTRO POLITÉCNICO E NO CAMPUS BOTÂNICO DA UNIVERSIDADE FEDERAL DO PARANÁ, CURITIBA, BRASIL.



Fonte: A autora (2020).

Para a formação desses corpos de frutificação, também chamados de basiodioma ou de cogumelo, as hifas fúngicas se modificam, formando pseudotecidos, os quais se distinguem nas diversas partes do cogumelo (píleo, estipe, lamelas, anel e volva) (PUTZKE; PUTZKE, 1998).

Os cogumelos comestíveis são, há séculos, utilizados na culinária como alimento e para aromatização, devido ao seu sabor único e sutil (RATHORE et al., 2019). Além disso, os mesmos são considerados uma iguaria devido às suas características sensoriais. Do ponto de vista nutricional, os cogumelos são alimentos valiosos, uma vez que possuem uma quantidade significativa de fibras dietéticas, baixa quantidade de gordura e baixo índice calórico (RAMOS; ANDRADE, 2017). Adicionalmente, esse alimento é fonte de diversos compostos nutraceuticos, como polissacarídeos, peptídeos, glicoproteínas, ácidos graxos insaturados, minerais, vitaminas, terpenos e antioxidantes como os compostos fenólicos. (WANI; BODHA; WANI, 2010; RAMOS; ANDRADE, 2017; RATHORE; PRASAD; SHARMA, 2017).

Esse fungos não são utilizados apenas como alimento, mas seu uso se expandiu a fins farmacêuticos, nutraceuticos e cosmeceuticos (PARDESHI;



PARDESHI, 2009 *apud* RATHORE; PRASAD; SHARMA, 2017). Devido a suas diversas propriedades farmacológicas, os mesmos tornaram-se objeto de interesse para a manutenção da saúde e prevenção de doenças (KUSHAIRI et al., 2020). As pesquisas farmacológicas confirmam grande parte do conhecimento tradicional a respeito dos efeitos medicinais dos cogumelos e demonstram que os compostos bioativos presentes nesses organismos resultam em diversas propriedades biológicas (WANI; BODHA; WANI, 2010). Dentre essas propriedades foram relatados o efeito antitumoral (GOMES et al., 2019; ZHAO; ZENG, 2021), imunomodulador (CHAIYAMA et al., 2020; YOO et al., 2020), anti-inflamatório (GOMES et al., 2019; KUSHAIRI et al., 2020) antifúngico (LIU et al., 2020; JALOOT et al., 2020), antibacteriano (HEARST et al., 2009; DUVNJAK et al., 2016), antioxidante (GOMES et al., 2019; KRÜZSELYI; MÓRICZ; VETTER, 2020), antiviral (HE et al., 2020; SILLAPACHAIYAPORN; CHUCHAWANKUL, 2020) e prebiótico (KHAN et al., 2018; SAWANGWAN, et al., 2018).

Muitos cogumelos tiveram o seu potencial de aplicação médica e industrial estudados, como o *Agaricus bisporus* (RAMOS et al., 2019; BLUMFIELD et al., 2020), *Agaricus brasiliensis* (CARVAJAL et al., 2012; ZHANG et al., 2018), *Lentinula edodes* (GARCIA et al., 2020; WANG et al., 2020b), *Ganoderma lucidum* (KHAN et al., 2018; WANG et al., 2020c) e *Pleurotus eryngii* (KIKUCHI et al., 2019; ABREU et al., 2021). Todavia, pode ainda haver milhares de espécies de cogumelos cujos possíveis benefícios para a humanidade devem ser investigados (BEULAH; MARGRET; NELSON, 2013)

#### 2.1.1.1 *Macrocybe titans*

O *Macrocybe titans* é um fungo saprófito, o que significa que o mesmo utiliza matéria vegetal morta em decomposição para o seu desenvolvimento e não causa doenças ou o apodrecimento de gramíneas e árvores vivas (DELONG; BREWER, 2013; KARLSEN-AYALA; SMITH, 2020).

O nome *Macrocybe titans* significa “cabeça gigante”, o que faz jus ao fato de esse ser o maior cogumelo lamelado conhecido no hemisfério ocidental (KARLSEN-AYALA; SMITH, 2020). Os corpos de frutificação (cogumelos) do gênero *Macrocybe* crescem como cespitosos (estipes unidos pela base) e atingem grandes dimensões.

Para a espécie *Macrocybe titans* já foram relatados espécimes de até 100 cm de diâmetro (PEGLER; LODGE; NAKASONE, 1998) (FIGURA 2). Esses corpos de frutificação possuem um aspecto robusto, coloração creme, cheiro intenso de farinha fresca e um agradável sabor adocicado em sua carne (CALONGE; MATA; UMAÑA, 2007).

O uso desse fungo como alimento já foi relatado, uma vez que os mesmos são coletados e utilizados pelo povo indígena Hoti na Amazônia venezuelana (ZENT; ZENT; ITURRIAGA, 2004). Devido ao tamanho desse cogumelo, o seu cultivo em grande escala pode ser interessante para a alimentação humana (CALONGE; MATA; UMAÑA, 2007).

FIGURA 2 – CORPOS DE FRUTIFICAÇÃO (COGUMELOS) DO FUNGO *Macrocybe titans*.



Fonte: A autora (2021).

Sobre sua distribuição geográfica, tal espécie se desenvolve em países tropicais e subtropicais (DELONG; BREWER, 2013; KARLSEN-AYALA; SMITH, 2020). Ela foi originalmente encontrada na Flórida em 1980 por Howard Bigelow e James Kimbrough, sendo descrita como *Tricholoma titans* (KARLSEN-AYALA; SMITH, 2020). Todavia, baseado em análises morfológicas, genéticas e ecológicas, a mesma foi reclassificada para o gênero *Macrocybe* (PEGLER; LODGE; NAKASONE, 1998). Espécimes desse fungo foram relatadas em diversas localidades, como na Índia (AMANDEEP; ATRI; MUNRUCHI, 2015), Costa Rica (CALONGE; MATA; UMAÑA, 2007), Colômbia (CORRALES; LOPEZ-QUINTERO, 2005), Geórgia

(DELONG; BREWER, 2013), Argentina (RAMIREZ et al., 2017) e Brasil (SAYKA, 2008; COIMBRA; GIBERTONI, 2009; TIBÉRIO et al., 2014).

Quanto aos estudos relativos a composição bioquímica dessa espécie, bem como possíveis aplicações farmacológicas ou industriais, algumas pesquisas foram realizadas até o momento, como a avaliação da citotoxicidade de seu extrato frente a artemia salina, além de potencial antioxidante de seus fenóis e flavonoides (KNAK; GUEDES; TAVARES, 2012), atividade anti-helmíntica (PEREIRA et al., 2014), potencial para a produção de enzimas oxidativas e hidrolíticas (ZILLY et al., 2012). Além disso, foi observado que uma fucogalactana isolada do mesmo apresentou bioatividade em células de melanoma murino B16F10 *in vitro*, reduzindo a migração celular sem causar citotoxicidade (MILHORINI et al., 2018). No entanto, outras frações polissacarídicas e extratos oriundos do *Macrocybe titans* foram obtidas por Silva (2017), porém, não tiveram sua estrutura química, propriedades biológicas e reológicas estudadas. Esses extratos e frações foram agora elucidados e os resultados serão apresentados no presente trabalho.

#### 2.1.1.2 *Ganoderma lucidum*

O cogumelo *Ganoderma lucidum*, também chamado de Reishi ou Ligzhi (ENSHASY; KAUL, 2013; MONEY, 2016), é considerado um cogumelo não comestível, devido ao seu corpo frutífero ser grosso e resistente, não possuindo uma estrutura carnuda como os cogumelos comumente utilizados na alimentação (BABY; JOHNSON; GOVINDAN, 2015), além de apresentar um sabor amargo (NIE et al., 2013) (FIGURA 2). Entretanto, desde a antiguidade o mesmo já era utilizado na medicina oriental, visando aumentar a longevidade e promover a saúde (SHEIKH et al., 2017), o que o fez ser popularmente denominado como cogumelo da imortalidade (AHMAD et al., 2020).



FIGURA 3 – CORPO DE FRUTIFICAÇÃO (COGUMELOS) DO FUNGO *Ganoderma lucidum*.



Fonte: <https://www.pinterest.pt/pin/408912841146778851/> Acesso: 10/10/2020.

Conforme Leskosek-Cukalovic et al., (2010), esse cogumelo é comercializado e pode ser encontrado na forma de chás, pó e extratos. Esses produtos podem ser sintetizados a partir do corpo de frutificação, micélio cultivado ou esporos, sendo utilizados para o tratamento de diversas doenças, incluindo, o câncer. O potencial medicinal desse fungo é decorrente da presença de compostos bioativos, sendo que, os polissacarídeos são considerados uma das principais substâncias bioativas do gênero *Ganoderma* spp. (NIE et al., 2013; AHMAD et al., 2020)

Os polissacarídeos de *Ganoderma lucidum* são majoritariamente  $\beta$ -glucanas ramificadas (Lu et al., 2020), embora glucanas lineares (WANG; ZHANG, 2009; WIATER et al., 2012) e heteropolissacarídeos (MIYAKAZI; NISHIJMA, 1982), já tenham sido reportados.

Estudos indicam que extratos e frações polissacarídicas do *Ganoderma lucidum* apresentam atividade imunomoduladora (SUI et al., 2016; LU et al., 2020), anti-inflamatória (KUSHAIRI et al., 2020) antioxidante (KAO et al., 2012; ZENG et al., 2019), prebiótica (KHAN et al., 2018), hipoglicêmica (XIAO et al., 2012), hipolipidêmica (XU et al., 2019) e antitumoral (SHEN et al., 2014; SOHRETOGLU; HUANG, 2018).

## 2.2 POLISSACARÍDEOS DE COGUMELOS: ESTRUTURA QUÍMICA, PROPRIEDADES REOLÓGICAS E BIOLÓGICAS

A maior parte dos carboidratos são encontrados na natureza na forma de polissacarídeos, que são polímeros de média e alta massa molecular (NELSON; COX, 2019). Essas moléculas diferem umas das outras quanto as suas unidades monossacarídicas, comprimento das cadeias, tipos de ligações, grau de ramificação, configuração do carbono anomérico e massa molecular (RUTHES; SMIDERLE; IACOMINI, 2015; RUTHES; SMIDERLE; IACOMINI, 2016).

Os polissacarídeos podem ocorrer na forma de homopolissacarídeos, quando apenas um tipo de monossacarídeo constitui o polímero, ou como heteropolissacarídeos, que são compostos por dois ou mais tipos de monossacarídeos. Essas moléculas possuem diferentes funções vitais, como por exemplo, função estrutural na parede celular ou armazenamento de monossacarídeos que são utilizados como combustíveis (NELSON; COX, 2019).

Diversas espécies de cogumelos já foram estudadas quanto a seus polissacarídeos, como o *Agaricus bisporus* (SMIDERLE et al., 2013; PIRES et al., 2017), *Amanita muscaria* (RUTHES et al., 2013), *Lentinus edodes* (MAITY et al., 2013; MORALES et al., 2020), *Pleurotus eryngii* (ABREU et al., 2021), *Hericium erinaceus* (CHEN et al., 2020), *Pholiota nameko* (SOVRANI et al., 2017; ABREU et al., 2019), *Macrocybe titans* (MILHORINI et al., 2018), *Ganoderma lucidum* (KUSHAIRI et al., 2020; LU et al., 2020), dentre outros.

Esses polímeros apresentam múltiplos efeitos biológicos e propriedades reológicas de interesse, o que instiga a comunidade científica a estudar as possibilidades da aplicação dos mesmos na área médica e industrial. Conforme Elisashvili (2012), essas atividades biológicas apresentadas pelos polissacarídeos estão diretamente relacionadas com a estrutura química dos mesmos. Diversos trabalhos demonstram que diferenças na estrutura de um polissacarídeo, seja quanto a sua massa molecular, ou grau de ramificação, por exemplo, podem resultar em uma diferença significativa na atividade biológica resultante (RUTHES et al., 2013a; RUTHES et al., 2013b). Devido a isso, torna-se interessante realizar a avaliação das características químicas da molécula em conjunto com as análises reológicas ou biológicas, visando compreender quais características químicas são necessárias para a obtenção de determinada atividade potencializada.

### 2.2.1 Homopolissacarídeos de cogumelos

Variados tipos de homopolissacarídeos já foram obtidos de distintas espécies de cogumelos, como:  $\beta$ -glucanas, as quais podem ser lineares (SILVEIRA et al., 2014; MORALES et al., 2020) ou ramificadas (SILVA, 2017; JESUS et al., 2018a);  $\alpha$ -glucanas, as quais também podem ser lineares (JESUS et al., 2018b; MORALES et al., 2020) ou ramificadas (KOMURA et al., 2014; CUI et al., 2020); além de mananas (CHENG et al., 2020) e galactanas (BRITO et al., 2018).

Essas moléculas apresentaram diferentes efeitos biológicos (RUTHES et al., 2013a; CASTRO-ALVES; NASCIMENTO, 2018; ABREU et al., 2021) e propriedades reológicas (SOVRANI et al., 2017; XU et al., 2016; ABREU et al., 2019), as quais serão discutidas a seguir.

#### 2.2.1.1 Propriedades biológicas de homopolissacarídeos obtidos de cogumelos

As glucanas são os principais componentes presentes nos extratos de cogumelos, portanto, provavelmente são as principais responsáveis pelos efeitos medicinais alcançados. A  $\beta$ -D-glucana mais comumente encontrada é constituída por uma cadeia principal de  $\beta$ -D-glucose ligada (1 $\rightarrow$ 3), frequentemente ramificada na posição O-6 por resíduos de  $\beta$ -D-glucose (RUTHES; SMIDERLE; IACOMINI, 2015).

Moléculas com essas características foram obtidas de diversos Agaricomycetes, como por exemplo, do cogumelo *Lactarius rufus*, a partir do qual os autores isolaram o polissacarídeo e observaram que o mesmo possui efeito anti-inflamatório e antinociceptivo (RUTHES et al., 2013c).

Uma  $\beta$ -glucana também constituída por ligações (1 $\rightarrow$ 3; 1 $\rightarrow$ 6), conhecida como lentinana, foi obtida do cogumelo *Lentinula edodes* e devido a sua significativa atividade imunomoduladora, além de seu efeito antioxidante, a mesma foi capaz de reduzir a tempestade de citocinas decorrente da infecção por COVID-19, o que possui grande significância terapêutica, uma vez que a liberação desregulada de citocinas leva ao quadro de síndrome do desconforto respiratório agudo (MURPHY et al., 2020). Além disso, essa molécula é amplamente conhecida pelo seu efeito antitumoral desde

a década de 60, quando foi isolada e testada pela primeira vez por Chihara et al., (1969).

Além das  $\beta$ -glucanas ramificadas, moléculas lineares também podem ser encontradas e já foram relatadas para *Cordyceps militaris* (SMIDERLE et al., 2014), *Pleurotus sajor-caju* (SILVEIRA et al., 2014), *Lentinula edodes* (MORALES et al., 2020), *Agaricus bisporus* e *Agaricus brasiliensis* (SMIDERLE et al., 2013). Esses polímeros apresentaram diferentes atividades biológicas, como anti-inflamatória (SILVEIRA et al., 2014; MORALES et al., 2020) imunomoduladora (SMIDERLE et al., 2013; SILVEIRA et al., 2014) e hipocolesterolêmica (MORALES et al., 2020).

Apesar de serem os mais abundantes, as  $\beta$ -glucanas não são os únicos homopolissacarídeos possíveis de serem obtidos dos Agaricomycetes e os efeitos biológicos de interesse médico não estão limitados a elas. Outros homopolissacarídeos, como as  $\alpha$ -glucanas, mananas e galactanas também já foram obtidas de diferentes espécies de cogumelo e apresentaram atividades biológicas relevantes.

A exemplo, uma  $\alpha$ -D-glucana ramificada obtida a partir do cogumelo *Volvariella volvacea*, apresentou efeito imunomodulador, aumentando significativamente a expressão do mRNA de TNF- $\alpha$ , IL-6 e IL-1 $\beta$  em macrófagos RAW264.7 *in vitro* (CUI et al., 2020). Por outro lado, uma  $\alpha$ -D-glucana linear ligada (1 $\rightarrow$ 4), obtida do *Lentinula edodes*, demonstrou os efeitos hipercolesterolêmico e anti-inflamatório (MORALES et al., 2020).

Lemieszek e colaboradores (2019), isolaram uma manana ramificada do cogumelo *Cantharellus cibarius* e observaram que a mesma foi capaz de suprimir a proliferação de células cancerosas do cólon e de destruir a integridade da membrana celular, porém, sem afetar adversamente as células epiteliais do cólon. Yang e autores (2019), por sua vez, isolaram uma galactana linear parcialmente metilada a partir do cogumelo *Cantharellus cibarius* e obtiveram que essa molécula possui efeito imunomodulador, atuando no aumento da fagocitose de macrófagos, na liberação de NO e secreção de TNF- $\alpha$ , IL-6 e IL-1 $\beta$ .

Uma relação de diferentes homopolissacarídeos obtidos de distintas espécies de cogumelos, bem como detalhes a respeito de suas estruturas químicas, como monossacarídeo constituinte, tipos de ligação, grau de ramificação e massa molecular, encontram-se dispostas na tabela 1.

TABELA 1 – EXEMPLOS DE HOMOPOLISSACARÍDEOS OBTIDOS DE COGUMELOS E SEUS RESPECTIVOS EFEITOS BIOLÓGICOS.

<b>Homopolissacarídeo</b>	<b>Cadeia principal</b>	<b>Ramificação</b>	<b>Massa molar</b>	<b>Efeito biológico</b>	<b>Fonte</b>	<b>Referência</b>
<b><math>\beta</math>-glucana</b>	$\rightarrow 3$ )- $\beta$ -D-Glcp-(1 $\rightarrow$	Substituição em O-6 por terminais de $\beta$ -D-Glcp-(1 $\rightarrow$ , ocorrendo 2 ramificações a cada 3 resíduos da cadeia principal.	$9,75 \times 10^5$ g.mol <sup>-1</sup>	Imunomodulador	<i>Pleurotus sajor-caju</i>	Carbonero et al., (2012)
	$\rightarrow 3$ )- $\beta$ -D-Glcp-(1 $\rightarrow$	Substituição em O-6 por terminais de $\beta$ -D-Glcp-(1 $\rightarrow$ e em menor proporção por oligossacarídeos formados por $\rightarrow 3$ )- $\beta$ -D-Glcp-(1 $\rightarrow$	$1,6 \times 10^4$ g.mol <sup>-1</sup>	Antinociceptivo	<i>Amanita muscaria</i>	Ruthes et al., (2013a)
	$\rightarrow 3$ )- $\beta$ -D-Glcp-(1 $\rightarrow$	Substituição em O-6 por $\beta$ -D-Glcp-(1 $\rightarrow$ , ocorrendo 4 ramificações a cada 100 resíduos da cadeia principal	$3,7 \times 10^4$ g.mol <sup>-1</sup>	Imunomodulador	<i>Pleurotus albidus</i>	Castro-Alves et al., (2017); Castro-Alves e Nascimento (2018)
	$\rightarrow 3$ )- $\beta$ -D-Glcp-(1 $\rightarrow$	Substituição em O-6 por $\beta$ -D-Glcp-(1 $\rightarrow$ , ocorrendo 1 ramificação a cada 5 resíduos da cadeia principal.	$1 \times 10^6$ g.mol <sup>-1</sup>	Cicatrizante	<i>Piptoporus betulinus</i>	Jesus et al., (2018b)
	$\rightarrow 6$ )- $\beta$ -D-Glcp-(1 $\rightarrow$	Ausente	$1,3 \times 10^4$ g.mol <sup>-1</sup>	Imunomodulador	<i>Pleurotus eryngii</i>	Abreu et al., (2021)
<b><math>\alpha</math>-glucana</b>	$\rightarrow 6$ )- $\beta$ -D-Glcp-(1 $\rightarrow$	Ausente	$6,536$ g.mol <sup>-1</sup>	Hipocolesterolêmico, anti-inflamatório, antioxidante e citotóxico em células tumorais MDA-MB-231	<i>Lentinula edodes</i>	Morales et al., (2020)
	$\rightarrow 3$ )- $\beta$ -D-Glcp-(1 $\rightarrow$	Ausente	$6,0 \times 10^4$ g.mol <sup>-1</sup>	Anti-inflamatório	<i>Pleurotus sajor-caju</i>	Silveira et al., (2014)
	$\rightarrow 4$ )- $\alpha$ -D-Glcp-(1 $\rightarrow$	Substituição em O-6 por terminais de $\beta$ -D-Glcp-(1 $\rightarrow$ , ocorrendo 1 ramificação a cada 8 resíduos da cadeia principal.	$1435,6 \times 10^4$ g.mol <sup>-1</sup>	Imunomodulador	<i>Volvariella volvacea</i>	Cui et al., (2020)

TABELA 1– EXEMPLOS DE HOMOPOLISSACARÍDEOS OBTIDOS DE COGUMELOS E SEUS RESPECTIVOS EFEITOS BIOLÓGICOS. (CONTINUAÇÃO)

<b>Manana</b>	→3)-α-D-Glcp-(1→	Ausente	3,9±0.8x10 <sup>5</sup> g.mol <sup>-1</sup>	Não avaliado	Fomitopsis betulina Lentinula edodes	Jesus et al., (2018a) Morales et al., (2020)
	→3)-α-D-Glcp-(1→	Ausente	Não avaliado	Hipocolesterolêmico, anti-inflamatório e citotóxico em células tumoriais MDA-MB-231		
	→3)-α-D-Manp- (1→ e →2)-α-D- Manp-(1→	Substituição no O-6 por →2)- α-D-Manp-(1→, por →6)-α-D- Manp-(1→ e por terminais de α-D-Manp-(1→.	9,43x10 <sup>4</sup> g.mol <sup>-1</sup>	Inibição da proliferação das células tumorais HepG2 e MCF-7 <i>in</i> <i>vitro</i> .	Phellinus igniarius	Cheng et al., (2020)
	→6)-α-D-Manp- (1→	Substituição em O-6 por →2)- β-D-Manp-(1→ e →3)- β-D- Manp-(1→ com diferentes comprimentos.	3,2x10 <sup>4</sup> g.mol <sup>-1</sup> – 4,6x10 <sup>4</sup> g.mol <sup>-1</sup>	Não avaliado	Pleurotus ostreatus var. florida	Komura et al., (2010)
	→6)-α-D-Manp- (1→	Substituição em O-2 por →2)- α-D-Manp-(1→ e por terminais de α-D-Manp-(1→, ocorrendo 1 ramificação a cada 2 unidades da cadeia principal.	67,1x10 <sup>4</sup> g.mol <sup>-1</sup>	Não avaliado	Cantharellus cibarius	Nyman et al (2016)
<b>Galactana</b>	→6)-α-D-Galp-(1→ e →6)-α-D-3-O- metil-Galp-(1→ em uma razão molar de 3: 1	Ausente	17,9x10 <sup>4</sup> g.mol <sup>-1</sup> ( <i>Pleurotus eryngii</i> ); 16,5x10 <sup>4</sup> g.mol <sup>-1</sup> ( <i>Pleurotus</i> <i>ostreatoroseus</i> )	Não avaliado	<i>Pleurotus eryngii</i> e <i>Pleurotus</i> <i>ostreatoroseus</i>	Carbonero et al., (2008)
	→6)-α-D-Galp-(1→ e →6)-α-D-3-O- metil-Galp-(1→. Duas moléculas foram isoladas, uma com razão molar de 2:1 e outra de 1:1	Ausente	37,6x10 <sup>3</sup> g.mol <sup>-1</sup> (para a molécula com razão molar 2:1); 28,5x10 <sup>3</sup> g.mol <sup>-1</sup> (para a molécula com razão molar 1:1)	Não avaliado	<i>Pleurotus</i> <i>citrinopileatus</i>	Brito et al., (2018)



### 2.2.1.2 Propriedades reológicas de $\beta$ -glucanas obtidas de cogumelos

Os homopolissacarídeos  $\beta$ -glucanas, além de apresentarem diversos efeitos biológicos, podem, dependendo de suas características físico-químicas, apresentarem propriedades de geleificação, espessamento e emulsificação (YANG et al., 2020), abrindo possibilidades de sua utilização na indústria alimentícia, farmacêutica e cosmética (ALVES et al., 2010).

Devido a isso, essas moléculas são utilizadas visando alcançar uma textura desejável nos produtos alimentícios (TONELI; MURR; PARK, 2005; YANG et al., 2020). Sua adição influencia ainda no sabor, qualidade e prazo de validade desses produtos (ABREU et al., 2019). Adicionalmente, devido as propriedades nutricionais e medicinais dos cogumelos, os mesmos são estabelecidos como alimentos nutracêuticos, o que se deve a presença de moléculas bioativas, como as  $\beta$ -glucanas. Por conseguinte, extratos contendo essas biomoléculas tem sido largamente testados pela indústria farmacêutica e de alimentos, para o desenvolvimento de alimentos funcionais (RATHORE; PRASAD; SHARMA, 2017).

Segundo Zhu, Du e Xu (2016), as  $\beta$ -glucanas podem ser utilizadas de diferentes formas. Quando na alimentação humana, por exemplo, esse polímero pode ser aplicado na fabricação de pães sem glúten, uma vez que esses produtos possuem um bom aceite pelas análises sensoriais; em lanches extrudados prontos para consumo, pois sua adição leva à manipulação da resposta glicêmica; em bebidas, levando ao controle da ingestão de alimentos, reduzindo o consumo de energia durante determinado período de tempo. Além disso, esses polissacarídeos podem ser utilizados como aditivo alimentar para animais de produção, ocasionando a melhora da resposta imune desses indivíduos. Quanto a aplicação na área médica, há possibilidade da utilização de poli-membranas contendo  $\beta$ -glucanas, visando acelerar o processo de cicatrização. Quando aplicada em produtos cosméticos, essas moléculas são efetivas no processo de hidratação, revitalização e cicatrização da pele, além de apresentarem efeito antienvelhecimento. Elas influenciam ainda as características físicas do produto, levando, por exemplo, a formação de filme após a aplicação de um hidratante.

A avaliação das propriedades físico-químicas dos polissacarídeos, como sua viscosidade e capacidade gelificante, visando investigar as possibilidades de utilização desses polímeros em produtos específicos, é realizada por meio de estudos

reológicos (NICKERSON; PAULSON; SPEERS, 2004; XU et al., 2016; SOVRANI et al., 2017; ABREU et al., 2019). Uma vez que as propriedades reológicas variam de acordo com as características químicas e concentração do polissacarídeo (BAO et al., 2016; ABREU et al., 2019), o estudo de suas estruturas químicas e das condições ideais para a obtenção do efeito mais adequado, podem ser avaliados. Uma relação de diferentes  $\beta$ -glucanas, obtidas de distintas espécies de cogumelos, bem como detalhes a respeito de suas estruturas químicas e propriedades reológicas, é encontrada na tabela 2.

TABELA 2 – EXEMPLOS DE  $\beta$ -GLUCANAS OBTIDAS DE COGUMELOS E SUAS RESPECTIVAS PROPRIEDADES REOLÓGICAS.

<b>Cadeia principal</b>	<b>Ramificação</b>	<b>Massa molar</b>	<b>Propriedades reológicas</b>	<b>Fonte</b>	<b>Referência</b>
<b><math>\rightarrow 3</math>)-<math>\beta</math>-D-Glcp-(1<math>\rightarrow</math></b>	Substituição em O-6 por uma unidade de $\beta$ -D-Glcp-(1 $\rightarrow$ , ocorrendo 2 ramificações a cada 5 resíduos da cadeia principal	3 frações: 160,1x10 <sup>-4</sup> g.mol <sup>-1</sup> ; 141,2x10 <sup>-4</sup> g.mol <sup>-1</sup> ; 137,0x10 <sup>-4</sup> g.mol <sup>-1</sup>	Viscosidade dependente da concentração e temperatura	<i>Lentinula edodes</i>	Zhang; Xu; Zhang (2008)
<b><math>\rightarrow 3</math>)-<math>\beta</math>-D-Glcp-(1<math>\rightarrow</math></b>	Substituição em O-6 por uma unidade de $\beta$ -D-Glcp-(1 $\rightarrow$ , ocorrendo 1 ramificação a cada 3 ou 4 resíduos da cadeia principal	7,2x10 <sup>5</sup> g.mol <sup>-1</sup>	Viscosidade dependente da concentração, temperatura e frequência	Cogumelo floral	Xu et al., (2016)
<b><math>\rightarrow 3</math>)-<math>\beta</math>-D-Glcp-(1<math>\rightarrow</math></b>	Substituição em O-6 por $\rightarrow 6$ )- $\beta$ -D-Glcp-(1 $\rightarrow$ ou por unidades de $\beta$ -D-Glcp-(1 $\rightarrow$ , ocorrendo 1 ramificação a cada 3 ou 4 resíduos da cadeia principal	Não avaliado	Não-Newtoniano; Viscosidade dependente da concentração e da frequência	<i>Pholiota nameko</i>	Sovrani et al., (2017)
<b><math>\rightarrow 3</math>)-<math>\beta</math>-D-Glcp-(1<math>\rightarrow</math></b>	Substituição em O-6 por $\rightarrow 6$ )- $\beta$ -D-Glcp-(1 $\rightarrow$ ou por unidades de $\beta$ -D-Glcp-(1 $\rightarrow$	1,4x10 <sup>6</sup> g.mol <sup>-1</sup>	Não Newtoniano; Viscosidade dependente da frequência; Estabilidade térmica sob simulação de pasteurização	<i>Pholiota nameko</i>	Abreu et al., (2019)



### 2.2.2 Heteropolissacarídeos de cogumelos

Os cogumelos têm sido amplamente estudados não apenas por suas  $\beta$ -D-glucanas, mas também por apresentarem uma classe de polissacarídeos mais complexos, denominados heteropolissacarídeos (RUTHES; SMIDERLE; IACOMINI, 2016).

Essas moléculas normalmente são heterogalactanas, como as fucogalactanas (MILHORINI et al., 2018; SAMUELSEN et al., 2019), manogalactanas (SILVEIRA et al., 2015; ABREU et al., 2021), fucomanogalactanas (OLIVEIRA et al., 2019) e glucogalactanas (SUN; LIU, 2009), que podem conter grupamentos metil (KOMURA et al., 2014; SILVEIRA et al., 2015). Além de heteromananas, como galactomananas (TEL-ÇAYAN et al., 2020), xilomanana (SMIDERLE et al., 2006) e glucuronoxilomananas (YUAN et al., 2020); ou heteroglucanas, como manogalactoglucanas (PIRES et al., 2017) e xiloglucana (MORADALI et al., 2007).

#### 2.2.2.1 Atividades biológicas de heteropolissacarídeos obtidos de cogumelos

Assim como os homopolissacarídeos, os heteropolissacarídeos são reportados por possuírem diferentes efeitos biológicos, como a atividade antitumoral (BISCAIA, 2016), imunomoduladora (LI et al., 2016; YUAN et al., 2019, anti-inflamatória e antinociceptiva (RUTHES et al., 2013b; SILVEIRA et al., 2015) e gastroprotetora (CHEN et al., 2020).

Uma fucogalactana obtida do cogumelo *Macrocybe titans*, quando testada em células de melanoma murino B16F10 *in vitro*, não apresentou citotoxicidade e não alterou a capacidade proliferativa e a morfologia das células. Todavia, esse polissacarídeo foi capaz de reduzir a migração celular *in vitro* em 40% (100  $\mu$ g/mL) e em 33% (250  $\mu$ g/mL) (MILHORINI et al., 2018). Uma fucogalactana isolada do *Ganoderma lucidum*, por sua vez, apresentou efeito imunomodulador, uma vez que a mesma estimulou a proliferação de linfócitos obtidos do baço de camundongos (YE et al., 2008). Essa heterogalactana foi também isolada do *Agaricus bisporus* e apresentou atividade anti-sepse, antinociceptiva e anti-inflamatória (RUTHES et al., 2013b) e do *Hericium erinaceus*, demonstrando efeito gastroprotetor (CHEN et al., 2020).

Abreu e colaboradores (2021) obtiveram, a partir do cogumelo *Pleurotus eryngii*, um extrato rico em manogalactana, o qual apresentou atividade imunomoduladora, estimulando a liberação de óxido nítrico, IL-1 $\beta$  e IL-10 por células THP-1. Uma manogalactana parcialmente metilada, obtida do *Pleurotus sajor-caju*, por Silveira e colaboradores (2015), foi capaz de reduzir a nocicepção nos testes de contorção e de formalina, além de reduzir o edema induzido por carragenina, indicando, portanto, possuir efeito antinociceptivo e anti-inflamatório.

Uma fucomanogalactana obtida de *Hypsizygus marmoreus* (OLIVEIRA et al., 2019), assim como a fucogalactana obtida do *Macrocybe titans* (MILHORINI et al., 2018), não foi citotóxica, nem alterou a morfologia e proliferação de células de melanoma murino B16-F10. No entanto, essa molécula foi capaz de inibir a capacidade de formação de colônias e de migração celular. Já uma fucomanogalactana do cogumelo *Amanita muscaria*, foi obtida e testada por Ruthes et al., (2013a) e apresentou uma potente inibição da dor inflamatória.

O cogumelo *Rhizopogon luteolus* foi utilizado por Tel-Çayan et al., (2020), para a obtenção de uma galactomanana e os autores observaram que essa biomolécula atuou como um agente anticolinesterase.

Pires e colaboradores (2017), isolaram uma manogalactoglucan do *Agaricus bisporus*, a qual foi capaz de reduzir a viabilidade de células HepG2, promover o aumento da liberação do citocromo C e diminuição do conteúdo de ATP, induzindo a apoptose pela via de morte mitocondrial.

Uma glucuronoxilomanana foi obtida do cogumelo *Tremella aurantialba*, e quando avaliada sobre macrófagos murinos RAW264.7, a mesma promoveu a secreção de óxido nítrico, IL-1 $\beta$  e TNF- $\alpha$ , sendo o receptor celular para essa molécula, identificado como TLR4 (YUAN et al., 2020).

Uma relação dos heteropolissacarídeos supracitados, dentre outros, bem como detalhes a respeito de suas estruturas químicas, se encontra listado na tabela 3.

TABELA 3 – EXEMPLOS DE HETEROPOLISSACARÍDEOS DE COGUMELOS E SEUS RESPECTIVOS EFEITOS BIOLÓGICOS.

Heteropolissacarídeo	Cadeia principal	Ramificação	Massa molar	Efeito biológico	Fonte	Referência
<b>Fucogalactana</b>	→3)-α-D-Galp-(1→	Substituição em O-6 por uma unidade de →3)-α-D-Galp-(1→ com terminal de α-L-Fucp-(1→, ou por um oligossacarídeo de →6)-α-D-Galp-(1→ com terminal de α-L-Fucp-(1→, ocorrendo as 2 ramificações a cada 5 unidades da cadeia principal	Cinco populações de polissacarídeos: 610,7; 40,7; 7,9; 1,6 e 0,3 kDa	Anti-inflamatório	<i>Poria cocos</i>	Lu et al., (2010)
	→6)-α-D-Galp-(1→	Substituição em O-2 por terminais de α-L-Fucp-(1→, ocorrendo 1 ramificação a cada 3 unidades da cadeia principal	1,5x10 <sup>4</sup> g.mol <sup>-1</sup>	Imunomodulador	<i>Hericium erinaceus</i>	Li et al., (2016)
	→6)-α-D-Galp-(1→	Substituição em O-2 por terminais de α-L-Fucp-(1→, ocorrendo 1 ramificação a cada 2-3 unidades da cadeia principal	14,2x10 <sup>3</sup> g.mol <sup>-1</sup>	Gastroprotetor e Anti-inflamatório Redução da migração de células tumorais B16F10, sem alterar a viabilidade celular	<i>Macrocybe titans</i>	Chen et al., (2020) Milhorini et al., (2018)
<b>Manogalactana</b>	→6)-α-D-Galp-(1→ e →6)-α-D-3-O-metil-Galp-(1→ em uma razão molar de 1,25:1	Substituição em O-2 por terminais de α-D-Manp-(1→	4,4x10 <sup>4</sup> g.mol <sup>-1</sup>	Não avaliado	<i>Pleurotus ostreatus</i>	Komura et al., (2014)
	→6)-α-D-Galp-(1→ (39,7 %) e →6)-α-D-3-O-Me-Galp-(1→ (23,3 %)	Substituição em O-2 por terminais de β-D-Manp-(1→ (33,7 %)	6,4x10 <sup>4</sup> g.mol <sup>-1</sup>	Anti-inflamatório; Antinociceptivo	<i>Pleurotus sajor-caju</i>	Silveira et al., (2015)
<b>Glucogalactana</b>	→6)-α-D-Galp-(1→	Substituição em O-2 por terminais de β-D-Glc-(1→, ocorrendo 1 ramificação a cada 2 unidades da cadeia principal.	2,4x10 <sup>4</sup> g.mol <sup>-1</sup>	Imunomodulador	<i>Pleurotus ostreatus</i>	Sun e Liu (2009)

TABELA 3– EXEMPLOS DE HETEROPOLISSACARÍDEOS DE COGUMELOS E SEUS RESPECTIVOS EFEITOS BIOLÓGICOS. (CONTINUAÇÃO)

<b>Fucomanogalactana</b>	→6)-α-D-Galp-(1→	Substituição em O-2 por terminais de α-L-Fucp-(1→ (21,6 %) e β-D-Manp-(1→ (8,9 %))	25,5x10 <sup>3</sup> g.mol <sup>-1</sup>	Inibição da dor inflamatória	<i>Amanita muscaria</i>	Ruthes et al., (2013a)
	→6)-α-D-Galp-(1→	Substituição em O-2 por terminais de α-L-Fucp-(1→ e β-D-Manp-(1→	17,1x10 <sup>4</sup> g.mol <sup>-1</sup>	Inibição da capacidade de migração e de formação de colônias das células de melanoma murino B16F10; Ausência de citotoxicidade	<i>Hypsizygus marmoreus</i>	Oliveira et al., (2019)
<b>Galactomanana</b>	→4)-β-D-Manp-(1→	Substituição por terminais de α-D-Galp-(1→ em O-6 e por β-D-Manp-(1→ em O2	5240 g.mol <sup>-1</sup>	Baixo efeito antioxidante	<i>Rhizopogon luteolus</i>	Tel-Çayan et al., (2020)
	→4)-β-D-Manp-(1→	Substituição por terminais de α-D-Galp-(2→ em O-6 e por β-D-Manp-(1→ em O2	5090 g.mol <sup>-1</sup>	Baixo efeito antioxidante; Agente anticolestérol	<i>Ganoderma adspersum</i>	
<b>Manogalactoglucana</b>	→6)-α-D-Galp-(1→ (32,8%) e →6)-β-D-Glcp-(1→ (37,0 %))	Substituição por α-D-Galp no O-2 de →6)-α-D-Galp-(1→ (3,3%) e no O-2 e O-4 de 6)-β-D-Glcp-(1→ (3,6 %). Unidades 2)-β-D-Glcp-(1→ e 2)-α-D-Galp-(1→ também estão presentes.	1,8x10 <sup>4</sup> g.mol <sup>-1</sup>	Citotóxica em células tumorais HepG2	<i>Agaricus bisporus</i>	Pires et al., (2017)
<b>Glucuronoxilomanana</b>	→3)-α-D-Manp-(1→ e →2)-α-D-Manp-(1→	Substituição no O-2 da α-D-Manp por unidades de →3)-β-Xylp-(1→ com um terminal de β-Xylp-(1→ (proporção de ramificação para unidade da cadeia principal: 1:5), e →4)-β-GlcpA-(1→ com um terminal de α-D-Manp-(1→ (proporção de 2:5), além de grupamentos acetil ligados a sexta hidroxila da α-D-Manp (proporção de 1:5)	~62,4x10 <sup>4</sup> g.mol <sup>-1</sup>	Imunomodulador	<i>Tremella aurantialba</i>	Yuan et al., (2020)

### 3- OBJETIVOS

#### 3.1 OBJETIVOS GERAIS

O presente trabalho tem como objetivo geral extrair, purificar e caracterizar polissacarídeos dos cogumelos *Macrocybe titans* e *Ganoderma lucidum*, bem como avaliar os efeitos biológicos e propriedades reológicas de alguns desses polímeros.

#### 3.2 OBJETIVOS ESPECÍFICOS

- a) Obter os polissacarídeos dos basidiomas dos diferentes cogumelos através de extrações aquosas e alcalinas;
- b) Purificar as frações polissacarídicas pela aplicação de diversas metodologias;
- c) Elucidar a estrutura química dos polissacarídeos purificados;
- d) Avaliar as propriedades reológicas dos polissacarídeos formadores de gel;
- e) Avaliar a atividade dos heteropolissacarídeos obtidos em células de melanoma murino B16F10 e de câncer de mama MCF-7 e MDA-MB-231.

**ARTIGO I**

**Different molecular weight fucogalactans from *Macrocybe titans* mushroom promote distinct effect on breast cancer cell death**

**Different molecular weight fucogalactans from *Macrocybe titans* mushroom promote distinct effect on breast cancer cell death**

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**Abstract**

Cancer is one of the deadliest diseases worldwide. Despite the several available therapies, the mortality rate is still high, and many side effects affect patients' quality of life. Due to this, there is an incessant search for new therapies, that united effectiveness with specificity, leading to higher survival rates and lower deleterious effects. In this context, two fucogalactans (F-1 and F-2) were obtained from *Macrocybe titans*. These polysaccharides showed a similar chemical structure, with a (1→6)-linked  $\alpha$ -D-Galp main chain partially substituted at O-2 by non-reducing end units of  $\alpha$ -L-Fucp. However, F-2 has a Mw 22 times higher than F-1 ( $1.42 \times 10^4$  g/mol and  $3.12 \times 10^5$  g/mol, respectively). Both fucogalactans were able to reduce the cell viability of MCF-7 and MDA-MB-231 without affecting the non-tumoral cell line VERO. F-1 induced the cell cycle arrest of both tumoral cell lines in G1 and G2/M phases. On the other hand, F-2 showed cell accumulation in G1 and G2/M phases only for MDA-MB-231, but with a most significant difference than the obtained with F-1 treatment. Additionally, apoptosis and necrosis were only observed in MDA-MB-231 cells treated with F-2. Both fucogalactans are promisors to be used in further studies on breast cancer therapy, however, the one with the highest Mw (F-2) showed the strongest effect on the triple-negative cells MDA-MB-231.

**Keywords:** Fucogalactans; Molecular weight; *Macrocybe titans*; Breast cancer cells.



## 1 Introduction

In 2020, 19.3 million new cancer cases were estimated worldwide, being breast cancer the most commonly diagnosed, since 2.3 million new cases had occurred (Sung et al., 2021). As reported by the National Cancer Institute, approximately 12.9 % of women will be diagnosed with breast cancer at some point during their lifetime, having a rate of relative survival of 90.3 %.

According to the American Cancer Society, about 2 of 3 breast cancers are formed by cells with estrogen and/or progesterone receptors, which help them grow and spread. Based on this, endocrine or hormone therapy (HT) is used to lower hormone levels or to prevent these hormones to bind the receptors on breast cancer cells. However, such therapeutic approach does not help patients whose tumors do not have hormone receptors, such as the triple-negative breast cancer cells. Besides that, HT presents marked side effects contributing to low rate of adherence and persistence during treatment (Peddie et al., 2021).

Based on the poor options for breast cancer treatments, it is necessary to find alternative therapies that could treat both: hormone sensitive and triple-negative breast cancers, with lower side effects. For this purpose, several cell lines are available for *in vitro* studies as a preliminary trial of potential antitumoral agents. For instance, MCF-7 is an estrogen and progesterone receptor-positive breast cell line, poorly aggressive and non-invasive, normally being considered to have low metastatic potential (Comşa, Cîmpean & Raica, 2015). On the other hand, MDA-MB-231 cells do not express such receptors (Flodrova et al., 2016). This triple-negative cell line is more aggressive than MCF-7, has and it presents high invasive and migration capacities (Comsa, Cîmpean & Raica, 2015; Razak et al., 2019).

In this context, mushrooms have been largely explored, once they possess several bioactive substances with antitumoral properties (Gomes et al., 2019; Yadav & Negi 2021). Water extracts from different mushroom species have been reported as antiproliferative, apoptotic, growth inhibitor, and reducer of colony formation in breast cancer cells (Gu & Leonard, 2006; Vaz et al., 2010; Lee et al., 2012; Yap et al., 2013; Zhang et al., 2017). Some of these extracts contain mainly carbohydrates (Vaz et al., 2010; Lee et al., 2012). It is an interesting feature once polysaccharides are the best known and most potent mushroom-derived compounds with antitumor properties (Smiderle, Ruthes & Iacomini, 2014).

Although some studies have reported the effect of isolated polysaccharides on MCF-7 and MDA-MB-231 cell lines, such as a  $\beta$ -glucan from *Lentinus edodes* (Xu, Zou & Xu, 2017) and an acidic polysaccharide from *Pleurotus abalonus* (Shi et al., 2013), several polymers that showed antitumor effect in other cancer cell types, were not evaluated about their potential in breast cancer cells. For instance, a fucogalactan obtained from the mushroom *Macrocybe titans* showed antimigratory effect in B16-F10 melanoma cell line, regardless of keeping their viability, proliferative capacity, and morphology with no alterations (Milhorini et al., 2018). However, its effect on breast cancer cells is still unknown.

Additionally, the biological effect of polysaccharides depends on their chemical characteristics, such as sugar composition, linkage types, branching degrees, and molecular weight. The chemical characterization is essential to determine the main features of polysaccharides structure related to bioactivity (Ferreira et al., 2015; Ruthes, Smiderle & Iacomini, 2015). For example, Komura et al., (2010) evaluated a fucogalactan from *Agaricus brasiliensis* (Mw  $19.4 \times 10^3$  g/mol) and a partially methylated fucogalactan from *Agaricus bisporus* var. *hortensis* (Mw  $31.1 \times 10^3$  g/mol). The authors presented that such structural differences affected their biological activities, once only the latter presented anti-inflammatory properties.

There is poor information in the literature comparing the chemical structures of mushroom polysaccharides related to their therapeutic properties. This study aimed to characterize a new fucogalactan from the mushroom *M. titans* and compare its chemical features with the one previously described (Milhorini et al., 2018). Besides, both heteropolysaccharides were evaluated on breast cancer cell lines to compare their biological effects and relate with their chemical differences.

## 2 Material and methods

### 2.1 Biological material

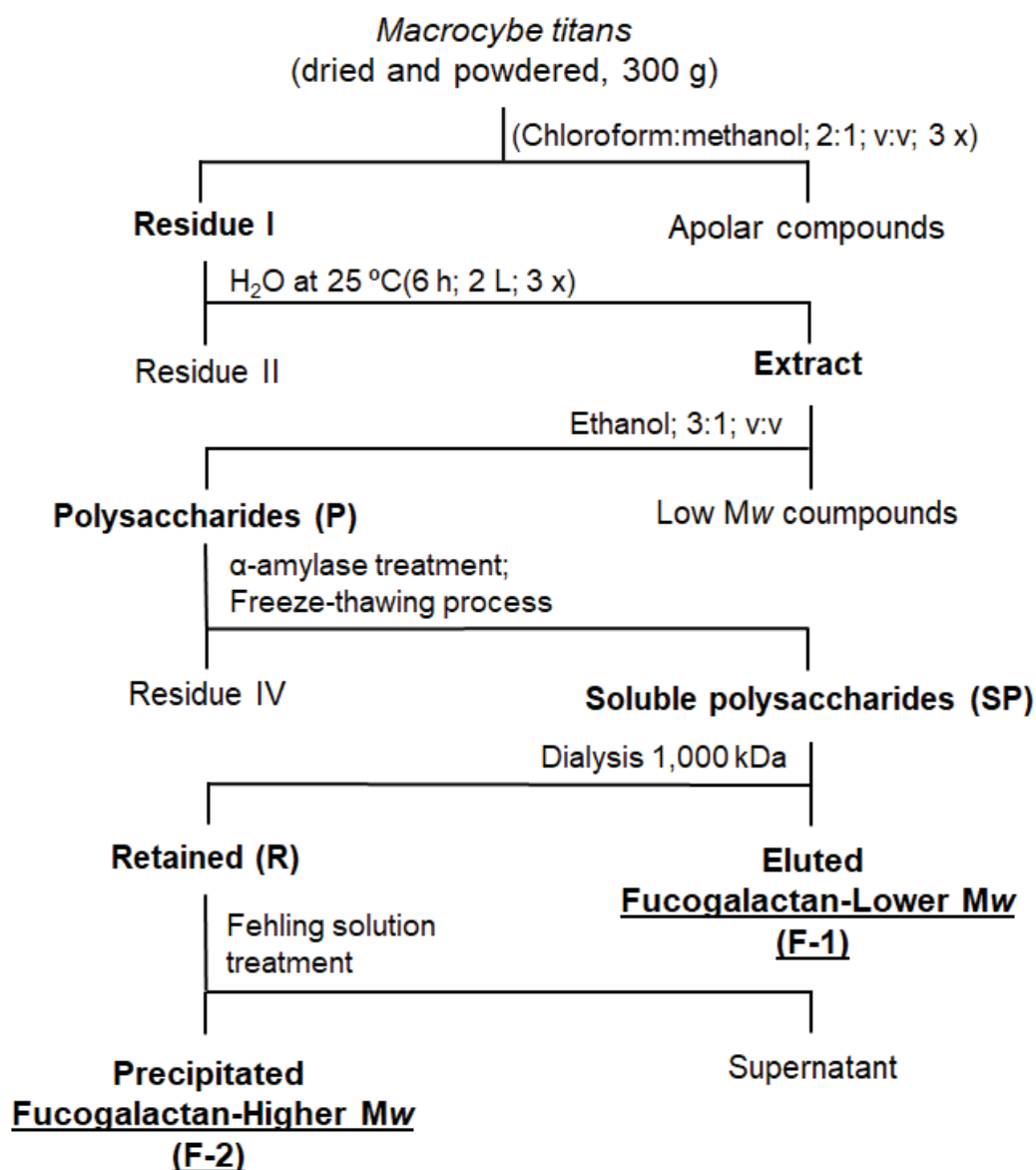
*Macrocybe titans* was a kind gift of Dr. Fábio Rogério Rosado from Federal University of Paraná, Palotina, Brazil. The mushroom identification was carried out by molecular analysis and the resulting nucleotide sequence was published in GenBank database (MZ519068). This species was registered on *Sistema Nacional de Gestão*

do Patrimônio Genético e do Conhecimento Tradicional Associado (SISGEN), with the number A3BA3B3.

## 2.2 Extraction and purification process

Fucogalactans were obtained through the steps represented in figure 1. The fucogalactan with lower molecular weight was extracted and purified according to Milhorini et al., (2018), being named as F-1. Briefly, to obtain this heteropolysaccharide, *M. titans* fruiting bodies (300 g) were freeze-dried, powdered, and defatted with chloroform and methanol (2:1; v:v; 60 °C; 3 h; 3x) under reflux. The delipidified residue was extracted with distilled water under mechanical stirring (2 L; 25 °C; 6 h; 3 x). The extract was filtered with nylon filter cloth and concentrated under reduced pressure. The polysaccharides were recovered from the extract by addition of ethanol (3:1; v:v), followed by centrifugation (10,000 rpm; 20 min; 4 °C), and dialysis against tap water for 24 h (6-8 kDa). Afterwards, they were treated with  $\alpha$ -amylase (Sigma-Aldrich), in phosphate buffer (0.08 M, pH 6.6) for 72 h at 85 °C. The non-degraded polysaccharides were obtained by ethanol precipitation (3:1; v:v), followed by dialysis against tap water for 24 h, and purified through the freeze-thawing procedure (Gorin & Iacomini, 1984). The soluble polysaccharides (SP) were obtained by centrifugation and dialysis against distilled water through a membrane of 1,000 kDa cut off. The eluted F-1 fraction was concentrated under reduced pressure and freeze-dried.

Fraction F-2 was purified by treating retained fraction (R) with Fehling solution according to Jones and Stoodley (1965) methodology, with adaptations. The sample (R, 6.0 g) was solubilized in an alkaline solution (86.5 g of potassium sodium tartrate; 62.5 g of potassium hydroxide; 250 mL of distilled water). Subsequently, the same volume of a solution containing copper sulfate (27.87 g in 250 mL of distilled water) was added. The mixture was submitted to magnetic stirring for 1 h followed by 12 h rest at 4 °C. After this period, the sample was centrifuged (10,000 rpm; 20 min; 4 °C) and the precipitated was neutralized with acetic acid, dialyzed against tap water for 48 h (6-8 kDa), treated with cationic resin, neutralized with 1 M NaOH, dialyzed for 24 h, and freeze-dried, giving rise to F-2 fraction.



**Figure 1:** Extraction and purification steps performed to obtain two fucogalactans (F-1 and F-2) from *M. titans* fruiting bodies.

### 2.3 Monosaccharide composition

Alditol acetates were prepared following Wolfrom and Thompson (1963a e 1963b), with modifications. F-2 (3 mg) was hydrolyzed with 2 M TFA (200  $\mu$ L; 8 h; 100 °C). Subsequently, the acid was removed by evaporation, distilled water was added (200  $\mu$ L), and reduction was performed with NaBH<sub>4</sub> (2 mg) overnight. The sample was evaporated to dryness and washed with methanol (200  $\mu$ L; 3 x). Acetylation was further carried out with anhydride acetic and pyridine (1:1; v:v; 200  $\mu$ L; 40 min; 100 °C). Alditol acetates were extracted with chloroform (1 mL), washed with 5 % copper sulfate

solution (2 mL; 4 x), and distilled water (2 mL; 2 x). Chloroform was evaporated and derivatives were resuspended in acetone (700  $\mu$ L). Analysis was performed in a gas chromatograph-mass spectrometer (GC-MS) (Shimadzu QP2020NX, quadrupole detector), with a VF-5MS column. The analysis started with the oven at 100 °C (held for 3 min), increasing to 220 °C (10 °C/min; held at 200 °C for 3 min), and subsequently to 250 °C (held for 3 min). Helium was used as carrier gas (2 mL/min). Alditol acetates were identified by their retention time and their electron ionization mass spectra, by comparison with standards.

## 2.4 Methylation analysis

F-2 was methylated according to Ciucani and Kerek (1984), with modifications. The sample was solubilized in dimethyl sulfoxide (3 mg/mL). Subsequently, powdered NaOH (20 mg) and methyl iodide (1 mL) were added. After stirring for 30 min the sample was let to rest for 24 h. Water was added (4 mL) to solubilize the mixture, which was neutralized with acetic acid and dialyzed against tap water (6-8 kDa) for 24 h. This process was repeated twice to guarantee the complete methylation of the polysaccharide. The methylated polysaccharide was hydrolyzed with formic acid (45 %; 100 °C; 8 h). After evaporation of the acid and reduction with NaBD<sub>4</sub> (2 mg), the sample was washed, acetylated, extracted, and analyzed as described in section 2.3.

## 2.5 Homogeneity and relative molecular weight ( $M_w$ )

The homogeneity of fucogalactans fractions (F-1 and F-2) was investigated by high-performance size-exclusion chromatography (HPSEC) coupled to a refractive index detector (Fig. 2). Four gel-permeation Ultrahydrogel columns were used:  $7 \times 10^6$ ,  $4 \times 10^5$ ,  $8 \times 10^4$ , and  $5 \times 10^3$  Da. Aqueous NaNO<sub>2</sub> (0.1 M) containing aqueous NaN<sub>3</sub> (200 ppm) was used as eluent at a flow rate of 0.6 mL/min. Samples were solubilized in the eluent (1 mg/mL), filtered (0.22  $\mu$ m), and injected (100  $\mu$ L) into the chromatograph. The relative molecular weight of F-2 was estimated by comparison with a curve of dextran patterns (487.0, 266.0, 124.0, 72.2, 40.2, 17.2, and 9.4 kDa).

## 2.6 Nuclear magnetic resonance (NMR) spectroscopy

NMR spectra (<sup>13</sup>C, HSQC-DEPT, and coupled HSQC) were obtained in a 400 MHz or 600 MHz Bruker model Avance spectrometer (Fig. 3). Samples were

solubilized in D<sub>2</sub>O (30 mg/mL) and analyses were performed at 70 °C. Chemical shifts were expressed in ppm ( $\delta$ ) relative to the resonance of the reference acetone ( $\delta$  30.2 and 2.22 for <sup>13</sup>C and <sup>1</sup>H, respectively).

## 2.7 Cell culture

The human breast cancer cell lines MCF-7 and MDA-MB-231 and the normal epithelial cell line VERO were cultured in supplemented DMEM medium (10% FBS and 1% penicillin-streptomycin). The culture medium was renewed twice a week and cells were maintained in a humidified atmosphere containing 5% CO<sub>2</sub> at 37 °C.

## 2.8 Cell viability assay

A preliminary evaluation was performed with F-1 (ESP) fraction to determine the cytotoxicity of fucogalactans to breast cancer cell lines (MCF-7 and MDA-MB-231) following MTT test, according to Mosmann (1983), with minor changes. For this experiment, tumoral cells (MCF-7:  $1.5 \times 10^4$  cells/well; MDA-MB-231:  $0.6 \times 10^4$  cells/well) were let to adhere into 24-well plates, for 24 h. After adherence, cells were incubated with F-1 (at 250, 500, 1000  $\mu$ g/mL), for 72 h, 96 h, and 120 h (Fig. 4A,C). In another plate, the procedure was repeated, however, after 72 h of treatment, a second dose of F-1 was added to the cells (at the same concentrations), which were incubated for more 48 h totalizing 120 h of treatment (Fig. 4B,D). Three hours before finishing the incubation period, MTT (5 mg/mL in PBS) was added to each well, and the cells were incubated for 3 h at 37 °C. Afterwards, the MTT solution was carefully removed and DMSO was added to solubilize purple formazan crystals. The absorbance was read at 595 nm in a microplate reader (Biotek®). Analyses were conducted in two independent experiments, in quadruplicate wells. Cell viability was expressed as a percentage of control cells (vehicle group treated with PBS) and the inhibitory concentration of 50% (IC<sub>50</sub>) was calculated.

## 2.9 Cell cycle analysis

Cell cycle was evaluated by flow cytometry, when MCF-7 and MDA-MB-231 tumor cells were treated with F-1 and F-2. VERO cells were used as control as they are a normal epithelial cell line. MCF-7 ( $0.6 \times 10^5$  cells/well), MDA-MB-231 ( $0.3 \times 10^5$  cells/well) and VERO ( $0.4 \times 10^4$  cells/well) were plated in 6-well plates and treated with

initial dose of 600 µg/mL, being cultured for 72 h, when they received a second dose of treatment (600 µg/mL). Cells were incubated for more two days and collected for analysis at 96 h (F-1) and 120 h (F-1 and F-2) (Figs. 5, 6, and S1). For this procedure, cells were detached with trypsin, washed once with PBS, and fixed with 70% ethanol overnight at 4 °C. Then the cells were collected by centrifugation and re-suspended in PBS containing 7-AAD (7-Aminoactinomycin D) plus 100 µg/mL RNase and incubated for 15 min in the dark. Analyses were acquired on a BD FACS Canto™ II (USA), using FlowJo X Software (BD, Becton, Dickinson & Company, USA).

## 2.10 Evaluation of apoptosis

Differences between early and late apoptotic, necrotic and living cells (MCF-7, MDA-MB-231, VERO) were observed by flow cytometry (Fig. 7). The experiment was performed as described in section 2.9, however after collecting cells, they were treated with Annexin V and 7-AAD for 15 min in the dark. Samples were acquired on a BD FACS Canto™ II (USA), and analyzed using Flowing Software (Turku Bioscience, Turku, Finland).

## 2.11 Statistical analyses

The results were expressed as mean ± SD using one-way analysis of variance (ANOVA) followed by Bonferroni's selected pairs comparison test or unpaired t-test when appropriate. A 95% confidence level ( $p < 0.05$ ) was considered as statistically significant. The graphs and analyses were developed using GraphPad Prism version 6 (GraphPad Software, San Diego, CA).

## 3 Results and discussion

After the extraction and purification procedures, F-1 fraction yielded 2.2 g while F-2 fraction yielded 1.2 g. The chemical characterization was performed to confirm that both samples were fucogalactans as expected and to compare their structures. Furthermore, the effects of both heteropolysaccharides were evaluated in breast cancer cell lines to investigate if differences in biological activities could be attributed to their chemical structures.



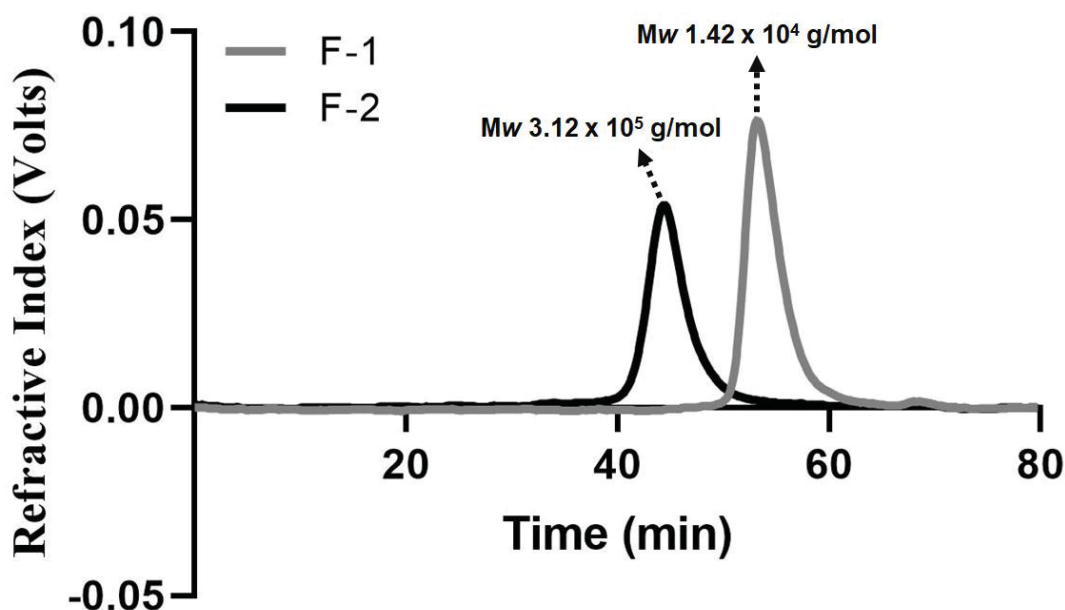
### 3.1 Characterization of fucogalactans

F-1 fraction has been previously characterized as a fucogalactan, however the polysaccharide F-2 present in the retained material (R) have not been studied so far.

The F-2 fraction presented mainly galactose (84.4 %) and fucose (15.6 %) in its composition, when analyzed by GC-MS. The ratio of galactose:fucose in F-2 (5.4:1) is approximated to that reported to F-1 (4.9:1), a fucogalactan obtained from *M. titans* (Milhorini et al., 2018). Other fucogalactans obtained from different mushroom species presented a ratio lower than the observed in F-1 and F-2 fractions. For instance, such heteropolysaccharide obtained from *Hericium erinaceus* (4.59:1) (Li et al., 2016), *Coprinus comatus* (4.57:1) (Li et al., 2013) and (4.02:1) (Fan et al., 2006), *Lactarius rufus* (2.44:1) (Ruthes et al., 2012), and *Agrocybe aegerita* (2.26:1) (Motoshima et al., 2018), although a molecule with a higher ratio was reported to *Agaricus brasiliensis* (6.19:1) (Komura et al., 2010).

The F-2 fraction showed a homogeneous elution profile in HPSEC and its  $M_w$  was estimated at  $3.12 \times 10^5$  g/mol (Figure 2). The  $M_w$  of F-2 was 22 times higher than the  $M_w$  of F-1 ( $1.42 \times 10^4$  g/mol) (Milhorini et al., 2018). The homogeneity of the F-1 obtained herein was confirmed by HPSEC analysis (Fig. 2). Various mushroom fucogalactans reported in the literature present similar molecular weight to the F-1 fraction, such as the one isolated from *Lactarius rufus* ( $1.4 \times 10^4$  g/mol) (Ruthes et al., 2012), *Agrocybe aegerita* ( $1.38 \times 10^4$  g/mol) (Motoshima et al., 2018), *Hericium erinaceus* ( $1.5 \times 10^4$  g/mol) (Li et al., 2016), *Coprinus comatus* ( $1.03 \times 10^4$  g/mol) (Fan et al., 2006), and *Agaricus brasiliensis* ( $1.94 \times 10^4$  g/mol) (Komura et al., 2010). However, a methylated fucogalactan isolated from *Macrolepiota dolichaula* ( $2.5 \times 10^5$  g/mol) presented similar  $M_w$  to F-2 fraction (Samanta et al., 2015).





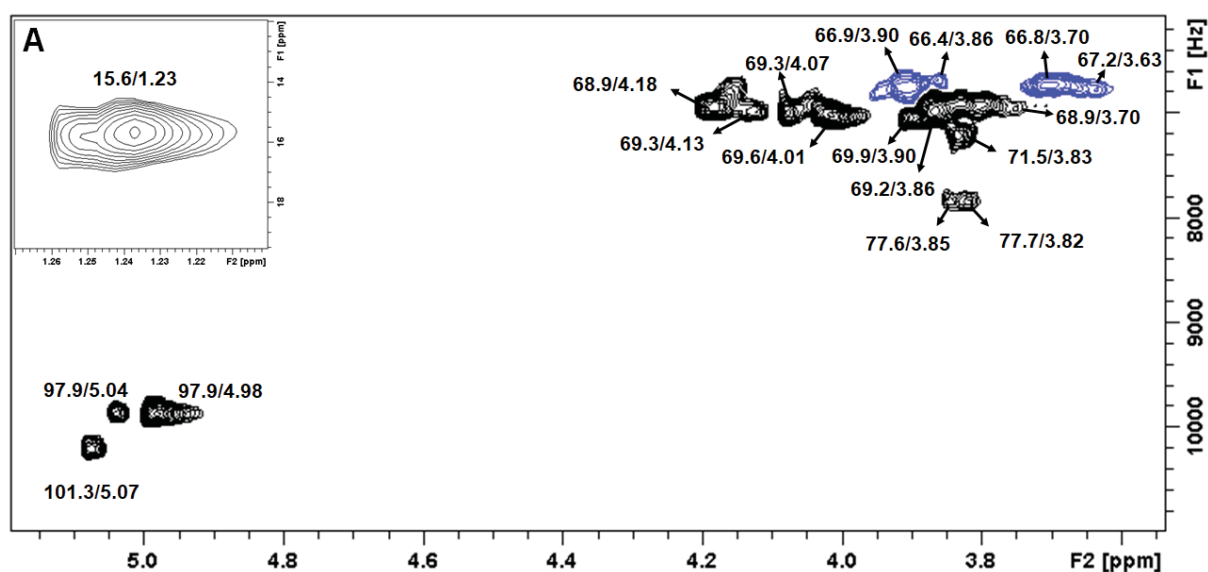
**Figure 2:** Elution profile of F-1 and F-2 fucogalactans in HPSEC coupled to a refractive index detector.

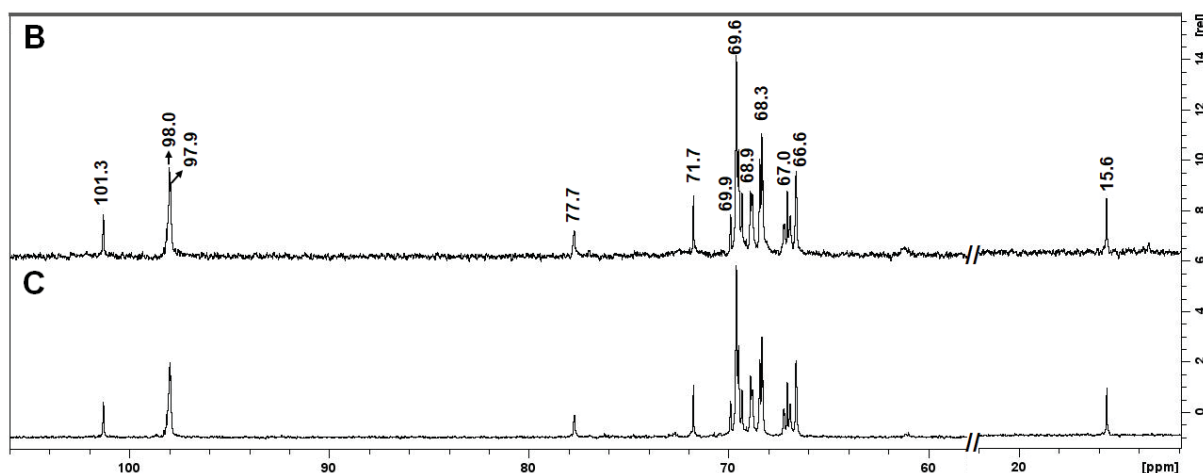
The HSQC-DEPT spectrum of F-2 (Fig. 3A) presented a great similarity with that reported to F-1 (Milhorini et al., 2018), presenting typical signals relative to a fucogalactan with a (1→6)-linked  $\alpha$ -D-Galp main chain partially substituted at O-2 by non-reducing end units of  $\alpha$ -L-Fucp. The assignments were determined by comparison with the literature data (Ruthes et al., 2012; Ruthes et al., 2013; Milhorini et al., 2018). The anomeric region showed signals (C1/H1) corresponding to Fucp ( $\delta$  101.3/5.07), 6-O-substituted Galp ( $\delta$  97.9/5.04), and 2,6-di-O-substituted Galp ( $\delta$  97.9/4.98). The coupling constants  $J_{C-1/H-1}$  obtained in the coupled-HSQC analysis (data not shown), attest the  $\alpha$ -configuration of Fucp and Galp, once it was 170.9 Hz for both units (Perlin & Casu, 1969). The CH<sub>3</sub> resonance of  $\alpha$ -L-Fucp appeared at ( $\delta$  15.6/1.23. Signals attributed to the substitutions of Galp at O-2 were observed at  $\delta$  77.6/3.85 and 77.7/3.82. The O-6 substituted units were evidenced by the negative phase resonance at  $\delta$  66.9/3.90, 66.8/3.70, and 67.2/3.63, 66.4/3.86. The <sup>13</sup>C spectrum of F-2 fraction corroborate with the HSQC-DEPT data (Fig. 3B).

To confirm the presence of fucogalactan in F-1 fraction, an analysis of <sup>13</sup>C-NMR was performed (Fig. 3C). The spectrum obtained confirmed that this fraction was successfully extracted and purified following Milhorini et al., (2018) methodology, once

the resonances observed corroborate to that observed on the previous study (Milhorini et al, 2018). Signals correspondent to the C-1 of  $\alpha$ -L-Fucp ( $\delta$  101.3) and  $\alpha$ -D-Galp ( $\delta$  98.0 and 97.9), as well as the O-6 ( $\delta$  66.6) and O-2 ( $\delta$  77.7) substitutions, were observed. Furthermore, the great similarity between F-1 (Figure 3C) and F-2 spectra (Fig. 2B) confirms that both fractions contain fucogalactans with the same chemical structure.

The methylation analysis confirmed the linkage types of the fucogalactan presented in F-2 and corroborated with NMR data. Derivatives correspondent to the main chain (2,3,4-Me<sub>3</sub>-Galp) totaled 74.0%, while the branching points (3,4-Me<sub>2</sub>-Galp) and the side chains Fucp-(1 $\rightarrow$  (2,3,4-Me<sub>3</sub>-Fucp) summed 17.6% and 7.8%, respectively. This data also corroborates with Milhorini et al., (2018) study, who found similar methylated derivatives in the fucogalactan.





**Figure 3:** NMR analyses of fucogalactans. (A) HSQC-DEPT and (B) <sup>13</sup>C spectra of F-2 fraction. (C) <sup>13</sup>C spectrum of F-1 fraction. The sample was analyzed in D<sub>2</sub>O at 70 °C and the results are expressed in ppm.

These data suggest that F-2 fraction as well as F-1 contains a fucogalactan with a (1→6)-linked α-D-Galp main chain partially substituted at O-2 by α-L-Fucp-(1→, with very similar amounts of fucose and galactose. The main difference observed between both fucogalactans is their  $M_w$ .

Similar fucogalactans were obtained from some mushroom species by different methods of extraction and purification. Fan et al., (2006) isolated this heteropolysaccharide from *Coprinus comatus* mycelia by hot water extraction and purified it by ethanol precipitation, DEAE-Sepharose Fast Flow column and Sephacryl S-300 High-Resolution column. The purification by chromatography columns was also used to get fucogalactans from *Hericium erinaceus* and *Albatrellus ovinus* (Li et al., 2016; Samuelsen et al., 2019). On the other hand, the treatments used to the obtention of F-1 and F-2 fractions, such as precipitation with Fehling solution, freeze-thawing, and dialysis process, were also applied to obtain fucogalactans from *Agaricus brasiliensis*, *Agaricus bisporus* var. *hortensis*, *Lactarius rufus*, and *Agrocybe aegerita* (Komura et al., 2010; Ruthes et al., 2013; Motoshima et al., 2018).

Although most of the published mushroom fucogalactans have a (1→6)-linked α-D-Galp main chain, a different fucogalactan has been isolated from mycelia of *Poria cocos* (Lu et al., 2010). It presents a (1→3)-linked main chain partially substituted at O-6 by (1→6)-linked α-D-Galp side chains with a non-reducing end unit of Fucp. Additionally, such heteropolysaccharide containing Galp units naturally methylated

were isolated from *Agaricus bisporus* var. *hortensis*, *A. bisporus*, and *Macrolepiota dolichaula* (Komura et al., 2010; Ruthes et al., 2012; Ruthes et al., 2013; Samanta et al., 2015).

Fucogalactans obtained from mushrooms were reported to have several biological activities, such as anti-inflammatory (Komura et al., 2010; Lu et al., 2010; Ruthes et al., 2013), antinociceptive (Komura et al., 2010; Ruthes et al., 2013), anti-sepsis (Ruthes et al., 2012; Ruthes et al., 2013), leishmanicidal (Motoshima et al., 2018), immunomodulatory (Mizuno et al., 2000; Samanta et al., 2015; Li et al., 2016), and antimelanoma effects (Milhorini et al., 2018).

### 3.2 Biological activities

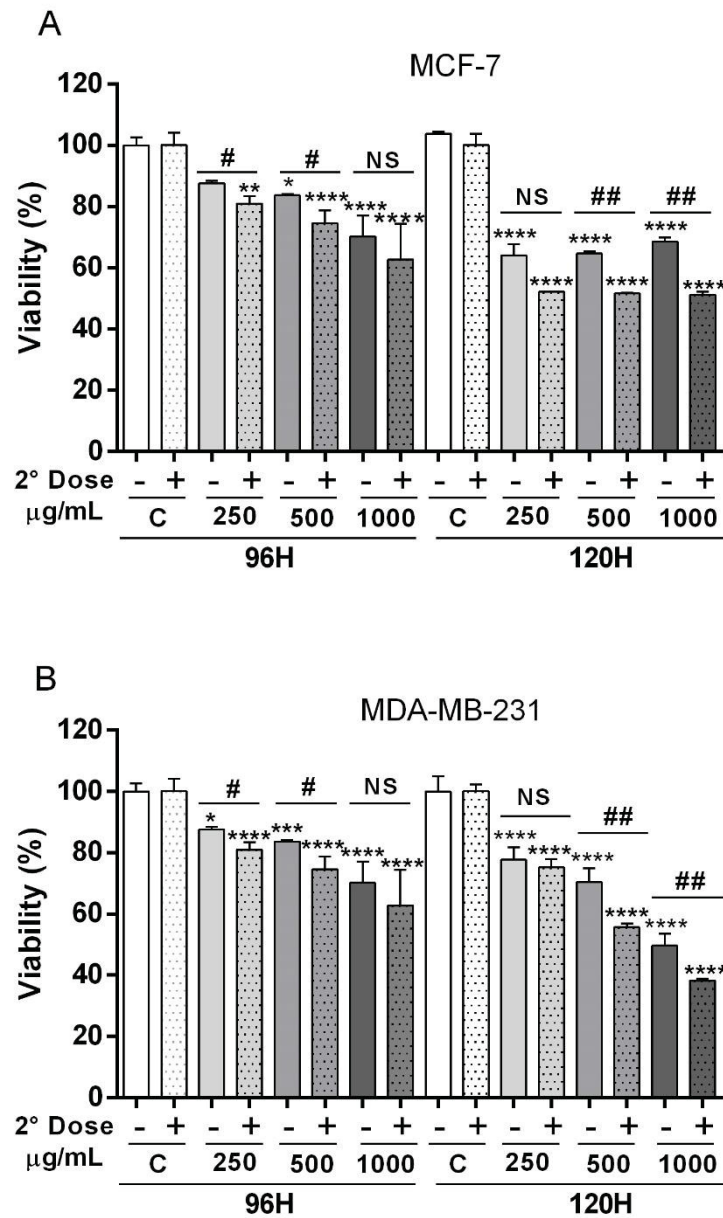
To compare if the different  $M_w$  of the fucogalactans could influence their activity on breast cancer cells, the first step was to determine the cell viability after treatment with F-1 and F-2. However, as F-1 (0.76%) presented the double yield when compared to F-2 (0.4%), a preliminary assay was performed with this fraction to determine the most effective incubation periods and concentrations of fucogalactans. For this purpose, MCF-7 and MDA-MB-231 cells were treated with different concentration of F-1 (1, 3, 10, 30, 100, 300, 1000  $\mu\text{g/mL}$ ) for 24 h and 48 h (data not shown). MCF-7 cell viability reduced 23.4% when they were treated, for 48 h, at 3  $\mu\text{g/mL}$ , however, prominent effects were observed at 100  $\mu\text{g/mL}$  or higher. In contrast, MDA-MB-231 cells reduced viability by 25% at 1,000  $\mu\text{g/mL}$  after 48 h of treatment, showing that these cells are more resistant to this treatment (data not shown).

From these results, extended exposure to the treatments and an additional dose were tested with F-1. On the second viability test, MCF-7 and MDA-MB-231 cells were treated with 250, 500, and 1,000  $\mu\text{g/mL}$  of F-1 for longer periods as shown in figure 4A,B. A second dose of F-1 was added to the cells and the evaluation was performed at two time points. After 72 h of treatment, it was observed a marked reduction on MCF-7 cell viability (40% for 250 and 500  $\mu\text{g/mL}$ ; and 37 % for 1,000  $\mu\text{g/mL}$ ), and slight reduction on cell viability of MDA-MB-231 cells (15.4 %, at 1,000  $\mu\text{g/mL}$ ). When tumoral cells were treated for longer periods (96 h and 120 h), the cell viability showed a marked reduction, especially at 120 h of treatment. More than 30% ( $p < 0.05$ ) of reduction was observed for MCF-7 when they were treated with one dose of F-1 (at 250, 500, and 1,000  $\mu\text{g/mL}$ ), while the MDA-MB-231 cells, that are more

aggressive, reduced their viability by 20% and 50% ( $p < 0.05$ ), at 500 and 1,000  $\mu\text{g/mL}$ , respectively. Furthermore, when the cells were treated with a second dose of F-1, totalizing 120 h, a prominent reduction on viability was achieved for both cell lines, at 500 and 1,000  $\mu\text{g/mL}$ , reaching 49% and 62% ( $p < 0.05$ ) of reduction at the highest concentration for MCF-7 and MDA-MB-231 cells, respectively (Fig. 4A,B). Interestingly, when the non-tumoral cell line VERO was treated with F-1, no alteration in their viability was observed (Fig. S1, supplementary material).

A ferment broth of the mushroom *Antrodia salmonea* was also able to reduce the cell viability of MCF-7 (at 150, 175, 200, and 400  $\mu\text{g/mL}$ ) and of MDA-MB-231, but with the highest concentrations (at 200 and 400  $\mu\text{g/mL}$ ). However, this treatment reduced the cell viability of the non-tumoral cells MCF-10 (Chang et al., 2017a). On the other hand, a cold-water extract from *Lignosus rhinoceros sclerotia*, containing 75 % of carbohydrate, reduced the cell viability of MCF-7, but did not affect the normal breast cells 184B5 (Lee et al., 2012).

Other mushroom extracts and polysaccharide fraction decreased MCF-7 viability. For instance, an acid polysaccharide ( $M_w$   $3.68 \times 10^5$ ) obtained from *Pleurotus abalonus*, which was composed mainly of galacturonic acid (41.7 %), glucose (29.8 %), and galactose (14.6 %), lead to a cytotoxic effect (at 50, 100, 200, and 400  $\mu\text{g/mL}$ ) in a dose-dependent manner, when cells received one dose of the treatment (Shi et al., 2013). Additionally, cells treated with water extracts from *Polycephalomyces nipponicus* (at 400  $\mu\text{g/mL}$ ) (Buranrat et al., 2019), or with water extracts from *Chalciporus piperatus*, *Fistulina hepatic*, *Gymnopus dryophilus*, *Infundibulicybe geotropa*, and *Kuehneromyces mutabilis* (at 10 and 30  $\mu\text{g/mL}$ ) (Ványolós et al., 2015), also resulted in growth inhibition.



**Figure 4:** Cell viability of MCF-7 (A) and MDA-MB-231 (B) cells treated with 1 dose (clean bars) and 2 doses (dotted bars) of F-1 in comparison to cells treated with PBS (white bars). Cells were incubated with F-1 (250, 500 or 1000 µg/mL) for 96 h and 120 h. Statistical analyses were performed by one-way analysis of variance (ANOVA) followed by Bonferroni test, selected pairs. The results represent the mean  $\pm$  SD of two independent experiments (n= 3). \*p< 0.05; \*\*p< 0.01; \*\*\*p< 0.001 when compared with vehicle control. #p< 0.05; ##p< 0.01; ###p< 0.001 when compared cells treated with 1 dose versus 2 doses, performed by unpaired t-test.

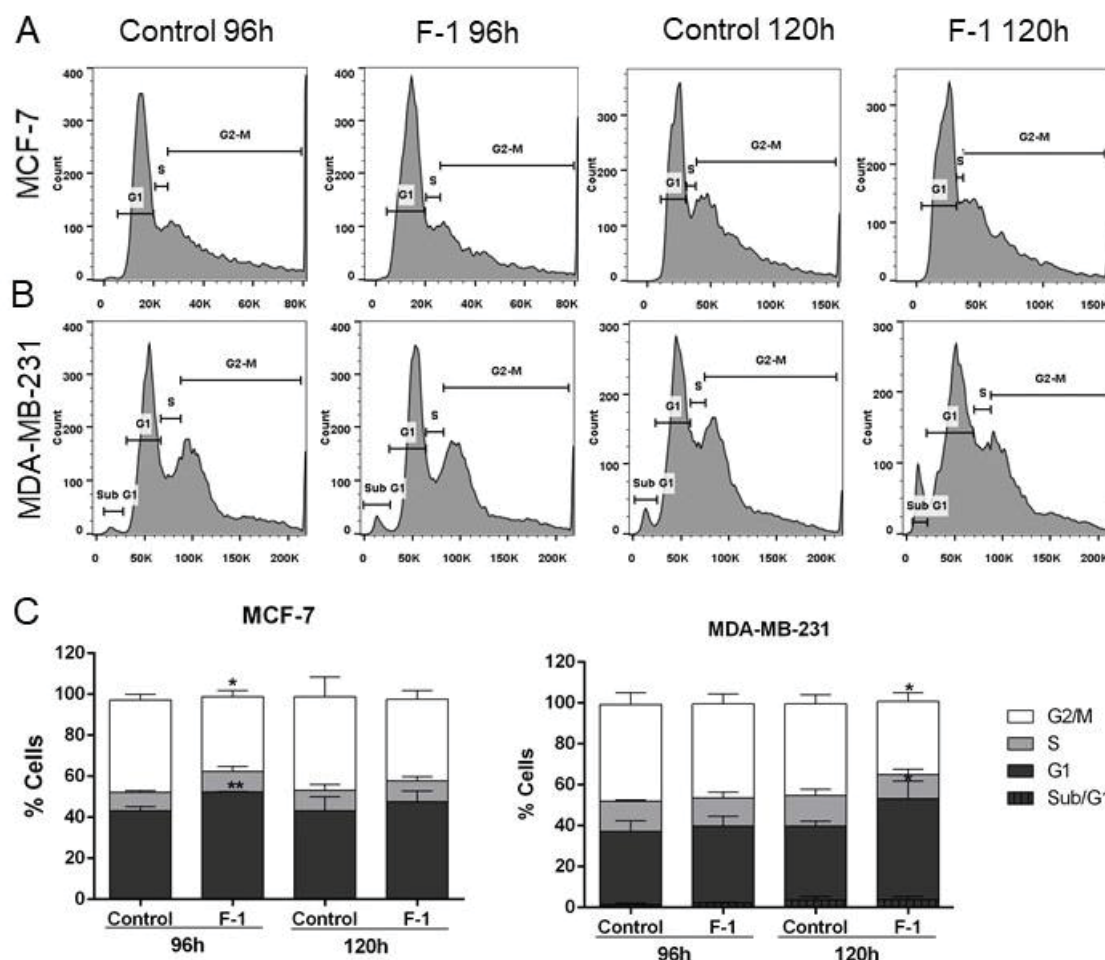
The IC<sub>50</sub> value, calculated based on cell viability assay of cells treated with two doses of F-1 (120 h), was 623 µg/mL and 639 µg/mL for MCF-7 and MDA-MB-231 cells, respectively (Fig. 4). Therefore, the further experiments were performed with both fucogalactan fractions (F-1 and F-2) at a concentration of 600 µg/mL. Preliminary tests using F-2 fraction were not performed due to its lower yield.

The IC<sub>50</sub> values obtained for F-1 in MCF-7 and MDA-MB-231 are higher than those usually found in the literature for other mushroom polysaccharides or water extracted compounds. For instance, water extracts from *Coprinus comatus* (450 µg/mL and 400 µg/mL, respectively), *Coprinellus sp* (120 µg/mL and 40 µg/mL, respectively), and *Flammulina velutipes* (150 µg/mL and 75 µg/mL, respectively) (Gu & Leonard, 2006), as well as the ferment broth of *Antrodia salmonea* (348 µg/mL and of 142 µg/mL, respectively) (Chang et al., 2017a) presented lower IC<sub>50</sub> values. Additionally, other compounds evaluated only using MCF-7 cells also showed a lower IC<sub>50</sub> than F-1, as the acid polysaccharide from *Pleurotus abalonus* (193 µg/mL) (Shi et al., 2013), polysaccharide extracts from *Clitocybe alexandri* (46.8 µg/mL) and *Lepista inversa* (137.4 µg/mL) (Vaz et al., 2010), the water extract from sclerotia of wild type strain (206 µg/mL) and cultivated strain (90 µg/mL) of *Lignosus rhinocerus* (Yap et al., 2013).

Analyses of cell cycle, cell death or association of both were performed by flow cytometry when breast tumoral cells and VERO cell line were treated with two doses of F-1 and F-2 (600 µg/mL x 2), for 96 h and/or 120 h. MCF-7 cells increased their population at G1 phase to 52.5% ( $p < 0.05$ ) and 48% (at 96 h and 120 h, respectively), compared with 43% of the controls (Fig. 5). For MDA-MB-231 cells, their population at G1 phase significantly increased to 49.4% ( $p < 0.05$ ) compared with 36% of the control, when they were exposed to F-1 for 120 h. At 96 h of treatment, no difference was observed with the control. In addition, a distinct sub-G1 peak was detected, but this increase was not statistically significant in relation to the control (Fig. 5).

On the other hand, MDA-MB-231 cells treated with a fermented broth of *A. salmonea* (200 µg/mL) for 12 h and 24 h, suffered a decrease in the G1 and S phases, while presented an increase in G2 and accumulation of subploid cells, which produced the sub-G1 peak (Chang et al., 2017b). At the same time, the acid polysaccharide (200 µg/mL) from *P. abalonus* induced strong S-phase arrest in a time-dependent manner, after treatments of 12, 24, and 48 h (Shi et al., 2013), and a water extract from *Polycephalomyces nipponicus* mycelia (500 µg/mL and 1000 µg/mL) lead MCF-7 cells to arrest at the G2/M phase after a treatment of 48 h (Buranrat et al., 2019).





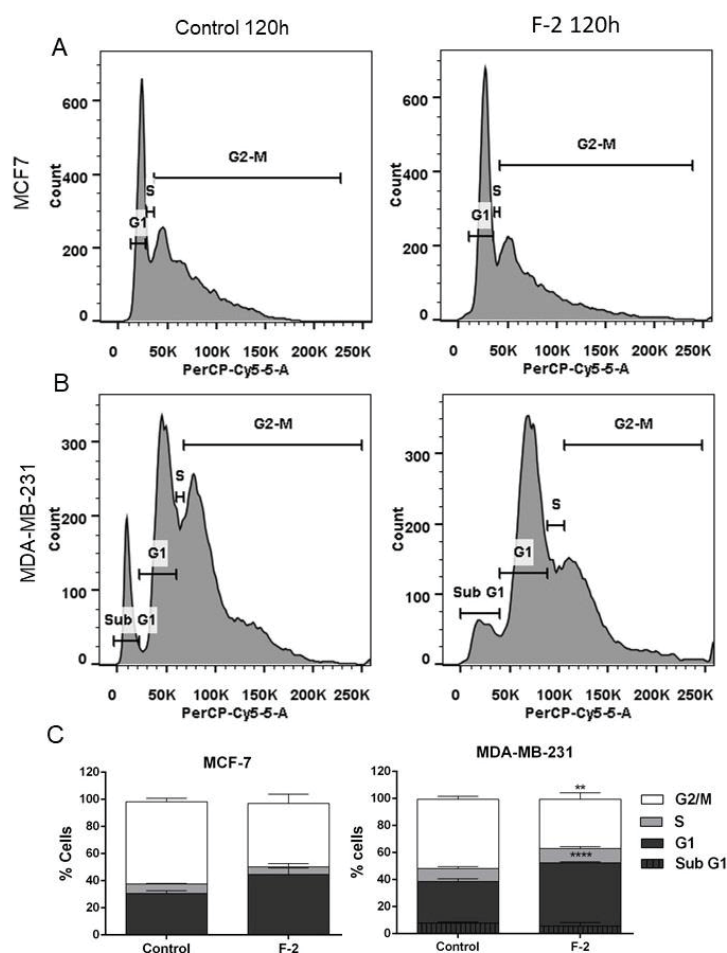
**Figure 5:** Effect of F-1 treatment on cell cycle progression of MCF-7 (A) and MDA-MB-231 (B) cells, analyzed by flow cytometry. The cells were treated with 2 doses (600  $\mu\text{g/mL}$ ) of F-1 for 96 h e 120 h. Cell cycle of F-1 treated cells compared to vehicle control treated with PBS (C). The results represent the mean  $\pm$  SD of two independent experiments ( $n=3$ ). Statistical analyzes were performed by unpaired t-tests. \* $p < 0.05$ ; \*\* $p < 0.01$  versus control.

When F-2 fraction was evaluated, cell cycle analysis of MDA-MB-231 cells treated for 120 h showed a significant number of cells accumulated in G1 phase (46.7%,  $p < 0.05$ ) compared with the control (30.8%) (Fig. 6). This effect was higher than the observed to the cells treated with F-1 (Fig. 5), which can be due to the highest  $M_w$  of F-2. It has been already reported that some polysaccharides presented more prominent biological properties when they have a higher  $M_w$ . For example, five fractions of  $\beta$ -glucan with different molecular weight were evaluated on THP-1 macrophages, and those with a higher molecular weight exhibited better activity on



enhancing the release of inflammatory cytokines (Liu et al., 2018). On the other side, when MCF-7 cells were treated for the same period with F-2 fraction, an increase of cells at G1 phase was observed (44.5%), however no significant difference was noted (Fig. 6).

The effect of F-1 and F-2 on the cell cycle of VERO cell line was evaluated as a control of non-tumorigenic cells (Fig. S1 A,B,C,D). No significant difference was obtained for both treatments. Molecules that are able to affect the tumoral cells, without causing damage to non-tumoral cells, are very promising as a candidate for cancer treatment, once, therapies that affect the healthy cells, lead to serious and several deleterious side-effects on the patients' organism.



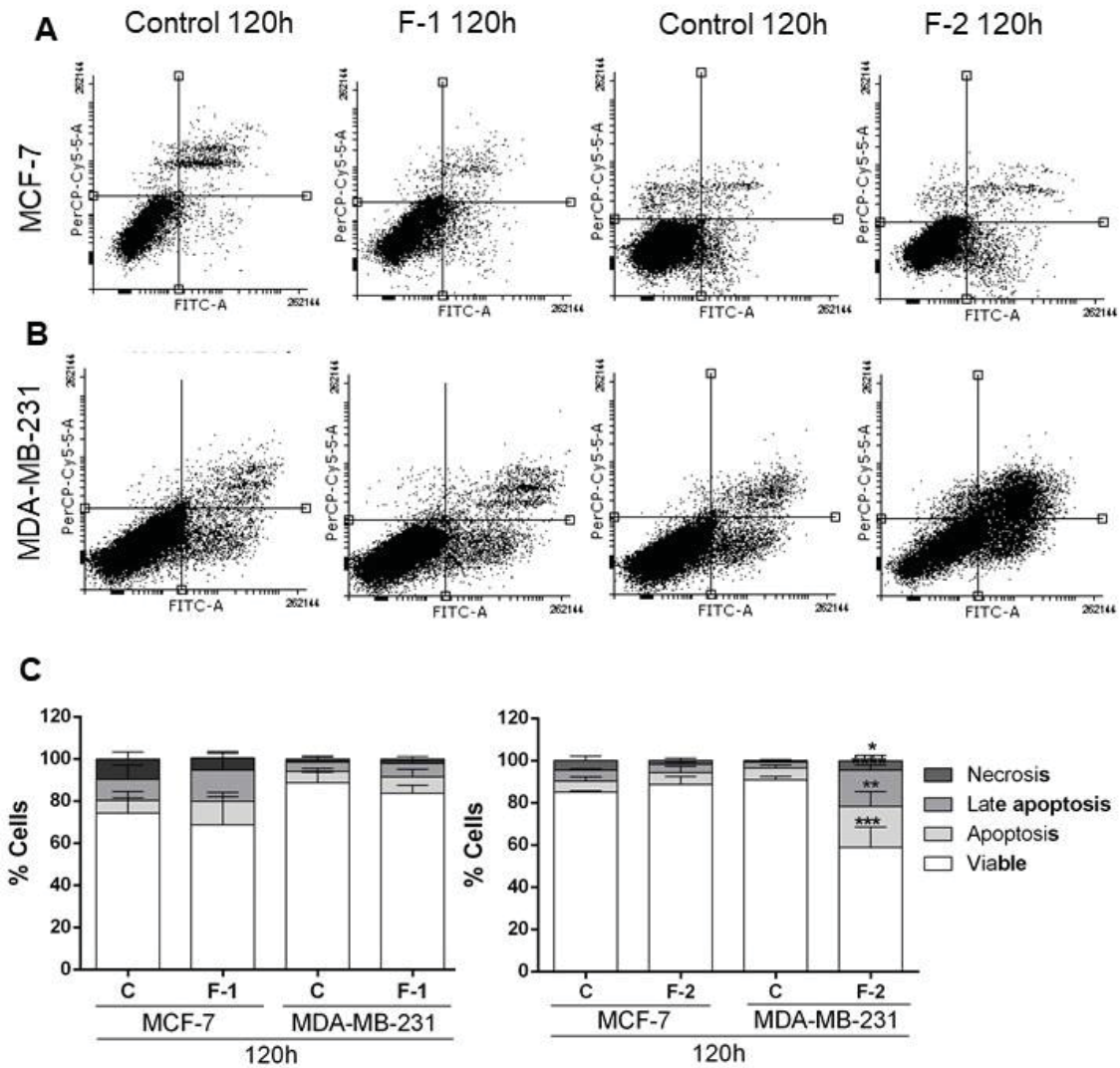
**Figure 6:** Effect of F-2 treatment on cell cycle progression of MCF-7 (A) and MDA-MB-231 (B) cells, analyzed by flow cytometry. The cells were treated with 2 doses (600  $\mu\text{g/mL}$ ) of F-2 for 120 h. Cell cycle of F-2 treated cells compared to vehicle control treated with PBS (C). The results represent the mean  $\pm$  SD of two independent

experiments (n= 3). Statistical analyzes were performed by unpaired t-tests. \*\*p< 0.01; \*\*\*p< 0.001 versus control.

Cell death was analyzed in both breast cancer cells, for 120 h of treatment with two doses of F-1 fraction. Interestingly, no significant increase in the stages of apoptosis was found for both cell lines compared to the control (Fig. 7C). The reduction in cell viability observed previously, on MTT assay (Fig. 4), is directly related to cell cycle arrest in the G-1 phase (Fig. 5), and it is an indication of upcoming apoptosis signaling. However, when cells were treated with F-1 for 120 h, no significant increase in apoptosis was observed for both breast tumor cells compared to the control (Fig. 7). A longer treatment (>120 h) with F-1 fraction could show a significant cell death, considering the results observed on MTT assay and cell cycle.

On the opposite, when the cells were treated with the high  $M_w$  fraction F-2, for the same period, MCF-7 cells showed no apoptosis, however MDA-MB-231 cells presented 4.3-fold increase in apoptosis compared to the control group (Fig. 7C). Increased necrosis induction was also observed. The results demonstrated that the effect of the high  $M_w$  fucogalactan (F-2) on cell cycle progression and apoptosis was more evident in MDA-MB-231 cells and promoted cell death at 120 h of treatment with two doses of 600  $\mu\text{g/mL}$ , while the low  $M_w$  fucogalactan (F-1) promoted cell cycle arrest at G1 phase and no cell death at this period. This result shows the great importance of studying the chemical characteristics of the polysaccharides, presenting that similar linkage types and different molecular weight may cause different biological effects.

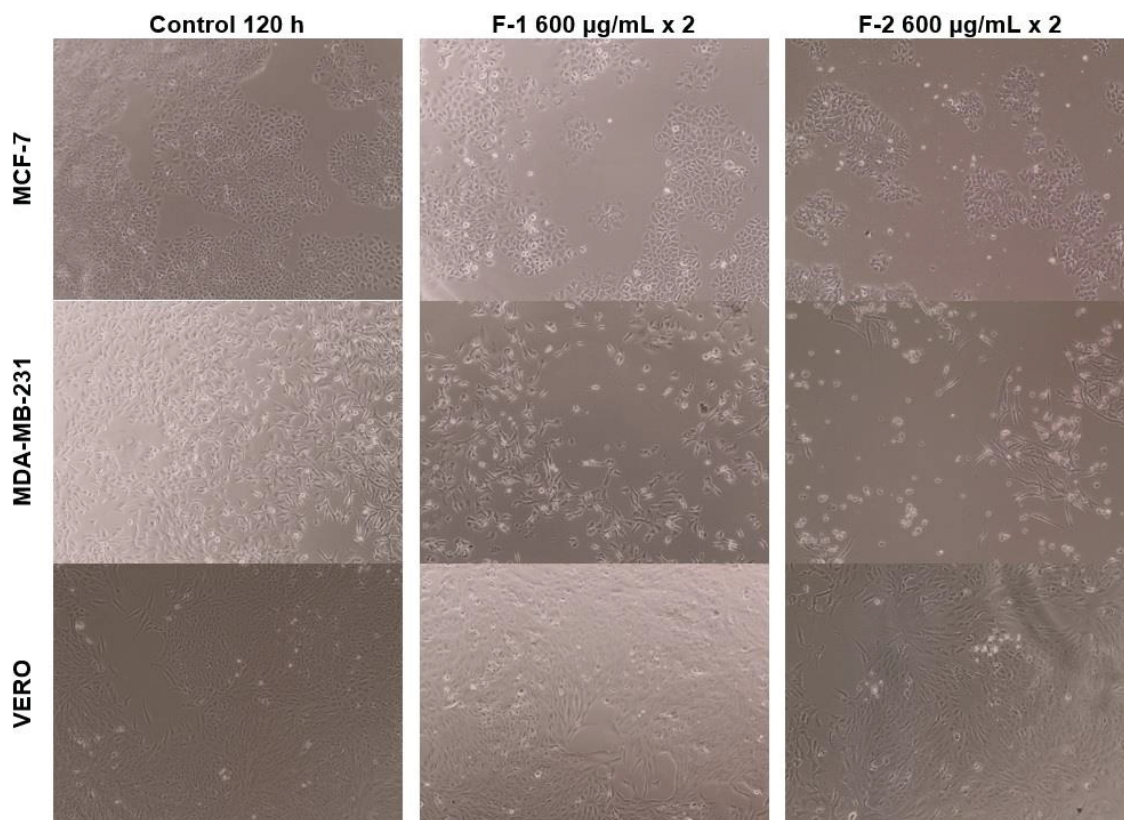
The induction of apoptosis was also observed on breast cancer cells treated with water extract from *Coprinellus sp* (Gu & Leonard, 2006). However, different from F-2 fraction, which was able to induce apoptosis only on MDA-MB-231 cells, this extract led to apoptosis in both MCF-7 and MDA-MB-231 cells within 2 h of treatment. When the authors performed the treatment at 112  $\mu\text{g/mL}$  in MCF-7 cells, about 72% of cells were in the early stage of apoptosis and >10% in the late stage of apoptosis. When a 2-fold dosage (225  $\mu\text{g/mL}$ ) was tested, advanced apoptosis was observed. This same behavior was observed when the extract was applied on MDA-MB-231 cells.



**Figure 7:** Analysis of apoptosis induction in MCF-7 (A) and MDA-MB-231 (B) cells using flow cytometry. The cells were treated with 2 doses (600  $\mu\text{g/mL}$ ) of F-1 or F-2 for 120 h. Bar chart (C) showing proportion of viable, early apoptotic, late apoptotic and necrotic cells after F-1 (left) and F-2 (right) treatment. The results represent the mean  $\pm$  SD of two independent experiments ( $n = 3$ ). Statistical analyzes were performed by unpaired t-test. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ , F-1 or F-2 treatment versus control.

It is important to mention that the control cells (VERO) exposed to F-1 and F-2 for 96 h and 120 h, presented no variation on cell cycle distribution and no cell death (Fig. S1, supplementary material).

The results reporting the effects of F-1 and F-2 on MCF-7, MDA-MB-231, and VERO cells, were confirmed by the pictures of cells on figure 8.



**Figure 8:** MCF-7, MDA-MB-231 and VERO cells after treatment of 2 doses (600 µg/mL) with F-1 and F-2 in comparison to control with PBS.

#### 4 Conclusions

The fucogalactans F-1 ( $1.42 \times 10^4$  g/mol) and F-2 ( $3.12 \times 10^5$  g/mol) have similar monosaccharide composition and linkage types, with a (1→6)-linked  $\alpha$ -D-Galp main chain partially substituted at O-2 by non-reducing end units of  $\alpha$ -L-Fucp. Both fucogalactans reduced the cell viability of MCF-7 and MDA-MB-231, without affecting the non-tumoral cell line VERO. Interesting, the fraction with the highest Mw (F2), showed the strongest effect on the triple-negative cells MDA-MB-231, leading to a bigger cycle cell arrest in G1 and G2/M phases. Additionally, only F-2 was able to promote cell death apoptosis and necrosis mediated in this cell line. These results suggest that the Mw of these molecules is an important feature to their biological properties.

#### Declaration of competing interest

The authors declare to have no competing interests.

## Acknowledgments

This work was supported by the Brazilian funding agency: Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

## References

- American Cancer Society (2021). *Hormone Therapy for Breast Cancer*. [Retrieved from <https://www.cancer.org/cancer/breast-cancer/treatment/hormone-therapy-for-breast-cancer.html>]
- Buranrat, B., Sangdee, K., & Sangdee, A. (2019). Comparative Study on the Effect of Aqueous and Ethanolic Mycelial Extracts from *Polycephalomyces nipponicus* (Ascomycetes) against Human Breast Cancer MCF-7 Cells. *International Journal of Medicinal Mushrooms*, 21, 671–681. <http://dx.doi.org/10.1615/IntJMedMushrooms.2019031140>.
- Ciucanu, I., & Kerek, F. (1984). A simple and rapid method for the permethylation of carbohydrates. *Carbohydrate Research*, 131, 2, 209–217. [http://dx.doi.org/10.1016/0008-6215\(84\)85242-8](http://dx.doi.org/10.1016/0008-6215(84)85242-8).
- Chang, C. T., Hseu, Y. C., Thiyagarajan, V., Huang, H. C., Hsu, L. S., Huang, P. J., Liu, J. Y., Liao, J. W., & Yang, H. L. (2017a). *Antrodia salmonea* induces G2 cell-cycle arrest in human triple-negative breast cancer (MDA-MB-231) cells and suppresses tumor growth in athymic nude mice. *Journal of Ethnopharmacology*, 196, 9-19. <http://dx.doi.org/10.1016/j.jep.2016.12.018>
- Chang, C. T., Korivi, M., Huang, H. C., Thiyagarajan, V., Lin, K. Y., Huang, P. J., Liu, J. Y., Hseu, Y. C., & Yang, H. L. (2017b). Inhibition of ROS production, autophagy or apoptosis signaling reversed the anticancer properties of *Antrodia salmonea* in triple-negative breast cancer (MDA-MB-231) cells. *Food and Chemical Toxicology*, 103, 1-17. <http://dx.doi.org/10.1016/j.fct.2017.02.019>
- Comşa, Ş., Cîmpean, A. M., & Raic, M. (2015). The Story of MCF-7 Breast Cancer Cell Line: 40 years of Experience in Research. *Anticancer research*, 35, 3147-3154.
- Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry*, 28, 350–356. <http://dx.doi.org/10.1021/ac60111a017>
- Fan, J. M., Zhang, J. S., Tang, Q. J., Liu, Y. F., Zhang, A. Q., & Pan, Y. J. (2006). Structural elucidation of a neutral fucogalactan from the mycelium of *Coprinus comatus*. *Carbohydrate Research*, 341, 1130–1134. <http://dx.doi.org/10.1016/j.carres.2006.03.039>
- Ferreira, I. C. F. R., Heleno, S. A., Reis, F. S., Stojkovic, D., Queiroz, M. J. R. P., Vasconcelos, M. H., & Sokovic, M. (2015). Chemical features of *Ganoderma* polysaccharides with antioxidant, antitumor and antimicrobial activities. *Phytochemistry*, 114, 38-55. <http://dx.doi.org/10.1016/j.phytochem.2014.10.011>
- Flodrova, D., Toporova, L., Macejova, D., Lastovickova, M., Brtko, J., & Bobalova, J. (2016). A comparative study of protein patterns of human estrogen receptor positive (MCF-7) and negative (MDA-MB-231) breast cancer cell lines. *General Physiology and Biophysics*, 35, 387–392. [http://dx.doi.org/10.4149/gpb\\_2016009](http://dx.doi.org/10.4149/gpb_2016009)
- Gomes, D. C. V., Alencar, M. V. O. B., Reis, A. C., Lima, R. M. T., Santos, J. V. O., Mata, A. M. O. F., Dias, A. C. S., Junior, J. S. C., Medeiros, M. G. F., Paz, M. F. C. J., Moreno, L. C. G. A. I., Sousa, J. M. C., Islam, M. T., & Cavalcante, A. A. C. M. (2019). Antioxidant, anti-inflammatory and cytotoxic/antitumoral bioactives from the phylum Basidiomycota and their possible mechanisms of action. *Biomedicine & Pharmacotherapy*, 112, 108643. <https://doi.org/10.1016/j.biopha.2019.108643>
- Gorin, P. A. J., & Iacomini, M. (1984). Polysaccharides of the lichens *Cetraria islandica* and *Ramalina usnea*. *Carbohydrate Research*, 128 (1), 119–132. [http://dx.doi.org/10.1016/0008-6215\(84\)85090-9](http://dx.doi.org/10.1016/0008-6215(84)85090-9)



- Gu, Y. H., & Leonar, J. (2006). In vitro effects on proliferation, apoptosis and colony inhibition in ER-dependent and ER-independent human breast cancer cells by selected mushroom species. *Oncology Reports*, 15, 417-423. <https://doi.org/10.3892/or.15.2.417>
- Jones, J. K. N., & Stoodley, R. J. Fractionation using copper complexes. *Methods in Carbohydrate Chemistry*, 5, 36-38, 1965.
- Komura, D. L., Carbonero, E. R., Gracher, A. H., Baggio, C. H., Freitas, C. S., Marcon, R., & Iacomini, M. (2010). Structure of *Agaricus* spp. fucogalactans and their anti-inflammatory and antinociceptive properties. *Bioresource Technology*, 101, 6192–6199. <http://dx.doi.org/10.1016/j.biortech.2010.01.142>
- Lee, M. L., Tan, N. H., Fung, S. Y., Tan, C. S., & Ng, S. T. (2012). The Antiproliferative Activity of Sclerotia of *Lignosus rhinocerus* (Tiger Milk Mushroom). *Evidence-Based Complementary and Alternative Medicine*, 2012, 697603. <http://dx.doi.org/10.1155/2012/697603>
- Li, B., Dobruchowsk, J. M., Gerwig, G. J., Dijkhuizen, L., & Kamerling, J. P. (2013). Structural investigation of water-soluble polysaccharides extracted from the fruit bodies of *Coprinus comatus*. *Carbohydrate Polymers*, 91, 314-321. <http://dx.doi.org/10.1016/j.carbpol.2012.08.045>
- Li, Q. Z., Wu, D., Zhou, S., Liu, Y. F., Li, Z. P., Feng, J., & Yang, Y. (2016). Structure elucidation of a bioactive polysaccharide from fruiting bodies of *Hericium erinaceus* in different maturation stages. *Carbohydrate Polymers*, 144, 196-204. <http://dx.doi.org/10.1016/j.carbpol.2016.02.051>
- Liu, Y., Tang, Q., Zhang, J., Xia, Y., Yang, Y., Wu, D., Fan, H., & Cu, S. W. (2018). Triple helix conformation of  $\beta$ -D-glucan from *Ganoderma lucidum* and effect of molecular weight on its immunostimulatory activity. *International Journal of Biological Macromolecules*, 114, 1064–1070. <https://doi.org/10.1016/j.ijbiomac.2018.03.054>
- Lu, M. K., Cheng, J. J., Lin, C. Y., & Chang, C. C. (2010). Purification, structural elucidation, and anti-inflammatory effect of a water-soluble 1,6-branched 1,3-a-D-galactan from cultured mycelia of *Poria cocos*. *Food Chemistry*, 118, 349–356. <https://doi.org/10.1016/j.foodchem.2009.04.126>
- Milhorini, S. S., Smiderle, F. R., Biscaia, S. M. P., Rosado, F. R., Trindade, E. S., & Iacomini, M. (2018). Fucogalactan from the giant mushroom *Macrocybe titans* inhibits melanoma cells migration. *Carbohydrate Polymers*, 190, 50–56. <https://doi.org/10.1016/j.carbpol.2018.02.063>
- Mizuno, M., Shiomi, Y., Minato, K. I., Kawakami, S., Ashida, H., & Tsuchida, H. (2000). Fucogalactan isolated from *Sarcodon aspratus* elicits release of tumor necrosis factor- $\alpha$  and nitric oxide from murine macrophages. *Immunopharmacology*, 46, 113–121. [http://dx.doi.org/10.1016/S0162-3109\(99\)00163-0](http://dx.doi.org/10.1016/S0162-3109(99)00163-0)
- Mosmann, T. (1983). Rapid Colorimetric Assay for Cellular Growth and Survival: Application to Proliferation and Cytotoxicity Assays. *Journal of Immunological Methods*, 65, 55–63. [https://doi.org/10.1016/0022-1759\(83\)90303-4](https://doi.org/10.1016/0022-1759(83)90303-4)
- Motoshima, R. A., Rosa, T. d. F., Mendes, L. d. C., Silva, E. V. d., Viana, S. R. F., Amaral, B. S. d., Souza, D. H. F. d., Lião, L. M., Silva, M. d. L. C. d., Sousa, L. R. F. d., & Carbonero, E. R. (2018). Inhibition of *Leishmania amazonensis* arginase by fucogalactan isolated from *Agrocybe aegerita* mushroom. *Carbohydrate Polymers*, 201, 532-538. <https://doi.org/10.1016/j.carbpol.2018.08.109>
- National Cancer Institute (2021). *Cancer Stat Facts: Female Breast Cancer*. [Retrieved from: <https://seer.cancer.gov/statfacts/html/breast.html>].
- Peddie, N., Agnew, S., Crawford, M., Dixon, D., MacPherson, I., & Fleming, L. (2021). The impact of medication side effects on adherence and persistence to hormone therapy in breast cancer survivors: A qualitative systematic review and thematic synthesis. *The breast*, 58, 147-159. <https://doi.org/10.1016/j.breast.2021.05.005>

- Perlin, A. S., & Casu, B. (1969). Carbon-13 and proton magnetic resonance spectra of D-glucose-<sup>13</sup>C. *Tetrahedron Letters*, 34, 2919–2924. [http://dx.doi.org/10.1016/S0040-4039\(01\)88308-8](http://dx.doi.org/10.1016/S0040-4039(01)88308-8).
- Razak, N. A., Abu, N., Ho, W. Y., Zambari, N. R., Tan, S. W., Alitheen, N. B., Long, K., & Yeap, S. K. (2019). Cytotoxicity of eupatorin in MCF-7 and MDA-MB-231 human breast cancer cells via cell cycle arrest, anti-angiogenesis and induction of apoptosis. *Scientific Reports*, 9(1), 1–12. <https://doi.org/10.1038/s41598-018-37796-w>
- Ruthes, A. C., Rattmann, Y. D., Carbonero, E. R., Gorin, P. A. J., & Iacomini, M. (2012). Structural characterization and protective effect against murine sepsis of fucogalactans from *Agaricus bisporus* and *Lactarius rufus*. *Carbohydrate Polymers*, 87, 1620–1627. <http://dx.doi.org/10.1016/j.carbpol.2011.09.071>
- Ruthes, A. C., Rattmann, Y. D., Malquevicz-Paiva, S. M., Carbonero, E. R., Córdova, M. M., Baggio, C. H., & Iacomini, M. (2013). *Agaricus bisporus* fucogalactan: Structural characterization and pharmacological approaches. *Carbohydrate Polymers*, 92, 184–191. <http://dx.doi.org/10.1016/j.carbpol.2012.08.071>
- 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>.
- Samanta, S., Nandi, A. K., Sen, I. K., Maity, P., Pattanayak, M., Devi, K. S. P., Khatua, S., Maiti, T. K., Acharya, K., & Islam, S. S. (2015). Studies on antioxidative and immunostimulating fucogalactan of the edible mushroom *Macrolepiota dolichaula*. *Carbohydrate Research*, 413, 22–29. <http://dx.doi.org/10.1016/j.carres.2015.05.006>
- Samuelsen, A. B. C., Rise, F., Wilkins, A. L., Teveleva, L., Nyman, A. A. T., & Aachmann, F. L. (2019). The edible mushroom *Albatrellus ovinus* contains a  $\alpha$ -L-fuco- $\alpha$ -D-galactan,  $\alpha$ -D-glucan, a branched (1→6)- $\beta$ -D-glucan and a branched (1→3)- $\beta$ -D-glucan. *Carbohydrate Research*, 471, 28–38. <https://doi.org/10.1016/j.carres.2018.10.012>
- Shi, X., Zhao, Y., Jiao, Y., Shi, T., & Yang, X. (2013). ROS-Dependent Mitochondria Molecular Mechanisms Underlying Antitumor Activity of *Pleurotus abalonus* Acidic Polysaccharides in Human Breast Cancer MCF-7 Cells. *Plos one*, 8, 5, e64266. <https://doi.org/10.1371/journal.pone.0064266>
- Smiderle, F. R., Ruthes, A. C., & Iacomini, M. (2014). Natural polysaccharides from mushrooms: anti-nociceptive and anti-inflammatory properties. In: K. G. Ramawat, & J. M. Mérillon. *Polysaccharides* (pp. 1–25). Springer
- Sung, H., Ferlay, J., Siegel, R. L., Laversanne, M., Soerjomataram, I., Jemal, A., & Bray, F. (2021). Global Cancer Statistics 2020: GLOBOCAN Estimates of incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA: A Cancer Journal for Clinicians*, 71, 3, 209–249. <https://doi.org/10.3322/caac.21660>
- Ványolós, A., Kovács, B., Bózsity, N., Zupkó, I., & Hohmanna, J. (2015). Antiproliferative Activity of Some Higher Mushrooms from Hungary against Human Cancer Cell Lines. *International Journal of Medicinal Mushrooms*, 17, 12, 1145–1149. [10.1615/IntJMedMushrooms.v17.i12.40](https://doi.org/10.1615/IntJMedMushrooms.v17.i12.40)
- Vaz, J. A., Heleno, S. A., Martins, A., Almeida, G. M., Vasconcelos, M. H., & Ferreira, I. C. F. R. (2010). Wild mushrooms *Clitocybe alexandri* and *Lepista inversa*: In vitro antioxidant activity and growth inhibition of human tumour cell lines. *Food and Chemical Toxicology*, 48, 2881–2884. <https://doi.org/10.1016/j.fct.2010.07.021>
- Wolf from, M. L & Thompson, A (1963a). Reduction with sodium borohydride. In: Whistler, R. L.; Wolf from, M. L. (Eds.). *Methods in Carbohydrate Chemistry* (p. 65–68). New York: Academic Press.
- Wolf from, M. L & Thompson, A (1963b). Acetylation. In: Whistler, R. L.; Wolf from, M. L. (Eds.). *Methods in Carbohydrate Chemistry* (p. 211– 215). New York: Academic Press.

Xu, H., Zou, S., & Xu, X. (2017). The  $\beta$ -glucan from *Lentinus edodes* suppresses cell proliferation and promotes apoptosis in estrogen receptor positive breast cancers. *Oncotarget*, 8, 49, 86693-86709. <https://doi.org/10.18632/oncotarget.21411>

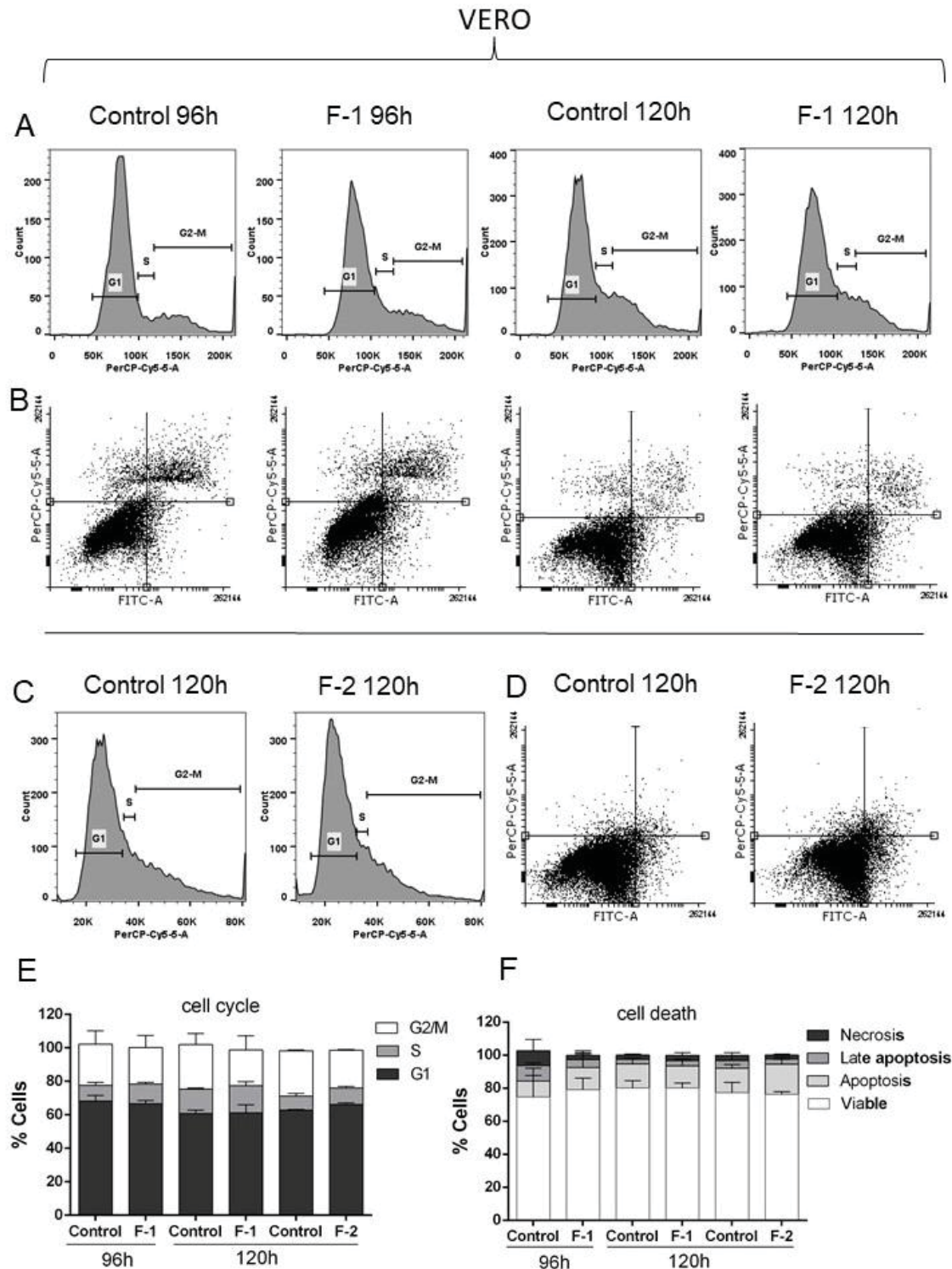
Yadav, D., & Negi, P. S. (2021). Bioactive components of mushrooms: Processing effects and health benefits. *Food Research International*, 148, 110599. <https://doi.org/10.1016/j.foodres.2021.110599>

Yap, Y. H. Y., Tan, N. H., Fung, S. Y., Aziz, A. A., Tan, C. S., & Ng, S. T. (2013). Nutrient composition, antioxidant properties, and anti-proliferative activity of *Lignosus rhinocerus* Cooke sclerotium. *Journal of the Science of Food and Agriculture*, 93, 2945–2952. <https://doi.org/10.1002/jsfa.6121>

Zhang, Y. (2017). *Ganoderma lucidum* (Reishi) suppresses proliferation and migration of breast cancer cells via inhibiting Wnt/b-catenin signaling. *Biochemical and Biophysical Research Communications*, 488, 679-684. <http://dx.doi.org/10.1016/j.bbrc.2017.04.086>



## Supplementary material



**Figure S1:** Effect of F-1 (A) and F-2 (C) treatment on cell cycle progression of VERO cells, analyzed by flow cytometry. The cells were treated with 2 doses (600  $\mu\text{g/mL}$ ) of F-1 or F-2 for 96 h and/or 120 h. Analysis of apoptosis induction in VERO cells, using flow cytometry. The cells were treated with 2 doses (600  $\mu\text{g/mL}$ ) of F-1 (B) or F-2 (D) for 120 h. Bar charts showing (E) cell cycle and (F) proportion of viable, early apoptotic,

late apoptotic and necrotic cells after F-1 and F-2 treatments. The results represent the mean  $\pm$  SD of two independent experiments (n= 3). Statistical analyzes were performed by unpaired t-test. \*p< 0.05; \*\*p< 0.01; \*\*\*p< 0.001, F-1 or F-2 treatment versus control.

**ARTIGO II**

**$\beta$ -Glucans from the giant mushroom *Macrocybe titans*: Chemical characterization and rheological properties**

**(Publicado na revista Food Hydrocolloids – MILHORINI *et al.*, 2022).**

**Doi:** <https://doi.org/10.1016/j.foodhyd.2021.107392>

**$\beta$ -Glucans from the giant mushroom *Macrocybe titans*: Chemical characterization and rheological properties**

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## Abstract

Regardless of the wealth of knowledge about the medicinal properties and industrial applications of fungal compounds, polysaccharides of some species of mushrooms remain unstudied. Therefore, fruiting bodies of the giant *M. titans* mushroom were chosen to extract polysaccharides and evaluate their physicochemical properties. Alkaline extraction yielded GFI fraction, which was composed of  $\beta$ -D-glucans and  $\alpha$ -D-glucans (glycogen). This fraction underwent amylase treatment giving rise to a fraction that left only  $\beta$ -D-glucans of different molar masses ( $\beta$ -GLC) that were composed of (1 $\rightarrow$ 3)-linked  $\beta$ -D-Glcp units substituted at O-6 by (1 $\rightarrow$ 6)-linked  $\beta$ -D-Glcp or single units of  $\beta$ -D-Glcp. Both GFI (at 2 % w/w) and  $\beta$ -GLC (at 1.5 % w/w), were analyzed about their rheological properties and presented a non-Newtonian shear-thinning behavior and gel-like behavior under low temperature. It was confirmed that the gel-like behavior of GFI was due to the presence of  $\beta$ -glucans, once the fraction with isolated  $\beta$ -glucans ( $\beta$ -GLC) produced a stronger gel than the mixture with glycogen. This is probably due to a better association and network formation among  $\beta$ -glucans. Furthermore, methylated analyses showed a great amount of (1 $\rightarrow$ 6)-linked Glcp units, which was observed to produce a weaker gel in comparison to other glucans. This study presented for the first time that glucans extracted from *M. titans* may form gel under suitable conditions, enabling their industrial application as thickening or gelling agents.

**Keywords:** *Macrocybe titans*; Polysaccharide;  $\beta$ -glucan; Rheology; Gel.

## 1. Introduction

Edible mushrooms have been widely consumed in eastern countries due to their nutritional value and medicinal properties. As they are considered functional foods, their use extends from cooking to folk medicine. The bioactive molecules present in mushroom fruiting bodies induce a remarkable effect on the prevention and treatment of several disorders (Ruthes, Smiderle & Iacomini, 2015). Among these bioactive compounds, polysaccharides are the best known and most potent agents with antitumor and immunomodulatory properties (Smiderle, Ruthes & Iacomini, 2014). Furthermore, such polymers have presented antioxidant (Zhang, Li, Wang, Zhang & Cheung, 2011), anti-inflammatory, antinociceptive (Abreu et al., 2019), and prebiotic effects (Synystya et al., 2009).

In addition to their medicinal potential, mushroom polysaccharides, mainly the  $\beta$ -glucans, may also show physicochemical characteristics such as gelling capacity, that enable their use in the food and cosmetic industry (Abreu et al., 2019; Ahmad, Munir & Abrar, 2012; Sovrani, Jesus, Simas-Tosin, Smiderle & Iacomini, 2019; Zhu, Du & Xu, 2016). Considering the notable development of the food and the pharmaceutical industries, molecules with rheological and biological properties are attractive agents to be used in several formulations as thickeners or gelling agents (Wang, Yin, Huang & Nie, 2020). A branched  $\beta$ -glucan obtained from *Pholiota nameko*, with (1 $\rightarrow$ 3)-linked main chain, exhibited a thermostable gel-like behavior in the range of 5-60 °C (at a concentration of 2 %, w/w), making it interesting to be used in the food industry to thicken or jellyfy products (Sovrani et al., 2017). Another  $\beta$ -glucan obtained from the same species and with the same main chain produced a more viscous dispersion and a gel-like behavior even at lower concentrations (0.5 and 1 % w/w) (Abreu et al., 2019). The difference between both  $\beta$ -glucans is based on the length of their side chains: the latter  $\beta$ -glucan presents shorter side chains than the first one. Interestingly, when the more viscous  $\beta$ -glucan was submitted to a pasteurization process simulation, the gel-like behavior was maintained, suggesting that it could be used as an additive in the food industry even in products that are submitted to this kind of thermal process. Other mushroom species have been described as sources of  $\beta$ -glucan with gel-like behavior, such as *Lentinus edodes* (at 0.3 and 0.6 % w/w) (Zhang, Xu & Zhang, 2008) and *Hericium erinaceus* (at 2 % w/v) (Wang et al., 2019). Rheological studies are an important evaluation to investigate the potential of

mushroom  $\beta$ -glucans and their applicability in the food industry, considering the gelling and thickening capacity of such molecules.

The giant mushroom *Macrocybe titans* (H.E. Bigelow & Kimbr.) Pegler, Lodge & Nakasone is a mushroom that grows in a caespitose pattern, which may exceed 30 kg of fresh weight (Pegler, Lodge, & Nakasone, 1998). Milhorini et al. (2018) isolated a fucogalactan from such species, which showed bioactivity in murine B16F10 melanoma cells *in vitro*, reducing cell migration without causing cytotoxicity. However, until recent knowledge, there are no studies reporting the structure and rheological properties of *M. titans*  $\beta$ -glucans. Therefore, this study aimed to isolate and characterize the structure and rheological properties of  $\beta$ -glucans-rich fractions from *M. titans* fruiting bodies.

## 2. Material and methods

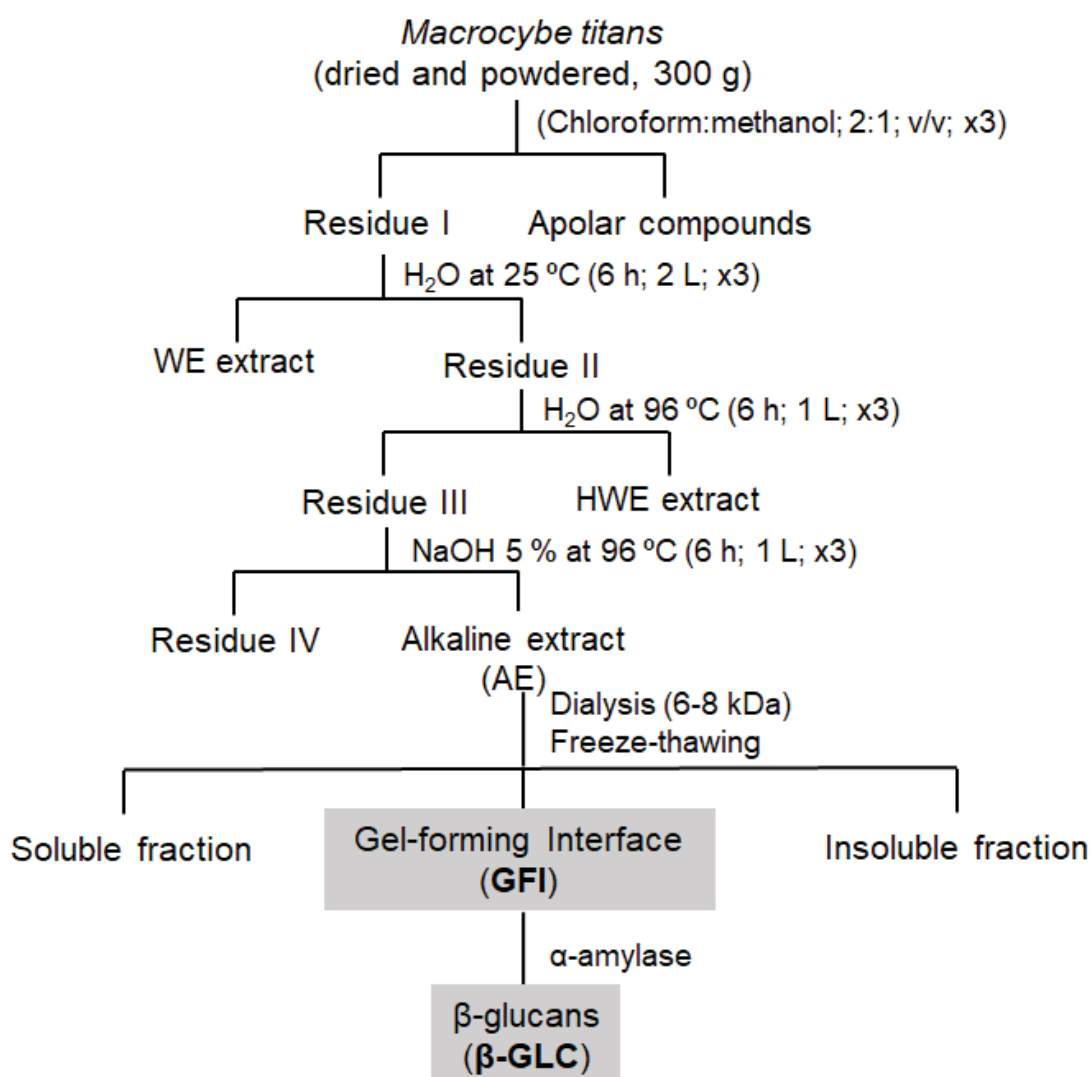
### 2.1 Biological material

The mushroom *Macrocybe titans* was collected in Jesuítas – PR, Brazil and kindly donated by Dr. Fábio Rogério Rosado from the Department of Bioscience, Federal University of Paraná – Palotina, PR, Brazil. This mushroom was identified by molecular analysis and the nucleotide sequence was published in GenBank database (MZ519068). The studied species was registered on *Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado* (SISGEN), number A3BA3B3.

### 2.2 Extraction and purification procedure

Extractions were carried out with freeze-dried and powdered fruiting bodies (300 g). Firstly, the material was delipidified using a mixture of chloroform and methanol (2:1; v/v) at 60 °C for 3 h (x3). The delipidified fraction was submitted to three sequential extractions, as shown in Fig. 1. The first of them was with distilled water and mechanical stirring at 25 °C (2 L; 6 h; x3), which was then centrifuged (10,000 rpm; 20 min; 15 °C) yielding the water extract (WE) and a precipitate (residue II). The residue II was submitted to hot water extraction (1 L; 6 h; 96 °C; x3;) under reflux and the hot water extract (HWE) was separated from a precipitate (residue III) under the same

centrifugation conditions described above. Finally, the third residue (residue III) was extracted with 5% (w/v) aq. NaOH containing NaBH<sub>4</sub> (1 L; 6 h; 96 °C; x3) under boiling reflux. After centrifugation to remove insoluble residues, the alkaline extract (AE) was neutralized with acetic acid, dialyzed against tap water (6-8 kDa) for 48 h, and freeze-dried. The dialyzed AE was then submitted to the freeze-thawing process (Gorin & Iacomini, 1984) yielding cold water-soluble and -insoluble polysaccharide fractions (not analyzed in this study), which were separated by centrifugation (9,000 rpm; 25 min; 4 °C). During the centrifugation process, a gel-forming interface (GFI) was observed, which was separated from the other ones, using a pipette. The GFI sample was treated with  $\alpha$ -amylase (Sigma-Aldrich), precipitated with ethanol (3:1; v/v), and dialyzed against tap water (6-8 kDa) for 24 h, giving rise to  $\beta$ -GLC sample.



**Figure 1:** Scheme of extraction and purification of *M. titans* β-glucans fraction.



### 2.3 Analysis of monosaccharide composition by GC-MS

Alditol acetates were prepared according to the methodology found in article by Sasaki et al. (2008), with small modifications. Briefly, samples (1 mg) were hydrolyzed with TFA 2 M (200  $\mu$ L) at 100 °C for different periods (8-18 h) to determine the best time for complete hydrolyzing  $\beta$ -glucans. The time of 18 h was chosen and after this period, the acid was evaporated off to dryness. Hydrolysis products were solubilized in 200  $\mu$ L of distilled water, reduced with NaBH<sub>4</sub> (pH 9-10) overnight at room temperature, neutralized with acetic acid, washed with methanol followed by evaporation (200  $\mu$ L; x3) and acetylated with pyridine–Ac<sub>2</sub>O (200  $\mu$ L; 1:1; v/v) at 100 °C for 30 min. The resulting alditol acetates were extracted with chloroform (1 mL), washed with copper sulfate 5 % (2.5 mL; x4), analyzed by GC-MS, and identified by their typical retention times and electron ionization profiles. For analyses, derivatized samples were solubilized in hexane (1 mL) and 1  $\mu$ L diluted by a factor of 10 was injected into a gas chromatograph (GC-2010 Plus) coupled to the TQ 8040 quadrupole triple mass spectrometer (Shimadzu) equipped with a Combipal autosampler (AOC 5000), using a SH-Rtx-5MS capillary column (30 m x 0.25 mm d.i.). The injector was maintained at 250 °C and analyses were performed starting at 100 °C with an increase to 280 °C (10 °C/min). Helium was used as carrier gas at a flow rate of 1.0 mL/min.

### 2.4 Methylation analysis

The purified  $\beta$ -glucan fraction ( $\beta$ -GLC) was firstly per-O-methylated with dimethyl sulfate according to the methodology adapted from Haworth (1915). The sample (15 mg) was solubilized in distilled water (1 mL), reduced with NaBH<sub>4</sub> (pH 9-10) overnight at room temperature, neutralized with HOAc, dialyzed against tap water (3.5 kDa) for 24 h, and lyophilized. Subsequently, the sample were solubilized in NaOH 40 % (3 mL) and dimethyl sulfate (3 mL) was gradually added (0.5 mL every 30 min). After that, the mixture remained under magnetic stirring overnight, the sample was dialyzed (3.5 kDa) for 24 h and lyophilized.

To guarantee that all free hydroxyls of the molecule were methylated, the sample was methylated for a second time using NaOH-Me<sub>2</sub>SO-MeI, a modified method of Ciucanu and Kerek (1984). Briefly, the sample was solubilized in dimethyl sulfoxide (1 mL), then powdered NaOH (20 mg) and methyl iodide (1 mL) were added. After 30 min under mechanical stirring, the sample was let to stand at room temperature for 24

h. Subsequently, it was solubilized in water, neutralized with HOAc, and dialyzed against tap water (3.5 kDa) for 24 h. This methylation process was repeated twice. In the last repetition, after the per-O-methylated sample was solubilized in water and neutralized it was extracted with chloroform. Thereafter, chloroform was evaporated, and methanolysis was carried out with 3 N MeOH-HCl at 80 °C for 2 h following methodology adapted from Woranovicz et al. (1999). Subsequently, hydrolysis with 2 N H<sub>2</sub>SO<sub>4</sub> was carried out for 24 h at 100 °C. Hydrolyzed samples were neutralized with BaCO<sub>3</sub>, reduced with NaBD<sub>4</sub> (pH 9-10) overnight at room temperature, and then neutralized, washed, acetylated, and analyzed in GC-MS as described above for composition analysis (topic 2.3).

## **2.5 Controlled Smith degradation of $\beta$ -glucans**

For oxidation, an aliquot of the sample  $\beta$ -GLC (100 mg) was solubilized in water and sodium periodate was added to a final concentration of 0.05 M. After 72 h under magnetic stirring and protected from light, samples were dialyzed (2 kDa cut-off) for 24 h. Oxidized materials were reduced with NaBH<sub>4</sub> overnight, neutralized with HOAc, and dialyzed (2 kDa cut-off) for 24 h, following methodology with modifications from Goldstein, Hay, Lewis & Smith, (1965). For partial hydrolysis, 2 M TFA was added until achieving pH 2.0 and the process was carried out at 100 °C for 30 min under reflux, followed by dialysis (2 kDa cut-off) for 24 h and freeze-drying (Abreu et al., 2019; Sovrani et al., 2017).

## **2.6 Colorimetric determination of triple helix with Congo Red**

Analyses were carried out following Ogawa & Hatano (1978) and Palacios, García-Lafuente, Guillamón & Villares, (2012), with minor modifications. According to Ogawa, Tsurugi & Watanabe (1972), glucans existing in an ordered three-dimensional conformation, generally triple helical structure, form a complex with Congo red in dilute NaOH solutions. This complex is stabilized by intermolecular interactions such as hydrogen bonds and/or hydrophobic interactions between the glucan and the dye. The complex formation can be observed by means of the shift in the maxima visible absorption (I<sub>max</sub>) of the Congo red spectrum.

Samples (GFI and  $\beta$ -GLC; 1 mg) and pattern Dextran M<sub>w</sub> 40,200 (Sigma-Aldrich; 1 mg), were solubilized in 0.05 M NaOH (980  $\mu$ L) and Congo Red 4,000  $\mu$ M

was added (20  $\mu$ L) to achieve a final concentration of 80  $\mu$ M. The blank was prepared with 0.05 M NaOH (980  $\mu$ l) plus Congo Red 4,000  $\mu$ M (20  $\mu$ L) and Dextran  $M_w$  40,200 was used as random-coil control, which shows similar absorbance to the Congo red. Absorbance readings were taken at a range of 420 to 640 nm with 10 nm intervals using a microplate reader.

## 2.7 Determination of protein and phenolic compounds

Protein amount was investigated by Bradford method (Bradford, 1976), following factures recommendations (Sigma-Aldrich). Bovine serum albumin (BSA) was used as standard. Phenolic compounds were estimated according to Singleton, Orthofer & Lamuela-Raventós (1999), using Folin & Ciocalteu phenol's reagent (Sigma-Aldrich). Gallic acid (GAE) was used as standard. For both analyses, samples were solubilized in distilled water at 2 mg/mL.

## 2.8 Nuclear magnetic resonance (NMR) spectroscopy

Mono- ( $^1\text{H}$ ,  $^{13}\text{C}$ -NMR, and DEPT-135) and two-dimensional (HSQC-DEPT) NMR analyses were performed using a BRUKER Avance-DRX-400 spectrometer. Glucan samples (30 mg) were solubilized in  $\text{Me}_2\text{SO}-d_6$  and analyzed at 70  $^\circ\text{C}$ . Chemical shifts were expressed in ppm ( $\delta$ ) relative to the  $\text{Me}_2\text{SO}-d_6$  signals ( $^{13}\text{C}$ :  $\delta$  39.70 and  $^1\text{H}$ :  $\delta$  2.40).

## 2.9 High-performance size-exclusion chromatography (HPSEC)

Analyses were performed in an HPSEC coupled to refractive index detector, using four gel-permeation Ultrahydrogel columns in series with exclusion sizes of  $7 \times 10^6$ ,  $4 \times 10^5$ ,  $8 \times 10^4$ , and  $5 \times 10^3$  Da. Aqueous  $\text{NaNO}_2$  (0.1 M) containing aqueous  $\text{NaN}_3$  (200 ppm) was used as eluent at a flow rate of 0.6 mL/min. Prior to analysis, fractions GFI and  $\beta$ -GLC were solubilized in the eluent (1 mg/mL) and filtered through a cellulose membrane (0.22  $\mu\text{m}$ ). Afterwards, samples were injected (100  $\mu\text{L}$  loop) into the chromatograph. To determinate the relative molecular weight of the  $\beta$ -glucan fraction ( $\beta$ -GLC), its retention time was compared with a curve of dextran patterns of different molecular masses (Sigma-Aldrich) ( $5.00 \times 10^3$ ;  $9.40 \times 10^3$ ;  $1.72 \times 10^4$ ;  $4.02 \times 10^4$ ;  $7.22 \times 10^4$ ;  $1.24 \times 10^5$ ;  $2.66 \times 10^5$ ;  $4.87 \times 10^5$ ;  $2.00 \times 10^6$  g/mol).

## 2.10 Rheological experiments

For rheological studies, it was used a HAAKE MARS II rheometer (Thermo Fisher Scientific, Karlsruhe, Germany) with a cone-plate measurement system (C60/2°TiL) and a 1 mm measurement gap. GFI and  $\beta$ -GLC were evaluated at concentrations of 1.5 % or 2 % w/w. The samples were solubilized in distilled water under magnetic stirring at room temperature for 12 h and let rest for 20 min before analyses. The temperature was controlled by a thermostatic bath (DC5, HAAKE) coupled to a Peltier thermal controller (TC 81, HAAKE). Prior to analysis, samples were kept on the rheometer plate for 300 s to equilibrate the sample temperature.

The flow behavior was obtained in the controlled shear rate mode by increasing shear rate ( $0.001$ - $1,000\text{ s}^{-1}$ ) during 600 s at 25 °C. Aiming to investigate the gelling temperature of glucan-rich fractions, the temperature-dependence of the elastic modulus ( $G'$ ) and viscous modulus ( $G''$ ) was analyzed based on a temperature ramp (from 24 °C to 3 °C) at a cooling rate of 2 °C/min, using fixed frequency (1 Hz) and fixed strain (1 %). Thereafter, the samples were maintained at 3 °C for 3,600 seconds and then frequency sweeps ( $0.02$ - $10\text{ Hz}$ ) with a fixed strain of 1 % were carried out to analyze their viscoelastic behavior.

All experiments were performed in independent triplicates and RheoWin 4 Data Manager software was used to analyze data.

## 3. Results and discussion

### 3.1 Chemical characterization of glucan fractions isolated from *M. titans*

*M. titans* fractions containing different glucans were purified as described in section 2.2. Monosaccharide composition analyses were carried out aiming to follow the purification process (Table 1).

**Table 1:** Yields and monosaccharide composition of fractions obtained from *M. titans* alkaline extract (AE).

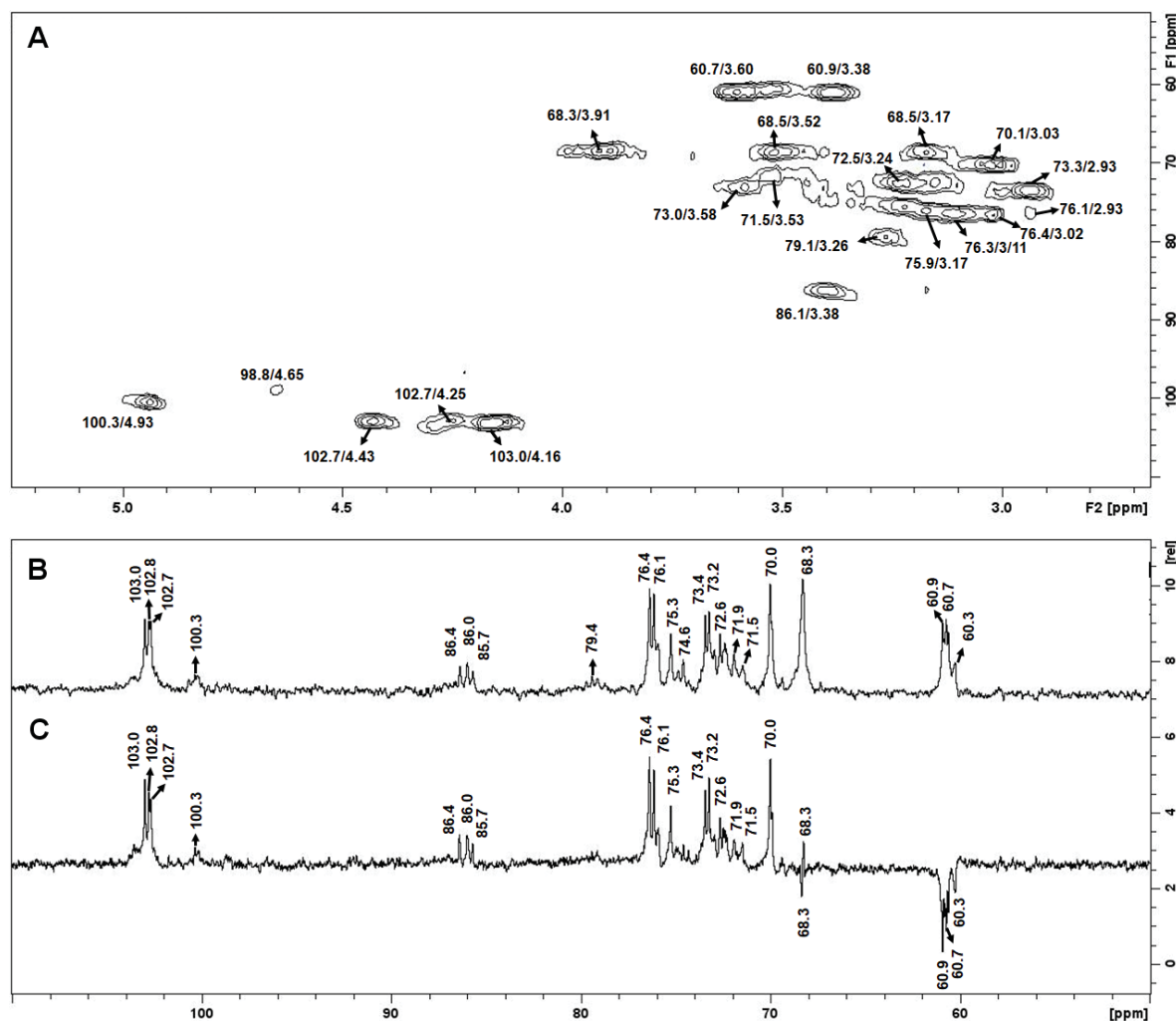
Fractions <sup>a</sup>	Yield <sup>b</sup> (%)	Monosaccharide Composition <sup>c</sup>					
		Glc	Man	Gal	Xyl	Ara	Fuc
<b>AE</b>	9.05	80.2	8.1	5.5	4.0	1.4	0.8
<b>GFI</b>	0.35	87.8	2.4	7.3	1.0	1.2	0.3
<b>β-GLC</b>	0.16	94.0	-	6.0	-	-	-

<sup>a</sup> AE: Alkaline extract. GFI: Gel-forming interface obtained from Alkaline Extract. β-GLC: Sample obtained from GFI and treated with α-amylase.

<sup>b</sup> Yields relative to dried weight of fungi.

<sup>c</sup> Alditol acetate derivatives obtained after hydrolysis, NaBH<sub>4</sub> reduction and acetylation.

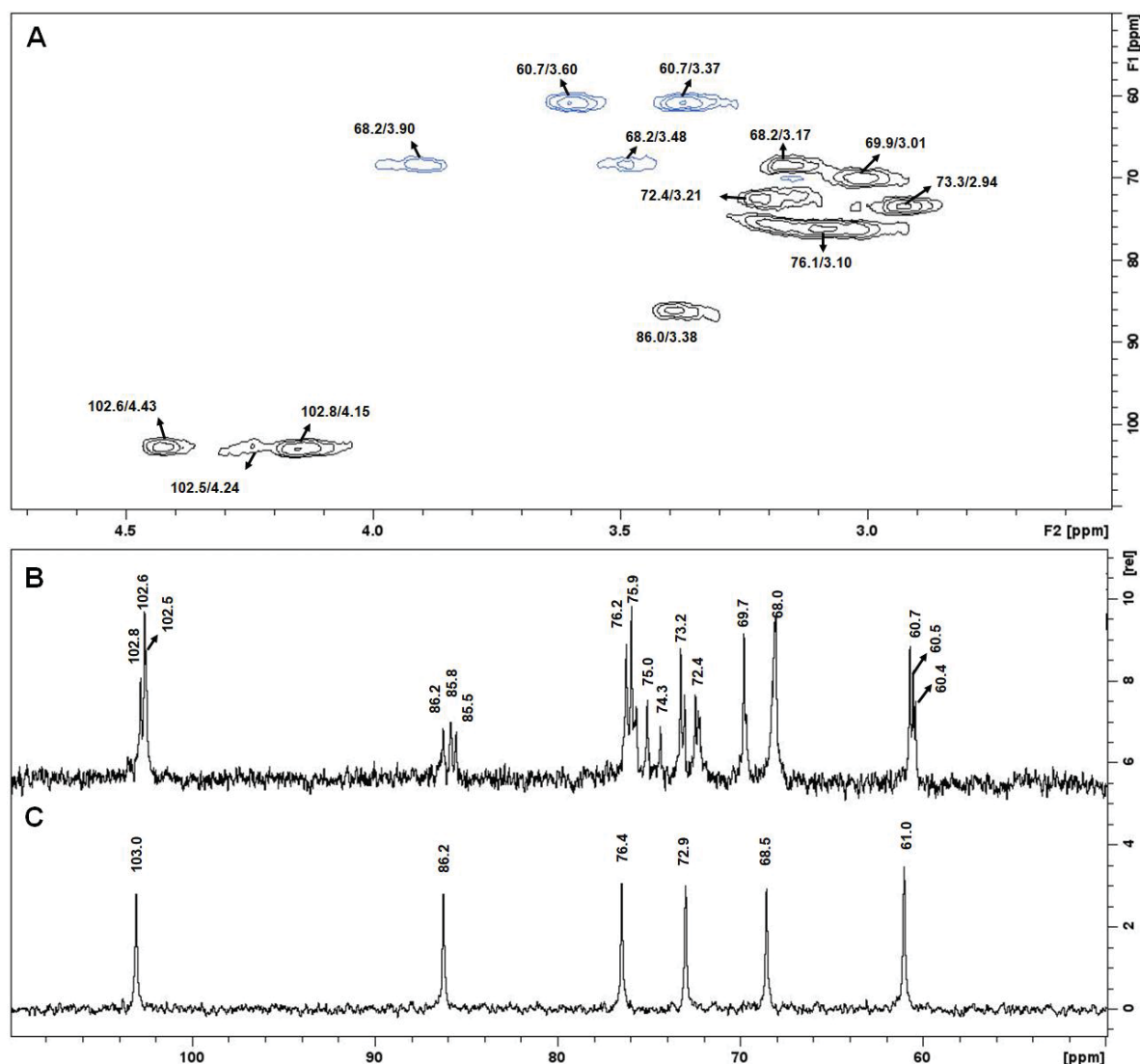
The glucose concentration constantly increased during the purification procedures, raising from 80.2 %, in the alkaline extract (AE), to 94 % in β-GLC fraction, strongly indicating that the purified fraction was composed of glucans. NMR analysis of GFI fraction (Fig. 2) showed prominent signals that indicate the presence of β-glucans and less intense signals of α-glucans. The NMR assignments observed are very similar to those reported in current literature and they were compiled in table 1 (supplementary material). Resonances observed in HSQC experiment (Fig. 2A and Table 1, supplementary material) corresponding to C-1 of β-D-Glcp were observed at δ 102.7/4.25, 102.7/4.43, and 103.0/4.16 (Abreu et al., 2019; Sovrani et al., 2017) and to C-1 of α-D-Glcp at δ 100.3/4.93 (Smiderle et al., 2010). A signal corresponding to glycosidic linkages was observed at δ 86.1/3.38, characteristic of O-3 substitution from (1→3)-linked β-D-glucans (Abreu et al., 2019; Sovrani et al., 2017); while the O-6 substitution was confirmed by signals at δ 68.3/3.91 and 68.5/3.52 (Moreno et al., 2016); and the presence of a signal at δ 79.1/3.26 confirmed O-4 substitution, probably relative to glycogen, the fungal energy storage (Smiderle et al., 2010). These assignments were confirmed by <sup>13</sup>C and DEPT-135 experiments (Fig. 2B and C). It was estimated that GFI was composed of 77.8 % β-glucan and 22.2 % α-glucan by integrating the area of the signals corresponding to the hydrogens linked to anomeric carbons (<sup>1</sup>H experiment; data not shown).



**Figure 2:** NMR spectra of GFI fraction. (A) HSQC; (B)  $^{13}\text{C}$  and (C) DEPT-135. The sample was solubilized in  $\text{Me}_2\text{SO}-d_6$  at  $70^\circ\text{C}$  (chemical shifts are expressed in ppm)

It is known that glycogen can easily be degraded by  $\alpha$ -amylase treatment (Synytsya & Novák, 2013), therefore, the fraction GFI was submitted to this enzymatic digestion, giving rise to  $\beta$ -GLC fraction, which was analyzed by NMR (Fig. 3). This analysis showed no signals relative to glycogen, indicating that the  $\alpha$ -amylase treatment was effective as a purification step. Accordingly, the HSQC-DEPT spectrum (Fig. 3A, Table 1, supplementary material) showed signals related to  $\beta$ -glucans containing  $\beta$ -D-Glcp units (1 $\rightarrow$ 3)- and/or (1 $\rightarrow$ 6)-linked. Resonances corresponding to C-1 of glucose in  $\beta$ -configuration were observed at  $\delta$  102.5/4.24, 102.6/4.43, and 102.8/4.15. No signals of  $\alpha$ -D-Glcp units were detected. The presence of O-3 substitution was evidenced by a signal at  $\delta$  86.0/3.38. Signals arising from non-substituted C-6 were observed at  $\delta$  60.7/3.37 and 60.7/3.60, while inverted signals

relative to O-substituted C-6 were observed at  $\delta$  68.2/3.48 and 68.2/3.90 (Abreu et al., 2019; Moreno et al., 2016; Smiderle et al., 2008; Sovrani et al., 2017). The  $^{13}\text{C}$  experiment confirmed the results (Fig. 3B).



**Figure 3:** NMR spectra of  $\beta$ -GLC fraction: (A) HSQC-DEPT and (B)  $^{13}\text{C}$ . NMR  $^{13}\text{C}$  spectrum of Smith-degraded  $\beta$ -GLC (C). The sample was solubilized in  $\text{Me}_2\text{SO}-d_6$  at  $70^\circ\text{C}$  (chemical shifts are expressed in ppm).

A controlled Smith Degradation was performed in  $\beta$ -GLC fraction to observe if there were fragments of (1 $\rightarrow$ 3)-linked chains, which are resistant to this oxidative treatment. The residual product was analyzed by NMR (Fig. 3C) and the spectrum showed six signals corresponding to a (1 $\rightarrow$ 3)-linked main chain at:  $\delta$  103.0 (C-1); 86.2



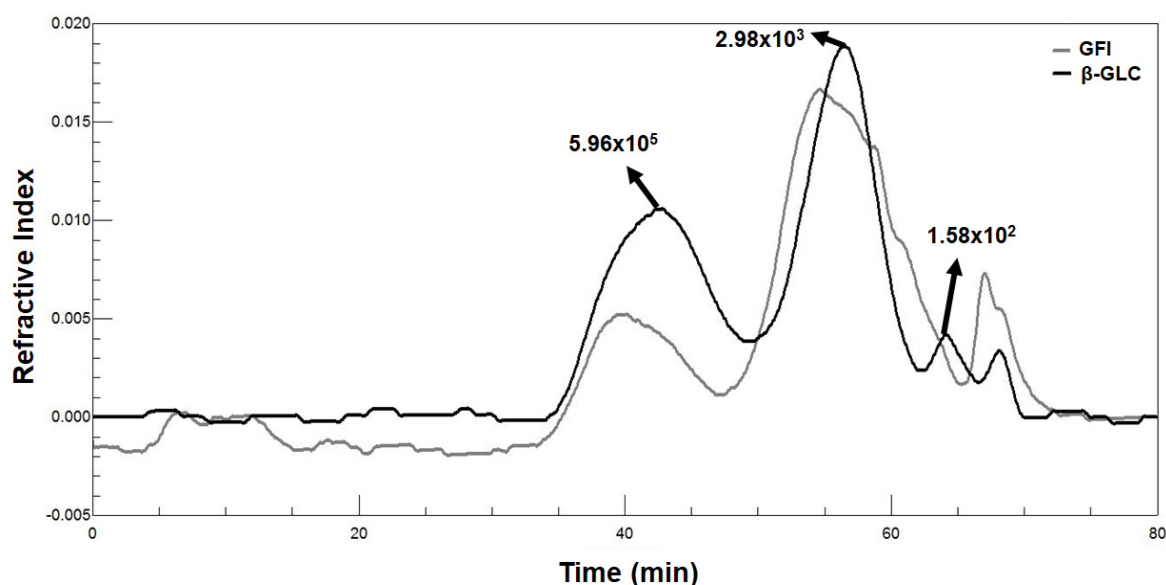
(C-3); 76.4 (C-5); 72.9 (C-2); 68.5 (C-4) and 61.0 (C-6). This data was confirmed by comparison with other studies (Carbonero et al., 2012; Moreno et al., 2016).

Methylation analysis of  $\beta$ -GLC corroborate with NMR results, once it presented derivatives corresponding to a (1 $\rightarrow$ 3)-linked D-glucan (2,4,6-Me<sub>3</sub>-Glc<sub>p</sub>; 13.5 %) substituted at O-6 (2,4-Me<sub>2</sub>-Glc<sub>p</sub>; 24.8 %), by branches that could be either D-Glc<sub>p</sub> units (2,3,4,6-Me<sub>4</sub>-Glc<sub>p</sub>; 9.0 %) or D-Glc<sub>p</sub> (1 $\rightarrow$ 6)-linked side chains (2,3,4-Me<sub>3</sub>-Glc<sub>p</sub>; 52.7 %).

Several  $\beta$ -glucans with similar structures were obtained from different mushroom species, such as *Pleurotus pulmonarius* (Smiderle et al., 2008), *Lentinus edodes* (Zhang et al., 2011), *Amanita muscaria* (Ruthes et al., 2013), and *Cookeina Tricholoma* (Moreno et al., 2016).

### 3.2 Relative molar mass and conformational studies of glucan fractions

Both fractions showed heterogeneous profiles when analyzed in HPSEC, indicating that these fractions are composed of glucans of different masses (Fig. 4). The  $\beta$ -GLC fraction presented three populations of  $\beta$ -glucans with  $M_w$   $5.96 \times 10^5$ ,  $2.98 \times 10^3$ , and  $1.58 \times 10^2$  g/mol.

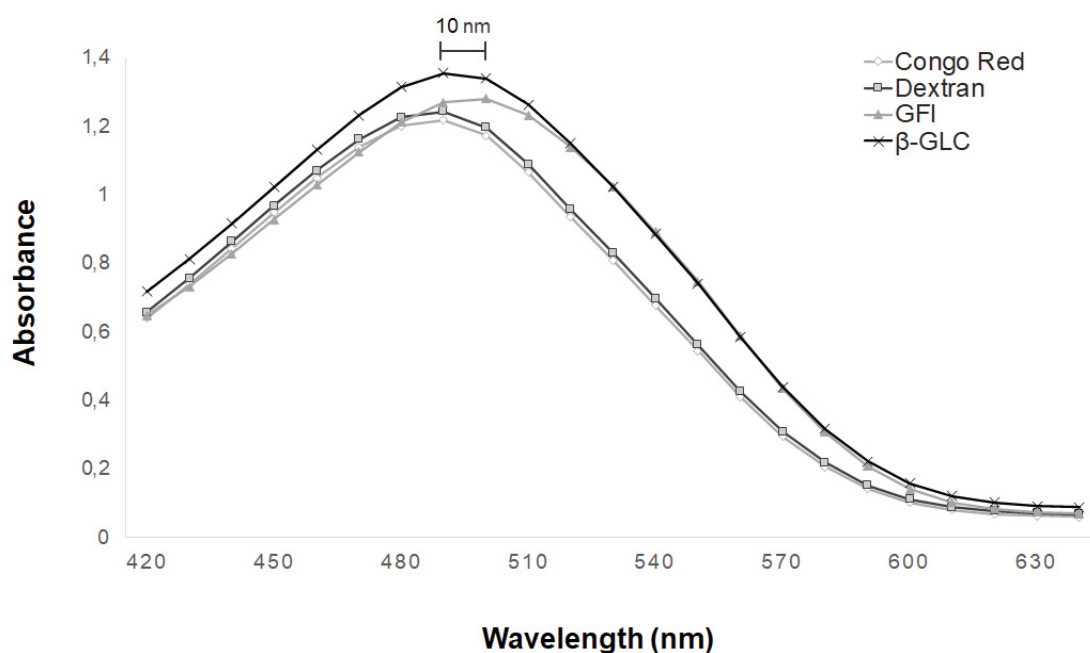


**Figure 4:** Elution profile of glucan fractions from *M. titans* (GFI and  $\beta$ -GLC) determined by HPSEC (refractive index detector), eluted in 0.1 M NaNO<sub>2</sub>.



A  $\beta$ -glucan (1 $\rightarrow$ 3),(1 $\rightarrow$ 6), named lentinan, obtained from *L. edodes*, is one of the best-characterized mushroom polysaccharides so far (Lemieszek & Rzeski, 2012). According to Zhang et al. (2011), many studies have been conducted and showed that lentinan was able to activate many cells of the immune system and presents antitumor activity only when they are in a triple-helical conformation. Both  $\alpha$ - and  $\beta$ -glucans had been reported to have a triple-helical conformation in aqueous or weakly alkaline solution (<0.15 M NaOH), and such conformation is maintained mainly by hydrogen bonds, which are also important to keep solutions with gel-like behavior state.

To evaluate the conformational structure of GFI and  $\beta$ -GLC, aliquots of each fraction were analyzed using the Congo red dye (Fig. 5). GFI showed a bathochromic shift (10 nm) when compared to Dextran, and this displacement indicates that some polysaccharides present in this sample have a triple-helical structure, that complex with the dye (Palacios et al., 2012; Smiderle et al., 2014).  $\beta$ -GLC fraction, in its turn, showed no displacement, revealing that  $\beta$ -glucans present in this fraction are not in triple-helical conformation. Molecular parameters obtained from laser light scattering showed that  $\alpha$ -(1 $\rightarrow$ 4)- and  $\beta$ -(1 $\rightarrow$ 3)-glucans have a flexible and helical conformation (Zhang, Cui, Cheung & Wang, 2007). On the other hand, glucose residues connected by (1 $\rightarrow$ 6)-linkages may present many possible conformations and great flexibility due to freedom of rotation of carbon-6, in comparison to the other carbons of the glucose ring (Zhang, Cui, Cheung & Wang, 2007). This characteristic may explain the bathochromic shift observed on GFI, which contains glycogen, an  $\alpha$ -(1 $\rightarrow$ 4)-linked glucan, and the loss of the helical conformation when the  $\beta$ -glucans were isolated on  $\beta$ -GLC fraction. Methylation results showed that  $\beta$ -GLC present high content of  $\beta$ -(1 $\rightarrow$ 6)-linkages (52.7 %), that could possibly be connected to a  $\beta$ -(1 $\rightarrow$ 3)-linked main chain and, therefore prevent the helical conformation to be formed.



**Figure 5:** Absorption spectra of Congo Red (control), Congo Red with dextran (random coil control), and Congo Red with  $\beta$ -Glucans fractions from *M. titans*.

To confirm that no other compounds were interfering in the conformational state, and consequently modifying rheological properties (Tudorache & Bordenave, 2019), protein and phenolic content were evaluated by colorimetric methods. None of these compounds were detected in GFI and  $\beta$ -GLC fractions.

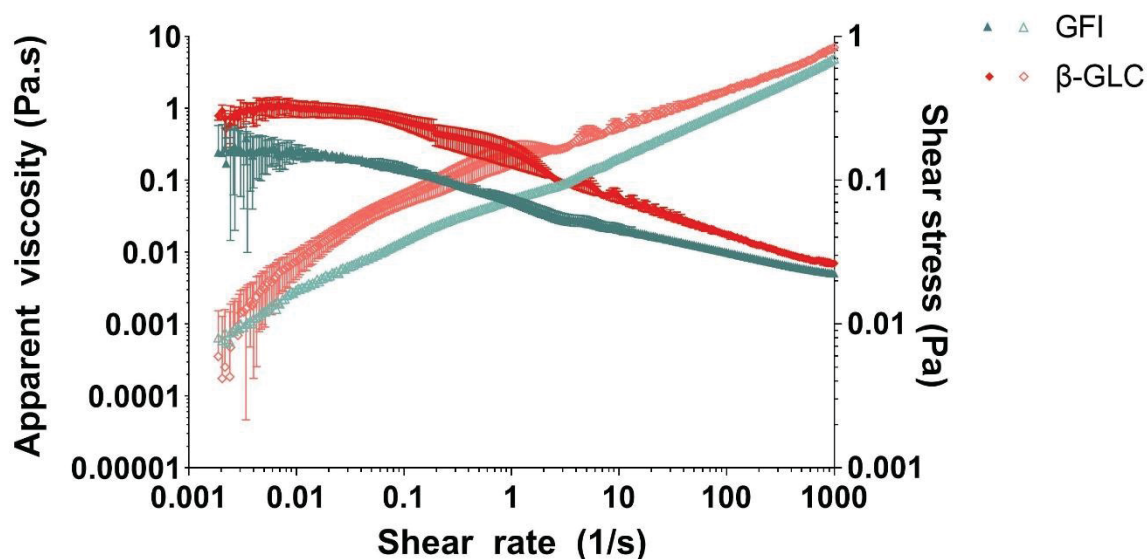
### 3.3 Rheological experiments

Rheological studies were carried out with GFI fraction at 2 % (w/w) and with  $\beta$ -GLC fraction at 1.5 % (w/w). The concentration of  $\beta$ -GLC fraction was chosen based on estimative of  $\beta$ -glucan percentual present in GFI fraction, which was 77.8 %. Thus, it was possible to infer if the rheological properties of GFI fraction arose from  $\beta$ -glucan content.

Viscosity curves of both samples showed that viscosity decreases with the increase of the shear rate, which is typical of non-Newtonian shear-thinning behavior (Fig. 6). This behavior is usually observed in polysaccharide solutions due to the alignment of polysaccharides or deformation along flow direction (Lapasin & Prici, 1995; Sovrani et al., 2017). Several purified  $\beta$ -glucans or extracts containing  $\beta$ -

glucans, obtained from different mushroom species such as *P. nameko* (Sovrani et al., 2017), *L. edodes* (Zhang; Xu; Zhang, 2008), *Agaricus brasiliensis* (Gonzaga et al., 2014), and *Dictyophora rubrovolvata* (Wang et al., 2020) also showed a non-Newtonian shear-thinning behavior. The  $\beta$ -GLC fraction showed higher viscosity than GFI fraction (Fig. 6). The viscosity of GFI and  $\beta$ -GLC was 0.3 Pa.s and 1.0 Pa.s, respectively, at a shear rate of  $0.01 \text{ s}^{-1}$ . This data suggests there is no synergistic effect between the glycogen and  $\beta$ -glucans on the solution viscosity, once  $\beta$ -GFI alone has a higher apparent viscosity than the mixture with glycogen. Such increase in apparent viscosity may also suggest glycogen was hampering  $\beta$ -glucans to form gel. It has been reported in the literature that the viscosity synergism occurs when  $\beta$ -glucan are in combination with some polysaccharides, such as xanthan, iota-carrageenan, and carboxymethyl cellulose. Nevertheless, the presence of alginate and gum Arabic together with  $\beta$ -glucans reduces viscosity when compared with  $\beta$ -glucan alone (Ghotra, Vasanthan & Temelli, 2009). By the results presented on this study, it was demonstrated that glycogen also reduces viscosity when associated with  $\beta$ -glucans. Furthermore, the triple-helical conformation observed on GFI (Fig. 5) does not seem to be associated with improvement of viscosity. On the contrary, it was observed that random-coil conformation detected on  $\beta$ -GFI could be related with the viscosity increase of this fraction.

Compared with other mushroom glucans previously described,  $\beta$ -GLC fraction showed lower viscosity. A  $\beta$ -D-glucan from *P. nameko*, which contains 8.9 % of (1 $\rightarrow$ 6)-linked Glcp units, presented an apparent viscosity of 31.6 Pa.s, at  $1 \text{ s}^{-1}$  shear rate (at 2 % w/w) (Abreu et al., 2019). Another  $\beta$ -D-glucan isolated from the same mushroom, with higher amount of (1 $\rightarrow$ 6)-linked Glcp units (16 %), showed 9x lower apparent viscosity when analyzed at the same conditions (3.5 Pa.s). Following the same pattern,  $\beta$ -D-glucan solution of this study ( $\beta$ -GLC) presented even lower apparent viscosity (0.15 Pa.s), which is probably due to the higher amount of (1 $\rightarrow$ 6)-linked Glcp units observed in this fraction (52.7 %).



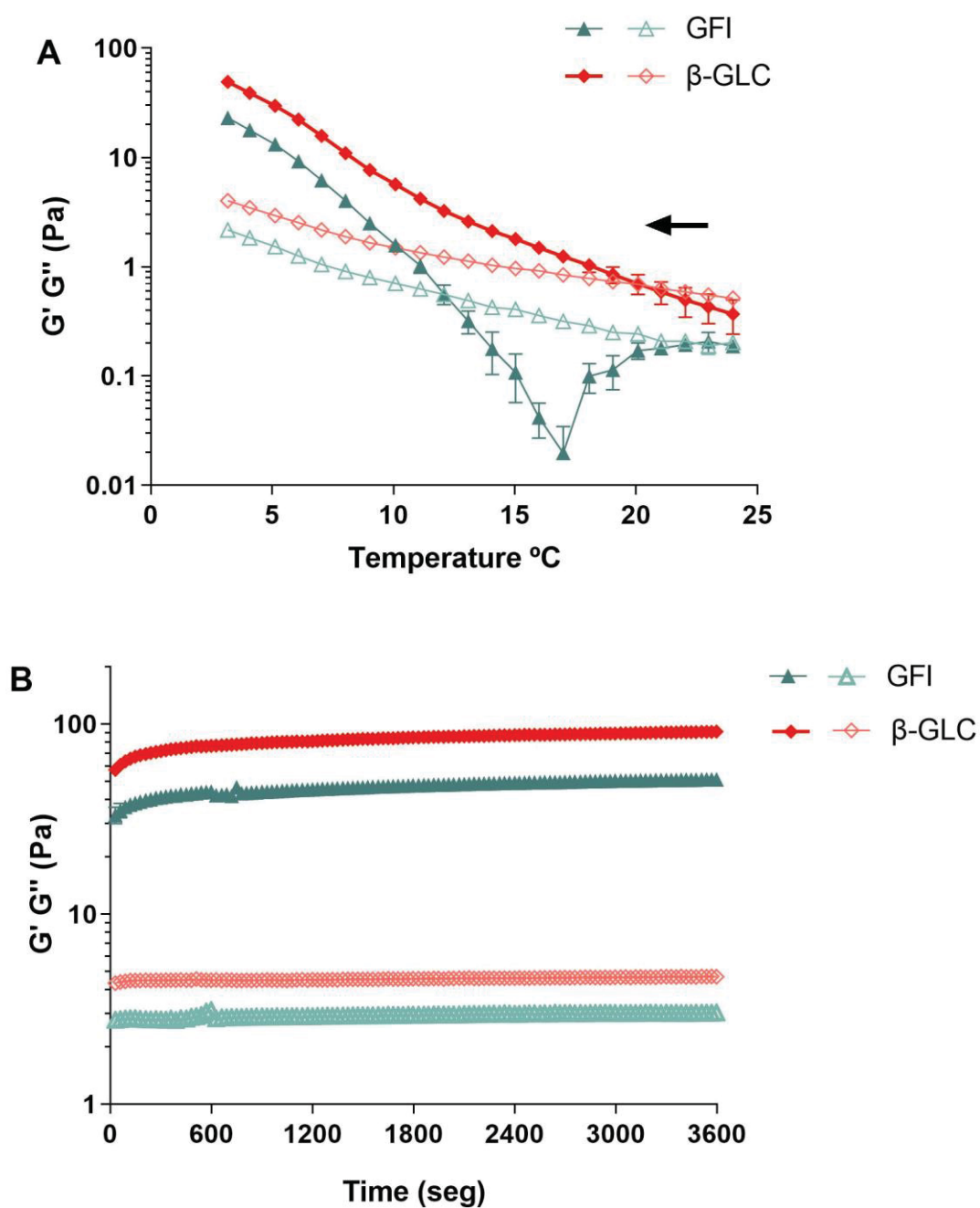
**Figure 6:** Flow (empty symbols) and viscosity (full symbols) curves at 25 °C of GFI (2 %) and  $\beta$ -GLC (1.5 %).

As observational analyses showed that the glucan-rich fractions (GFI and  $\beta$ -GLC) increased their viscosity when left at 3 °C, the samples were submitted to a cooling ramp (from 24 °C to 3 °C) to verify which temperature induces the gelling state of each fraction (Fig. 7A). GFI showed a crossover of elastic modulus ( $G'$ ) and viscous modulus ( $G''$ ) at 11 °C, when  $G'$  became higher than  $G''$  and gradually increased while temperature decreases, suggesting the sol-gel transition. On the other hand,  $\beta$ -GLC showed the crossover at 20 °C. These results demonstrated that both fractions are thermo-responsive and tend to form gel under low temperatures, which was further confirmed by frequency sweeps (Fig. 8).

Dispersion containing isolated  $\beta$ -glucans ( $\beta$ -GLC) showed an earlier moduli crossover (at 20 °C) suggesting that even at higher temperatures the self-association of similar polymers, which can increase junction zones, were more favorable and pronounced than the association of glycogen and  $\beta$ -glucans as observed in GFI mixture. The glycogen present in GFI fraction probably interacts with  $\beta$ -glucans and interferes the formation of junction zones among the  $\beta$ -glucans, promoting lower gelling effect. The behavior transition at temperatures lower than 25 °C seems to be a consequence of favorable interchain association among the molecules when the temperature decreases, favoring a tridimensional gel network (Fittolani, Seeberger & Delbianco, 2020; Rinaudo, 1993).

A (1→3),(1→6)-linked  $\beta$ -glucan obtained from Huangshan Floral mushroom (considered a special variety of *L. edodes*) showed a similar behavior to that of  $\beta$ -GLC fraction. That glucan had a gelling point at 20.6 °C, at a concentration of 1.4 % (w/v) (Xu, Zhang, Liu, Sun & Wang, 2016). Similar properties were described to lentinan, a typical  $\beta$ -glucan obtained from *L. edodes*, which presented an increase in the sol-gel transition temperature with increasing polymer concentrations: at 0.3 % (w/w) gel was formed at 21.5 °C while at 0.6 % (w/w) this transition occurred at 26.2 °C (Zhang, Xu & Zhang, 2008). On the other hand, two  $\beta$ -glucans obtained from *P. nameko* showed different behavior: they kept gel-like behavior at large temperature ranges and presented strong thermal stability (Abreu et al., 2019; Sovrani et al., 2017). The first  $\beta$ -glucan, with longer side chains, showed gel-like behavior from 5 to 60 °C, at 2 % (w/w), being stable when glucan was again refrigerated to 5 °C (Sovrani et al., 2017). The second  $\beta$ -glucan, with shorter side chains, showed gel-like behavior in a decreasing temperature ramp (from 90 °C to 4 °C), even at lower concentrations (0.5, 1, and 2 % w/w) (Abreu et al., 2019). Therefore, it can be concluded that gelation of polysaccharides depends on different chemical features such as branching degree, molar mass, concentration of polymer, linkages, and molecular tridimensional conformation in a solvent. All of the factors lead the polymers to establish more junction zones, such as hydrogen bonds and Van der Waals forces, or to disrupt them when dispersions are submitted to variations of temperature, giving rise to sol-gel transitions (Fittolani, Seeberger & Delbianco, 2020; Tako, 2015).

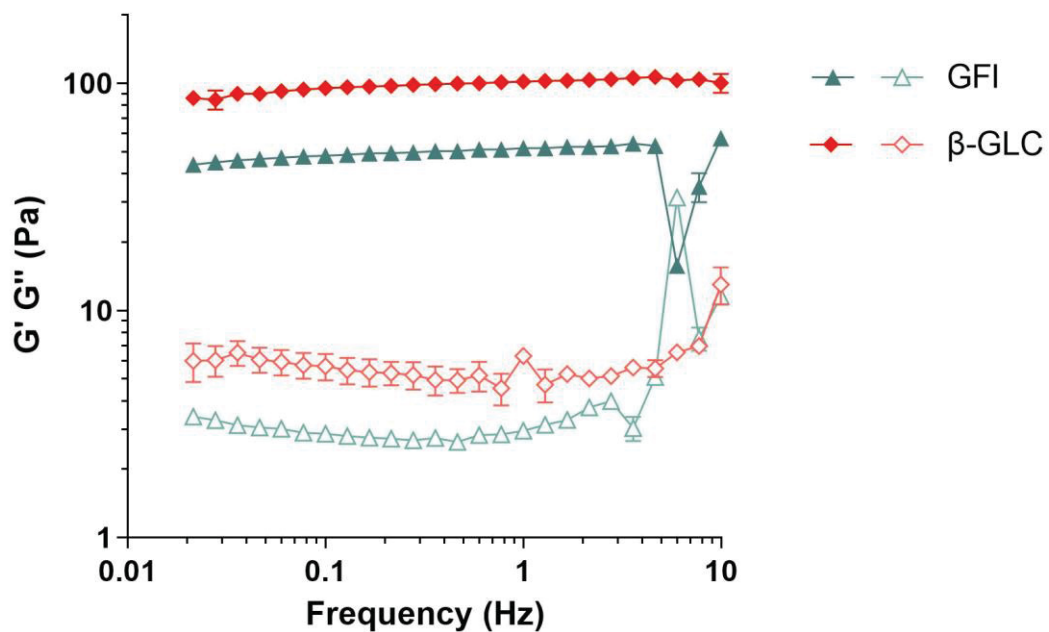
To understand if  $G'$  and  $G''$  moduli varied after temperature ramp, these moduli were observed during 60 min after refrigeration of dispersions. Both moduli were maintained with minor variations, showing no time-dependence during the period analyzed for both GFI and  $\beta$ -GLC fractions (Fig. 7B).



**Figure 7:** Elastic modulus ( $G'$ , full symbols) and viscous modulus ( $G''$ , empty symbols) of GFI (2 %) and  $\beta$ -GLC (1.5 %) as a function of temperature (A) and as a function of time (at 3 °C) (B). All analyses were performed with a fixed frequency at 1 Hz and strain of 1%.

The frequency sweeps of GFI (at 2 %, w/w) and  $\beta$ -GLC (at 1.5 %, w/w) fractions were evaluated to characterize the viscoelastic behavior of samples after cooling (Fig. 8). For this procedure, both samples were maintained for 60 min at 3 °C

and later they were submitted to frequency sweeps, showing a gel-like behavior, with  $G'$  values higher than  $G''$  at almost all ranges of the applied frequencies (Fig. 8). The mechanical spectrum of GFI fraction showed that an increase in frequency above 4 Hz, induced a destabilization of moduli and their frequency dependence was observed (Fig. 8), suggesting that GFI form a weaker gel-structure in comparison with  $\beta$ -GLC sample, that presented independent  $G'$  values frequency (Morgan, 2000). By this result, it can be suggested that the presence of glycogen interferes the gel formation capacity of  $\beta$ -glucans, and consequently produces a weaker gel-structure as observed in the frequency sweep profile of GFI fraction. According to Lapasin & Prici (1995), when  $G'$  values are 1-2 orders of magnitude greater than  $G''$ , the sample shows a typical gel-like behavior. Based on  $G'/G''$  ratio of both GFI and  $\beta$ -GLC fractions, they can be classified as gels (table 2).



**Figure 8:** Elastic modulus ( $G'$ , full symbols) and viscous modulus ( $G''$ , empty symbols) over a frequency sweep of GFI (2 %) and  $\beta$ -GLC (1.5 %). Analyses were carried out at 3 °C, with a strain of 1 %.



**Table 2:** Elastic modulus values ( $G'$ ) and ratio ( $G'/G''$ ) of fractions GFI and  $\beta$ -GLC over a range of frequencies at 3 °C.

Frequency (Hz)	GFI (2 %)		$\beta$ -GLC (1.5 %)	
	$G'$ (Pa) $\pm$ sd	$G'/G''$	$G'$ (Pa) $\pm$ sd	$G'/G''$
0.1	48.0 $\pm$ 1.9	16.7	95.1 $\pm$ 8.2	16.7
1	51.8 $\pm$ 2.4	17.6	93.9 $\pm$ 7.4	14.9
10	57.2 $\pm$ 1.64	4.9	85.9 $\pm$ 13.5	9.1

The gel-like behavior was also reported to  $\beta$ -glucans from other mushrooms, such as *H. erinaceus* (at 2 % w/v; at 25 °C) (Wang et al., 2019), *P. nameko* (at 0.5; 1 and 2 % w/w; at 25 °C) (Abreu et al., 2019), and Huangshan Floral mushroom (at 1.4 % w/v; at 10, 15, and 25 °C) (Xu et al., 2016). Based on the results from this study and those from the literature, it is clear  $\beta$ -glucans have interesting functional and physicochemical properties, beyond those health benefits. Also, their rheological properties can vary depending on the chemical structure of  $\beta$ -glucans as linkage-type, degree of branching, size of side chains (Bai et al., 2017), molar mass (Wang et al., 2021), and consequently the adopted conformation in solution (Wei, Guo, Li, Ma & Zhang, 2021). Such observations provide evidence of the importance of evaluating all these features.

#### 4. Conclusions

Two fractions (GFI and  $\beta$ -GLC) were obtained from the mushroom *M. titans*. Chemical analyses showed that GFI was composed of  $\beta$ -D-glucan and  $\alpha$ -D-glucan (glycogen), while  $\beta$ -GLC was constituted by three populations of (1 $\rightarrow$ 3)-linked  $\beta$ -D-glucan, substituted at O-6 by (1 $\rightarrow$ 6)-linked  $\beta$ -D-Glcp side chains or single units of  $\beta$ -D-Glcp. The Congo red assay showed that the mixture of  $\beta$ -glucans and glycogen (GFI) presented a helicoidal conformation while  $\beta$ -GLC, with only  $\beta$ -glucans population, presented a random coil pattern. Rheological analysis showed that GFI and  $\beta$ -GLC presented a non-Newtonian shear-thinning behavior and that  $\beta$ -GLC fraction formed a more viscous dispersion than the one formed by GFI. Furthermore, both fractions produced thermo-responsive gels which were favored below 11 °C and 20 °C for GFI and  $\beta$ -GLC, respectively. Fraction with isolated  $\beta$ -glucans ( $\beta$ -GLC) produced stronger gel than GFI fraction, suggesting that glycogen interferes in the association of  $\beta$ -glucans to form a gel network, and that the helical conformation does not increase gel-

formation capacity. Methylation analysis revealed the presence of a great amount of (1→6)-linked Glcp units (52.7 %), which in comparison to other studies reduces the viscosity and gel-like behavior. In conclusion, for the first time,  $\beta$ -glucans isolated from *M. titans* were evaluated on their rheological properties and showed potential to be applied as a thickener or gelling agent in the food or cosmetic industries.

### Declaration of competing interest

The authors declare to have no competing interests.

### Acknowledgments

This work was supported by the Brazilian funding agency: Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

### References

- Abreu, H., Simas, F. F., Smiderle, F. R., Sovrani, V., Dallazen, L., Maria-Ferreira, D., Werner, M.F., Cordeiro, L. M. C., & Iacomini, M. (2019). Gelling functional property, anti-inflammatory and antinociceptive bioactivities of  $\beta$ -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>.
- Ahmad, A., Munir, B., & Abrar, M (2012). Perspective of  $\beta$ -Glucan as Functional Ingredient for Food Industry. *Journal of Nutrition & Food Sciences*, 02, 02. <https://doi.org/10.4172/2155-9600.1000133>.
- Bai, L., Liu, F., Xu, X., Huan, S., Gu, Y., & McClements, D. J. (2017). Impact of polysaccharide molecular characteristics on viscosity enhancement and depletion flocculation. *Journal of Food Engineering*, 207, 35–45. <https://doi.org/10.1016/j.jfoodeng.2017.03.021>.
- 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](https://doi.org/10.1016/0003-2697(76)90527-3).
- Carbonero, E. R., Ruthes, A. C., Freitas, C. S., Utrilla, P., Gálvez, J., Da Silva, E. V., Sassaki, G. L., Gorin, P. A. J., & Iacomini, M. (2012). Chemical and biological properties of a highly branched  $\beta$ -glucan from edible mushroom *Pleurotus sajor-caju*. *Carbohydrate Polymers*, 90, 814–819. <https://doi.org/10.1016/j.carbpol.2012.06.005>.
- 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](https://doi.org/10.1016/0008-6215(84)85242-8).
- Fittolani, G., Seeberger, P. H., & Delbianco, M. (2020). Helical polysaccharides. *Peptide Science*, 112, 1, Article e24124. <https://doi.org/10.1002/pep2.24124>.
- Goldstein, J., Hay, G. W., Lewis, B. A., & Smith, F. (1965). Controlled degradation of polysaccharides by periodate oxidation, reduction, and hydrolysis. In: R. L. Whistler (Eds.), *Methods in Carbohydrate Chemistry*, 5 (pp. 361–370), Academic Press.
- Gonzaga, M. L. C., Menezes, T. M. F., Rebêlo, L. M., Souza, J. R. R., Ricardo, N. M. P. S., & Soares, S. A. (2014). *Agaricus brasiliensis* mushroom betaglucans solutions: Physicochemical properties and

griseofulvin solubilization by self-assembly micro-nano particles formation. *Bioactive carbohydrates and dietary fibre*, 4, 144-154. <https://doi.org/10.1016/j.bcdf.2014.09.004>.

Gorin, P. A. J., & Iacomini, M. (1984). Polysaccharides of the lichens *Cetraria islandica* and *Ramalina usnea*. *Carbohydrate Research*, 128, 1, 119–132. [https://doi.org/10.1016/0008-6215\(84\)85090-9](https://doi.org/10.1016/0008-6215(84)85090-9).

Ghotra, B. S., Vasanthan, T., & Temelli, F. (2009). Rheological properties of aqueous blends of high purity barley  $\beta$ -glucan with high purity commercial food gums. *Food Chemistry*, 117, 417-425. <https://doi.org/10.1016/j.foodchem.2009.04.027>.

Jesus, L. I., Smiderle, F. R., Ruthes, A. C., Vilaplana, F., Dal'Lin, F. T., Werner, M. F., Van Griensven, L. J. L. D & Iacomini, M. (2018). Chemical characterization and wound healing property of a  $\beta$ -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>.

Hawort, W. N. (1915). New Method of Preparing Alkylated Sugars. *Journal of the Chemical Society*, 107, 8-16. <https://doi.org/10.1039/CT9150700008>.

Lapasin, R., & Prici, S. (1995). Rheology of polysaccharide systems. In: R. Lapasin, & S. Prici (Eds.), *Rheology of industrial polysaccharides: Theory and applications* (pp. 250-494). Springer Science+Business Media Dordreeh.

Lemieszek, M., & Rzeski, W. (2012). Anticancer properties of polysaccharides isolated from fungi of the Basidiomycetes class. *Wspolczesna Onkol*, 16, 4, 285-289. <https://doi.org/10.5114/wo.2012.30055>.

Milhorini, S. S., Smiderle, F. R., Biscaia, S. M. P., Rosado, F. R., Trindade, E. S., & Iacomini, M. (2018). Fucogalactan from the giant mushroom *Macrocybe titans* inhibits melanoma cells migration. *Carbohydrate Polymers*, 190, 50–56. <https://doi.org/10.1016/j.carbpol.2018.02.063>.

Morales, D., Rutckeviski, R., Villalva, M., Abreu, H., Soler-Rivas, C., Santoyo, S., Iacomini, M., & Smiderle, F. R. (2020). Isolation and comparison of  $\alpha$ - and  $\beta$ -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  $\beta$ -D-glucans from *Cookeina tricholoma*. *Carbohydrate Polymers*, 141, 220–228. <https://doi.org/10.1016/j.carbpol.2016.01.001>.

Morgan, K. (2000). Cereal  $\beta$ -glucans. In: Phillips, G. O., & Williams, P. A (Eds.) *Handbook of hydrocolloids* (Chapter 16). CRC Press LCC.

Ogawa, K., & Hatano, M. (1978). Circular dichroism of the complex of a (1-3)- $\beta$ -D-glucan with Congo Red. *Carbohydrate Research*, 67, 527-535. [https://doi.org/10.1016/S0008-6215\(00\)84144-0](https://doi.org/10.1016/S0008-6215(00)84144-0).

Ogawa, K., Tsurugi J., & Watanabe, T (1972). Complex of gel-forming  $\beta$ -1,3-D- glucan with congored in alkaline solution. *Chemistry Letters*, 689–692. <https://doi.org/10.1246/cl.1972.689>.

Palacios, I., García-Lafuente, A., Guillamón, E., & Villares, A. (2012). Novel isolation of water-soluble polysaccharides from the fruiting bodies of *Pleurotus ostreatus* mushrooms. *Carbohydrates Research*, 358, 72–77. <https://doi.org/10.1016/j.carres.2012.06.016>.

Pegler, D. N., Lodge, D. J., & Nakasone, K. K. (1998). The pantropical genus *Macrocybe* gen. nov. *Mycologia*, 90, 3, 494-504. <https://doi.org/10.2307/3761408>.

Rinaudo, M. (1993). Gelation of Polysaccharides. *Journal of Intelligent Material Systems and Structures*, 4, 210-215. <https://doi.org/10.1177/1045389X9300400210>.

- Ruthes, A. C., Carbonero, E. R., Córdova, M. M., Baggio, C. H., Sassaki, G. L., Gorin, P. A., Santos, A. R.; & 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., 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, 215–222. <https://doi.org/10.1016/j.chroma.2008.08.083>.
- Singleton, V. L., Orthofer, R., & Lamuela-Raventos, R. M. (1999). Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. *Methods Enzymol*, 299, 152-178. [https://doi.org/10.1016/S0076-6879\(99\)99017-1](https://doi.org/10.1016/S0076-6879(99)99017-1).
- 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  $\beta$ -glucan isolated from *Pleurotus pulmonarius*. *European Journal of Pharmacology*, 597, 86–91. <https://doi.org/10.1016/j.ejphar.2008.08.028>.
- Smiderle, F. R., Sassaki, G. L., Arkel, J. V., Iacomini, M., Wichers, H. J., & Griensven, L. J. L. D. (2010). High Molecular Weight Glucan of the Culinary Medicinal Mushroom *Agaricus bisporus* is an  $\alpha$ -Glucan that Forms Complexes with Low Molecular Weight Galactan. *Molecules*, 15, 5818-5830. <https://doi.org/10.3390/molecules15085818>.
- Smiderle, F. R., Ruthes, A. C., & Iacomini, M. (2014). Natural polysaccharides from mushrooms: anti-nociceptive and anti-inflammatory properties. In: K. G. Ramawat, & J. M. Mérillon. *Polysaccharides* (pp. 1-25). Springer.
- Smiderle, F. R., Baggio, C. H., Borato, D. G., Santana-Filho, A. S., Sassaki, G. L., Iacomini, M., & Griensven, L. J. L. D. V. (2014). Anti-Inflammatory Properties of the Medicinal Mushroom *Cordyceps militaris* Might Be Related to Its Linear (1→3)- $\beta$ -D-Glucan. *Plos One*, 9, Article e110266. <https://doi.org/10.1371/journal.pone.0110266>.
- Sovrani, V., Jesus, L. I., Simas-Tosin, F. F., Smiderle, F. R., & Iacomini, M. (2017). Structural characterization and rheological properties of a gel-like  $\beta$ -D-glucan from *Pholiota nameko*. *Carbohydrate Polymers*, 169, 1–8. <https://doi.org/10.1016/j.carbpol.2017.03.093>.
- Synytsya, A.; Mičková, K.; Synytsya, A.; Jablonsky, I.; Spěvák, J.; Erban, V.; Kovářiková, E.; & Čopíková, J. (2009). Glucans from fruit bodies of cultivated mushrooms *Pleurotus ostreatus* and *Pleurotus eryngii*: Structure and potential prebiotic activity. *Carbohydrate Polymers*, 76, 548–556. <https://doi.org/10.1016/j.carbpol.2008.11.021>.
- Synytsya, A., & Novák, M. (2013). Structural diversity of fungal glucans. *Carbohydrate Polymers*, 92, 792–809. <https://doi.org/10.1016/j.carbpol.2012.09.077>.
- Tako, M. (2015). The principle of polysaccharide gels. *Advances in Bioscience and Biotechnology*, 6, 22-36. <https://doi.org/10.4236/abb.2015.61004>.
- Tudorachea, M., & Bordenave, N. (2019). Phenolic compounds mediate aggregation of water-soluble polysaccharides and change their rheological properties: Effect of different phenolic compounds. *Food Hydrocolloids*, 97, 105193. <https://doi.org/10.1016/j.foodhyd.2019.105193>.
- Wang, X. Y., Xu, R., Yin, J. Y., Wang, Y. X., Ma, L. Y., Nie, S. P., Xiong, T., & Xie, M. Y. (2019). Physicochemical, structural and rheological properties of alkali-extracted polysaccharide from fruiting body of *Hericium erinaceus*. *LWT - Food Science and Technology*, 115, 108330. <https://doi.org/10.1016/j.lwt.2019.108330>.

- Wang, Y. X., Yin, J. Y., Huang, X. J., & Nie, S. P. (2020). Structural characteristics and rheological properties of high viscous glucan from fruit body of *Dictyophora rubrovolvata*. *Food Hydrocolloids*, 101, 105514. <https://doi.org/10.1016/j.foodhyd.2019.105514>.
- Wang, Y. X., Yin, J. Y., Zhang, T., Xin, Y., Huang, X. J., & Nie, S. P. (2021). Utilizing relative ordered structure theory to guide polysaccharide purification for structural characterization. *Food Hydrocolloids*, 115, 106603. <https://doi.org/10.1016/j.foodhyd.2021.106603>.
- Wei, Y., Guo, Y., Li, R., Ma, A., & Zhang, H. (2021). Rheological characterization of polysaccharide thickeners oriented for dysphagia management: Carboxymethylated curdlan, konjac glucomannan and their mixtures compared to xanthan gum. *Food Hydrocolloids*, 110, 106198. <https://doi.org/10.1016/j.foodhyd.2020.106198>.
- Woranovicz, S. M., Pinto, B. M., Gorin, P. A. J., & Iacomini, M. (1999). Novel structures in galactoglucomannans of the lichens *Cladonia substellata* and *Cladonia ibitipocae*: significance as chemotypes. *Phytochemistry*, 51, 395-402. [https://doi.org/10.1016/S0031-9422\(99\)00005-9](https://doi.org/10.1016/S0031-9422(99)00005-9).
- Xu, J. L., Zhang, J. C., Liu, Y., Sun, H. J., & Wang, J. H. (2016). Rheological properties of a polysaccharide from floral mushrooms cultivated in Huangshan Mountain. *Carbohydrate Polymers*, 139, 43-49. <https://doi.org/10.1016/j.carbpol.2015.12.011>.
- Zavadinack, M., Bellan, D. L., Bertage, J. L. R., Milhorini, S. S., Trindade, E. S., Simas, F. F., Sassaki, G. L., Cordeiro, L. M. C., & Iacomini, M. (2021). An  $\alpha$ -D-galactan and a  $\beta$ -D-glucan from the mushroom *Amanita muscaria*: Structural characterization and antitumor activity against melanoma. *Carbohydrate Polymers*, 274, 118647. <https://doi.org/10.1016/j.carbpol.2021.118647>.
- Zhang, M., Cuia, 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 & Technology*, 18, 4-19. <https://doi.org/10.1016/j.tifs.2006.07.013>.
- Zhang, Y., Xu, X., & Zhang, L. (2008). Dynamic viscoelastic behavior of triple helical Lentinan in water: Effect of temperature. *Carbohydrate Polymers*, 73, 26-34. <https://doi.org/10.1016/j.carbpol.2007.10.020>.
- Zhang, Y., Li, S., Wang, X., Zhang, L., & Cheung, P. C. K. (2011). Advances in lentinan: Isolation, structure, chain conformation and bioactivities. *Food Hydrocolloids*, 25, 196-206. <https://doi.org/10.1016/j.foodhyd.2010.02.001>.
- Zhu, F., Du, B., & Xu, B. (2016). A critical review on production and industrial applications of beta-glucans. *Food Hydrocolloids*, 52, 275-288. <https://doi.org/10.1016/j.foodhyd.2015.07.003>.

**ARTIGO III**

**Isolation and characterization of a  $\beta$ -glucan from the giant mushroom  
*Macrocybe titans***

**Isolation and characterization of a  $\beta$ -glucan from the giant mushroom  
*Macrocybe titans***

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**Abstract**

*Macrocybe titans*, known as giant mushroom, has been poorly studied about its polysaccharides so far. This species could contain interesting molecules with biological and rheological properties, deserving more attention from the scientific community. In this work, a  $\beta$ -glucan (GLC) was isolated from *M. titans* fruiting bodies through alkaline extraction, freeze-thawing fractionation, and treatment with Fehling solution. GLC showed 93 % of glucose, a Mw  $1.1 \times 10^4$  g/mol, and a random-coil conformation. NMR analyses of complete and partially degraded polysaccharide indicated that GLC is composed of a  $\beta$ -D-glucan, probably with a (1 $\rightarrow$ 3)-linked main chain, branched at O-6 position by (1 $\rightarrow$ 6)-linked  $\beta$ -D-Glcp side chains or  $\beta$ -D-Glcp-(1 $\rightarrow$  non-reducing end units. Once  $\beta$ -glucans are known for their large possibility of application in medical and industrial fields, the GLC extraction and purification approach developed in this study could be used to guide further studies about GLC biological properties.

**Keywords:** *Macrocybe titans*;  $\beta$ -glucan; Extraction; Purification; Characterization.

## 1. Introduction

$\beta$ -glucans have been attracting the attention of researchers and industries worldwide due to their specific physical properties such as water solubility, viscosity, and gelation. These features enable their use as thickener, stabilizer, and gelling agents in food, pharmaceutical, and cosmetic industries (Zhu, Du & Xu, 2016; Sovrani et al., 2017; Abreu et al., 2019). Besides, these polysaccharides have several biological activities, being promisors to medical sectors due to their diversity of health-promoting properties (Zhu, Du & Xu, 2016; Maheshwari, Sowrirajan & Joseph, 2019). It is known that their use as an adjuvant improves the immune system and reduces the adverse effects of chemotherapy and radiotherapy (Steimbach et al., 2021; Yang & Huang, 2021).

$\beta$ -Glucans are present in different sources like plants, bacteria, seaweed, and fungi (such as yeast and mushrooms), however they differ in chemical structure (Maheshwari, Sowrirajan & Joseph, 2019; Yang & Huang, 2021). Among these, mushrooms have been extensively studied about their glucans in the last decades. These polysaccharides have been extracted from different mushroom species such as *Lentinula edodes* (Morales et al., 2019), *Schizophyllum commune* (Chen et al., 2020), *Agaricus bisporus* (Pires et al., 2017), *Coriolus versicolor* (Zhang et al., 2021), *Ganoderma lucidum* (Liu, Amakye & Ren, 2021), *Lactarius volemus* Fr. (Zhong et al., 2021), *Pleurotus eous* (Berk.) Sacc. (Gunasekaran, Govindan & Ramani, 2021), and *Fomitopsis betulina* (Jesus et al., 2018a).

Mushroom  $\beta$ -glucans have been investigated for their biological properties and show different activities such as immunomodulatory (Zhang et al., 2021), antimicrobial (Ahmad et al., 2021), antitumoral (Zhang et al., 2011), antinociceptive (Moreno et al., 2016), prebiotic (Ruthes et al., 2021), antidiabetic (Zhang et al., 2018), anti-sepsis (Ruthes et al., 2013a), hypocholesterolemic and antioxidant effects (Morales et al., 2020).

Despite the extensive investigation about  $\beta$ -glucans, some mushroom species have still been minimally explored. For instance, *Macrocybe titans* (H.E. Bigelow & Kimbr.), which is known as giant mushroom due to its large caespitose clusters which may exceed 30 kg of fresh weight (Pegler, Lodge & Nakasone, 1998), was reported to have a fucogalactan with antimelanoma effect (Milhorini et al., 2018). However, to our best knowledge, there are few reports of *M. titans*  $\beta$ -glucans

characterization or biological activity investigation. Therefore, this present study aimed to extract and purify a  $\beta$ -glucan fraction from the fruiting bodies of *M. titans* as well as to characterize its chemical structure.

## 2. Material and methods

### 2.1 Biological material

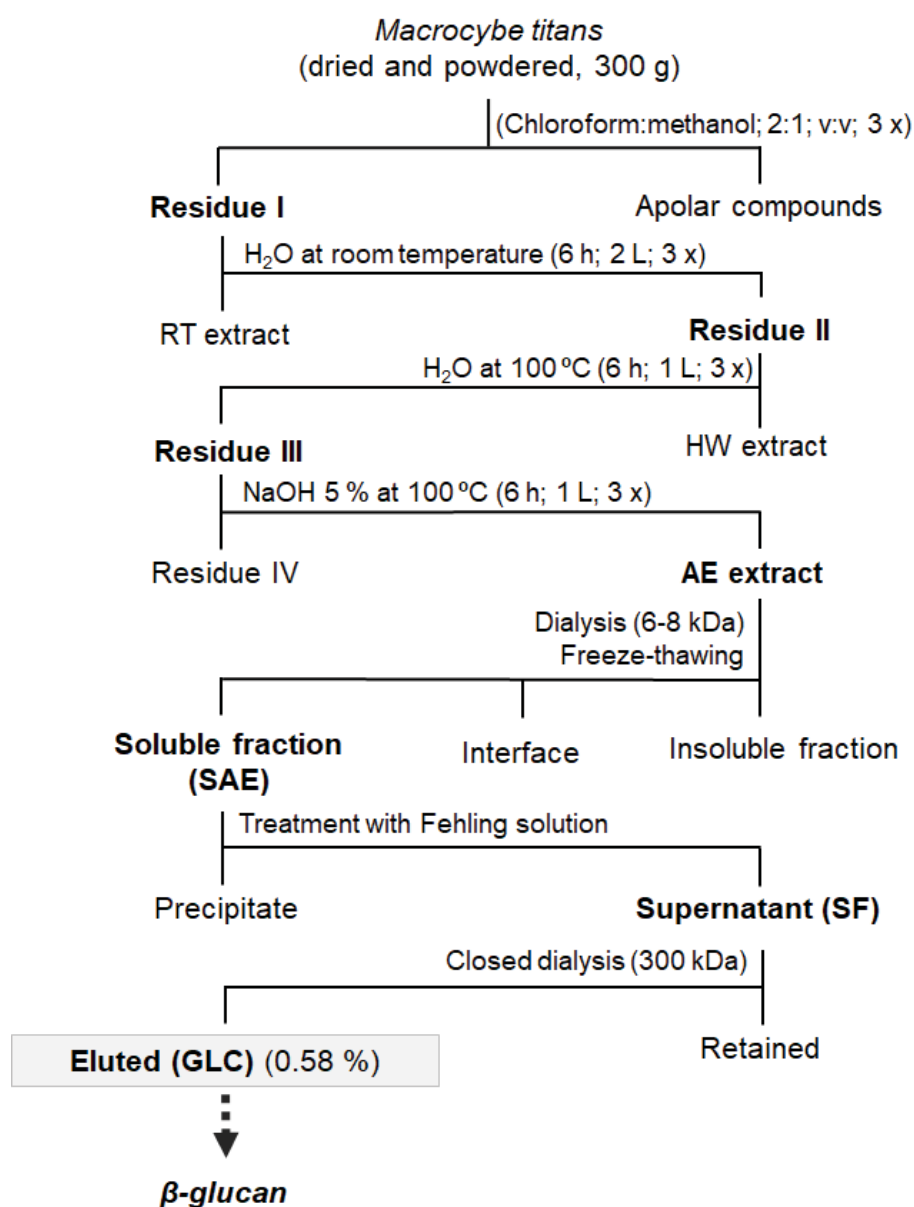
*Macrocybe titans* fruiting bodies was donated by Dr. Fábio Rogério Rosado from the Department of Bioscience of Federal University of Paraná, Brazil, Palotina. This specie was registered with the number A3BA3B3 on *Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado* (SISGEN). Molecular identification was carried out and the nucleotide sequence was published in GenBank database (MZ519068).

### 2.2 Extraction and purification procedures

*Macrocybe titans* fruiting bodies (300 g) were dried and powdered to be submitted to sequential extractions procedures (Fig. 1). Firstly, the material was defatted with chloroform and methanol (2:1; v:v; 60 °C; 3 h; 3 x). Subsequently, the delipidified residue (residue I) was extracted under mechanical stirring with distilled water at room temperature (6 h; 3x). The Room Temperature Extract (RT) was recovered by centrifugation (10,000 rpm; 20 min) and the obtained precipitated (residue II) was extracted under reflux with boiling distilled water (6 h; 3 x). The Hot Water Extract (HW) was also recovered by centrifugation and the resulting precipitated (residue III) was finally extracted with NaOH 5 % in the presence of NaBH<sub>4</sub> (6 h; 3 x; 100 °C), under reflux. The Alkaline Extract (AE) was obtained by centrifugation, neutralized with acetic acid, dialyzed against tap water for 48 hours (6-8 kDa), concentrated under reduced pressure, and freeze-dried.

AE was submitted to the freeze-thawing fractionation (Gorin & Iacomini, 1984) and the water-soluble polysaccharides (SAE) were obtained in the supernatant of a centrifugation process (10,000 rpm; 20 min; 4 °C). SAE was treated with Fehling solution according to a methodology adapted from Jones and Stoodley (1965). The sample was solubilized in solution A (86.5 g of potassium sodium tartrate and 62.5 g

of potassium hydroxide in distilled water; 250 ml). Subsequently, solution B was added (27.87 g of copper sulfate in distilled water; 250 ml). The mixture was kept under magnetic stirring for 1 hour and let rest for 12 hours at 4 °C. The supernatant of this treatment (SF) was obtained by centrifugation (10,000 rpm; 20 min; 4 °C), neutralized with acetic acid, and dialyzed against tap water for 48 hours (6-8 kDa). Further, was treated with cationic resin, neutralized with NaOH 1 M, dialyzed against tap water for 24 hours (6-8 kDa), and concentrated under reduced pressure. SF was finally dialyzed against distilled water in a closed system. The eluted material, named GLC, was concentrated and freeze-dried.



**Figure 1:** Extractions and purifications steps performed in *M. titans* fruiting bodies.

### 2.3 Monosaccharides composition determination

GLC fraction (3 mg) was hydrolyzed with TFA 2 M (400  $\mu$ L) for 8 hours. The acid was evaporated to dryness and the sample was solubilized in distilled water (200  $\mu$ L). NaBH<sub>4</sub> was added to perform the reduction process of molecules (Wolfrom & Thompson, 1963a). After resting for 12 hours, GLC was neutralized with acetic acid, evaporated to dryness, and washed with methanol (200  $\mu$ L; 3 x). Acetylation of the reduced sample was carried out with pyridine–Ac<sub>2</sub>O (200  $\mu$ L; 1:1; v:v) at 100 °C for 30 minutes (Wolfrom & Thompson, 1963b). The alditol acetates were extracted with chloroform (1 mL), washed with copper sulfate 5 % (2.5 mL; 4 x), and with distilled water (2.5 mL; 2 x). After total evaporation of chloroform, the sample was resuspended in acetone and injected in a gas chromatograph-mass spectrometer (GC-MS). A Shimadzu (QP2020NX, quadrupole detector) GC with VF5-M5 capillary column (30 m x 0.25 mm) was used. Helium was used as a carrier gas (2.0 mL/min). The injector temperature was 250 °C and the oven was kept at 100 °C for 3 minutes, following an increase to 220 °C, 250 °C, and 280 °C (10 °C/min; held for 3 min). Monosaccharides were identified by comparing their retention time and electron ionization profiles with standards (Sasaki et al., 2008).

### 2.4 Methylation analysis

GLC (3 mg) was methylated with NaOH-Me<sub>2</sub>SO-MeI, following a modified method of Ciucanu and Kerek (1984). Briefly, after solubilization of GLC in Me<sub>2</sub>SO (1 mL), powdered NaOH (20 mg) and methyl iodide (1 mL) were added. The fraction was kept under mechanical stirring for 30 min and subsequently let to stand at room temperature for 24 h. After this period, the sample was solubilized in water, neutralized with acetic acid, and dialyzed against tap water (6-8 kDa) for 24 hours. This methylation process was repeated twice to guarantee that all free hydroxyls of the polysaccharide were methylated. In the second methylation process, the per-O-methylated polysaccharides, instead of being dialyzed, were extracted with chloroform. After chloroform evaporation, formolysis was performed (formic acid 90 %; 100 °C; 2 h). The acid was evaporated to dryness and hydrolysis with H<sub>2</sub>SO<sub>4</sub> 2 N (100 °C; 16 h) was carried out. The sample was neutralized with BaCO<sub>3</sub> and reduced with NaBD<sub>4</sub> (pH 9-10) at room temperature for 12 hours. Subsequently, it was neutralized, washed,

acetylated, and analyzed in GC-MS as described above for composition analysis (topic 2.3).

## **2.5 Controlled Smith degradation**

Controlled degradation of polysaccharide by periodate oxidation was carried out following Goldstein, Hay, Lewis & Smith, (1965), with modifications. GLC (100 mg) was treated with sodium periodate (0.05 M) under magnetic stirring in absence of light for 72 hours. The oxidated material was dialyzed against tap water for 24 hours (2 kDa) and reduced with NaBH<sub>4</sub> at room temperature for 12 hours. Subsequently, the partial hydrolysis was carried out with TFA (pH 2.0), under reflux for 30 minutes (100 °C). The sample was neutralized with NaOH (1 M), dialyzed against tap water for 24 hours (2 kDa), and freeze-dried.

## **2.6 Congo red test**

Analysis was carried out following Ogawa & Hatano (1978). GLC (1 mg) was solubilized in NaOH 0.05 M (980 µL) and Congo Red 4.000 µM (20 µL) was added to achieve the final concentration of 80 µM. Dextran (487,000 Da) was used as a random-coil patten. Blank was prepared with NaOH 0.05 M (980 µL) and Congo Red 4.000 µM (20 µL). The absorbance values were obtained in a microplate reader at a range of 420 to 620 nm with 10 nm intervals.

## **2.7 Determination of protein and phenolic compounds**

The presence of protein was evaluated by Bradford method (Bradford, 1976) according to manufactures recommendation (Sigma-Aldrich). Bovine serum albumin (BSA) was used as standard. The phenolic compounds content was investigated with Folin & Ciocalteu phenol's reagent (Sigma, F9252) (Singleton, Orthofer & Lamuela-Raventós, 1999). Gallic acid (GAE) was used as standard. Both analyses were carried out with sample solubilized in distilled water (2 mg/mL).

## **2.8 Homogeneity and relative molecular weight**

GLC was solubilized (1 mg/mL) in aqueous NaNO<sub>2</sub> (0.1 M) containing aqueous NaN<sub>3</sub> (200 ppm). After filtration through a cellulose membrane (0.22 µm), the sample was injected (100 µL) in a high-performance size-exclusion chromatograph (HPSEC).

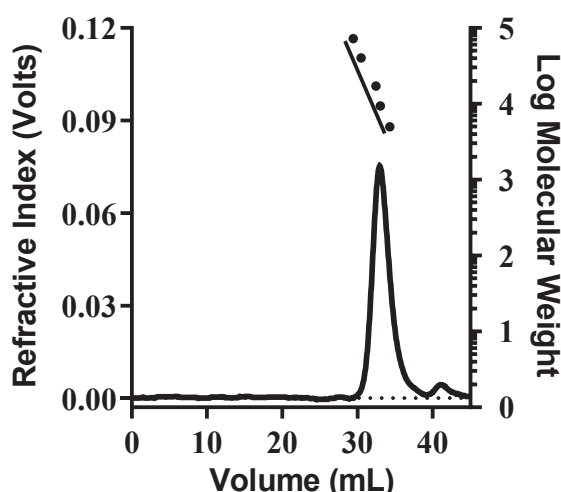
Four gel-permeation Ultrahydrogel columns in series with exclusion sizes of  $7 \times 10^6$ ,  $4 \times 10^5$ ,  $8 \times 10^4$ , and  $5 \times 10^3$  Da were used. The eluent was aqueous  $\text{NaNO}_2$  (0.1 M) with aqueous  $\text{NaN}_3$  (200 ppm) (0.6 mL/min). The relative molecular weight was estimated by comparing GLC retention time with a curve of dextran patterns (5,000; 9,400; 17,200; 40,200 and 72,200 Da).

## 2.9 Nuclear Magnetic Resonance

NMR mono ( $^{13}\text{C}$ ) and two-dimensional (HSQC-DEPT) analyses were performed in a BRUKER spectrometer, model Avance-DRX-400. GLC (20 mg) was solubilized in deuterated dimethylsulfoxide ( $\text{Me}_2\text{SO}-d_6$ ) and analyses were performed at 70 °C. The chemical shifts were expressed in ppm ( $\delta$ ) relative to the  $\text{Me}_2\text{SO}-d_6$  resonance ( $\delta$  39.70/2.40 for  $^{13}\text{C}$  and  $^1\text{H}$ , respectively).

## 3. Results and discussion

GLC fraction showed 93 % of glucose and 7 % of galactose in its composition. Protein and phenolic compounds were not detected. HPSEC analysis presented a homogeneous peak of this fraction. The relative molecular weight was  $1.1 \times 10^4$  g/mol (Fig. 2).



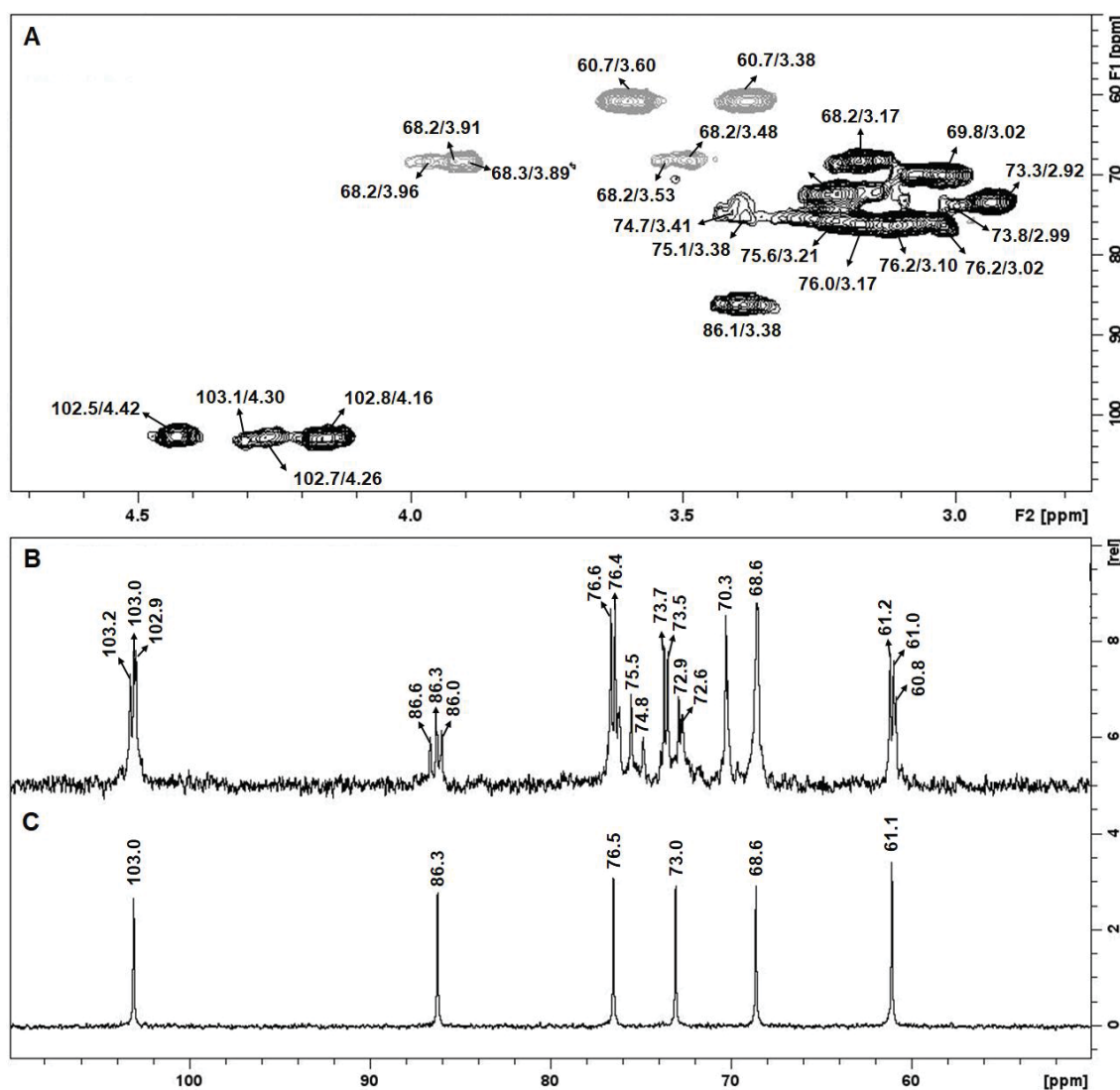
**Figure 2:** Elution profile of GLC in HPSEC coupled to a refractive index detector. Elution time of dextran standards of Mw 5,000; 9,400; 17,200; 40,200 and 72,200 Da (right to left) were employed to plot the calibration curve.



The HSQC-DEPT spectrum showed resonances at  $\delta$  102.5/4.42, 102.7/4.26, 102.8/4.16, and 103.1/4.30, that corresponded to C-1 of  $\beta$ -D-Glcp (Morales et al., 2020; Zavadinack et al., 2021) (Fig. 3A). The presence of substituted C-3 units was confirmed by the signal at  $\delta$  86.1/3.38 (Jesus et al., 2018b; Moreno et al., 2016). Resonances revealing the C-6 substitution were observed as inverted signals at  $\delta$  68.2/3.96, 68.2/3.91, 68.3/3.89, 68.2/3.53, and 68.2/3.48 (Sovrani et al., 2017), while non-substituted C-6 units were seen at  $\delta$  60.7/3.60 and 60.7/3.38 (Zavadinack et al., 2021). The  $^{13}\text{C}$  experiment corroborates with HSQC-DEPT data, with resonances of the C-1 of  $\beta$ -D-Glcp at  $\delta$  103.2, 103.0 and 102.9; C-3 substitution at  $\delta$  86.6, 86.3 and 86.0; C-6 substitution at  $\delta$  68.6; and non-substituted C-6 at  $\delta$  61.2, 61.0 and 60.8 (Sovrani et al., 2017; Zavadinack et al., 2021) (Fig. 3B). When compared to literature these data indicate the presence of a  $\beta$ -glucan with (1 $\rightarrow$ 3) and (1 $\rightarrow$ 6) linkage types (Jesus et al., 2018b; Morales et al., 2020; Moreno et al., 2016; Sovrani et al., 2017; Zavadinack et al., 2021). This kind of polysaccharide is commonly found in mushroom species, such as *Lentinula edodes* (Liu et al., 2014; Zhang et al., 2011; Morales et al., 2020); *Amanita muscaria* (Ruthes et al., 2013b), *Amanita muscaria* (L.:Fr) (Zavadinack et al., 2021), *Cookeina tricholoma* (Moreno et al., 2016), and *Pholiota nameko* (Sovrani et al., 2017).

GLC was submitted to controlled Smith degradation and the resistant material was analyzed by NMR (Fig. 3C). The spectrum suggests that GLC probably has a (1 $\rightarrow$ 3)-linked main chain, once only six signals were observed at  $\delta$  103.0 (C-1), 86.3 (C-3) 76.5 (C-5), 73.0 (C-2), 68.6 (C-4), and 61.1 (C-6), which are correspondent to a linear  $\beta$ -(1 $\rightarrow$ 3)-glucan (Sovrani et al., 2017; Zavadinack et al., 2021).  $\beta$ -glucans with a (1 $\rightarrow$ 3)-linked main chain are usually found in mushroom fruiting bodies. Such molecules have been studied about their biological properties and it has been observed the immunomodulatory (Morales et al., 2020), anti-inflammatory and anti-nociceptive (Abreu et al., 2019), anti-melanoma (Zavadinack et al., 2021), and wound healing effects (Jesus et al., 2018b).

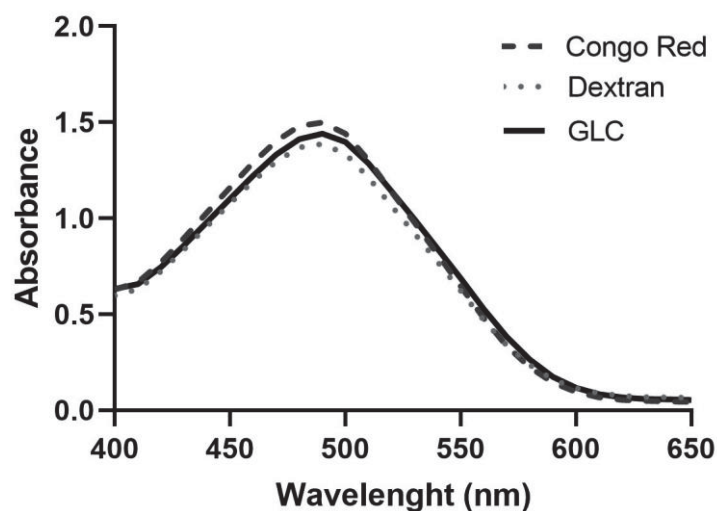
Methylation analysis performed on GLC corroborate with NMR results, once it presented derivatives corresponding to Glcp-(1 $\rightarrow$  (2,3,4,6-Me<sub>4</sub>-Glcp; 29.3%), (1 $\rightarrow$ 3)-Glcp (2,4,6-Me<sub>3</sub>-Glcp; 14.3 %), (1 $\rightarrow$ 6)-Glcp (2,3,4-Me<sub>3</sub>-Glcp; 41.7 %), and (1 $\rightarrow$ 3,6)-Glcp (2,4-Me<sub>2</sub>-Glcp; 14.7 %).



**Figure 3:** GLC analyses in NMR: (A) HSQC-DEPT and (B)  $^{13}\text{C}$ . (C) GLC after controlled Smith degradation. Samples were solubilized in  $\text{Me}_2\text{SO}-d_6$  at  $70^\circ\text{C}$  (chemical shifts are expressed in ppm).

The structural conformation of GLC was investigated by Congo red analysis (Fig. 4). According to Ogawa, Tsurugi and Watanabe (1972) a  $\beta$ -glucan presenting an ordered three-dimensional structure, generally triple-helical conformation, can form a complex with Congo red dye in alkaline solution, leading to a bathochromic shift of the maximum visible absorbance. No displacement was observed to GLC when compared to the blank Congo red or the random-coil pattern dextran, indicating that GLC has a random-coil conformation. It was reported that a  $\beta$ -glucan with (1 $\rightarrow$ 3),(1 $\rightarrow$ 6) linkages obtained from *Lentinula edodes* showed a helical conformation (Morales et al., 2020).

On the other hand, a fraction of (1→3),(1→6)- $\beta$ -glucans obtained from *M. titans* presented a random-coil conformation (Milhorini et al., 2021).



**Figure 4:** Absorption spectra of GLC, Congo red (control), and Dextran (random coil control).

#### 4. Conclusions

A polysaccharide fraction (GLC) was purified from *M. titans* fruiting bodies. GLC has a Mw  $1.1 \times 10^4$  g/mol and 93 % of glucose. This structure has a random-coil conformation and probably a (1→3)-linked  $\beta$ -D-Glcp main chain, branched at O-6 position by (1→6)-linked  $\beta$ -D-Glcp or by non-reducing end units of  $\beta$ -D-Glcp. This polysaccharide could be further applied in biological studies, such as investigating its antitumor and immunomodulatory effects.

#### Declaration of competing interest

The authors declare to have no competing interests.

#### Acknowledgments

This work was supported by the Brazilian funding agency: Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

## References

- Abreu, H., Simas, F. F., Smiderle, F. R., Sovrani, V., Dallazen, L., Maria-Ferreira, D., Werner, M.F., Cordeiro, L. M. C., & Iacomini, M. (2019). Gelling functional property, anti-inflammatory and antinociceptive bioactivities of  $\beta$ -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>
- Ahmad, R., Sellathoroe, S., Ngadi, E., Saharuddin, T. S. T., Zakaria, I. I., Selvakumaran, S., & Wan-Mohtar, W. A. A. Q. I. (2021). Isolation, identification, cultivation and determination of antimicrobial  $\beta$ -glucan from a wild-termite mushroom *Termitomyces heimii* RFES 230662. *Biocatalysis and Agricultural Biotechnology*, 37, 102187. <https://doi.org/10.1016/j.bcab.2021.102187>
- 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](https://doi.org/10.1016/0003-2697(76)90527-3)
- Chen, Z., Yin, C., Fan, X., Ma, K., Yao, F., Zhou, R., Shi, D., Cheng, W., & Gao, H. (2020). Characterization of physicochemical and biological properties of *Schizophyllum commune* polysaccharide extracted with different methods. *International Journal of Biological Macromolecules*, 156, 1425–1434. <https://doi.org/10.1016/j.ijbiomac.2019.11.183>
- 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](https://doi.org/10.1016/0008-6215(84)85242-8)
- Goldstein, J., Hay, G. W., Lewis, B. A., & Smith, F. (1965). Controlled degradation of polysaccharides by periodate oxidation, reduction, and hydrolysis. In: R. L. Whistler (Eds.), *Methods in Carbohydrate Chemistry*, 5 (pp. 361–370), Academic Press.
- Gunasekaran, S., Govindan, S., & Ramani, P. (2021). Investigation of chemical and biological properties of an acidic polysaccharide fraction from *Pleurotus eous* (Berk.) Sacc. *Food Bioscience*, 42, 101209.
- Jesus, L. I., Smiderle, F. R., Cordeiro, L. M. C., Freitas, R. A. de., Van Griensven, L. J. L. D., & Iacomini, M. (2018a). Simple and effective purification approach to dissociate mixed water insoluble  $\alpha$ - and  $\beta$ -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>
- 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. (2018b). Chemical characterization and wound healing property of a  $\beta$ -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>
- Liu, Y., Zhang, J., Tang, Q., Yang, Y., Guo, Q., Wang, Q., Wu, D., & Cui, S. W. (2014). Physicochemical characterization of a high molecular weight bioactive  $\beta$ -D-glucan from the fruiting bodies of *Ganoderma lucidum*. *Carbohydrate Polymers*, 101, 968–974. <https://doi.org/10.1016/j.carbpol.2013.10.024>
- Liu, H., Amakye, W. K., & Ren, J. (2021). *Codonopsis pilosula* polysaccharide in synergy with dacarbazine inhibits mouse melanoma by repolarizing M2-like tumor-associated macrophages into M1-like tumor-associated macrophages. *Biomedicine & Pharmacotherapy*, 142, 112016. <https://doi.org/10.1016/j.biopha.2021.112016>
- Maheshwari, G., Sowrirajan, S., & Joseph, B. (2019).  $\beta$ -Glucan, a dietary fiber in effective prevention of lifestyle diseases – An insight. *Bioactive Carbohydrates and Dietary Fibre*, 19, 100187. <https://doi.org/10.1016/j.bcdf.2019.100187>
- Milhorini, S. S., Smiderle, F. R., Biscaia, S. M. P., Rosado, F. R., Trindade, E. S., & Iacomini, M. (2018). Fucogalactan from the giant mushroom *Macrocybe titans* inhibits melanoma cells migration. *Carbohydrate Polymers*, 190, 50–56. <https://doi.org/10.1016/j.carbpol.2018.02.063>
- Milhorini, S. S., Simas, F. F., Smiderle, F. R., Jesus, L. I., Rosado, F. R., & Iacomini, M. (2021).  $\beta$ -Glucans from the giant mushroom *Macrocybe titans*: Chemical characterization and rheological properties. Unpublished.

- Morales, D., Rutckeviski, R., Villalva, M., Abreu, H., Soler-Rivas, C., Santoyo, S., Iacomini, M., & Smiderle, F. R. (2020). Isolation and comparison of  $\alpha$ - and  $\beta$ -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  $\beta$ -D-glucans from *Cookeina tricholoma*. *Carbohydrate Polymers*, 141, 220–228. <https://doi.org/10.1016/j.carbpol.2016.01.001>.
- Ogawa, K., & Hatano, M. (1978). Circular dichroism of the complex of a (1-3)- $\beta$ -D-glucan with Congo Red. *Carbohydrate Research*, 67, 527-535. [https://doi.org/10.1016/S0008-6215\(00\)84144-0](https://doi.org/10.1016/S0008-6215(00)84144-0)
- Ogawa, K., Tsurugi J., & Watanabe, T (1972). Complex of gel-forming  $\beta$ -1,3-D-glucan with congo red in alkaline solution. *Chemistry Letters*, 1, 8, 689–692. <https://doi.org/10.1246/cl.1972.689>
- Pegler, D. N., Lodge, D. J., & Nakasone, K. K. (1998). The pantropical genus *Macrocybe* gen. nov. *Mycologia*, 90, 3, 494-504. <https://doi.org/10.2307/3761408>
- Pires, A. do. R. A., Ruthes, A. C., Cadena, S. M. S. C., & Iacomini, M. (2017) Cytotoxic effect of a mannogalactoglucan extracted from *Agaricus bisporus* on HepG2 cells. *Carbohydrate Polymers*, 170, 33–42. <https://doi.org/10.1016/j.carbpol.2017.04.050>
- Ruthes, A. C., Carbonero, E. R., Córdova, M. M., Baggio, C. H., Santos, A. R. S., Sasaki, G. L., Cipriani, T. R., Gorin, P. A. J., & Iacomini, M. (2013a). *Lactarius rufus* (1→3),(1→6)- $\beta$ -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., Cordova, M. M., Baggio, C. H., Sasaki, G. L., Gorin, P. A. J., & Iacomini, M. (2013b). 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., Cantu-Jungles, T. M., Cordeiro, L. M. C., & Iacomini, M. (2021). Prebiotic potential of mushroom D-glucans: implications of physicochemical properties and structural features. *Carbohydrate Polymers*, 262, 117940. <https://doi.org/10.1016/j.carbpol.2021.117940>
- Sasaki, 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, 215–222. <https://doi.org/10.1016/j.chroma.2008.08.083>
- Sovrani, V., Jesus, L. I., Simas-Tosin, F. F., Smiderle, F. R., & Iacomini, M. (2017). Structural characterization and rheological properties of a gel-like  $\beta$ -D-glucan from *Pholiota nameko*. *Carbohydrate Polymers*, 169, 1–8. <https://doi.org/10.1016/j.carbpol.2017.03.093>
- Steimbach, L., Borgmann, A. V., Gomar, G. G., Hoffmann, L. V., Rutckeviski, R., Andrade, D. P., & Smiderle, F. R. (2021). Fungal beta-glucans as adjuvants for treating cancer patients: A systematic review of clinical trials. *Clinical Nutrition*, 40, 3104-3113. <https://doi.org/10.1016/j.clnu.2020.11.029>
- Singleton, V. L., Orthofer, R., & Lamuela-Raventos, R. M. (1999). Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. *Methods Enzymol*, 299, 152-178. [https://doi.org/10.1016/S0076-6879\(99\)99017-1](https://doi.org/10.1016/S0076-6879(99)99017-1)
- Wolfson, M. L., & Thompson, A. (1963a). Reduction with sodium borohydride. In: Whistler, R. L.; Wolfson, M. L. (Eds.). *Methods in Carbohydrate Chemistry*, 2, (pp. 65-68), Academic Press.
- Wolfson, M. L., & Thompson, A. (1963b). Acetylation. In: Whistler, R. L.; Wolfson, M. L. (Eds.). *Methods in Carbohydrate Chemistry*, v. 2, (pp. 211- 215), Academic Press.

- Yang, W., & Huang, G. (2021). Extraction methods and activities of natural glucans. *Trends in Food Science & Technology*, 112, 50-57. <https://doi.org/10.1016/j.tifs.2021.03.025>
- Zavadinack, M., Bellan, D. L., Bertage, J. L. R., Milhorini, S. S., Trindade, E. S., Simas, F. F., Sassaki, G. L., Cordeiro, L. M. C., & Iacomini, M. (2021). An  $\alpha$ -D-galactan and a  $\beta$ -D-glucan from the mushroom *Amanita muscaria*: Structural characterization and antitumor activity against melanoma. *Carbohydrate Polymers*, 274, 118647. <https://doi.org/10.1016/j.carbpol.2021.118647>
- Zhang, Y., Li, S., Wang, X., Zhang, L., & Cheung, P. C. K. (2011). Advances in lentinan: Isolation, structure, chain conformation and bioactivities. *Food Hydrocolloids*, 25, 196-206. <https://doi.org/10.1016/j.foodhyd.2010.02.001>
- Zhang, L., Liu, Y., Ke, Y., Liu, Y., Luo, X., Li, C., Zhang, Z., Liu, A., Shen, L., Chen, H., Hu, B., Wu, H., Wu, W., Lin, D., & Li, S. (2018). Antidiabetic activity of polysaccharides from *Suillellus luridus* in streptozotocin-induced diabetic mice. *International Journal of Biological Macromolecules*, 119, 134-140. <https://doi.org/10.1016/j.ijbiomac.2018.07.109>
- Zhang, X., Cai, Z., Mao, H., Hu, P., & Li, X. (2021). Isolation and structure elucidation of polysaccharides from fruiting bodies of mushroom *Coriolus versicolor* and evaluation of their immunomodulatory effects. *International Journal of Biological Macromolecules*, 166, 1387-1395. <https://doi.org/10.1016/j.ijbiomac.2020.11.018>
- Zhong, R. F., Yang, J. J., Geng, J. H., & Chen, J. (2021). Structural characteristics, anti-proliferative and immunomodulatory activities of a purified polysaccharide from *Lactarius volemus* Fr. *International Journal of Biological Macromolecules*, 192, 967-977. <https://doi.org/10.1016/j.ijbiomac.2021.10.049>
- Zhu, F., Du, B., & Xu, B. (2016). A critical review on production and industrial applications of beta-glucans. *Food Hydrocolloids*, 52, 275-288. <https://doi.org/10.1016/j.foodhyd.2015.07.003>

**ARTIGO IV**

**Antimelanoma effect of a fucoxylomannan isolated from *Ganoderma lucidum* fruiting bodies**



**Antimelanoma effect of a fucoxylomannan isolated from *Ganoderma lucidum* fruiting bodies**

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**Abstract**

A fucoxylomannan (FXM) was isolated from the mushroom *Ganoderma lucidum* through alkaline extraction followed by dialysis, freeze-thawing, and fractionation by Fehling's solution. The main chain of FXM presented  $\alpha$ -D-Manp-(1 $\rightarrow$ 4)-linked units, and some of them were branched at O-6 position by  $\alpha$ -L-Fucp-(1 $\rightarrow$ 2)- $\beta$ -D-Xylp groups. Its  $M_w$  was  $3.59 \times 10^4$  g.mol<sup>-1</sup>. FXM was tested on melanoma B16-F10 cells and it showed cell viability and cell density reduction, as well as antiproliferative effect, through cell cycle arrest. Additionally, the anchorage-independent clonogenic capacity of such cells was significantly reduced by FXM, decreasing the number of cells by colony and the colonies area. No effect on viability neither in proliferation of non-tumoral Balb c/3T3 fibroblasts was observed. These results indicate that FXM is a promising anti-proliferative compound impairing pivotal tumorigenic mechanisms, eliciting this polysaccharide to be further explored as an antimelanoma drug.

**Keywords:** *Ganoderma lucidum*; fucoxylomannan; heteropolysaccharide; antimelanoma; antitumor activity.

## 1. Introduction

*Ganoderma lucidum* is a popular mushroom known as Lingzhi (in China) and Reishi (in Japan) (Money, 2016). Traditionally, it is called immortality mushroom due to its use in Asian countries for over 2000 years, aiming the health endorsement as well as longevity improvement (Ahmad, 2020). According to Liang et al. (2019), the most attractive properties of *G. lucidum* are the immunomodulatory and antitumor effects.

It is known that these biological effects are related to some bioactive compounds present in Linghzi mushroom, being the polysaccharides one of the major active components (Ahmad, 2020; Liang et al., 2019). Many studies have reported different biological effects that could be attributed to *G. lucidum* polysaccharides, such as promotion of neurogenesis (Huang et al., 2017), improvement of the intestinal barrier functions (Jin et al., 2017), enhancement of insulin sensibility (Xu et al., 2017), antifatigue (Cai et al., 2021), hypolipidemic and antioxidant activities (Xu et al., 2019), prebiotic (Guo et al., 2021), immunomodulatory (Liu et al., 2018), antidiabetic (Xiao et al., 2017), and antitumor activity (Fu, Shi & Ding, 2019; Liu, Amakye & Ren, 2021).

Most of the *G. lucidum* polysaccharides are branched  $\beta$ -glucans (Lu et al., 2020), however heteropolysaccharides have also been isolated from this fungus (Ruthes, Smiderle & Iacomini, 2016), and could exhibit biological properties.

Totalizing more than 9.9 million deaths in 2020, cancer is one of the leading causes of death worldwide with an incidence rate on the rise (Sung et al., 2021). Cancer cells arise from normal tissue through an intricate natural selection process, acquiring advantageous characteristics, such as resistance to cell death and immune evasion, besides a unique capacity to metastasize to different tissues (Hanahan & Weinberg, 2011), originating the major cause of cancer death – the metastatic process (Lambert, Pattabiraman, & Weinberg, 2016). In order to successfully form a metastasis, cancer cells must migrate and invade other tissues, completing the intravasation into the transit compartment, subsequently, the extravasation into the new site and its colonization. At the same time, cancer cells finely modulate the adhesion in different microenvironments that lack the previous stimulus found in the primary tumor (Welch & Hurst, 2019).

Those steps are developed in a particular rapid progression for some types of cancer, such as melanoma. For this reason, melanoma is considered the most lethal form of skin cancer (Davis, Johnson, Sosman, & Chandra, 2018; Ward & Farma, 2017).

Recent advances in cancer therapeutics such as target and immunotherapies have improved metastatic melanoma treatment, increasing patient's overall survival, significantly suppressing the limited benefits of the conventional chemotherapy intervention (Domingues, Lopes, Soares, & Populo, 2018). Despite of this, metastatic melanoma treatment is still a scientific and clinical challenge, once both cited treatments (target and immunotherapy) are dependent on specific genetic and immune panoramas of each organism, being ineffective for a range of different patients. Additionally, mild, severe and even life threatening adverse effects have been observed for both interventions (Kroschinsky et al., 2017; Seidel, Otsuka, & Kabashima, 2018).

Polysaccharides have been largely studied as an interesting alternative to improve the treatment of highly metastatic cancers, as showed by the diverse antitumor activities previously reported for such molecules (Khan, Date, Chawda, & Patel, 2019).

*G. lucidum* crude polysaccharides were reported to have antimelanoma effect when administrated to C57BL/6 mice inoculated with B16 cells (240 mg/kg/day). A combination treatment containing *G. lucidum* polysaccharides reduced the tumor volume of melanoma when compared with the dacarbazine alone (Liu et al., 2021). Polysaccharides from the same mushroom *efficiently* decreased metastasis of B16 cells in C57BL/6J mice (Zheng, Jia, Zhao, Wei & Liu, 2012).

The therapeutic effects observed for mushroom polysaccharides and the ever-growing interest in finding antitumor compounds justifies the importance of this study that aimed to isolate *G. lucidum* polysaccharides for an accurate antitumoral investigation. Antimelanoma properties using extracts of polysaccharides have been previously investigated; however, the isolated molecules were not assessed. This study has purified and characterized the chemical structure of a fucoxylomannan and observed its effect in murine melanoma cells (B16-F10), compared to a non-tumorigenic fibroblast cell line (BALB/3T3).

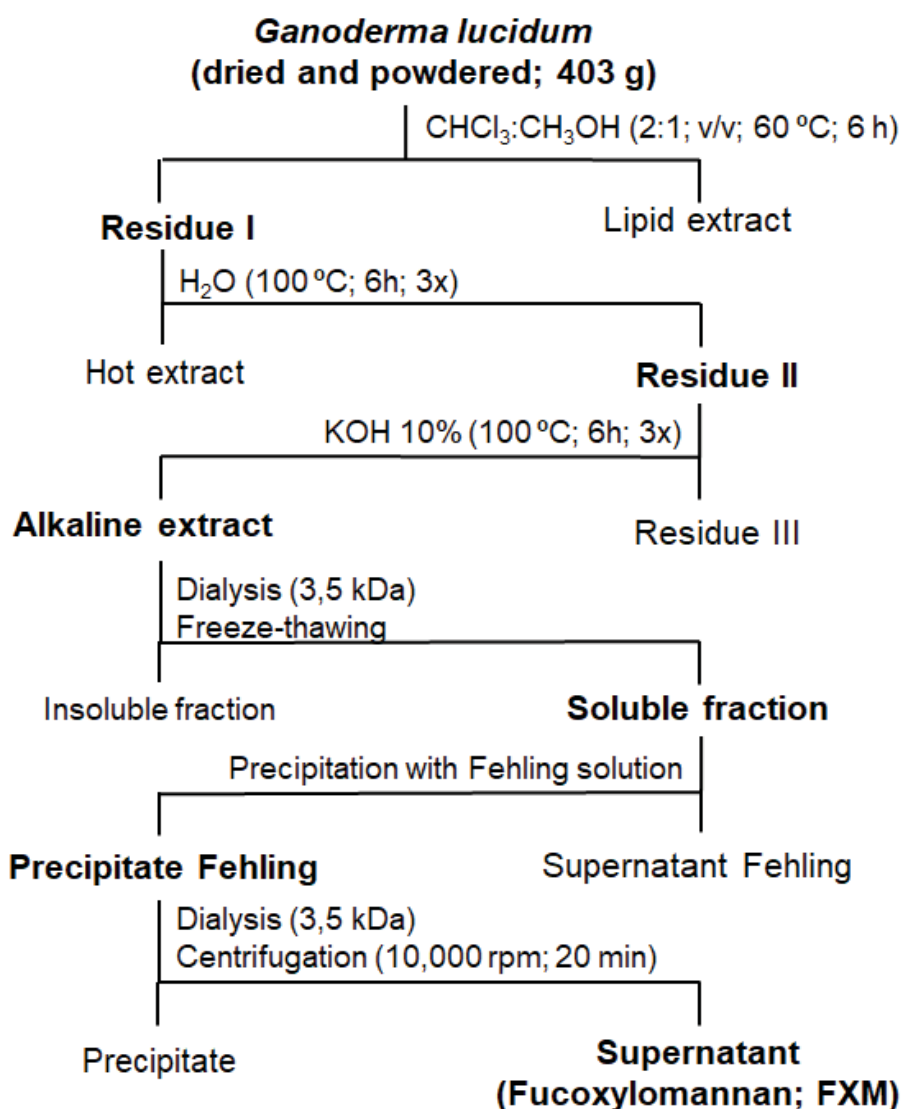
## 2. Material and methods

### 2.1 Biological material

*G. lucidum* fruiting bodies were donated by the company Juncao Brazil, located in the town Taboão da Serra, State of São Paulo, Brazil. The study of this species was registered on *Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado* (SISGEN), with the number A3BA3B3.

### 2.2 Extraction and purification processes

Extraction and purification procedures were carried out as represented in Fig. 1. The dried fruiting bodies (403 g) were milled and defatted with chloroform-methanol (2:1; v/v) (at 60 °C; 6 h; 3 x) using a Soxhlet apparatus. The remaining residue was subsequently extracted with distilled water and with 10 % KOH (containing 1 mg NaBH<sub>4</sub>). Both extractions were carried out under reflux (at 100 °C; 6 h; 3 x). The alkaline extract was recovered by filtration with nylon filter cloth, neutralized with acetic acid and dialyzed (MWCO 3.5 kDa) against tap water for 72 h. Further, the sample was concentrated under reduced pressure and the freeze-thawing process was carried out (Gorin & Iacomini, 1984). The supernatant obtained was treated with Fehling solution, following methodology adapted from Jones and Stoodley (1965). For this assay, the sample was solubilized in a solution containing potassium sodium tartrate (86.5 g) and potassium hydroxide (62.5 g) in distilled water (250 mL). Subsequently, a solution of copper sulfate (27.9 g) in distilled water (250 mL) was added. After magnetic stirring (1 h), the sample rest in refrigerator (~4 °C), overnight. The precipitate was recovered by centrifugation (10,000 rpm; 20 min; 4 °C), neutralized with acetic acid, dialyzed against tap water for 48 h, treated with cationic resin, neutralized with 1 M NaOH and dialyzed against tap water for 24 h. Finally, the dialyzed sample was centrifugated and the obtained supernatant was named FXM.



**Figure 1:** Scheme of extraction and purification of a fucoxylomannan (FXM) from *G. lucidum* fruiting bodies.

### 2.3 Monosaccharide composition

FXM (1 mg) was hydrolyzed with TFA (2 M) at 100 °C for 8 h. After total remotion of the acid by evaporation, distilled water (200  $\mu\text{L}$ ) was added and the reducing process was carried out with 1 mg of  $\text{NaBH}_4$ , overnight at room temperature (Wolfrom & Thompson, 1963a). Subsequently, the sample was neutralized with acetic acid, dried and washed with methanol (200  $\mu\text{L}$ ; 3 x). Acetylation was performed with pyridine– $\text{Ac}_2\text{O}$  (200  $\mu\text{L}$ ; 1:1; v/v) at 100 °C for 30 min (Wolfrom & Thompson, 1963b). Alditol acetates were extracted with chloroform (1 mL) and washed with 5 % copper sulfate (2.5 mL; x 3). After total evaporation of chloroform, the sample was solubilized

in acetone and 1  $\mu$ L was injected (split mode 1:10) in a gas chromatography-mass spectrometer operating at 70 eV, coupled to a single-quadrupole apparatus (QP2020, Shimadzu), using a capillary column, RTX-5-MS (30 m x 0.25 mm x 0.25  $\mu$ m film thickness) and equipped with an AOC-6000 autosampler (Pal Systems). Helium was used as a carrier gas (45 cm/sec), at a flow rate of 1.54 mL/min. A non-linear heat ramp was used, starting at 50 °C and after 1 min being increased to 150 °C (20 °C/min), then reaching to 200 °C (5 °C/min), being hold for 3 min. Lastly, a heat of 20 °C/min was carried out until reach 250 °C, a temperature which was held for 3.5 min. Monosaccharides were identified by their typical retention time and electron impact profiles.

## 2.4 Linkage type evaluation

FXM was methylated according to Ciucanu and Kerek (1984), with modifications. Briefly, the sample (3 mg) was solubilized in dimethyl sulfoxide (1 mL) and finely powdered NaOH (20 mg) was added. Then, methyl iodide (1 mL) was used as methylating agent, and the reaction was maintained under magnetic stirring for 30 min. Subsequently, the sample was let to rest for 24 h, solubilized in water, neutralized with acetic acid, and dialyzed against tap water (MWCO 3.5 kDa) for 24 h. This methylation process was repeated twice aiming the complete methylation of the polysaccharide. In the last repetition, after the time of rest, the sample was extracted with chloroform (1 mL) and this solvent was evaporated to dryness. The per-O-methylated derivative was hydrolyzed with 45 % formic acid (1 mL) for 6 h at 100 °C, followed by evaporation to dryness. Then, the sample was dissolved in 2 M TFA and maintained at 100 °C for 20 h. Afterwards, the material was evaporated to dryness, resuspended in 200  $\mu$ L of distilled water and reduced with NaBD<sub>4</sub> (24 h, 25 °C), followed by acetylation, generating the partially O-methylated acetate alditol derivatives, which were analyzed by GC-MS Shimadzu single quadrupole using a QP2020 NX model gas chromatograph-mass spectrometer, with He as the carrier gas at flow rate 1.0 mL/min. The results are expressed as a relative percentage of each component, and the derivatives were identified by the ion  $m/z$  by comparing their positive ions with standards (Sasaki et al., 2005).



## 2.5 Partial hydrolysis

An aliquot of FXM (25 mg) was partially hydrolyzed. Using 3 mL of 0.2 M TFA, the sample was maintained for 2 h at 100 °C and then neutralized with 0.2 M NaOH, followed by precipitation with ethanol (3:1 v/v), and centrifugation (10,000 rpm, 4 °C). The precipitate fraction FXM-H was submitted to NMR analysis (<sup>1</sup>H and HSQC-DEPT) using a Bruker Avance 600 MHz spectrometer. The NMR conditions are described in section 2.9.

## 2.6 Homogeneity and relative molar mass

Analyses were carried out in a high-performance size-exclusion chromatography (HPSEC) coupled to refractive index detector. Four gel-permeation Ultrahydrogel columns in series with exclusion sizes of  $7 \times 10^6$ ,  $4 \times 10^5$ ,  $8 \times 10^4$ , and  $5 \times 10^3$  Da were used. The eluent was aqueous NaNO<sub>2</sub> (0.1 M) containing aqueous NaN<sub>3</sub> (200 ppm), at a flow rate of 0.6 mL/min. FXM and FXM-H (1 mg.mL<sup>-1</sup>) were solubilized in the eluent, filtered through a cellulose membrane (0.22 µm) and injected (100 µL loop) into the chromatograph. The relative molar mass of FXM was determined by the comparison of the sample retention time with a curve of dextran patterns of different molecular masses (Sigma-Aldrich) (487.0, 266.0, 124.0, 72.2, 40.2, 17.2 and 9.4 kDa).

## 2.7 Congo red test

The conformational structure of FXM was investigated by helix-coil transition analysis according to Ogawa, Tsurugi & Watanabe (1972), with minor modifications. Briefly, FXM (1 mg) was solubilized in NaOH (0.05 mol.L<sup>-1</sup>; 980 µL). Subsequently, Congo red solution (4 mM) was added (20 µL) to achieve a final concentration of 80 µM. Dextran M<sub>w</sub> 487.0 kDa (Sigma) (1 mg) was used as random coil pattern and blank was prepared with the alkaline solution plus Congo red (4 mM). The visible absorption spectrum was taken using a microplate reader within the wavelength range of 400-650 nm in intervals of 10 nm.

## 2.8 Protein, phenolic compounds, and carbohydrate content

Protein concentration was investigated using Bradford method (Bradford, 1976), according to specification recommended by the manufacturer BioAgency

(product 500-0009N). Bovine serum albumin (BSA) was used as standard. Phenolic compounds content was evaluated with Folin & Ciocalteu phenol's reagent (Sigma, F9252) following Singleton, Orthofer & Lamuela-Raventós (1999) methodology. Gallic acid (GAE) was used as standard. For both analyses, FXM was solubilized in distilled water at 3 mg.mL<sup>-1</sup>. Total sugar content was determined spectrophotometrically following the method of Dubois (Dubois et al., 1956) using a mixture of mannose, xylose and fucose as standard. Samples and patterns were solubilized at 1 mg.mL<sup>-1</sup>.

## 2.9 Nuclear magnetic resonance

NMR spectra of mono- (<sup>13</sup>C; <sup>1</sup>H, and DEPT135) and two-dimensional (HSQC-DEPT; COSY; TOCSY; HSQC-TOCSY, coupled-HSQC, HMBC, and NOESY) experiments were obtained using a BRUKER Avance III HD 400 MHz equipped with a BBI probe and in a BRUKER Avance III HD 600 MHz spectrometer equipped with a TCI CryoProbe Prodigy (Sasaki et al., 2013). The samples were solubilized in D<sub>2</sub>O (60 mg.mL<sup>-1</sup>). Analyses were carried out at 70 °C. Chemical shifts are expressed in ppm (δ) relative to resonance of the external reference acetone (at δ 32.77 and 2.21 for <sup>13</sup>C and <sup>1</sup>H signals, respectively) based on DSS (2,2-dimethyl-silapentane-3,3,4,4,5,5-*d*<sub>6</sub>-5 sulfonate sodium salt; δ = 0.0 for <sup>13</sup>C and <sup>1</sup>H) (Smiderle et al., 2012).

## 2.10 *In vitro* antitumoral activity analysis

### 2.10.1 Polysaccharide preparation

For *in vitro* experiments, FXM was dissolved at a concentration of 5 mg.mL<sup>-1</sup> using Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Cat. 12800-017, Massachusetts, USA) without fetal bovine serum (FBS) (Gibco, Cat. 12657029, Massachusetts, USA) and sterilized in 0.22 μm membranes (Millipore, Cat. SLGV033RS, New Jersey, USA). DMEM without FBS was used as control in the same volume as the polysaccharide solution. FXM dilutions were prepared in the desired concentrations.

### 2.10.2 Cell culture

Murine melanoma B16-F10 (BCRJ, Code 0046) and the non-tumorigenic fibroblast BALB/3T3 clone A31 (ATCC, Code CCL-163) cell lines were used. Cells were maintained in a humidified incubator at 37 °C and 5 % CO<sub>2</sub>, and were cultivated

using DMEM supplemented with 10 % FBS, 0.25  $\mu\text{g.mL}^{-1}$  penicillin/streptomycin (Thermo Fisher, Cat. 15140122, Massachusetts, USA) and 1.57  $\text{g.L}^{-1}$  sodium bicarbonate (Merck, Cat. 36486, New Jersey, USA). For subculturing and experimental seeding, the cell confluence was not higher than 80 %. Cells were detached with trypsin/EDTA (Gibco, Cat. 15400054, Massachusetts, USA) and counted in hemocytometer.

### **2.10.3 Cytotoxicity and cell density assays**

FXM effects on cell viability and cell density were analyzed in the murine melanoma B16-F10 and in the non-tumorigenic fibroblast BALB/3T3 cell lines. B16-F10 (500 cells/well) or BALB/3T3 cells (2,000 cells/well) were plated in 96 well plates and exposed to 10, 100, 250, 500 or 1,000  $\mu\text{g.mL}^{-1}$  of FXM for 72 h. Neutral Red (NR) (Borenfreund & Puerner, 1985) and Crystal Violet (CV) (Gillies, Didier, & Denton, 1986) assays were performed to analyze cell cytotoxicity and proliferation, respectively. At the end of the treatment period, dye coloring and elution were performed according to the experimental protocol applied. NR and CV resulting absorbances were read using a microplate reader (Biotek Epoch, Vermont, USA) at 540 and 570 nm, respectively.

### **2.10.4 Cell cycle analysis**

Effects of FXM in the B16-F10 cell cycle were analyzed by flow cytometry. B16-F10 (12,000 cells/well) were plated in 6 well plates and rest without FBS for 24 h, following treatment with 250 and 500  $\mu\text{g.mL}^{-1}$  FXM for 72 h. After the treatment period, cells were detached with trypsin/EDTA and processed accordingly to BD PI/RNase kit protocol (BD Biosciences, Cat. 550825, California, USA). Samples were acquired on a FACS Calibur cytometer (BD Biosciences, California, USA) and analyzed using Flowing Software (Turku Bioscience Centre, Turku, Finland).

### **2.10.5 Anchorage-independent colony formation assay**

The capacity of FXM to affect the anchorage-independent colony formation capacity of B16-F10 cells was analyzed using the soft-agar method, as described by Borowicz et al., (2014). Two sterile solutions of agarose (Sigma-Aldrich, Cat. A9539, Missouri, USA) diluted in ultra-pure water were prepared at the concentrations of 1 % and 0.6 % and powder DMEM was prepared twice concentrated (DMEM2) in ultra-pure

water. In 12 well plates, 500  $\mu\text{L}$ /well of the 1 % agarose solution was mixed with 500  $\mu\text{L}$ /well of DMEM2 to produce the bottom layer and allowed to solidify at room temperature for 30 min. For the upper layer, 500  $\mu\text{L}$ /well of 0.6 % agarose solution was mixed with 350  $\mu\text{L}$ /well of DMEM2 with 20 % FBS, FXM (150  $\mu\text{L}$ /well at a final concentration of 250 or 500  $\mu\text{g}\cdot\text{mL}^{-1}$ ) and B16-F10 cells (450 cells/well). The upper layer was seeded above the previously solidified layer and it was allowed to solidify as previously cited. DMEM2 without FBS was used as control. After preparing the plate, 500  $\mu\text{L}$ /well of FXM (at 250  $\mu\text{g}\cdot\text{mL}^{-1}$  or 500  $\mu\text{g}\cdot\text{mL}^{-1}$ ) diluted in DMEM2 with 20 % FBS was plated on the top of the agarose. Control wells received the same solution but without FXM. Plates were then placed in the incubator for 72 h. Following this period, the supernatant of each well was removed and substituted with 1 mL/well of DMEM 10 % FBS. Every 5 days, 500  $\mu\text{L}$  of DMEM was added to each well to prevent drying. Colonies were allowed to grow for 14 days, and after this period, they were fixed with 2 % paraformaldehyde for 1 h and stained with CV overnight. The wells were photographed for colony counting and area measurement; the analysis were performed using ImageJ Fiji Software (Schindelin et al., 2012).

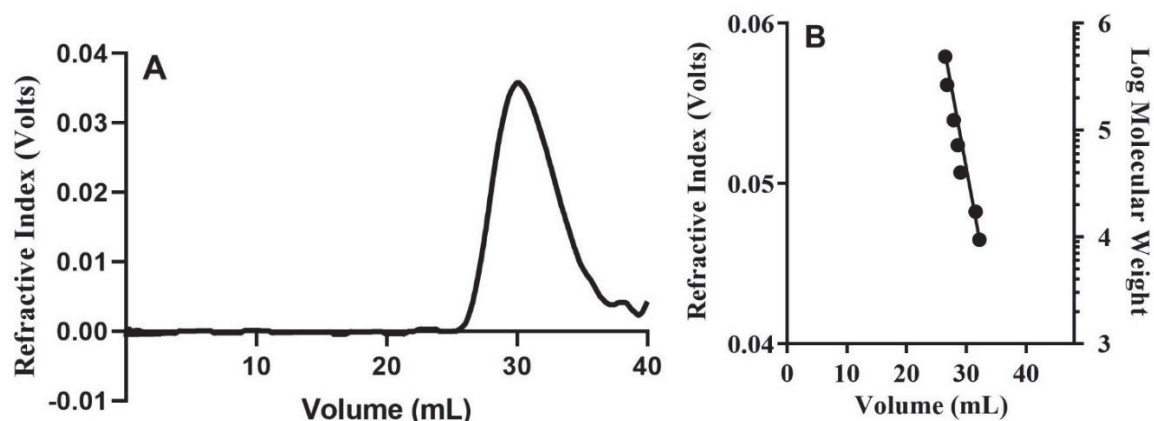
### 2.10.6 Statistical analysis

Significant differences between experimental groups were determined by unpaired t-test with Welch's correction, using GraphPad Prism 6 software. Data is presented as mean  $\pm$  SD, and  $p < 0.05$  was considered significant.

## 3. Results and discussions

### 3.1 Structural characterization of fucoxylomannan from *G. lucidum*

The purified fraction (FXM; 0.350 g) from *G. lucidum* fruiting bodies was obtained by sequential steps shown in Fig. 1. Its analysis of alditol acetates derivatives presented fucose (26.6 %), xylose (26.0 %), mannose (44.7 %), and a residual amount of glucose (2.7 %) (Fig. S1, supplementary material). The total sugar content was 96.7 %; while proteins and phenolic compounds were not detected. The elution profile of FXM obtained in HPSEC was homogeneous (Fig. 2A) and its relative molecular weight, estimated by comparison with a curve of dextran pattern (Fig. 2B), was  $3.59 \times 10^4 \text{ g}\cdot\text{mol}^{-1}$ .



**Figure 2:** (A) Elution profile of FXM in HPSEC coupled to a refractive index detector. (B) Calibration curve of dextran standards of  $M_w$  487.0, 266.0, 124.0, 72.2, 40.2, 17.2 and 9.4 kDa (left to right).

The methylation analysis showed 2,3,4-Me<sub>3</sub>-Fuc, 3,4-Me<sub>2</sub>-Xyl, 2,3-Me<sub>2</sub>-Man, 2,3,6-Me<sub>3</sub>-Man and 2,3,4,6-Me<sub>4</sub>-Man derivatives, suggesting the presence of an heteropolysaccharide, with the major content of mannosyl residues. A small amount of 2,3,4-Me<sub>3</sub>-Glc, 2,4,6-Me<sub>3</sub>-Glc and 2,4-Me<sub>2</sub>-Glc derivatives was also observed, probably related to a residual fraction of D-glucan, very common in mushroom extracts (Table 1).

**Table 1.** Partially O-methylated alditol acetates obtained on methylation analysis of FXM.

Partially O-methylated alditol acetates <sup>a</sup>	$Rt^b$	% Area of fragments <sup>c</sup>	Linkage types <sup>d</sup>
2,3,4-Me <sub>3</sub> -Fuc	13.233	27	Fucp-(1→
3,4-Me <sub>2</sub> -Xyl	14.021	23	2→)-Xylp-(1→
2,3,4,6-Me <sub>4</sub> -Man	14.414	4	Manp-(1→
2,4,6-Me <sub>3</sub> -Glc	15.412	1	3→)-Glc p-(1→
2,3,6-Me <sub>3</sub> -Man	15.529	20	4→)-Manp-(1→
2,3,4-Me <sub>3</sub> -Glc	16.063	Tr <sup>e</sup>	6→)-Glc p-(1→
2,3-Me <sub>2</sub> -Man	16.236	24	4,6→)-Manp-(1→
2,4-Me <sub>2</sub> -Glc	16.921	Tr	3,6→)-Glc p-(1→

<sup>a</sup> Analyzed by GC-MS, after methylation, total acid hydrolysis, reduction with NaBD<sub>4</sub> and acetylation.

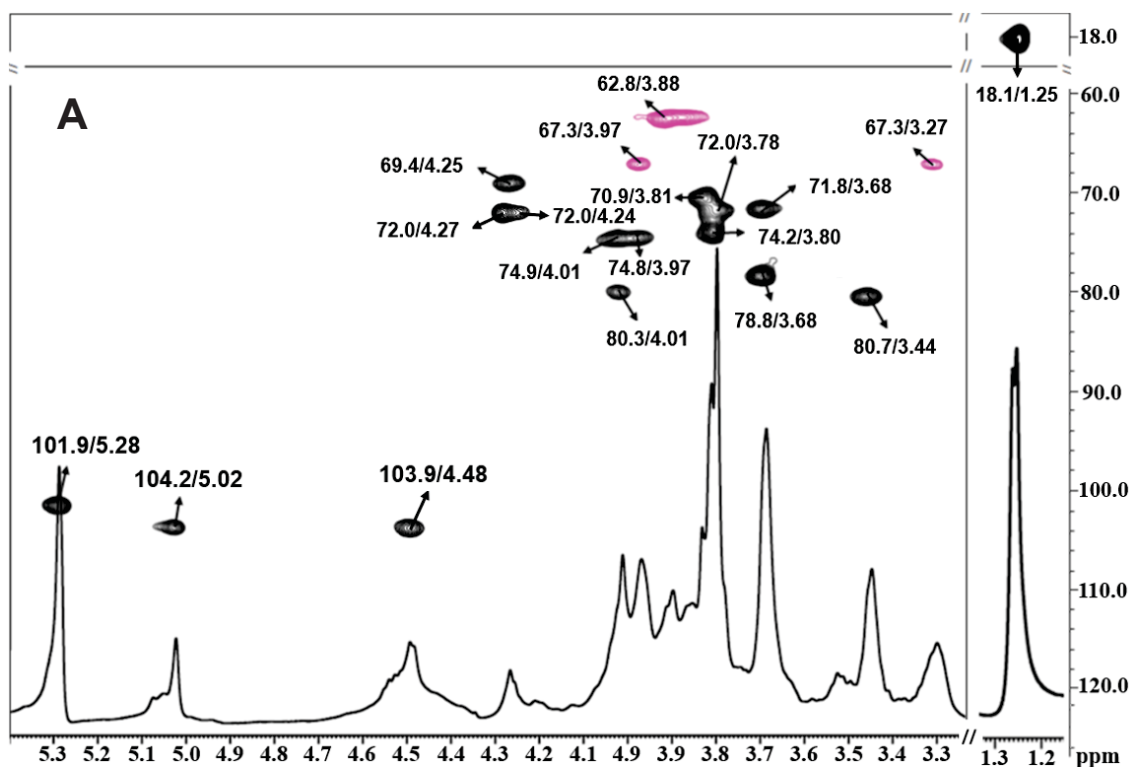
<sup>b</sup> % of peak area relative to total peak area.

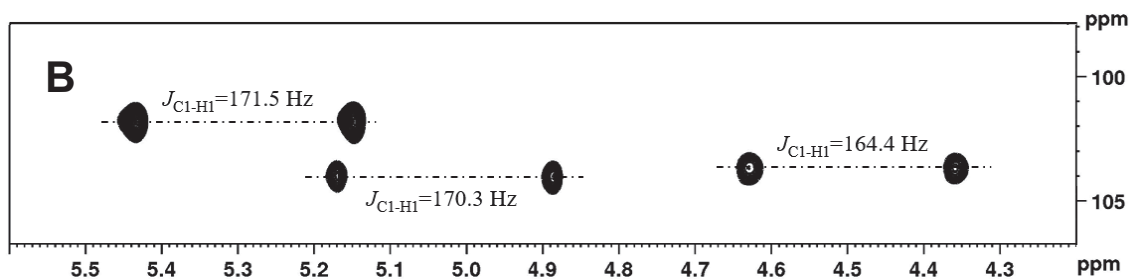
<sup>c</sup> Retention time.

<sup>d</sup> Based on derived O-methyl alditol acetates.

<sup>e</sup> Traces: <1%.

NMR analyses ( $^{13}\text{C}$ ; DEPT135;  $^1\text{H}$ ; HSQC-DEPT; HSQC-TOCY; COSY; TOCSY, coupled-HSQC, HMBC and NOESY) were performed to elucidate the chemical structure of FXM. The HSQC-DEPT spectrum (Fig. 3A) showed signals in the anomeric region (C1/H1) at  $\delta$  104.2/5.02, 103.9/4.48 and 101.9/5.28, corresponding to  $\alpha$ -L-Fucp,  $\beta$ -D-Xylp, and  $\alpha$ -D-Manp, respectively (Ruthes, Rattmann, Carbonero, Gorin, & Iacomini, 2012; Smiderle, Sassaki, Van Griensven, & Iacomini, 2013; Cordeiro, Almeida, & Iacomini, 2015). The coupling constant values were calculated from the coupled-HSQC spectra (Milhorini et al., 2018; Wang et al., 2021). The Xylp  $J_{\text{C1-H1}}$  at 164.4 Hz confirmed the  $\beta$  configuration of this glycosyl, while the values for Fucp and Manp (170.3 Hz and 171.5 Hz, respectively), revealed the  $\alpha$  configuration of both units (Fig. 3B) (Perlin & Casu, 1969). Additionally, resonances of substituted C-6 were observed at  $\delta$  67.3/3.97 and 67.3/3.27, while non-substituted C-6 was noted at  $\delta$  62.8/3.88 (Sovrani et al., 2017; Milhorini et al., 2018). The signal relative to -CH<sub>3</sub> of  $\alpha$ -L-Fucp was observed at  $\delta$  18.1 (Fig. 3A) (Milhorini et al., 2018).





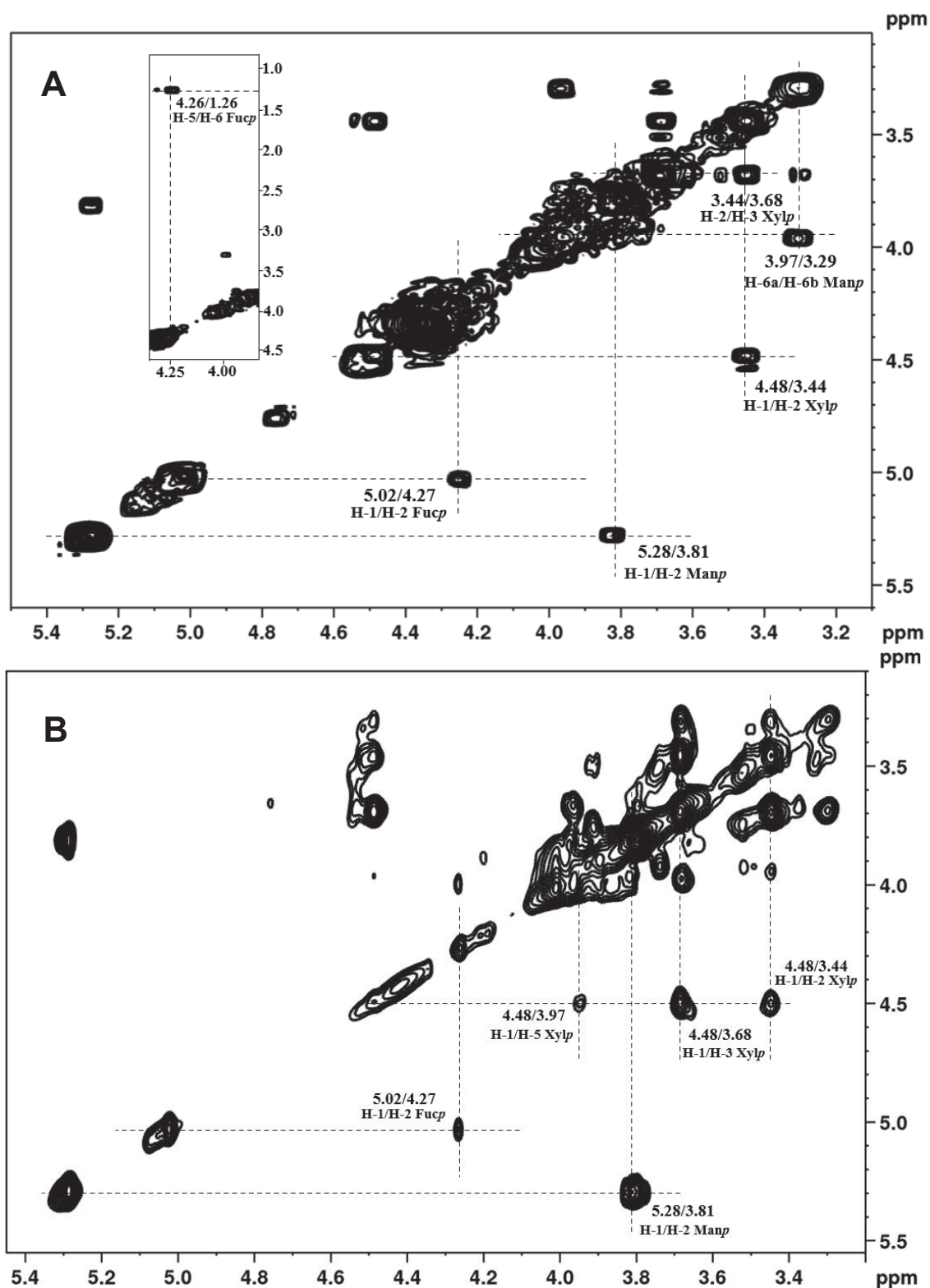
**Figure 3:** (A) HSQC-DEPT and  $^1\text{H}$  (B) coupled-HSQC of analyses of FXM. The sample was analyzed in  $\text{D}_2\text{O}$  at  $70^\circ\text{C}$ .

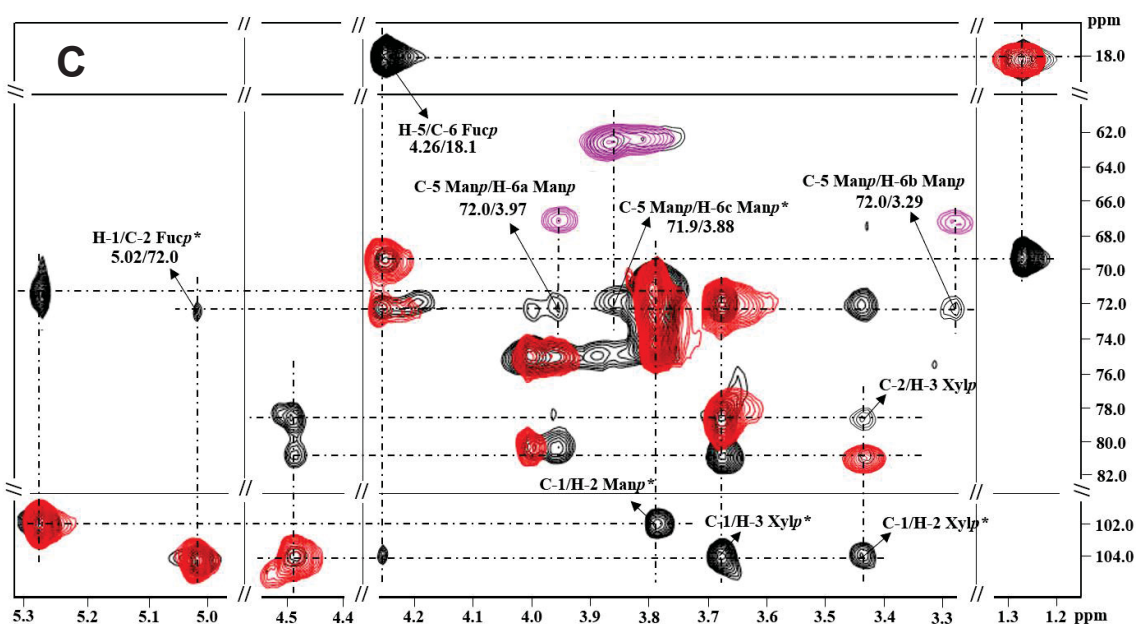
These results corroborate with  $^{13}\text{C}$  and DEPT-135 spectra (Fig. S2 A and B, supplementary material), once similar signals relative to the C-1 of  $\alpha\text{-L-Fucp}$  ( $\delta$  104.2),  $\beta\text{-D-Xylp}$  ( $\delta$  103.9), and  $\alpha\text{-D-Manp}$  ( $\delta$  101.9), as well as the substituted C-6 ( $\delta$  67.3), non-substituted C-6 ( $\delta$  62.8), and the  $-\text{CH}_3$  of  $\alpha\text{-L-Fucp}$  ( $\delta$  18.1) were observed (Ruthes et al., 2012; Smiderle et al., 2013; Cordeiro et al., 2015).

Correlations between adjacent glycosyls were observed in COSY spectrum (Fig. 4A) at  $\delta$  5.28/3.81, 5.02/4.27 and 4.48/3.44, for H-1/H-2 of the  $\alpha\text{-D-Manp}$ ,  $\alpha\text{-L-Fucp}$  and  $\beta\text{-D-Xylp}$  units, respectively. Compatible with the DEPT-135 spectra (Fig. S2 B, supplementary material), it is possible to observe the correlation between the H-6a and H-6b of the O-6 substituted  $\alpha\text{-D-Manp}$  units at  $\delta$  3.97/3.29 (Zavadinack et al., 2021). In addition, H-5/H-6 from  $\alpha\text{-L-Fucp}$  units is indicated at  $\delta$  4.26/1.26 (Oliveira et al., 2018) and the H-2/H-3 of  $\beta\text{-D-Xylp}$  appear at  $\delta$  3.44/3.68. In TOCSY analysis (Fig. 4B), it is possible to observe the correlations between H-1/H-2, H-1/H-3 and H-1/H-5 of the Xylp units at  $\delta$  4.48/3.44,  $\delta$  4.48/3.68 and  $\delta$  4.48/3.97, respectively. Only one correlation signal of Fucp units was observed at  $\delta$  5.02/4.27. For Manp units, a high intense correlation signal at  $\delta$  5.28/3.81 was observed, justified by the overlapping of  $\alpha\text{-D-Manp}$  units signals of H-1/H-2, H-1/H-3 and H-1/H-4 – for the  $\alpha\text{-D-Manp}$  non reducing end units (Gorin & Mazurek, 1975). Another overlapping was observed for C-4/H-4 of the Fucp non reducing end units,  $4\rightarrow)\text{-}\alpha\text{-D-Manp-)}1\rightarrow$  and the  $4,6\rightarrow)\text{-}\alpha\text{-D-Manp-)}1\rightarrow$  units at  $\delta$  80.3/4.02;4.00;4.01, respectively, confirmed by COSY, HSQC-TOCSY and HSQC analysis (Fig. S3, S4 and S5 supplementary material). The signals observed in the figure S5 (supplementary material) were separately integrated and showed a prevalence of the mannose signal (65 %), as expected. The signals were



confirmed by comparison with literature data (Ruthes et.al., 2012; Ruthes et.al., 2013; Oliveira et.al., 2018) and the experiments of HSQC-TOCSY superimposed on HSQC-DEPT spectra (Fig. 4C), HMBC, and 2D NOESY (Fig. 5). The details are demonstrated in Table 2.





**Figure 4:** 2D-NMR analyses of the FXM fraction. (A) COSY, (B) TOCSY, and (C) HSQC-TOCSY – in black – superimposed on HSQC-DEPT – in red and purple - correlation maps. The sample was analyzed in D<sub>2</sub>O at 70 °C. \* Signs confirmed in TOCSY and/or COSY analysis.

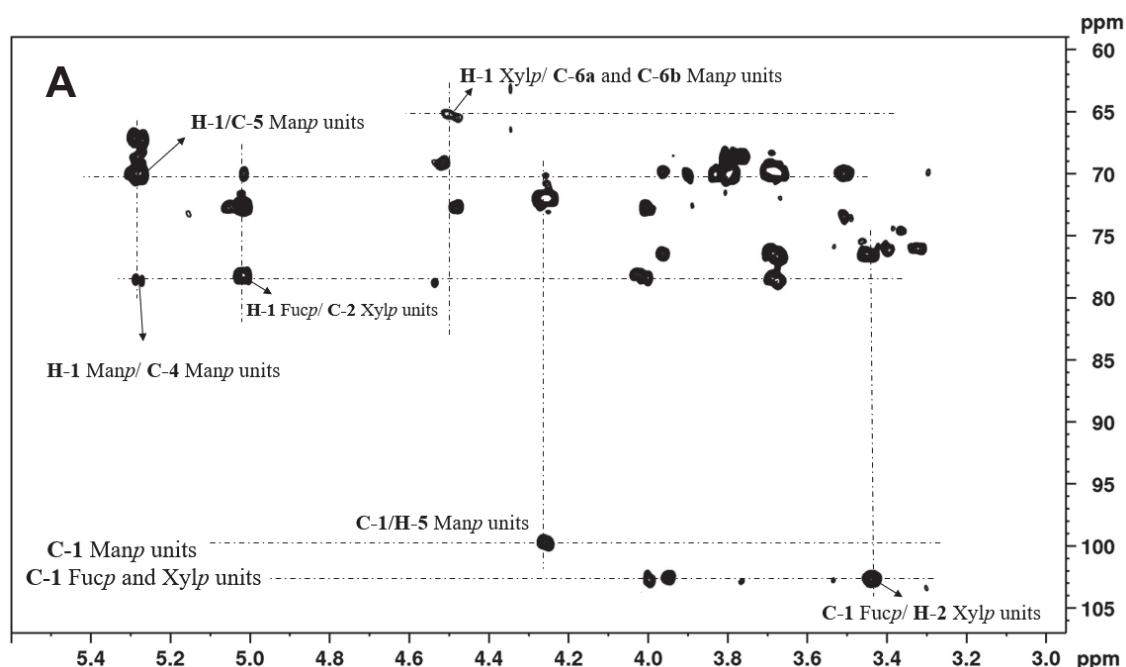
**Table 2.** <sup>1</sup>H and <sup>13</sup>C chemical shifts<sup>a</sup> of the FXM polysaccharide.

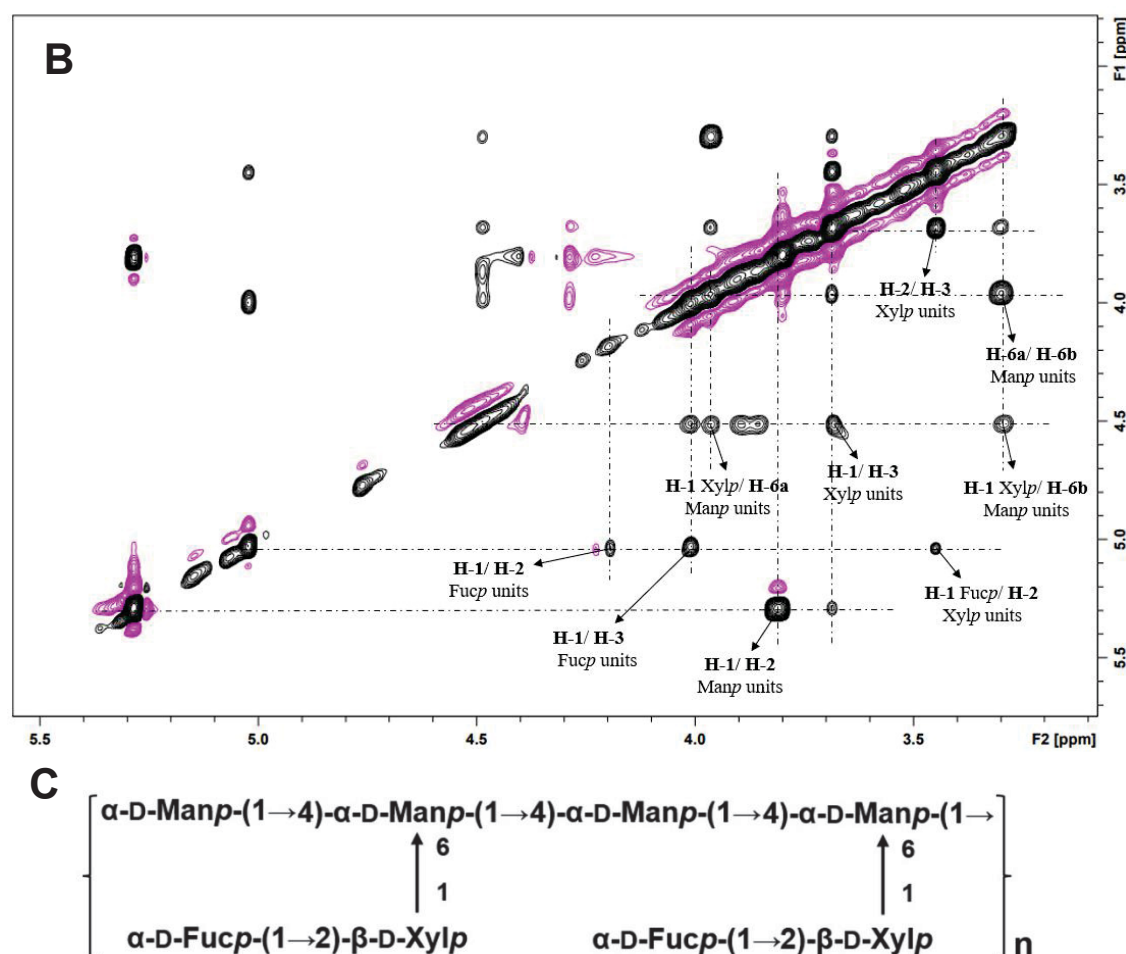
Units			1	2	3	4	5	6	
								a	b
α-D-Manp-(1→	<sup>13</sup> C		101.1	70.4	74.0	72.0	71.9	62.8	62.8
	<sup>1</sup> H		5.27	3.80	3.79	3.78	4.22	3.85	N.D. <sup>b</sup>
4→)-α-D-Manp-(1→	<sup>13</sup> C		101.9	70.9	74.1	80.3	72.0	62.8	62.8
	<sup>1</sup> H		5.28	3.81	3.80	4.00	4.24	3.88	N.D. <sup>b</sup>
4,6→)-α-D-Manp-(1→	<sup>13</sup> C		101.8	71.1	74.1	80.3	72.0	67.3	67.3
	<sup>1</sup> H		5.30	3.81	3.82	4.01	4.25	3.97	3.29
2→)-β-D-Xylp-(1→	<sup>13</sup> C		103.9	80.7	78.8	71.8	74.8	-	-
	<sup>1</sup> H		4.48	3.44	3.68	3.68	3.97	-	-
α-L-Fucp-(1→	<sup>13</sup> C		104.2	72.0	74.9	80.3	69.4	18.1	18.1
	<sup>1</sup> H		5.02	4.27	4.01	4.02	4.25	1.26	1.26

<sup>a</sup>Assignments are based on <sup>13</sup>C, <sup>1</sup>H, HSQC-DEPT, HSQC-TOCSY, COSY, TOCSY, HMBC and 2D NOESY NMR analyses and are expressed as ppm.

<sup>b</sup>Not determined.

Long range correlation techniques (HMBC and 2D NOESY, Fig. 5) were performed to confirm the linkage types of the fucoxylomannan. In the HMBC spectra (Fig. 5A), it is possible to observe the correlation between the H-1 of the  $\beta$ -D-Xylp units with the C-6a and C-6b of the  $\alpha$ -D-Manp units at  $\delta$  4.48/67.3 and the (1 $\rightarrow$ 4) linkage between the Manp units at  $\delta$  5.28/80.3, confirmed by the presence of partially methylated acetate derivatives 3,4-Me<sub>2</sub>-Xyl and 2,3-Me<sub>2</sub>-Man, respectively (Table 1). The Fucp-(1 $\rightarrow$ 2)-Xylp fragment was confirmed by the correlation signal at  $\delta$  5.02/80.7, compatible with the presence of the 3,4-Me<sub>2</sub>-Xylp and the 2,3,4-Me<sub>3</sub>-Fucp derivatives. The Nuclear Overhauser enhancement experiment (2D NOESY) (Fig. 5B), which indicates a spatial molecular proximity relationship between the units cited above, contributed to prove the linkage types in the FXM, where it is possible to observe a correlation between H-1 Fucp/H-2 Xylp and H-1 Xylp/6a and 6b Manp units. Additionally, other signals in accordance with the results obtained were observed for H-2/H-3, H-1/H-3 of Xylp units, H-1/H-2, H-6a/H-6b of Manp units and H-1/H-2, H-1/H-3 of Fucp units.





**Figure 5:** Analyses of HMBC (A), 2D NOESY (B), and the presumed structure (C) of the FXM fraction. Sample was analyzed in D<sub>2</sub>O at 70 °C in a Bruker Avance III 600 MHz (chemical shifts are expressed in  $\delta$  ppm).

To confirm the main chain composition of the polysaccharide, a partial hydrolysis was developed, giving rise the FXM-H sample (Fig. S6). The percentage of monosaccharides in FXM-H was determined by integrating <sup>1</sup>H signals of the spectrum, which showed 81 % of mannose and 19 % of xylose. The presence of fucosyl signal was not observed, indicating that this monosaccharide was fully hydrolyzed, confirming its external position in the molecule. The less intense signals of substituted C-6/H-6 suggests that the remaining xylose units are branched in the main chain at O-6. The elution profile of FXM-H in HPSEC was heterogeneous and contains three peaks (Fig. S7). This heterogeneity was expected once the main chain of FXM could be hydrolyzed into small fragments, besides the remotion of external fucosyl units.

Considering all the results presented about methylation (Table 1) and NMR analyses (Fig. 3, 4, and 5), as well as the fact that FXM-H has a majority percentage of mannose, it can be concluded that the main chain of FXM is composed of  $\alpha$ -Manp-(1 $\rightarrow$ 4)-linked, and the substitution occur at O-6 position by disaccharides of Fucp-(1 $\rightarrow$ 2)-Xylp, confirming the chemical structure of the fucoxylomannan (Fig. 5C).

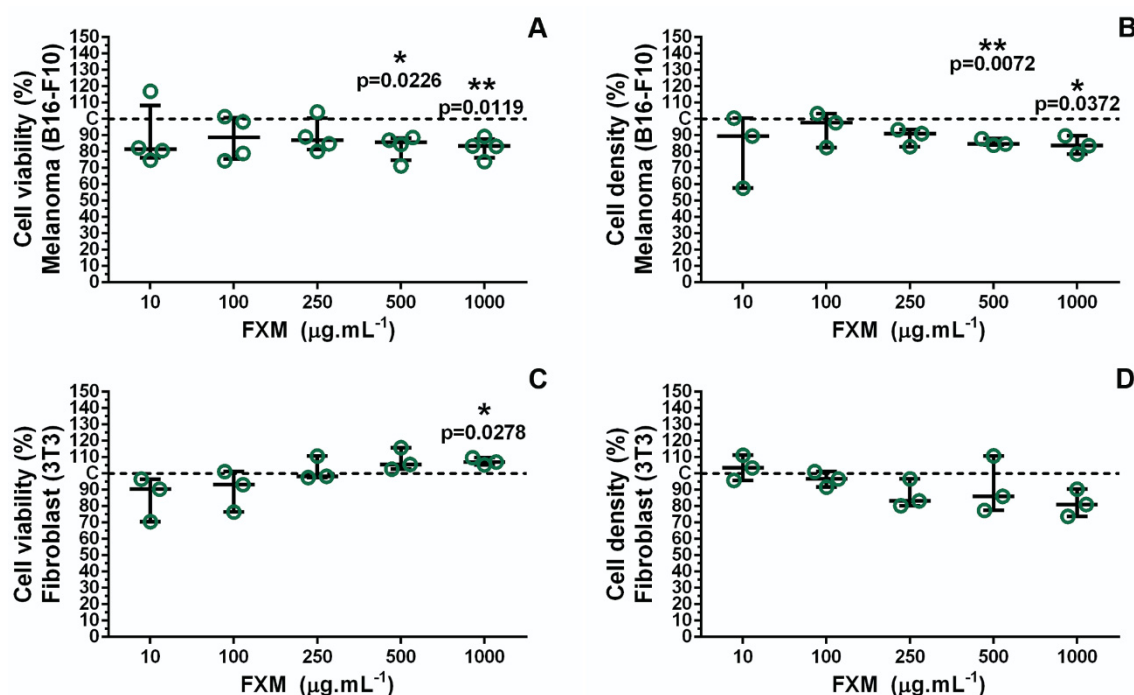
Despite a fucoxylomannan have been previously found in *G. lucidum* fruiting bodies (Miyakazi & Nishijma, 1982), the heteropolysaccharide isolated and characterized in the present work has features that were not reported. Miyakazi & Nishijma obtained a structure with a main chain of 1 $\rightarrow$ -D-Manp-(4 $\rightarrow$  units, however, they observed branches at O-3 position by D-Fucp-(1 $\rightarrow$ 4)-D-Xylp-(1 $\rightarrow$ . On the other hand, the FXM isolated in this study is branched at O-6 position by the disaccharide, as clarified by two-dimensional NMR studies and methylation analysis.

Other fucoxylomannans were isolated and characterized from fruiting bodies of the mushrooms *Polyporus pinicola* (Axelsson, Björndal & Lindberg, 1969; Fraser, Karacsonyi & Lindberg., 1967) and *Fomitopsis officinalis* (Quinine conk) (Golovchenko et al., 2020). However, the chemical structures of such fucoxylomannans were different from those found for *G. lucidum* heteropolysaccharide.

The structural conformation of FXM was analyzed with Congo red dye according to the methodology developed by Ogawa et al., (1972). The analysis did not show any bathochromic shift when compared to the random coil pattern of dextran, or the blank of Congo red, indicating that this molecule does not have a triple helix conformation (Fig. S8, supplementary material). Literature data show that fractions containing mushroom heteropolysaccharides generally do not present helical structure when evaluated in Congo red analysis, as observed with the xylose-rich heteropolysaccharide from the mushroom *Phellinus linteus*, which also showed a random-coil conformation (Wang, Pei, Ma, Cai, & Yan, 2014). At the same time, Abreu et al. (2021) obtained a  $\beta$ -glucans-rich extract and another extract with heteropolysaccharide (mannogalactan) from *Pleurotus eryngii*. The first one showed a triple-helical conformation, while the latter did not show displacement in the Congo red experiment.

### 3.2 FXM selectively affects B16-F10 cell proliferation

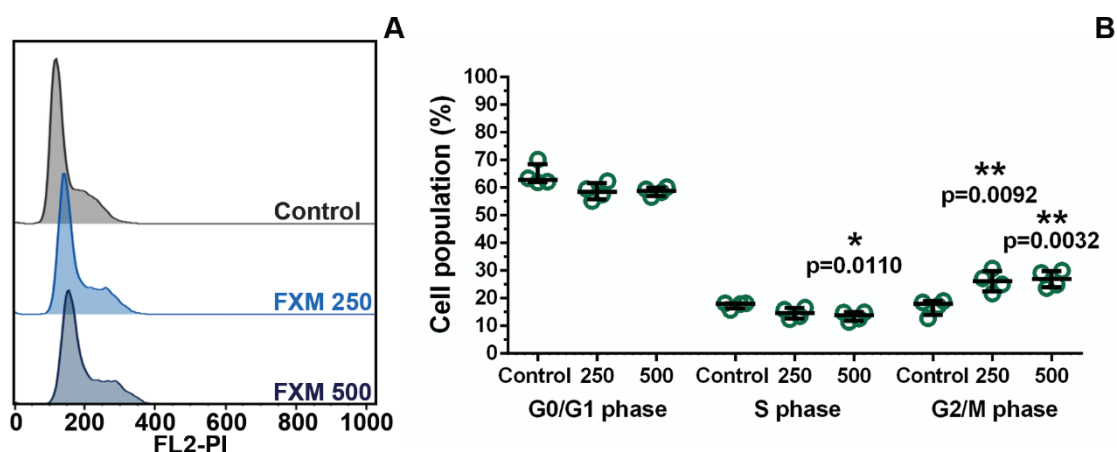
Melanoma cells exposed to FXM reduced their capacity to uptake and retain neutral red (NR) by 17.1 % and 17.5 % (at 500 and 1000  $\mu\text{g.mL}^{-1}$  FXM, respectively), when compared to control group (Fig. 6A). Interestingly, NR uptake capacity of non-tumor fibroblasts was not affected by FXM in any of the tested concentrations (Fig. 6C). Crystal violet (CV) assay resulted in a similar trend as observed for NR. Melanoma cell density was reduced by 14.5 % and 16.0 % when exposed to FXM (at 500 and 1000  $\mu\text{g.mL}^{-1}$  FXM, respectively; Fig. 6B). In contrast, cell density of non-tumor fibroblasts was not affected by the polysaccharide treatment (Fig. 6D). To further investigate the decreasing effect of FXM on cell density, the cell cycle of melanoma cells was observed after treatment with the polysaccharide. For such assay, the concentrations were selected based on NR and CV results: 250  $\mu\text{g.mL}^{-1}$  (when occurred no reduction in cell viability) and 500  $\mu\text{g.mL}^{-1}$  (the smallest concentration that affected cell density).



**Figure 6:** (A) Cell viability (NR uptake) and (B) Cell density (CV stained) in Melanoma cells (B16-F10), after treatment with FXM. (C) Cell viability (NR uptake) and (D) Cell density (CV stained) in Fibroblast cells (3T3), after treatment with FXM. These results represent the set of at least three biologically independent experiments with each cell line. Data was normalized with control group (represented here as a dashed line).



The analysis showed that FXM was able to interfere with melanoma cell cycle, in both concentrations evaluated (Fig. 7A and B). Cells treated with 500  $\mu\text{g.mL}^{-1}$  FXM resulted in 22.9 % less cells in S phase when compared to control group. Additionally, FXM (at 250 and 500  $\mu\text{g.mL}^{-1}$ ) significantly induced an accumulation of melanoma cells in G2/M phase (54.7 % and 59.3 %, respectively). These observations together with the previous results of cell viability and cell density indicate that FXM effect on melanoma cells is related to an anti-proliferative activity. The absence of effects on non-tumor cells is in accordance with this biological mechanism, since anti-proliferative drugs have a more pronounced effect against highly proliferative cells (Zhang et al., 2020).



**Figure 7:** Melanoma (B16-F10) cell cycle is affected by FXM treatment. (A) Cell cycle histograms – FL2/PI. (B) Cell cycle analysis. These results represent the set of four biologically independent experiments.

The anti-proliferative effect induced by mushroom heteropolysaccharides is commonly attributed to such compounds in a large range of concentrations and in different cell lines (Kothari, Patel, & Kim., 2018). Three polysaccharide fractions obtained from *Tricholoma matsutake* presented anti-proliferative activity against the human liver carcinoma HepG2 cell line and human lung carcinoma A549 cell line (You et al., 2013). Such fractions contained glucose, galactose, mannose and fucose, however their activity was noticeable at 2  $\text{mg.mL}^{-1}$ , a concentration 4 times higher than the observed in this study for FXM. Four water-soluble heteropolysaccharides obtained from *Lepista sordida*, composed of glucose, mannose, galactose, rhamnose, and arabinose, presented anti-proliferative effects against human laryngocarcinoma Hep-

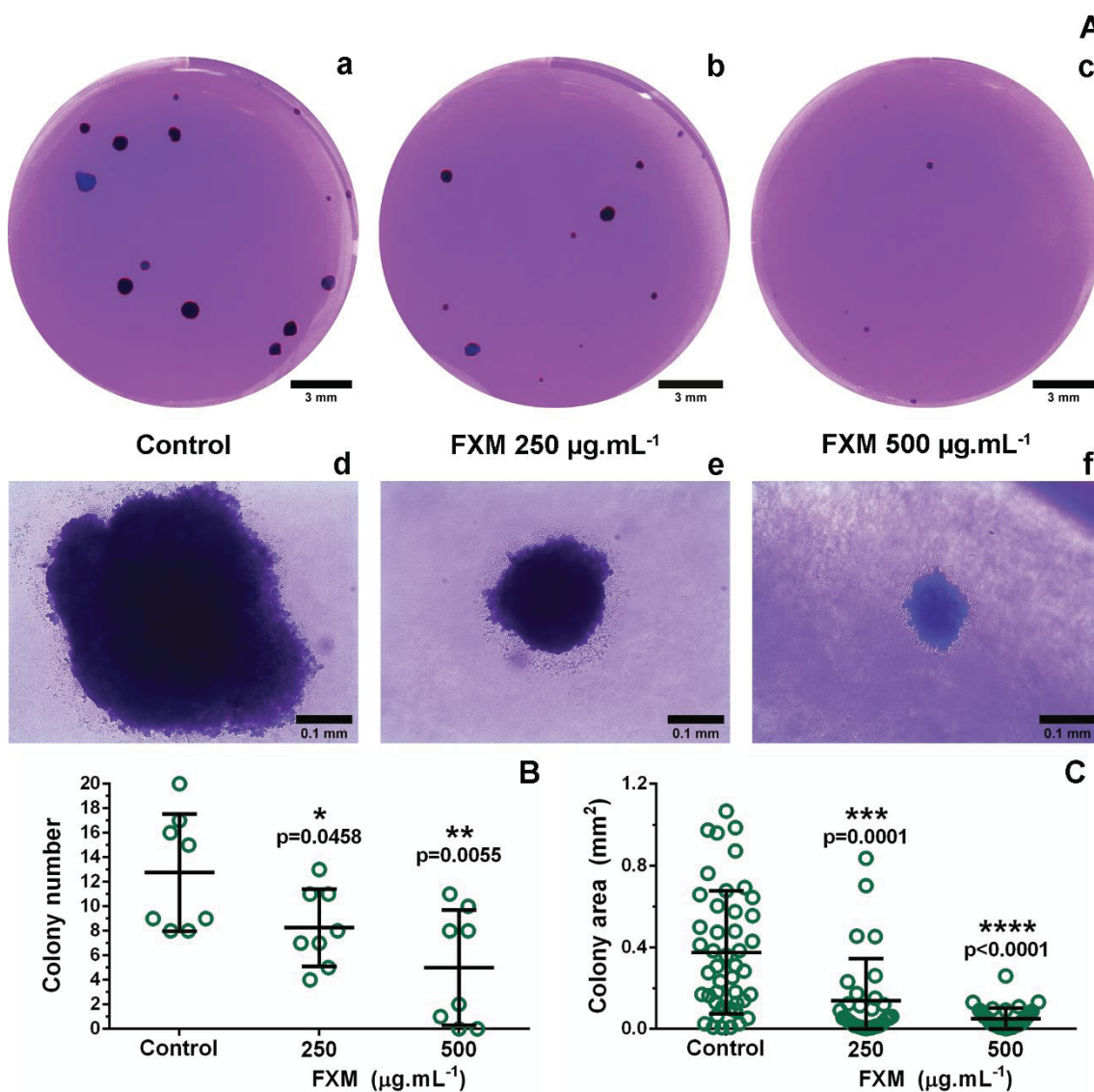


2 cell line (Miao et al., 2013) although in smaller concentrations than FXM (10 to 200  $\mu\text{g.mL}^{-1}$ ). Interestingly, the most pronounced anti-proliferative effect was induced by the polysaccharide with the smallest molecular weight – 57 kDa, which is close to the  $M_w$  of FXM (35 kDa). Heteropolysaccharide fractions from *Boletus edulis*, which contained fucose, glucose, galactose, and mannose, induced similar effects as FXM against human colon adenocarcinoma LS180 cell line, reducing its proliferation, while also generating accumulation in G0/G1 cell cycle phase (Lemieszek et al., 2013). Anti-proliferative effects have been attributed to mushroom extracts containing polysaccharides and triterpenes from *G. lucidum*, called ReishiMax, widely used as dietary supplement and studied – including in clinical trials - for its anticancer activities (Jin, Beguerie, Sze, & Chan, 2016). *In vitro* studies in different cancer cell lines indicate that ReishiMax had an inhibitory effect in the signaling cascade of mTOR kinase – a large signaling pathway associated with diverse cellular mechanisms, such as cell survival, migration, cell growth and proliferation (Sohretoglu, Zhang, Luo, & Huang, 2019; Suarez-Arroyo et al., 2013). It is possible to hypothesize that part of *G. lucidum* extract activities are linked to its polysaccharide portion, and in a similar manner to those extracts, FXM is impairing cellular mechanisms related to cell proliferation.

It is worth noticing that although anti-proliferative effects are widely described for mushroom polysaccharides, FXM selective effects stands out as a differential, as such characteristic is less explored in the literature, not being reported in any of the previously cited studies. As cancer treatment induce adverse effects because of lack of selectivity (between cancer cells and normal cells), finding other treatment options is an urgent issue, and the observed results incited by FXM seems to be interesting initial indicator of this polysaccharide potential. Commonly known adverse effects of cancer treatment such as anemia, nausea, dermatitis, and organ damage are still present in new developed interventions. All treatments tumble upon the difficulty to target cancer cells and differentiate them from normal tissue (Nurgali, Jagoe, & Abalo, 2018; Yu et al., 2019). Although the effects described here for FXM are an *in vitro* setting, hence far from the complexity of *in vivo* models, the selective reduction of melanoma cell proliferation with no harm to normal fibroblast cells points towards an interesting activity of FXM to be further explored *in vivo* using tumor models.

### 3.3 FXM significantly impairs anchorage-independent colony formation capacity

Given the observed FXM activity on melanoma cells proliferation, FXM was analyzed in one of the pivotal points for metastasis formation – the colony formation capacity. The treatment with the polysaccharide extensively affected formation of melanoma colonies in an anchorage-absent microenvironment. When cells were treated with FXM at 250 and 500  $\mu\text{g.mL}^{-1}$ , melanoma cells decreased their clonogenic capacity by 35.3 % and 60.8 % respectively when compared to non-treated cells (Fig. 8A a-f and 8B). FXM treatment also reduced colonies area. When compared to non-treated colonies, the polysaccharide strikingly decreased the colony area by 63.1 % and 86.4 %, at 250 and 500  $\mu\text{g.mL}^{-1}$ , respectively (Fig. 8A a-f and 8C).



**Figure 8:** Anchorage-independent colony formation melanoma (B16-F10) cell capacity is significantly reduced. (A) Colonies imaging – (a and d) Control, (b and e) FXM 250  $\mu\text{g.mL}^{-1}$ , (c and f) FXM 500  $\mu\text{g.mL}^{-1}$ . Images captured from the same biologically independent experiment. Images d, e, and f are representative of each group's colony area mean ( $\text{mm}^2$ ). (B) Colony number. (C) Colony area. The results represent the set of three biologically independent experiments, with at least two technical replicates in each experiment.

Similar to the whole metastatic process, the colonization of new tissues by metastatic cells is an inefficient process, rate-limited by diverse biological barriers and with impactful consequences related to treatment resistance and cancer relapse (Lambert et al., 2016). Released by the primary tumor, growth factors, inflammatory and immune suppressive cytokines and chemokines prepare possible colonization sites, known as the premetastatic niches. Once in the new tissue, metastatic cells release autocrine and paracrine factors to keep modulating the microenvironment, dampening the tissue defenses, increasing the extracellular matrix stiffness and promoting a supportive niche (Massagué & Obenauf, 2016; Pachmayr, Treese, & Stein, 2017). To successfully complete the process and originate a metastasis, cancer cells need to enable sustained proliferative capacity, a hallmark of cancer that is simulated by anchorage independent colony formation assays and can potentially predict *in vivo* tumorigenicity (Franken, Rodermond, Stap, Haveman, & Van Bree, 2006; Menyhart et al., 2016). In this context, FXM reduction of melanoma cells colony formation capacity demonstrates the impairment of an important ability of metastasis formation cells, being a promising activity that can potentially be translated in positive results using *in vivo* models.

#### 4. Conclusions

In the search for new approaches for cancer and specifically metastatic melanoma treatment, the activities observed for FXM – a fucoxylomannan obtained from the worldwide distributed mushroom *G. lucidum* – are of much interest. This molecule has  $M_w$  of  $3.59 \times 10^4 \text{ g.mol}^{-1}$  and an  $\alpha\text{-Manp-(1}\rightarrow\text{4)}$ -linked main chain branched at O-6 position by  $\alpha\text{-L-Fucp-(1}\rightarrow\text{2)-}\beta\text{-D-Xylp}$  fragments. Although the

obtained results are initial observations from *in vitro* models, FXM demonstrated the potential to be selective on its effects, affecting melanoma cells proliferation and even reducing their capacity to form colonies in an anchorage independent assay, hence interfering with a pivotal point in the metastatic process. *In vivo* studies using FXM in metastatic models could reveal a promising drug to selectively affect cancer cells and diminish their metastatic life-threatening capacity.

### Declaration of competing interest

The authors declare to have no competing interests.

### Acknowledgments

This work was supported by the Brazilian funding agencies: Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (166515/2017-5) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) (Funding Code 001). We thank the company Juncao Brazil for donating *G. lucidum* for this study.

### References

- Abreu, H., Zavadinack, M., Smiderle, F. R., Cipriani, T. R., Cordeiro, L. M. C., & Iacomini, M. (2021). Polysaccharides from *Pleurotus eryngii*: Selective extraction methodologies and their modulatory effects on THP-1 macrophages. *Carbohydrate Polymers*, 252, 117177. <https://doi.org/10.1016/j.carbpol.2020.117177>
- Ahmad, M. F. (2020). *Ganoderma lucidum*: A rational pharmacological approach to surmount cancer. *Journal of Ethnopharmacology*, 260, 11304. <https://doi.org/10.1016/j.jep.2020.113047>
- Axelsson, K., Björndal, H., & Lindberg, B. (1969). Structure of the fucoxylomannan from *Polyporus pinicola* (Fr). *Acta chemica scandinavica*, 23, 1579-1600. <https://doi.org/10.3891/acta.chem.scand.23-1597>
- Borenfreund, E., & Puerner, J. a. (1985). A simple quantitative procedure using monolayer cultures for cytotoxicity assays (HTD/NR-90). *Journal of Tissue Culture Methods*, 9(75101), 7–9. <https://doi.org/10.1007/BF01666038>
- Borowicz, S., Van Scoyk, M., Avasarala, S., Karuppusamy Rathinam, M. K., Tauler, J., Bikkavilli, R. K., & Winn, R. A. (2014). The soft agar colony formation assay. *Journal of Visualized Experiments*, 92, 1–6. <https://doi.org/10.3791/51998>
- 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](https://doi.org/10.1016/0003-2697(76)90527-3)

- Cai, M., Xing, H., Tian, B., Xu, J., Li, S., Zhu, H., Yang, K., & Sun, P. (2021). Characteristics and antifatigue activity of graded polysaccharides from *Ganoderma lucidum* separated by cascade membrane technology. *Carbohydrate Polymers*, 269, 11832. <https://doi.org/10.1016/j.carbpol.2021.118329>
- Cordeiro, L. M. C.; Almeida, C. P. de.; & Iacomini, M. (2015). Unusual linear polysaccharides: (1→5)- $\alpha$ -L-Arabinan, (1→3)-(1→4)- $\alpha$ -D-glucan and (1→4)- $\beta$ -D-xylan from pulp of buriti (*Mauritia flexuosa*), an edible palm fruit from the Amazon region. *Food Chemistry*, 173, 141–146. <https://doi.org/10.1016/j.foodchem.2014.10.020>
- 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](https://doi.org/10.1016/0008-6215(84)85242-8)
- Davis, E. J., Johnson, D. B., Sosman, J. A., & Chandra, S. (2018). Melanoma: What do all the mutations mean? *Cancer*, 124(17), 3490–3499. <https://doi.org/10.1002/cncr.31345>
- Domingues, B., Lopes, J., Soares, P., & Populo, H. (2018). Melanoma treatment in review. *ImmunoTargets and Therapy*, Volume 7, 35–49. <https://doi.org/10.2147/itt.s134842>
- Franken, N. a P., Rodermond, H. M., Stap, J., Haveman, J., & van Bree, C. (2006). Clonogenic assay of cells in vitro. *Nature Protocols*, 1(5), 2315–2319. <https://doi.org/10.1038/nprot.2006.339>
- Fraser, R. N., Karacsonyi, S., & Lindberg, B. (1967). Polysaccharides elaborated by *Polyporus pinicola* (Fr). *Acta chemica Scandinavica*, 21, 1783-1789. <https://doi.org/10.3891/acta.chem.scand.21-1783>
- Fu, Y., Shi, L., & Ding, K. (2019). Structure elucidation and anti-tumor activity in vivo of a polysaccharide from spores of *Ganoderma lucidum* (Fr.) Karst. *International Journal of Biological Macromolecules*, 141, 693-699. <https://doi.org/10.1016/j.ijbiomac.2019.09.046>
- Gillies, R. G., Didier, N., & Denton, M. (1986). Determination of cell number in monolayer cultures. *Analytical Biochemistry*, 159, 109–113. [https://doi.org/10.1016/0003-2697\(86\)90314-3](https://doi.org/10.1016/0003-2697(86)90314-3)
- Golovchenko, V. V., Naranmandakh, S., Ganbaatar, J., Prilepskii, A. Y., Burygin, G. L., Chizhov, A. O., & Shashkov, A. S. (2020). Structural investigation and comparative cytotoxic activity of water-soluble polysaccharides from fruit bodies of the medicinal fungus quinine conk. *Phytochemistry*, 175, 112313. <https://doi.org/10.1016/j.phytochem.2020.112313>
- Gorin, P. A. J., & Iacomini, M. (1984). Polysaccharides of the lichens *Cetraria islandica* and *Ramalina usnea*. *Carbohydrate Research*, 128(1), 119–132. [https://doi.org/10.1016/0008-6215\(84\)85090-9](https://doi.org/10.1016/0008-6215(84)85090-9)
- Gorin, P. A. J., & Mazurek, M. (1975). Further Studies on the Assignment of Signals in  $^{13}\text{C}$  Magnetic Resonance Spectra of Aldoses and Derived Methyl Glycosides. *Canadian Journal of Chemistry*, 53(8), 1212-1223. <https://doi.org/10.1139/v75-168>
- Guo, C., Guo, D., Fang, L., Sang, T., Wu, J., Guo, C., Wang, Y., Wang, Y., Chen, C., Chen, J., Chen, R., & Wang, X. (2021). *Ganoderma lucidum* polysaccharide modulates gut microbiota and immune cell function to inhibit inflammation and tumorigenesis in colon. *Carbohydrate Polymers*, 267, 118231. <https://doi.org/10.1016/j.carbpol.2021.118231>
- Hanahan, D., & Weinberg, R. A. (2011). Hallmarks of cancer: the next generation. *Cell*, 144(5), 646–674. <https://doi.org/10.1016/j.cell.2011.02.013>
- Huang, S., Mao, J., Ding, K., Zhou, Y., Zeng, X., Yang, W., Wang, P., Zhao, C., Yao, J., Xia, P., & Pei, G. (2017). Polysaccharides from *Ganoderma lucidum* Promote Cognitive Function and Neural Progenitor Proliferation in Mouse Model of Alzheimer's Disease. *Stem Cell Reports*, 8, 84-94. <https://doi.org/10.1016/j.stemcr.2016.12.007>
- Human ( $\alpha 2 \rightarrow 6$ ) and Avian ( $\alpha 2 \rightarrow 3$ ) Sialylated Receptors of Influenza A Virus Show Distinct Conformations and Dynamics in Solution. Guilherme L. Sasaki, Stefano Elli, Timothy R. Rudd,



- Eleonora Macchi, Edwin A. Yates, Annamaria Naggi, Zachary Shriver, Rahul Raman, R. Sasisekharan, Giangiacomo Torri, Marco Guerrini. *Biochemistry* 52 (41), 7217-7230, 2013.  
<https://doi.org/10.1021/bi400677n>
- Jin, X., Beguerie, J. R., Sze, D. M., & Chan, G. C. (2016). *Ganoderma lucidum* (Reishi mushroom) for cancer treatment. *Cochrane Database of Systematic Reviews*, 1, 1–12.  
<https://doi.org/10.1002/14651858.CD007731.pub3>
- Jin, M., Zhu, Y., Shao, D., Zhao, K., Xu, C., Li, Q., Yanga, H., Huang, Q., & Shi, J. (2017). Effects of polysaccharide from mycelia of *Ganoderma lucidum* on intestinal barrier functions of rats. *International Journal of Biological Macromolecules*, 94, 1–9. <https://doi.org/10.1016/j.ijbiomac.2016.09.099>
- Jones, J. K. N., & Stoodley, R. J. Fractionation using copper complexes. *Methods in Carbohydrate Chemistry*, v. 5, pp. 36-38, 1965.
- Khan, T., Date, A., Chawda, H., & Patel, K. (2019). Polysaccharides as potential anticancer agents—A review of their progress. *Carbohydrate Polymers*, 210(January), 412–428.  
<https://doi.org/10.1016/j.carbpol.2019.01.064>
- Kothari, D., Patel, S., & Kim, S. K. (2018). Anticancer and other therapeutic relevance of mushroom polysaccharides: A holistic appraisal. *Biomedicine and Pharmacotherapy*, 105(May), 377–394.  
<https://doi.org/10.1016/j.biopha.2018.05.138>
- Kroschinsky, F., Stölzel, F., von Bonin, S., Beutel, G., Kochanek, M., Kiehl, M., & Schellongowski, P. (2017). New drugs, new toxicities: Severe side effects of modern targeted and immunotherapy of cancer and their management. *Critical Care*, 21(1), 1–11. <https://doi.org/10.1186/s13054-017-1678-1>
- Lambert, A. W., Pattabiraman, D. R., & Weinberg, R. A. (2016). Emerging Biological Principles of Metastasis. *Cell*, 168(4), 670–691. <https://doi.org/10.1016/j.cell.2016.11.037>
- Lemieszek, M. K., Cardoso, C., Nunes, F. H. F. M., Barros, A. I. R. N. A., Marques, G., Pożarowski, P., & Rzeski, W. (2013). *Boletus edulis* biologically active biopolymers induce cell cycle arrest in human colon adenocarcinoma cells. *Food and Function*, 4(4), 575–585. <https://doi.org/10.1039/c2fo30324h>
- Liang, C., Tian, D., Liu, Y., Li, H., Zhu, J., Li, M., Xin, M., & Xia, J. (2019) Review of the molecular mechanisms of *Ganoderma lucidum* triterpenoids: Ganoderic acids A, C2, D, F, DM, X and Y. *European Journal of Medicinal Chemistry*, 174, 130-141. <https://doi.org/10.1016/j.ejmech.2019.04.039>
- Liu, Y., Tang, Q., Zhang, J., Xia, Y., Yang, Y., Wu, D., Fan, H., & Cu, S. W. (2018). Triple helix conformation of  $\beta$ -D-glucan from *Ganoderma lucidum* and effect of molecular weight on its immunostimulatory activity. *International Journal of Biological Macromolecules*, 114, 1064–1070.  
<https://doi.org/10.1016/j.ijbiomac.2018.03.054>
- Liu, H., Amakye, W. K., & Ren, J. (2021). *Codonopsis pilosula* polysaccharide in synergy with dacarbazine inhibits mouse melanoma by repolarizing M2-like tumor-associated macrophages into M1-like tumor-associated macrophages. *Biomedicine & Pharmacotherapy*, 142, 112016.  
<https://doi.org/10.1016/j.biopha.2021.112016>
- Lu, J., He, R., Sun, P., Zhang, F., Linhardt, R. J., & Zhang, A. (2020). Molecular mechanisms of bioactive polysaccharides from *Ganoderma lucidum* (Lingzhi), a review. *International Journal of Biological Macromolecules*, 150, 765–774. <https://doi.org/10.1016/j.ijbiomac.2020.02.035>
- Massagué, J., & Obenauf, A. C. (2016). Metastatic Colonization. *Nature*, 529(7586), 298–306.  
<https://doi.org/10.1038/nature17038>
- Menyhárt, O., Harami-Papp, H., Sukumar, S., Schäfer, R., Magnani, L., de Barrios, O., & Györfy, B. (2016). Guidelines for the selection of functional assays to evaluate the hallmarks of cancer. *Biochimica et Biophysica Acta - Reviews on Cancer*, 1866(2), 300–319.  
<https://doi.org/10.1016/j.bbcan.2016.10.002>

- Miao, S., Mao, X., Pei, R., Miao, S., Xiang, C., Lv, Y., Yang, X., Sun, J., Jia, S., & Liu, Y. (2013). Antitumor activity of polysaccharides from *Lepista sordida* against laryngocarcinoma in vitro and in vivo. *International Journal of Biological Macromolecules*, 60, 235–240. <https://doi.org/10.1016/j.ijbiomac.2013.05.033>
- Milhorini, S. da. S.; Smiderle, F. R.; Biscaia, S. M. P.; Rosado, F. R.; Trindade, E. S.; & Iacomini, M. (2018). Fucogalactan from the giant mushroom *Macrocybe titans* inhibits melanoma cells migration. *Carbohydrate Polymers*, 190, 50–56. <https://doi.org/10.1016/j.carbpol.2018.02.063>
- Miyakazi, T., & Nishijima, M. (1982). Structural examination of an alkali-extracted, water-soluble heteroglycan of the fungus *Ganoderma lucidum*. *Carbohydrate Research*, 109, 290–294. [https://doi.org/10.1016/0008-6215\(82\)84047-0](https://doi.org/10.1016/0008-6215(82)84047-0)
- Money, N. P. (2016). Are mushroom medicinal? *Fungal biology*, v. 120, 449–453. <https://doi.org/10.1016/j.funbio.2016.01.006>
- Nurgali, K., Jagoe, R. T., & Abalo, R. (2018). Editorial: Adverse effects of cancer chemotherapy: Anything new to improve tolerance and reduce sequelae? *Frontiers in Pharmacology*, 9(MAR), 1–3. <https://doi.org/10.3389/fphar.2018.00245>
- Ogawa, K., Tsurugi J., & Watanabe, T. (1972). Complex of gel-forming  $\beta$ -1,3-D- glucan with congo red in alkaline solution. *Chemistry Letters*, 689–692. <https://doi.org/10.1246/cl.1972.689>
- Oliveira, G. K. F., Silva, E. V. da, Ruthes, A. C., Lião, L. M., Iacomini, M., & Carbonero, E. R. (2018). Chemical structure of a partially 3-O-methylated mannofucogalactan from edible mushroom *Grifola frondosa*. *Carbohydrate Polymers*, 187, 110–117. <https://doi.org/10.1016/j.carbpol.2018.01.080>
- Pachmayr, E., Treese, C., & Stein, U. (2017). Underlying Mechanisms for Distant Metastasis - Molecular Biology. *Visceral Medicine*, 33(1), 11–20. <https://doi.org/10.1159/000454696>
- Perlin, A. S., & Casu, B. (1969). Carbon-13 and proton magnetic resonance spectra of D-glucose-13C. *Tetrahedron Letters*, 34, 2919–2924. [http://dx.doi.org/10.1016/S0040-4039\(01\)88308-8](http://dx.doi.org/10.1016/S0040-4039(01)88308-8)
- Ruthes, Andrea 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(1), 761–769. <https://doi.org/10.1016/j.carbpol.2013.06.061>
- Ruthes, Andrea C., Rattmann, Y. D., Malquevicz-Paiva, S. M., Carbonero, E. R., Córdova, M. M., Baggio, C. H., Santos, A. R. S., Gorin, P. A. J., & Iacomini, M. (2013). *Agaricus bisporus* fucogalactan: Structural characterization and pharmacological approaches. *Carbohydrate Polymers*, 92(1), 184–191. <https://doi.org/10.1016/j.carbpol.2012.08.071>
- Ruthes, A. C., Rattmann, Y. D., Carbonero, E. R., Gorin, P. A. J., & Iacomini, M. (2012). Structural characterization and protective effect against murine sepsis of fucogalactans from *Agaricus bisporus* and *Lactarius rufus*. *Carbohydrate Polymers*, 87, 1620–1627. <http://dx.doi.org/10.1016/j.carbpol.2011.09.071>
- 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>
- 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., Elli, S., Rudd, T. R., Macchi, E., Yates, E. A., Naggi, A., Shriver, Z., Raman, R., Sasisekharan, R., Torri, G., Guerrin, M. (2013). Human ( $\alpha 2 \rightarrow 6$ ) and avian ( $\alpha 2 \rightarrow 3$ ) sialylated receptors



of influenza A virus show distinct conformations and dynamic in solution. *Biochemistry*, 41, 7217–7230. <https://doi.org/10.1021/bi400677n>

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J. Y., White, D. J., Hartenstein, V., Eliceiri, K., Tomancak, P., & Cardona, A. (2012). Fiji: An open-source platform for biological-image analysis. *Nature Methods*, 9(7), 676–682. <https://doi.org/10.1038/nmeth.2019>

Seidel, J. A., Otsuka, A., & Kabashima, K. (2018). Anti-PD-1 and anti-CTLA-4 therapies in cancer: Mechanisms of action, efficacy, and limitations. *Frontiers in Oncology*, 8(MAR), 1–14. <https://doi.org/10.3389/fonc.2018.00086>

Singleton, V. L., Orthofer, R., & Lamuela-Raventos, R. M. (1999). Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. *Methods Enzymol*, 299, 152–178. [https://doi.org/10.1016/S0076-6879\(99\)99017-1](https://doi.org/10.1016/S0076-6879(99)99017-1)

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. (2012). Exopolysaccharides, proteins and lipids in *Pleurotus pulmonarius* submerged culture using different carbon sources. *Carbohydrate Polymers*, 87, 368–376. <https://doi.org/10.1016/j.carbpol.2011.07.063>

Smiderle, F. R.; Sassaki, G. L.; Van Griensven, L. J. L. D.; & Iacomini, M. (2013) Isolation and chemical characterization of a glucogalactomannan of the medicinal mushroom *Cordyceps militaris*. *Carbohydrate Polymers*, 97, 74– 80. <https://doi.org/10.1016/j.carbpol.2013.04.049>

Sohretoglu, D., Zhang, C., Luo, J., & Huang, S. (2019). Reishimax inhibits mtorc1/2 by activating AMPK and inhibiting IGFR/PI3K/RHEB in tumor cells. *Signal Transduction and Targeted Therapy*, 4(1), 1–8. <https://doi.org/10.1038/s41392-019-0056-7>

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

Suarez-Arroyo, I. J., Rosario-Acevedo, R., Aguilar-Perez, A., Clemente, P. L., Cubano, L. A., Serrano, J., Schneider, R. J., & Martínez-Montemayor, M. M. (2013). Anti-Tumor Effects of *Ganoderma lucidum* (Reishi) in Inflammatory Breast Cancer in In Vivo and In Vitro Models. *PLoS ONE*, 8(2). <https://doi.org/10.1371/journal.pone.0057431>

Sung, H., Ferlay, J., Siegel, R. L., Laversanne, M., Soerjomataram, I., Jemal, A., & Bray, F. (2021). Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA: A Cancer Journal for Clinicians*, 0(0), 1–41. <https://doi.org/10.3322/caac.21660>

Wang, J., Wang, Y., Yang, X., Lin, P., Liu, N., Li, X., Zhang, B., & Guo, S. (2021). Purification, structural characterization, and PCSK9 secretion inhibitory effect of the novel alkali-extracted polysaccharide from *Cordyceps militaris*. *International Journal of Biological Macromolecules*, 179, 407–417. <https://doi.org/10.1016/j.ijbiomac.2021.02.191>

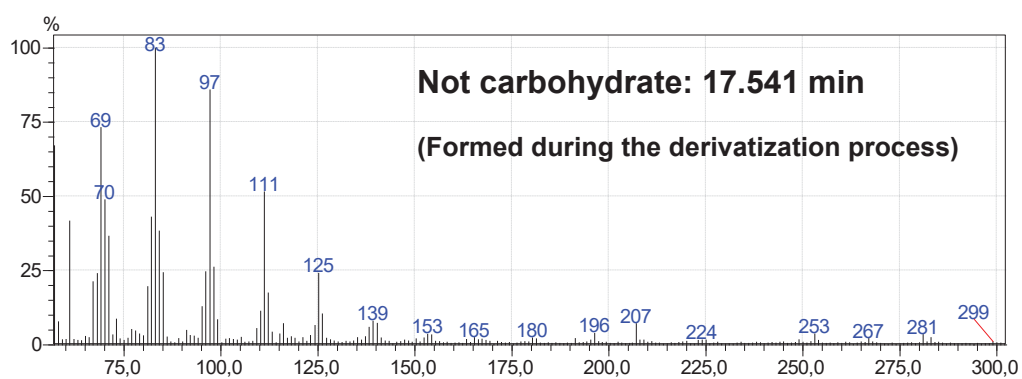
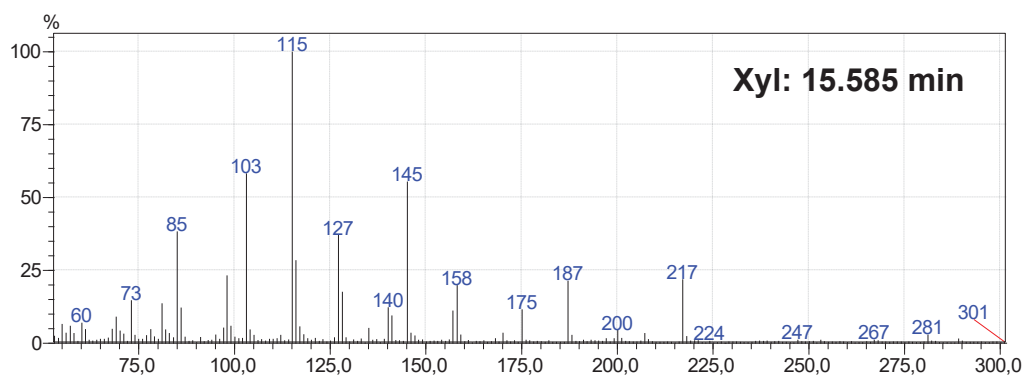
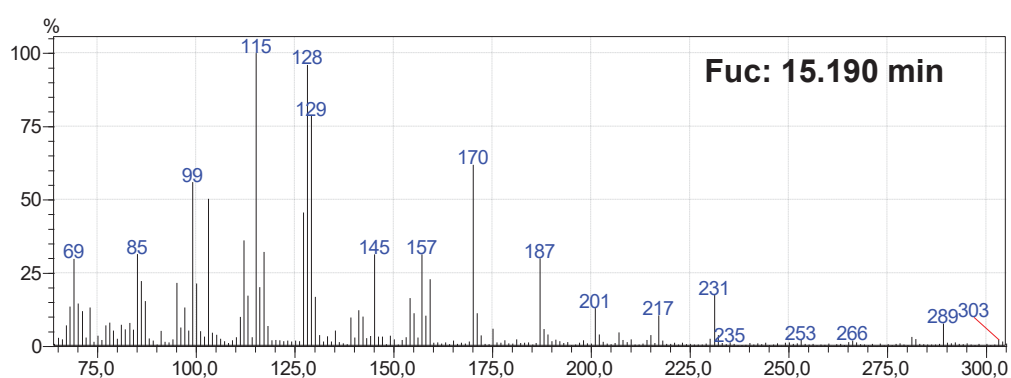
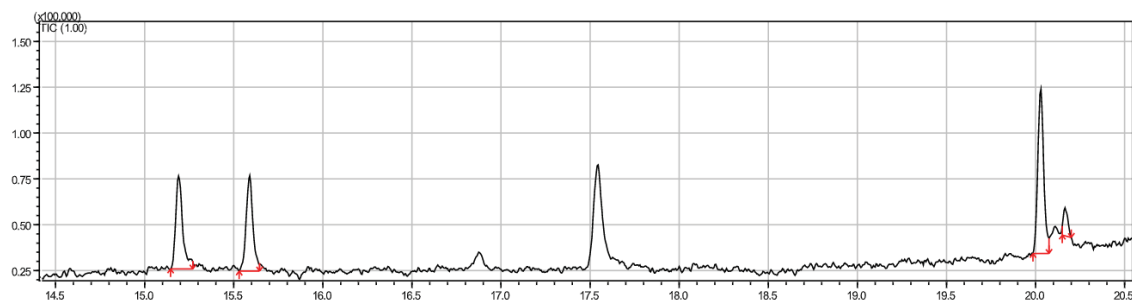
Wang, Z. B., Pei, J. J., Ma, H. L., Cai, P. F., & Yan, J. K. (2014). Effect of extraction media on preliminary characterizations and antioxidant activities of *Phellinus linteus* polysaccharides. *Carbohydrate polymers*, 109, 49–55. <https://doi.org/10.1016/j.carbpol.2014.03.057>

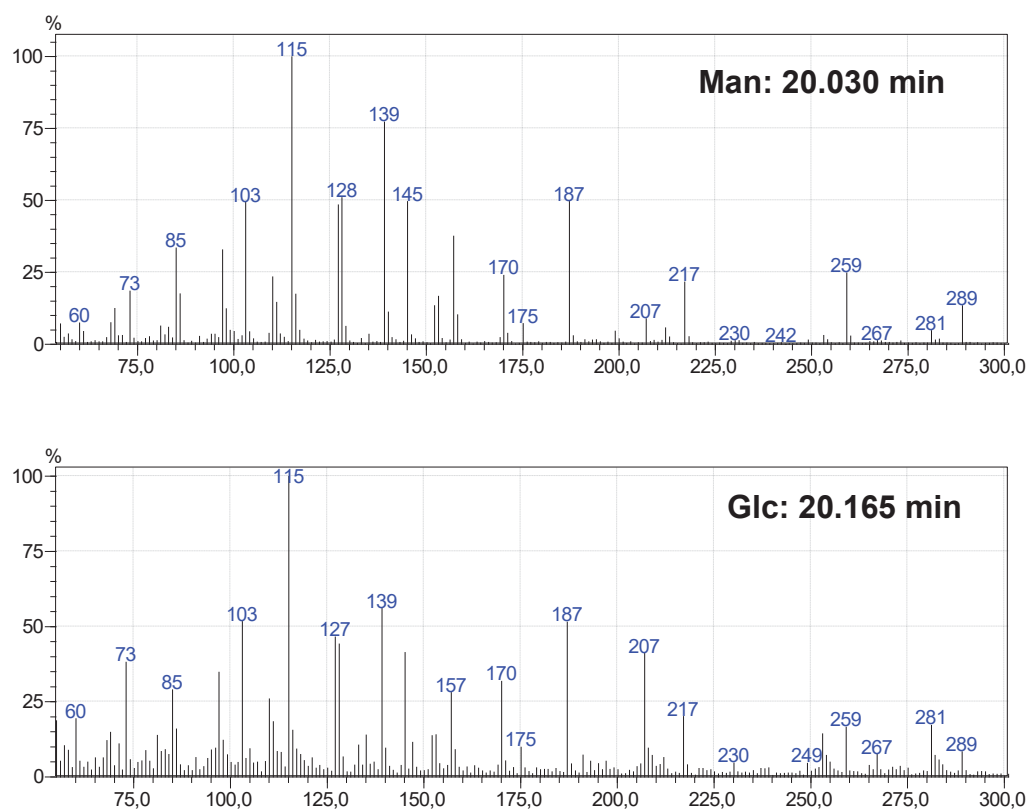
Ward, W. H., & Farma, J. M. (2017). *Cutaneous Melanoma: Etiology and Therapy* (W. H. Ward & J. M. Farma (eds.); Vol. 6, Issue 12). Codon Publications. <https://doi.org/10.15586/codon.cutaneousmelanoma.2017>

Welch, D. R., & Hurst, D. R. (2019). Defining the Hallmarks of Metastasis. *Cancer Research*, 79(12), 3011–3027. <https://doi.org/10.1158/0008-5472.CAN-19-0458>

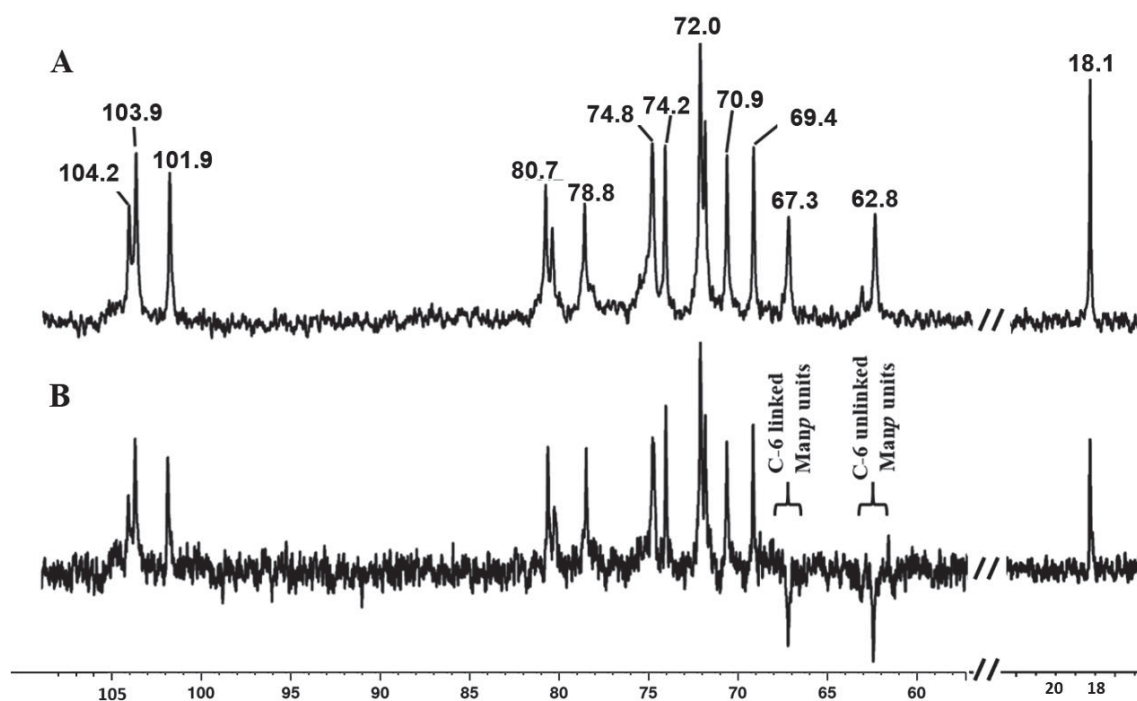
- Wolfson, M. L & Thompson, A (1963a). Reduction with sodium borohydride. In: Whistler, R. L.; Wolfson, M. L. (Eds.). *Methods in Carbohydrate Chemistry* (p. 65-68). New York: Academic Press.
- Wolfson, M. L & Thompson, A (1963b). Acetylation. In: Whistler, R. L.; Wolfson, M. L. (Eds.). *Methods in Carbohydrate Chemistry* (p. 211- 215). New York: Academic Press.
- Xu, S., Dou, Y., Ye, B., Wu, Q., Wang, Y., Hu, M., Ma, F., Rong, X., & Guo, J. (2017). *Ganoderma lucidum* polysaccharides improve insulin sensitivity by regulating inflammatory cytokines and gut microbiota composition in mice. *Journal of Functional Foods*, 38, 545-552. <https://doi.org/10.1016/j.jff.2017.09.032>
- Xu, Y., Zhang, X., Yan, X. H., Zhang, J. L., Wang, L. Y., Xue, H., Jiang, G. C., Ma, X. T., & Liu, X. J. (2019). Characterization, hypolipidemic and antioxidant activities of degraded polysaccharides from *Ganoderma lucidum*. *International Journal of Biological Macromolecules*, 135, 706–716. <https://doi.org/10.1016/j.ijbiomac.2019.05.166>
- Xiao, C., Wu, Q., Zhang, J., Xie, Y., Cai, W., & Tan, J. (2017). Antidiabetic activity of *Ganoderma lucidum* polysaccharides F31 down-regulated hepatic glucose regulatory enzymes in diabetic mice. *Journal of Ethnopharmacology*, 196, 47–57. <https://doi.org/10.1016/j.jep.2016.11.044>
- You, L., Gao, Q., Feng, M., Yang, B., Ren, J., Gu, L., Cui, C., & Zhao, M. (2013). Structural characterisation of polysaccharides from *Tricholoma matsutake* and their antioxidant and antitumour activities. *Food Chemistry*, 138(4), 2242–2249. <https://doi.org/10.1016/j.foodchem.2012.11.140>
- Yu, J. B., Jairam, V., Lee, V., Park, H. S., Thomas, C. R., Melnick, E. R., Gross, C. P., Presley, C. J., & Adelson, K. B. (2019). Treatment-Related Complications of Systemic Therapy and Radiotherapy. *JAMA Oncology*, 5(7), 1028–1035. <https://doi.org/10.1001/jamaoncol.2019.0086>
- Zavadinack, M., Bellan, D. L., Bertage, J. L. R., Milhorini, S. S., Trindade, E. S., Simas, F., Sassaki, G. L., Cordeiro, L. M. C., & Iacomini, M. (2021). An  $\alpha$ -D-galactan and a  $\beta$ -D-glucan from the mushroom *Amanita muscaria*: Structural characterization and antitumor activity against melanoma. *Carbohydrate Polymers*, 274, 118647. <https://doi.org/10.1016/j.carbpol.2021.118647>
- Zhang, Z., Zhou, L., Xie, N., Nice, E. C., Zhang, T., Cui, Y., & Huang, C. (2020). Overcoming cancer therapeutic bottleneck by drug repurposing. *Signal Transduction and Targeted Therapy*, 5(1). <https://doi.org/10.1038/s41392-020-00213-8>
- Zheng, S., Jia, J., Zhao, J., Wei, Q., & Liu, Y. (2012). *Ganoderma lucidum* polysaccharides eradicates the blocking effect of fibrinogen on NK cytotoxicity against melanoma cells. *Oncology Letters*, 3, 613-616. <https://doi.org/10.3892/ol.2011.515>

## Supplementary material

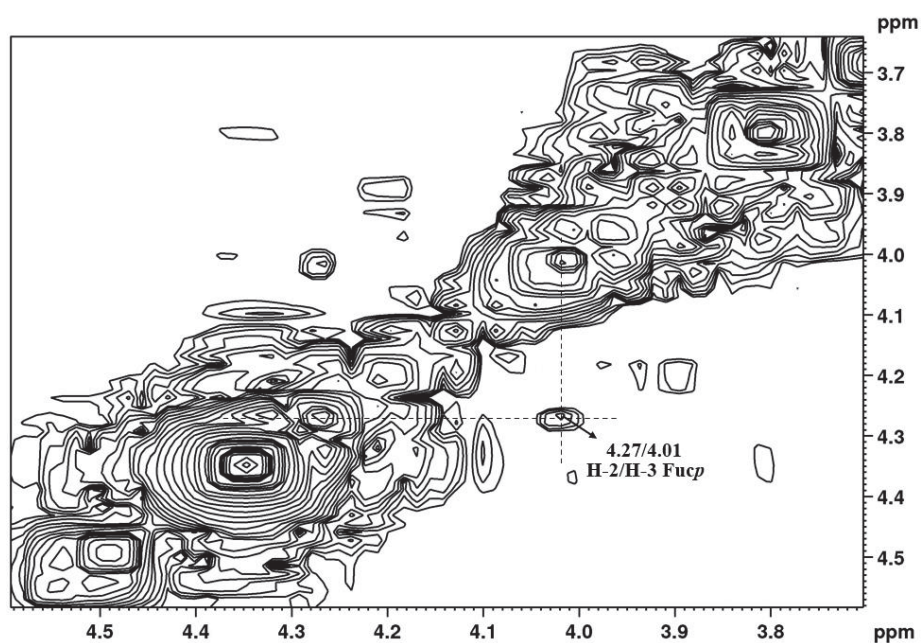




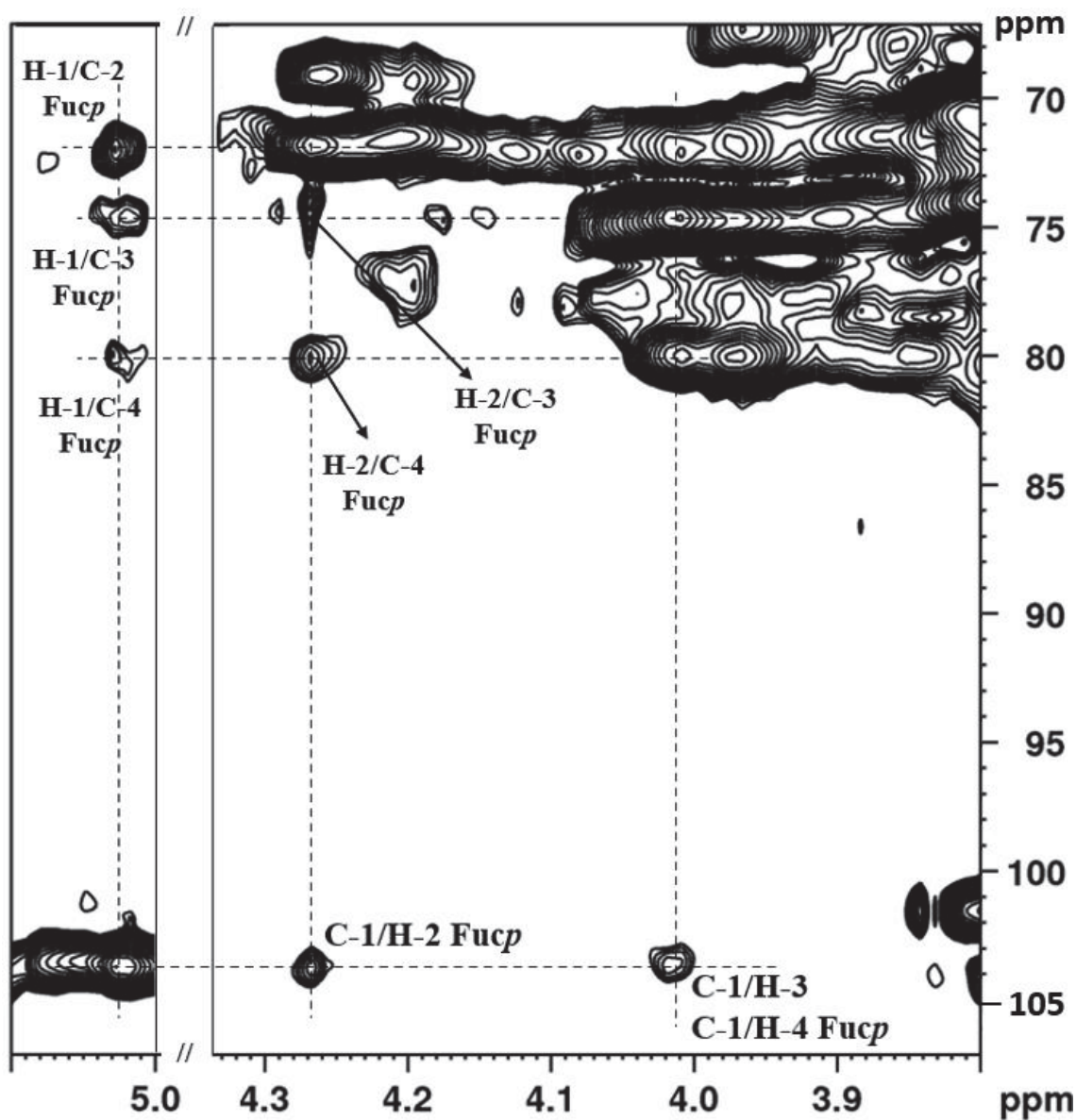
**Figure S1:** Monosaccharide chromatogram of the FXM fraction and electron impact profile of the acetate alditol derivatives obtained after hydrolysis, reduction and acetylation.



**Figure S2:** NMR analyses of the FXM. (A)  $^{13}\text{C}$  and (B) DEPT135. The sample was analyzed in  $\text{D}_2\text{O}$  at 70 °C and the results are expressed in ppm.

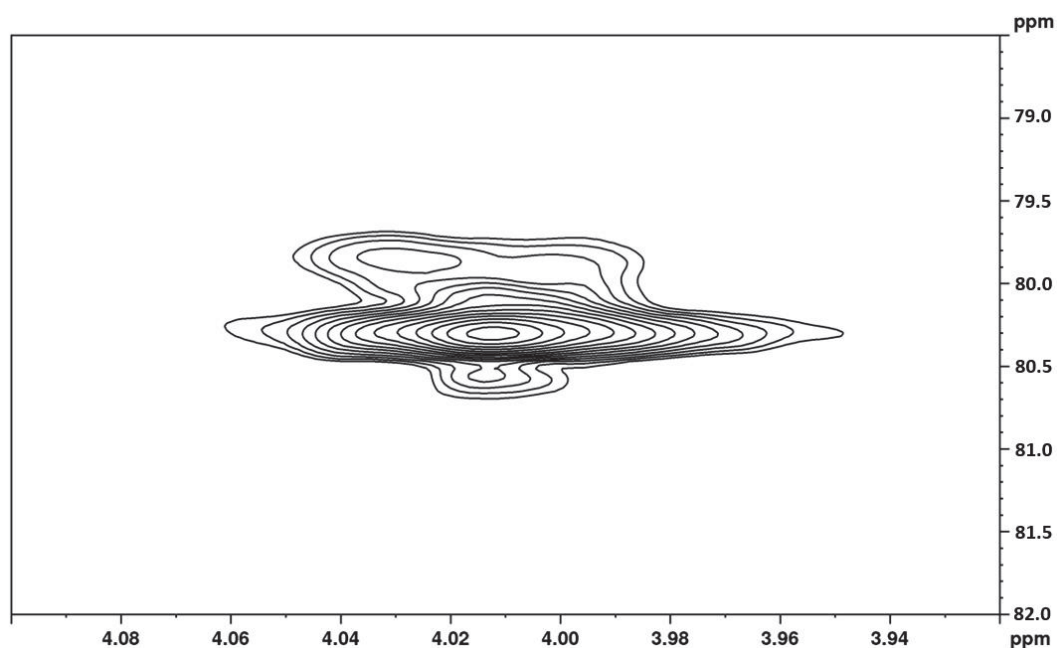


**Figure S3:** COSY spectrum of the FXM polysaccharide. The sample was analyzed in  $\text{D}_2\text{O}$  at 70 °C and the results are expressed in ppm.

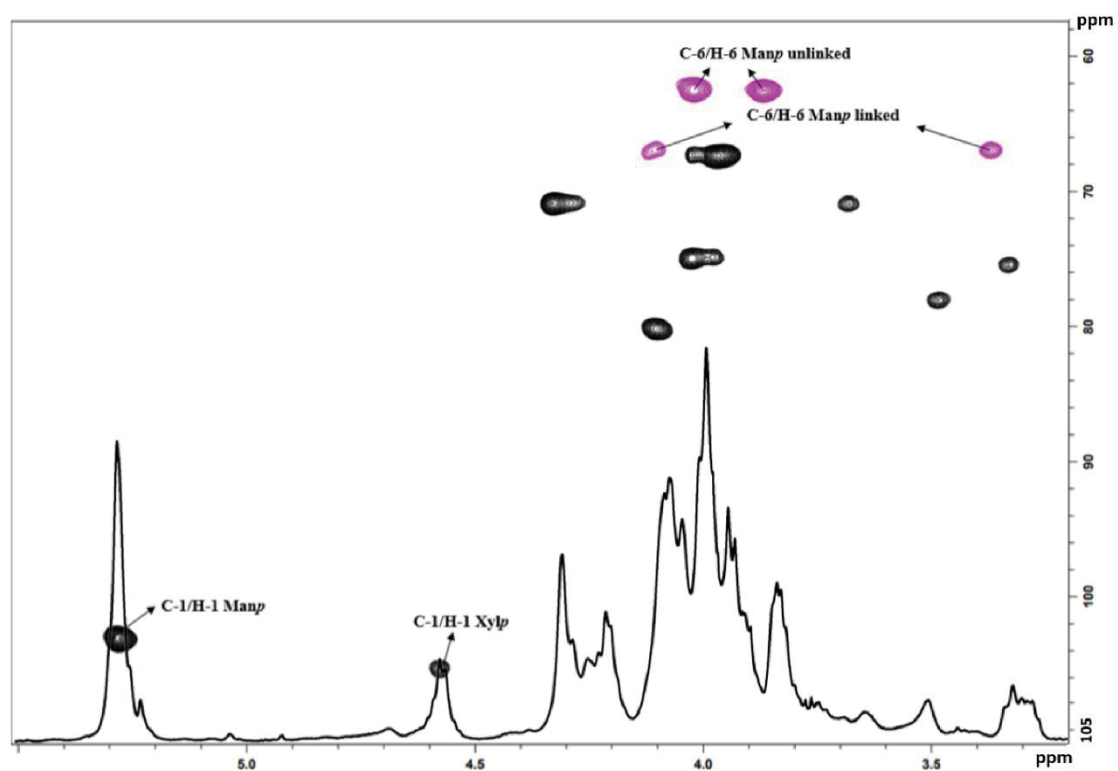


**Figure S4:** HSQC-TOCSY spectrum of the FXM polysaccharide. The sample was analyzed in  $\text{D}_2\text{O}$  at 70 °C and the results are expressed in ppm.



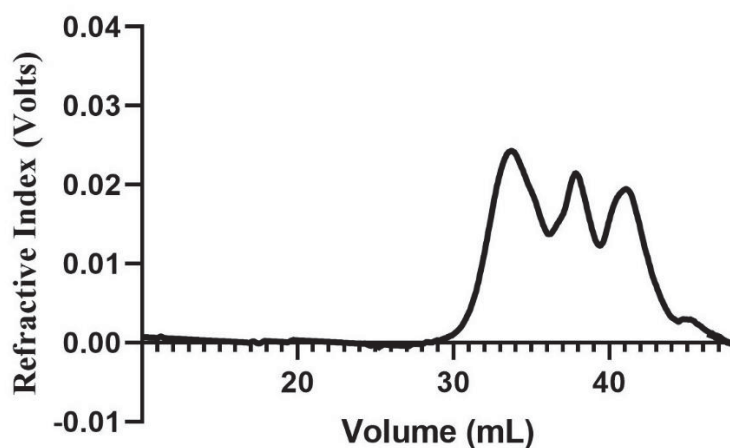


**Figure S5:**  $^1\text{H}$ - $^{13}\text{C}$  HSQC NMR spectrum of the FXM polysaccharide. The  $^{13}\text{C}$  resolution was improved through a SW of 80 ppm and a TD of 512 on indirect dimension. The sample was analyzed in  $\text{D}_2\text{O}$  at 70 °C and the results are expressed in ppm.

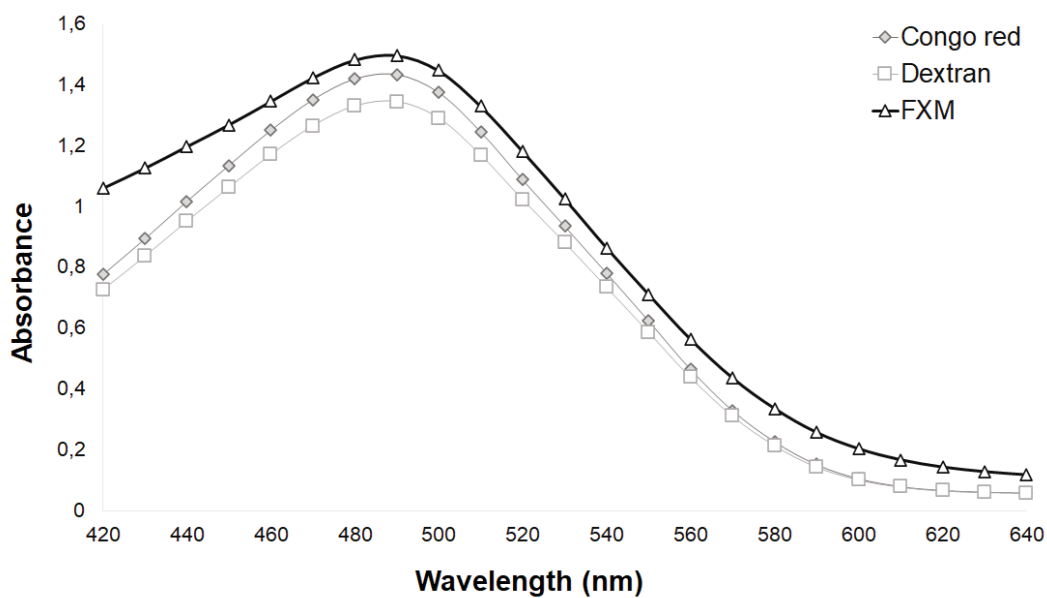




**Figure S6:** HSQC-DEPT and  $^1\text{H}$  spectra analysis from FXM-H fraction after partial acid hydrolysis. The sample was analyzed in  $\text{D}_2\text{O}$  at 70 °C in a Bruker Avance III 600 MHz (chemical shifts are expressed in  $\delta$  ppm).



**Figure S7:** Elution profile of FXM-H in HPSEC coupled to a refractive index detector.



**Figure S8:** Absorption spectra of FXM, Congo red (control), and Dextran (random coil control).

**ARTIGO V**

**Branched  $\beta$ -glucans with different Mw obtained from *Ganoderma lucidum* fruiting bodies**

**Branched  $\beta$ -glucans with different Mw obtained from *Ganoderma lucidum*  
fruiting bodies**

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**Abstract**

*Ganoderma lucidum* have been used in the traditional medicine for over millennia due to its biological properties. It is known that one of the responsible for these interesting medicinal effects are the polysaccharides. Due to this, the obtention and chemical investigation of such molecules are of pharmaceutical interest. Based on this, two fractions containing  $\beta$ -glucans (R12 and E12) were obtained from *G. lucidum* fruiting bodies, through alkaline extraction, freeze-thawing process, treatment with Fehling solution, and dialysis. Both samples have glucose as their main monosaccharide constituent. NMR analysis suggested that these fractions are composed of a  $\beta$ -glucan with (1 $\rightarrow$ 3), (1 $\rightarrow$ 4), and (1 $\rightarrow$ 6) linkage types. Both polysaccharides present a random-coil conformation. Additionally, both fractions showed homogeneous profiles in HPSEC, and have different Mw, presenting  $1.3 \times 10^4$  and  $5 \times 10^3$  g/mol, respectively. These molecules can be further applied in biological studies aiming to investigate how their chemical differences may affect their biological properties.

**Keywords:** *Ganoderma lucidum*;  $\beta$ -glucans; Extraction; Purification; Characterization.

## 1 Introduction

*Ganoderma lucidum* is one of the most studied mushroom species worldwide (Ferreira et al., 2015). This fungus has been used for millennia in traditional medicine aiming longevity improvement (Ahmad, 2020). Several studies supported that *G. lucidum* can prevent and treat different types of cancers (Liang et al., 2019; Ahmad, 2020). Additionally, this mushroom also showed antidiabetic (Ma, Hsieh & Chen, 2015), immunomodulatory (Liu et al., 2018), and anti-inflammatory (Guo et al., 2021) effects. Polysaccharides are known as one of the main contributors to these biological properties (Ferreira et al., 2015; Wan et al., 2022).

One of the most abundant polysaccharides in mushrooms are the  $\beta$ -glucans, which can have different chemical structures (Mironczuk-Chodakowska, Kujawowicz & Witkowska, 2021). The most common type of  $\beta$ -glucans has a (1 $\rightarrow$ 3)-linked main chain frequently branched at O-6 by  $\beta$ -D-Glcp residues or by (1 $\rightarrow$ 6)-linked- $\beta$ -D-Glcp side chains (Ruthes, Smiderle & Iacomini, 2015). This kind of polysaccharide has been obtained from several mushroom species, including *G. lucidum* (Liu et al., 2014; Zhang et al., 2018; Li et al., 2020). However, more unusual  $\beta$ -glucans with a (1 $\rightarrow$ 3) main chain were found in different mushrooms, such as one obtained from *Calocybe indica*, which was branched at O-4 position by  $\beta$ -D-Glcp residues (Mandal et al., 2010). Such unusual polysaccharide was also found in *G. lucidum* mycelium and showed branches at O-6 by (1 $\rightarrow$ 6)-linked- $\beta$ -D-Glcp side chains or at O-2 by (1 $\rightarrow$ 4)-linked- $\beta$ -D-Glcp side chains (Sone et al., 1985).

Nonetheless,  $\beta$ -glucans with another type of main chain, such as (1 $\rightarrow$ 6)-linked- $\beta$ -D-Glcp, were observed in some other mushroom species. For instance, this polysaccharide was branched at O-3 by  $\beta$ -D-Glcp residues and was obtained from *Albatrellus ovinus* (Samuelsen et al., 2019). On the other hand, structures branched at O-4 by  $\beta$ -D-Glcp residues or by (1 $\rightarrow$ 4)-linked- $\beta$ -D-Glcp side chains were obtained from *Calocybe gambosa* (Villares, 2013), *Calocybe indica* (Mandal et al., 2010), and *G. lucidum* (Dong et al., 2012).

$\beta$ -Glucans with different structures are reported in the literature due to a variety of biological activities, such as immunomodulatory (Wang et al., 2017; Zhang et al., 2021), antitumor (Sone et al., 1985; Zavadinack et al., 2021), antinociceptive (Moreno et al., 2016), and antioxidant effects (Morales et al., 2020). It is known that differences in some physical-chemical features of the polysaccharide could result in a different biological effect. For instance, Liu et al., (2018) fractionated a  $\beta$ -glucan from *G. lucidum*

into five fractions with different molecular weights and observed that those with higher molecular weight exhibited better activity on enhancing the release of inflammatory cytokines by THP-1 macrophages. Based on this fact, it is important to elucidate the chemical structure before investigating the biological properties of such polymers. According to Ferrari et al., (2015), the chemical features of a molecule could give clues to the chemical aspects involved in its respective bioactivities.

Therefore, this study aimed to obtain different  $\beta$ -glucans fractions from *G. lucidum* and elucidate their chemical structure. The molecules and data obtained in this investigation could be further applied in analyses of structure–biological properties relationships.

## 2 Material and methods

### 2.1 Biological material

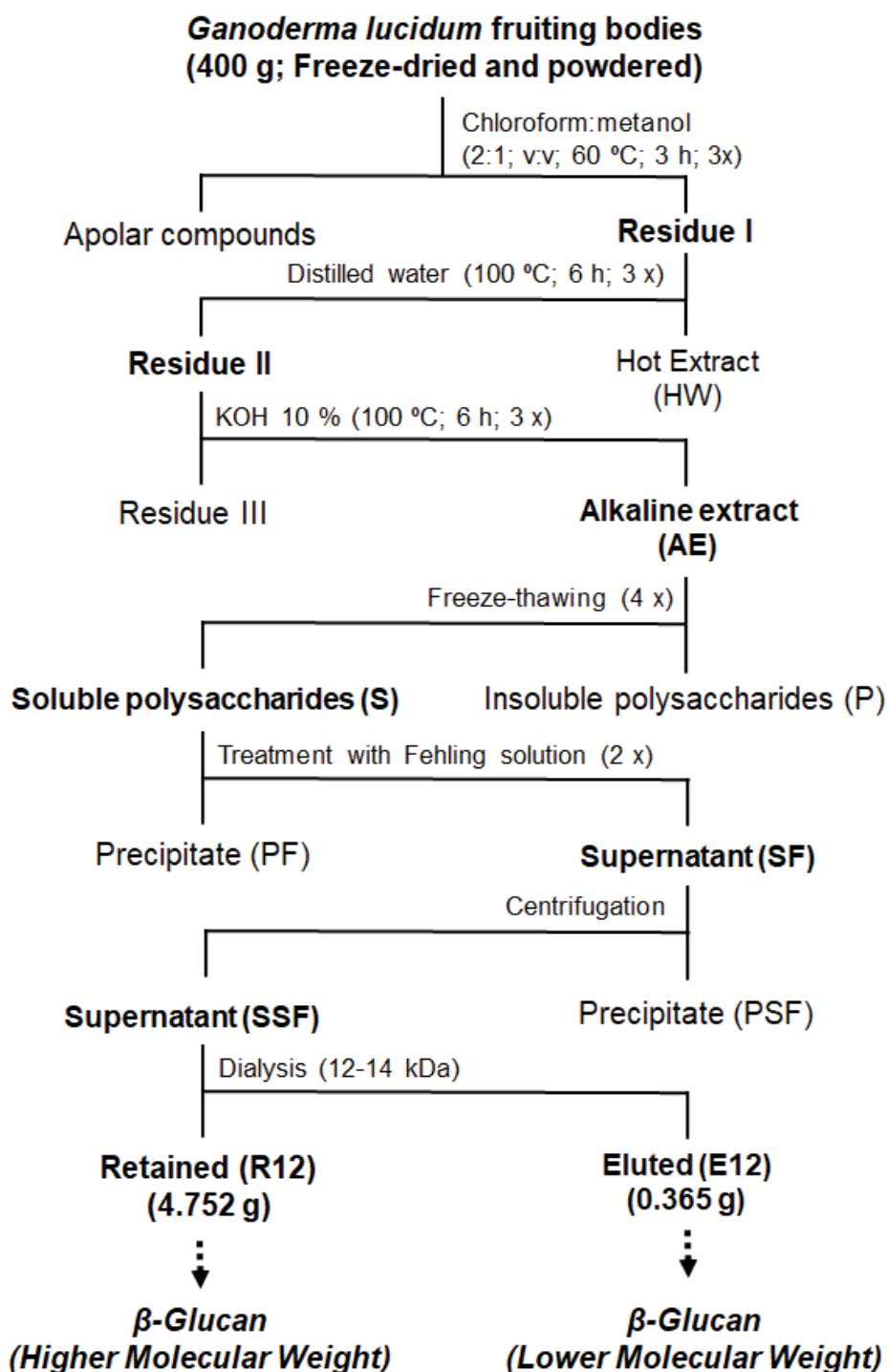
The mushroom *Ganoderma lucidum* was donated by the company Juncao Brazil, from Taboão da Serra, State of São Paulo, Brazil. The species was registered on Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado (SISGEN), with the number A3BA3B3.

### 2.2 Extraction and purification process

The extraction and purification procedures applied to the obtention of *G. lucidum* polysaccharides were represented in figure 1. Briefly, the milled fruiting bodies were delipidified with chloroform and methanol (2:1; v:v; 60 °C; 3 h; 3 x) using a Soxhlet apparatus. The resulting defatted residue (Residue I) was extracted with distilled water and with KOH 10 % in the presence of NaBH<sub>4</sub>, subsequently. Both extractions were carried out under reflux (at 100 °C; 6 h; 3 x). The alkaline extract (AE) was used in this study. For this, AE was recovered by filtration with nylon filter cloth, neutralized with acetic acid, and dialyzed against tap water (3,5 kDa; 72 h). Afterward, it was concentrated under reduced pressure and fractionated by the freeze-thawing procedure (Gorin & Iacomini, 1984). The water-soluble polysaccharides (S) were recovered by centrifugation and treated with Fehling solution, following Jones and Stoodley (1965) methodology, with modifications. In summary, the freeze-dried sample

was solubilized in an alkaline solution (86.5 g of potassium sodium tartrate and 62.5 g of potassium hydroxide in 250 mL of distilled water) and centrifugated (10.000 rpm; 20 min; 4 °C) to remove any insoluble content. Subsequently, a solution of copper sulfate was added (27.87 g of copper sulfate in 250 mL of distilled water) and the mixture was kept under magnetic stirring for 1 hour. After resting in a refrigerator (~4 °C) overnight, the sample was centrifugated (10.000 rpm; 20 min; 4 °C), and the supernatant (SF) was neutralized with acetic acid, dialyzed against tap water (3,5 kDa; 48 h), treated with cationic resin, neutralized with NaOH 1 M, and dialyzed against tap water (3,5 kDa; 24 h). The treatment with Fehling solution was repeated twice and in the last repetition, the final dialyzed material was centrifugated, giving rise to a supernatant fraction (SSF), which was concentrated under reduced pressure and dialyzed against distilled water (12-14 kDa). Dialysis was carried out until the eluted material (E12) showed no sugar when analyzed by the phenol-sulfuric acid method (Dubois et al., 1956). Both eluted (E12) and retained (R12) were concentrated under reduced pressure and freeze-dried.





**Figure 1:** Extractions and purification process applied on *G. lucidum* fruiting bodies.

### 2.3 Monosaccharide composition

The R12 and E12 fractions (3 mg) were hydrolyzed with TFA (2 M; 8 h; 100 °C). The acid was evaporated to dryness, water was added (200 µL), and reduction was carried out with NaBH<sub>4</sub> for 12 hours (Wolfson & Thompson, 1963a). After

neutralization with acetic acid, samples were washed with methanol (200  $\mu$ L; 3 x) and water (200  $\mu$ L; 2 x). Acetylation was performed with acetic anhydride and pyridine (1:1; 200  $\mu$ L; 40 min; 100  $^{\circ}$ C) (Wolfrom & Thompson, 1963b). The alditol acetates were extracted with chloroform (1 mL), washed with copper sulfate (5 %; 2.5 mL; 4 x), and water (200  $\mu$ L; 3 x). Chloroform was evaporated and samples were solubilized in acetone (700  $\mu$ L) to be injected in a gas chromatograph-mass spectrometer (GC-MS) (Shimadzu QP2020NX, quadrupole detector), with a VF-5MS column (30 m x 0.25 mm). The injector was at 250  $^{\circ}$ C and analyses started with the oven at 100  $^{\circ}$ C (held for 3 min). The temperature was increased subsequently to 220  $^{\circ}$ C, 250  $^{\circ}$ C, and 280  $^{\circ}$ C (10  $^{\circ}$ C/min; held for 3 min). Helium was used as carrier gas (2 mL/min). Alditol acetates were identified by their retention time and their electron ionization mass spectra, by comparison with standards.

## **2.4 Congo red test**

The conformational structure of R12 and E12 was investigated by helix-coil transition analysis according to Ogawa, Tsurugi & Watanabe (1972), with adaptations. Samples were solubilized in NaOH 0.05 M (980  $\mu$ L) and Congo Red 4.000  $\mu$ M (20  $\mu$ L) was added to achieve the final concentration of 80  $\mu$ M. Dextran Mw 487.000 (Sigma) (1 mg) was used as a random coil pattern and blank was prepared NaOH 0.05 M (980  $\mu$ L) plus Congo red 4.000  $\mu$ M (20  $\mu$ L). The ultraviolet-visible absorption spectrum was taken using a microplate reader at a range of 400 to 650 nm with 10 nm intervals.

## **2.5 Determination of protein and phenolic compounds**

The presence of protein was investigated by Bradford method (Bradford, 1976), following to manufactures recommendation (Sigma-Aldrich). Bovine serum albumin (BSA) was used as standard. Phenolic compounds content was investigated with Folin & Ciocalteu phenol's reagent (Sigma, F9252), according to Singleton, Orthofer & Lamuela-Raventós (1999) methodology. Gallic acid (GAE) was used as standard. Phenolic analyses were carried out with R12 and E12 solubilized in distilled water at 2 mg/mL, while for Bradford experiment samples were at 5 mg/mL or 10 mg/mL, respectively.

## 2.6 Homogeneity and relative molecular weight determination

Analyses were carried out in a high-performance size-exclusion chromatograph (HPSEC), using four gel-permeation Ultrahydrogel columns in series with exclusion sizes of  $7 \times 10^6$ ,  $4 \times 10^5$ ,  $8 \times 10^4$ , and  $5 \times 10^3$  Da. The eluent was aqueous  $\text{NaNO}_2$  (0.1 M) with aqueous  $\text{NaN}_3$  (200 ppm) (0.6 mL/min). For analyses, R12 and E12 were solubilized in the eluent (1 mg/mL), filtrated through a cellulose membrane (0.22  $\mu\text{m}$ ), and injected (100  $\mu\text{L}$ ) in the chromatograph. The relative molecular weight (Mw) was estimated by comparing the retention time of R-12 and E12 with a curve of dextran patterns (5,000; 9,400; 17,200; 40,200 and 72,200 Da).

## 2.7 Nuclear magnetic resonance (NMR) spectroscopy

NMR mono ( $^{13}\text{C}$ ; DEPT-135) and two-dimensional (HSQC) analyses were performed in a 600 MHz Bruker model Avance spectrometer. R12 (in  $\text{D}_2\text{O}$ ) and E12 (in  $\text{Me}_2\text{SO}-d_6$ ) were analyzed at 70 °C. Both samples were solubilized at 30 mg/550  $\mu\text{L}$ . The chemical shifts were expressed in ppm ( $\delta$ ) relative to the  $\text{Me}_2\text{SO}-d_6$  resonance ( $\delta$  39.70 [ $^{13}\text{C}$ ] and 2.40 [ $^1\text{H}$ ]), for E12, or to the resonance of the reference acetone ( $\delta$  30.2 [ $^{13}\text{C}$ ] and 2.22 [ $^1\text{H}$ ]), for R12.

## 3 Results and discussion

Monosaccharide analyses of both isolated fractions showed a very similar composition, containing glucose as the main monosaccharide (93.9 and 94.0, respectively), which suggests that both fractions are composed of glucans (Table 1). The content of phenolic compounds and proteins was higher in R12 fraction than in E12 fraction (Table 1). The yield of R12 was 13.1 times higher than E12.

**Table 1:** Yield, monosaccharides, proteins, and phenolic compounds content of fractions obtained from *G. lucidum* alkaline extract.

Fractions	Yield <sup>a</sup> (%)	Monosaccharide Composition <sup>b</sup>			Proteins <sup>c</sup>	Phenolic compounds <sup>d</sup>
		Glc	Gal	Man		
<b>R12</b>	1.18	95.5	3.5	1.0	1.36	1.92
<b>E12</b>	0.09	95.6	3.1	1.3	0.18	0.93

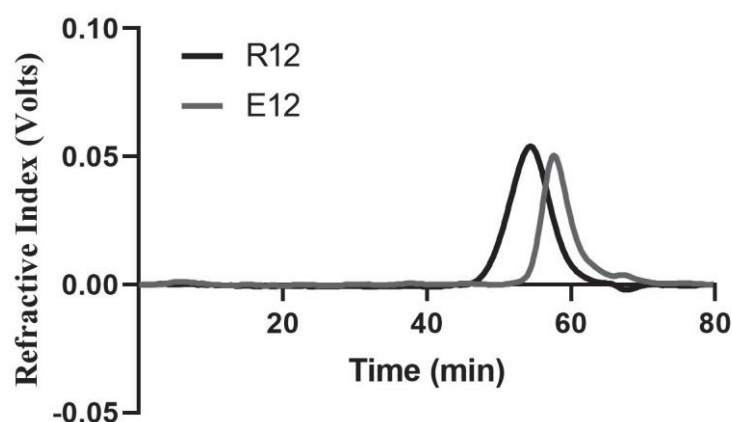
<sup>a</sup> Yields relative to dried weight of fungi.

<sup>b</sup> Alditol acetate derivatives obtained after hydrolysis, NaBH<sub>4</sub> reduction and acetylation.

<sup>c</sup> Relative to g BSA/100 g sample.

<sup>d</sup> Relative to g GAE/100 g sample.

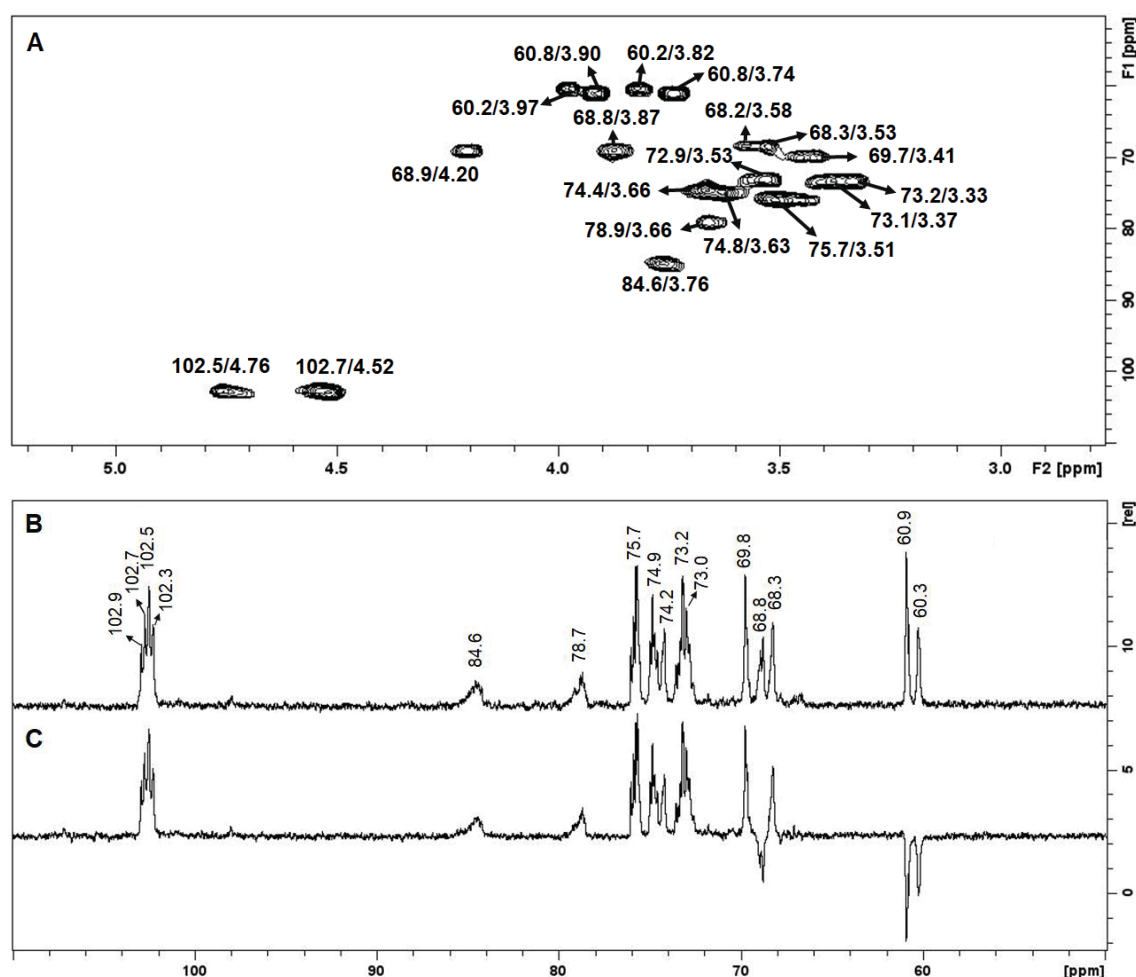
Both fractions showed a homogeneous elution profile when analyzed by HPSEC-RI (Figure 2). The fraction R12 contains a polysaccharide 2.6 times bigger than the one present in E12, since their Mw was  $1.3 \times 10^4$  and  $5 \times 10^3$  g/mol, respectively.



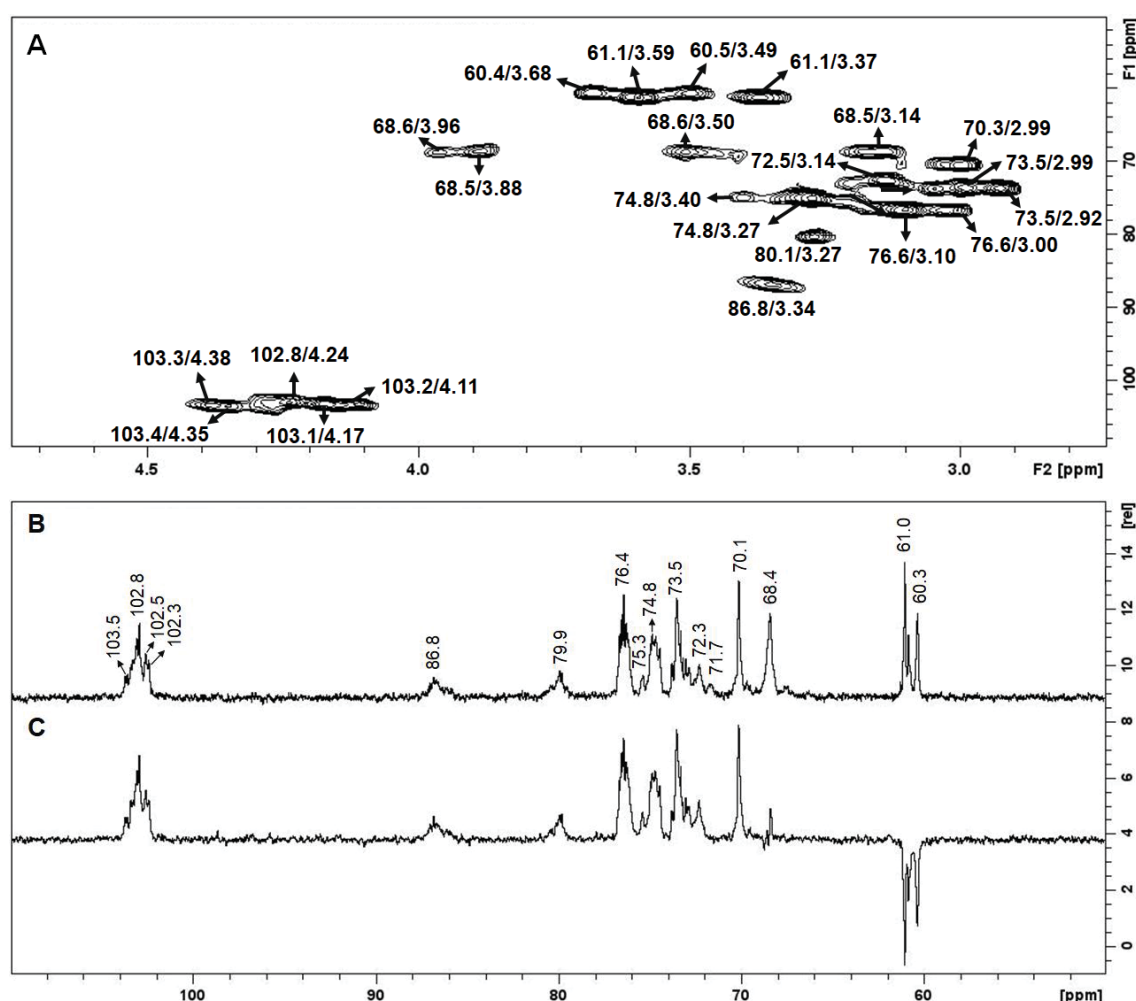
**Figure 2:** Elution profile of R12 and E12 fractions in HPSEC coupled to a refractive index detector.

In accordance with the monosaccharide composition, HSQC analyses of R12 and E12 (Figures 3 and 4, A) presented signals corresponding to a  $\beta$ -glucan (Villares et al., 2013; Wang et al., 2017; Milhorini et al., 2021). The anomeric region showed resonances relatives to  $\beta$ -D-Glcp units ( $\delta$  102.5/4.76 – 103.2/4.11) (Wang et al., 2017; Milhorini et al., 2021; Zavadinack et al., 2021). It can also be observed signals of substituted C-3 units at  $\delta$  84.6/3.76 – 86.8/3.34 and of substituted C-4 units at  $\delta$

78.9/3.66 – 80.1/3.27 (Amaral et al., 2008; Mandal et al., 2010; Dong et al., 2012; Villares et al., 2013; Wang et al., 2017; Milhorini et al., 2021). The data from HSQC analyses corroborate with  $^{13}\text{C}$  and DEPT-135 experiments (Figure 3 and 4, B and C). Signals referring to C-1 of  $\beta$ -D-Glcp ( $\delta$  102.3 – 103.5), and substituted C-3 ( $\delta$  84.6 – 86.8) and C-4 ( $\delta$  78.7 – 79.9) were observed. Additionally, the inverted signals in DEPT-135 at  $\delta$  68.8 – 68.4 reveal a C-6 substitution (confirmed by HSQC-DEPT for E12 fraction, data not shown), although non-substituted C-6 was also observed ( $\delta$  60.3 – 61.0) (Sovrani et al., 2017; Zavadinack et al., 2021; Milhorini et al., 2021). These data indicate that both glucan fractions are composed of a  $\beta$ -glucans with (1 $\rightarrow$ 3), (1 $\rightarrow$ 4), and (1 $\rightarrow$ 6) linkages.



**Figure 3:** R12 analyses in NMR: (A) HSQC, (B)  $^{13}\text{C}$ , and (C) DEPT-135. Samples were solubilized in  $\text{D}_2\text{O}$  and analyses were performed at 70 °C (chemical shifts are expressed in ppm).

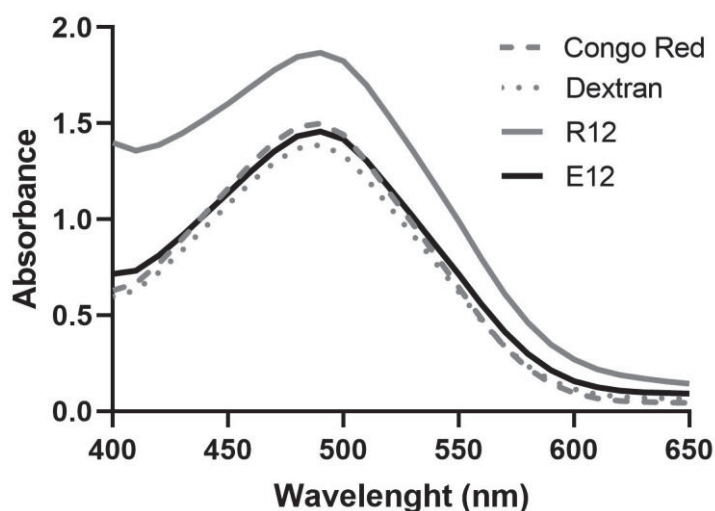


**Figure 4:** E12 analyses in NMR: (A) HSQC, (B)  $^{13}\text{C}$ , and (C) DEPT-135. Samples were solubilized in  $\text{Me}_2\text{SO}-d_6$  and analyses were performed at 70 °C (chemical shifts are expressed in ppm).

$\beta$ -glucans with these features are unusual and not often found in mushrooms. However, a  $\beta$ -glucan containing the same linkage types observed in this study was obtained from *Ganoderma resinaceum*. This molecule, with  $\text{Mw } 2.6 \times 10^4 \text{ g/mol}$ , has a (1 $\rightarrow$ 3)-linked main chain, which was branched at O-6 by (1 $\rightarrow$ 4)-linked  $\beta$ -D-Glcp side chains (Amaral et al., 2008). On the other hand, two  $\beta$ -glucans with  $\text{Mw } 2.97 \times 10^4$  and  $5.08 \times 10^4 \text{ g/mol}$ , were isolated from *Coriolus versicolor* and showed a backbone composed of both (1 $\rightarrow$ 4) and (1 $\rightarrow$ 3)-linked types, with branches of  $\beta$ -D-Glcp units or (1 $\rightarrow$ 4)-linked side chains attached at O-6 (Zhang et al., 2021). To confirm the main chain, as well as elucidate the linkages present in R12 and E12 fractions, further chemical analyses such as methylation and partial degradation should be performed.

The R12 and E12 fractions were evaluated on their structural conformation, using Congo red dye. It is known that  $\beta$ -glucans with a triple-helical structure can form a complex with Congo red in alkaline solutions, which lead to a shift in the maxima visible absorption (Ogawa, Tsurugi & Watanabe, 1972). Glucans that do not have an ordered three-dimensional conformation, such as dextran, do not form a complex and no displacement can be observed. Both glucan fractions displayed no bathochromic shift, showing a random coil conformation such as the control of dextran and the Congo red solution.

$\beta$ -Glucans with a random coil conformation have been obtained from several mushrooms, such as a linear  $\beta$ -(1 $\rightarrow$ 3)-glucan isolated from *Lentinula edodes* (Morales et al., 2020) and a  $\beta$ -(1 $\rightarrow$ 3)-glucan branched at O-6 by  $\beta$ -D-Glcp or by (1 $\rightarrow$ 6)-linked- $\beta$ -D-Glcp, purified from *Macrocybe titans* (Milhorini et al., 2021).



**Figure 5:** Absorption spectra of R12 and E12, Congo red (control), and Dextran (random coil control).

#### 4 Conclusions

Two fractions (R12 and E12) containing unusual  $\beta$ -glucans, which have (1 $\rightarrow$ 3), (1 $\rightarrow$ 4), and (1 $\rightarrow$ 6) linkage types, were obtained from *G. lucidum* fruiting bodies. Both have mainly glucose in their monosaccharide composition and displayed a random coil conformation. Their Mw presented a difference of 2.6 times, since it was  $1.3 \times 10^4$  and  $5 \times 10^3$  g/mol, respectively. Such polysaccharides can further be applied in different



investigations of biological properties, especially R12, which presented the higher yield.

### Declaration of competing interest

The authors declare to have no competing interests.

### Acknowledgments

This work was supported by the Brazilian funding agency: Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

### References

- Ahmad, M. F. (2020). *Ganoderma lucidum*: A rational pharmacological approach to surmount cancer. *Journal of Ethnopharmacology*, 260, 11304. <https://doi.org/10.1016/j.jep.2020.113047>
- Amaral, A. E., Carbonero, E. R., Simão, R. C. G., Kadowaki, M. K., Sassaki, G. L., Osaku, C. A., Gorin, P. A. J., & Iacomini, M. (2008). An unusual water-soluble b-glucan from the basidiocarp of the fungus *Ganoderma resinaceum*. *Carbohydrate Polymers*, 72, 473–478. <https://doi.org/10.1016/j.carbpol.2007.09.016>
- 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](https://doi.org/10.1016/0003-2697(76)90527-3)
- Dong, Q., Wang, Y., Shi, L., Yao, J., Li, J., Ma, F., & Ding, K. (2012). A novel water-soluble b-D-glucan isolated from the spores of *Ganoderma lucidum*. *Carbohydrate Research*, 353, 100–105. <http://dx.doi.org/10.1016/j.carres.2012.02.029>
- Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry*, 28, 350–356. <http://dx.doi.org/10.1021/ac60111a017>
- Ferreira, I. C. F. R., Heleno, S. A., Reis, F. S., Stojkovic, D., Queiroz, M. J. R. P., Vasconcelos, M. H., & Sokovic, M. (2015). Chemical features of *Ganoderma* polysaccharides with antioxidant, antitumor and antimicrobial activities. *Phytochemistry*, 114, 38–55. <http://dx.doi.org/10.1016/j.phytochem.2014.10.011>
- Gorin, P. A. J., & Iacomini, M. (1984). Polysaccharides of the lichens *Cetraria islandica* and *Ramalina usnea*. *Carbohydrate Research*, 128, 1, 119–132. [https://doi.org/10.1016/0008-6215\(84\)85090-9](https://doi.org/10.1016/0008-6215(84)85090-9)
- Guo, C., Guo, D., Fang, L., Sang, T., Wu, J., Guo, C., Wang, Y., Wang, Y., Chen, C., Chen, J., Chen, R., & Wang, X. (2021). *Ganoderma lucidum* polysaccharide modulates gut microbiota and immune cell function to inhibit inflammation and tumorigenesis in colon. *Carbohydrate Polymer*, 267, 118231. <https://doi.org/10.1016/j.carbpol.2021.118231>
- Jones, J. K. N., & Stoodley, R. J. Fractionation using copper complexes. *Methods in Carbohydrate Chemistry*, v. 5, pp. 36–38, 1965.
- Li, J., Gu, F., Cai, C., Hu, M., Fan, L., Hao, J., & Yu, G. (2020). Purification, structural characterization, and immunomodulatory activity of the polysaccharides from *Ganoderma lucidum*. *International Journal of Biological Macromolecules*, 143, 806–813. <https://doi.org/10.1016/j.foodchem.2020.127645>

- Liang, C., Tian, D., Liu, Y., Li, H., Zhu, J., Li, M., Xin, M., & Xia, J. (2019). Review of the molecular mechanisms of *Ganoderma lucidum* triterpenoids: Ganoderic acids A, C2, D, F, DM, X and Y. *European Journal of Medicinal Chemistry*, 174, 130-141. <https://doi.org/10.1016/j.ejmech.2019.04.039>
- Liu, Y., Zhang, J., Tang, Q., Yang, Y., Guo, Q., Wang, Q., Wu, D., & Cui, S. W. (2014). Physicochemical characterization of a high molecular weight bioactive  $\beta$ -D-glucan from the fruiting bodies of *Ganoderma lucidum*. *Carbohydrate Polymers*, 101, 968-974. <https://doi.org/10.1016/j.carbpol.2013.10.024>
- Liu, Y., Tang, Q., Zhang, J., Xia, Y., Yang, Y., Wu, D., Fan, H., & Cu, S. W. (2018). Triple helix conformation of  $\beta$ -D-glucan from *Ganoderma lucidum* and effect of molecular weight on its immunostimulatory activity. *International Journal of Biological Macromolecules*, 114, 1064–1070. <https://doi.org/10.1016/j.ijbiomac.2018.03.054>
- Ma, H. T., Hsieh, J. F., & Chen, S. T. (2015). Anti-diabetic effects of *Ganoderma lucidum*. *Phytochemistry*, 109-113. <https://doi.org/10.1016/j.phytochem.2015.02.017>
- Mandal, S., Maity, K. K., Bhunia, S. K., Dey, B., Patra, S., Sikdar, S. R., & Islam, S. S. (2010). Chemical analysis of new water-soluble (1-6)-, (1-4)- $\alpha$ ,  $\beta$ -glucan and water-insoluble (1-3)-, (1-4)- $\beta$ -glucan (Calocyban) from alkaline extract of an edible mushroom, *Calocybe indica* (Dudh Chattu). *Carbohydrate Research*, 345, 2657-2663. <https://doi.org/10.1016/j.carres.2010.10.005>
- Milhorini, S. S., Simas, F. F., Smiderle, F. R., Jesus, L. I., Rosado, F. R., & Iacomini, M. (2021).  $\beta$ -Glucans from the giant mushroom *Macrocybe titans*: Chemical characterization and rheological properties. Unpublished.
- Mironczuk-Chodakowska, I., Kujawowicz, K., & Witkowska, A. M. (2021). Beta-Glucans from Fungi: Biological and Health-Promoting Potential in the COVID-19 Pandemic Era. *Nutrients* 2021, 13, 3960. <https://doi.org/10.3390/nu13113960>
- Morales, D., Rutckeviski, R., Villalva, M., Abreu, H., Soler-Rivas, C., Santoyo, S., Iacomini, M., & Smiderle, F. R. (2020). Isolation and comparison of  $\alpha$ - and  $\beta$ -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  $\beta$ -D-glucans from *Cookeina tricholoma*. *Carbohydrate Polymers*, 141, 220–228. <https://doi.org/10.1016/j.carbpol.2016.01.001>
- Ogawa, K., Tsurugi J., & Watanabe, T (1972). Complex of gel-forming  $\beta$ -1,3-D-glucan with congo red in alkaline solution. *Chemistry Letters*, 1, 8, 689–692. <https://doi.org/10.1246/cl.1972.689>
- 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>
- Samuelsen, A. B. C., Rise, F., Wilkins, A. L., Teveleva, L., Nyman, A. A. T., & Aachmann, F. L. (2019). The edible mushroom *Albatrellus ovinus* contains a  $\alpha$ -L-fuco- $\alpha$ -D-galactan,  $\alpha$ -D-glucan, a branched (1 $\rightarrow$ 6)- $\beta$ -D-glucan and a branched (1 $\rightarrow$ 3)- $\beta$ -D-glucan. *Carbohydrate Research*, 471, 28-38. <https://doi.org/10.1016/j.carres.2018.10.012>
- Singleton, V. L., Orthofer, R., & Lamuela-Raventos, R. M. (1999). Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. *Methods Enzymol*, 299, 152-178. [https://doi.org/10.1016/S0076-6879\(99\)99017-1](https://doi.org/10.1016/S0076-6879(99)99017-1)
- Sone, Y., Okuda, R., Wada, N., Kishida, E., & Misaki, A. (1985). Structures and Antitumor Activities of the Polysaccharides Isolated from Fruiting Body and the Growing Culture of Mycelium of *Ganoderma lucidum*. *Agricultural and Biological Chemistry*, 49, 9, 2641-2653. <https://doi.org/10.1080/00021369.1985.10867134>

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

Villares, A. (2013). Polysaccharides from the edible mushroom *Calocybe gambosa*: Structure and chain conformation of a (1→4),(1→6)-linked glucan. *Carbohydrate Research*, 375, 153-157. <http://dx.doi.org/10.1016/j.carres.2013.04.017>

Wan, X., Yin, Y., Zhou, C., Hou, L., Cui, Q., Zhang, X., Cai, X., Wang, Y., Wang, L., & Tian, J. (2022). Polysaccharides derived from Chinese medicinal herbs: A promising choice of vaccine adjuvants. *Carbohydrate Polymers*, 276, 118739. <https://doi.org/10.1016/j.carbpol.2021.118739>

Wang, Y., Liu, Y., Yu, H., Zhou, S., Zhang, Z., Wu, D., Yan, M., Tang, Q., Zhang, J. (2017). Structural characterization and immuno-enhancing activity of a highly branched water-soluble  $\beta$ -glucan from the spores of *Ganoderma lucidum*. *Carbohydrate Polymers*, 167, 337-344. <http://dx.doi.org/10.1016/j.carbpol.2017.03.016>

Wolf from, M. L & Thompson, A (1963a). Reduction with sodium borohydride. In: Whistler, R. L.; Wolf from, M. L. (Eds.). *Methods in Carbohydrate Chemistry* (p. 65-68). New York: Academic Press.

Wolf from, M. L & Thompson, A (1963b). Acetylation. In: Whistler, R. L.; Wolf from, M. L. (Eds.). *Methods in Carbohydrate Chemistry* (p. 211- 215). New York: Academic Press.

Zavadinack, M., Bellan, D. L., Bertage, J. L. R., Milhorini, S. S., Trindade, E. S., Simas, F. F., Sasaki, G. L., Cordeiro, L. M. C., & Iacomini, M. (2021). An  $\alpha$ -D-galactan and a  $\beta$ -D-glucan from the mushroom *Amanita muscaria*: Structural characterization and antitumor activity against melanoma. *Carbohydrate Polymers*, 274, 118647. <https://doi.org/10.1016/j.carbpol.2021.118647>

Zhang, H., Nie, S., Guo, Q., Wang, Q., Cui, S. W., & Xie, M. (2018). Conformational properties of a bioactive polysaccharide from *Ganoderma atrum* by light scattering and molecular modeling. *Food Hydrocolloids*, 84, 16-25. <https://doi.org/10.1016/j.foodhyd.2018.05.023>

Zhang, X., Cai, Z., Mao, H., Hu, P., & Li, X. (2021). Isolation and structure elucidation of polysaccharides from fruiting bodies of mushroom *Coriolus versicolor* and evaluation of their immunomodulatory effects. *International Journal of Biological Macromolecules*, 166, 1387-1395. <https://doi.org/10.1016/j.ijbiomac.2020.11.018>

**ARTIGO VI**

**Linear  $\beta$ - and  $\alpha$ -glucans from *Ganoderma lucidum* fruiting bodies**

**Linear  $\beta$ - and  $\alpha$ -glucans from *Ganoderma lucidum* fruiting bodies**

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## Abstract

*Ganoderma lucidum*, known as Reishi, has biological properties that resulted in its widespread use in traditional medicine. Polysaccharides are known as one of the main bioactive compounds of this fungus. Due to this, the obtention of these polymers is of scientific and pharmaceutical interest. Herein, we obtained water-insoluble polysaccharides by alkaline extraction, after removing the main soluble polysaccharides by aqueous extraction. The insoluble polysaccharides obtained were fractionated by alkaline and acid precipitation. Subsequently, they were extracted with Me<sub>2</sub>SO (at 40 °C and 100 °C), resulting in four samples (GLC-1, GLC-2, GLC-3, and GLC-4), which were analyzed by NMR and GC-MS. The fractions obtained from the alkaline precipitate (GLC-1 and GLC-2) contain a  $\beta$ -(1→3)-glucan. On the other hand, the fractions obtained from the acid precipitate showed mainly an  $\alpha$ -(1→3)-glucan (GLC-3), or a mixture of three linear polysaccharides:  $\beta$ -(1→3)-glucan,  $\alpha$ -(1→3)-glucan, and  $\alpha$ -(1→4)-glucan (GLC-4). This research reports the variability of linear polysaccharides present in *Ganoderma lucidum* fruiting bodies. These fractions could be obtained with a satisfactory yield, enabling their application for biological studies.

**Keywords:** *Ganoderma lucidum*; polysaccharides; linear glucans.

## 1 Introduction

*Ganoderma lucidum* known as Reishi, the immortality mushroom, is a traditional fungus that has been artificially cultivated and commercialized since the 1980s (Wasser, 2005). This species has been consumed for millenniums in popular medicine as a folk remedy for promoting health and longevity (Ahmad, 2020). Several biological properties have been reported to this mushroom, such as antitumoral (Zhu, Yao, Ahmad & Chang, 2019; Shi et al., 2021), prebiotic (Khan et al., 2018; Guo et al., 2021), antioxidant (Zhu, Yao, Ahmad & Chang, 2019; Ryu et al., 2021), anti-diabetic (Ma, Hsieh & Chen, 2015; Ryu et al., 2021), and immunomodulatory effects (Li et al., 2020; Ren, Zhang & Zhang, 2021). This is the reason that *G. lucidum* is currently used in the formulation of nutraceuticals and as functional foods (Saltarelli et al., 2009).

Polysaccharides are one of the main bioactive compounds in *Ganoderma lucidum* (Nie, Zhang, Li & Xie, 2013; Ahmad, 2020; Wan et al., 2022). Such molecules are classified as homo or heteropolysaccharides, according to their composition (Nie, Zhang, Li & Xie, 2013; Ruthes, Smiderle & Iacomini, 2016). The homopolysaccharide group comprises the  $\beta$ - and  $\alpha$ -glucans, which are found in mushrooms usually as branched structures (Shi, Li, Yin & Nie, 2019; Xie et al., 2019; Li et al., 2020; Sang et al., 2021), although linear glucans can also be isolated from such fungi (Wang & Zhang, 2009; Jesus et al., 2018; Nowak et al., 2019). Linear glucans have been isolated from *Ganoderma lucidum*. An  $\alpha$ -(1 $\rightarrow$ 3)-glucan was obtained from its spores (Bao, Duan, Fang & Fang, 2001) and fruiting bodies (Chen, Zhang, Nakamura & Norisuye, 1998; Chen, Zhou, Zhang, Nakamura & Norisuye, 1998; Wiater et al., 2012); while a  $\beta$ -(1 $\rightarrow$ 3)-glucan was obtained from its fruiting bodies (Wang & Zhang, 2009).

Linear  $\alpha$ - and  $\beta$ -glucans with different linkage types (1 $\rightarrow$ 3; 1 $\rightarrow$ 4; or 1 $\rightarrow$ 6) were also obtained from other mushroom species, such as *Ganoderma tsugae* (Peng, Zhang, Zhang, Xu & Kennedy, 2005), *Pleurotus ostreatus* (Synytsya et al., 2009; Palacios, Garc  a-Lafuente, Guilla  n & Villares, 2012), *Pleurotus sajor-caju* (Silveira et al., 2014), *Fomitopsis betulina* (Jesus et al., 2018), *Lentinula edodes* (Jeff et al., 2013; Morales et al., 2020), *Poria cocos* (Jin et al., 2003; Jin et al., 2004; Zhang et al., 2005), *Amanita muscaria* (Kiho et al., 1994), *Cordyceps militaris* (Smiderle et al., 2014), *Agaricus bisporus* and *Agaricus brasiliensis* (Smiderle et al., 2013). Some of these linear glucans showed different biological activities, such as anti-proliferative (Jeff et al., 2013), hypocholesterolemic, antitumoral, antioxidant (Morales et al., 2020),



and anti-inflammatory effects (Smiderle et al., 2014; Silveira et al., 2014; Morales et al., 2020).

In summary, polysaccharides are strongly associated with the biological effects reported to *G. lucidum*, especially the glucans. It is essential to have an established methodology for obtaining such molecules to apply them in pharmacological approaches. Based on this, this study aimed to report the extraction and isolation of linear glucan-enriched fractions from this basidiomycete.

## 2 Material and methods

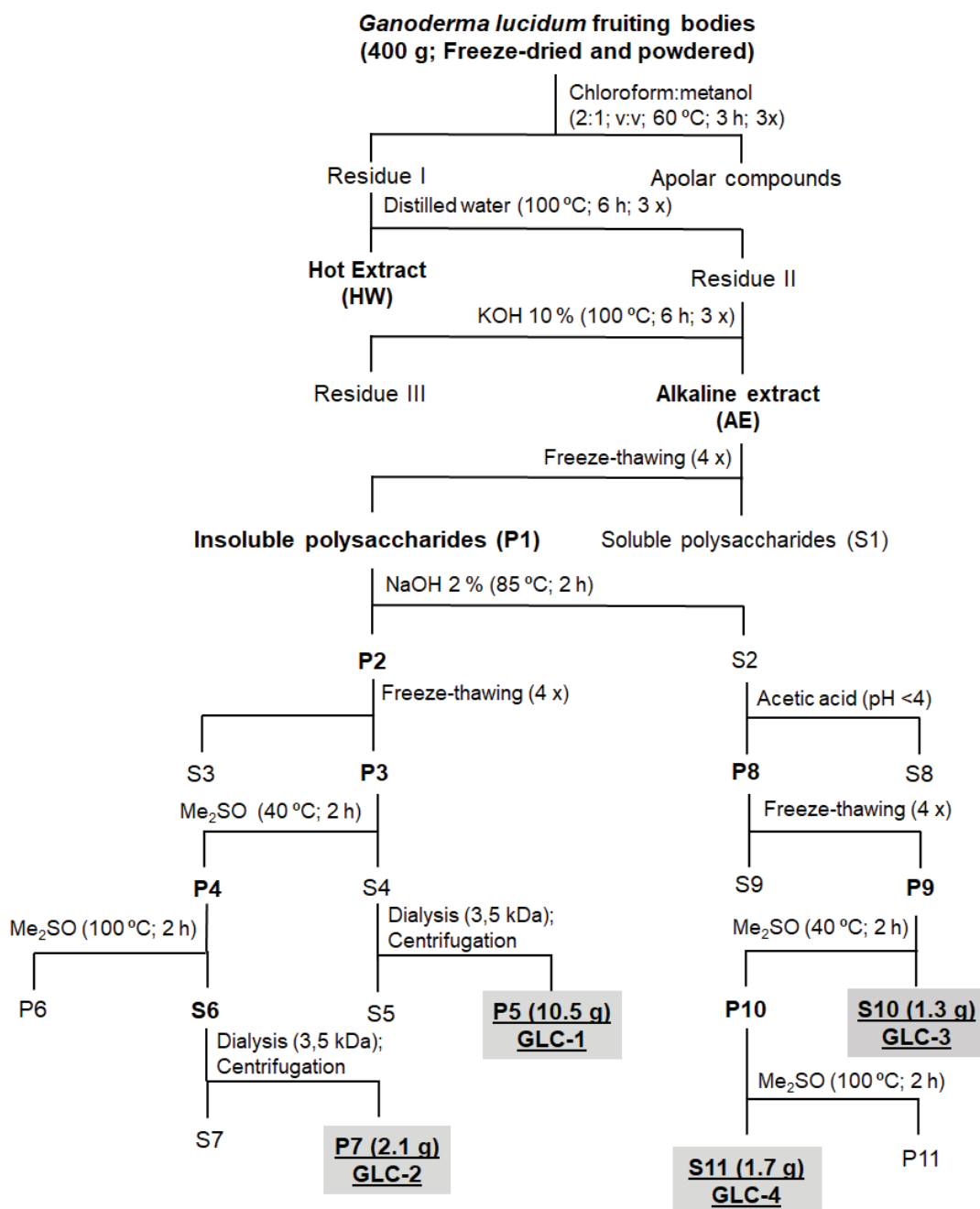
### 2.1 Biological material

*G. lucidum* fruiting bodies were donated by the company Juncao Brazil, Taboão da Serra, State of São Paulo, Brazil. This species was registered on *Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado* (SISGEN), with the number A3BA3B3.

### 2.2 Extraction and purification process

Extraction and purification of polysaccharides from *G. lucidum* were carried out as represented in figure 1. Firstly, the dried fruiting bodies were defatted with chloroform and methanol (2:1; v:v; 60 °C; 3 h; 3 x) using a Soxhlet apparatus, resulting in a delipidified residue (Residue I). The residue I was extracted with distilled water (at 100 °C; 6 h; 3 x) and subsequently with 10 % KOH in the presence of NaBH<sub>4</sub> (at 100 °C; 6 h; 3 x). Both extractions were performed under reflux. The alkaline extract (AE) was used in this research. This extract was neutralized with acetic acid, dialyzed (3,5 kDa; 72 h), concentrated under reduced pressure, and fractionated by the freeze-thawing procedure (Gorin & Iacomini, 1984). Cold water-insoluble polysaccharides (P1) were obtained in the precipitate by centrifugation (10,000 rpm; 20 min; 4 °C). Aiming to remove the protein content, P1 fraction was treated with 2 % NaOH containing NaBH<sub>4</sub> (at 80 °C; 2 h) (Smiderle et al., 2008). The partially deproteinated soluble polysaccharides (S2) were separated from the insoluble (P2) by centrifugation. P2 fraction was resuspended in distilled water, neutralized with acetic acid, dialyzed against tap water (3,5 kDa; 48 h), and concentrated under reduced pressure.

Subsequently, P2 was resubmitted to the freeze-thawing process (Gorin & Iacomini, 1984) to remove any residual water-soluble polysaccharide. The resulting precipitate (P3) was submitted to sequential extractions with Me<sub>2</sub>SO (55 mg/mL), firstly at 40 °C (2 h) and subsequently at 100 °C (2 h), under reflux. Both Me<sub>2</sub>SO-soluble extracts (S4 and S6, respectively) were dialyzed against tap water (3,5 kDa) for 48 h and centrifuged (8,000 rpm; 20 min). The final precipitates (P5 and P6, respectively) were freeze-dried and further denominated GLC-1 and GLC-2 fractions, respectively. At the same time, the soluble polysaccharide fraction obtained in the treatment with 2 % NaOH (S2) was added with acetic acid until achieving pH <4.0, to remove the residual proteins. The deproteinate soluble polysaccharide (S8), obtained by centrifugation, was not used in this study. On the other hand, the precipitated fraction (P8) was neutralized with 1 M NaOH, dialyzed against tap water (3,5 kDa; 48 h), concentrated under reduced pressure, and fractionated by the freeze-thawing process (Gorin & Iacomini, 1984). The residual water-soluble polysaccharides (S9) were removed by centrifugation. The precipitate (P9) was submitted to the sequential extractions with Me<sub>2</sub>SO for 2 h (at 40 °C and at 100 °C, subsequently). The Me<sub>2</sub>SO-soluble polysaccharides (S10 and S11) were dialyzed against tap water (3,5 kDa; 48 h), concentrated under reduced pressure, freeze-dried, and denominated GLC-3 and GLC-4, respectively.



**Figure 1:** Scheme of extraction and isolation of linear  $\beta$ - and  $\alpha$ -glucans from *Ganoderma lucidum* fruiting bodies.

### 2.3 Monosaccharide composition

GLC-1, GLC-2, GLC-3, and GLC-4 (3 mg) were hydrolyzed with 2 M TFA (100 °C; 8 h). Subsequently, the acid was evaporated to dryness. Derivatization of monosaccharides to alditol acetates was carried out following Wolfrom & Thompson (1963 a,b), with modifications. Briefly, distilled water was added (200  $\mu$ L) and reduction

was carried out with  $\text{NaBH}_4$  at room temperature for 12 h. Samples were neutralized with acetic acid, evaporated to dryness, washed with methanol (200  $\mu\text{L}$ ; 3 x), and acetylated with acetic anhydride and pyridine (200  $\mu\text{L}$ ; 1:1; v:v; 100  $^\circ\text{C}$ ; 30 min). The alditol acetates were extracted with chloroform (1 mL), washed with copper sulfate 5 % (2.5 mL; 4 x), and dried by evaporation. Samples were finally resuspended in acetone and injected in a gas chromatograph-mass spectrometer (GC-MS) (Shimadzu QP2020NX, quadrupole detector), with a VF-5MS column (30 m x 0.25 mm). The injector temperature was kept at 250  $^\circ\text{C}$ . The oven was initially at 100  $^\circ\text{C}$  (kept for 3 minutes) and increased to 220  $^\circ\text{C}$ , 250  $^\circ\text{C}$ , and 280  $^\circ\text{C}$  (10  $^\circ\text{C}/\text{min}$ ; held for 3 min). Helium was the carrier gas (2.0 mL/min). Monosaccharides were identified by comparing their retention time and electron ionization profiles with standards (Sasaki et al., 2008).

#### **2.4 Protein and phenolic compounds content**

Protein content was estimated using the Bradford method (Bradford, 1976), according to manufacturer recommendations (Sigma Aldrich). Bovine serum albumin (BSA) was used as standard. Phenolic compounds content was investigated with Folin & Ciocalteu phenol's reagent (Sigma, F9252), according to Singleton, Orthofer & Lamuela-Raventós (1999) methodology. Gallic acid (GAE) was used as standard. For the Bradford experiment samples were at 5 mg/mL. Phenolic analyses were carried out with GLC-1 and GLC-2 at 5 mg/mL, while GLC-3 and GLC-4 were at 1 mg/mL.

#### **2.5 Nuclear magnetic resonance (NMR) spectroscopy**

NMR analyses ( $^{13}\text{C}$ ; DEPT-135) were performed in a BRUKER spectrometer, model Avance 600. Analyses were carried out at 70  $^\circ\text{C}$  with samples (30 mg) solubilized in deuterated dimethylsulfoxide ( $\text{Me}_2\text{SO}-d_6$ ). The chemical shifts were expressed in ppm ( $\delta$ ) relative to the  $\text{Me}_2\text{SO}-d_6$  resonance ( $\delta$  39.70/2.40 for  $^{13}\text{C}$  and  $^1\text{H}$ , respectively).

### **3 Results and discussion**

Samples GLC-1, GLC-2, GLC-3, and GLC-4 presented mainly glucose in their monosaccharide composition (table 1). GLC-1 and GLC-2 are very similar and showed

the highest amounts of glucose (95.3 and 95.0, respectively), while the following obtained fractions GLC-3 and GLC-4 presented reduced amount (74.3 and 69.2, respectively). Monosaccharide composition results indicate that the glucan fractions obtained from the alkaline precipitate were more pure than those obtained from the acid precipitation. Phenolic compounds were found in a minor quantity in GLC-1 and increased in the subsequent fractions (Table 1). The highest protein content was found in the samples extracted with Me<sub>2</sub>SO at 100 °C (GLC-2 and GLC-4) (Table 1).

**Table 1:** Yield, monosaccharides, proteins, and phenolic compounds content of fractions obtained from *G. lucidum* alkaline extract.

Fractions	Yield <sup>a</sup> (%)	Monosaccharide Composition <sup>b</sup>						Proteins <sup>c</sup>	Phenolic compounds <sup>d</sup>
		Glc	Man	Gal	Xyl	Ara	Fuc		
<b>GLC-1</b>	2.6	95.3	3.5		1.2			1.3	0.6
<b>GLC-2</b>	0.5	95.0	3.7		1.3			2.8	1.3
<b>GLC-3</b>	0.3	74.3	11.4	2.5	6.0	1.1	4.7	1.2	4.7
<b>GLC-4</b>	0.4	69.2	17.0		7.7	1.0	5.1	1.9	6.1

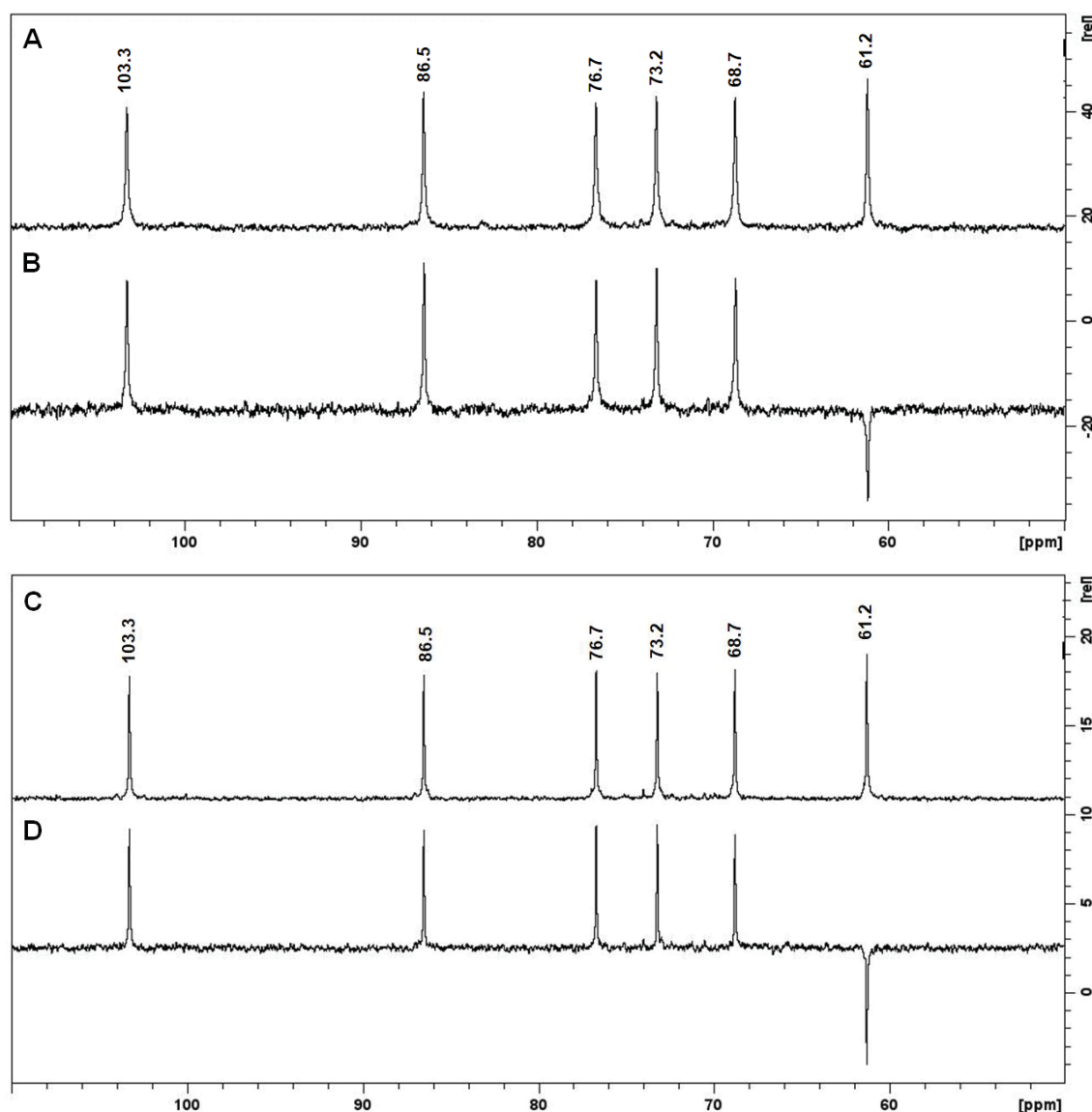
<sup>a</sup> Yield relative to dried weight of fungi.

<sup>b</sup> Alditol acetate derivatives obtained after hydrolysis, NaBH<sub>4</sub> reduction and acetylation.

<sup>c</sup> Relative to g BSA/100 g sample.

<sup>d</sup> Relative to g GAE/100 g sample.

Fractions were subsequently analyzed by NMR (Figures 2, 3, 4, and 5 A, B). GLC-1 and GLC-2 showed similar spectra containing only six signals in <sup>13</sup>C and DEPT-135 experiments (Figure 2 A, B, C, D). The signals were determined by comparison with reported data and are corresponding to a linear β-D-(1→3)-glucan (Carbonero et al., 2012; Bhanja et al., 2014; Silveira et al., 2014; Smiderle et al., 2014; Moreno et al., 2016; Sovrani et al., 2017). The low-field signal of C-1 (δ 103.3) and the C-3 (δ 86.5) indicates the β-configuration of Glcp and the C-3 substitution, respectively. The other signals refer to the C-5 (δ 76.7), C-2 (δ 73.2), C-4 (δ 68.7), and non-substituted C-6 (δ 61.2). The CH<sub>2</sub> (C-6) was confirmed by the inverted signal in DEPT-135 analysis (Figure 2 B).



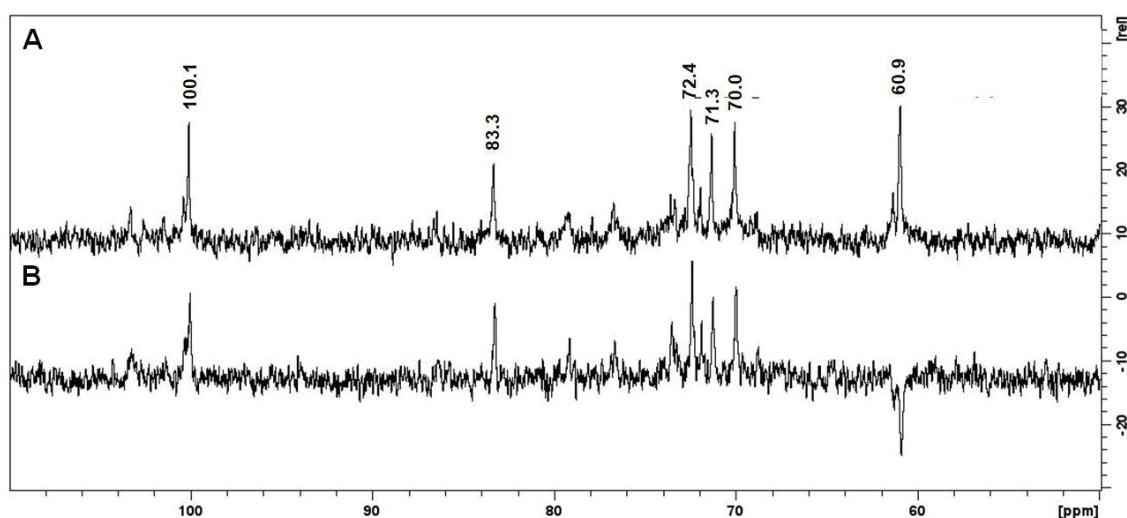
**Figure 2:** Analyses of GLC-1 (A, B) and GLC-2 (C, D) by NMR. (A), (C):  $^{13}\text{C}$ ; (B), (D): DEPT-135. Samples were solubilized in  $\text{Me}_2\text{SO}-d_6$  and analyses were performed at 70 °C. Chemical shifts are expressed in ppm.

Wang and Zang (2009) isolated a linear  $\beta$ -(1 $\rightarrow$ 3)-glucan using extraction with 1 M NaOH. The authors have performed this alkaline extraction after extracting the fruiting bodies with 0.9 % NaCl solution (at 25 °C, 40 °C, and 95 °C), which previously removed the water-soluble polysaccharides. The linear polysaccharide was purified by  $\text{Me}_2\text{SO}$  extraction. Similarly, in this study the linear  $\beta$ -glucan was obtained from the residual material, after removing the main water-soluble polysaccharides through aqueous extraction. This confirms that the linear  $\beta$ -glucans are located in the innermost layer of the mushrooms cell wall (Netea et al., 2008). Therefore, the best approach to

achieve such molecules is to perform the removal of the outer cell wall layer polysaccharides previously by mild extractions. This was the approach used in this study followed by a partial purification process to remove the remained content of water-soluble polysaccharides. The purification procedures included freeze-thawing and centrifugations, before performing the Me<sub>2</sub>SO extraction.

Linear  $\beta$ -(1 $\rightarrow$ 3)-glucans were also obtained from other mushroom species, including *Termitomyces eurhizus* (Chakraborty, Mondal, Rout & Islam, 2006), *Pleurotus sajor-caju* (Silveira et al., 2014), *Cordyceps militaris* (Smiderle et al., 2014), and *Laetiporus sulphureus* (Bull.: Fr.) Murr (Alquini et al., 2004).

GLC-3 fraction, obtained through acid precipitation, was analyzed by NMR (<sup>13</sup>C and DEPT-135) and showed six main signals (Figure 3 A, B) indicating a linear polysaccharide. However, differently from GLC-1 and GLC-2, the signals refer to a linear  $\alpha$ -D-(1 $\rightarrow$ 3)-glucan, corresponding to C-1 ( $\delta$  100.1), C-3 ( $\delta$  83.3), C-5 ( $\delta$  72.4), C-2 ( $\delta$  71.3), C-4 ( $\delta$  70.0), and C-6 ( $\delta$  60.9) (Peng et al., 2005; Jesus et al., 2018; Nowak et al., 2019).



**Figure 3:** GLC-3 analyses in NMR: (A) <sup>13</sup>C; (B) DEPT-135. The sample was solubilized in Me<sub>2</sub>SO-*d*<sub>6</sub> and analyses were performed at 70 °C. Chemical shifts are expressed in ppm.

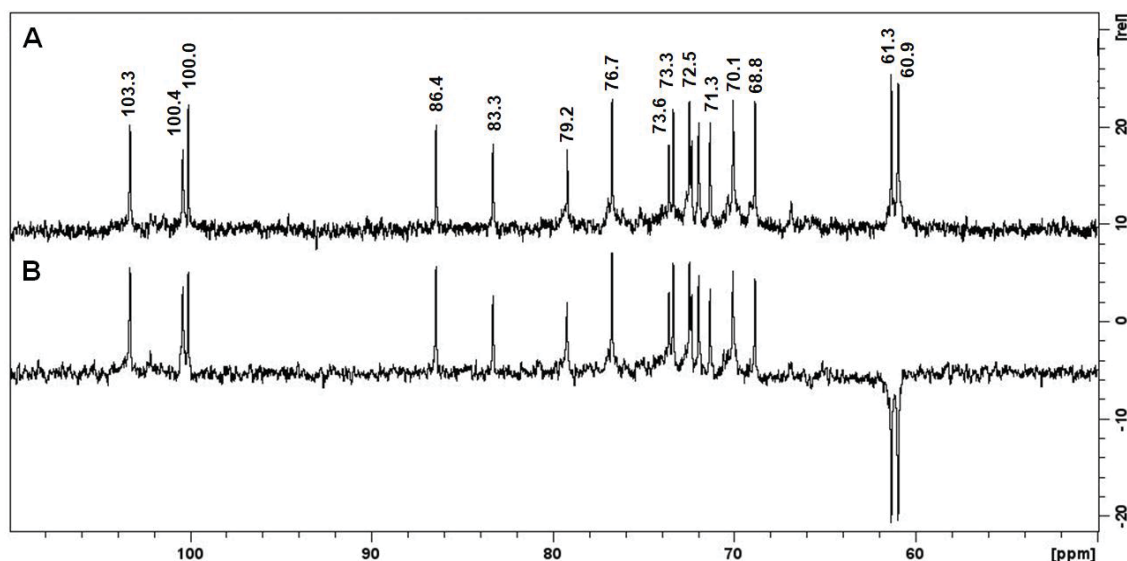
Linear  $\alpha$ -(1 $\rightarrow$ 3)-glucans were obtained from *G. lucidum* fruiting bodies by Chen et al., (1998) and Wiater et al., (2012). Both authors isolated this linear polysaccharide through alkaline extraction 1 M NaOH after performing aqueous extractions with saline



solution or distilled water. Other mushrooms were reported to have  $\alpha$ -(1 $\rightarrow$ 3)-glucan, such as *Ganoderma tsugae* (Peng et al., 2005), *Ramaria botrytis* (Bhanja et al., 2014), *Fomitopsis betulina* (Jesus et al., 2018), *Poria cocos* (Jin et al., 2004), *Amanita muscaria* (Kiho et al., 1994), *Pleurotus ostreatus* (Synytsya et al., 2009), and *Lentinula edodes* (Morales et al., 2020). Such linear glucans are very insoluble and present an extended chain conformation (Zhang et al., 2007). This characteristic confers resistance to the fungal cell wall and according to Ruiz-Herrera and Ortiz-Castellanos (2019), the linear  $\alpha$ -(1 $\rightarrow$ 3)-glucans may be related to virulence of pathogenic fungi because they block the recognition of  $\beta$ -glucans by dectin. However, their role in mushrooms is still incomplete (Synytsya & Novak, 2013).

GLC-4 fraction NMR analyses ( $^{13}\text{C}$  and DEPT-135) showed resonances of three different linear molecules (Figure 4 A, B). The signals at  $\delta$  103.3 (C-1), 86.4 (C-3), 76.7 (C-5), C-2 (73.3), C-4 (68.8), and (C-6) 61.3 indicate the presence of a  $\beta$ -(1 $\rightarrow$ 3)-glucan, while signals at  $\delta$  100.0 (C-1), 83.3 (C-3), 72.5 (C-5), 71.3 (C-2), 70.1 (C-4), and 60.9 (C-6) showed an  $\alpha$ -(1 $\rightarrow$ 3)-glucan (Sovrani et al., 2017; Jesus et al., 2018; Silveira et al., 2014; Nowak et al., 2019). Different from GLC-1, GLC-2, and GLC-3, this fraction always presented another resonance in the anomeric region at  $\delta$  100.4, referring to an  $\alpha$ -Glc<sub>p</sub>. The signal at  $\delta$  79.2 evidences the presence of C-4 substitution (Smiderle et al., 2010; Sovrani et al., 2017). These data suggest the presence of an  $\alpha$ -(1 $\rightarrow$ 4)-glucan. A similar linear  $\alpha$ -(1 $\rightarrow$ 4)-glucan was obtained from *Pleurotus ostreatus* (Palacios et al., 2012).

These data show that GLC-1 and GLC-2 were composed of a linear  $\beta$ -(1 $\rightarrow$ 3)-glucan. On the other hand, GLC-3 contains mainly an  $\alpha$ -(1 $\rightarrow$ 3)-glucan, while GLC-4 presented a mixture of  $\alpha$ -(1 $\rightarrow$ 3)-glucan,  $\alpha$ -(1 $\rightarrow$ 4)-glucan, and  $\beta$ -(1 $\rightarrow$ 3)-glucan.



**Figure 4:** GLC-4 analyses in NMR: (A)  $^{13}\text{C}$ ; (B) DEPT-135. The sample was solubilized in  $\text{Me}_2\text{SO}-d_6$  and analyses were performed at 70 °C. Chemical shifts are expressed in ppm.

#### 4 Conclusions

Three types of linear polysaccharides were isolated from *G. lucidum* fruiting bodies. Two fractions obtained were very similar (GLC-1 and GLC-2), showed the highest purity, and were composed of a  $\beta$ -(1 $\rightarrow$ 3)-glucan. The third fraction (GLC-3) contains mainly an  $\alpha$ -(1 $\rightarrow$ 3)-glucan. The fourth one (GLC-4) presented a mixture of the three linear molecules:  $\alpha$ -(1 $\rightarrow$ 3)-glucan,  $\alpha$ -(1 $\rightarrow$ 4)-glucan, and  $\beta$ -(1 $\rightarrow$ 3)-glucan. These data didactically represent the structural variability of linear polysaccharides present in *G. lucidum*. The glucans were obtained with a satisfactory yield and can be further applied in biological studies to investigate their bioactivities.

#### Declaration of competing interest

The authors declare to have no competing interests.

#### Acknowledgments

This work was supported by the Brazilian funding agency: Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

## References

- Ahmad, M. F. (2020). *Ganoderma lucidum*: A rational pharmacological approach to surmount cancer. *Journal of Ethnopharmacology*, 260, 11304. <https://doi.org/10.1016/j.jep.2020.113047>.
- Alquini, G., Carbonero, E. R., Rosado, F. R., Cosentino, C., & Iacomini, M. (2004). Polysaccharides from the fruit bodies of the basidiomycete *Laetiporus sulphureus* (Bull.: Fr.) Murr. *Federation of European Microbiological Societies*, 230, 47-52. [https://doi.org/10.1016/S0378-1097\(03\)00853-X](https://doi.org/10.1016/S0378-1097(03)00853-X)
- Bao, X., Duan, J., Fang, X., & Fang, J. (2001). Chemical modifications of the (1→3)- $\alpha$ -D-glucan from spores of *Ganoderma lucidum* and investigation of their physicochemical properties and immunological activity. *Carbohydrate Research*, 336, 127–140. [https://doi.org/10.1016/S0008-6215\(01\)00238-5](https://doi.org/10.1016/S0008-6215(01)00238-5)
- 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, 52–58. <http://dx.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, 248-254. [https://doi.org/10.1016/0003-2697\(76\)90527-3](https://doi.org/10.1016/0003-2697(76)90527-3)
- Carbonero, E. R., Ruthes, A. C., Freitas, C. S., Utrilla, P., Gálvez, J., Da Silva, E. V., Sasaki, G. L., Gorin, P. A. J., & Iacomini, M. (2012). Chemical and biological properties of a highly branched  $\beta$ -glucan from edible mushroom *Pleurotus sajor-caju*. *Carbohydrate Polymers*, 90, 814-819. <https://doi.org/10.1016/j.carbpol.2012.06.005>.
- Chakraborty, I., Mondal, S., Rout, D., & Islam, S. S. (2006). A water-insoluble (1-3)- $\beta$ -D-glucan from the alkaline extract of an edible mushroom *Termitomyces eurhizus*. *Carbohydrate Research*, 341, 2990-2993. <https://doi.org/10.1016/j.carres.2006.09.009>
- Chen, J., Zhang, L., Nakamura, Y., & Norisuye, T. (1998). Viscosity behavior and chain conformation of a (1→3)- $\alpha$ -glucan from *Ganoderma lucidum*. *Polymer Bulletin*, 41, 471–478. <https://doi.org/10.1007/s002890050389>
- Chen, J., Zhou, J., Zhang L., Nakamura, Y., & Norisuye, T. (1998). Chemical Structure of the Water-Insoluble Polysaccharide Isolated from the Fruiting Body of *Ganoderma lucidum*. *Polymer Journal*, 30, 838-842. <https://doi.org/10.1295/polymj.30.838>
- Gorin, P. A. J., & Iacomini, M. (1984). Polysaccharides of the lichens *Cetraria islandica* and *Ramalina usnea*. *Carbohydrate Research*, 128, 1, 119–132. [https://doi.org/10.1016/0008-6215\(84\)85090-9](https://doi.org/10.1016/0008-6215(84)85090-9).
- Guo, C., Guo, D., Fang, L., Sang, T., Wu, J., Guo, C., Wang, Y., Wang, Y., Chen, C., Chen, J., Chen, R., & Wang, X. (2021). *Ganoderma lucidum* polysaccharide modulates gut microbiota and immune cell function to inhibit inflammation and tumorigenesis in colon. *Carbohydrate Polymer*, 267, 118231. <https://doi.org/10.1016/j.carbpol.2021.118231>
- Jeff, I. B., Yuan, X., Sun, L., Kassim, R. M. R., Foday, A. D., & Zhou, Y. (2013). Purification and in vitro anti-proliferative effect of novel neutral polysaccharides from *Lentinus edodes*. *International Journal of Biological Macromolecules*, 52, 99-106. <http://dx.doi.org/10.1016/j.ijbiomac.2012.10.007>
- Jesus, L. I., Smiderle, F. R., Cordeiro, L. M. C., Freitas, R. A. de., Van Griensven, L. J. L. D., & Iacomini, M. (2018). Simple and effective purification approach to dissociate mixed water insoluble  $\alpha$ - and  $\beta$ -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>
- Jin, Y., Zhang, L., Chen, L., Chen, Y., Cheung, P. C. K., & Chen, L. (2003). Effect of culture media on the chemical and physical characteristics of polysaccharides isolated from *Poria cocos* mycelia. *Carbohydrate Research*, 338, 1507-1515. [https://doi.org/10.1016/S0008-6215\(03\)00197-6](https://doi.org/10.1016/S0008-6215(03)00197-6)

- Jin, Y., Zhanga, L., Tao, Y., Zeng, C., Chen, Y., & Cheung, P. C. K. (2004). Solution properties of a water-insoluble (1-3)- $\alpha$ -D-glucan isolated from *Poria cocos* mycelia. *Carbohydrate Polymers*, 57, 205–209. <https://doi.org/10.1016/j.carbpol.2004.04.013>
- Khan, T., Date, A., Chawda, H., & Patel, K. (2019). Polysaccharides as potential anticancer agents—A review of their progress. *Carbohydrate Polymers*, 210, 412–428. <https://doi.org/10.1016/j.carbpol.2019.01.064>
- Kiho, T., Yoshida, I., Katsuragawa, M., Sakushima, M., Usui, S., & Ukai, S. (1994). Polysaccharides in fungi XXXIV. A polysaccharide from the fruiting bodies of *Amanita muscaria* and the antitumoral activity of its carboxymethylated product. *Biological and Pharmaceutical Bulletin*, 17, 11, 1460–1462. <https://doi.org/10.1248/bpb.17.1460>
- Li, J., Gu, F., Cai, C., Hu, M., Fan, L., Hao, J., & Yu, G. (2020). Purification, structural characterization, and immunomodulatory activity of the polysaccharides from *Ganoderma lucidum*. *International Journal of Biological Macromolecules*, 143, 806–813. <https://doi.org/10.1016/j.foodchem.2020.127645>
- Ma, H. T., Hsieh, J. F., & Chen, S. T. (2015). Anti-diabetic effects of *Ganoderma lucidum*. *Phytochemistry*, 109–113. <https://doi.org/10.1016/j.phytochem.2015.02.017>
- Morales, D., Rutkevicki, R., Villalva, M., Abreu, H., Soler-Rivas, C., Santoyo, S., Iacomini, M., & Smiderle, F. R. (2020). Isolation and comparison of  $\alpha$ - and  $\beta$ -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  $\beta$ -D-glucans from *Cookeina tricholoma*. *Carbohydrate Polymers*, 141, 220–228. <https://doi.org/10.1016/j.carbpol.2016.01.001>
- Netea, M. G., Brown, G. D., Kullberg, B. J., Gow, N. A. R. (2008). An integrated model of the recognition of *Candida albicans* by the innate immune system., *Nature Reviews Microbiology*, 6, 67–78. <https://doi.org/10.1038/nrmicro1815>
- Nie, S., Zhang, H., Li, W., & Xie, M. (2013). Current development of polysaccharides from *Ganoderma*: Isolation, structure and bioactivities. *Bioactive Carbohydrates and Dietary Fibre*, 1, 1, 10–20. <https://doi.org/10.1016/j.bcdf.2013.01.001>
- Nowak, K., Wiater, A., Choma, A., Wiącek, D., Bieganski, A., Siwulski, M., & Waśko, A. (2019). Fungal (1 $\rightarrow$ 3)- $\alpha$ -D-glucans as a new kind of biosorbent for heavy metals. *International Journal of Biological Macromolecules*, 137, 960–965. <https://doi.org/10.1016/j.ijbiomac.2019.07.036>
- Palacios, I., García-Lafuente, A., Guillaumon, E., & Villares, A. (2012). Novel isolation of water-soluble polysaccharides from the fruiting bodies of *Pleurotus ostreatus* mushrooms. *Carbohydrates Research*, 358, 72–77. <https://doi.org/10.1016/j.carres.2012.06.016>
- Peng, Y., Zhang, L., Zhang, Y., Xu, X., & Kennedy, J. F. (2005). Solution properties of water-insoluble polysaccharides from the mycelium of *Ganoderma tsugae*. *Carbohydrate Polymers*, 59, 351–356. <https://doi.org/10.1016/j.carbpol.2004.10.004>
- Ren, L., Zhang, J., & Zhang, T. (2021). Immunomodulatory activities of polysaccharides from *Ganoderma* on immune effector cells. *Food Chemistry*, 340, 127933. <https://doi.org/10.1016/j.foodchem.2020.127933>
- Ruiz-herrera, J., Ortiz-castellanos, L. (2019). Cell wall glucans of fungi. A review. *The Cell Surface*, 5, 100022. <https://doi.org/10.1016/j.tcs.2019.100022>
- 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. <http://dx.doi.org/10.1016/j.carbpol.2015.08.061>

- Ryu, D. H., Cho, J. Y., Sadiq, N. B., Kim, J. C., Lee, B., Hamayun, M., Lee, T. S., Kim, H. S., Park, S. H., Nho, C. W., & Kim, H. Y. (2021). Optimization of antioxidant, anti-diabetic, and anti-inflammatory activities and ganoderic acid content of differentially dried *Ganoderma lucidum* using response surface methodology. *Food Chemistry*, 335, 127645. <https://doi.org/10.1016/j.foodchem.2020.127645>
- Saltarelli, R., Ceccaroli, P., Iotti, M., Zambonelli, A., Buffalini, M., Casadei, L., Vallorani, L., & Stocchi, V. (2009). Biochemical characterisation and antioxidant activity of mycelium of *Ganoderma lucidum* from Central Italy. *Food Chemistry*, 116, 143-151. <https://doi.org/10.1016/j.foodchem.2009.02.023>
- Sang, T., Guo, C., Guo, D., Wu, J., Wang, Y., Wang, Y., Chen, J., Chen, C., Wu, K., Na, K., Li, K., Fang, L., Guo, C., & Wang, X. (2021). Suppression of obesity and inflammation by polysaccharide from sporoderm-broken spore of *Ganoderma lucidum* via gut microbiota regulation. *Carbohydrate Polymers*, 256, 117594. <https://doi.org/10.1016/j.carbpol.2020.117594>
- Shi, X. D., Li, O. Y., Yin, J. Y., & Nie, S. P. (2019). Structure identification of  $\alpha$ -glucans from *Dictyophora echinovolvata* by methylation and 1D/2D NMR spectroscopy. *Food Chemistry*, 271, 338-344. <https://doi.org/10.1016/j.foodchem.2018.07.160>
- Shi, Y. J., Zheng, H. X., Hong, Z. P., Wang, H. B., Wang, Y., Li, W. Y., & Li, Z. H. (2021). Antitumor effects of different *Ganoderma lucidum* spore powder in cell- and zebrafish-based bioassays. *Journal of Integrative Medicine*, 19, 177-184. <https://doi.org/10.1016/j.joim.2021.01.004>
- Silveira, M. L. L., Smiderle, F. R., Agostini, F., Pereira, E. M., Bonatti-Chaves, M., Wisbeck, E., Ruthes, A. C., Sasaki, G. L., Cipriani, T. R., Furlan, S. A., & Iacomini, M. (2014). Exopolysaccharide produced by *Pleurotus sajor-caju*: Its chemical structure and anti-inflammatory activity. *International Journal of Biological Macromolecules*, 75, 90-96. <http://dx.doi.org/10.1016/j.ijbiomac.2015.01.023>
- Singleton, V. L., Orthofer, R., & Lamuela-Raventos, R. M. (1999). Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. *Methods Enzymol*, 299, 152-178. [https://doi.org/10.1016/S0076-6879\(99\)99017-1](https://doi.org/10.1016/S0076-6879(99)99017-1)
- 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 $\rightarrow$ 3), (1 $\rightarrow$ 6)-linked  $\beta$ -glucan isolated from *Pleurotus pulmonarius*. *European Journal of Pharmacology*, 597, 86-91. <https://doi.org/10.1016/j.ejphar.2008.08.028>
- Smiderle, F. R., Sasaki, G. L., Arkel, J. V., Iacomini, M., Wichers, H. J., & Griensven, L. J. L. D. (2010). High Molecular Weight Glucan of the Culinary Medicinal Mushroom *Agaricus bisporus* is an  $\alpha$ -Glucan that Forms Complexes with Low Molecular Weight Galactan. *Molecules*, 15, 5818-5830. <https://doi.org/10.3390/molecules15085818>
- Smiderle, F. R., Alquin, G., Tadra-Sfeir, M. Z., Iacomini, M., Wichers, H. J., & Van Griensven, L. J. L. D. (2013). *Agaricus bisporus* and *Agaricus brasiliensis* (1 $\rightarrow$ 6)- $\beta$ -D-glucans show immunostimulatory activity on human THP-1 derived macrophages. *Carbohydrate Polymers*, 94, 91-99. <http://dx.doi.org/10.1016/j.carbpol.2012.12.073>
- Smiderle, F. R., Baggio, C. H., Borato, D. G., Santana-Filho, A. S., Sasaki, G. L., Iacomini, M., & Griensven, L. J. L. D. V. (2014). Anti-Inflammatory Properties of the Medicinal Mushroom *Cordyceps militaris* Might Be Related to Its Linear (1 $\rightarrow$ 3)- $\beta$ -D-Glucan. *Plos One*, 9, 10, e110266. <https://doi.org/10.1371/journal.pone.0110266>
- Sovrani, V., Jesus, L. I., Simas-Tosin, F. F., Smiderle, F. R., & Iacomini, M. (2017). Structural characterization and rheological properties of a gel-like  $\beta$ -D-glucan from *Pholiota nameko*. *Carbohydrate Polymers*, 169, 1-8. <https://doi.org/10.1016/j.carbpol.2017.03.093>
- Synytsya, A.; Mičková, K.; Synytsya, A.; Jablonsky, I.; Spěváček, J.; Erban, V.; Kovářiková, E.; & Čopíková, J. (2009). Glucans from fruit bodies of cultivated mushrooms *Pleurotus ostreatus* and *Pleurotus eryngii*: Structure and potential prebiotic activity. *Carbohydrate Polymers*, 76, 548-556. <https://doi.org/10.1016/j.carbpol.2008.11.021>



- Synytsya, A., Novák, M. (2013). Structural diversity of fungal glucans. *Carbohydrate Polymers*, 92, 792–809. <https://doi.org/10.1016/j.carbpol.2012.09.077>
- Wan, X., Yin, Y., Zhou, C., Hou, L., Cui, Q., Zhang, X., Cai, X., Wang, Y., Wang, L., & Tian, J. (2022). Polysaccharides derived from Chinese medicinal herbs: A promising choice of vaccine adjuvants. *Carbohydrate Polymers*, 276, 118739. <https://doi.org/10.1016/j.carbpol.2021.118739>
- Wang, J., & Zhang, L. (2009). Structure and chain conformation of five water-soluble derivatives of a  $\beta$ -D-glucan isolated from *Ganoderma lucidum*. *Carbohydrate Research*, 344, 105-112. <https://doi.org/10.1016/j.carres.2008.09.024>
- Wasser, P. W. (2005). Reishi or Ling Zhi (*Ganoderma lucidum*). *Encyclopedia of Dietary Supplements*, 1, 603-622. <https://doi.org/10.1201/b13959-62>
- Wiater, A., Paduch, R., Choma, A., Pleszczyńska, M., Siwulski, M., Dominik, J., Janusz, G., Tomczyk, M., & Szczodrak, J. (2012). Biological study on carboxymethylated (1→3)- $\alpha$ -D-glucans from fruiting bodies of *Ganoderma lucidum*. *International Journal of Biological Macromolecules*, 51, 1014-1023. <https://doi.org/10.1016/j.ijbiomac.2012.08.017>
- Wolfson, M. L. & Thompson, A (1963a). Reduction with sodium borohydride. In: Whistler, R. L.; Wolfson, M. L. (Eds.). *Methods in Carbohydrate Chemistry* (p. 65-68). New York: Academic Press.
- Wolfson, M. L. & Thompson, A (1963b). Acetylation. In: Whistler, R. L.; Wolfson, M. L. (Eds.). *Methods in Carbohydrate Chemistry* (p. 211- 215). New York: Academic Press.
- Xie, J., Liu, Y., Chen, B., Zhang, G., Ou, S., Luo, J., & Peng, X. (2019). *Ganoderma lucidum* polysaccharide improves rat DSS-induced colitis by altering cecal microbiota and gene expression of colonic epithelial cells. *Food & nutrition research*, 63, 1559. <https://doi.org/10.29219/fnr.v63.1559>
- Zhang, L., Chen, L., Xu, X., Lin, Y., Cheung, P. C. K., Kennedy, J. F. (2005). Comparison on chain stiffness of a water-insoluble (1-3)- $\alpha$ -D-glucan isolated from *Poria cocos* mycelia and its sulfated derivative. *Carbohydrate Polymers*, 59, 257–263. <https://doi.org/10.1016/j.carbpol.2004.09.017>
- Zhang, M., Cui, S. W. W., Cheung, P. C. K. C. K., Wang, Q. (2007). Antitumor polysaccharides from mushrooms: a review on their isolation process, structural characteristics and antitumor activity. *Trends Food Science Technology*, 18, 4–19. <https://doi.org/10.1016/j.tifs.2006.07.013>
- Zhu, L. F., Yao, Y., Ahmad, Z., & Wei Chang, M. W. (2019). *Ganoderma lucidum* spore powder based proteoglycan and its application in hyperglycemic, antitumor and antioxidant function. *Process Biochemistry*, 84, 103-111. <https://doi.org/10.1016/j.procbio.2019.05.025>

#### 4- CONCLUSÃO

As espécies fúngicas aqui estudadas apresentaram distintos polissacarídeos em sua composição, sendo que, em ambos cogumelos foram encontrados homopolissacarídeos e heteropolissacarídeos. Os homopolissacarídeos obtidos foram as glucanas, especialmente, as  $\beta$ -glucanas, todavia, o *G. lucidum* apresentou uma maior variedade estrutural desses polímeros quando comparado ao *M. titans*. Os heteropolissacarídeos encontrados no *M. titans* foram heterogalactanas, enquanto no *G. lucidum*, uma heteromanana foi obtida. Os experimentos reológicos e biológicos realizados tanto com os heteropolissacarídeos, quanto com os homopolissacarídeos do *M. titans*, relevaram que as características químicas de um polissacarídeo ou fração polissacarídica influenciam grandemente nas propriedades físico-químicas ou biológicas obtidas. Adicionalmente, os heteropolímeros obtidos de ambos cogumelos demonstraram atividade significativa sobre diferentes linhagens de células tumorais, revelando uma possibilidade de aplicação dessas moléculas na área médica.

A partir do cogumelo *Macrocybe titans*, os seguintes resultados foram obtidos:

- a) Extrato aquoso obtido a 25 °C:
  - 1) Heteropolissacarídeo: Duas frações contendo fucogalactanas (F1 e F2,  $1,42 \times 10^4$  g/mol e  $3,12 \times 10^5$  g/mol, respectivamente). Ambas possuem uma estrutura similar, sendo compostas por uma cadeia principal de  $\alpha$ -D-Galp (1 $\rightarrow$ 6)-ligada, contendo algumas substituições em O-2 por unidades de  $\alpha$ -L-Fucp. Ambas apresentaram citotoxicidade em células tumorais MCF-7 e MDA-MB-231, todavia, apenas a de maior Mw (F-2) foi capaz de causar apoptose nas células MDA-MB-231.
- b) Extrato alcalino (NaOH 5%) obtido a 100 °C:
  - 1) Homopolissacarídeo: Uma fração composta por uma mistura de  $\beta$ -glucanas e glicogênio (*Gel-forming fraction* - GFI) e uma contendo apenas  $\beta$ -glucanas de diferentes Mw ( $\beta$ -GLC), as quais foram avaliadas quanto a sua capacidade geleificante. Ambas mostraram um comportamento não newtoniano, pseudoplástico. A fração mista apresentou ser dependente de frequência, enquanto a  $\beta$ -GLC se mostrou estável nas frequências testadas. Adicionalmente,  $\beta$ -GLC formou um gel mais forte do que a mistura GFI. Esses



dados indicam que a propriedade de geleificação das amostras é decorrente da presença das  $\beta$ -glucanas, não sendo dependente do glicogênio.

2) Homopolissacarídeo: Uma fração (GLC) contendo uma  $\beta$ -glucana de  $M_w$   $1,1 \times 10^4$  g/mol. Esse polissacarídeo possui uma cadeia principal é (1 $\rightarrow$ 3)-ligada, contendo algumas substituições em O-6 por  $\beta$ -D-Glcp ou por unidades de  $\beta$ -D-Glcp (1 $\rightarrow$ 6)-ligadas. Adicionalmente, apresenta conformação não helicoidal.

A partir do cogumelo *Ganoderma lucidum*, os seguintes resultados foram obtidos:

a) Extrato alcalino (KOH 10%) obtido a 100 °C:

1) Heteropolissacarídeo: Uma fração contendo fucoxilomanana (FXM;  $M_w$  of  $3,59 \times 10^4$  g.mol<sup>-1</sup>), a qual apresentou efeito antiproliferativo nas células de melanoma B16-F10, além de induzir a parada do ciclo celular (redução na fase S e aumento na fase G2/M). Adicionalmente, reduziu a capacidade clonogênica independente de ancoragem e diminuiu a área das colônias formadas por essa linhagem celular.

2) Homopolissacarídeo: Duas frações de  $\beta$ -glucana (R12 e E12;  $1,3 \times 10^4$  e  $5 \times 10^3$  g/mol, respectivamente) possivelmente compostas por  $\beta$ -glucanas com ligações do tipo (1 $\rightarrow$ 3), (1 $\rightarrow$ 4) e (1 $\rightarrow$ 6).

3) Homopolissacarídeo: Duas frações compostas por  $\beta$ -glucana linear ligada-(1 $\rightarrow$ 3) (GLC-1 e GLC-2), uma fração contendo principalmente  $\alpha$ -glucana linear ligada-(1 $\rightarrow$ 3) (GLC-3), e uma possuindo uma mistura de três  $\beta$ -glucanas lineares: ligadas por  $\beta$ -(1 $\rightarrow$ 3),  $\alpha$ -(1 $\rightarrow$ 3) e  $\alpha$ -(1 $\rightarrow$ 4) (GLC-4).

## 7- REFERÊNCIA

- ABREU, H.; SIMAS, F. F.; SMIDERLE, F. R.; SOVRANI, V.; DALLAZEN, L.; MARIA-FERREIRA, D.; WERNER, M.F.; CORDEIRO, L. M. C.; IACOMINI, M. Gelling functional property, anti-inflammatory and antinociceptive bioactivities of  $\beta$ -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, 117177, 2021.
- AHMAD, M. F. *Ganoderma lucidum*: A rational pharmacological approach to surmount cancer. **Journal of Ethnopharmacology**, 260, 11304, 2020.
- ALVES, V. D.; FREITAS, F.; COSTA, N.; CARVALHEIRA, M.; OLIVEIRA, R.; GONÇALVES, M. P.; REIS, M. A. M. Effect of temperature on the dynamic and steady-shear rheology of a new microbial extracellular polysaccharide produced from glycerol byproduct. **Carbohydrate Polymers**, v. 79, p. 981–988, 2010.
- AMANDEEP.; ATRI, N. S.; MUNRUCHI, K. A Checklist of Coprophilous Agarics of India. **Current Research in Environmental & Applied Mycology**, v. 5, n. 4, p. 322–348, 2015.
- BABY, S., JOHNSON, A. J., GOVINDAN, B. Secondary metabolites from *Ganoderma*. **Phytochemistry**, v. 114, p. 66-101, 2015.
- BAO, X.; DUAN, J.; FANG, X.; FANG, J. Chemical modifications of the (1→3)- $\alpha$ -D-glucan from spores of *Ganoderma lucidum* and investigation of their physicochemical properties and immunological activity. **Carbohydrate Research**, n. 336, v. 127–140, 2001.
- BEULAH, G. H.; MARGRET, A. A.; NELSON, J. Marvelous medicinal mushrooms. **International journal of pharmacy and biological sciences**, v. 3, n. 1, p. 611-615, 2013.
- BLUMFIELD, M.; ABBOTT, K.; DUVE, E.; CASSETTARI, T.; MARSHALL, S.; FAYET-MOORE, F. Examining the health effects and bioactive components in *Agaricus bisporus* mushrooms: a scoping review. **Journal of Nutritional Biochemistry**, v. 84, 108453, 2020.
- BISCAIA, S. M. P. **Manogalactana parcialmente metilada de *Pleurotus eryngii* possui atividade antimelanoma sem efeito colateral**. Tese (Doutorado em Biologia Celular e Molecular) – Setor de Ciências Biológicas, Universidade Federal do Paraná, Curitiba, 2016.
- BISCAIA, S. M. P.; CARBONERO, E. R.; BELLAN, D. L.; BORGES, B. S.; COSTA, C. R.; ROSSI, G. R.; TRINDADE, E. S. Safe Therapeutics of Murine Melanoma Model Using A Novel Antineoplastic, The Partially Methylated Mannogalactan From *Pleurotus eryngii*. **Carbohydrate Polymers**, 2017.
- 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**, 29e34, p. 458-459, 2018.
- CALONGE, D. F.; MATA, M.; UMAÑA, L. *Macrocybe titans*, um hongo espectacular presente em la Costa Rica, América Central. **Bull. Famm**, v. 32, p. 21-24, 2007.
- 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  $\alpha$ -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.; DA SILVA, E. V.; SASSAKI, G. L.; GORIN, P. A. J.; IACOMINI, M. Chemical and biological properties of a highly branched  $\beta$ -glucan from edible mushroom *Pleurotus sajor-caju*. **Carbohydrate Polymers**, v. 90, p. 814-819, 2012.

CORRALES, A; LÓPEZ-Q, C. A. *Macrocybe titans* (Bigelow and Kimbr.) Pegler, Lodge, and Nakasone, a new report for Colombia. **Actual Biol.**, v. 27, n. 82, p. 93-97, 2005.

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, 493e499, 2012.

CASTRO-ALVES, V. C.; GOMES, D.; MENOLLI JR, N.; SFORÇA, M. L.; JOÃO NASCIMENTO, J. R. O. do. Characterization and immunomodulatory effects of glucans from *Pleurotus albidus*, a promising species of mushroom for farming and biomass production. **International Journal of Biological Macromolecules**, v. 95, p. 215–223, 2017.

CASTRO-ALVES, V. C.; NASCIMENTO, J. R. O. do.  $\alpha$ - and  $\beta$ -D-Glucans from the edible mushroom *Pleurotus albidus* differentially regulate lipid-induced inflammation and foam cell formation in human macrophage-like THP-1 cells. **International Journal of Biological Macromolecules**, v. 111, p. 1222–1228, 2018.

CHAIYAMA, V.; KEAWSOMPONG, S.; LEBLANC, J. G.; LEBLANC, A. DE. M. DE.; CHATEL, J. M.; CHANPUT, W. Action modes of the immune modulating activities of crude mushroom polysaccharide from *Phallus atrovolvatus*. **Bioactive Carbohydrates and Dietary Fibre**, v. 23, 100216, 2020.

CHANG, S.T.; MILES, P.G. Mushroom biology: a new discipline, **Mycologist**, v. 6, n.2, 64–65, 1992.

CHEN, W.; WU, D.; JIN, Y.; LI, Q.; LIU, Y.; QIAO, X.; ZHANG, J.; DONG, G.; LI, Z.; LI, T.; YANG, Y. Pre-protective effect of polysaccharides purified from *Herichium erinaceus* against ethanol-induced gastric mucosal injury in rats. **International Journal of Biological Macromolecules**, v. 159, p. 948–956, 2020.

CHENG, J.; SONG, J.; LIU, Y.; LU, N.; WANG, Y.; HU, C.; HE, L.; WEI, H.; LV, G.; YANG, S.; ZHANG, Z. Conformational properties and biological activities of  $\alpha$ -D-mannan from *Sanghuangporus sanghuang* in liquid culture. **International Journal of Biological Macromolecules**, v. 164, p. 3568–3579, 2020.

CHIHARA G, MAEDA Y, HAMURO J, SASAKI T, FUKUOKA F. Inhibition of Mouse Sarcoma 180 by Polysaccharides from *Lentinus edodes* (Berk.) Sing. **Nature**, v. 222, p. 687 – 688, 1969.

COIMBRA, V. R. M.; GIBERTONI, T. B. Diversidade de Agaricales (basidiomycota) no campus da UFPE. **XVII Congresso de Iniciação Científica, I Congresso de Iniciação em Desenvolvimento Tecnológico e Inovação**, Pernambuco, 2009.

CUI, F. J.; JIANG, L. H.; QIAN, L. S.; SUN, W. J.; TAO, T. L.; ZAN, X. Y.; YANG, Y.; WU, D.; ZHAO, X. A macromolecular  $\alpha$ -glucan from fruiting bodies of *Volvariella volvacea* activating RAW264. 7 macrophages through MAPKs pathway. **Carbohydrate Polymers**, v. 230, 115674, 2020.

DELONG, J.; BREWER, M. T. *Macrocybe titans*: Largest Mushroom Species in the Western Hemisphere Found Growing in Georgia. **University of Georgia Extension**, 2013.

DUVNJAK, D.; PANTIĆ, M.; PAVLOVIĆ, V.; NEDOVIĆ, V.; LEVIĆ, S.; MATIJAŠEVIĆ, D.; SKNEPNEK, A.; NIKŠIĆ, M. Advances in batch culture fermented *Coriolus versicolor* medicinal mushroom for the production of antibacterial compounds. **Innovative Food Science and Emerging Technologies**, v. 34, p. 1–8, 2016.

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

ENSHASY, H. A. EL.; HATTI-KAUL, R. Mushroom immunomodulators: unique molecules with unlimited applications. **Trends in Biotechnology**, v. 31, n. 12, p. 668-67, 2013.

FERREIRA, I. C. F. R.; HELENO, S. A.; REIS, F. S.; STOJKOVIC, D.; QUEIROZ, M. J. R. P.; VASCONCELOS, M. H., & SOKOVIC, M. Chemical features of Ganoderma polysaccharides with antioxidant, antitumor and antimicrobial activities. **Phytochemistry**, 114, 38-55, 2015.

GARCIA, J.; AFONSO, A.; FERNANDES, C.; NUNES, F. M.; MARQUES, G.; SAAVEDRA, M. J. Comparative antioxidant and antimicrobial properties of *Lentinula edodes* Donko and Koshin varieties against priority multidrug-resistant pathogens. **South African Journal of Chemical Engineering**, 2020.

GOMES, D. C. V.; ALENCAR, M. V. O. B. de.; REIS, A. C. dos.; LIMA, R. M. T. de.; SANTOS, J. V. de. O.; MATA, A. M. O. F. da.; DIAS, A. C. S.; JUNIOR, J. S. da. C.; MEDEIROS, M. das. G. F. de.; PAZ, M. F. C. J.; GAYOSO, L. C.; MORENO, A. I.; SOUSA, J. M. de. C. e.; ISLAM, M. T.; CAVALCANTE, A. A. de. C. M. Antioxidant, anti-inflammatory and cytotoxic/antitumoral bioactives from the phylum Basidiomycota and their possible mechanisms of action. **Biomedicine & Pharmacotherapy**, v. 112, 108643, 2019.

HE, X.; FANG, J.; GUO, Q.; WANG, M.; LI, Y.; MENG, Y.; HUANG, L. Advances in antiviral polysaccharides derived from edible and medicinal plants and mushrooms. **Carbohydrate Polymers**, v. 229, 115548, 2020.

HEARST, R.; NELSON, D.; MCCOLLUM, G.; B. MILLAR, C.; MAEDA, Y.; GOLDSMITH, C. E.; ROONEY, P. J.; LOUGHREY, A.; RAO, J. R.; MOORE, J. E. An examination of antibacterial and antifungal properties of constituents of Shiitake (*Lentinula edodes*) and Oyster (*Pleurotus ostreatus*) mushrooms. **Complementary Therapies in Clinical Practice**, v. 15, p. 5–7, 2009.

HIBBETT, D. S. A phylogenetic overview of the Agaricomycotina. **Mycologia**, v. 98, n. 6, p. 917–925, 2006.

JALOOT, A. S.; OWAID, M. N.; NAEEM, G. A.; MUSLIM, R. F. Mycosynthesizing and characterizing silver nanoparticles from the mushroom *Inonotus hispidus* (Hymenochaetaceae), and their antibacterial and antifungal activities. **Environmental Nanotechnology, Monitoring & Management**, v. 14, 100313, 2020.

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  $\beta$ -D-glucan from edible mushroom *Piptoporus betulinus*. **International Journal of Biological Macromolecules**, v. 117, p. 1361–1366, 2018a.

JESUS, L. I.; SMIDERLE, F. R.; CORDEIRO, L. M. C.; FREITAS, R. A. de.; VAN GRIENSVEN, L. J. L. D.; IACOMINI, M. Simple and effective purification approach to dissociate mixed water insoluble  $\alpha$ - and  $\beta$ -D-glucans and its application on the medicinal mushroom *Fomitopsis betulina*. **Carbohydrate Polymers**, v. 200, p. 353–360, 2018b.

KAO, P. F.; WANG, S. H.; HUNG, W. T.; LIAO, Y. H.; LIN, C. M.; YANG, W. B. Structural Characterization and Antioxidative Activity of Low-Molecular-Weights Beta-1,3-Glucan from the Residue of Extracted *Ganoderma lucidum* Fruiting Bodies. **Journal of Biomedicine and Biotechnology**, v. 2012, 2012.

KARLSEN-AYALA, E.; SMITH, M. E. *Macrocybe titans*: The Mushroom Giant of the Western Hemisphere. **IFAS Extension, University of Florida**, 2020.

KHAN, I.; HUANG, G.; LI, X.; LEONG, W.; XIA, W.; HSIAO, W. W. L. Mushroom polysaccharides from *Ganoderma lucidum* and *Poria cocos* reveal prebiotic functions. **Journal of Functional Foods**, v. 41, p. 191–201, 2018.

KIKUCHI, T.; ISOBE, M.; UNO, S.; IN, Y.; ZHANG, J.; YAMADA, T. Strophasterols E and F: Rearranged ergostane-type sterols from *Pleurotus eryngii*. **Bioorganic Chemistry**, v. 89, 103011, 2019.

- KNAK, M. U.; GUEDES, A.; TAVARES, L. B. B. Atividade citotóxica frente a *Artemia salina* e antioxidante do fungo *Macrocybe titans*. **Dynamis revista tecno-científica**, v. 5, p. 81, 2009.
- KOMURA, D. L.; CARBONERO, E. R.; GRACHER, A.H.; BAGGIO, C.H.; FREITAS, C.S.; MARCON, R.; SANTOS, A.R.S.; GORIN, P.A.J.; IACOMINI, M. Structure of *Agaricus* spp. fucogalactans and their anti-inflammatory and antinociceptive properties. **Bioresource Technology**, v. 101, p. 6192-6199, 2010.
- KOMURA, D. L.; RUTHES, A. C.; CARBONERO, E. R.; GORIN, P. A. J.; IACOMINI, M. Water-soluble polysaccharides from *Pleurotus ostreatus* var. florida mycelial biomass. **International Journal of Biological Macromolecules**, v. 70, p. 354–359, 2014.
- KRÜZSELYI, D.; MÓRICZ, A. M.; VETTER, J. Comparison of different morphological mushroom parts based on the antioxidant activity. **LWT - Food Science and Technology**, v. 127, 109436, 2020
- KUSHAIRI, N.; TARMIZI, N. A. K. A.; PHAN, C. W.; MACREADIE, I.; SABARATNAM, V.; NAIDU, M.; DAVID, P. Modulation of neuroinflammatory pathways by medicinal mushrooms, with particular relevance to Alzheimer's disease. **Trends in Food Science & Technology**, v. 104, p. 153–162, 2020.
- LEMIESZEK, M. K.; NUNES, F. M.; MARQUES, G.; RZESKI, W. *Cantharellus cibarius* branched mannans inhibits colon cancer cells growth by interfering with signals transduction in NF- $\kappa$ B pathway. **International Journal of Biological Macromolecules**, v. 134, p. 770–780, 2019.
- LESKOSEK-CUKALOVIC, I.; DESPOTOVIC, S.; LAKIC, N.; NIKSIC, M.; NEDOVIC, V.; TESEVIC, V. *Ganoderma lucidum* — Medical mushroom as a raw material for beer with enhanced functional properties. **Food Research International**, v. 43, p. 2262–2269, 2010.
- LI, Q. Z.; WU, D.; ZHOU, S.; LIU, Y. F.; LI, Z. P.; FENG, J.; YANG, Y. Structure elucidation of a bioactive polysaccharide from fruiting bodies of *Hericium erinaceus* in different maturation stages. **Carbohydrate Polymers**, v. 144, p. 196–204, 2016.
- LIRA, C. R. S. de. **Diversidade de Agaricomycetes lignocelulolíticos (Basidiomycota) em áreas do sertão de Pernambuco**. 73 f. Dissertação (Mestrado em Biologia de Fungos) – Departamento de Micologia do Centro de Ciências Biológicas, Universidade Federal de Pernambuco, Recife, 2012.
- LESKOSEK-CUKALOVIC, I.; DESPOTOVIC, S.; LAKIC, N.; NIKSIC, M.; NEDOVIC, V.; TESEVIC, V. *Ganoderma lucidum* — Medical mushroom as a raw material for beer with enhanced functional properties. **Food Research International**, v. 43, p. 2262–2269, 2010.
- LIANG, C., TIAN, D., LIU, Y., LI, H., ZHU, J., LI, M., XIN, M., & XIA, J. Review of the molecular mechanisms of *Ganoderma lucidum* triterpenoids: Ganoderic acids A, C2, D, F, DM, X and Y. **European Journal of Medicinal Chemistry**, 174, 130-141, 2019.
- LIU, Q.; KONG, W.; HU, S.; KANG, Y.; ZHANG, Y.; BUN, T. Effects of *Oudemansiella radicata* polysaccharide on postharvest quality of oyster mushroom (*Pleurotus ostreatus*) and its antifungal activity against *Penicillium digitatum*. **Postharvest Biology and Technology**, v. 166, 111207, 2020.
- LU, J.; HE, R.; SUN, P.; ZHANG, F.; LINHARDT, R. J.; ZHANG, A. Molecular mechanisms of bioactive polysaccharides from *Ganoderma lucidum* (Lingzhi), a review. **International Journal of Biological Macromolecules**, v. 150, p. 765–774, 2020.
- MAO, H.; WANG, H. Resolution of deep divergence of club fungi (phylum Basidiomycota). **Synthetic and Systems Biotechnology**, v. 4, p. 225–231, 2019.
- MAITY, S.; MANDAL, E. K.; MAITY, K.; BHUNIA, S. K.; BEHERA, B.; MAITI, T. K.; MALLICK, T. K.; SIKDAR, S. R.; ISLAM, S. S. Structural study of an immunoenhancing polysaccharide isolated from an edible hybrid mushroom of *Pleurotus florida* and *Lentinula edodes*. **Bioactive Carbohydrate Dietary Fibre**, v. 1, p. 72-80, 2013.



MILHORINI, S. da. S.; SMIDERLE, F. R.; BISCAIA, S. M. P.; ROSADO, F. R.; TRINDADE, E. S.; IACOMINI, M. Fucogalactan from the giant mushroom *Macrocybe titans* inhibits melanoma cells migration. **Carbohydrate Polymers**, v. 190, p. 50–56, 2018.

MIYAKAZI, T.; NISHIJIMA, M. Structural examination of an alkali-extracted, water-soluble heteroglycan of the fungus *Ganoderma lucidum*. **Carbohydrate Research**, 109, 290-294, 1982.

MONEY, N. P. (2016). Are mushroom medicinal? **Fungal biology**, v. 120, 449-453.

MORADALI, M. F.; MOSTAFAVI, H.; GHODS, S.; HEDJAROUDE, G. A. Immunomodulating and anticancer agents in the realm of macromycetes fungi (macrofungi). **International Immunopharmacology**, v. 7, n. 6, p. 701–724, 2007.

MORALES, D.; RUTCKEVISKI, R.; VILLALVA, M.; ABREU, H.; SOLER-RIVAS, C.; SANTOYO, S.; IACOMINI, M.; SMIDERLE, F. R. Isolation and comparison of  $\alpha$ - and  $\beta$ -D-glucans from shiitake mushrooms (*Lentinula edodes*) with different biological activities. **Carbohydrate Polymers**, v. 229, 115521, 2020.

MURPHY, E. J.; MASTERSON, C.; REZOAGLI, E.; O'TOOLE, D.; MAJOR, I.; STACK, G. D.; LYNCH, M.; LAFFEY, J. G.; ROWAN, N. J.  $\beta$ -Glucan extracts from the same edible shiitake mushroom *Lentinus edodes* produce differential in-vitro immunomodulatory and pulmonary cytoprotective effects—Implications for coronavirus disease (COVID-19) immunotherapies. **Science of the Total Environment**, v. 732, 139330, 2020.

NELSON, D. L.; COX, M. M. **Princípios de bioquímica de Lehninger**. 7. ed. Porto Alegre: Artmed, 2019.

NICKERSON, M. T.; PAULSON, A.T.; SPEERS, R. A. Time–temperature studies of gellan polysaccharide gelation in the presence of low, intermediate and high levels of co-solute. **Food Hydrocolloids**, v. 18, p. 783–794, 2004.

NIE, S.; ZHANG, H.; LI, W.; XIE, M. Current development of polysaccharides from *Ganoderma*: Isolation, structure and bioactivities. **Bioactive Carbohydrates and Dietary Fibre**, v. 1, p. 10–20, 2013.

NYMAN, A. A. T.; AACHMANN, F. L.; RISE, F.; BALLANCE, S.; SAMUELSEN, A. B. C. Structural characterization of a branched (1→6)- $\alpha$ -mannan and  $\beta$ -glucans isolated from the fruiting bodies of *Cantharellus cibarius*. **Carbohydrate Polymers**, v. 146, p. 197–207, 2016.

OLIVEIRA, R. S.; BISCAIA, S. M. P.; BELLAN, D. L.; VIANA, S. R. F.; LEAL, M. C. D. M.; VASCONCELOS, A. F. D.; LIÃO, L. M.; TRINDADE, E. S.; ELAINE R. CARBONER, E. R. Structure elucidation of a bioactive fucomannogalactan from the edible mushroom *Hypsizygus marmoreus*. **Carbohydrate Polymers**, v. 225, 115203, 2019.

PEGLER, D. N., LODGE, D. J., NAKASONE, K. K. The pantropical genus *Macrocybe* gen. nov. **Mycologia**, v. 90, n. 3, p. 494-504, 1998.

PEREIRA, F. C. B. N.; SNAK, A.; SCHEMMER, J. N.; SILVA, S.; TIBÉRIO, A. E. G.; ROSADO, F. R.; FERNANDES, N. L. M. Avaliação do efeito do extrato aquoso de fungos basidiomicetes em larvas de Strongyloidea. **5º Simpósio de Biotecnologia na Agroindústria**, Palotina, 2015.

PIRES, A. DO. R. A.; RUTHES, A. C.; CADENA, S. M. S. C.; IACOMINI, M. Cytotoxic effect of a mannogalactoglucan extracted from *Agaricus bisporus* on HepG2 cells. **Carbohydrate Polymers**, v. 170, p. 33–42, 2017.

PUTZKE, J.; PUTZKE, M. T. L. **Os Reinos dos Fungos**. Santa Cruz do Sul: EDUNISC, 1998.

RAMIREZ, N. A.; NIVEIRO, N.; MICHLIG, A.; POPOFF, O. F. First record of *Macrocybe titans* (Tricholomataceae, Basidiomycota) in Argentina. **The journal of biodiversity data**, v. 13, n. 4, p. 153-158, 2017.

RAMOS, I. R.; ANDRADE, C. D. The beneficial role of edible mushrooms in human health. **Current Opinion in Food Science**, v. 14, p. 122–128, 2017.

RAMOS, M.; BURGOS, N.; BARNARD, A.; EVANS, G.; PREECE, J.; GRAZ, M.; RUTHES, A. C.; JIMÉNEZ-QUERO, A.; MARTÍNEZ-ABAD, A.; VILAPLANA, F.; NGOC, L. P.; BROUWER, A.; VAN DER BURG, B.; GARRIGÓS, M. del. C.; JIMÉNEZ, A. *Agaricus bisporus* and its by-products as a source of valuable extracts and bioactive compounds. **Food Chemistry**, v. 292, p. 176–187, 2019.

RATHORE, H.; PRASAD, S.; SHARMA, S. Mushroom nutraceuticals for improved nutrition and better human health: A review. **Pharma Nutrition**, v. 5, p. 35–46, 2017.

RATHORE, H.; PRASAD, S.; KAPRI, M.; TIWARI, A.; SHARMA, S. Medicinal importance of mushroom mycelium: Mechanisms and applications. **Journal of Functional Foods**, v. 56, p. 182–193, 2019.

ROMÁN, O. B.; ALONSO, E.; COCERO, M. J.; GOTO, M. (2016).  $\beta$ -Glucan recovery from *Ganoderma lucidum* by means of pressurized hot water and supercritical CO<sub>2</sub>. **Food and Bioproducts Processing**, 98, 21-28.

RUTHES, A. C.; CARBONERO, E. R.; CÓRDOVA, M. M.; BAGGIO, C. H.; SASSAKI, G. L.; GORIN, P. A.; SANTOS, A. R.; IACOMINI, M. Fucomannogalactan and glucan from mushroom *Amanita muscaria*: structure and inflammatory pain inhibition. **Carbohydrate Polymers**, v. 98, p. 761-769, 2013a.

RUTHES, A. C.; RATTMANN, Y. D.; MÁLQUEVICZ-PAIVA, S. M.; CARBONERO, E. R.; CÓRDOVA, M. M.; BAGGIO, C. H.; SANTOS, A. E. S.; GORIN, P. A. J.; IACOMINI, M. *Agaricus bisporus* fucogalactan: Structural characterization and pharmacological approaches. **Carbohydrate Polymers**, v. 92, p. 184-191, 2013b.

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. *Lactarius rufus* (1→3),(1→6)- $\beta$ -d-glucans: Structure, antinociceptive and anti-inflammatory effects. **Carbohydrate Polymers**, v. 94, p. 129-136, 2013c.

RUTHES, A. C.; 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.

RUTHES, A. 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.

ROMÁN, Y.; IACOMINI, M.; SASSAKI, G. L.; CIPRIANI, T. R. Optimization of chemical sulfation, structural characterization and anticoagulant activity of *Agaricus bisporus* fucogalactan. **Carbohydrate Polymers**, v. 146, p. 345–352, 2016.

SAYKA, R. A. **Determinação do perfil químico e bioquímico de *Macrocybe titans***. 83 f. Dissertação (Mestrado em Química) –Programa de Pós-Graduação em Química, Universidade Estadual de Ponta Grossa, Ponta Grossa, 2008.

SAMUELSEN, A. B C.; RISE, F.; WILKINS, A. L.; TEVELEVA, L.; NYMAN, A. A. T.; AACHMANN, F. L. The edible mushroom *Albatrellus ovinus* contains a  $\alpha$ -L-fuco- $\alpha$ -D-galactan,  $\alpha$ -D-glucan, a branched (1→6)- $\beta$ -D-glucan and a branched (1→3)- $\beta$ -D-glucan. **Carbohydrate Research**, v. 471, p. 28–38, 2019.

SANTOS, R. A. C. dos. **Análise genômica da levedura xilanolítica *Pseudozyma brasiliensis* GHG001**. 154 f. Dissertação (Mestrado em Genética e Biologia Molecular) – Instituto de Biologia, Universidade Estadual de Campinas, Campinas, 2018.

SANTOS-NEVES, J. C. **Caracterização estrutural dos polissacarídeos obtidos do Basidioma de *Pleurotus ostreatus* variedade *florida***. 79 f. Dissertação (Mestrado em Ciências-Bioquímica) – Setor de Ciências Biológicas, Universidade Federal do Paraná, Curitiba, 2007.



SAWANGWAN, T.; WANSANIT, W.; PATTANI, L.; NOYSANG, C. Study of prebiotic properties from edible mushroom extraction. **Agriculture and Natural Resources**, v. 52, 519e524, 2018.

SHEIKH, B. Y.; SARKER, M. M. R.; KAMARUDIN, M. N. A.; ISMAIL, A. Prophetic medicine as potential functional food elements in the intervention of cancer: A review. **Biomedicine & Pharmacotherapy**, v. 95, p. 614–648, 2017.

SHEN, J.; PARK, H. S.; XIA, Y. M.; KIM, G. S.; CUI, S. W. The polysaccharides from fermented *Ganoderma lucidum* mycelia induced miRNAs regulation in suppressed HepG2 cells. **Carbohydrate Polymers**, v. 103, p. 319–324, 2014.

SHI, X.; ZHAO, Y.; JIAO, Y.; SHI, T.; YANG, X. ROS-Dependent Mitochondria Molecular Mechanisms Underlying Antitumor Activity of *Pleurotus abalonus* Acidic Polysaccharides in Human Breast Cancer MCF-7 Cells. **Plos one**, 8, 5, e64266, 2013.

SILLAPACHAIYAPORN, C.; CHUCHAWANKUL, S. HIV-1 protease and reverse transcriptase inhibition by tiger milk mushroom (*Lignosus rhinocerus*) sclerotium extracts: *In vitro* and *in silico* studies. **Journal of Traditional and Complementary Medicine**, v. 10, 396e404, 2020.

SILVA, S. **POLISSACARÍDEOS DO COGUMELO *Macrocybe titans*: CARACTERIZAÇÃO ESTRUTURAL E ATIVIDADE BIOLÓGICA**. 101 f. Dissertação (Mestrado em Ciências-Bioquímica) – Setor de Ciências Biológicas, Universidade Federal do Paraná, Curitiba, 2017.

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  $\beta$ -d-glucan isolated from *Pleurotus sajor-caju*. **Carbohydrate Polymers**, v. 113, p. 588-596, 2014.

SILVEIRA, M. L. L. **Caracterização estrutural e ação antinociceptiva e anti-inflamatória de Polissacarídeos Isolados de *Pleurotus sajor-caju***. 196 f. Tese (Doutorado em Ciências-Bioquímica) – Setor de Ciências Biológicas, Universidade Federal do Paraná, Curitiba, 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.

SMIDERLE, F. R.; CARBONERO, E. R.; MELLINGER, C. G.; SASSAKI, G. L.; GORIN, P. A. J.; IACOMINI, M. Structural characterization of a polysaccharide and a  $\beta$ -glucan isolated from the edible mushroom *Flammulina velutipes*. **Phytochemistry**, v. 67, p. 2189–2196, 2006.

SMIDERLE, F. R.; ALQUINI, G.; TADRA-SFEIR, M. Z.; IACOMINI, M.; WICHES, H. J.; VAN G.; LEO J.L.D. *Agaricus bisporus* and *Agaricus brasiliensis* (1→6)- $\beta$ -d-glucans show immunostimulatory activity on human THP-1 derived macrophages. **Carbohydrate Polymers**, v. 94, p. 91-99, 2013.

SMIDERLE, F. R.; ALQUINI, G.; TADRA-SFEIR, M. Z.; IACOMINI, M.; WICHES, H. J.; VAN G.; LEO J.L.D. *Agaricus bisporus* and *Agaricus brasiliensis* (1→6)- $\beta$ -d-glucans show immunostimulatory activity on human THP-1 derived macrophages. **Carbohydrate Polymers**, v. 94, p. 91-99, 2013.

SMIDERLE, F. R.; RUTHES, A. C.; IACOMINI, M. Natural polysaccharides from mushrooms: anti-nociceptive and anti-inflammatory properties. RAMAWAT, K. G.; MÉRILLON, J. M. **Polysaccharides**, Springer, 2014.


SMIDERLE, F. R.; BAGGIO, C. H.; BORATO, D. G.; SANTANA-FILHO, A. S.; SASSAKI, G. L.; IACOMINI, M.; GRIENSSEN, L. J. L. D. V. Anti-Inflammatory Properties of the Medicinal Mushroom *Cordyceps militaris* Might Be Related to Its Linear (1→3)- $\beta$ -D-Glucan. **Plos One**, v. 9, p. e110266, 2014.

SOHRETOGLU, D.; HUANG, S. *Ganoderma lucidum* Polysaccharides as an anti-cancer agent. **Anti-Cancer Agents in Medicinal Chemistry**, v. 18, n. 5, p. 667-674, 2018.

- SOVRANI, V.; JESUS, L. I. de.; SIMAS-TOSIN, F. F.; SMIDERLE, F. R.; IACOMINI, M. Structural characterization and rheological properties of a gel-like  $\beta$ -D-glucan from *Pholiota nameko*. **Carbohydrate Polymers**, v. 169, p. 1–8, 2017.
- SUN, Y.; LIU, J. Purification, structure and immunobiological activity of a water-soluble polysaccharide from the fruiting body of *Pleurotus ostreatus*. **Bioresource Technology**, v. 100, p. 983–986, 2009.
- SUI, X-C.; GUO, Q-B.; XIA, Y-M.; CUI, S. W.; SHEN, J.; ZHANG, J.; DING, Z-Y. Structure features of the intracellular polysaccharide from *Ganoderma lucidum* and the irrelative immune-anticancer activities of GLPs. **Bioactive Carbohydrates and Dietary Fibre**, v. 8, p. 43–50, 2016.
- TEHRANI, M. H. H.; FAKHREHOSEINI, E.; NEJAD, M. K.; MEHREGAN, H.; HAKEMI-VALA, M. Search for Proteins in the Liquid Extract of Edible Mushroom, *Agaricus bisporus*, and Studying their Antibacterial Effects. **Iranian Journal of Pharmaceutical Research**, Winter, v. 11, n. 1, p. 145–150, 2012.
- TEL-ÇAYAN, G.; MUHAMMAD, A.; DEVECI, E.; DURU, M. E.; ÖZTÜRK, M. Isolation, structural characterization, and biological activities of galactomannans from *Rhizopogon luteolus* and *Ganoderma adspersum* mushrooms. **International Journal of Biological Macromolecules**, v. 165, p. 2395–2403, 2020.
- TIBÉRIO, A. E. G.; PEREIRA, F. C. B. N.; SILVA, S.; SCHEMMER, J. N.; PAULERT, R.; FERNANDES, N. L. M.; ROSADO, F. R.; CORTEZ, V. G. *Macrocybe titans* (Basidiomycota): Cogumelo com Potencial Biotecnológico e sua Ocorrência no Oeste do Paraná. **IV Simpósio de Bioquímica e Biotecnologia**, Londrina, 2014.
- TONELI, J. T. de. C. L.; XIDIEH MURR, F. E. X.; KIL JIN PARK, K. J. Estudo da reologia de polissacarídeos utilizados na indústria de alimentos. **Revista Brasileira de Produtos Agroindustriais, Campina Grande**, Especial, v.7, n.2, p.181-204, 2005.
- TORTORA, G.J.; FUNKE, B.R.; CASE, C.L. **MICROBIOLOGIA**, 12<sup>a</sup> Ed. Artmed, 2017.
- WANG, J.; ZHANG, L. Structure and chain conformation of five water-soluble derivatives of a  $\beta$ -D-glucan isolated from *Ganoderma lucidum*. **Carbohydrate Research**, 344, p. 105–112, 2009.
- WANG, Y. X.; YIN, J. Y.; HUANG, X. J.; NIE, S. P. Structural characteristics and rheological properties of high viscous glucan from fruit body of *Dictyophora rubrovolvata*. **Food Hydrocolloids**, v. 101, 105514, 2020a.
- WANG, J.; ZHOU, Z.; DAN, D.; HU, G. Physicochemical properties and bioactivities of *Lentinula edodes* polysaccharides at different development stages. **International Journal of Biological Macromolecules**, v. 150, p. 573–577, 2020b.
- WANG, X.; WANG, B.; ZHOU, L.; WANG, X.; VEERARAGHAVAN, V. P.; MOHAN, S. K.; XIN, F. *Ganoderma lucidum* put forth anti-tumor activity against PC-3 prostate cancer cells via inhibition of Jak-1/STAT-3 activity. **Saudi Journal of Biological Sciences**, v. 27, p. 2632–2637, 2020c.
- WANI, B. A.; BODHA, R. H.; WANI, A. H. Nutritional and medicinal importance of mushrooms. **Journal of Medicinal Plants Research**, v. 4, n. 24, p. 2598–2604, 2010.
- WIATER, A.; PADUCH, R.; CHOMA, A.; PLESZCZYNSKA, M.; SIWULSKI, M.; DOMINIK, J.; JANUSZ, G.; TOMCZYK, M.; SZCZODRAK, J. Biological study on carboxymethylated (1→3)- $\alpha$ -D-glucans from fruiting bodies of *Ganoderma lucidum*. **International Journal of Biological Macromolecules**, 51, p. 1014–1023, 2012.
- XIAO, C.; WU, Q. P.; CAI, W.; TAN, J. B.; YANG, X. B.; ZHANG, J. M. Hypoglycemic Effects of *Ganoderma lucidum* Polysaccharides in Type 2 Diabetic Mice. **Archives of Pharmacal Research**, v. 35, n. 10, 1793–1801, 2012.

- XU, J. L., ZHANG, J. C., LIU, Y., SUN, H. J., WANG, J. H. Rheological properties of a polysaccharide from floral mushrooms cultivated in Huangshan Mountain. **Carbohydrate Polymers**, v. 139, p. 43–49, 2016.
- XU, Y.; ZHANG, X.; YAN, X. H.; ZHANG, J. L.; WANG, L. Y.; XUE, H.; JIANG, G. C.; MA, X. T.; LIU, X. J. Characterization, hypolipidemic and antioxidant activities of degraded polysaccharides from *Ganoderma lucidum*. **International Journal of Biological Macromolecules**, v. 135, p. 706–716, 2019.
- YANG, G.; QU, Y.; MENG, Y.; WANG, Y.; SONG, C.; CHENG, H.; LI, X.; SUN, L.; ZHOU, Y. A novel linear 3-O-methylated galactan isolated from *Cantharellus cibarius* activates macrophages. **Carbohydrate Polymers**, v. 214, n. 34–43, 2019.
- YANG, X.; LI, A.; LI, X.; SUN, L.; GUO, Y. An overview of classifications, properties of food polysaccharides and their links to applications in improving food textures. **Trends in Food Science & Technology**, v. 102, p. 1–15, 2020.
- YE, L. B.; ZHANG, J. S.; ZHOU, K.; YANG, Y.; ZHOU, S.; JIA, W.; HAO, R. X.; PAN, Y. J. Purification, NMR Study and Immunostimulating Property of a Fucogalactan from the Fruiting Bodies of *Ganoderma lucidum*. **Planta Med**, v. 74, p. 1730–1734, 2008.
- YUAN, Q.; ZHANG, X.; MA, M.; LONG, T.; XIAO, C.; ZHANG, J.; LIU, J.; ZHAO, L. Immunoenhancing glucuronoxylomannan from *Tremella aurantialba* Bandoni et Zang and its low-molecular-weight fractions by radical depolymerization: Properties, structures and effects on macrophages. **Carbohydrate Polymers**, v. 238, 116184, 2020.
- YOO, J. H.; LEE, Y. S.; KU, S.; LEE, H. J. *Phellinus baumii* enhances the immune response in cyclophosphamide-induced immunosuppressed mice. **Nutrition research**, v. 75, p. 15–31, 2020.
- ZENT, E. L.; ZENT, S.; ITURRIAGA, T. Knowledge and use of fungi by a mycophilic society of the venezuelan Amazon. **Economic Botany**, v. 58, n. 2, p. 214–226, 2004.
- ZENG, X.; LI, P.; CHEN, X.; KANG, Y.; XIE, Y.; LI, X.; XIE, T.; ZHANG, Y. Effects of deproteinization methods on primary structure and antioxidant activity of *Ganoderma lucidum* polysaccharides. **International Journal of Biological Macromolecules**, v. 126, p. 867–876, 2019.
- ZHAO, Y.; ZHENG, W. Deciphering the antitumoral potential of the bioactive metabolites from medicinal mushroom *Inonotus obliquus*. **Journal of Ethnopharmacology**, v. 265, 113321, 2021.
- ZHANG, Y., XU, X., ZHANG, L. Dynamic viscoelastic behavior of triple helical Lentinan in water: Effect of temperature. **Carbohydrate Polymers**, v. 73, p. 26–34, 2008.
- ZHANG, J.; GAO, X.; PAN, Y.; XU, N.; JIA, L. Toxicology and immunology of *Ganoderma lucidum* polysaccharides in Kunming mice and Wistar rats. **International Journal of Biological Macromolecules**, v. 85, p. 302–310, 2016.
- ZHANG, Y.; LIU, D.; FANG, L.; ZHAO, X.; ZHOU, A.; XIE, J. A galactomannoglucan derived from *Agaricus brasiliensis*: Purification, characterization and macrophage activation via MAPK and I $\kappa$ B/NF $\kappa$ B pathways. **Food Chemistry**, v. 239, p. 603–611, 2018.
- ZHU, F.; DU, B.; XU, B. A critical review on production and industrial applications of beta-glucans. **Food Hydrocolloids**, v. 52, 275e288, 2016.
- ZILLY, A.; BAZANELLA, G. C. S.; HELM, C. V.; ARAÚJO, C. A. V.; SOUZA, C. G. M.; BRACHT, A.; PERALTA, R. M. Solid-State Bioconversion of Passion Fruit Waste by White-Rot Fungi for Production of Oxidative and Hydrolytic Enzymes. **Food Bioprocess Technology**, v. 5, p. 1573–1580, 2012.

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