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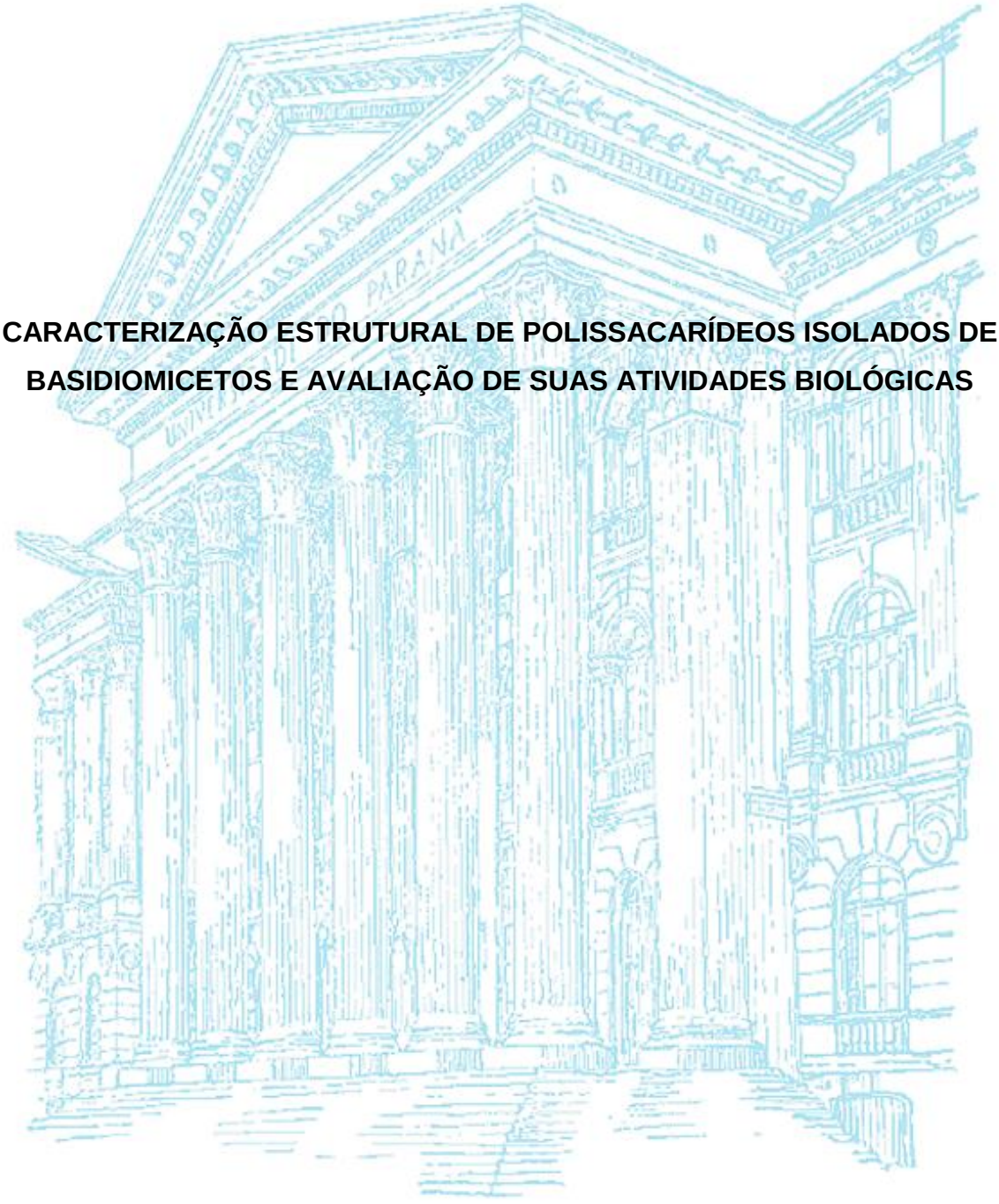

CARACTERIZAÇÃO ESTRUTURAL DE POLISSACARÍDEOS ISOLADOS DE BASIDIOMICETOS E AVALIAÇÃO DE SUAS ATIVIDADES BIOLÓGICAS

CURITIBA

2017

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CARACTERIZAÇÃO ESTRUTURAL DE POLISSACARÍDEOS ISOLADOS DE BASIDIOMICETOS E AVALIAÇÃO DE SUAS ATIVIDADES BIOLÓGICAS

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Orientador: Prof. Dr. Marcello Iacomini
Coorientadora: Dra. Fhernanda Ribeiro Smiderle

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Entrega ao Senhor as tuas obras, e teus desígnios serão estabelecidos.
(Provérbios 16:4)

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RESUMO

Os cogumelos despertam o interesse da comunidade científica porque são considerados como fonte de alimentos e de compostos naturais que possuem um amplo espectro de atividade biológica. Os polissacarídeos são apontados como os compostos mais bioativos presentes nos fungos. Dessa forma, este trabalho apresenta os resultados obtidos a partir da extração, purificação e caracterização estrutural de polissacarídeos das espécies *Piptoporus betulinus*, *Phellinus igniarius* e *Pholiota nameko*. Adicionalmente, foram avaliadas algumas frações em relação às suas atividades biológicas *in vitro* e as propriedades reológicas. A extração dos polissacarídeos foi realizada com água na temperatura ambiente e a 100 °C, e ao resíduo das extrações aquosas foi empregado uma solução de hidróxido de potássio a 5%. Os polissacarídeos extraídos foram purificados a partir dos processos de congelamento e descongelamento, diálise, tratamentos de Fehling e com solução alcalina de hidróxido de sódio na concentração de 0,1 M. A caracterização química foi feita a partir da utilização de métodos analíticos como cromatografia gasosa, ressonância magnética nuclear e cromatografia de exclusão por tamanho para determinar a composição monossacarídica, tipo de ligação, grau de ramificação, configuração da cadeia, massa molecular e homogeneidade. Três glucanas foram isoladas e caracterizadas de *P. betulinus*, sendo a primeira uma β-D-glucana do extrato aquoso quente com conformação em espiral aleatória e com característica de gel. Este polissacarídeo foi testado em relação à sua capacidade de cicatrização *in vitro*, com células de adenocarcinoma de cólon humano (Caco-2). Os resultados mostraram que a β-D-glucana é capaz de acelerar o processo de migração celular para o fechamento da lesão em 55%. Desta forma, este polissacarídeo apresenta um potencial de aplicação para a formulação de dispositivos com o objetivo de diminuir o tempo de cicatrização de cortes e feridas, por exemplo. Os outros dois polímeros foram uma α-D-glucana e uma β-D-glucana insolúveis em água que foram separadas a partir de uma mesma fração do extrato alcalino através do tratamento com hidróxido de sódio 0,1 M. Uma α-D-galactana parcialmente metilada foi obtida a partir da extração aquosa fria do corpo de frutificação de *P. igniarius*. O polissacarídeo obtido apresentou potencial imunomodulador ao estimular a secreção de citocinas TNF-α e IL-10 após incubação com células THP-1. Uma β-D-glucana com alto grau de ramificação foi isolada de *P. nameko* e sua propriedade reológica foi estudada. Os resultados indicaram que o polímero apresenta comportamento de gel e que não há diferença significativa no comportamento entre a fração bruta e a β-D-glucana na mesma concentração em solução, sendo que este comportamento não é alterado frente a variações de temperatura. Em vista disto, extratos de *P. nameko* contendo β-D-glucana possuem potencial para ser utilizados na indústria de alimentos como agentes espessantes, com a possibilidade de promover estabilidade térmica dos produtos diante das variações de temperatura que ocorrem durante os processos de produção, armazenagem e transporte.

Palavras-chave: *Piptoporus betulinus. Phellinus igniarius. Pholiota nameko.* Glucanas. Galactana. Caracterização química. Cicatrização. Atividade Imunomodularora, Reologia.

ABSTRACT

Mushrooms have gained the scientific community interest because they are considered as food and source of natural compounds with wide spectrum of biological activities. Polysaccharides are the most bioactive compounds present in fungi. This work presents results from the extraction, purification and structural characterization of polysaccharides from *Piptoporus betulinus*, *Phellinus igniarius* and *Pholiota nameko* species. Moreover, some fractions were evaluated about their biological activity *in vitro* and rheological properties. The extraction of polysaccharides was sequentially performed with water at room temperature and at 100 °C and to the residue an alkaline extraction was employed with 5% potassium hydroxide. The extracted polysaccharides were purified with freeze and thawing process, dialysis, Fehling solution and 0.1 M sodium hydroxide treatment. The chemical characterization was done with analytical methods such as gas chromatography, nuclear magnetic resonance and high performance size exclusion chromatography to determine the monosaccharide composition, linkage type, branching degree, anomeric configuration, molecular mass and homogeneity. Three glucans were isolated and chemical characterized from *P. betulinus*; the first one is a β-D-glucan from hot aqueous extract with random coil conformation and gel characteristic. This polysaccharide was submitted to scratch *in vitro* assay with human epithelial colorectal adenocarcinoma cells (Caco-2) to verify its wound healing capacity. The results showed that β-D-glucan is capable to accelerate the cells migration to wound closure in 55%. This represents the potential of this polymer to develop wound device to treat lesions in lesser time. The other two polymers isolated from *P. betulinus* are water insoluble β-D-glucan and α-D-glucan which were obtained in the same alkaline fraction and separated from each other by 0.1 M sodium hydroxide treatment. A partially methylated α-D-galactan was obtained from the fruiting bodies of *P. igniarius.* The obtained polysaccharide showed immunomodulatory potential by stimulation of TNF-α and IL-10 secretion after incubation with THP-1 cells. One β-D-glucan with high branching degree was isolated from *P. nameko* and its rheological property was evaluated. The results indicated that the polymer has a gel-like behavior similar to the crude extract at the same concentration and presented thermal stability without altering its rheological behavior. Such characteristics suggested a potential application of *P. nameko* extracts with β-D-glucan to increase thickness of food products and also to promote resistance of products submitted to different temperatures during its processing, storage and transport.

Keywords: *Piptoporus betulinus. Phellinus igniarius. Pholiota nameko.* Glucans. Galactan. Chemical characterization. Wound healing. Immunomodulatory activity. Rheology.

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LISTA DE ABREVIATURAS, SIGLAS E SÍMBOLOS

Solventes e reagentes

Ac_2O	-	Anidrido acético
EtOH	-	Etanol
HOAc	-	Ácido acético
KOH	-	Hidróxido de potássio
Me_2SO	-	Dimetilsulfóxido
Me_2SO-d_6,	-	Dimetilsulfóxido deuterado
MeOH	-	Metanol
MTT	-	Brometo de (3-metil-[4-5-dimetiltiazol-2-il]-2,5 difeniltetrazólio)
$NaBD_4$	-	Borohidreto de sódio deuterado
$NaBH_4$	-	Borohidreto de sódio
NaOH	-	Hidróxido de sódio
PBS	-	Solução salina tamponada
PMA	-	12-miristato 13-acetato de forbol
TFA	-	*Trifluoroacetic acid* (Ácido trifluoacético)

Frações obtidas do cogumelo *Piptoporus betulinus*

HW	-	Extrato polissacarídico obtido após extração aquosa quente do corpo de frutificação do cogumelo
IK5	-	Fração precipitada do congelamento e degelo, após extração alcalina do corpo de frutificação do cogumelo
K5	-	Extrato polissacarídico obtido após extração alcalina do corpo de frutificação do cogumelo
PK5	-	Fração precipitada do tratamento com NaOH 0,1 M, após

congelamento e degelo do extrato polissacarídico K5

R1M - Fração retida na diálise em membrana de 1000 kDa, após congelamento e degelo do extrato polissacarídico HW

SHW - Fração sobrenadante do congelamento e degelo, após extração aquosa quente do corpo de frutificação do cogumelo

SK5 - Fração solúvel do tratamento com NaOH 0,1 M, após congelamento e degelo do extrato polissacarídico K5

Frações obtidas do cogumelo *Phellinus igniarius*

CW - Extrato polissacarídico obtido após extração aquosa fria do corpo de frutificação do cogumelo

PCW - Fração precipitada do congelamento e degelo, após extração alcalina do corpo de frutificação do cogumelo

SCW - Fração sobrenadante do congelamento e degelo, após extração aquosa fria do corpo de frutificação do cogumelo

SCWE3 - Fração eluída na diálise em membrana de 3,5 kDa, após congelamento e degelo do extrato polissacarídico CW-PI

SCWR3 Fração retida na diálise em membrana de 3,5 kDa, após congelamento e degelo do extrato polissacarídico CW-PI

Frações obtidas do cogumelo *Pholiota nameko*

bG-PN - Fração precipitada do congelamento e degelo, após tratamento com solução de Fehling

CW - Extrato polissacarídico obtido após extração aquosa fria do corpo de frutificação do cogumelo

FSCW - Fração sobrenadante de Fehling após extração aquosa a frio e congelamento e degelo

SCW - Fração sobrenadante do congelamento e degelo, após extração aquosa fria do corpo de frutificação do cogumelo

Termos associados à estrutura de polissacarídeos

P	-	Piranosídica
Fuc*p*	-	Fucopiranose
Gal*p*	-	Galactopiranose
Glc*p*	-	Glucopiranose
kDa	-	Quilodaltons
Man*p*	-	Manopiranose

Métodos analíticos

^{13}C-NMR	-	Ressonância magnética nuclear de carbono treze
^{1}H-NMR	-	Ressonância magnética nuclear de hidrogênio
DEPT	-	*Distortionless Enhancement by Polarization Transfer*
GC-MS	-	Cromatografia gasosa acoplada à espectrometria de massa
HPSEC	-	*High pressure size exclusion chromatography* (Cromatografia de exclusão estérica de alta performance)
HSQC	-	*Heteronuclear Single Quantum Coherence* (coerência heteronuclear simples quântica)
MALLS	-	Detector de espalhamento de luz laser em multiângulos
MHz	-	Megahertz
NMR	-	*Nuclear magnetic resonance* (Ressonância magnética nuclear)
Ppm	-	Partes por milhão

Termos associados à análise reológica

G"	-	Módulo de perda ou viscoso
G´	-	Módulo de armazenamento ou elástico
Pa	-	Pascal

Termos associados à análise conformacional

Vg	-	Inclinação dos pontos logarítmicos relacionados com a conformação em solução de acordo com a escala teórica
M*w*	-	Massa molecular
Rg	-	Raio de giro
SEC	-	Cromatografia de exclusão por tamanho

Atividade biológica

Caco-2	-	Células de adenocarcinoma de cólon humano
FBS	-	Soro fetal bovino
DMEN/F-12	-	*Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12*
RPMI	-	Meio Roswell Park Memorial Institute
THP-1	-	Células de leucemia monocítica aguda humana

Análises Estatísticas

ANOVA	-	Análise de variância

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1. INTRODUÇÃO

Cogumelos integraram parte da cultura e história da humanidade por milhares de anos por serem considerados fonte de alimentos e compostos com propriedades farmacológicas. Em muitas partes do mundo os cogumelos são alimentos populares devido a textura e sabor, por apresentarem baixo valor calórico e possuírem carboidratos, lipídios e proteínas em sua composição (VALVERDE; HERNÁNDEZ-PÉREZ; PAREDES-LÓPEZ, 2015). Outros compostos como triterpenos, lactonas, alcalóides e vitaminas estão presentes também, o que reforça sua importância nutricional (WASSER, 2014). A composição química favorece o uso dos cogumelos como alimento funcional e nutracêutico devido a capacidade de aumentar o valor nutritivo do alimento e potencial terapêutico (CHANG, 2005).

Estudos demonstraram que inúmeros cogumelos possuem uma ampla faixa de atividade biológica dentre as quais se destacam a antioxidante, antitumoral, antiviral, anti-inflamatória e imunomoduladora. Recentemente os cogumelos despertaram o interesse da comunidade científica para a utilização como agentes naturais potentes para a prevenção e tratamento de diversas doenças como câncer, diabetes mellitus e doenças cardiovasculares e neurodegenerativas (ZHANG et al., 2016). As propriedades medicinais são atribuídas a diversas substâncias que têm sido isoladas e identificadas a partir dos corpos de frutificação, dos micélios e caldo fermentado (ELISASHVILI, 2012).

Polissacarídeos derivados de fungos pertencem a um grande grupo de biopolímeros dentre os quais alguns fazem parte da estrutura da parede celular fúngica. Além de possuir função estrutural, algumas moléculas também são capazes de formar inclusões intracelulares e anexar outras superfícies. E, há também outros compostos que podem servir como fonte de reserva de energia ou ser excretados para o meio extracelular caracterizando um mecanismo de proteção celular. Muitos polissacarídeos são derivados de cogumelos comestíveis, o que representa uma grande vantagem para o seu emprego no desenvolvimento de alimentos funcionais ou nutracêuticos e medicamentos (GIOVASIS, 2014).

A atividade biológica dos polissacarídeos está diretamente relacionada com a estrutura química. Diferentes tipos de ligação, grau de ramificação, massa molecular e solubilidade influenciam no mecanismo de ação exercido pela molécula resultando em diferentes efeitos farmacológicos. A análise química deve ser

criteriosa para determinar a melhor relação entre estrutura química e propriedade farmacológica para melhor compreensão dos efeitos terapêuticos atribuídos aos polissacarídeos fúngicos (GIOVASIS, 2014; RUTHES, SMIDERLE e IACOMINI, 2015).

Os basidiomicetos escolhidos para o presente trabalho foram: *Piptoporus betulinus, Phellinus igniarius e Pholiota nameko* por apresentarem valor nutricional e excelentes propriedades medicinais. Dessa forma, o presente estudo teve como finalidade caracterizar os polissacarídeos, estudar a atividade biológica e avaliar o comportamento reológico de alguns dos polissacarídeos isolados.

2. REVISÃO BIBLIOGRÁFICA

2.1 FILO BASIDIOMYCOTA

Os fungos pertencentes ao filo Basidiomycota (popularmente conhecidos como basidiomicetos) apresentam como principal característica a existência de um basídio que carrega esporos (basidiósporos) (ESPOSITO e AZEVEDO, 2004). A maioria é terrestre e seus esporos são dispersos pela ação do vento, mas há também um pequeno grupo que habita em águas doces e marinhas. Muitos são organismos saprófitas porquanto se alimentam de matéria orgânica em decomposição. Dessa forma, desempenham um papel importante na reciclagem de biomoléculas ao atuarem no processo de decomposição do substrato e produção de substâncias que favorecem o aproveitamento por outros organismos, além de beneficiar a fertilização do solo (WEBSTER e WEBER, 2007).

Desde a antiguidade, os cogumelos são considerados como alimento devido ao seu comprovado valor nutritivo apresentando alto teor de proteínas, fibras e carboidratos e baixos teores de gordura, contribuindo para uma dieta balanceada. Seu uso na culinária também é favorecido pelo sabor e aroma que apresentam. Além da importância gastronômica, os cogumelos são empregados há milhares de anos nos países orientais como fonte medicinal para tratamento de diversas enfermidades (SMIDERLE, 2012; FURLANI e GODOY, 2005).

Basidiomicetos contêm compostos biologicamente ativos presentes nos corpos de frutificação, nos micélios e no caldo fermentado. Eles são popularmente utilizados na forma de extratos ou em pó para prevenir e tratar doenças. Estudos

realizados com extratos de cogumelos comprovaram que estes possuem propriedades antibacteriana, antifúngica, antiviral, antioxidante e que podem ser também empregados como nematicidas (WASSER, 2014; WANI, BODHA e WANI, 2010). Dentre os compostos existentes nos basidiomicetos, os polissacarídeos se destacam por demonstrar um grande espectro de atividade biológica, sendo em sua maioria representados por glucanas. D-Glucanas são responsáveis principalmente por ampliar a resposta do sistema imune por células e exibir efeito antitumoral em animais e humanos (ZONG, CAO e WANG, 2012; WASSER, 2014).

Diante das propriedades medicinais e nutricionais apresentadas pelos compostos presentes nos fungos do filo Basidiomycota, os estudos científicos para o seu emprego no desenvolvimento de fármacos e alimentos funcionais se intensificaram nas últimas décadas (GIOVASIS, 2014). Dessa forma, esse filo desperta grande interesse para o isolamento, purificação e caracterização de substâncias para estabelecer uma relação entre a estrutura química e atividade biológica (LINDEQUIST *et al*., 2014). Para ampliar esse conhecimento, nesse trabalho, foram estudados alguns gêneros de basidiomicetos: *Piptoporus, Phellinus e Pholiota*.

2.1.1 Gênero *Piptoporus*

Piptoporus betulinus é considerado um fungo parasita comum e importante de árvores da família Betulaceae (conhecidas como Bétulas), normalmente encontradas na Europa, América do Norte e Ásia. Esse fungo é responsável pela decomposição da madeira de Bétulas envelhecidas e em estado frágil e é comumente conhecido por Bétula poliporácea (do inglês, *Birch polypore*), nome que faz alusão ao gênero da planta que parasita (*Betula*) e da ordem que pertence o fungo (*Polyporales*). Seu corpo de frutificação possui coloração branco-marrom (FIGURA 1) e quando se apresenta na fase jovem é comestível, sendo que essa parte do cogumelo é utilizada há muito tempo na medicina popular devido às reconhecidas propriedades medicinais (PLESZCZYNSKA *et al*., 2016; GRIENKE *et al*., 2014).

FIGURA 1. ESPÉCIE *Piptoporus betulinus*.
Fonte: amadej.trnkoczy[AT]siol.net., acesso em 09/05/2017

A evidência mais antiga do uso de *P. betulinus* pela humanidade data de 5300 anos atrás. Esse fungo foi achado nos pertences carregados pela múmia Ötzi (conhecida como homem do gelo) o qual foi encontrado na região de Tirol na Itália em 1991. Cientistas acreditam que esse cogumelo era usado pelo homem do gelo como purgativo e remédio para combater parasitas intestinais (LEMINIESK *et al*., 2009).

Suas propriedades nutricionais e medicinais são reconhecidas em diversos países e, consequentemente, seu emprego é muito popular para a manutenção de uma dieta balanceada e para o tratamento de diversas enfermidades. Como exemplo, países como a Rússia, Hungria e Romênia utilizam infusões elaboradas a partir dos corpos de frutificação de *P. betulinus* para obter efeitos calmantes e como suplemento para uma dieta equilibrada. Na Sibéria e na Finlândia, os chás feitos a partir desse cogumelo são empregados para o tratamento de diversos tipos de câncer. Na América do Norte e na Sibéria o pó obtido de *P. betulinus* é usado para aliviar a dor. Outras atividades biológicas relatadas na literatura para extratos e compostos isolados de *P. betulinus* estão relacionadas com os efeitos anti-inflamatórios, antiviral e imunomodulador (PLESZCZYNSKA *et al*., 2017; GRIENKE *et al*., 2014; DRESCH *et al*., 2015).

Dentre os compostos bioativos já identificados nesta espécie, podemos destacar os metabólitos secundários, em especial os triterpenos. ALRESLY e colaboradores (2016) isolaram 10 compostos a partir de um extrato de acetato de etila como ácido betulínico, betulina, peróxido de ergosterol entre outros, sendo que

todos apresentaram atividade antimicrobiana e antifúngica. KAMO e colaboradores (2013) isolaram substâncias semelhantes do corpo de frutificação e testes *in vitro* e *in vivo* demonstraram atividade antitumoral e anti-inflamatória das mesmas. SCHLEGEL e colaboradores (2000) extraíram um antibiótico denominado piptamina (FIGURA 2) através do cultivo submerso do cogumelo e o composto apresentou atividade antimicrobiana para bactérias gram-positivas, especialmente *Staphylococcus aureus* e *Enterococcus faecalis*, além de abranger fungos patogênicos também. α-D-Glucanas foram isoladas a partir do emprego de soluções alcalinas de hidróxido de sódio: OLENNIKOV e colaboradores (2012) realizaram a caracterização química do polissacarídeo obtido, enquanto WIATER e colaboradores (2011) realizaram uma reação de carboximetilação do polímero para aumentar sua solubilidade e testá-lo quanto ao seu potencial antioxidante.

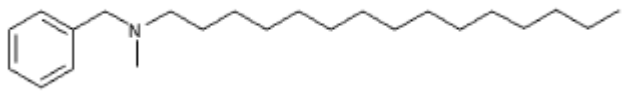

FIGURA 2. ESTRUTURA QUÍMICA DA PIPTAMINA ISOLADA DE *Piptoporus betulinus.*
Fonte: Adaptado de PLESZCZYNSKA *et al.* (2017)

Há poucos relatos na literatura que descrevem o isolamento, a caracterização química e a atividade biológica de compostos isolados do fungo *P. betulinus*. Grande parte dos estudos está relacionada aos extratos brutos obtidos a partir de diferentes métodos analíticos e a avaliação de suas atividades biológicas. Consequentemente, os efeitos farmacológicos observados não podem ser atribuídos exclusivamente a uma ou outra molécula isolada deste cogumelo. Em vista disso, é necessário aprimorar a pesquisa que envolve a identificação de compostos bioativos desse fungo e também estabelecer a relação entre estrutura química e atividade biológica contribuindo para elevar seu potencial de aplicação (PLESZCZYNSKA *et al*., 2017).

2.1.2 Gênero *Phellinus*

O gênero *Phellinus* pertence à família Hymenochaetaceae e possui 138 espécies identificadas no mundo todo. Muitas espécies como *P. igniarius*, *P. linteus*, *P. gilvus*, *P. pini* e *P. hartigii* são conhecidas pela variedade de propriedades medicinais que apresentam sendo muito utilizadas na medicina tradicional chinesa. *P. iginiarius* geralmente cresce em árvores saudáveis e de clima temperado sendo muito comum em países da Europa, na China e no Japão. Apresenta corpo de frutificação na coloração marrom no estágio jovem (FIGURA 3), o qual vai escurecendo com o envelhecimento até adquirir a cor negra, além de mostrar consistência dura e aspecto lenhoso (ZAPORA *et al*., 2016).

FIGURA 3. ESPÉCIE *Phellinus igniarius*.
Fonte: http://out-grow.com/fungi-cultures-c-2/10ml-liquid-culture-syringes-false-tinder-conk-phellinus-igniarius-p-257.html, acesso em 09/05/2017.

Há uma demanda muito grande por esta espécie na Ásia e, consequentemente, houve considerável declínio de sua população na natureza por causa do excesso de coleta realizada nos últimos anos. Há pouca informação disponível sobre sua reprodução e distribuição no seu habitat natural o que dificulta as ações voltadas para sua conservação e manejo. Somado a isso, diversos métodos testados para o seu cultivo artificial não têm sido bem sucedidos, o que acarreta no aumento de seu valor de mercado. Sua alta procura está no fato de *P. iginiarius* ser rico em compostos bioativos como esteroides, flavonoides, cumarinas,

macrolídeos, sesquiterpenos e polissacarídeos (ZAPORA *et al*., 2016; YUAN *et al*., 2015; GUO, ZOU e MIN, 2010).

Extratos obtidos a partir do corpo de frutificação, do micélio e do filtrado de cultura exibiram efeitos neuroprotetores na isquemia cerebral e na doença de Alzheimer, inibição da enzima xantina oxidase (responsável pela produção de ácido úrico) e ação antioxidante através da inibição de radicais livres. Há estudos que relatam também o potencial terapêutico dos extratos em impedir a progressão de doenças cardiovasculares e esclerose múltipla. Além desses efeitos, diversas pesquisas constataram o potencial do uso desses extratos para tratar diferentes tipos de câncer (ZAPORA *et al*., 2016; YIN *et al*., 2015; JIN *et al.*, 2014).

Também foram descritos na literatura alguns relatos sobre as propriedades medicinais exibidas por compostos isolados desse fungo. WU e colaboradores (2014) isolaram um composto fenólico de *P. igniarius* denominado hispolona, o qual apresentou potente ação antitumoral contra a linhagem celular humana de câncer de pulmão. KIM e colaboradores (2016) identificaram duas substâncias no extrato metanólico desse cogumelo cuja estrutura química refere-se às feligridinas E e G (FIGURA 4). Esses sesquiterpenos diminuíram a capacidade de proliferação e infecção do vírus influenza devido à inibição da glicoproteína neuraminidase.

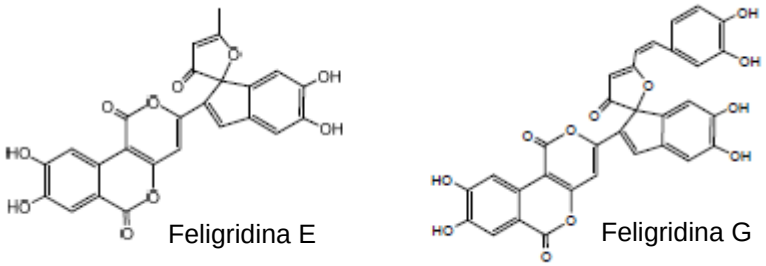

FIGURA 4. ESTRUTURA QUÍMICA DAS FELIGRIDINAS E e G ISOLADAS DE *Phellinus igniarius.*
Fonte: Adaptado de ZAPORA *et al.* (2016).

Os polissacarídeos são considerados uma das classes de compostos medicinais mais ativas presentes nesse fungo, sendo sua grande maioria representada por β-glucanas. Nas últimas décadas, os polissacarídeos isolados das espécies de *Phellinus* apresentaram principalmente potencial inibidor para o

crescimento de tumores e metástases com baixa toxicidade, sendo moléculas candidatas para o desenvolvimento de medicamentos antitumorais naturais (ZAPORA *et al*., 2016; GUO, ZOU e MIN, 2010). Extratos de polissacarídeos obtidos do corpo de frutificação desse cogumelo apresentaram também atividades antioxidante, anti-inflamatória e imunomoduladora (SUABJAKYONG *et al*., 2015; LUNG, TSAI e HUANG *et al*., 2009). Entretanto, pouca atenção foi concedida para a extração, purificação e caracterização dos polissacarídeos de *P. iginiarius* até o momento, o que pode ser constatado pelo baixo número de estudos publicados (GUO; ZOU e MIN, 2010).

2.1.3 Gênero *Pholiota*

O gênero *Pholiota* pertence à família Strophariaceae sendo uma das espécies mais conhecidas o *Pholiota nameko*. Esse fungo comestível é um dos mais cultivados no Japão juntamente com *Lentinus edodes*, *Flammulina velutipes* e *Pleurotus ostreatus*. O nome nameko, que significa cogumelo viscoco em japonês, é devido à camada gelatinosa que apresenta sobre seu corpo de frutificação. Esse fungo desenvolve-se em troncos de árvores em decomposição, apresenta coloração caramelo e seu habitat natural são as florestas densas da Ásia, sendo também amplamente cultivado na China (FIGURA 5). Por apresentar sabor agradável e aspecto gelatinoso, é muito utilizado na culinária oriental, exibindo elevado valor nutricional pela presença de diversos nutrientes como vitaminas, aminoácidos essenciais, proteínas, lipídios e polissacarídeos. Além da presença de componentes importantes para uma dieta balanceada e saudável, também apresenta compostos que possuem diferentes atividades biológicas (ARITA, 1978; NEDA, 2007; SOVRANI, 2016).

FIGURA 5. ESPÉCIE *Pholiota nameko.*
Fonte: https://ekokube.com/en/pholiota.html, acesso em 09/05/2017.

RODRIGUES e colaboradores (2016) empregaram diferentes métodos de extração e obtiveram frações do cogumelo *P. nameko* que apresentaram atividade antidiabética e prebiótica. JI e colaboradores (2012) realizaram extração aquosa e etanólica desse cogumelo, sendo que os extratos obtidos exibiram atividade antioxidante. Proteínas obtidas deste mesmo fungo apresentaram atividade antitumoral contra a linhagem celular humana de câncer de mama (ZHANG *et al*., 2014; QIAN *et al*., 2016). Dentre as substâncias de *P. nameko* estudadas, os polissacarídeos isolados ou complexados com outros compostos se destacam por apresentar um amplo espectro de atividades biológicas (LI *et al*., 2012).

ZHENG e colaboradores (2014) utilizaram um polissacarídeo obtido do micélio de *P. nameko* como vetor para reagir com zinco com o objetivo de formar um complexo zinco-polissacarídeo para empregar no desenvolvimento de um composto com propriedades anti-hiperlipidêmica e hepatoprotetora. Polissacarídeos isolados desse mesmo cogumelo apresentaram também atividade anti-inflamatória (LI *et al*., 2008), imunomoduladora e antitumoral (CHEN e XIANG, 2013). Os polissacarídeos mais abundantes em *P. nameko* são as D-glucanas, as quais atuam como fibras insolúveis, possibilitando que esse fungo seja empregado na formulação de alimentos funcionais com propriedades prebióticas (RODRIGUES *et al*., 2016). Outra finalidade para as D-glucanas desse cogumelo é seu emprego na indústria

alimentícia como agente espessante ou gelificante em diferentes preparações (SOVRANI *et al*., 2017).

Dessa forma, o cogumelo *P. nameko* se constitui uma excelente fonte para extração de diversos compostos bioativos de interesse para a indústria alimentícia e farmacêutica a ser explorada no campo científico (RODRIGUES *et al*., 2016).

2.2 POLISSACARÍDEOS FÚNGICOS

Os polissacarídeos compreendem um grande grupo de biopolímeros de alta massa molecular, formados por unidades monossacarídicas unidas entre si por ligações glicosídicas. O carbono anomérico, que está envolvido na ligação, pode apresentar configuração α ou β, dependendo da posição da hidroxila. Sua estrutura química difere quanto à composição monossacarídica, tipo de ligações, tamanho da cadeia, grau de ramificação e conformação espacial. Além disso, outros fatores como fonte, métodos de extração e procedimentos de purificação podem influenciar de forma significativa nas propriedades físico-químicas e estruturais dos polissacarídeos (SILVEIRA, 2015; WANG *et al*., 2015).

Os polissacarídeos podem ser encontrados na célula do fungo exercendo função estrutural e de reserva de energia, além de prover um mecanismo de proteção ou adesão celular com outras superfícies. Muitos são derivados de cogumelos comestíveis o que oferece uma grande vantagem em relação ao seu uso em alimentos e no tratamento de diversas doenças (GIOVASIS, 2014).

No entanto, apenas alguns polissacarídeos têm sido comercializados na forma de pó ou em cápsulas para tratamento de doenças. O alto custo de produção e purificação e o rendimento baixo faz com que poucos cheguem ao mercado. Um dos principais problemas está relacionado com a produção desses biopolímeros a partir do corpo de frutificação, entretanto essa dificuldade pode ser amenizada pelo cultivo submerso, o qual permite produzir micélio em larga escala, com o uso de biorreatores, por exemplo (GIOVASIS, 2014).

Os polissacarídeos constituem os principais compostos bioativos presentes nos fungos e suas atividades biológicas estão relacionadas principalmente com o aumento da resposta imune inata e mediada por células, além da ação antitumoral exibida em animais e humanos. Dentre os polissacarídeos produzidos por cogumelos estão a quitina e as glucanas, os quais formam a parede celular e,

heteropolissacarídeos e glicopeptídeos existentes na matriz celular (FIGURA 6). Desses, as glucanas se destacam por estarem relacionadas com atividades anti-inflamatória, antioxidante, imunomoduladora e antitumoral (WASSER, 2014; SMIDERLE, 2012; MANZI E PIZZOFERRATO, 2000).

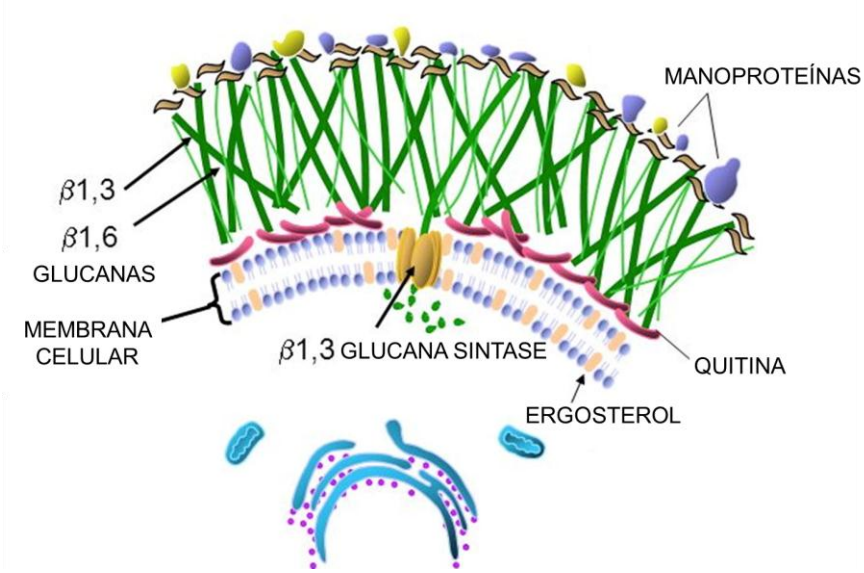

FIGURA 6. ESTRUTURA DA PAREDE CELULAR FÚNGICA.
Fonte: Adaptado de http://www.nammex.com/redefining-medicinal-mushrooms/, acesso em 09/05/2017.

2.3 GLUCANAS DE FUNGOS

Apesar de sua composição monossacarídica ser simples, as glucanas possuem ampla variabilidade estrutural. Elas se diferenciam em relação à sua massa molecular, posição e distribuição das ligações glicosídicas, grau de ramificação e configuração anomérica. Esses polímeros podem apresentar unidades em duas configurações estruturais (α ou β), ou ainda mesclar ambas na mesma estrutura. As glucanas em configuração β são a forma mais comumente encontrada em fungos, sendo que as mais isoladas de basidiomicetos são constituídas por resíduos de β-D-Glc*p* unidas por ligações (1→3) na cadeia principal, sendo algumas dessas unidades substituídas em O-6 por resíduos de β-D-Glc*p* ou cadeias laterais de β-D-Glc*p*-(1→3)-β-D-Glc*p*. Pelo alto teor de hidroxilas e capacidade de formar pontes de hidrogênio, glucanas fúngicas podem formar complexos com outros

polissacarídeos, proteínas, compostos fenólicos, entre outros compostos. Essas interações intermoleculares dificultam sua purificação, entretanto esse pode ser um dos fatores que influenciam suas estruturas secundárias e características físico-químicas, alterando consequentemente o desempenho de suas atividades biológicas (SYNYTSYA e NOVAK, 2014; RUTHES, SMIDERLE e IACOMINI, 2015; ZHU et al., 2015). A principal atividade biológica atribuída às D-glucanas é o efeito imunomodulador, o qual é mediado por receptores presentes na superfície de macrófagos, células dendríticas e neutrófilos, os quais reconhecem essas moléculas. Os receptores frequentemente encontrados nestas células são dectina-1, receptores Toll-like (TLR-2, TLR-4, TLR-6), receptor do sistema complemento três (CR3), lactosilceramida (LacCer) e receptores de varredura (FIGURA 7).

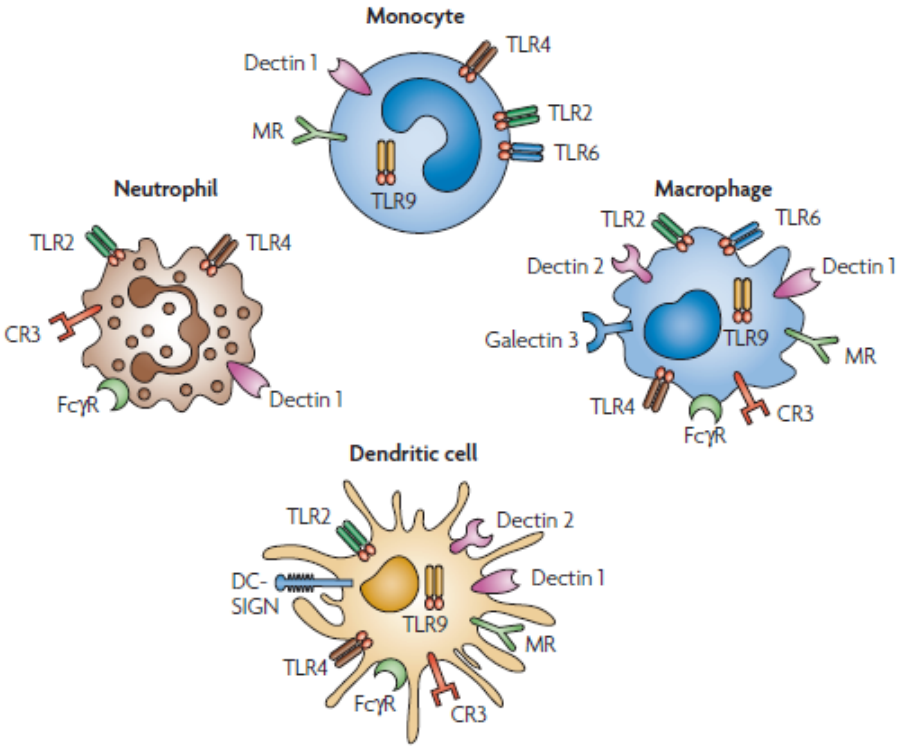

FIGURA 7. REPRESENTAÇÃO ESQUEMÁTICA DOS RECEPTORES DAS CÉLULAS DO SISTEMA IMUNE.
Fonte: Adaptado de NETEA *et al* (2008).

A interação entre estes receptores e os polissacarídeos desencadeia uma série de eventos que modulam a resposta imune inata e adaptativa através da liberação de citocinas pró-inflamatórias (IL-1α/β, IL-6, IL-8, IL-12, TNF-α) assim

como também moléculas citotóxicas que atuam como mediadores inflamatórios como o óxido nítrico e o peróxido de hidrogênio. Em determinadas situações, a produção de citocinas e mediadores inflamatórios pode auxiliar o sistema imune a combater doenças infecciosas, tumorais e autoimunes como o diabetes mellitus tipo 1 (CHANPUT et al., 2012; VANNUCCI et al., 2013). A tabela 1 mostra exemplos de diferentes atividades biológicas relatadas para glucanas isoladas de fungos.

TABELA 1 – Atividades biológicas de glucanas extraídas de fungos

Efeito Biológico	Experimento	Fonte	Glucana	Referência
Imunomodulatório	*In vivo*	*Agaricus blazei*	α-glucana (1→4), (1→6)	Mizuno *et al.*, (1998)
	In vivo	*Pleurotus sajor caju*	β-glucana-(1→3),	Silveira *et al.*, (2014)
Antitumoral	*In vivo* *In vitro e* *In vivo*	*Lentinus edodes*	Lentinana β-glucana-(1→3), (1→6)	Ng; Yap (2002) Markova *et al.*, (2003)
	In vivo	*Grifola frondosa*	Grifolan β-glucana-(1→3), (1→6)	Nanba; Kubo (1997).
	In vivo	*Agaricus blazei*	Extrato rico em glucanas	Hardy (2008)
Anti-inflamatório	*In vivo*	*Pleurotus pulmonaris*	β-glucana-(1→3), (1→6)	Smiderle *et al.*, (2008)
	In vivo	*Lactarius rufus*		Ruthes *et al.*, (2013)
Pró-inflamatório	*In vitro*	*Pleurotus sajor caju*	β-glucana-(1→3), (1→6)	Carbonero *et al.* (2012)
Antinociceptivo	*In vivo*	*Pleurotus pulmonaris*	β-glucana-(1→3), (1→6)	Smiderle *et al.*, (2008) Baggio *et al.*, (2012)
	In vivo	*Lactarius rufus*		Ruthes *et al.*, (2013)
	In vivo	*Cookeina tricholoma*		Moreno *et al.*, (2016)
Antidiabético	*In vivo*	*Agaricus blazei*	β-glucana complexada com proteínas	Kim *et al.*, (2005)
Antioxidante	*In vitro*	*Entoloma lividoalbum*	β-glucana-(1→3), (1→6)	Maity *et al.*, (2014)
	In vitro	*Russula albonigra*		Nandi *et al.*, (2014)
Prebiótico	*In vitro*	*Pleurotus ostreatus e Pleurotus. eryngii*	β-glucana-(1→3), (1→6) e α-glucana-(1→3)	Synytisya *et al.*, (2009)
Antiviral	*In vitro*	*Pleurotus tuber-regium*	β-glucana-(1→3), (1→6)	Zhang *et al.*, (2004)
	In vitro e *In vivo*	*Lentinus edodes, Ganoderma lucidum, Inonotus obliquus*	Extrato rico em glucanas	Vetvicka e Vetvickova (2015)

Fonte: Adaptado de Smiderle (2012).

2.4 GALACTANAS DE FUNGOS

Galactanas são definidas como polímeros de galactose, e podem ser encontradas em algas, fungos e líquens. Na literatura há poucos relatos de galactanas derivadas de fungos, sendo que o gênero *Pleurotus* é o mais conhecido quanto à produção desses polissacarídeos (DELATTRE, FENORADOSOA e MICHAUD, 2011). α-Galactanas parcialmente metiladas foram isoladas de *Pleurotus ostreatoroseus* Sing e *Pleurotus eryngii* (ROSADO *et al*., 2002; CARBONERO *et al*., 2008), sendo uma com ligações α-(1→4) e a outra com ligações α-(1→6). Os polissacarídeos mais comumente encontrados em cogumelos contendo galactose são as heterogalactanas como manogalactanas, fucomanogalactanas e glucogalactanas (RUTHES, SMIDERLE e IACOMINI, 2016). LU e colaboradores (2010) caracterizaram uma α-galactana-(1→3),(1→6) de *Poria cocos* com resíduos de fucose, enquanto VINOGRADOV e WASSER (2005) extraíram uma galactana parcialmente metilada com resíduos de glucose de *Inonotus levis*. Uma heterogalactana mais complexa foi obtida de *Phellinus igniarius* por YANG e colaboradores (2007), cuja estrutura apresentava resíduos de 3-*O*-metil-D-galactose ligados (1→6), contendo glucose, manose e fucose. O estudo de galactanas de origem fúngica ainda é pouco expressivo e a pesquisa que envolve o isolamento, caracterização e determinação de atividades farmacológicas requer maior atenção (PIERRE et al., 2014).

2.5 ESTUDO DA ATIVIDADE IMUNOMODULADORA *IN VITRO* COM CÉLULAS THP-1

2.5.1 Macrófagos

Os macrófagos desempenham um papel importante na imunidade porque representam a primeira linha de defesa do organismo após a barreira epitelial. Eles são responsáveis por processar o material antigênico antes de apresentá-lo a outras células do sistema imune. A ativação de macrófagos por polissacarídeos ocorre por consequência da ligação desses polímeros com os receptores presentes na superfície destas células. Esta interação resulta na ativação da fagocitose, do sistema complemento, do *burst* respiratório, na produção de citocinas como TNF-α e

IL-10 e de enzimas ciclooxigenases como a COX-2. Estes efeitos favorecem a diferenciação e a proliferação celular possibilitando que o hospedeiro defenda-se contra patógenos e o desenvolvimento de tumores. Desta forma, os efeitos farmacológicos destes polímeros resultam na habilidade de modular a resposta imune celular mediada por macrófagos (SILVEIRA, 2015; VANNUCCI *et al*., 2013; MOSSER e EDWARDS, 2008).

2.5.2 Células THP-1 e diferenciação por PMA (forbol 12-miristato 13-acetato)

THP-1 é uma linhagem celular de monócitos humanos comumente utilizada para avaliar a habilidade de compostos em modular a atividade de macrófagos. Devido à circulação de monócitos no sangue, estas células podem acessar facilmente o local da infecção e responder rapidamente ao reconhecimento de substâncias derivadas de patógenos ou invasores e se diferenciar em macrófagos. Nos experimentos *in vitro*, estas células são descritas no estado ativado/diferenciado, o qual pode ser alcançado pela estimulação exercida pelos ativadores inflamatórios (LPS ou citocinas) ou ainda pela presença de forbol 12-miristato 13-acetato (PMA) (LUND *et al*., 2016; AUWERX, 1991, CHANPUT, MES e WICHERS, 2013).

Sob a influência do PMA, as células THP-1 paralisam sua proliferação e se diferenciam em macrófagos. A diferenciação está associada a uma drástica mudança em sua morfologia, quando se observa um núcleo irregular e os vacúolos podem ser visualizados no citoplasma. O processo de diferenciação é caracterizado também pelo aumento da aderência das células na placa de cultura celular. Após o tratamento com PMA, as células THP-1 se diferenciam em macrófagos mimetizando o comportamento do macrófago nativo em diversos aspectos (AUWERX, 1991).

2.5.3 Ativação das células THP-1 por LPS

O LPS (lipopolissacarídeo) é uma toxina presente na membrana externa de bactérias gram-negativas, sendo considerado um potente estimulador da resposta imune, por ligar-se ao complexo CD14/TLR4/MD2, promovendo a secreção de citocinas pró-inflamatórias. Após a exposição das células diferenciadas THP-1 ao LPS, ocorre a ativação do fator de trascrição NF-κB, o que favorece a expressão de

genes que conduzem à inflamação, proliferação, diferenciação e migração celular. Consequentemente ocorre o aumento da aderência celular, da atividade fagocítica e da expressão de genes para a produção de citocinas (IL-1β, IL-6, IL-8, IL-10 e TNF-α). Esta resposta inflamatória pode causar danos ao organismo quando estimulada em níveis elevados e, portanto, sua inibição ou manutenção em níveis basais é requerida para o tratamento de diversas enfermidades (SILVEIRA, 2015; LUND *et al*., 2016; CHANPUT, MES e WICHERS, 2013). Por seus efeitos, o LPS é comumente utilizado nos testes *in vitro* em THP-1 como controle positivo e parâmetro de comparação entre as amostras testadas.

2.6 ESTUDO DA MIGRAÇÃO CELULAR (*WOUND HEALING* OU *CELL SCRATCH ASSAY*)

2.6.1 Lesão tecidual e cicatrização

A lesão tecidual é o estímulo inicial para o desenvolvimento do processo de cicatrização, sendo um processo sistêmico e dinâmico que resulta em uma cascata de eventos celulares para a reconstituição do tecido. A cicatrização possui três fases: inflamatória, proliferação e remodelação e este processo está representado na FIGURA 8 (CAMPOS, BORGES-BRANCO e GROTH, 2007).

A fase inflamatória é caracterizada pela agregação plaquetária e coagulação sanguínea que formam uma matriz para a migração celular e serve como reservatório para as citocinas e fatores de crescimento que serão liberados nas fases seguintes. Na degranulação plaquetária são liberados fatores de crescimento e glicoproteínas adesivas que, juntamente, com a cascata de coagulação produz mediadores químicos que recrutam células inflamatórias (neutrófilos e monócitos) para o local da lesão. Estas células fagocitam bactérias, fragmentos celulares e corpos estranhos. No caso dos monócitos, a fagocitose aliada ao contato dos fatores liberados pelas plaquetas contribui para a sua ativação transformando-os em macrófagos. Os macrófagos são as principais células envolvidas no controle do processo de cicatrização que, além de fagocitar componentes celulares, produz citocinas que atuam na formação do tecido de granulação (MENDONÇA e COUTINHO-NETTO, 2009).

A fase proliferativa é a etapa que ocorre o fechamento da lesão e compreende a reepitelização (migração das células epiteliais), fibroplasia (proliferação de fibroblastos) e angiogênese (proliferação de células endoteliais dos capilares). Essa proliferação celular é ativada por mediadores vasoativos e fatores quimiotáticos liberados na fase inflamatória, resultando no desenvolvimento de uma nova matriz extracelular com vasos sanguíneos novos denominada de tecido de granulação, o qual irá ocupar o local da lesão (MENDONÇA e COUTINHO-NETTO, 2009).

Na fase de remodelação, destaca-se a reorganização da matriz que é substituída pela deposição de colágeno. A proliferação celular diminui de intensidade e a maioria das células sofre apoptose ou migra da região da lesão. A degradação da matriz por enzimas proteolíticas é muito importante para o remodelamento do tecido, sendo que a área passa a ser constituída por poucas células, por colágeno e outras proteínas. A maturação e o remodelamento da matriz é um processo gradual e que envolve diversas etapas de produção, digestão e orientação das fibras de colágeno (JEREMIAS, 2013).

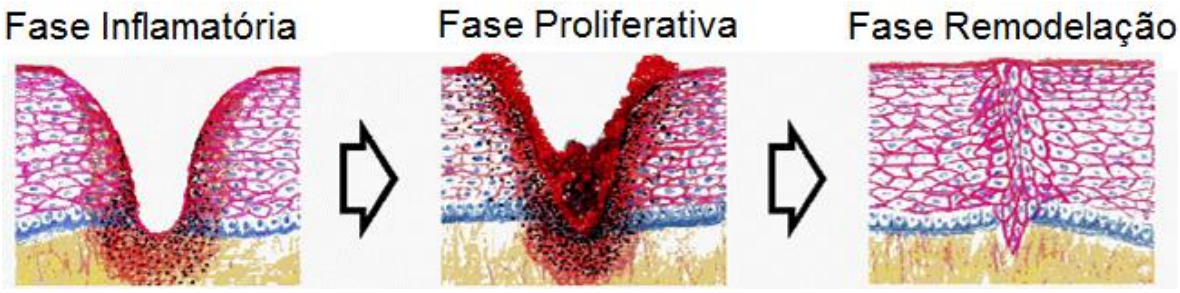

FIGURA 8. REPRESENTAÇÃO ESQUEMÁTICA DAS FASES DE CICATRIZAÇÃO. Fonte: http://www.misodor.com/cicatrizacao.html, acesso em 20/11/2017.

2.6.2 Migração celular *in vitro*

O ensaio de migração celular *in vitro* é um método para estudar o processo de cicatrização. O método é baseado na criação de uma lacuna denominada de "arranhão" (do inglês, *scratch*) em uma monocamada de células confluentes, e na observação do movimento das células que estão nas extremidades em direção à lacuna formada para fechar esse "arranhão" até novo contato célula-célula ser novamente estabelecido. As principais etapas envolvem a criação do "arranhão" na

monocamada celular, captura das imagens no início e em intervalos regulares do movimento das células até o fechamento do “arranhão” e a comparação com as imagens do controle para determinar a taxa de migração celular. O acompanhamento individual da migração das células das extremidades para a lacuna é monitorado com o auxílio de um microscópio e da análise das imagens por um programa de computador. Para avaliar o potencial de um composto em promover a cicatrização, a monocamada celular contendo o “arranhão” é tratada com a substância de estudo e as células são comparadas com as células controle, sob as mesmas condições experimentais. A partir dessa comparação é possível determinar se o composto atuou favoravelmente no direcionamento da migração celular durante o ensaio *in vitro* ou não (LIANG, PARK e GUAN, 2007).

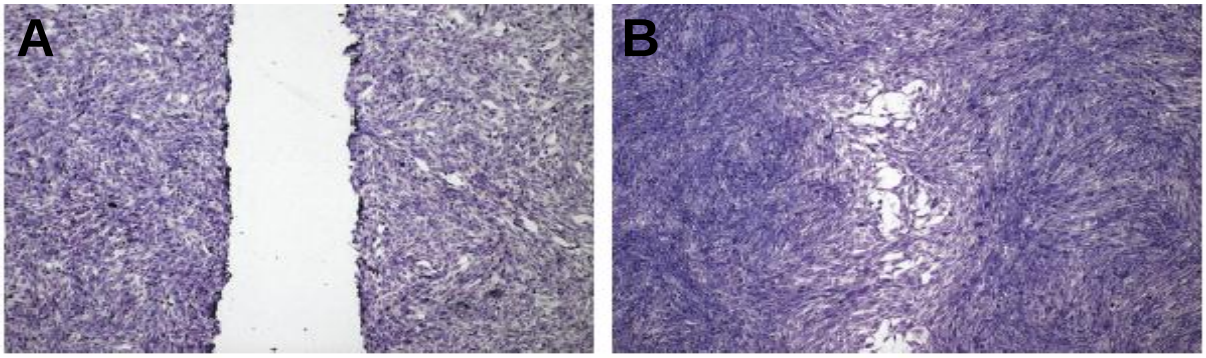

FIGURA 9. ESQUEMA DO ENSAIO DE MIGRAÇÃO CELULAR *IN VITRO* REALIZADO COM CÉLULAS DE GLIOMA DE CAMUNDONGO (C6) SENDO (A) LOGO APÓS A FORMAÇÃO DA LESÃO E (B) APÓS 24 HORAS DA LESÃO
Fonte: Adaptado de LEMIESZEK *et al.* (2009).

2.7 REOLOGIA

A reologia consiste na ciência que estuda a deformação e fluxo dos materiais avaliando a resposta que apresentam frente à aplicação de uma tensão ou deformação, sendo que um dos objetivos é estipular a relação entre as propriedades reológicas do material e sua estrutura química (VIANNA FILHO, 2009).

Recentemente, os estudos sobre as propriedades reológicas de polissacarídeos têm despertado a atenção da comunidade científica pelo potencial de aplicação na indústria de alimentos, cosmética e farmacêutica como agentes geleificantes, estabilizantes e espessantes na fabricação de produtos. Na tecnologia

de alimentos, esses polímeros podem ser empregados para melhorar a consistência e textura desejada e aumentar o tempo de prateleira ao proteger contra processos físicos que promovem a cristalização, sedimentação e desagregação mecânica (LIU *et al*., 2016; DO NASCIMENTO, 2017).

A pululana é um exemplo de polissacarídeo empregado na indústria alimentícia. É uma α-glucana produzida pelo fungo *Aureobasidium pullulans* que em solução aquosa forma um filme polimérico com baixa permeabilidade ao oxigênio que pode servir para o revestimento de alimentos e dessa forma conservar seu sabor e aparência por mais tempo. Outra aplicação é no desenvolvimento de alimentos funcionais conferindo uma ação prebiótica ao promover o crescimento de forma seletiva de diferentes linhagens de bactérias no intestino como *Bifidobacterium spp, Lactobacillus ssp* e *Enterococcus faecium* (BARBOSA *et al*., 2004; SYNYTSYA *et al*., 2009). As glucanas não são clivadas por enzimas presentes no corpo humano, sendo consideradas fibras alimentares que podem favorecer a motilidade intestinal e a formação de fezes, diminuindo a absorção de substâncias tóxicas e carcinogênicas e, consequentemente diminuindo a incidência de câncer (RUTHES, SMIDERLE e IACOMINI, 2015).

A obtenção de polissacarídeos a partir de fontes naturais como algas, plantas, fungos e culturas microbianas apresenta-se mais vantajosa em relação ao sintetizado em laboratório devido seu amplo espectro químico e propriedades reológicas que dificilmente podem ser reproduzidas artificialmente, além da disponibilidade e abundância na natureza (VIANNA FILHO, 2009).

2.7.1 Propriedade dos fluidos

Isaac Newton foi o primeiro pesquisador que introduziu o conceito básico de viscosidade e elaborou hipóteses para o escoamento dos fluidos entre duas placas paralelas. O fluido é definido como substância que se deforma quando submetida a uma tensão. O fluido ideal ou newtoniano deforma-se irreversivelmente e escoa, sendo a energia empregada na deformação dissipada na forma de calor. Como exemplo, temos os gases e os líquidos que apresentam esse comportamento. A propriedade de um líquido em resistir ao fluxo frente à aplicação de uma tensão de cisalhamento é caracterizada pela viscosidade, a qual é dependente da natureza físico-química do composto, da temperatura, da pressão, da taxa e do tempo de

cisalhamento aplicado. Na reologia dos fluidos, a propriedade mais importante é a viscosidade (PORTO, 2009; DO NASCIMENTO, 2017; VRIESMANN, 2008).

Para fluidos ideais, viscosidade é expressa de acordo com a lei de Newton pela seguinte equação:

$$\tau = \eta \dot{\gamma}$$

Onde:

τ = tensão de cisalhamento (Pa)

η = viscosidade (Pa.s)

γ = taxa de cisalhamento (s^{-1})

O estudo da reologia envolve a relação entre a taxa de cisalhamento e a tensão de cisalhamento. Quando essa relação é linear, o fluido é considerado newtoniano e sua viscosidade é constante. Na maioria dos fluidos, a viscosidade varia de acordo com a taxa de cisalhamento e a relação é não linear, sendo dessa forma considerados não newtonianos. Os fluidos não newtonianos em determinadas condições de tensão e taxa de cisalhamento, independentemente do tempo, podem apresentar comportamento pseudoplástico, dilatante ou plástico. No comportamento pseudoplástico, a viscosidade diminui conforme aumenta a taxa de cisalhamento, no dilatante ocorre o contrário; a viscosidade aumenta com o aumento da taxa de cisalhamento e no plástico o fluido somente escoa se exceder a uma tensão de cisalhamento crítica (PORTO, 2009; MATHIAS *et al*., 2013; VRIESMANN, 2008).

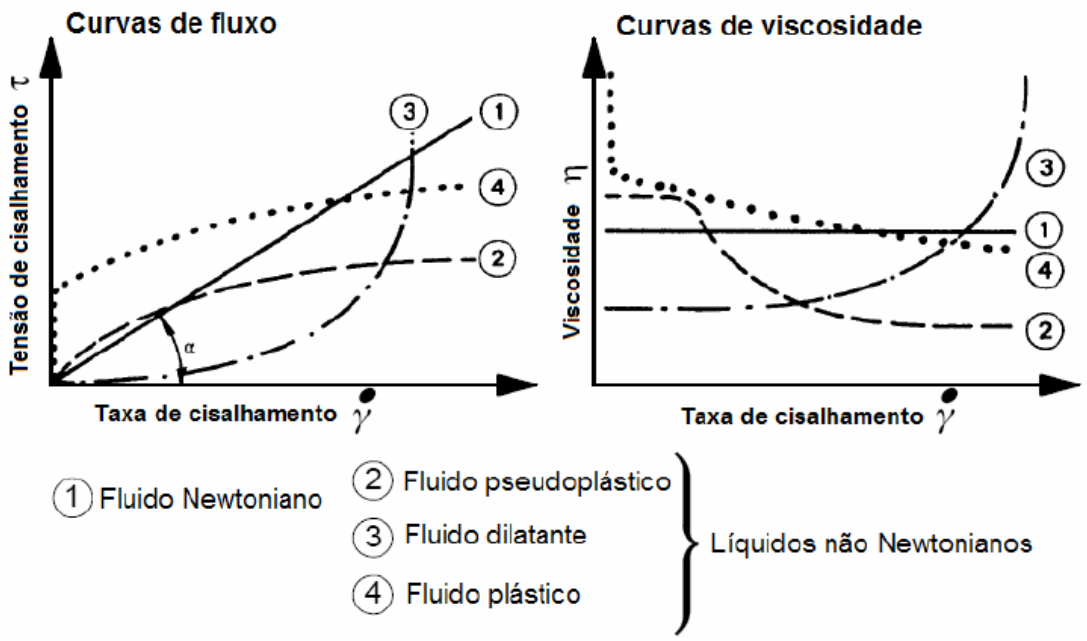

FIGURA 10. COMPORTAMENTO DE FLUXO DOS FLUÍDOS NEWTONIANOS E NÃO NEWTONIANOS**.**

Fonte: VRIESMANN (2008).

Geralmente, as soluções de polissacarídeos apresentam comportamento pseudoplástico; esse comportamento é crescente com o aumento da concentração do polímero e diminuído com o aumento da temperatura. Quando a taxa de cisalhamento é muito baixa, as moléculas estão desordenadas na solução, mas conforme aumenta o valor da tensão aplicada, as moléculas que estavam dispersas alinham-se na direção do fluxo e a viscosidade diminui (PROVIN, 2012; VRIESMANN, 2008).

Para descrever o comportamento reológico de fluidos não newtonianos são usados modelos matemáticos que representam a forma como a tensão de cisalhamento varia com a taxa de cisalhamento sendo possível estimar a viscosidade aparente. O modelo mais geral é o de Herschel-Bulkley já que pode ser aplicado para fluidos pseudoplásticos, dilatantes e plásticos (VIANNA FILHO, 2009).

A equação de Herschel-Bulkley é expressa da seguinte forma:

$$\tau - \tau_0 = K\left(\dot{\gamma}\right)^n$$

Onde:

τ = tensão de cisalhamento (Pa)

τ_0 = tensão de cisalhamento (Pa)

K = índice de consistência ($Pa.s^m$)

γ = taxa de cisalhamento (s^{-1})

n = índice de comportamento de fluxo (adimensional)

As curvas de fluxo descrevem a dependência da viscosidade aparente em relação à taxa de cisalhamento, sendo os principais meios para avaliar os diferentes perfis de fluidos. Grande parte dos fluidos é caracterizada por um comportamento reológico que os classifica entre o líquido e o sólido denominado de viscoelástico, sendo que nas soluções poliméricas é o comportamento predominante. Para investigar as soluções viscoelásticas, uma tensão oscilatória é aplicada na amostra e a resistência à deformação é avaliada. O comportamento viscoelástico pode ser quantificado através dos módulos de armazenamento ou elástico (G') e de perda ou viscoso (G") tendo ambos como unidade matemática Pascal (Pa). Os módulos G' e G" fazem parte do módulo de cisalhamento complexo (G*) que expressa a resistência total à deformação (VRIESMANN, 2008). O estudo do comportamento viscoelástico é fundamentado pela relação de G' e G" em função da frequência.

Se uma amostra apresentar módulo elástico (G') superior ao módulo viscoso (G") e ambos independentes da frequência, o material é caracterizado como um gel forte. Valores de G" maiores que G' em baixa frequência significa que o material possui característica de solução concentrada ou gel fraco. Uma amostra é classificada de solução concentrada ou gel fraco também se ocorrer uma inversão dos módulos em alta frequência e os valores de G' forem maiores que G". Em soluções diluídas, os valores de G" são consideravelmente maiores que G' em qualquer faixa de frequência (DO NASCIMENTO, 2017).

A variação da temperatura afeta também as propriedades viscoelásticas dos polímeros por influenciar as interações hidrofóbicas e pontes de hidrogênio das soluções. Os valores dos módulos G' e G" podem variar de acordo com a faixa de temperatura. Se houver dependência dos módulos em relação à temperatura, a solução revela-se termicamente instável, mas se esses valores não forem influenciados com o aumento ou diminuição da temperatura a substância apresenta estabilidade térmica (PROVIN, 2012; SOVRANI et al., 2017).

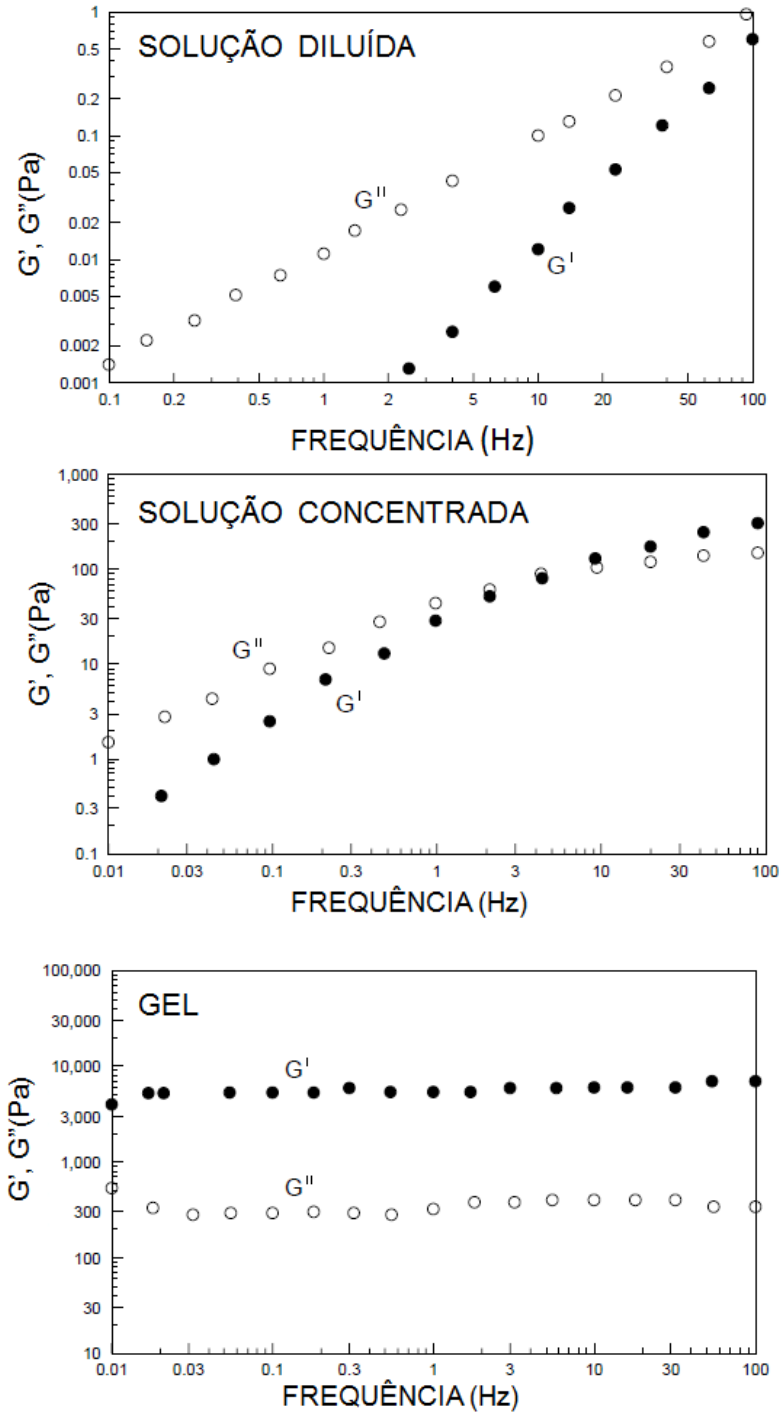

FIGURA 11. REPRESENTAÇÃO DOS COMPORTAMENTOS REOLÓGICOS DAS SOLUÇÕES DILUÍDA, CONCENTRADA E DE GEL.

Fonte: Adaptado de STEFFE (1996).

3. OBJETIVOS

3.1 OBJETIVO GERAL

- Caracterizar estruturalmente os polissacarídeos presentes nos cogumelos *Piptoporus betulinus*, *Phellinus igniarius* e *Pholiota nameko* e avaliar seus potenciais efeitos biológicos e propriedades reológicas.

3.2 OBJETIVOS ESPECÍFICOS

- Extrair os polissacarídeos dos cogumelos *Piptoporus betulinus*, *Phellinus igniarius* e *Pholiota nameko* por extrações aquosas e alcalinas;
- Purificar as frações polissacarídicas obtidas utilizando diferentes metodologias;
- Caracterizar a estrutura química fina dos polissacarídeos purificados;
- Investigar o potencial efeito cicatrizante do polissacarídeo purificado de *Piptoporus betulinus*;
- Estudar as propriedades reológicas do polissacarídeo purificado de *Pholiota nameko.*
- Avaliar o potencial imunomodulador do polissacarídeo isolado de *Phellinus igniarius*.

ARTIGO I

CHEMICAL CHARACTERIZATION AND WOUND HEALING PROPERTY OF A β-D-GLUCAN FROM EDIBLE MUSHROOM *Piptoporus betulinus*

(Submetido na revista International Journal of Biological Macromolecules)

CHEMICAL CHARACTERIZATION AND WOUND HEALING PROPERTY OF A β-D-GLUCAN FROM EDIBLE MUSHROOM *Piptoporus betulinus*

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ABSTRACT

A water-soluble β-D-glucan was obtained from fruiting bodies of *Piptoporus betulinus*, by hot aqueous extraction followed by freeze-thawing procedure and dialysis. Its molar mass distribution and conformational behavior in solution was assessed by size-exclusion chromatography, showing a polysaccharide with an average molecular weight of 2.5 x 10^5 Da with a random coil conformation for molecular weights below 1 x 10^6 Da. Typical signals of β-(1→3)-linkages were observed in NMR spectrum (δ 102.6/5.14; 102.9/5.08; 102.9/4.89; and δ 85.4/4.13; 85.5/4.10) and also signals of O-6 substitution at δ 69.4/4.56 and 69.3/4.24. The analysis of partially *O*-methylated alditol acetates corroborates with the NMR results, indicating the presence of a β-D-glucan with a main chain (1→3)-linked, substituted at O-6 by single-units of glucose. The β-D-glucan showed no toxicity on human colon carcinoma cell line (Caco-2) up to 1000 µg mL^{-1} and promoted cell migration on *in vitro* scratch assay, demonstrating a potential wound healing capacity.

Keywords: β-D-glucan; random coil; wound healing.

1. INTRODUTION

Mushrooms are well known to possess a diversity of medicinal properties due to the biological active compounds present in their fruiting bodies, cultured mycelium and broth. Polysaccharides isolated from medicinal and edible mushrooms notably exhibit therapeutic properties such as immunomodulating, anticancer, antimicrobial, hypocholesterolemic and hypoglycemic activities [1,2].

Piptoporus betulinus is a polypore mushroom belonging to basidiomycetes that grows on birch trees (*Betula* sp.), and is restricted to the northern hemisphere. This fungus has been used as a folk medicine and it showed many applications such as anti-fatiguing, immune-enhancing, antitumoral and analgesic properties. The polypore is also used as an antiparasitic, antimicrobial agent and for wound healing processes [3,4]. Its fruiting body fragment was encountered in the belongings of a natural mummy found in Europe in 1991, known as Ötzi the Iceman. It is believed that the fungus was used as tinder and possibly for medical purposes [5].

Among the molecules found in the fungal cell wall there are a variety of polysaccharides, including D-glucans that are one of the most common polysaccharides found in fungi and considered to be the main compound responsible for the therapeutic benefits described in the literature [6,7]. They present various anomeric configurations (α-, β- or mixed α,β), linkage types (1,3-; 1,4-; 1,6-linkages), branching degree, molecular weight and solubility, and such variability of their chemical structure may influence their biological activity [8-11].

There are few studies about D-glucans extracted from *P. betulinus*. Vunduk et al. [12] obtained β-glucan-rich extracts that presented a large screening of biological activities such as antioxidant, antimicrobial and especially angiotensin-converting enzyme (ACE) inhibitory ability. Olennikov et al. [13] isolated and chemically characterized a (1→3),(1→6)-α-D-glucan. Wiater et al. [14] extracted water-insoluble and alkali-soluble (1→3)-α-D-glucans, which were carboxymethylated to improve their solubility for *in vitro* antitumoral tests. The molecules presented cytotoxic effect against tumor cell cultures but no free radical scavenging activity [14].

β-D-Glucans present a versatile biological activity spectrum and many studies have focused on the therapeutics of such polysaccharides [15,10]. Most of the studies show the classical effect of β-D-glucans on the stimulation of the immune system. However, their capacity of forming a cross-linked network by hydrogen

bonds conferring rigidity or gelling aspects may be useful for other medical purposes, such as wound dressing material, and such capacity is still poorly studied [16]. The most common method chosen to evaluate the wound healing is the *in vitro* scratch assay, which is easily handled and low-cost, being considered a suitable method to measure cell migration *in vitro* [17]. Only few authors have described the influence of β-D-glucans on cell monolayer regeneration [16]. Therefore, in this study, the *in vitro* scratch assay was employed to evaluate the effect of a β-D-glucan from *P. betulinus* on the wound healing process. Besides, the isolation and chemical characterization of this polysaccharide as well as its conformational properties were described.

2. METHODS

2.1 FUNGAL MATERIAL

Fresh fruiting bodies of the polypore *P. betulinus* were collected from the wild in the municipality of Deurne, The Netherlands in 2014. After collection, they were identified by Prof. Leo van Griensven (Plant Research International, Wageningen University and Research Centre) according to the methods of classical herbarium taxonomy.

2.2 EXTRACTION AND PURIFICATION

The scheme of extraction and purification of the studied polysaccharide is showed at figure 1. Dried and milled *Piptoporus betulinus* (PB) fruiting bodies (127 g) were extracted with chloroform-methanol (2:1 v/v) at 60 ºC for 3 h (x 3 - each), to remove apolar compounds. The residue was dried and submitted to successive cold and hot aqueous extraction for 6 h (x 3). Each aqueous extract was evaporated to a small volume and the polysaccharide was precipitated by adding ethanol (3:1 v/v) and centrifuged at 8000 rpm at 10 ºC for 20 min. The sediment was dialyzed (6-8 kDa M_r cut-off membrane) against tap water for 24 h, concentrated under reduced pressure and freeze-dried.

The resultant fraction (HW) from hot aqueous extraction was partially purified by freeze-thawing process [10], followed by centrifugation (8000 rpm, at 10 ºC, for 20 min) to separate the soluble fraction (SHW) from the insoluble one. Afterwards, a

dialysis (1000 kDa M_r cut-off) was performed against distilled water, in a closed system, to separate the β-D-glucan, that remained, present in the retained fraction (R1M), from other polysaccharides.

2.3 ANALYSIS OF MONOSACCHARIDE COMPOSITION BY GC-MS

The polysaccharide fractions (1 mg) were hydrolyzed with 2 M TFA at 100 ºC for 8 h, followed by evaporation to dryness. The dried carbohydrate samples were dissolved in distilled water (100 µL) and 1 mg $NaBD_4$ was added. The solution was held at room temperature overnight to reduce aldoses into alditols [18]. The product was dried and excess $NaBD_4$ was neutralized by the addition of acetic acid, and removed following the addition of methanol (x 2) under a compressed air stream in a fume hood. Acetylation of the alditols was performed in pyridine–Ac_2O (200 µL; 1:1, v/v) for 30 min at 100 ºC. The pyridine was removed by 5% $CuSO_4$ solution and the resulting alditol acetates were extracted with $CHCl_3$. The resulting derivatives were analyzed by GC-MS, (Varian CP-3800 gas chromatograph coupled to an Ion-Trap 4000 mass spectrometer), using a VF5 column (30 m x 0.25 mm i.d.) programmed from 100 to 280 °C at 10 °C min^{-1}, with He as carrier gas. The correspondent monosaccharides were identified by their typical retention time, in comparison with standards, and the results were expressed as mol%, calculated according to Pettolino et al. [18].

2.4 METHYLATION ANALYSIS

Per-*O*-methylation of the isolated polysaccharide (5 mg) was carried out using NaOH-Me_2SO-MeI [19]. After isolation of the products by neutralization (HOAc), dialysis, and evaporation, the methylation process was repeated. The per-*O*-methylated derivatives were hydrolyzed using 2 N sulphuric acid (1 mL) for 16 h at 100 °C followed by neutralization with $BaCO_3$, filtration, reduction with $NaBD_4$ and acetylation as described above, to give a mixture of partially *O*-methylated alditol acetates, which was analyzed by GC-MS using a VF5 capillary column. The derivatives were identified by their typical retention time and electron impact profile, in comparison to standards according to Sassaki et al. [20]. The results were given as mol%, calculated according to Pettolino et al. [18].

2.5 MOLAR MASS DISTRIBUTION BY SIZE EXCLUSION CHROMATOGRAPHY (SEC)

The molar mass distribution of the isolated β-D-glucan from *P. betulinus* was analyzed by size exclusion chromatography (SECcurity 1260, Polymer Standard Services, Mainz, Germany coupled to a multiple-angle laser light scattering detector (MALLS; BIC-MwA7000, Brookhaven Instrument Corp., New York), and a refractive index detector (SECcurity 1260, Polymer Standard Services, Mainz, Germany) thermostatted at 45 °C, as described by Moreno et al. [22].

2.6 NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY STUDIES

NMR spectra were obtained using a 400MHz Bruker Avance III spectrometer with a 5 mm inverse probe. 2D NMR (HSQC) analyses were performed at 70 °C in D_2O. Chemical shifts are expressed in δ (ppm) relative to acetone (δ 30.2 for ^{13}C signal and 2.22 for ^{1}H signal) used as internal standard.

2.7 CELL LINES

Human colon carcinoma cell line (Caco-2) was purchased from the Cell Bank of Rio de Janeiro, Brazil. Cells were cultivated in Dulbecco's modified Eagle's medium (DMEM) and Ham´s-F12 (1:1), supplemented with 10% fetal bovine serum (FBS), 100 IU mL^{-1} penicillin/streptomycin. Cultures were maintained in humidified atmosphere (95% air and 5% CO_2) at 37 °C.

2.8 CELL VIABILITY ASSAY

Caco-2 cells were cultured in 96-well plates, at a density of 2 x 10^4 cells/well, and incubated overnight as described above. Subsequently, culture medium was removed and cells were treated with increasing concentrations (10, 100, 500, 1000 and 2000 µg mL^{-1}) of *P. betulinus* β-D-glucan, diluted in FBS free medium. After 24 h of incubation, the solution was discarded and 100 µL of MTT solution (0.5 mg mL^{-1}) was added to each well and incubated for 3 h at 37 °C. Then, MTT solution was

aspired and 100 µL of dimethyl sulfoxide (Me_2SO) was added to solubilize the formazan crystals. Cell survival was assessed through absorbance determination at 570 nm using a microplate reader (Synergy^{TM} HT, Biotek®, USA). Medium alone was used as control and the cell viability was expressed as a percentage of control cells.

2.9 *IN VITRO* SCRATCH ASSAY

Caco-2 cells were seeded at a density of 2 x 10^5 cells/well and grown to a confluent monolayer in 6-well culture plate. A linear scratch was made in each well using a 200 µL sterile plastic pipette tip, perpendicular to a black line drawn on the underside of the plate for reference, creating a cell-free area. Posteriorly, wounded monolayers were washed with PBS to remove detached cells and debris, and incubated in 3 mL FBS-free culture medium containing *P. betulinus* β-D-glucan at 10, 100 and 1000 µg mL^{-1} at 37°C. Images of each scratch were captured at 0 and 24 h with a digital camera on an inverted microscope (Olympus) at 10 x magnification. The wound closure analysis was made from edge to edge using the ImageJ software. For all treatments, the wound at time 0 was arbitrarily assigned as 100% and the percentage of wound healing at 24 h was compared to each cell treatment at the initial time [17].

2.10 STATISTICAL ANALYSIS

All samples were analyzed in triplicate and the experiments were repeated three times. Data were expressed as mean ± standard error of the mean (SEM). Statistical significance was determined using one-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test. Calculations were performed using GraphPad Prism version 5 (GraphPad Prism Software, San Diego, USA). In all cases, differences were considered to be significant when $P < 0.05$.

3. RESULTS AND DISCUSSION

3.1 CHEMICAL CHARACTERIZATION OF β-D-GLUCAN

The dried fruiting bodies of *Piptoporus betulinus* (127 g) were submitted to deslipidification followed by hot water extraction, giving a crude extract (HW, 1.8 g) composed mainly by glucose. A partial precipitation occurred after freeze-thawing process, and the soluble fraction (SHW, 0.5 g) was separated from the insoluble one by centrifugation (Fig. 1). The monosaccharide composition showed that the soluble fraction presented also glucose as major component (69.8%). SHW was submitted to a dialysis against distilled water using a 1,000 kDa M_r cut-off membrane. The retained fraction (R1M, 0.1 g) showed only glucose in its composition, indicating that the purification process was successful. This fraction presented high viscosity in aqueous solution. To determine its chemical linkages an aliquot of R1M fraction was submitted to methylation reaction and analyzed by GC-MS. The relative percentage of the partially *O*-methylated alditol acetates obtained are described in table 1. The methylation analysis indicated that the backbone of the isolated D-glucan is (1→3)-linked (67%) and nearly 15% of its main chain is substituted at *O*-6 by D-Glc*p* residues.

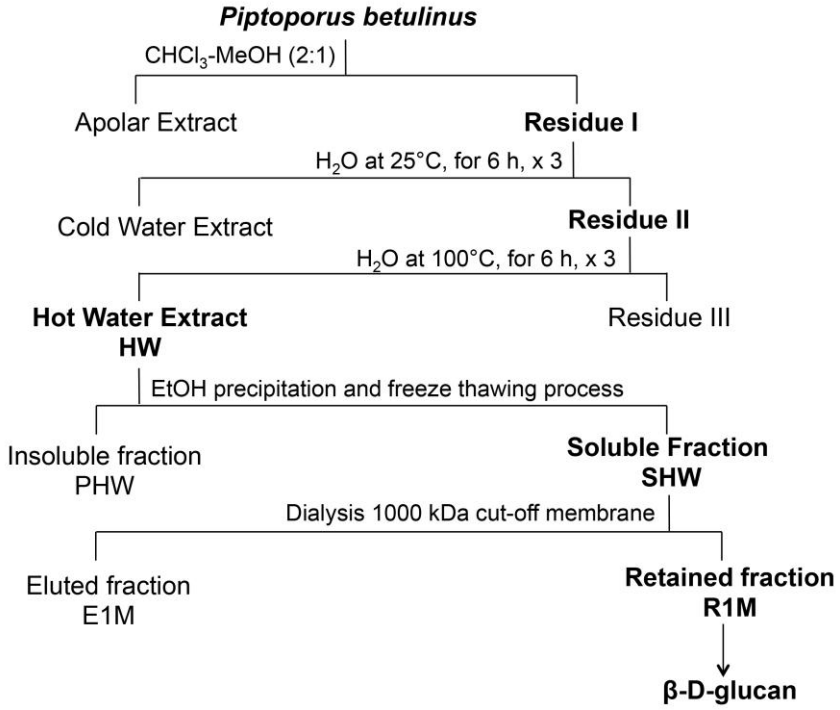

FIGURE 1. Scheme of extraction and purification of β-D-glucan.

Table 1 - Partially *O*-methylated alditol acetates of β-D-glucan.

Partially *O*-methylalditol acetates	Mol%[a]	Linkage type[b]
2,3,4,6-Me_4-Glc	17	Glcp-(1→
2,4,6-Me_3-Glc	68	3→)-Glc-(1→
2,4-Me_2-Glc	15	3,6→)-Glc-(1→

[a] Relative percentage of each derivative was calculated according to Pettolino et al. (2012).

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

The molar mass distribution, macromolecular size and the results from the conformation studies of the (1→3),(1→6)-linked D-glucan are presented in Fig. 2. The polysaccharide fraction exhibit monomodal SEC weight distributions *w (*log V_h) for a wide range of hydrodynamic sizes comprised between 1 and 200 nm, indicating an homogeneous distribution of molecular populations (Fig. 2a), with a M_w of 2.5 x 10^5 kDa. The conformational plot shown in figure 2b represents the relation between the weight-average molar mass $\overline{M}w$ and the radius of gyration (*R*g). According to the scaling theory, the slope of the logarithmic plots is related to the conformation of the polysaccharide in solution. The conformation in solution of a compact sphere would present a theoretical value of 0.33 for, 0.5–0.6 for a flexible random coil and 1 for a stiff rod [23]. The fraction R1M showed a conformation in Me_2SO solution that can be assigned as a random coil for molar masses below 1 x 10^6 Da; however, a transition towards a stiffer macromolecular conformation can be observed above M_w > 2 x 10^6 Da (Figure 2b).

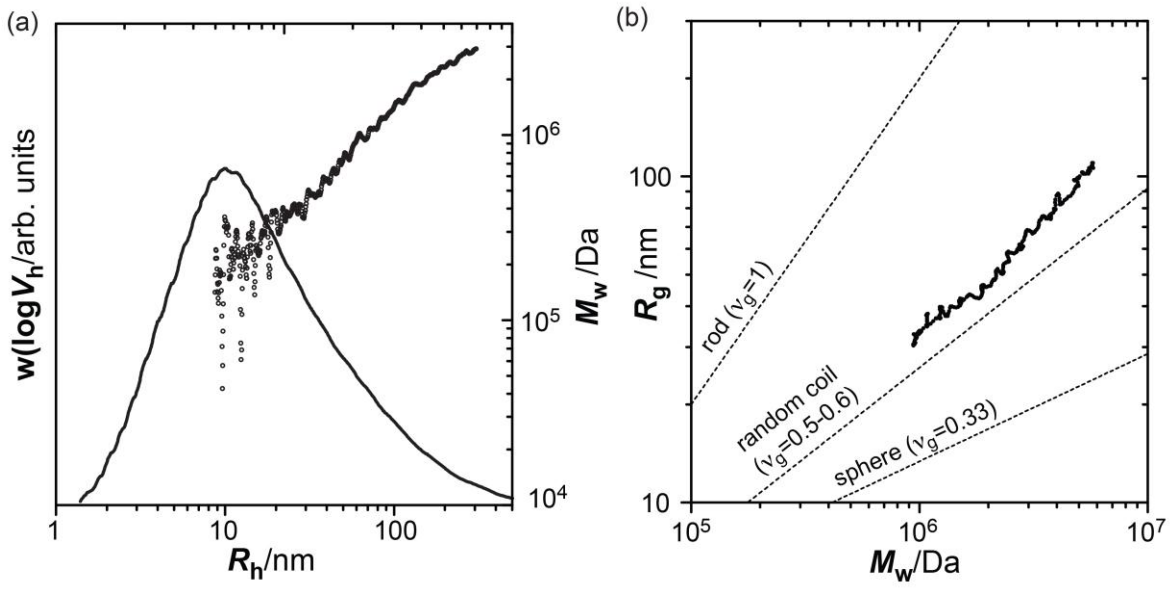

FIGURE 2. Molar mass distribution, macromolecular size (A) and conformational studies (B) of *P. betulinus* β-D–glucan.

The HSQC-NMR spectrum (Fig. 3) of sample R1M showed that the D-Glc*p* units are in β-configuration due to the presence of high frequency carbon-1 signals (δ 102.6/5.14; 102.9/5.08 and 102.9/4.89) [24]. Besides, (1→3) and (1→6)-linkages were confirmed by the presence of typical signals at δ 85.4/4.13 and 85.5/4.10 (substituted C-3) and δ 69.4/4.56 and 69.3/4.24 (substituted C-6). The other carbon/hydrogen frequencies were assigned as C-2 (δ 73.3/3.90), C-4 (δ 68.9/3.89), C-5 (δ 76.3/3.88), and non-substituted C-6 (61.4/4.28 and 61.4/4.11). These results were assigned according to the correlation among the NMR spectrum and comparasion to literature values of similar polysaccharides [21,25-27].

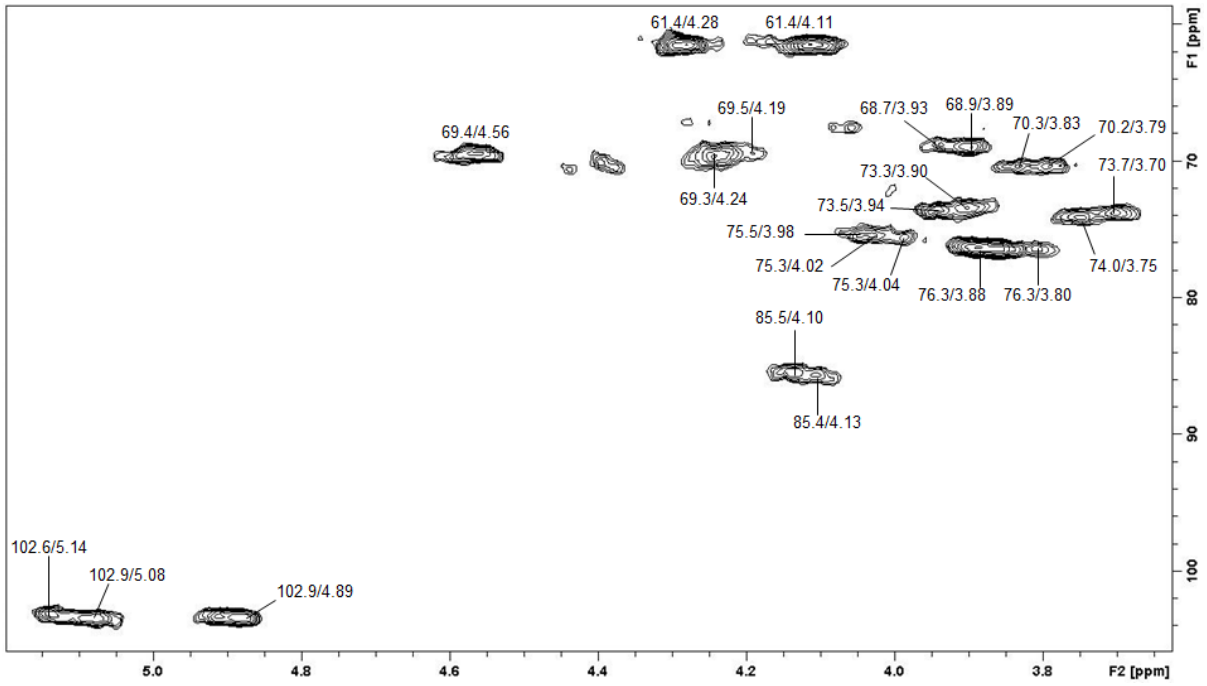

FIGURE 3. HSQC NMR spectrum of the β-D-glucan (R1M) from *P. betulinus* in D_2O at 70°C (chemical shifts are expressed in ppm).

3.2 BIOLOGICAL EFFECTS OF β-D-GLUCAN

The MTT assay is a colorimetric method used for assessing cell viability, based on reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Cells with active mitochondrial metabolism convert MTT into a purple colored formazan product, a marker of viable cells [28]. As shown in figure 4, the results from MTT assay demonstrated that 24 h incubation of Caco-2 cells with the β-D-glucan (R1M) 10-1000 µg mL^{-1} did not change cell viability when compared to control medium. Only at 2000 µg mL^{-1}, the β-D-glucan reduced the cell viability in 85%. Based on this data, the β-D-glucan present very low cytotoxic effect against Caco-2 cells, therefore 10, 100 and 1,000 µg mL^{-1} of β-D-glucan were tested in the wound healing assay.

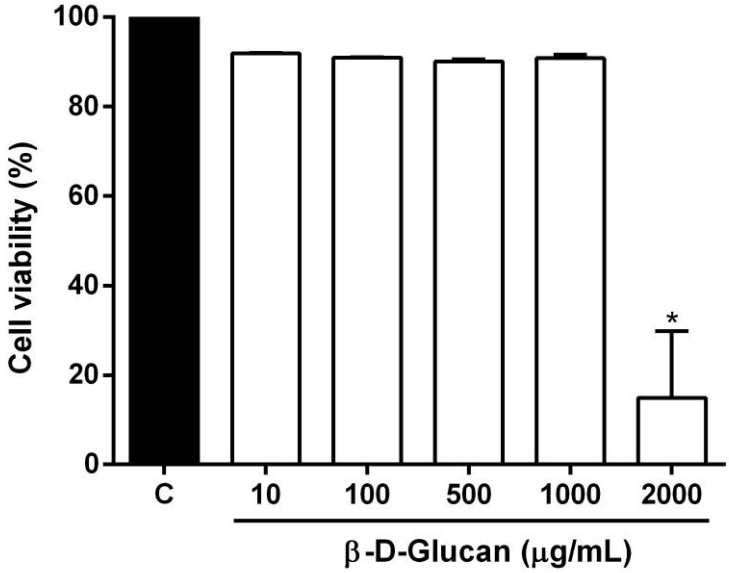

FIGURE 4. Effects of β-D-glucan (R1M) on Caco-2 cell viability, determined by MTT assay. Results are expressed as percentage of control (C). Data on graph are representative of experiments performed at least three times, in triplicate. *$p < 0.05$, One Way Anova followed by Bonferroni`s post hoc test.

It had been reported that in addition to the well-known variety of pharmacological and biological effects of mushrooms, a regenerative property gives new insight into epithelial wound healing [29,30]. In the gastrointestinal tract, epithelial wound healing is a persistent and important physiological process, and at pathophysiological conditions such as gastric ulcers and colitis, an impaired epithelial healing may result in immunogenic responses and disequilibrium of the homeostasis, due to improper or impaired tissue repair [31].

It was demonstrated that a mushroom bioactive polysaccharide facilitates the *in vitro* wound healing process by epithelial intestinal cell migration. In the scratch assay, the β-D-glucan at a non-cytotoxic concentration of 1000 μg mL^{-1}, accelerated the Caco-2 cells migration to wound closure in 55% (Fig. 5), reinforcing the potential application of mushroom polysaccharides in wound healing process.

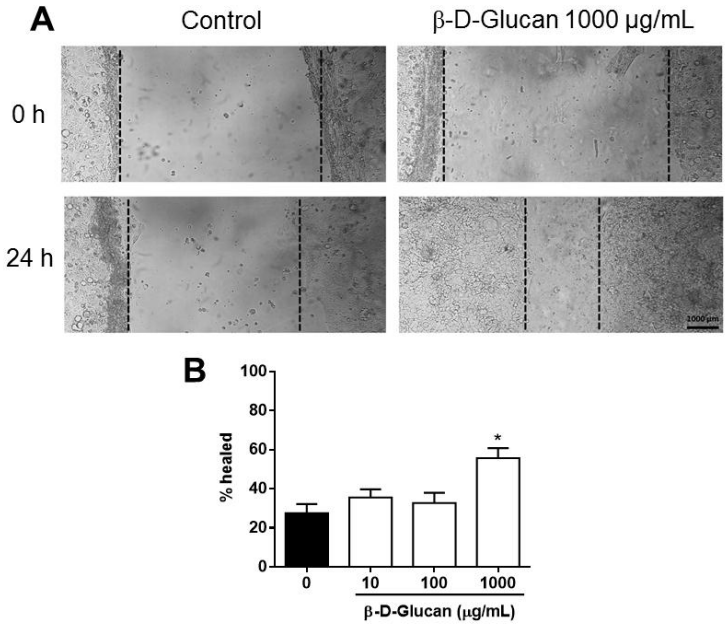

Figure 5. Effects of β-D-Glucan (R1M) on Caco-2 cells migration in the wound healing assay. Confluent cell monolayers were wounded with a pipette tip and incubated with medium alone or with β-D-Glucan (10-1,000 µg/ml) for 24 h. Representative images of scratched areas in confluent Caco-2 cells layers treated with medium or β-D-Glucan 1,000 µg/mL (Panel A). Wound healing was photographed at 0 and 24 h after wounding. Data on graph are representative of experiments performed at least three times, in triplicate, and results are expressed as percentage of healing comparing to 0 h (Panel B). *P< 0.05, One way ANOVA followed by Bonferroni post hoc test.

D-Glucans have the capacity to form different helical structures such as single- and triple-helix or to behave as random coil. These conformational states, separately or combined, can convert a solution into gel under heat and certain conditions of humidity [9]. Such a gelling characteristic can be useful for the development of wound dressing material, that is designed to cover the wound, promote protection and accelerate the healing process [32, 33]. Gels are applied as primary dressings and recommended for slightly exuding wounds. They are considered a modern material compared to the traditional wound healing agents due to their ability to produce a moisture environment that can also contain therapeutic substances [34]. A variety of D-glucans and their derivatives are employed in the

wound dressings because of their innate biological activity and because of the possibility to produce dressings with the desired physical property [35]. The β-D-glucan isolated from *P. betulinus* exhibited a random coil conformation and presented high viscosity in solution. Our results demonstrated that the purified β-D-glucan promoted Caco-2 cells migration in an *in vitro* wound closure model, thereby showing to be a good candidate to be used as wound dressing material.

CONCLUSION

The present study reported the isolation and chemical characterization of a (1→3),(1→6)-β-D-glucan from the mushroom *P. betulinus*. The analyses showed that this β-D-glucan exhibits a random coil conformation in solution. Its wound healing capacity was assessed by an *in vitro* scratch approach that showed the β-D-glucan increased the *in vitro* cellular response to injury by promoting cell migration. The results observed emphasize the importance of mushroom polysaccharides in different therapeutic areas, showing that besides acts as biological response modifiers, β-D-glucans may be used also for wound healing as wounding dressing materials.

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ARTIGO II

EFFECTIVE APPROACH TO SEPARATE D-GLUCANS COMPLEX FROM THE EDIBLE FUNGUS *Piptoporus betulinus*

Effective approach to separate D-glucans complex from the edible fungus *Piptoporus betulinus*

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ABSTRACT

Two insoluble water glucans (SK5 and PK5) were isolated from the fruiting bodies of *Piptoporus betulinus* mushroom through successive water and KOH 5% extraction. The polysaccharides were separated from the same fraction by alkaline treatment with NaOH 0.1 M aqueous solution. The difference of branching degree between the two glucans influenced the distinct solubility presented by each compound due to intermolecular association. Both polysaccharides were characterized by GC-MS analysis and NMR spectroscopy. The results indicated that SK5 fraction is constituted by a main chain of (1→3)-linked β-D-Glc*p* units substituted at *O*-6 by (1→6)-linked β-D-Glc*p* units and PK5 fraction is composed by a linear α-(1→3) glucan. According to literature review there are few isolated glucans from this fungus and the chemical characterization of these compounds are poorly explored. The polymers observed in this work presented different chemical structure compared to previous studies and can be considered innovative for *P. betulinus* mushroom**.**

Keywords: *Piptoporus betulinus*; glucan; NaOH treatment; structural characterization

1. INTRODUCTION

Mushrooms have attracted great attention in the last decades for their nutritional and medicinal values; they are well known in the folk medicine and consumed as food due to their desirable texture and flavor (Chowdhury, Kubra & Ahmed, 2015). Edible mushrooms present a diversity of bioactive compounds which can be used as a source for the development of functional food, nutraceuticals and drugs (Heleno et al., 2015; Wasser, 2014). The diversity of their chemical content offers strong ability to prevent and treat a variety of pathologies such as tumors, microbial and viral infections, immune disorders, diabetes and neurodegenerative diseases (Phan et al., 2014).

Piptoporus betulinus is a polypore mushroom that grows as trunk parasite on birch species trees. It was found in the items of the iceman “Ötzi”, a mummified man encountered in Hauslabjoch, near the Austria/Italian border, in 1991, who was probably carrying it for medical purposes (Alresly et al., 2016; Dresch et al., 2015). Europeans and Chinese people have also appreciated such fungus as medicine, furthermore some studies have showed that extracts from *P. betulinus* presented antitumoral (Pleszczyńska et al., 2016), anti-inflammatory (Kamo, Asanoma, Shibata & Hirota, 2003), antimicrobial (Suay et al., 2000), cardiovascular (Vunduk et al., 2015), anti-fatiguing and immune-enhancing effects (Grienke, Zöll, Peintner & Rollinger, 2014).

The potential of fungi compounds was previously demonstrated in several studies, being the polysaccharides the most active substances present in mushrooms (Zong, Cao & Wang, 2012). D-Glucans represent the most investigated group due to their importance to the immune system. They have been reported as biological response modifiers and such characteristic is related to its capacity to bind to macrophage receptors and modulate the immune function (Gründemann et al., 2015; Manzi & Pizzoferrato, 2000; Mueller et al., 2000).

D-Glucans are found in the fungi cell wall and present a diversity of chemical structures with different linkage types (1,3-; 1,4-; 1,6-linkages), molecular configuration (α, β- or mixed α,β), branching degrees, molecular mass and solubility (Ruthes, Smiderle & Iacomini, 2015; Chema et al., 2015). Such variety may confuse the researchers if a careful purification procedure has not been carried out, because polysaccharides are able to interact to each other by hydrogen bonds, forming

complexes of one or more different molecules. Furthermore, different physicochemical parameters may influence their biological activity and thus result in a large spectrum of applications in food, biomedical, pharmaceutical and cosmetic areas (Kagimura et al., 2015).

Birch polypore polysaccharides, such as in *P. betulinus*, have not been studied in deep concerning to their chemical structure and biological activity (Pleszczyńska et al., 2017). Herein, we show the separation of α and β glucans from the fruiting bodies of *P. betulinus* by using an effective, simple and low cost treatment with NaOH 0.1 M solution. After separation, the different D-glucans were chemically characterized.

2. METHODS

2.1 FUNGAL MATERIAL

Piptoporus betulinus was furnished by Dr. Leo van Griensven, from Plant Research International, Wageningen University and Research Centre, The Netherlands.

2.3 ANALYSIS OF MONOSACCHARIDE COMPOSITION BY GC-MS

The polysaccharide fractions (1 mg) were hydrolyzed with 2 M TFA at 100 °C for 8 h, followed by evaporation to dryness. The dried carbohydrate samples were dissolved in distilled water (100 µL) and 1 mg $NaBH_4$ was added. The solution was held at 23 °C overnight to reduce aldoses into alditols (Sassaki et al., 2008). The product was dried and excess $NaBH_4$ was neutralized by the addition of acetic acid, which was removed following the addition of methanol (2 times) under a compressed air stream in a fume hood. Acetylation of the alditols was performed in pyridine–Ac_2O (200 µL; 1:1, v/v), heated for 30 min at 100 °C. The pyridine was removed by 5% $CuSO_4$ solution and the resulting alditol acetates were extracted with $CHCl_3$. These were analyzed by GC-MS, and identified by their typical retention times and the results were given as mol%, calculated according to Pettolino et al. (2012). The identification of correspondent monosaccharides was made by comparison of the standards with a Varian CP-3800 gas chromatograph coupled to an Ion-Trap 4000

mass spectrometer, using a VF5 column (30 m x 0.25 mm i.d.) programmed from 100 to 280 °C at 10 °C min^{-1}, with He as carrier gas.

2.4 METHYLATION ANALYSIS

Per-*O*-methylation of isolated polysaccharide (5 mg) was carried out using NaOH-Me_2SO-MeI (Ciucanu & Kerek, 1984). After isolation of the products by neutralization (HOAc), dialysis, and evaporation, the methylation process was repeated. The per-*O*-methylated derivatives were hydrolyzed using 2 N sulphuric acid (1 mL) for 16 h at 100 °C followed by neutralization with $BaCO_3$, filtered, then reduced with $NaBD_4$ and acetylated as described above, to give a mixture of partially *O*-methylated alditol acetates, which was analyzed by GC-MS using a VF5 capillary column. The derivatives were identified by their typical retention times and electron impact spectra, compared to partially *O*-methylated alditol acetates according to Sassaki [20]. The results were given as mol%, calculated according to Pettolino et al. (2012).

2.5 CONTROLLED SMITH DEGRADATION OF β-D-GLUCANS

The purified fractions (SK5 and PK5; 30 mg) were oxidized with 0.05 M aqueous $NaIO_4$ (20 mL) at room temperature (23 °C) protected from light for 72 h. Ethylene glycol was added to stop the reaction, the material was dialyzed (2 kDa M_r cut-off membrane) and the resulting polyaldehyde was reduced with $NaBH_4$ for 12 h, neutralized with HOAc, dialyzed and concentrated to 50 mL (Goldstein, Hay, Lewis, & Smith, 2005). The residue was submitted to partial hydrolysis with TFA, pH 2.0, for 30 minutes at 100 °C, and dialysed (2 kDa M_r cut-off membrane). The final solution was freeze-dried. The material resistant to oxidation was then analyzed by ^{13}C-NMR spectroscopy.

2.6 NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY STUDIES

NMR spectra were obtained using a 400 MHz Bruker Avance III spectrometer with a 5 mm inverse probe. NMR analyses (HSQC and ^{13}C) were performed at 70 °C

in Me_2SO-d_6. Chemical shifts being expressed in δ (ppm) relative to Me_2SO-d_6 (δ 39.4 for ^{13}C signal and 2.4 for ^{1}H signal).

3. RESULTS AND DISCUSSION

3.1 ISOLATION OF THE D-GLUCANS COMPLEX

The dried fruiting bodies of *Piptoporus betulinus* (127 g) were submitted to successive cold and hot water extractions and the remained residue was extracted with 5% NaOH giving a crude extract (K5, 1.8 g) composed of mannose (8.2%) and glucose (91.8%). A partial precipitation occurred after freeze-thawing process, and the insoluble fraction (IK5) was separated from the soluble one by centrifugation (Fig. 1). The monosaccharide composition (Table 1) showed that the insoluble fraction consisted of mannose (3.8%) and glucose (96.2%). IK5 (1.4 g) was submitted to an alkaline treatment employing 0.1 M NaOH solution giving rise to insoluble (PK5) and soluble (SK5) alkaline fractions. SK5 (0.3 g) sample showed to be composed only of glucose while PK5 (0.8 g) fraction presented glucose as majoritary component, indicating that the purification process was successful.

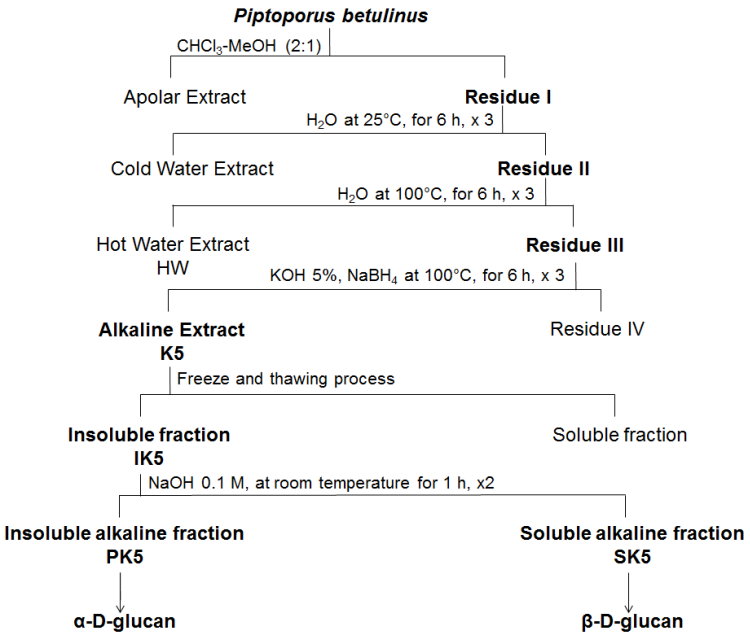

Figure 1: Scheme of extraction and purification of α-D-glucan and β-D-glucan obtained from fruiting bodies of *Piptoporus betulinus*.

TABLE 1. Monosaccharide composition of fractions obtained from *Piptoporus betulinus*.

Fractions	Monosaccharide Composition (%)[a]	
	Man	**Glc**
K5	8.2	91.8
IK5	3.8	96.2
PK5	Tr[b]	99.0
SK5	-	100

[a] % of peak area relative to total peak areas, determined by GC–MS.

[b] Trace amounts < 1%.

3.2 CHEMICAL CHARACTERIZATION OF D-GLUCANS

An aliquot of SK5 and PK5 fractions were submitted to methylation reaction and analyzed by GC-MS, giving as partially *O*-methylated alditol acetates derivatives as described in table 2. The result from SK5 fraction indicated that the main derivative is related to D-glucan (1→3)-linked (55%) and 19% of its units is substituted at O-6 by D-Glc*p* residues. The PK5 fraction presented 2,4,6-tri-*O*-methyl-Glc*p* (99%) and 2,3,4,6-tetra-*O*-methyl-Glc*p* (<1%) related to a linear (1→3)-linked glucan structure.

TABLE 2. Partially *O*-methylated alditol acetates formed on linkage analysis of the D-glucans isolated from *P. betulinus* fruiting bodies

Partially *O*-methylated alditol acetates [a]	% Area of Fragments [b]		Linkage Type
	SK5	PK5	
2,3,4,6-Me_4-Glc	19.1	Tr[c]	Glcp-(1→
2,4,6-Me_3-Glc	55.0	99.0	3→)-Glc-(1 →
2,3,4-Me_3-Glc	6.9	-	6→)-Glc-(1 →
2,4-Me_2-Glc	19.0	-	3,6→)-Glc-(1→

[a] According to Pettolino et al. (2012).

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

[C] Trace amounts <1%.

^{13}C and HSQC NMR spectra of crude (K5 and IK5) and purified fractions (PK5 and SK5) are shown in Fig. 2 and Fig. 3. ^{13}C NMR spectrum of the K5 fraction (Fig. 2A) contained four distinct signals in the anomeric region corresponding to C-1 signals of β-D-Glc*p* at δ 103.6 and 102.6 (Carbonero et al., 2006), β-D-Man*p* at δ 101.4 (Silveira et al., 2015) and α-D-Glc*p* at δ 99.4 (Li et al., 2016). Its HSQC NMR spectrum (Fig. 3A) showed similar signals with the frequency of the carbon and hydrogen at δ 102.8/4.14 and 102.2/4.43 related to C-1 of β-D-Glc*p*, δ 101.0/4.21 correlated to C-1 of β-D-Man*p* and δ 99.1/4.98 associated to α-D-Glc*p* (Gorin, 1981). The signal observed for mannose could indicate the presence of mannans, although polysaccharides containing only mannose are not usual in basidiomycetes and are considered typical of yeasts such as *Saccharomyces cerevisiae* (Komura et al., 2010). Or it could be some residue of the commonly found mannogalactans that were isolated from a variety of mushroom species (Silveira et al., 2015; Smiderle et al., 2008).

Both ^{13}C and HSQC spectra suggested two possibilities concerning the glucans composition: K5 fraction may be composed of distinct glucans with different anomeric configurations; or it is composed of one glucan type which presents both α- and β-configurations in its structure. Smiderle et al., 2011 isolated from the same mushroom extract an α-(1,4),(1,6)-glucan and β-(1,6)-glucan of *Agaricus bisporus* and *Agaricus brasiliensis*, while Santos-Neves (2008) and Rout (2005) isolated a branched α,β-glucan from the edible mushroom *Pleurotus florida*. To confirm these possibilities and finally elucidate the chemical structure of *P. betulinus* glucans, a careful purification procedure was performed.

After freeze and thawing step the fraction showing the highest yield (IK5) was analyzed by NMR. The spectroscopy experiments (Figs. 2B and 3B) of IK5 fraction presented similar signals when compared to K5 spectra, although it was possible to observe a small reduction of intensity of signals relative to C-1 of β-D-Man*p* (Fig. 2B and 3B) accompanied of reduction of signals from 60 to 86 ppm. This result associated to the increase in the amount of glucose (Table 1) indicated that the mannose was not covalently linked to the glucans.

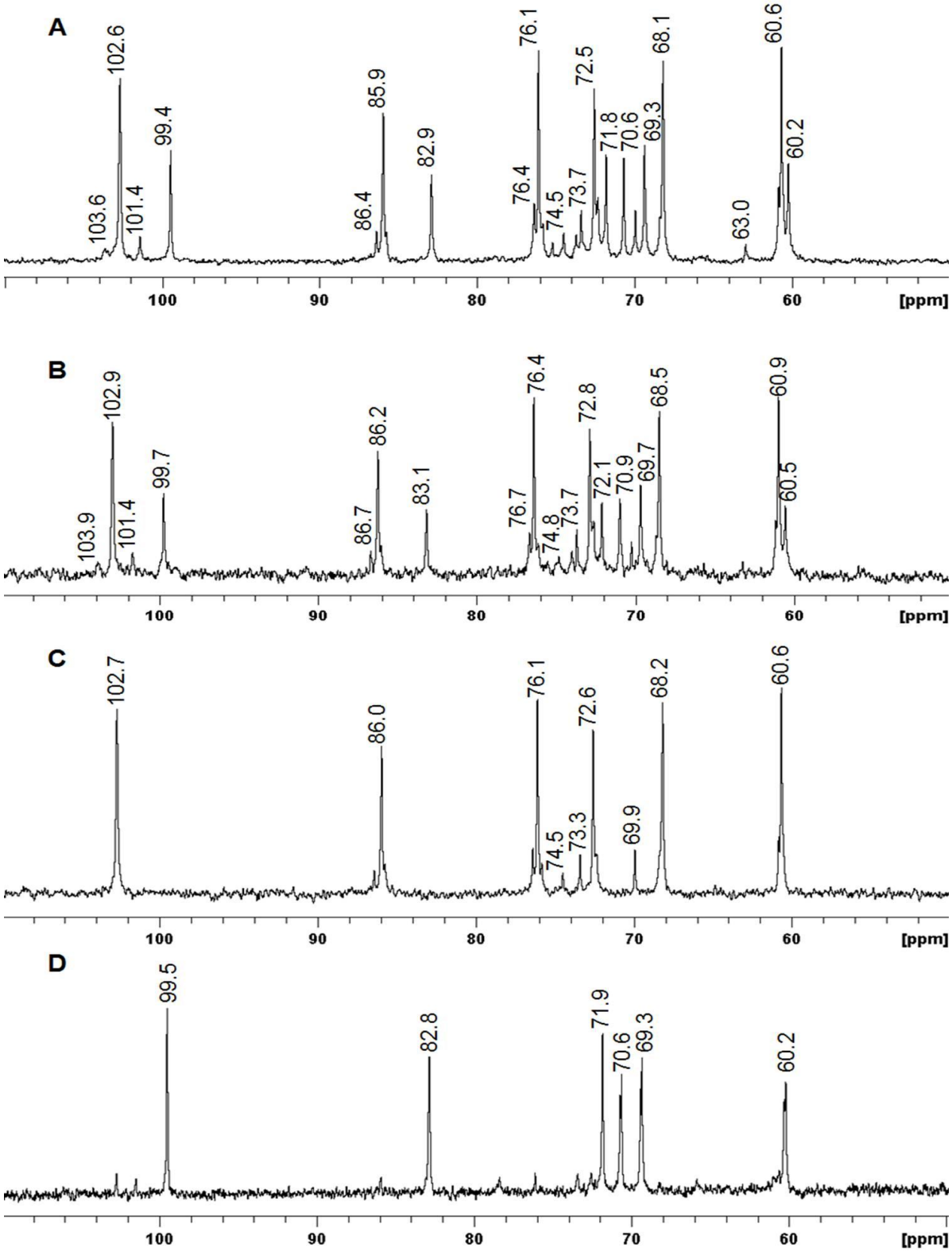

FIGURE 2. ^{13}C-NMR spectrum of K5 (A), IK5 (B), SK5 (C) and PK5 (D) fractions in Me_2SO-d_6 at 70 °C (chemical shifts are expressed in δ ppm).

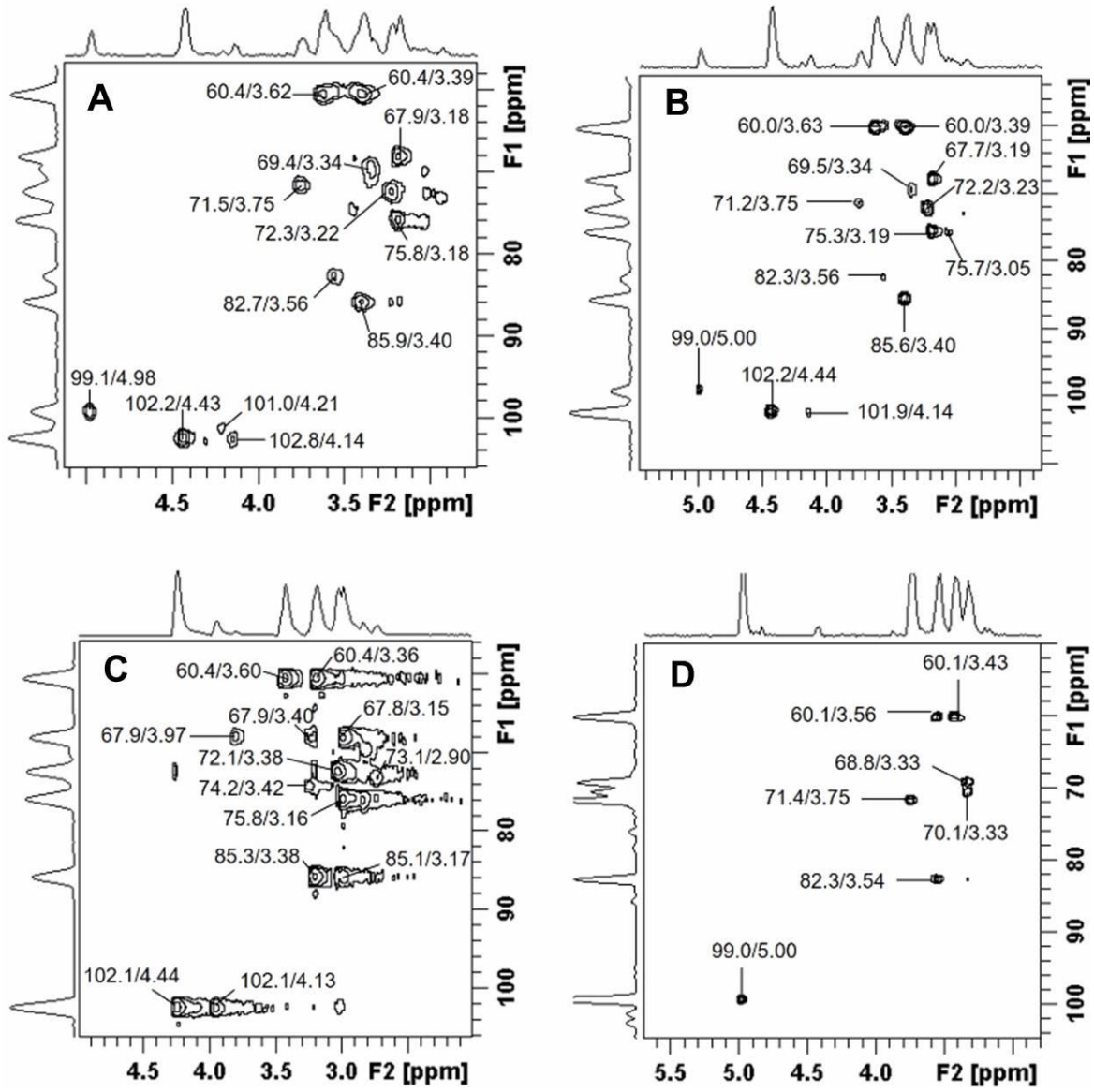

FIGURE 3. HSQC NMR spectrum of K5 (A), IK5 (B), SK5 (C) and PK5 (D) fractions in Me_2SO-D_6 at 70 °C (chemical shifts are expressed in δ ppm).

To continue with the purification process, a treatment with 0.1 M NaOH solution was performed with the aim of removing possible interactions such as hydrogen bonds formed between one or more polysaccharides. The resultant SK5 and PK5 fractions showed different solubilities in the alkaline solution and also different signals were observed in the NMR spectra. The HSQC-NMR spectrum (Fig. 3C) of sample SK5 showed signals at the anomeric region related to C-1 of D-Glc*p* units in β-configuration (δ 102.1/4.44 and 102.1/4.13). Moreover, (1→3) and (1→6)-linkages were confirmed by the presence of their typical signals at δ 85.3/3.38 and

85.1/3.17 (substitution at *O*-3) and δ 67.9/3.97 and 67.9/3.40 ppm (substitution at *O*-6). The ^{13}C NMR analysis showed six main signals at δ 102.7 (C-1), 86.0 (C-3), 76.1 (C-5), 72.6 (C-2), 68.2 (C-4) and 60.6 (C-6), corresponding to the main chain (1→3)-linked in β-configuration. The signals were assigned according to the correlation among the NMR spectra and literature values of similar polysaccharides (Ruthes et al., 2013; Chen et al., 2015; Moreno et al., 2016). To confirm the structure of the main chain, SK5 fraction was submitted to controlled smith degradation and the resultant fraction resistant to $NaIO_4$ oxidation presented six signals characteristic of β-D-glucan (1→3)-linked (data not show). Based on the results of monosaccharide composition, methylation and spectroscopy analysis, it can be assumed that SK5 fraction has a main chain formed by β-D-Glc*p* units (1→3)-linked and substituted at O-6 by non-reducing β-D-Glc*p* or β-D-Glc*p*-(1→6)-linked side chains.

The most insoluble fraction isolated was PK5 that remained as a precipitate after the alkaline treatment. Its ^{13}C-NMR spectrum (Fig. 2D and 3D) showed intense signals at δ 99.5 (C-1), 82.8 (C-2), 71.9 (C-5); 70.6 (C-2), 69.3 (C-4) and 60.2 (C-6) corresponding to α-D-glucan (1→3)-linked. The HSQC spectrum (Fig. 3D) demonstrated in the anomeric region characteristic signals of D-Glc*p* units in the α-configuration which is attributed to the low frequency of C-1 at 99.0 ppm and H-1 high field at 5.00 ppm. The other signals in HSQC spectrum at δ 82.3/3.54; 71.4/3.75; 70.1/3.33; 68.8/3.33 are attributed to C-3/H-3, C-5/H-5, C-2/H-2 and C-4/H-4 respectively. The non-substitute C-6/H-6 related signals are at δ 60.1/3.56 and 60.1/3.43 (Synytsya et al., 2009; Roy et al., 2009; Smiderle et al., 2010; Li et al., 2016).

D-Glucans are usually isolated from mushrooms employing water or alkaline solutions in different concentrations and most of them are β-D-glucans while α-D-glucans identification in basidiomycetes is less usual (Ruthes, Smiderle & Iacomini, 2015). The NaOH 0.1 M treatment proved to be a good purification method to separate two glucans with different anomeric configurations that were strongly interacting by intermolecular forces. Bhanja et al. (2014) have also separated α- and β-glucans from the water insoluble crude extract of *Ramaria botrytis* fungus, using a similar procedure with different concentrations of NaOH solution. In both cases, the separation occurred due to the difference of behavior between α-D-linear-glucan and β-D-branched-glucan in aqueous alkaline solution. Homoglycans that possess higher

branching degree are more soluble in aqueous solutions than linear polymers because branching reduces the probability of intermolecular association and enhance the tendency to form stable solutions. Otherwise, linear polysaccharides have potential to present stable intermolecular binding and form ordered arrays with the development of crystalline regions which are difficult to dissolve (Whistler, 1973).

P. betulinus fungi usually exhibited very high content of β-glucans in its dry mass, although it is also found α-glucan in minor proportion (Vunduk et al., 2015). In the present work, the amount of α-glucan (0.8 g) was higher than β-glucan (0.3 g) found in the extract which is uncommon to observe for this mushroom. There are few studies that isolated glucans from *P. betulinus* (Sari, Prange, Lelley & Hambitzer, 2017). Olennikov et al. (2012) isolated and characterized a branched α-D-glucan-(1→3) substituted at the C-6 by β-D-glucopyranose residues and Wiater et al. (2011) isolated and carboxymethylated an α-D-glucan (1→3)-linked and studied its free radical scavenging activity. The present study showed a purification method of D-glucans complex, as well as the isolation and structural characterization of two glucans from *P. betulinus* mushroom. The β-(1→3),(1→6)-branched-glucan and α-(1→3)-linear-glucan were obtained from the same fraction and separated after NaOH 0.1 M treatment. The biological activity of the isolated polysaccharides will be tested in further studies.

CONCLUSION

The present study reported a careful procedure of purification to separate D-glucans complex from *P. betulinus* mushroom extract. The NaOH 0.1 M treatment demonstrated to be efficient, simple and low-cost method. The obtained α- and β-D-glucans were chemically characterized. The investigation of the primary structure of these glucans will support to elucidate the relationship between structure-biological activities in further studies.

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ARTIGO III

PARTIALLY 3-*O*-METHYLATED (1→6)-LINKED A-D-GALACTAN FROM *PHELLINUS IGNIARIUS*: CHEMICAL CHARACTERIZATION AND IMMUNOMODULATORY ACTIVITY

Partially 3-*O*-methylated (1→6)-linked α-D-galactan from *Phellinus igniarius*: chemical characterization and immunomodulatory activity

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ABSTRACT

Phellinus igniarius is one of the most used mushrooms in traditional Chinese medicine for the treatment of many diseases. Its dried fruiting body was treated with chloroform-methanol to remove apolar compounds and after that extracted with cold water. The extract was purified by forced freeze-thawing treatment and dialysis, resulting in a homogeneous eluted fraction (M_w 3,000 Da), composed of galactose (67.4%) and 3-*O*-methyl galactose (32.6%). The HSQC analysis indicated the presence of a linear polysaccharide, which was confirmed by only one signal in the anomeric region, relative to C-1/H-1 (δ 98.5/5.34), presenting α-configuration. The spectrum also showed signals at δ 66.2/3.75 and 56.6/3.82 relative to 6-*O* substituted units and natural 3-*O*-methyl group respectively. Methylation analysis confirmed the 6-*O*-substitution by the presence of 2,3,4-Me_3-Gal*p* residue. These results suggest the presence of a partially 3-*O*-methylated (1→6)-linked α-D-galactan with low molecular weight. The immunomodulatory activity of the purified polysaccharide was evaluated and it showed strong ability to stimulate THP-1 macrophages.

Keywords: *Phellinus igniarius*; galactan; chemical characterization; immunomodulatory activity.

1. INTRODUCTION

Mushrooms from genus *Phellinus* are distinguished from other basidiomycetes due to their relevant biological effects described in Chinese folk medicine [1]. *Phellinus igniarius* is traditionally considered as food and medicine in China and it is also recognized as Sanghuang (yellow polyporus) [2]. Antiviral and antioxidant activities were reported for extracts produced from this species [3,4]. Polysaccharides are one of the main compounds of *P. igniarius* that exhibit interesting pharmacological effects such as immunomodulatory and antitumor activities [5]. Extracellular polysaccharides obtained from *P. igniarius* mycelia showed tumor-inhibitory and liver-protective effects in mice [6].

The literature featured isolation and characterization of galactans and heterogalactans from mushrooms. The latter were isolated from *Agaricus bisporus* (fucogalactan) [7], *Pleurotus sajor-caju* (mannogalactan) [8], and *Lentinus edodes* (fucomannogalactan) [9]. Studies have demonstrated that these heterogalactans presented anticoagulant, anti-inflammatory and antinociceptive activities, respectively [7,8,9]. Partially 3-*O*-methylated-α-galactans were isolated from *P. eryngii* and *P. ostreatoroseus*, although their biological effects were not tested [10].

Polysaccharides from mushrooms are considered biologically active molecules and have been reported to improve innate and cell-mediated immune responses [11,12]. Their immunostimulatory activities involve mostly monocytes, macrophages and neutrophils responses, such as activation, proliferation and cytokine production by these cells. A considerable number of studies are related to macrophage function which includes enhance of phagocytosis, proliferation and differentiation. Moreover, immunostimulatory polysaccharides can also increase the production of reactive oxygen species, nitric oxide and secretion of cytokines by macrophages through the recognition of such polymers by macrophage membrane receptors [13].

THP-1 cells are widely used to evaluate the ability of compounds to modulate the macrophage activity. Such cells express different membrane receptors that is able to recognize polysaccharides and, therefore initiate signaling pathways by the production of some cytokines [14,15].

Considering that mushroom polysaccharides have showed intense biological activities and that they are recognized by macrophage membrane receptors, this

study aimed to isolate and characterize a galactan from *P. igniarius*, and later evaluate its effect on THP-1 differentiated macrophages.

2. EXPERIMENTAL

2.1. FUNGAL MATERIAL

The fungal material was furnished by Dr. Leo J. L. D. van Griensven, from Plant Research International, Wageningen University and Research Centre, The Netherlands. *P. igniarius* was collected from the wild and its fruiting bodies have been identified by Dr. Usa Klinhom (Mahasarakham University, Thailand). The sample has been deposited in the collection of the Natural Medicinal Mushroom Museum of the Faculty of Biology of Mahasarakham University.

2.2. EXTRACTION AND PURIFICATION OF GALACTAN FROM *P. igniarius*

The dried and milled mushroom (157.7 g) was firstly extracted with chloroform-methanol (2:1; v/v) at 60 ºC for 3 h (x 3), to remove apolar compounds (Fig. 1). The residue was dried and submitted to successive cold aqueous extraction (1 L) for 6 h (x 3). After centrifugation (8,000 rpm, 20 ºC, 15 min) to recover the aqueous extract, it was evaporated to a small volume and the polysaccharide was precipitated by addition of ethanol (3:1 v/v) and centrifuged at 10,000 rpm at 10 ºC, for 15 min. The sediment was dialyzed (2 kDa M_r cut-off) against tap water for 24 h, concentrated under reduced pressure and freeze-dried.The resultant fraction CW was purified by freeze-thawing process, [16] which consists in dissolving the sample to reduced volume of water, and then submit it to freeze and slowly thaw until observe separation of the soluble and insoluble polysaccharides. The soluble fraction (SCW) was acquired after centrifugation (10,000 rpm, at 10 ºC, for 15 min) and the freeze-thawing process was repeated with reduced volume to guarantee complete separation. The remained soluble fraction (SCWS) was dialyzed (3.5 kDa M_r cut-off) against distilled water, in a closed system for 10 days, with constant water renovation. The eluted fraction (E3) was concentrated under reduced pressure and lyophilized for the chemical characterization analyses.

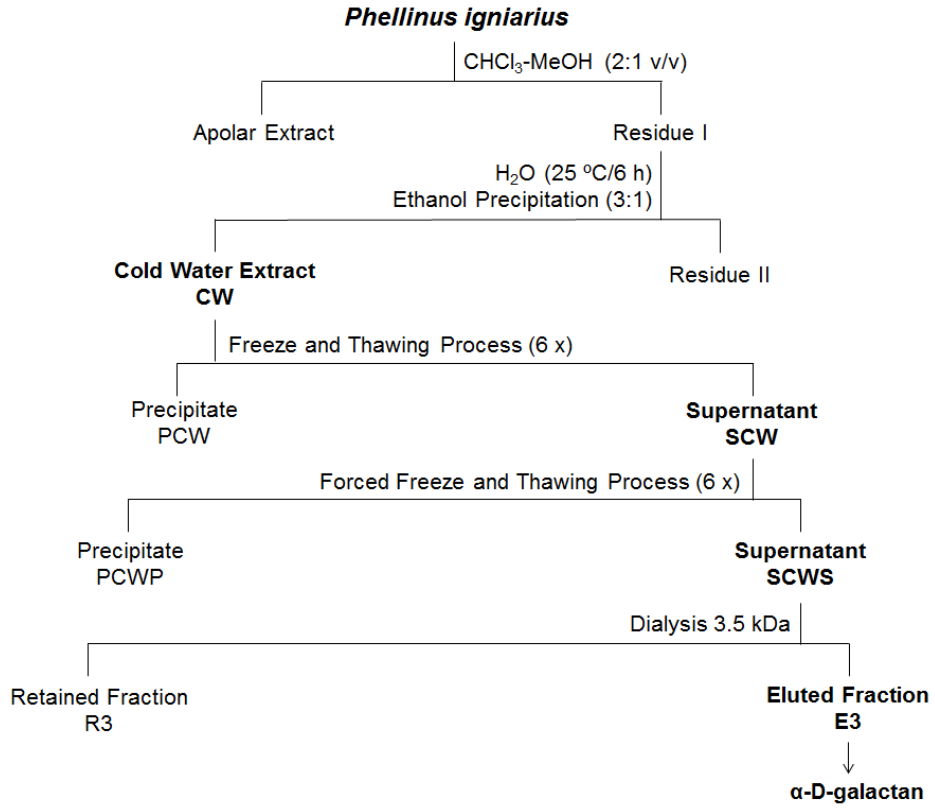

FIGURE 1. General scheme of isolation and purification of 3-*O*-methylated (1→6)-linked α-D-galactan.

2.3. ANALYSIS OF MONOSACCHARIDE COMPOSITION BY GC-MS

The analyzed samples (1 mg) were hydrolyzed with 2 M TFA at 100 ºC for 8 h, followed by evaporation to dryness. The dried carbohydrate samples were dissolved in distilled water (100 µL) and 1 mg $NaBH_4$ was added. The solution was held at room temperature overnight to reduce aldoses into alditols [17]. The product was dried and excess $NaBH_4$ was neutralized by the addition of acetic acid, which was removed following the addition of methanol (x 2) under a compressed air stream in a fume hood. Acetylation of the alditols was performed in pyridine–Ac_2O (200 µL; 1:1, v/v), heated for 30 min at 100 ºC. The pyridine was removed by 5% $CuSO_4$ solution and the resulting alditol acetates were extracted with $CHCl_3$. The derivatives were analyzed by GC-MS Varian CP-3800 gas chromatograph coupled to an Ion-Trap 4000 mass spectrometer, using a VF5 column (30 m x 0.25 mm i.d.) programmed from 100 to 280 °C at 10 °C min^{-1}, with He as carrier gas. The correspondent monosaccharides were identified by their typical retention time, in

comparison with standards, and the results were expressed as mol%, calculated according to Pettolino et al. [18].

2.4. METHYLATION ANALYSIS

The galactan (5 mg) was per-*O*-methylated according to the method of Ciucanu & Kerek [19], using powdered NaOH in Me_2SO-MeI. The per-*O*-methylated products were submitted to methanolysis with MeOH-HCl 3 N (1 mL) for 2 h at 80 °C [20] followed by hydrolysis using 2 N H_2SO_4 (1 mL) for 16 h at 100 °C. The acid was neutralized with $BaCO_3$, which was removed by centrifugation (10,000 rpm, at 20 ºC, for 15 min) and filtration. The sample was reduced with $NaBD_4$ and acetylated as described above, to give a mixture of partially *O*-methylated alditol acetates. The derivatives were identified by their typical retention time and electron impact profile, in comparison to standards according to Sassaki et al., [21]. The results were given as mol%, calculated according to Pettolino et al., [18].

2.5. HPSEC ANALYSIS

The relative molecular weight (M_w) of the galactan was determined by high performance size exclusion chromatography (HPSEC). The elution time of the polysaccharide was compared with a calibration curve obtained using standard dextrans (670 kDa, 487 kDa, 266 kDa, 124 kDa, 72.2 kDa, 40.2 kDa, 17.2 kDa, 9.4 kDa and 5.2 kDa, from Sigma-Aldrich). HPSEC analyses were performed using Waters ultrahydrogel columns in series with exclusion sizes of 7×10^6, 4×10^5, 8×10^4 and 5×10^3 Da and a refractive index detector. The eluent was 0.2 M aq. $NaNO_2$ containing 200 ppm aq. NaN_3 at 0.6 mL/min. The sample and dextran standards were dissolved in 0.2 M aq. $NaNO_2$ and filtered through a membrane (0.22 µm) before injection (100 µL loop). HPSEC data was collected and analyzed by the Wyatt Technology ASTRA program.

2.6. NMR SPECTROSCOPY

NMR spectra were obtained using a 400 MHz Bruker model Avance III spectrometer with a 5 mm inverse probe, at 70 °C in D_2O. Chemical shifts (δ) were

expressed in ppm relative to acetone, at δ 30.2 (^{13}C) and 2.22 (^{1}H). NMR signals were assigned on the basis of 2D NMR experiment (HSQC) and literature data.

2.7. CELL LINE

THP-1 cells (human acute monocytic leukemia cell line) were maintained in RPMI 1640 liquid culture medium (Sigma-Aldrich), supplemented with 10% fetal calf serum, streptomycin (100 µg/mL) and penicillin (100 U/mL), at 5% CO_2, at 37 ºC.

2.8. DIFFERENTIATION OF THP-1 CELLS

The mature macrophage-like state was induced by treating THP-1 cells (2 x 10^5 cells/mL) with 5 ng/mL phorbol 12-myristate 13-acetate (PMA), for 48 h, in 24-wells polystyrene tissue culture plates, containing 1 mL cell suspension/well. After differentiation, cells adhered to the surface allowing removal of the culture medium containing PMA. The wells were then washed with PBS (phosphate buffered saline) and PMA free fresh medium was added. The THP-1 macrophages (differentiated cells) were let to rest for 24 h, at 37 ºC in 5% CO_2 atmosphere.

2.9. CYTOTOXICITY ASSAY

Metabolic activity of THP-1 macrophages was evaluated by MTT (3,4,5-dimethyl-2-thiazolyl-2,5-diphenyl-2H-tetrazolium) assay. The culture medium of the THP-1 macrophages was removed and replaced by fresh medium containing polysaccharide fraction (SCWE3) at different concentrations (10, 50, 100 and 250 µg/mL) or PBS (50 µL). The cells were then incubated for 24 h, at 37 ºC in 5% CO_2 atmosphere. Three hours before completing the incubation period, MTT solution (20 µL; at 5 mg/mL) was added. At the end of incubation, unreacted MTT was removed, formazan crystals were solubilized in Me_2SO/EtOH (200 µL/well) and optical density was measured at 595 nm.

2.10. MACROPHAGE STIMULATION AND CYTOKINE EVALUATION

After differentiation, THP-1 macrophages (2 x 10^5 cells/well) were incubated with fresh medium containing polysaccharide fraction (SCWE3) at different concentrations (5, 10, 50 µg/mL) or PBS (50 µL) or lipopolysaccharide (LPS; 500 ng/mL) as negative and pro-inflammatory controls, respectively. After 24 h of incubation at 37 ºC in 5% de CO_2 atmosphere, the cell medium of each condition was collected and kept at - 80 ºC for 24 h. Pro-inflammatory (TNF-α) and anti-inflammatory (IL-10) cytokines were determined and quantified by ELISA (eBioscience kits), according to the manufacturer's instructions.

2.11. STATISTICAL ANALYSIS

The results are expressed as mean ± standard deviation of quadruplicate conditions of three representative experiments. Statistical significance was determined using one-way analysis of variance (ANOVA) followed by Tukey's test. Data were considered different at a significance level of $p < 0.05$. The graphs were drawn and the statistical analyses were performed using GraphPad Prism version 5.01 for Windows.

3. RESULTS AND DISCUSSION

3.1. CHEMICAL STRUCTURE OF THE PURIFIED GALACTAN

As described in section 2.2, extraction processes followed by different purification procedures were performed to isolate a galactan from *P. igniarius*. The purification was accompanied by chromatographic analyses, as follows. The crude extract (CW) presented glucose, mannose and galactose as major components and, in minor proportion, xylose, arabinose and 3-*O*-methyl-galactose. The most common polysaccharides obtained from mushrooms are D-glucans and heterogalactans [22], therefore the composition of CW are coherent to the hypothesis of more than one polysaccharide being present in this fraction. The HSQC spectrum of CW (Fig. 3A) reinforced this hypothesis by the presence of typical signals of D-glucan and galactan. The spectrum exhibited signals that could be relative to C-1/H-1 (δ

102.4/4.93 and 102.4/4.24) and *O*-substituted C3/H3 of β-D-glucose (δ 83.8/3.65); and signals relative to C1/H1 of α-D-galactose (δ 98.4/4.80).

Taking into account that D-glucans are usually insoluble in comparison to heterogalactans, a freeze-thawing process was chosen as first purification step and a partial precipitation occurred, generating a soluble fraction (SCW) and an insoluble one, which was not analyzed in this study (Fig. 1). The monosaccharide composition analysis showed that SCW contained arabinose (10.1%), xylose (12.7%), 3-*O*-methyl-galactose (26.2%), galactose (25.3%), mannose (12.4%), and glucose (13.4%). The remaining residue of glucose in SCW could be a persistent contamination of D-glucans. Moreover, the presence of xylose and especially arabinose are not common in mushroom polysaccharides and could be a contamination of plants, since *P. igniarius* was collected from the wild. The HPSEC chromatogram of SCW showed a blunt peak with a shoulder eluting after 55 min (Fig. 2), confirming the necessity of more purification steps. The HSQC spectrum of this fraction showed less signals than CW spectrum, although the glucose signal relative to C1/H1 (δ 102.0/5.37) was still present. The signal of C1/H1 α-D-mannose (δ 101.7/5.41) was also observed. The signal of C1/H1 of α-D-galactose (δ 98.0/5.26) was presented in high intensity. No signals relative to xylose or arabinose were observed in any of the spectra. A repetition of freeze-thawing process with reduced volume of water was done with the aim of purifying this sample, resulting in a soluble fraction (SCWS) that was recovered after centrifugation, dialysis and lyophilization. This procedure was successful in removing the glucose contaminant and the sample presented mainly 3-*O*-methyl-galactose (32.6%) and galactose (49.7%) with minor proportions of xylose and arabinose

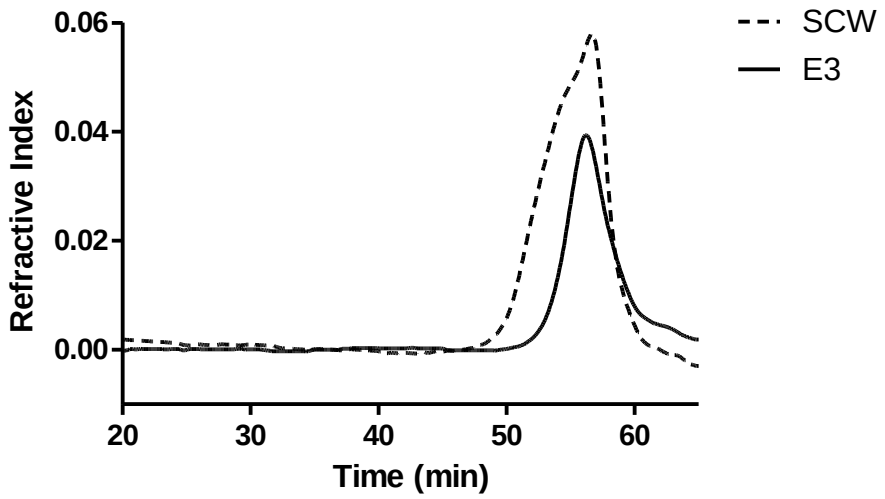

FIGURE 2. Elution profiles of SCW (dashed line) and E3 (solid line) fractions by HPSEC.

With the aim of removing xylose and arabinose contaminants, fraction SCWS was dialyzed against distilled water, in a closed system, using a 3.5 kDa M_r cut-off membrane. The eluted fraction (E3) showed only 3-*O*-methyl-galactose (32.6%) and galactose (67.4%) as monosaccharides. The chromatographic profile observed on HPSEC analysis was homogeneous (Fig. 2), with a well defined peak, and its M_w was estimated approximately at 3.0 x 10^3 g/mol (relative to dextrans). Such low molecular weight galactan has not been yet described for mushrooms. Considering that a variety of mushroom polysaccharides have presented immunomodulatory properties and, that they have been extracted from edible mushrooms [12], it could be an advantage if they were easily absorbed. Low molecular weight polysaccharides and low branching degree may be more bio-available to easily get in contact with macrophages of the gut-associated lymphatic tissue [23].

The linkage type of the isolated galactan was evaluated by analysis of its methyl derivatives and confirmed by NMR spectroscopy. An aliquot of the purified fraction (E3) was submitted to methylation reaction and analyzed by GC-MS, showing as derivatives only 2,3,4-Me_3-Gal*p* (88%) and 2,3,4,6-Me_4-Gal*p* (12%). This result confirmed that this molecule is linear and the galactopyranose units are *O*-6 linked.

The spectrum of the purified fraction (E3) showed only one signal, in the anomeric region, at δ 98.9/4.74, which corresponded to C-1/H-1 of galactose in α-

configuration. The other carbon/hydrogen frequencies of E3 were assigned at δ 69.8/3.79 (C-2/H-2), δ 77.4/3.28 (C-3/H-3), δ 67.0/3.57 (C-4/H-4), δ 69.5/3.91 (C-5/H-5), δ 67.4/3.57 (C-6/H-6) (Fig 3C). A resonance arising from the O-CH_3-group was observed at δ 56.7/3.36, confirming the result obtained by GC-MS analysis of the presence of 3-*O*-Me-α-Gal*p*. These chemical shifts are in agreement with literature [8,10]. The results obtained suggested that the purified polysaccharide is a (1→6)-linked α-D-galactan which presents 32.6% of naturally methylated galactose units. As observed in other mushroom polysaccharides, the methyl group is located at the O-3 position [8,10]. A heterogalactan of *P. igniarius* was previously described [24] and it presented a complex structure composed of galactose, 3-*O*-methyl-galactose, mannose, glucose and fucose. However the authors did not present a chromatographic profile to confirm the homogeneity of the sample. In the present work, the crude samples (CW and SCW) were composed by a diversity of monossacharides (arabinose, xylose, 3-*O*-methyl-galactose, mannose, galactose and glucose), although it was confirmed that arabinose, xylose, mannose and glucose were not covalently linked to the α-D-galactan, as they were removed after simple purification methods. Polysaccharides may complex with each other by inter-molecular interactions, which hampers their purification, requiring careful analysis and interpretation of NMR and methylation data [22]. Other polysaccharides containing galactose in their structures were isolated from the fruiting bodies of *Pleurotus eryngii* [10], *P. pulmonarius* [25], from submerged culture of *Inonotus levis* [26] and an extracellular polysaccharide was produced by *P.ostreatoroseus* [27].

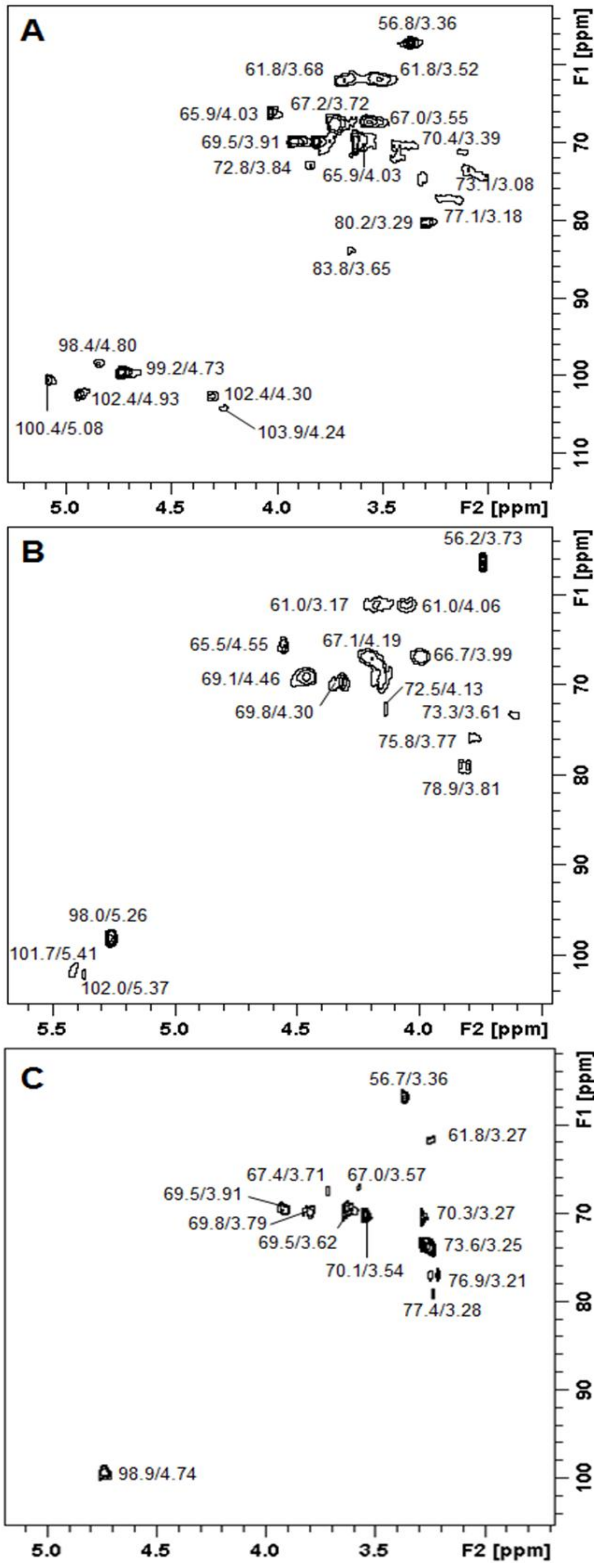

FIGURE 3. HSQC NMR Spectrum of CW (A), SCW (B) and E3 (C) fractions. CW and E3 were analysed in Me_2SO-d_6 at 70 °C and SCW was analysed in D_2O at 70 °C (chemical shifts are expressed in δ ppm).

The immunostimulatory activity of the purified α-galactan was performed through evaluation of cytokines secretion (TNF-α and IL-10) by macrophage-differentiated THP-1 cells, after incubation with different concentrations of the polysaccharide. Firstly, the citotoxicity of E3 fraction was evaluated by MTT assay, which showed that it did not altered the viability of THP-1 macrophages at the range of 10 to 50 µg/mL (Fig. 4).

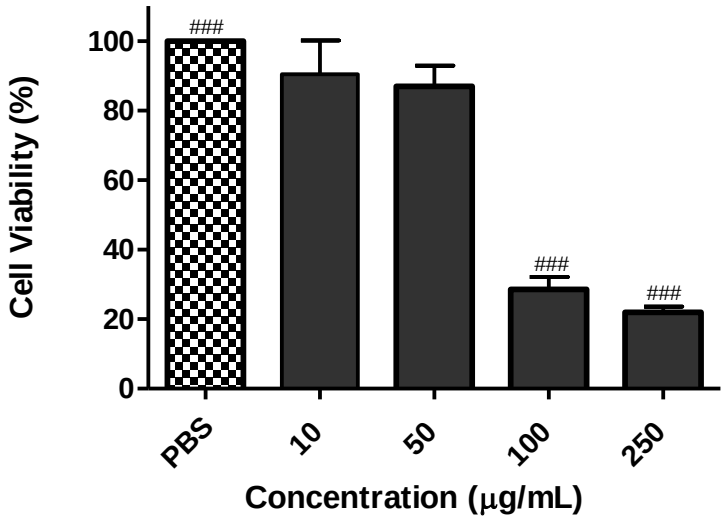

FIGURE 4. Effect of E3 on the viability of THP-1 macrophages. THP-1 macrophages were exposed for 24 h at the indicated concentrations. Cell viability was determined by MTT assay. Culture medium with PBS was used as negative control, corresponding to 100% viability. Statistical analyses were performed by means of one-way analysis of variance (ANOVA) followed by Turkey's test. The results represent the mean ± SD of quadruplicate conditions of three representative experiments. ### $p < 0.001$ versus negative control.

After 24 h incubation with THP-1 macrophages, the partially 3-*O*-methylated-α-galactan from *P. igniarius* was able to stimulate cytokines secretion by these cells at 5, 10 and 50 µg/mL (Fig. 5).

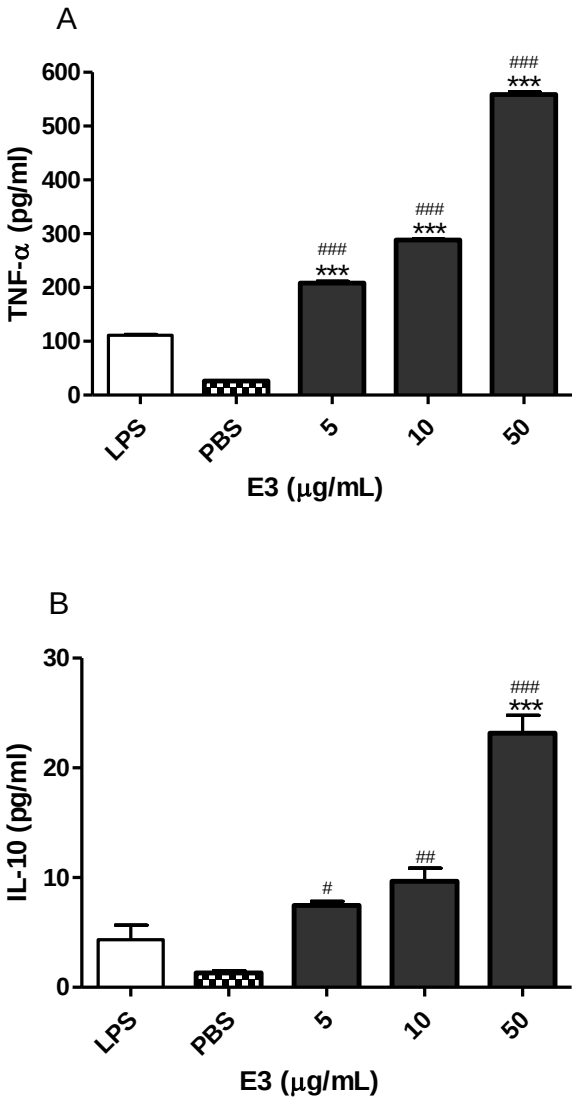

FIGURE 5. Ability of E3 to stimulate the secretion of TNF-α (A) and lL-10 (B) by THP-1 macrophages. Cells were incubated with positive control (LPS; 500 ng/mL), negative control (PBS) and α-galactan (E3; 5, 10 and 50 μg/mL) for 24 h. Statistical analyses were performed by means of one-way analysis of variance (ANOVA) followed by Turkey's test. The results represent the mean ± SD of quadruplicate conditions of three representative experiments. Asterisks (*) represents statistically significant difference from the positive control-LPS (*** $p < 0.001$). Hash marks ($^{\#}$) represents statistically significant difference from the negative control-PBS ($^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$, $^{\#\#\#}$ $p < 0.001$).

At the highest tested concentration, the mushroom polysaccharide stimulated the secretion of TNF-α and IL-10 5.0 times higher than LPS stimulation. A crescent increase of TNF-α secretion was detected with crescent concentrations of α-galactan (Fig. 5A). TNF-α is a pro-inflammatory cytokine and its over-expression can induce inflammation processes like fever and tissue damages; on the other hand TNF-α show a crucial role against invasive microorganisms and tumor cells [28, 29]. There is scientific evidence in the literature about inflammatory cytokine induction by mushroom extracts, which is an indicative that mushroom compounds may help activate the immune system cells [30-34]. The IL-10 secretion promoted by α-galactan incubation was statistically higher than LPS stimulation, but only at 50 µg/mL (Fig. 5B); there was no statistical difference between LPS and galactan at 5 and 10 µg/mL. IL-10 is an immunesuppressive cytokine that promotes the equilibrium between the inflammatory and anti-inflammatory responses and its stimulation promoted by mushroom polysaccharides indicates the beneficial effects of such fungi compounds [35-38]. From the results obtained in this study it is possible to suggest that *P. igniarius* α-galactan may be a potent immunomodulatory agent, although further tests are required to determine its mechanism of action.

4. CONCLUSION

The present study showed the isolation and purification of a polysaccharide from the aqueous extract of the edible mushroom *P. igniarius*. NMR and GC-MS analyses confirmed that this polymer is a linear galactan with a backbone composed exclusively of (1→6)-linked-α-D-Gal*p* units, some of them being naturally methylated at O-3 position. Its low molecular weight (3,000 Da) is not common for mushroom polysaccharides, and it may confer an advantage in absorption by the gastrointestinal tract. The immunomodulatory activity of the purified polysaccharide was investigated and the preliminary studies showed that it presented the ability of stimulating the production of TNF-α and IL-10 at non-toxic concentrations (lower than 50 µg/mL). The α-galactan from *P. igniarius* showed interesting effects on the stimulation of THP-1 macrophages, being an interesting candidate to advance the studies on its pharmacological properties.

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ARTIGO IV

STRUCTURAL CHARACTERIZATION AND RHEOLOGICAL PROPERTIES OF GEL-LIKE β-D-GLUCAN FROM *PHOLIOTA NAMEKO*

(Publicado na revista *Carbohydrate Polymers* – SOVRANI *et al*., 2017)

Structural characterization and rheological properties of a gel-like β-D-glucan from *Pholiota nameko*

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ABSTRACT

A crude fraction (SCW) was extracted with cold water from *Pholiota nameko* and showed mannose (24.1%), galactose (44.9%) and glucose (31%). Purification procedures resulted in a polysaccharide fraction (bG-PN), that showed only glucose. NMR and methylation analyses of bG-PN indicated a β-D-glucan-(1→3)-linked, substituted at O-6 by β-D-Glc*p* or (1→6)-linked β-D-Glc*p* side chains. Rheological studies of crude and purified fractions at the same concentration showed similar shear-thinning behavior and gel-like structure which indicates no need to isolate the polymer to achieve some desirable rheological properties. SCW (at 1% and 2%) and bG-PN (at 2%) presented thermal stability during heating and cooling, suggesting that the physical structure of gels (SCW and bG-PN at 2%) and viscoelastic fluid (SCW at 1%) formed were not altered in the tested temperature range. Our results suggest that *P. nameko* β-D-glucans can be applied in different food preparations as thickener or gelling agents modifying their rheological properties.

Keywords: *Pholiota nameko,* structural characterization, β-D-glucan, rheology.

1. INTRODUCTION

Edible mushrooms have been considered for many years as functional foods due to their high nutritional value (Ren, Perera, & Hemar, 2012). They present an attractive chemical composition, being rich in proteins, carbohydrates, fibers, vitamins and unsaturated fatty acids (Wani, Bodha, & Wani, 2010). Mushrooms have showed a great interest in biomedical area due to the presence of bioactive substances, including polysaccharides (Zhao, Wang, & Lu, 2009). *Pholiota nameko* is a well known edible mushroom rich in biological compounds, being extensively cultivated to be used as food and as traditional medicine in China and Japan (Neda, 2008; Zhang et al., 2014).

Studies showed that *P. nameko* polysaccharides demonstrated strong anti-inflammatory activity in rodents through paw edema model (Li, Lu, Zhang, Lu, & Liu, 2008) and hypolipidemic effect after orally treatment in hyperlipidemic rats (Li, Zhang, & Ma, 2010). Furthermore, this mushroom can be useful for food industry to design supplements due to its potential as prebiotic (Rodrigues et al., 2017). Fungal polysaccharides comprise a large group of polymers with pharmaceutical and biotechnological applications and such properties are closely related to their chemical structures (Bot et al., 2001; Ruthes, Smiderle, & Iacomini, 2015). The most studied class of polysaccharides present in edible mushrooms are the β-D-glucans which frequently consist of a backbone composed of β-(1→3)-linked glucopyranose, partially substituted at O-6 position by glucopyranose units (Ren, Reynisson, Perera, & Hemar, 2013; Synytsya & Novak, 2014).

Mushroom nutritional values associated with their therapeutic benefits stimulate the application of such organisms or their extracts in food industry as additives, supplements and emulsifiers (Liu et al., 2016; Xu, Xu, Zhang, & Zhang, 2008; Xu, Zhang, Liu, Sun, & Wang, 2016). β-D-Glucans can present special physicochemical properties such as gelling capability, which might be employed in food, pharmaceutical and cosmetic industry (Laroche & Michaud, 2007). The determination of their rheological characteristics is helpful to predict gelation, thickening and emulsification properties. β-D-Glucans are abundant polysaccharides in edible mushrooms and have potential to increase water viscosity and provide desirable appearance and texture for food products (Bot, Smorenburg, Vreeker, Pâques, & Clark, 2001; Synytsya & Novak, 2014). The viscosity promoted by such

molecules in solution is related to their molecular weight, chemical structure, concentration and food matrix (Zhu, Bin, Bian, & Xu, 2015).

Few reports have mentioned some food industry applications using β-glucan, such as rice noodles prepared with β-glucan-rich fractions from *L. edodes* (Heo et al., 2013); and wheat flour mixture containing β-glucan-enriched material from the same mushroom to prepare cakes (Kim et al., 2011). In both cases, the addition of mushroom β-glucans enhanced the quality of the products by improving texture characteristics and increasing the fiber content.

Observing the potential use of mushroom β-glucan-rich fractions for food industry, this study aimed to isolate, purify and structurally characterize a gel-like β-D-glucan from *Pholiota nameko*, including studies on rheological properties of the crude and purified fractions.

2. MATERIAL AND METHODS

2.1 FUNGAL MATERIAL

Pholiota nameko was kindly provided by the company Nayumi Cogumelos, located in the city of Mogi das Cruzes, São Paulo, Brazil.

2.2 EXTRACTION AND PURIFICATION

Pholiota nameko (PN) (2.5 kg) was freeze dried until total dryness (226.0 g) and milled in a blender (Fig. 1). The apolar compounds were extracted using a chloroform-methanol solution (2:1 v/v) at 60 °C for 5 h (3 times). Subsequently, the residue was dried and submitted to sequential aqueous extractions at room temperature (23 °C) under mechanical stirring for 6 h (3 times). The aqueous extract was concentrated under reduced pressure and the polysaccharide was precipitated with ethanol (3:1 v/v). The ethanolic solution was centrifuged at 8,500 rpm at 10 °C for 20 min and the precipitate was dialyzed (12-14 kDa M_r cut-off membrane) against tap water for 24 hours and freeze dried.

The resultant fraction (CW; 37.4 g) was submitted to freeze-thawing process (Gorin & Iacomini, 1984). This method consists of solubilizing the sample in small volume of distilled water followed by freeze and slowly thawing at room temperature

(23 °C) until the formation of precipitates. The soluble fraction (SCW; 17.9 g) was separated from the insoluble one by centrifugation (8,500 rpm, 4 °C, 20 min) and treated with Fehling solution (Jones & Stoodley, 1965). The fraction (FSCW; 1.2 g) which did not complex with Cu^{2+} ions was recovered after centrifugation (8,500 rpm, 4°C, 20 min) and separated from the insoluble Cu^{2+} complex, which was not used in this study.

The obtained fractions were neutralized with HOAc, dialyzed against tap water for 48 hours (12-14 kDa M_r cut-off membrane) and deionized on a cationic resin ion exchange and then freeze dried. The FSCW fraction was submitted to another freeze–thawing process and centrifuged (8,500 rpm, 4°C, 20 min): resulting in a soluble fraction (not used in this study) and a gel fraction (bG-PN; 0.52 g), that was used for the chemical and rheological analysis.

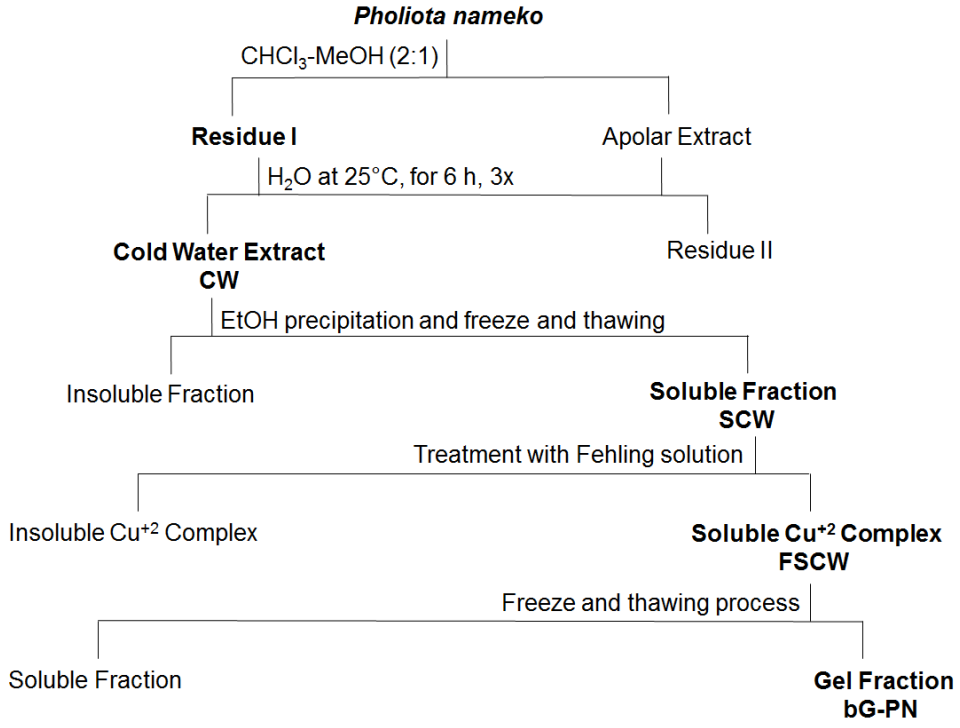

FIGURE 1. Scheme of extraction and purification of β-D-glucan obtained from fruiting bodies of *Pholiota nameko*.

2.3 ANALYSIS OF MONOSACCHARIDE COMPOSITION BY GC-MS

Aliquots of polysaccharide fractions (1.0 mg) were hydrolyzed with 2 M TFA for 8 hours at 100 °C following the method of Sassaki et al. (2008). The acid was removed by evaporation to dryness. The hydrolyzed products were dissolved in distilled water (100 µL) and 1 mg of $NaBH_4$ was added. The solution was held overnight in room temperature (23 °C) to reduce aldoses into alditols, neutralized with HOAc and later evaporated to dryness. The residue was removed following the addition of methanol (2 times) under a compressed air stream in a fume hood. The samples were acetylated with pyridine–Ac_2O (200 µL; 1:1, v/v), heated for 30 min at 100 °C. The resulting alditol acetates were extracted with chloroform and analyzed by gas chromatography-mass spectrometry (GC-MS) using a Varian model CP-3800 gas chromatograph coupled to an Ion-Trap 4000 mass spectrometer, with a VF5 column (30 m x 0.25 mm i.d.) programmed from 100 to 280 °C at 10 °C/3 min, with He as carrier gas. The alditol acetates were identified by their typical retention times and electron impact profiles.

2.4 METHYLATION ANALYSIS

Per-*O*-methylation of purified polysaccharide (bG-PN) fraction was carried out using NaOH–Me_2SO–MeI according to Ciucanu and Kerek (1984). After isolation of the products by neutralization (HOAc), dialysis, and evaporation, the methylation process was repeated. The per-*O*-methylated products were submitted to methanolysis with MeOH-HCl 3 N (1 mL) for 2 h at 80 °C (Jannson, Kenne, Liedgren, Lindberg, & Lonngren, 1976) followed by hydrolysis using 2 N sulphuric acid (1 mL) for 16 h at 100 °C. The sample was reduced with $NaBD_4$ and acetylated to give a mixture of partially *O*-methylated alditol acetates, which was analyzed by GC-MS. Partially *O*-methylated alditol acetates were identified from m/z by comparing their positive ions with standards and expressed as a relative percentage of each component.

2.5 CONTROLLED SMITH DEGRADATION OF β-D-GLUCAN (BG-PN)

The purified fraction (bG-PN; 50 mg) was oxidized with 0.05 M aqueous $NaIO_4$ (20 mL) at room temperature (23 °C) protected from light for 72 hours. Ethylene glycol was added to stop the reaction, the material was dialyzed (2 kDa M_r

cut-off membrane) and the resulting polyaldehyde was reduced with $NaBH_4$ for 12 hours, neutralized with HOAc, dialyzed and concentrated to 50 mL (Goldstein, Hay, Lewis, & Smith, 2005). The residue was submitted to partial hydrolysis with TFA, pH 2.0, for 30 minutes at 100 °C and dialysis (2 kDa M_r cut-off membrane). The final solution was freeze dried the material resistant to oxidation was analyzed by ^{13}C-NMR spectroscopy.

2.6 NUCLEAR MAGNETIC RESONANCE

^{13}C- and HSQC-NMR spectra were obtained using a 400 MHz Bruker model Avance III spectrometer with a 5 mm inverse probe. The analyses were performed at 70 °C and polysaccharide samples (40 mg) were dissolved in Me_2SO-d_6. Chemical shifts are expressed in ppm (δ) relative to Me_2SO-d_6 ^{13}C and ^{1}H signals at δ 39.40 and 2.40, respectively.

2.7 RHEOLOGICAL MEASUREMENTS

Rheological analysis were made on crude fraction SCW (at 1% and 2% w/w), that presented 48.4% yield and on the isolated β-D-glucan fraction (bG-PN), at 2% (w/w) due its low yield (1.4%). Samples were solubilized in distilled water being maintained under magnetic stirring for 5 days at 70 °C and rest during 20 minutes before analyses. Rheological measurements were carried out using a HAAKE MARS II rheometer (Thermo Fisher Scientific, Karlsruhe, Germany), at 25 °C with a cone-plate (C60/2° TiL) measurement system. A 1 mm measurement gap was used. The temperature was controlled by a circulating water bath (DC5, Haake) coupled to a Peltier temperature control device (TC 81, Haake). During measurements on temperature variation, the system containing sample was covered with a sample hood (POM 222-1903) to prevent water evaporation. Before all rheological measurements, samples were maintained on the plate for 300 s, in order to allow temperature equilibrium. Viscosity curves were obtained in the CR mode (controlled shear rate) by applying an increasing shear rate (0.005 to 500 s^{-1}) during 300 s. Viscoelastic behavior of samples were evaluated using frequency sweeps (0.02 to 10 Hz) with strain of 1%. On thermostability studies, temperature sweeps were

performed by heating (5 to 60 °C) and subsequent cooling (60 to 5 °C) the samples at a rate of 2 °C/min, at a frequency of 1 Hz and strain of 1%. The software RheoWin 4 Data Manager was employed to obtain the rheological parameters. All the analyses were performed in triplicates and graphs show the mean values and their corresponding standard error of the mean (SEM).

3. RESULTS AND DISCUSSION

3.1 PREPARATION OF POLYSACCHARIDE EXTRACTS AND ISOLATION OF β-D-GLUCAN

Polysaccharide fractions were obtained from *Pholiota nameko* fruiting bodies after successive cold water extractions as explained above (section 2.2). Table 1 presents the monosaccharide composition of each fraction obtained over the purification process.

TABLE 1. Monosaccharide composition of fractions obtained from *Pholiota nameko*.

Fractions	**Monosaccharide Composition (%)[a]**		
	Man	**Gal**	**Glc**
CW	14.1	6.3	79.6
SCW	24.1	44.9	31.0
bG-PN	-	-	100.0

[a] % of peak area relative to total peak areas, determined by GC–MS.

All fractions analyzed presented mannose, galactose and glucose, being this last monosaccharide the most abundant in CW (79.6%) and bG-PN (100%) fractions. The composition observed for CW and SCW indicated the presence of a mixture containing a D-glucan and an heteropolysaccharide composed of mannose and galactose as observed in previous reports that isolated mannogalactans from basidiomycetes (Smiderle et al., 2008; Silveira et al., 2015). Fehling solution treatment was effective to separate the D-glucan from the other heteropolysaccharide, as confirmed by the monosaccharide composition of bG-PN (Table 1).

3.2 CHEMICAL COMPOSITION OF SCW FRACTION AND CHARACTERIZATION OF β-D-GLUCAN

The SCW fraction presented a mixture of monosaccharide components (Table 1) and was submitted to treatment with Fehling solution and freeze-thawing purification process giving rise to bG-PN fraction. NMR spectra of crude and purified fractions are shown in Fig. 2. ^{13}C-NMR spectrum of the crude extract SCW (Fig. 2A) indicated a mixture of polysaccharides due to the presence of various anomeric signals.

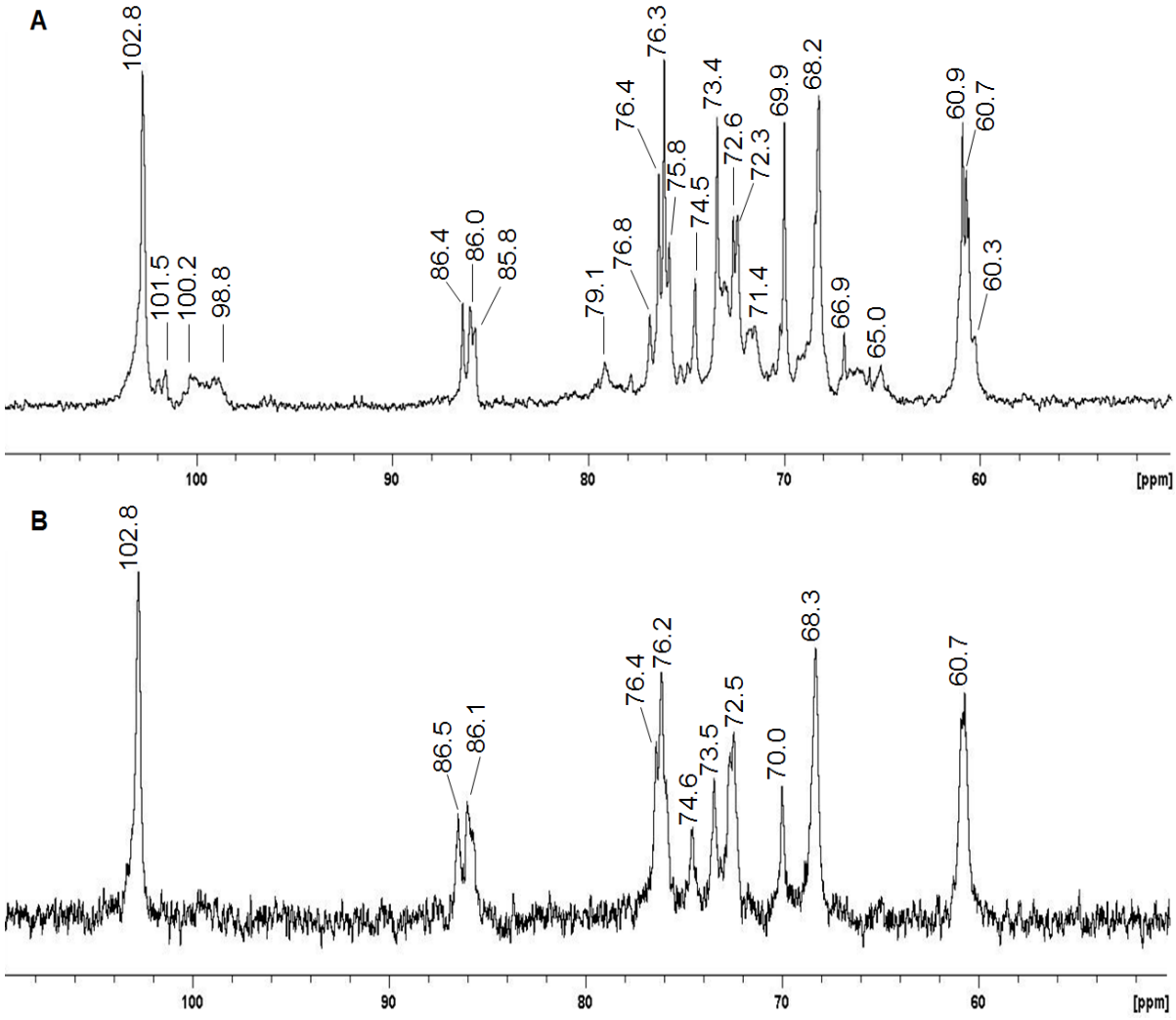

FIGURE 2. ^{13}C-NMR spectra of SCW (A) and BG-PN (β-D-GLUCAN) (B) fractions in Me_2SO-D_6 at 70°C (chemical shifts are expressed in δ ppm).

The main C-1 signals that correspond to β-D-Glc*p* (δ 102.8), β-D-Man*p* (δ 101.5), α-D-Glc*p* (δ 100.2) and α-D-Gal*p* (δ 98.8) were identified by comparison with previous reports (Rosado et al., 2003; Smiderle et al., 2006; Smiderle et al., 2010). The signal at δ 79.1 arises from O-4 substitution of α-D-Glc*p* residues characteristic of glycogen-like polysaccharides, which are normally encountered in mushrooms (Smiderle et al., 2010; Stanek, Falk, Huber, 1998). The presence of mannogalactans in SCW fraction was confirmed by the signals at δ 76.8 and δ 66.9, corresponding to O-2 and O-6 substitutions of α-D-Gal*p* residues (Silveira et al., 2015). Furthermore, signals at δ 85.8; 86.0; and 86.4 indicated O-3 substitution of β-D-Glc*p* residues (Moreno et al., 2016).

Methylation analysis of bG-PN showed the presence of 2,3,4,6-Me_4-Glc*p* (27%); 2,4,6-Me_3-Glc*p* (30%); 2,3,4-Me_3-Glc*p* (16%); and 2,4-Me_2-Glc*p* (27%) derivatives. These data indicate that bG-PN presents a backbone composed of Glc*p*-(1→3)-linked, highly substituted at O-6 by Glc*p* residues and/or side chains of Glc*p*-(1→6)-linked. A similar D-glucan, with the same proportion of side-branches was isolated previously from *Cookeina tricholoma* by alkaline extraction (Moreno et al. 2016). Interestingly, in the present study, the D-glucan was easily extracted by water. To confirm methylation results, bG-PN was analyzed by NMR spectroscopy (Figs. 2B and 3).

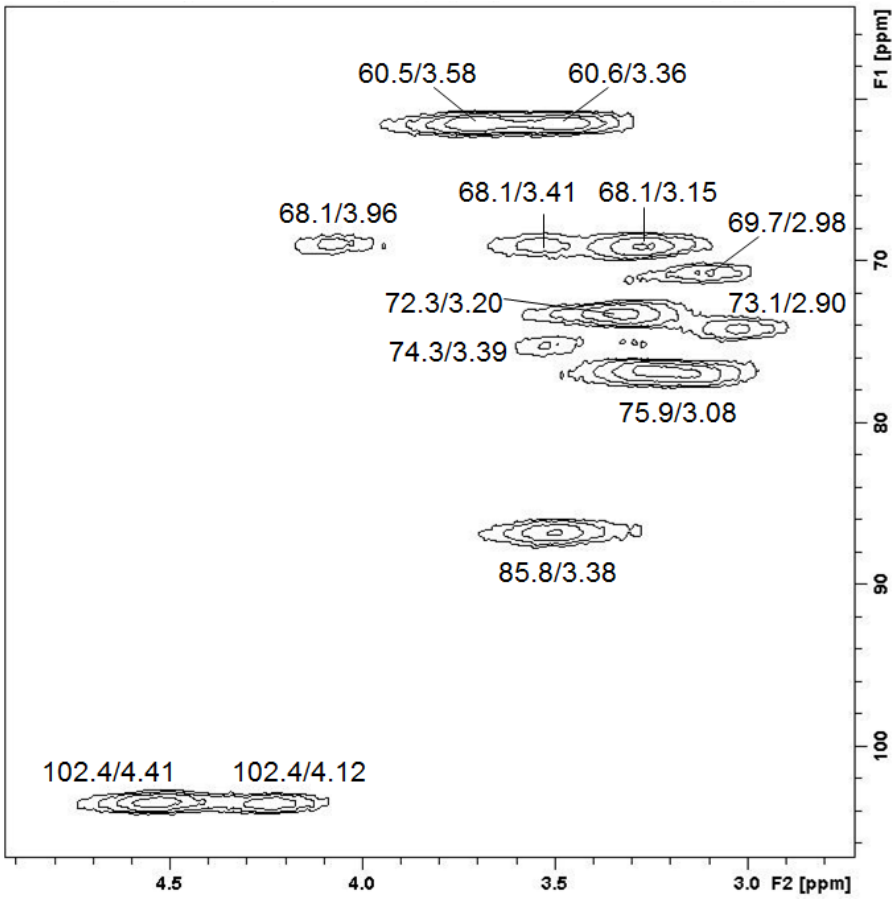

FIGURE 3. HSQC-NMR spectrum of BG-PN in Me_2SO-D_6 at 70 °C (chemical shifts are expressed in δ ppm).

The ^{13}C-NMR spectrum contained only one anomeric signal at δ 102.8 (Fig. 2B), while it was observed two distinct signals (C-1/H-1) in HSQC experiment, one at δ 102.4/4.12 corresponding to non-reducing end-units, and another at δ 102.4/4.41 from 3-*O*- and 3,6-di-*O*-substituted residues. The β-configuration was confirmed by low frequency H-1 (δ 4.12 and 4.41) and high-frequency C-1 signals (δ 102.4) (Hall & Johnson, 1969). The glycosidic linkages suggested by the methylation analyses were confirmed by the presence of 3-*O*-substitution signals at δ 86.5, 86.1 and 85.8 (Figs. 2B and 3) and 6-*O*- substitution signals at δ 68.3 (Fig. 2B), which appeared as doublet in HSQC spectrum at δ 68.1; 3.96/3.41 (Fig. 3) and was confirmed by RMN-^{13}C-DEPT experiment (data not shown). Non-substituted C-6 signals were also observed and are assigned at δ 60.7 (Fig. 2B), 60.5/3.58 and 60.6/3.36 (Fig. 3) (Carbonero et al., 2012). The other signals in HSQC spectrum at δ 72.3/3.20;

68.1/3.15; 75.9/3.08 are attributed to C2/H2, C4/H4, and C5/H5 respectively, of β-D-Glc*p*-(1→3)-linked main chain. Similar signals relative to mushroom β-D-glucans were observed by Carbonero et al. (2006), Ruthes et al. (2013) and Moreno et al. (2016).

The main chain of the isolated β-D-glucan (bG-PN) was confirmed after performing a controlled Smith degradation that consists of $NaIO_4$ oxidation followed by mild acid hydrolysis. Only (1→3)-linked residues are resistant and all branching residues were totally oxidized by this procedure and after analyzing the residual product by ^{13}C-NMR, six intense signals were observed (Fig. 4). It proved to be a linear (1→3)-linked β-D-glucan with six typical signals at δ 102.8; 86.1; 76.3; 72.8; 68.3 and 60.7 ppm corresponding to C-1, C-3, C-5, C-2, C-4, and C-6, respectively (Gorin, 1981).

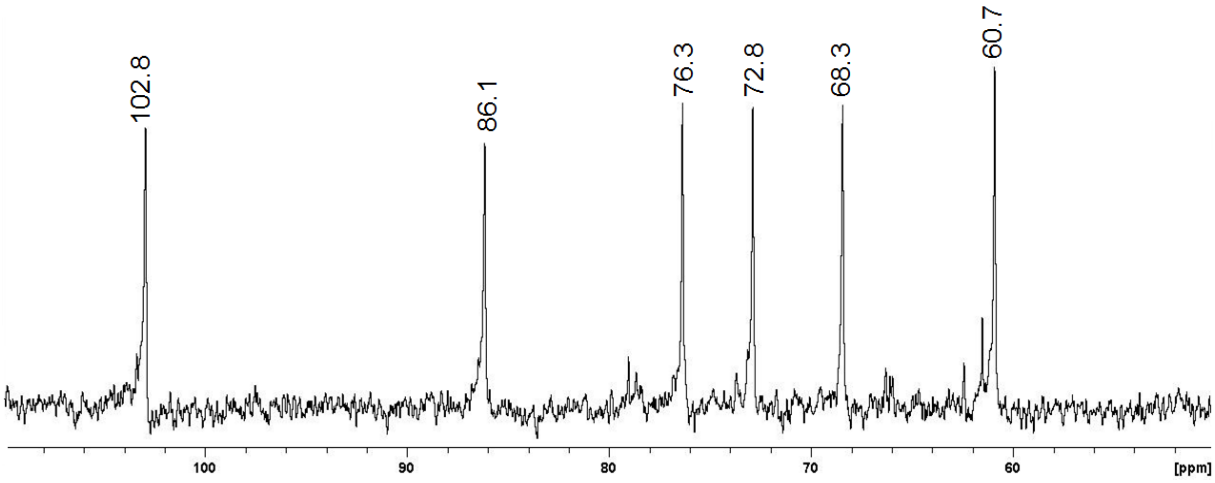

FIGURE 4. ^{13}C-NMR spectrum of partially degraded glucan from *Pholiota nameko* in Me_2SO-D_6 at 70 °C (chemical shifts are expressed in δ ppm).

The results obtained by the chemical analyses are in agreement and confirmed the presence of a β-D-glucan with a main chain (1→3)-linked and highly substituted at O-6 by non-reducing β-D-Glc*p* or β-D-Glc*p*-(1→6)-linked side chains. Similar β-D-glucans from *Pleurotus florida* (Rout, Mondal, Chakraborty, Pramanik, & Islam, 2005), *Pleurotus pulmonarius* (Smiderle et al., 2008) and *Cantharellus cibarius* (Nyman, Aachmann, Rise, Balance, & Samuelsen, 2016) have been studied. Rodrigues et al. (2015) observed evidences of β-glucans, α-glucans and glucan-

protein complexes by FTIR-ATR in five species of mushrooms including *P. nameko*. However, the chemical characterization of such molecules had not been reported. Studies on biological activities of crude polysaccharide extracts from genus *Pholiota* are commonly found, although there is a lack of information relating the chemical structure of such polymers with their biological effects or rheological properties (Li, Lu, Zhang, Lu, & Liu, 2008; Li, Zhang, & Ma, 2010). A purified (1→3),(1→6)-β-D-glucan from *P. nameko* fungi has not been reported up to this date.

3.3 RHEOLOGICAL CHARACTERIZATION OF SCW AND β-D-GLUCAN FRACTIONS

High degree of polysaccharide branching promotes higher interaction between water and polysaccharides by hydrogen bonds. Such inter- and intramolecular interactions may also influence solution behavior by increasing viscosity or forming gels (Yalpani & Hall, 1984). Fraction SCW and the highly branched β-D-glucan isolated from *P. nameko* showed gelling appearance; therefore their rheological properties were evaluated.

The flow behavior of SCW fraction (at 1% and 2% w/w) was compared with the behavior of isolated β-D-glucan (at 2% w/w) (Fig. 5).

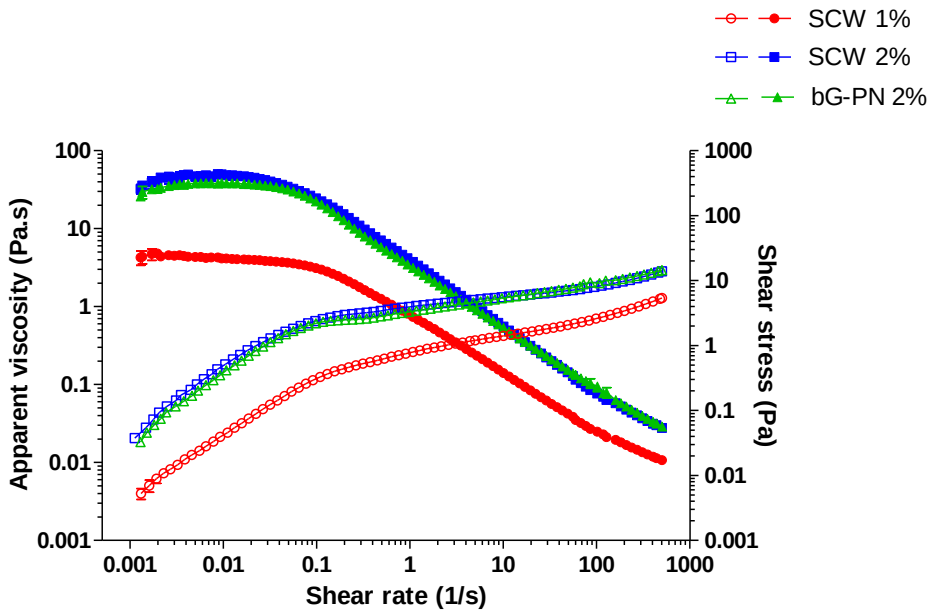

FIGURE 5. Flow (empty symbols) and viscosity (full symbols) curves of aqueous SCW fraction at 1% and 2% and aqueous BG-PN fraction at 2%.

The apparent viscosity of the first one (SCW) was concentration dependent, as zero shear viscosity value increase when the polymer concentration rises. The viscosity observed for 1% SCW dispersion at a shear rate of 0.01 s^{-1} was 4.1 Pa.s and increased to 49.5 Pa.s at 2% of concentration. It was observed that at higher concentrations there is more intermolecular connections promoting “junction zones”, which limit movement and stretching of polymers in solution and, consequently increase viscosity (Freitas et al., 2009). SCW aqueous dispersions showed lower viscosity than those observed for aqueous extract from *Flammulina velutipes* (Du et al., 2016) and *Auricularia auricular-judae* (Bao et al., 2016). These could be related to the composition of the extracts, considering that all of them were composed by galactose, mannose and glucose in different proportions. The crude fraction SCW presented higher amounts of galactose (44.9%) in comparison to *A. auricular-judae* and *F. velutipes* fractions, that contained 5.4% (Bao et al., 2016) and 12% of galactose residues (Yang et al., 2012), respectively.

Both tested concentrations of SCW fraction (1% and 2% w/w) showed shear-thinning behavior, as there was an increase of the shear stress and a pronounced decrease of their viscosities, especially after 0.05 s^{-1}, with the increasing of the shear rate (Fig. 5). This is a common behavior of polysaccharide dispersions, where disordered polysaccharides seem to align or deform along the flow direction, decreasing the dispersion viscosity (Lapasin & Pricl, 1995). Such behavior was identified for other polysaccharides from edible mushrooms as *F. velutipes* (Du et al., 2016), floral mushrooms (Xu, Zhang, Liu, Sun, & Wang, 2016), *A. auricular-judae* (Bao et al., 2016), and *Ganoderma lucidum* (Liu et al., 2016).

The flow and viscosity curves of fraction bG-PN at 2% (Fig. 5) were very similar with those observed for SCW at the same concentration, for all tested shear rates, suggesting that it is not necessary to completely purify the β-D-glucan to obtain the desirable viscosity. This result is interesting for the application in industry, which requires less purification steps to avoid unnecessary expenses. Besides, SCW yield was 34 times higher than bG-PN. The purified β-D-glucan from *P. nameko* formed less viscous aqueous dispersion than those isolated from floral mushrooms (Xu, Zhang, Liu, Sun, & Wang, 2016) and *G. lucidum* (Liu et al., 2016), although all of them showed shear-thinning behaviors. The fraction bG-PN (at 2%) presented viscosity of 3.5 Pa.s at 1 s^{-1} while that from floral mushrooms cultivated in Huangshan Mountain have similar viscosity at half of concentration (Xu, Zhang, Liu,

Sun, & Wang, 2016). Initial viscosity of bG-PN (at 2%) at first Newtonian plateau, at 0.01 s^{-1}, was 38.4 Pa.s, while β-D-glucan from *G. lucidum* showed almost 150 Pa.s at the same shear rate (Liu et al, 2016). β-D-Glucan from *P. nameko* showed a highly branched structure, containing longer side chains in comparison to β-D-glucans from floral mushrooms (Xu, Zhang, Liu, Sun, & Wang, 2016) and *G. lucidum* (Liu et al., 2016), that present only non-reducing end units of Glc*p* as branches. Such chemical characteristics may be responsible for the differences observed on viscosity of the three β-D-glucans.

The viscoelastic behaviors of fraction SCW (at 1% and 2% w/w) and isolated β-D-glucan (at 2% w/w) at 25 °C are shown in Fig. 6.

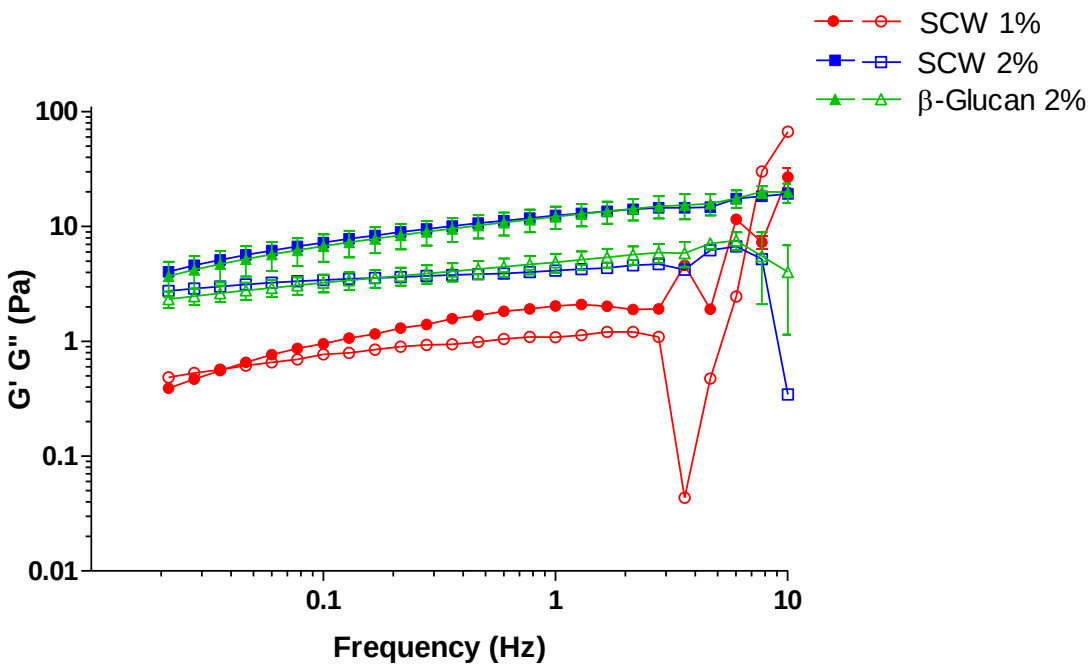

FIGURE 6. Frequency sweeps at 25°C of fractions SCW at 1% and 2% and BG-PN at 2%. Elastic modulus (G') is represented by full symbols while viscous modulus (G") by open symbols. Fixed strain of 1%.

G' and G" of SCW at 1% were crossed-over at low frequency (< 0.05 Hz) characterizing it as concentrated solution and entangled systems (Clark & Ross-Murphy, 1987). When the concentration reached 2% (w/w), SCW showed a gel-like

behavior with G' higher than G", both slightly frequency dependent, in all tested frequency range. The increase of concentration promoted the formation of more entanglements and junction points between the polymer chains resulting in formation of a gel network (Brito, Sierakowski, Reicher, Feitosa, & Paula, 2005). The gel-like behavior was also noticed in aqueous extract from mushroom *A. auricular-judae* (named AP) at 0.5%, 1%, and 2%, and gel strength increased with increment of AP concentration (Bao et al., 2016). Our results showed that bG-PN (2% w/w) has similar gel-like behavior than that from aqueous extract SCW at the same concentration (Fig. 6). The β-D-glucan from *P. nameko* formed lower strength gel than that from *G. lucidum*, as G' and G" values of bG-PN at 2% were comparable with those from *G. lucidum* at 1%, at the same temperature (Liu et al., 2016). In contrast, β-D-glucan isolated from floral mushrooms (Xu, Zhang, Liu, Sun, & Wang, 2016) (at 1%) did not form gel at 25 °C (Xu, Zhang, Liu, Sun, & Wang, 2016).

With the aim of investigating thermal stability of SCW and bG-PN, dispersions were heated (from 5 to 60 °C) and cooled (from 60 to 5 °C) and their viscoelastic moduli were determined. SCW (at 1% and 2%) and bG-PN (at 2%) showed strong thermal stability over the temperature course (Fig. 7). During heating and cooling, G' and G" reached almost the same values as the initial temperatures, suggesting that the temperatures tested did not affect the physical structure of gels and viscoelastic fluids formed by both samples.

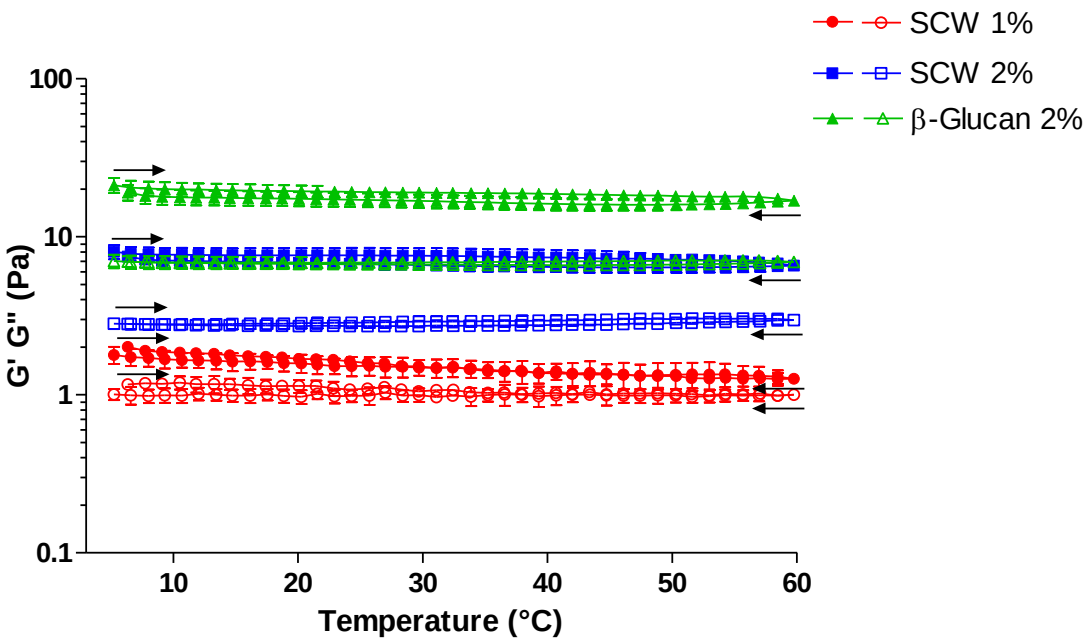

FIGURE 7. Elastic (G', full symbols) and viscous moduli (G", open symbols) of fractions SCW (at 1% and 2%) and BG-PN (at 2%) as a function of temperature. Fixed frequency at 1 Hz and strain of 1%.

At the temperature ramps, bG-PN (2% w/w) showed higher G' and G" values than those observed for SCW fraction at the same concentration (Fig. 7). This behavior was different from that observed at frequency sweeps suggesting that at 5 °C, there were more intra- and intermolecular associations between the isolated β-D-glucan molecules, resulting in more junction zones and consequently in more strength gel than that formed at 25 °C. The β-D-glucan (bG-PN) gel showed to be thermal stable, without strength loss, during the temperature ramp tested.

CONCLUSION

Polysaccharide extracts from *P. nameko* were obtained by cold water extractions and a highly branched glucan was purified and chemically characterized. Its backbone was composed of (1→3)-linked β-D-Glc*p* units, being 27% substituted at O-6 by single units of β-D-Glc*p* or (1→6)-linked β-D-Glc*p* side chains. The crude extract (SCW) and purified β-D-glucan (bG-PN) showed similar non-Newtonian

shear-thinning behavior, and viscosity of SCW fraction was higher when there was an increase in its concentration. SCW and bG-PN (at 2%) showed gel-like behavior and were thermal stable between the range of 5 to 60 °C. Such characteristics suggested a potential application of *P. nameko* fractions to increase thickness of food products and also to improve resistance of products submitted to different temperatures during its processing, storage and/or transport. The shear-thinning behavior and gel-like structure of SCW and bG-PN fractions at 25 °C were similar at the same concentration which suggested there is no need to purify the β-D-glucan to obtain some desirable rheological properties. The results obtained indicate a potential use of β-D-glucan-rich fractions from *P. nameko* in food industry as thickening agents to improve desirable characteristics of food products associated with other therapeutic benefits that are attributed to β-D-glucans.

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4. CONCLUSÕES

Nesta tese foram abordados os seguintes aspectos: 1) Caracterização química de polissacarídeos presentes nos corpos de frutificação dos cogumelos *Piptoporus betulinus*, *Phellinus igniarius* e *Pholiota nameko* obtidos por extrações aquosas e alcalinas; 2) Avaliação do efeito cicatrizante pelo ensaio de migração celular *in vitro* da fração do extrato aquoso quente de *Piptoporus betulinus*; 3) Avaliação do potencial imunomodulador *in vitro* da fração do extrato aquoso frio; 4) Avaliação do comportamento reológico da fração do extrato aquoso frio de *Pholiota nameko*.

As seguintes conclusões podem ser propostas:

- Uma β-(1→3),(1→6)-glucana com baixo grau de ramificação foi isolada de *P. betulinus*, a qual apresenta uma conformação em espiral aleatória em solução, sendo que essa característica favorece sua capacidade para formar gel. O estudo de migração celular *in vitro* demonstrou sua capacidade de promover o processo de cicatrização por aumentar a resposta celular à lesão através da indução da migração das células, diminuindo o tempo de cicatrização previsto. Pode ser considerada uma molécula promissora para o desenvolvimento de formulações cicatrizantes de origem natural.
- Uma β-(1→3),(1→6)-glucana pouco ramificada e uma α-(1→3)-glucana linear foram obtidas da fração alcalina de *P. betulinus*. Ambos os polímeros apresentaram insolubilidade em água fria após procedimento de congelamento e descongelamento. A separação das duas glucanas da fração insolúvel foi através de uma metodologia pouco empregada em que uma solução de hidróxido de sódio na concentração de 0,1 M foi utilizada. Essa separação foi realizada com base no comportamento que ambas apresentaram em solução alcalina. Devido as ramificações presentes na β-(1→3),(1→6)-glucana, sua solubilidade na solução alcalina foi maior porque o polímero apresentou menor interação intermolecular em relação a α-(1→3)-glucana linear. Diferenças na estrutura química como conformação e o grau de ramificação na cadeia de um polissacarídeo pode acarretar em diferentes respostas biológicas. Futuramente, essas moléculas serão avaliadas

em um estudo comparativo *in vitro* sobre a influência que a diferença estrutural pode exercer na atividade imunomoduladora.

- Uma α-(1→6)-galactana parcialmente 3-*O*-metilada de baixa massa molecular foi obtida de *P. igniarius*. Galactanas não são comumente encontradas em cogumelos, sendo que há poucos relatos na literatura a respeito do isolamento e caracterização química desses polímeros. As atividades biológicas descritas estão normalmente associadas à galactanas isoladas de algas. O tamanho da galactana encontrada no fungo *P. igniarius* pode favorecer a solubilidade e a absorção no organimo. Esse homopolímero apresentou efeito imunomodulador sobre células THP-1 diferenciadas em macrófagos através da estimulação da secreção das citocinas TNF-α e IL-10.
- Uma β-(1→3),(1→6)-glucana com alto grau de ramificação com cadeia principal β-(1→3) foi isolada a partir da extração aquosa fria de *P. nameko*. Devido à solução contendo este polissacarídeo apresentar viscosidade, seu comportamento reológico foi avaliado. Na concentração de 2%, as curvas de fluxo apresentaram valores de G' maiores que G" o que caracteriza um comportamento de gel. Um comportamento semelhante foi observado para o extrato bruto na mesma concentração. O experimento de rampa de temperatura (5 a 60 °C) mostrou que as soluções de extrato bruto e a solução com a glucana pura apresentaram estabilidade térmica, pois os módulos G' e G" apresentaram pouca diferença, o que sugere que não houve alteração físico-química das amostras. Pelo fato das propriedades reológicas do extrato aquoso serem muito semelhantes aos da glucana pura, não há necessidade de isolar o composto para seu emprego na indústria de alimentos, já que taxas de viscosidade parecida podem ser obtidas com o extrato bruto deste cogumelo.
- Este trabalho mostrou que os cogumelos estudados apresentaram compostos com diferentes características estruturais, o que comprova a diversidade química desses fungos. Os polissacarídeos isolados apresentam diferentes aplicações farmacológicas e biotecnológicas de acordo com os experimentos realizados, o que os tornam muito interessantes para diferentes aplicações.

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ANEXO

TERMO DE LICENÇA – REVISTA CARBOHYDRATE POLYMERS - ARTIGO IV