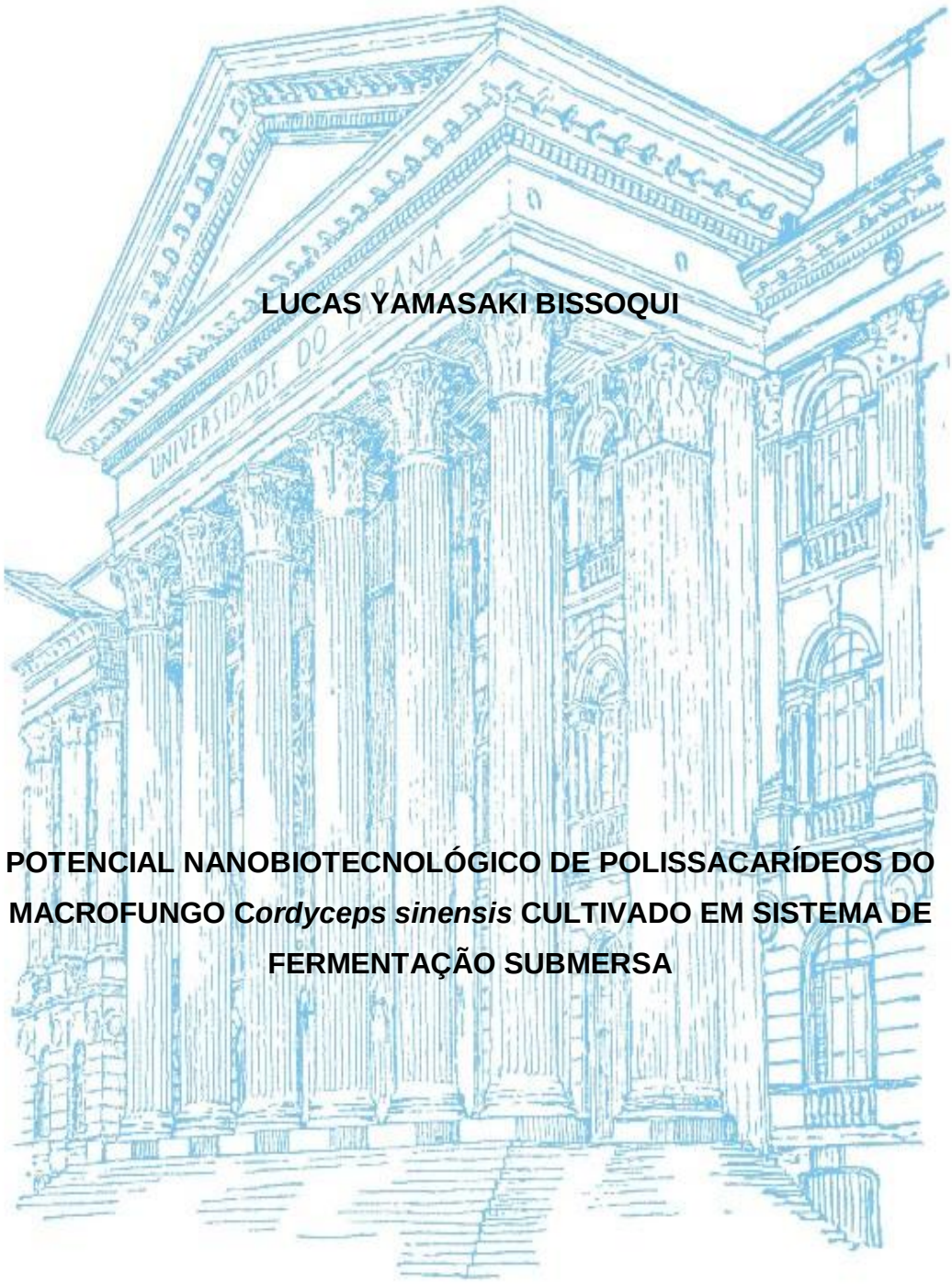
UNIVERSIDADE FEDERAL DO PARANÁ



LUCAS YAMASAKI BISSOQUI

POTENCIAL NANOBIOTECNOLÓGICO DE POLISSACARÍDEOS DO MACROFUNGO C*ordyceps sinensis* CULTIVADO EM SISTEMA DE FERMENTAÇÃO SUBMERSA

CURITIBA

2017

LUCAS YAMASAKI BISSOQUI

POTENCIAL NANOBIOTECNOLÓGICO DE POLISSACARÍDEOS DO MACROFUNGO *Cordyceps sinensis* CULTIVADO EM SISTEMA DE FERMENTAÇÃO SUBMERSA

Tese apresentada como requisito parcial à obtenção do grau de Doutor em Engenharia de Bioprocessos e Biotecnologia, no Curso de Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia, área de concentração: Saúde Humana e Animal, Setor de Tecnologia, da Universidade Federal do Paraná.

Orientador: Prof. Dr. Carlos Ricardo Soccol
Co-orientadoras: Profª. Drª. Rosália Rubel
Profª. Drª. Valcineide O Tanobe

CURITIBA

2017

Programa de Pós-Graduação ENGENHARIA DE BIOPROCESSOS E BIOTECNOLOGIA

TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em ENGENHARIA DE BIOPROCESSO BIOTECNOLOGIA da Universidade Federal do Paraná foram convocados para realizar a arguição da tese de Doutorado de **LUCAS YAMA BISSOQUI** intitulada: **Potencial nanobiotecnológico de polissacarídeos do macrofungo Cordyceps sinensis cultivado em sistem fermentação submersa.**, após terem inquirido o aluno e realizado a avaliação do trabalho, são de parecer pela APROVAÇÃO no rito de defesa.

A outorga do título de doutor está sujeita à homologação pelo colegiado, ao atendimento de todas as indicações e correções solicitadas pela ba ao pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

Curitiba, 13 de Julho de 2017.

CARLOS RICARDO SOCCOL
Presidente da Banca Examinadora (UFPR)

SUSAN GRACE KARP
Avaliador Interno (UFPR)

ROSÁLIA RUBEL
Avaliador Externo (UFPR)

VALCINEIDE OLIVEIRA DE ANDRADE TANOBE
Avaliador Externo (UFPR)

SANDRO JOSÉ RIBEIRO BONATTO
Avaliador Externo (IPP)

Dedico este trabalho à minha filha Ana Sofia, presente de Deus e amor da minha vida. Também dedico a minha esposa Sheila por suas orações, por sempre me apoiar, ouvir, incentivar, e às vezes criticar quando necessário. Obrigado pela paciência, pelo suporte, pela atenção e dedicação neste período de intensa atividade. Sempre presente na elaboração e execução deste trabalho.

Benção de Deus para mim!

AGRADECIMENTOS

Ao Prof. Carlos Ricardo Soccol pela orientação e confiança para a realização desse trabalho.

À Profª. Rosália Rubel pela orientação, acompanhamento e confiança nos momentos difíceis em que alguns resultados não vieram.

Ao Prof. Sandro Bonatto pelo constante diálogo, incentivo e apoio na bancada durante os experimentos no Instituto Pelé Pequeno Príncipe.

À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) pela concessão de bolsa de doutorado.

Ao Prof. Dr. Miguel Noseda pelo diálogo, atenção e orientações preciosas que contribuiram para os experimentos relacionados com os polissacarídeos.

À Pós doutoranda Valcineide Tanobe, minha amiga química e companheira de bancada que co-orientou este trabalho, pela amizade, diálogo, incentivo e ensinamentos.

Aos professores do Programa de Pós Graduação em Engenharia de Bioprocessos e Biotecnologia, Dra. Adenise L. Woiciechowski, Dra. Adriane B. P. Medeiros, Dr. Júlio C. de Carvalho, Dra. Luciana P. S. Vandenberghe, Dr. Luiz A. J. Letti, Dra. Cristine Rodrigues e Dr. Gilberto Vinicius Pereira de Melo, pelo auxílio e colaboração em diversos aspectos.

Ao meu veterano de fermentações, Leandro Freire dos Santos, pela amizade, acolhimento, apoio e sugestões na realização deste trabalho.

Aos funcionários do Departamento de Engenharia de Bioprocessos e Biotecnologia (DEBB/UFPR): Mityo Miyaoka, Otacílio Pires, Natacha Amálio, Agatha Leão e Marisa Borges pela amizade, colaboração e ajuda.

Aos amigos, colegas de curso, de laboratório e estagiários, Andre Luis Lopes, André Gollo, Jeferson Luz, Khiomara, Mario, Marcela, Liliana, Antonio, David, Claudia, Lina, Estefania, Omar, Luiz, Emerson, Luis Zevallos, Walter, Bianni, pela amizade e companheirismo no decorrer do curso e no dia-a-dia.

Aos meus pastores, Ismael Machado Correa, Fábio Alcantara, Andréia Ribeiro de Souza e Paulo Sergio Zucolli, pelo companheirismo e direção espiritual nesta caminhada.

Agradeço aos meus familiares: ao meu pai Jubrair, à minha mãe Maria, à minha irmã Jéssica e ao meu irmão Matheus, pelo amor, apoio e ajuda no momento mais difícil da minha vida.

A todos que colaboraram para a realização deste trabalho.

RESUMO

A nanobiotecnologia é o resultado da combinação de técnicas e aplicações do campo de pesquisa da nanotecnologia para biologia, biotecnologia, veterinária ou saúde. Devido à variedade de aplicações e a possibilidade da combinação de novas idéias e abordagens a nanobiotecnologia é um campo de grande potencial para criação de produtos e processos inovadores. A síntese verde de nanopartículas é um campo relativamente novo que envolve rotas menos tóxicas, mais seguras e eficiente energeticamente. Dessa forma, devido à sua natureza não tóxica e boa solubilidade em água muitos polissacarídeos são reconhecidos como estabilizantes e agentes redutores “verdes” na síntese de nanopartículas. Por essa razão o objetivo desse trabalho foi avaliar o potencial nanobiotecnológico de macromicetos (*Cordyceps sinensis, Ganoderma lucidum* e *Pleorotus ostretaus)* produzidos por sistema de fermentação submersa na produção de nanopartículas de prata e paládio com atividade imunomodulatória e antitumoral, respectivamente. As nanopartículas foram caracterizadas por espectroscopia ultravioleta-visível (UV-VIS) e no infravermelho por transformada de Fourier (FTIR), difração de raios X (XRD), espalhamento dinâmico de luz (DLS) e microscopia eletrônica de transmissão (TEM). Nanopartículas de prata conjugadas com frações de polissacarídeos (F1A-AgNPs, F2A-AgNPs e EPS2-AgNPs) demonstraram potencial atividade imunossupressora em macrófagos de camundongos (RAW 264.7), visto que foi observado um efeito de diminuição significativa ($p<0,05$) nas capacidades de fagocitose, retenção lisossomal e adesão de macrófagos, que por sua vez é um fator crítico na determinação da resposta imune inflamatória. Nanopartículas de paládio conjugadas com a fração F2A de polissacarídeos (F2A-PdNPs) aumentaram significativamente ($p<0,05$) a inibição do crescimento de células tumorais de carcinoma adrenocortical (H295R) de 12 para 26%, quando comparado com o controle, o que representa em média um aumento em torno de 100% da citotoxicidade da fração de polissacarídeos F2A. Os resultados mostraram que os polissacarídeos de *C. sinensis* avaliados podem ser utilizados como um agente de redução e estabilizantes eficazes, não tóxicos para a síntese verde de nanopartículas, que por sua vez podem ser aplicáveis a criação e inovação de diversos dispositivos médicos.

Palavras-chave: Nanobiotecnologia. Polissacarídeos. Nanopartículas. *Cordyceps sinensis. Ganoderma lucidum. Pleurotus ostreatus*. Nanopartículas de Prata. Nanopartículas de Paládio.

ABSTRACT

Nanobiotechnology is the result of the combination of techniques and applications from the field of nanotechnology research to biology, biotechnology, veterinary or health. Due to the variety of applications and the possibility of combining new ideas and approaches, nanobiotechnology is a field of great potential for creating innovative products and processes. Green nanoparticle synthesis is a relatively new field that involves less toxic, safer, and energy efficient routes. Thus, due to its non-toxic nature and good water solubility many polysaccharides are recognized as stabilizers and "green" reducing agents in the synthesis of nanoparticles. For this reason, the objective of this work was to evaluate the nanobiotechnological potential of macromycets (*Cordyceps sinensis, Ganoderma lucidum and Pleorotus ostretaus*) produced by submerged fermentation system in the production of silver and palladium nanoparticles with immunomodulatory and antitumor activity, respectively. The nanoparticles were characterized by ultraviolet-visible (UV-VIS) spectroscopy and fourier transform infrared (FTIR), X-ray diffraction (XRD), dynamic light scattering (DLS) and transmission electron microscopy (TEM). Silver nanoparticles conjugated with polysaccharide fractions (F1A-AgNPs, F2A-AgNPs and EPS2-AgNPs) demonstrated potential immunosuppressive activity in macrophages (RAW 264.7), since a significant decrease effect ($p<0.05$) was observed in the phagocytosis, lysosomal retention capacities and macrophage adhesion, which in turn is a critical factor in the determination of the inflammatory immune response. Palladium nanoparticles conjugated with F2A fraction of polysaccharides (F2A-PdNPs) significantly increased ($p<0.05$) inhibition of tumor cell growth (H295R) from 12 to 26% as compared to the control, which represents in mean an increase of about 100% in the cytotoxicity of the F2A polysaccharide fraction. The results showed that polysaccharides of *C. sinensis* evaluated could be used as a reducing agent and effective stabilizers, non-toxic to the green synthesis of nanoparticles, which in turn may be applicable to the creation and innovation of various medical devices.

Key-words: Nanobiotechnology. Polysaccharides. Nanoparticles. Cordyceps sinensis. Ganoderma lucidum. Pleurotus ostreatus. Silver nanoparticles. Palladium nanoparticles.

SUMÁRIO

1 INTRODUÇÃO

A nanotecnologia engloba uma capacidade cada vez mais sofisticada de manipular a matéria em nanoescala, resultando em novos materiais, dispositivos e produtos que demonstram diferentes propriedades. Nos últimos anos essa área tem sido reconhecida como um campo revolucionário de ciência e tecnologia, comparável à introdução de revoluções promovidas pela energia elétrica, biotecnologia e informação digital.

Em 1959, o físico Richard Feynman deu início à era nanotecnológica ao considerar a manipulação de átomos como forma de criar novos materiais a partir de uma nova organização estrutural (Feyman, 1960). Desde então, com os avanços tecnológicos e a produção de novos conhecimentos, a nanotecnologia é definida como o entendimento, o controle e a exploração de materiais e sistemas cujas estruturas e componentes apresentam propriedades e fenômenos físicos, químicos e biológicos que são significativamente novos e/ou modificados devido à sua escala nanométrica (1 a 100 nm) (NSF, 2001; ISO/TS 80004-1:2015).

De acordo com um levantamento realizado por Kay e Shapira (2009), nos anos de 1990 a 2006 o Brasil foi o líder de pesquisa em nanotecnologia na América Latina com 5.456 publicações, no entanto, considerando o mesmo período, ainda ficou muito atrás de países como EUA (101.205), China (51.620), Japão (47.894) e Alemanha (41.793) em termos de publicações. Países desenvolvidos como os Estados Unidos, Japão e membros da União Européia, investiram bilhões de dólares anuais em pesquisa em nanotecnologia na última década (Roco, 2011).

Por se tratar de um campo de conhecimento multidisciplinar que engloba as áreas da física, química, engenharia, biologia e medicina, a área de aplicação da nanotecnologia é muito ampla, no entanto, grandes avanços e desenvolvimentos são mais evidentes na nanoeletrônica, nanomateriais e nanobiotecnologia (Steele et al., 2017; Xing et al., 2017).

A nanobiotecnologia é o resultado da combinação de técnicas e aplicações do campo de pesquisa da nanotecnologia para biologia, biotecnologia, veterinária ou saúde (ISO/TS 80004-5:2011). Devido à variedade de aplicações e à possibilidade da combinação de novas ideias e abordagens, a

nanobiotecnologia é um campo de grande potencial para criação de produtos e processos inovadores. No entanto, até 2014 foram identificadas apenas 507 companhias que atuam em nível global na área de saúde e que possuem simultaneamente competências/capacidade nas áreas de biotecnologia e nanotecnologia (Maine et al., 2014).

Nanopartículas e nanomateriais existem naturalmente no ambiente como a poeira vulcânica. Também podem ser produzidos sem intenção como as partículas produzidas pela combustão de um motor a diesel, e também podem ser produzidas intencionamente através da manipulação do carbono, metais, e polímeros em nível molecular (Custance et al., 2009).

A aplicação comercial de nanopartículas (NP) metálicas é promissora e apresenta um grande potencial de inovação em diferentes áreas como cosmética, alimentos, indústria química, catálise, mecânica e ambiental entre outras (Hedayatnasab et al., 2017; Kaushnik et al., 2016; Yukuyama et al., 2016; Usmani et al., 2017).

Existem muitas aplicações para nanopartículas metálicas nas áreas da medicina e farmácia (Jain et al., 2007). Nanopartículas de prata são reconhecidas por sua ação antimicrobiana e muitos produtos estão comercialmente disponíveis para o uso clínico como Acticoat®, Silverline®, Silvasorb® e Silversoaker® (Rai et al., 2009; Chaloupka et al., 2010). Nanopartículas de paládio têm sido descritas com potencial atividade antibacteriana, antitumoral e também usadas na construção de sensores analíticos (Medici et al., 2015; Safavi et al., 2013; Sharmila et al., 2017).

Diversos métodos químicos e físicos tem sido desenvolvidos para a síntese de nanopartículas metálicas como redução química, tratamento fotoquímico, tratamento a laser, eletroquímica e aplicação de campos ultrasônicos, no entanto, esses processos ou podem usar compostos orgânicos como hidrazina, borohidreto de sódio, tiofenol, tiouréia e acetato de mercapto que quando usados em larga escala podem causar poluição ambiental, ou ainda são processos considerados caros para produção industrial (Mafuné et al., 2002; Natsuki et al., 2015).

Nesse contexto, há uma preocupação crescente com o desenvolvimento de métodos baratos, simples, ecologicamente corretos e sustentáveis para produção de nanopartículas (Takkar et al., 2010; Virkutyte e

Varma, 2010). Em um processo de síntese verde de nanopartículas existem três fatores principais que devem ser observados como a escolha do solvente, o uso de um agente redutor ambientalmente seguro e um estabilizante da nanopartícula não tóxico (Raveendran et al., 2003).

Dessa forma, uma alternativa muito interessante é o emprego de biomoléculas de várias espécies de plantas e de microorganismos, que são produzidas naturalmente, e têm sido usadas com sucesso para a síntese de nanopartículas metálicas (Park Y. et al., 2011).

1.1 JUSTIFICATIVA

A síntese verde de nanopartículas é um campo relativamente novo que envolve rotas menos tóxicas, seguras e eficientes energeticamente. Dessa forma, devido à sua natureza não tóxica e boa solubilidade em água muitos polissacarídeos são reconhecidos como estabilizantes e agentes redutores "verdes" na síntese de nanopartículas (Duan et al., 2015). Polissacarídeos possuem grupos hidroxilas e um terminal redutor na molécula que desempenha um papel chave na biorredução de sais precursores (Mata et al., 2009).

Assim sendo, polissacarídeos produzidos por macromicetos (*Cordyceps sinensis, Ganoderma lucidum* e *Pleurotus ostreatus*) em sistema de cultivo de fermentação submersa poderiam ser agentes verdes e não tóxicos para produção de nanopartículas metálicas. Além disso, polissacarídeos de macromicetos são moléculas biologicamente ativas que podem apresentar efeitos antioxidantes, imunomoduladores e antitumorais (Yan et al., 2013). Estes polissacarídeos podem ser extraidos da biomassa micelial produzida por fermentação submersa (intrapolissacarídeos) ou a partir do caldo de fermentação (exopolissacarídeos).

Existem poucos trabalhos publicados na literatura sobre nanopartículas de paládio na área da saúde em comparação com o grande número de artigos publicados na área de síntese, produção de sensores e aplicações tecnológicas, sendo que não há nenhuma publicação que analisou e/ou empregou polissacarídeos de cogumelos para síntese verde de nanopartículas de paládio.

Com relação as nanopartículas de prata, apesar de existirem muitos estudos referentes à síntese e sua aplicação como antimicrobiano, há poucos e

controversos estudos que abordam o efeito imunomodulador das nanopartículas de prata.

1.2 OBJETIVOS

1.2.1 Objetivo Geral

Avaliar o potencial nanobiotecnológico do macrofungo *Cordyceps sinensis* produzido por sistema de fermentação submersa na produção de nanopartículas com atividade imunomodulatória e/ou antitumoral.

1.2.2 Objetivos Específicos

a) Avaliar o potencial da atividade antioxidante e antitumoral das espécies de macromicetos *C. sinensis, G. lucidum* e *P. ostreatus* produzidos por sistema de fermentação submersa;
b) Produzir e caracterizar nanopartículas de prata e paládio empregando polissacarídeos de *Cordyceps sinensis* como agente redutor e estabilizante da partícula;
c) Avaliar o potencial imunomodulatório *in vitro* das partículas de prata em macrófagos de camundongo linhagem RAW 264.7;
d) Avaliar o potencial antitumoral *in vitro* das partículas de paládio em células humanas de tumor do córtex adrenal linhagem H295R.

CHAPTER I

PHARMACOLOGICAL PROPERTIES OF BIOCOMPOUNDS FROM SPORES OF THE LINGZHI OR REISHI MEDICINAL MUSHROOM *Ganoderma lucidum* (AGARICOMYCETES): A REVIEW

Pharmacological Properties of Biocompounds from Spores of the Lingzhi or Reishi Medicinal Mushroom *Ganoderma lucidum* (Agaricomycetes): A Review

Carlos Ricardo Soccol[1,*], Lucas Yamasaki Bissoqui[1], Cristine Rodrigues[1], Rosália Rubel[2], Sandra R.B.R. Sella[3], Fan Leifa[4], Luciana Porto de Souza Vandenberghe[1], Vanete Thomaz Soccol[1]

[1] Department of Bioprocess Engineering and Biotechnology, Federal University of Paraná (UFPR), CEP 81531-980, Curitiba, Paraná, Brazil.

[2] Hospital de Clínicas, UFPR, Curitiba, Paraná, Brazil.

[3] Center for the Production of and Research into Immunobiological Products, Paraná State Department of Health, Piraquara, PR, Brazil.

[4] Associate professor, Institute of Horticulture, Academy of Agronomy Sciences of Zhejiang, Zhejiang Province, China.

**Address for Correspondence*: Carlos Ricardo Soccol. Bioprocess Engineering and Biotechnology Department, UFPR, CEP 81531-980, Curitiba, Paraná, Brazil. E-mail: soccol@ufpr.br

Abstract

Ganoderma lucidum is well-known representative of higher basidiomycetes that has been used in traditional Chinese medicine for centuries. New discoveries related to this medicinal mushroom and its biological properties are frequently reported. However, it was only in recent decades that scientists started to pay special attention to *G. lucidum* spores. This is in part due to the recent development of methods for breaking the spore wall and extracting biocompounds from the spore. Although there are some research groups working with *G. lucidum* spores, there is still limited data in the literature and the methods used have not been systematized. This review therefore describes the main advances in techniques for breaking the spore wall and extracting biocompounds from the spore. In addition, the major active components identified and their biological properties, such as neurological activity and anti-aging and cell-protective effects are investigated as these are of importance for potential drug development.

Keywords: *Ganoderma lucidum*; spore; triterpenes; sterols; fatty acids; anti-aging; protective effect; neurological activity.

ABBREVIATION: BHT, Butylated hydroxytoluene; **CAM**, Complementary and alternative medicine; **CP**, Classical pathway of the complement system; **CPT**, Carnitine palmitoyl transfrase; **DPPH**, 1,1 diphenyl-2- picrylhydrazyl radical; **DTH**, Delayed-type hypersensitivity; **EC_{50}**, Effective concentration; **ED_{50}**, Median effective dose; **EPO**, European Patent Office; **GA**, Ganoderic acid A; **GLS**, Ganoderma lucidum spores; **IC_{50}**, Inhibition concentration; **ICV**, Intracerebroventricularly; **LLC**, Lewis lung carcinoma; **MAPKs**, Mitogen-activated protein kinases; **MCAE**, Mechanochemical-assisted

extraction; **MNCs**, Splenic mononuclear cells; **MNU**, N-metyl-N-nitrosourea; **NGK2D**, Natural killer group 2D receptor; **NOD**, Non-obese diabetic; **PBMCs**, Peripheral blood mononuclear cells; **PMNs**, Polymorphonuclear neutrophils; **PPAR**, Peroxisome proliferator-activated receptor; **SRBC**, Sheep red blood cells; **STZ**, Streptozotocin; **TAN**, Tumor associated neutrophils.

I. INTRODUCTION

Complementary and alternative medicine (CAM) has been described as "diagnosis, treatment and/or prevention that complements mainstream medicine by contributing to a common whole, satisfying a demand not met by orthodoxy, or diversifying the conceptual frameworks of medicine".[1] The application of CAM has increased in recent decades and in traditional Chinese medicine, *Ganoderma lucidum* is an agent which is used in CAM for more than two thousand years to promote health and longevity.[2]

Polysaccharides, proteins and triterpenoids obtained from the fruiting bodies of *G. lucidum* are among the key compounds in this fungus studied to date that are responsible for its biological effects.[3] However, in recent decades it has been reported that these biomolecules can also be obtained from *G. lucidum* spores (GLS) and, even more interestingly, that they may exhibit greater biological effects than biomolecules from other parts of the fungus.[4]

One of the challenges when extracting biomolecules from spores is to break the sturdy thick wall known as the sporoderm, which protects the contents of the spore from the external environment.[5] Considerable research has been undertaken in an attempt to discover new methods for breaking the sporoderm and obtaining higher yields using cheaper processes.[6]

In view of the large number of active biocompounds extracted from GLS with greater biological effects than biocompounds extracted from other parts of this fungus and the dearth of literature available on the subject, this review discusses the importance of using specific techniques to break the spore wall, the main methods of biomolecule extraction and the pharmacological potential of GLS.

II. FINE STRUCTURE AND CHEMICAL COMPOSITION OF GANODERMOID SPORES

Ganoderma lucidum (Leyss.: Fr.) P. Karst., as described by *Karsten*, was characterized as having a pileus covered by a shiny crust and yellowish brown, ovate or elliptical, warty spores.[7] It is basidiomycete that, although little known in the West, has attracted much attention from researchers for its biological properties. GLS are tiny particles about 6.5 – 8.0 μm to 9.6 – 12.6 μm in size enwrapped with outer bilayers of sporoderm.[8] The spore walls are bitunicate with pillars contacted among exosporium and endosporium, with a thickness of 0.8-1.1 μm and 11-14 μm, respectively. Some inorganic elements such as Mg, Al, Si, P, S, Cl, K, Ca, Fe and Ni were detected in the sporoderm of *G. lucidum.* Silicon (19.01%) and calcium (24.31%) were detected at higher levels.[9] The sporoderm is extremely hard and resilient and acts as a barrier for the release of components inside the spores.[10]

A. SPORODERM BREAKING TECHNIQUES

It has been reported that GLS can be administered in a dormant or germinative phase. Germinating spores had higher bioactivity than dormant spores and suggested that these effects depend on whether the sporoderm has been broken or not.

In practice GLS can be used in the whole or broken form, but reports have shown that broken GLS have higher activity than whole spores.[6,11,12] Whole spores have been found in human and animal faeces, indicating that the *G. lucidum* sporoderm may be indestructible *in vivo;*[11] however, it was reported that spores with intact sporoderm may exhibit activity when administered orally *in vivo.*[4]

Some studies have shown that the quality and, consequently, biological effects of extracted biocompounds depend on whether the sporoderm has been broken, as fragmentation of the spore wall facilitates biomolecule extraction by allowing greater contact with the extraction solvent.[4,11,12]. Ma et al.[6] increased the yield of aqueous polysaccharide extract by 40.08% and observed a greater biological effect after the spore wall had been broken down. Yue et al.[4] reported that the percentage of carbohydrate content in sporoderm-broken spores (1.57 ± 0.06) was higher than in extracts of sporoderm-unbroken spores (0.41 ± 0.01). Fu et al.[13] also increased the extraction yield of polysaccharides from 0.94% using whole spores to 2.98 % using sporoderm-broken spores.

It appears that the method chosen to break the sporoderm, which can be physical, physicochemical and/or enzymatic (Table 1), influences the biological effects of GLS. Xie et al.[14] reported that sporoderm-broken GLS had a greater inhibitory effect on cancer cell growth than intact GLS and that spores broken by enzymatic methods were most effective in inhibiting cancer cell growth.

Table 1. Methods to obtain sporoderm-broken spores of *G. lucidum*

Method	**Ratio of broken (%)**	**Reference**
Physical		
Ultrasonography	60 - 80	8, 38
Grinding	-	37, 44
Ultra-fine grinding	100	4
High speed centrifugal shearing (HSCS) pulverizer	100	6
Physical/Chemical		
Supercritical extraction with CO_2	-	13
Microwave and alcohol	-	42
Enzymatic		
Inoculating spores with *G. lucidum* mycelia*	-	44, 48
Not Specified	99.8	11

(-) Data not reported by the author.

(*) The enzymatic method was conducted by inoculating the heat-treated *G. lucidum* spores with *G. lucidum* mycelia.

The search for new methods for breaking spores intensified between the years 1995 and 2013, when 26 patent applications for GLS-breaking techniques were filed on Espacenet, a worldwide database developed by the European Patent Office (EPO) and the member states of the European Patent Organization.

A search carried out in Espacenet with the words “ganoderma” and “spore” in Smart search found 180 patents filed between 1995 and 2013 (Fig. 1).

As research into GLS progresses, new active compounds and their biological properties become known. It is therefore important to develop rapid, low-cost, high- efficiency methods for spore breaking to ensure that compounds that could be used in the development of new drugs can be extracted more efficiently.

B. EXTRACTION OF BIOCOMPOUNDS FROM GLS

The yield in the extraction process varies according to the solvents, concentrations and extraction conditions used (e.g., time and temperature). The success or failure of the process is monitored with different types of chemical and biological assays.

A variety of solvents are used to extract biocompounds from GLS; for example, methanol and chloroform are commonly used to extract triterpenes, water to extract polysaccharides, dichloromethane for lipids and ethanol for lipids and polysaccharides.[14-18] The time and temperature used vary depending on the purpose of the extraction (Table 2).

Table 2. Biocompound´s extraction from *G. lucidum* spores

Extraction	**Biocompounds**	**Condition**	**Reference**
Methanolic	Triterpenes	Methanol by refluxing for 3 h; extraction with hexane; concentrated and extracted with $CHCl_3$.	14,19
	Ganodermasides A and B	Dried spores were extracted with methanol, separated by filtration and concentrated.	28
Ethanolic	Crude extract	Rinsed in absolute ethanol for 10 h in a shaking bath at 30°C and concentrated under vacuum.	12
	Crude extract	Spores were dissolved in ethanol, shaken for 24 h and centrifuged evaporated and the yield of the spore extract was 10% of the original weight.	27
	Crude extract	Spore powder in absolute ethanol for 3 days. Re-extracted the pellet after centrifugation for another 3 days.	39
	C19 fatty acids	Extraction in ethanol at room temperature for 40 h; the extract was filtered, concentrated and fractionated into five fractions according to differences of solubility in ethanol and $CHCl_3$.	18

Aqueous	Polysaccharide	Defatted by 95% ethanol the residue was decocted for 4 h with 2×20 vols of boiling water. The combined aqueous extract was deproteinated with trichloroacetic acid.	38,40
	Polysaccharide	After treatment with ethanol to remove lipids, the dried spores were extracted with boiling water, and the water extract was concentrated, and dialyzed.	15
	Polysaccharide	The spores were dissolved in 10 volumes of water and stirred at 4°C for 12 h. Centrifuged for 30 min., supernatant was concentrated and precipitated with 6 volumes of water-free ethanol.	42
	Crude extract	The spores of *G. lucidum* were extracted with water first at room temperature and then at 70°C. The filtrates were concentrated.	41
	Crude extract	Boiled water at a concentration of 50 mg/ml; extract was stored at 4 ºC and reheated to 70ºC for 10 min before every experiment.	49
$CHCl_3$ (chloroform)	Triterpenes	Extracted three times with 20 volumes of $CHCl_3$ by refluxing in a boiling water bath for 3 h. After filtration, the combined solutions were evaporated to dryness *in vacuum*.	22

CH_2Cl_2 (dichloromethane)	Lipids Crude extract	Extracted ultrasonically with CH_2Cl_2 for 2 h. The suspension was filtered, and the residue was re-extracted (2X). Solvent was removed with a rotary evaporator at 30°C.	16
Supercritical carbon dioxide	Ergosterol Sterols Lipids	The lipids in spores were extracted using supercritical carbon dioxide. Ethanol was added to elevate the effectiveness of extraction.	17,25,28
Mechanochemical-assisted extraction (MCAE)	Polysaccharide	After co-grinding for several minutes (5–30 min), the powder was extracted with an appropriate volume of water (10–30 mL/g) with certain temperature (50–90 °C) and time (30–150 min). Then the mixture was centrifuged (4,200 x g / 10 min) and the supernatant was concentrated.	34

C. ACTIVE CHEMICAL COMPOUNDS IN GLS

C1. Triterpenes

Studies have shown that triterpenes, particularly lanostane-type triterpenes, are one of the most important bioactive constituents of GLS.[19] Lanostane-type triterpenes are a group of molecules whose structure is based on lanosterol (Fig. 2), an intermediate in steroid and triterpene biosynthesis. Ganoderic acid A (GA) is an oxygenated triterpene.[20]

While there are more than 130 different types of triterpenes in *G. lucidum*, to our knowledge only 27 had been identified in GLS by 2013.[14,19,20-23] Some physiological activities of this mushroom have been found to be dependent on its triterpene content and composition, which vary with the strain and conditions under which it has been cultivated (Table 3).

Table 3. Triterpenes identified in *G. lucidum* spores until the year 2013

Triterpenes	**µg/g extract**	
	Min et al.[14]	Gao et al.[22]
Lucidumol A	376.7 ± 2.6	317.6 ± 24.5
Ganoderiol A	17.4 ± 40.9	-
Ganodermanontriol	94.9 ± 2.2	875.4 ± 69.2
Ganodermatriol	-	27.7 ± 2.9
Lucidumol B	85.1 ± 0.6	19.1 ± 1.4
Ganoderiol F	122.4 ± 6.0	186.2 ± 22.9
Ganodermanondiol	313.1 ± 6.6	76.4 ± 8.6
Ganoderic acid B	3092.8 ± 82.2	309.2 ± 26.1
Ganoderic acid A	8408.3 ± 250.0	801.4 ± 46.8
Ganoderic acid α	5577.1 ± 131.4	64.4 ± 6.4

Ganoderic acid H and C1	6583.5 ± 228.9	701.2 ± 15.0
Ganolucidic acid A	736.3 ± 36.9	90 ± 4.9
Ganoderic acid β	179.3 ± 18.3	-
Ganoderic acid γ	-	549.7 ± 38.3
Ganoderic acid ε	-	160.3 ± 12.0
Ganoderic acid η	-	172.5 ± 11.41
Ganoderic acid θ	-	63.9 ± 9.0
Ganoderic acid C2	-	754.7 ± 73.2
Ganoderic acid C6	-	122.3 ± 42.5
Ganoderic acid G	-	257.3 ± 13.9

(-) Data not reported by the author.

C2. Sterols

Ergosterol (Fig. 3) is a sterol precursor of vitamin D2. It is converted by the action of ultraviolet light. Ergosterol and its peroxidation products may exhibit significant pharmacological properties such as pro-apoptotic activity and anti-inflammatory effect.[24]

Yuan et al.[25] detected 1.202 ± 0.031 (mg/g) and 2.267 ± 0.048 (mg/g) of free ergosterol and total ergosterol, respectively, in GLS. Another important observation was that the relative abundances of free and esterified ergosterol were different in the different parts of *G. lucidum*. The spores and tubes have higher percentage of ergosteryl esters (41.9% and 39.7% total ergosterol, respectively) than the pileus and stipe tissues (3.6% and 6.2%, respectively).[26]

C3. Fatty acids

Gas-liquid chromatography of spore lipids obtained by supercritical CO_2 fluid extraction (SFE-CO_2) from sporoderm-broken spores detected 15 kinds of fatty acids, the

main compounds being palmitic acid (6.12%), stearic acid (4.97%), oleic acid (67.11%) and linoleic acid (9.63%).[17] These data are consistent with those reported by Chen et al.[21] and Liu et al.[16].

Chen et al.[21] identified and quantified nine fatty acids, the most predominant of which were oleic acid ($C_{18:1,}$ 57.5%), linoleic acid ($C_{18:2}$, 13.4%), palmitic acid ($C_{16:0}$, 19.6%), hexadecenoic acid ($C_{16:1}$, 2.2%) and linolenic acid ($C_{18:3}$, 0.5%). Liu et al.[16] also identified 18 fatty acids and determined their concentrations using gas chromatography-mass spectrometry in six GLS samples with $C_{16:0}$, $C_{18:0}$, $C_{18:1}$ and $C_{18:2}$ as major constituents ($C_{n:m}$, where "n" is the carbon number and "m" the number of unsaturated bonds in the molecule).

Mixtures of long-chain fatty acids in GLS have been reported to inhibit proliferation of various human cancer cells.[27] Furthermore, C-19 fatty acids such as nonadecanoic acid and cis-9-nonadecenoic acid were recently identified as active compounds in GLS.[18]

Chen et al.[21] also identified some characteristic components in the essential oil of GLS, such as monoterpenes (e.g., α-pinene, D-limonene), aromatic aldehydes (e.g., nonanal, 2-heptenal, 2-decenal and 2, 4-decadienal) and several alkanes.

C4. Carbohydrates

Yue et al.[4] analyzed the amount of polysaccharides in extracts from different parts of the fruiting body of *G. lucidum* and from GLS. The percentage carbohydrate content in the various *G. lucidum* extracts was similar: 3.59% ± 0.24, 3.29% ± 0.16 and 3.45% ± 0.16 for whole fruiting bodies, pilei and stipes, respectively. In contrast, the content in sporoderm-broken spores was 1.57% ± 0.06, which was higher than in sporoderm-unbroken spores (0.41% ± 0.01). All the extracts had the following natural sugars: ribose, rhamnose, arabinose, xylose, mannose, glucose and galactose. Mannose and glucose were

the main sugar components in spore extracts, while in whole-fruiting-body extracts the main sugar components were arabinose and glucose. Although this is an interesting result, it should be noted that the whole fruiting bodies, pilei and stipes analyzed in the study were purchased in Hong Kong, while the spores were bought in China and may therefore have been cultivated under different conditions.

A neutral polysaccharide known as GLSA50-1B was isolated from the boiling water extract of sporoderm-broken GLS. This polysaccharide was characterized as a β-D-glucan with a molecular weight of 103 kDa, a 1,6-linked backbone and an average of one branch attached to O-4 of every other glucosyl residue in the backbone consisting of 1,4-linked β-D-glucosyl residues.[15]

III. PHARMACOLOGICAL EFFECTS OF BIOACTIVE COMPOUNDS FROM *G. lucidum* SPORES

A. ANTIVIRAL

In one study into the effects of antiviral compounds extracted from GLS, ten lanostane-type triterpenes obtained from the chloroform-soluble fraction in a methanolic extract were assessed and characterized. Ganoderic acid β, lucidumol B, ganodermanondiol and ganolucid acid A showed the best inhibitory activity against HIV-1 protease, with IC_{50} values of 20, 50, 90 and 70 μM, respectively.[14]

B. ANTI-COMPLEMENT ACTIVITY

Triterpenoids from the methanolic extract of GLS showed anti-complement activity against the classical pathway (CP) of the complement system. The triterpenoid concentrations causing 50% inhibition of the CP (IC_{50}), were 4.8, 16.2 and 41.7 μM for ganoderiol F, ganodermanontriol and ganodermanondiol, respectively. An important

finding is the importance of carbonyl and hydroxymethyl groups in the side chain of the molecule to increase anti-CP activity.[19]

C. ANTI-AGING ACTIVITY

Anti-aging studies are commonly performed in a variety of organisms, such as yeasts, nematodes, fruit flies, mice and rats. Studies with the *UTH1* gene, a yeast-aging gene, found that ergosterols and ganodermasides A, B, C and D isolated from methanolic GLS extracts exhibited anti-aging properties.[28,29]

D. EFFECT ON ENERGY METABOLISM

Unsaturated fatty acids from GLS extract obtained by supercritical CO_2 were reported to induce the transcriptional activity of peroxisome proliferator-activated receptor alpha (PPARα), as well as PPARγ and PPARδ, which are key regulators of energy metabolism. According to Huang et al.,[30] GLS lipid may regulate key enzymes of energy metabolism such as carnitine palmitoyl transferase 1a (CPT 1a) by PPARα in β-oxidation.

E. PROTECTIVE EFFECT

It has been reported that GLS lipids block N-methyl-N-nitrosourea (MNU), which has been shown to induce photoreceptor cell apoptosis in studies using rats as animal models. Lipids from GLS may regulate the expression of Bax, Bcl-xl and Caspase-3, inhibiting MNU-induced rat photoreceptor cell apoptosis and protecting retinal function.[31]

The radio-protective effects of GLS polysaccharides were investigated in a mouse animal model exposed to ^{60}Co gamma irradiation. In this study a reduction in micronuclei

formation, an increase in antioxidant enzyme activity, an increase in the number of nucleated cells in bone marrow and protection against lipid peroxidation were observed.[32]

Hepatoprotective effects of GLS extract has been observed on cadmium hepatotoxicity in mice. GLS extract induced an eightfold increase in expression of hepatic metallothionein-1 mRNA and an increase in metallothionein protein, which is responsible to sequester the cadmium in cytosol and acts against cadmium toxicity.[33]

F. ANTIOXIDANT ACTIVITY

Significant antioxidant activity was reported after mechanochemical-assisted extraction (MCAE) of polysaccharides from GLS. The 1,1 diphenyl-2- picrylhydrazyl radical (DPPH)-scavenging ability (EC_{50} value) of the GLS polysaccharides, with ascorbic acid and butylated hydroxytoluene (BHT) used as controls, were 0.36 mg/mL, 0.25 mg/mL and 0.43 mg/mL, respectively.[34]

Analysis of the antioxidant activity of polysaccharide extracts from spores, fruiting bodies and mycelia using different culture media revealed that spores provided the best results. The DPPH ($EC_{50\ value}$) of the GLS polysaccharide extract was 0.015 mg/mL.[35]

G. NEUROLOGICAL ACTIVITY

According to some studies, GLS powder has a neuroprotective effect and could be an option for the treatment of neurodegenerative disorders such as epilepsy, Alzheimer's disease and depression.

In rats, the number of somatostatin immunoreactive cells in the cerebral cortex and hippocampus were decreased in a GLS-treated group, 6.37±0.91 and 7.37±0.91 respectively compared with an epilepsy-model group, 10.25±2.05 and 12.75±1.90 respectively. The level of neuropeptide Y in the cerebral cortex and hippocampus were

also decreased in GLS treated group somastatin and neuropeptide Y have been implicated on seizures and epilepsy.[36]

An interesting study shows that GLS has great potential for use in the treatment of neurodegenerative disorders, such as Alzheimer's disease. An oral dose of GLS powder at 2, 4 and 8 mg/Kg was administered in rats which hippocampi were injected intracerebroventricularly (ICV) with streptozotocin (STZ). The results showed that GLS could alleviate oxidative stress and mitochondrial dysfunction in the hippocampus of rats injected ICV with STZ, could protect neurons from apoptosis and could improve cognitive dysfunction.[37]

H. IMMUNOLOGICAL ACTIVITIES

One of the main substances in GLS that are responsible for much of their immunostimulatory effect are polysaccharides. This biological effect seems to be related to certain characteristics of the carbon chain, the β-D configuration, the degree of substitution in the main chain and the length of the side chain.

The addition of ionic groups, the structure of glucans and the density of negative charges on a polysaccharide appears to be related to immunostimulatory activity.[38]

Ethanolic extract of GLS had either no effect or a suppressive rather than a proliferative effect on peripheral blood mononuclear cells (PBMCs) at dosages ranging from 1 μg/mL to 1 mg/mL. It also showed a growth-inhibiting effect on monocytes with only approximately 70% cell viability at four different concentrations (1 μg/mL, 10 μg/mL, 100 μg/mL and 1 mg/mL) after 48 hours of incubation. As for sporoderm unbroken GLS, at doses between 1 μg/mL and 100 μg/mL, the expression patterns of co-stimulatory molecules on dendritic cells (DCs) were similar to those of negative controls.[39]

Sporoderm-broken spores can significantly enhance delayed-type hypersensitivity (DTH) and phagocytic index in mice. In one study a 1 g/kg dose significantly increased DTH in mice.[6]

GLS at 1 and 2 g/kg were found to inhibit significantly the growth of implanted S-180 sarcoma in mice. Spleen lymphocytes isolated from the mice and proliferative responses at a dose range of 2 to 4 g/kg of sporoderm-broken GLS were significantly different from those of untreated control mice.[4]

Although the immunostimulatory effect of *G. lucidum* polysaccharides are well known, little is known about the molecular mechanism of stimulation. The proliferation of splenic mononuclear cells (MNCs) treated with 200, 400, or 800 μg/mL of GLS for 72 h was significantly increased compared with that of control cells, and there was also significantly increased production of IL-2 and TNF-α. A proteomic technique was used to determine which proteins are influenced by GLS; ten were identified as having a possible stimulatory effect on MNCs proliferation and cytokine production.[40]

Another probable molecular pathway through which extracts of sporoderm-broken germinating GLS activate human polymorphonuclear neutrophils (PMNs) were also investigated. At concentrations of 40 mg/mL and 80 mg/mL, GLS enhanced the phagocytic activity of PMNs in a dose-dependent manner. This effect was significantly attenuated by treatment with P38 mitogen-activated protein kinase (p38 MAPK) inhibitor.[41] These results indicate that GLS may act on the p38 MAPK pathway in a dose-dependent manner.

A water-soluble polysaccharide extracted from GLS stimulated dose-dependently at 50, 100 and 200 μg/mL TNF-α and IL-6 secretion in murine resident peritoneal macrophages and induced phosphorylation of ERK1/2, p38 and JNK, three important mitogen-activated protein kinases (MAPKs) that regulate immune response.[42] These

signaling pathways besides NGK2D/NCRs (natural-killer group 2D receptor) are also crucial to activation of NK (Natural Killer) cells by water extract obtained of dried *G. lucidum* mycelium.[43]

Although *G. lucidum* biocompounds have been described with immunomodulatory activity such as neutrophil activation, there is no notice that correlates the induction of TAN (tumor-associated neutrophils) with the exposure of *G. lucidum* biocompounds.

I. ANTITUMOR EFFECT

In the last fifteen years, the use of herbal therapies in alternative medicine has been increasing, evidence suggests that increasing use is being made of dietary supplements in cancer treatment. Many studies have shown that GLS can have an effect on different types of tumors. This is due to the presence of different molecules such as triterpenoids, polysaccharides and fatty acids in its composition.

Among the different triterpenes found in the chloroform-soluble fraction of the methanolic extract of GLS, lucidumol A exhibited the most potent cytotoxicity (ED_{50} value, 2.3 µg/mL) against Lewis lung carcinoma (LLC) cells, and ganodermanondiol (ED_{50}, 3.4 µg/ml) the most potent cytotoxicity against Meth-A cells (sarcoma) in mouse tumor cell lines.[23]

Lipids extracted from germinating spores and sporoderm-broken GLS had remarkable dose-dependent antitumor effects and a significant effect on mouse hepatoma, sarcoma S-180 and reticulocyte sarcoma L-II cells, with inhibition of 80% to 90%.[24] Active fatty acids, nonadecanoic acid and cis-9-nonadecenoic acid from GLS inhibited tumor cell proliferation and induced apoptosis in a promyelocytic cell line (HL-60).[18]

Other studies have shown that addition of GLS to malignant human breast carcinoma MT-1 cells at a final concentration of 1 mg/mL followed by incubation of the

cultures for two days resulted in detachment of these cells from the tissue culture plates and their death. Proliferation of adherent cells was also inhibited.[44]

When GLS extract (final concentration 100 to 300 µg/mL) was added to different blood cancer cell lines (Jurkat, Raji, HL-60 and MOLT4) and incubated for 24 h, proliferation of these cancer cells was significantly inhibited in a dose-dependent manner in the MTT assay.[27]

Sporoderm-unbroken GLS were administered orally to sarcoma-bearing mice at 1 and 2 g/kg and were found to inhibit the growth of the implanted S-180 sarcoma. Oral administration of 2 g/kg was shown to be most effective. The efficacies of sporoderm-broken spores and sporoderm-unbroken spores were also compared at this concentration. The weights of sarcomas in mice treated with sporoderm-broken spores and those treated with sporoderm-unbroken spores were reduced by 31.5% and 22.4%, respectively, compared with untreated controls.[4]

IV. CLINICAL EXPERIENCE

Some toxicological studies have shown that *G. lucidum* is safe for *in vivo* and clinical use. However, more toxicological information is required for GLS to be approved for clinical use.

Wicks et al.[45] evaluated the safety and tolerability of *G. lucidum* extract containing spore in 16 health human volunteers. 2 g of extract were administered orally for 10 consecutive days and no significant adverse effects were observed. Another study reported that *GLS powder* may have beneficial effects on cancer-related fatigue without any relevant adverse effects. The two most common discomforts were dizziness (16.0%) and dry mouth (12.0%).[46]

However, abnormally elevated serum levels of a glycoprotein associated with tumors (CA72-4) were reported in patients who took GLS powder while receiving treatment for gastrointestinal cancer.[47] Elevation of serum levels of CA72-4, has been reported in a variety of malignancies, including gastrointestinal, ovarian, and cancer of the breast.

V. FUTURE DEVELOPMENTS

As many laboratories are investigating the pharmacological effects of GLS, other biological properties of this substance and potential applications for it are likely to be discovered in the near future. With the rapid nanobiotechnology development is expected the production and application of many nanoparticles containing biomolecules from *G. lucidum*, however, continued effort is required to develop efficient methods for breaking the spore wall and extracting biocompounds so that high yields can be achieved at low cost. Controlled clinical studies to evaluate the safety of formulations containing GLS are also required. Furthermore, it is necessary to consider some hurdles to the use of GLS in practice, such as the time related in the spore production in a solid state culture under specific conditions for growth of the fruit bodies and sporulation, as well as the amount of spores in relation to other parts of *G. lucidum* in clinical trials, despite its high added value.

ACKNOWLEDGEMENT

The authors would like to thank CAPES and CNPq for providing financial support.

REFERENCES

1. Ernst E, Resch KL, White AR. Complementary medicine. What physicians think of it: a meta-analysis. Arch Intern Med. 1995;155(22):2405-2408.

2. McMeekin D. The perception of Ganoderma lucidum in Chinese and Western culture. Mycologist. 2004;18(4):165-169.

3. Batra P, Sharma AK, Khajuria R. Probing Lingzhi or Reishi Medicinal Mushroom Ganoderma lucidum (Higher Basidiomycetes): A Bitter Mushroom with Amazing Health Benefits. Int J Med Mushrooms. 2013;15(2):127-143.

4. Yue GGL, Fung KP, Leung PC, Lau CBS. Comparative studies on the immunomodulatory and antitumor activities of the different parts of fruiting body of Ganoderma lucidum and Ganoderma spores. Phytother Res. 2008;22(10):1282-1291.

5. Liu X, Wang JH, Yuan JP. Pharmacological and anti-tumor activities of Ganoderma spores processed by top-down approaches. J Nanosci Nanotechnol. 2005;5(12):2001-2013.

6. Ma J, Fu Z, Ma P, Su Y, Zhang Q. Breaking and characteristics of Ganoderma lucidum spores by high speed entrifugal shearing pulverizer. J Wuhan Univ Technol. 2007;22(4):617-621.

7. Coleman LC. Structure of spore wall in Ganoderma. Bot Gaz. 1927;83:48-60.

8. Zhao D, Chang MW, Li JS, Suen W, Huang J. Investigation of ice-assisted sonication on the microstructure and chemical quality of Ganoderma lucidum spores. J Food Sci. 2014;79(11):2253-2265.

9. Chen T, Li K, Chen C, Chen X, Wen Q, Lin X. Ultrastructure of basdiospores and sporoderm of Ganoderma lucidum and G.sinense. Fujian Journal of Agricultural Sciences. 1998;4:33-38.

10. Mims CW, Seabury F. Ultrastructure of tube formation and basidiopore development

in Ganoderma lucidum. Mycologia; 1989:754-764.

11. Liu X, Yuan JP, Chung CK, Chen XJ. Antitumor activity of the sporoderm-broken germinating spores of Ganoderma lucidum. Cancer Lett. 2002;182(2):155-161.

12. Zhu HS, Yang XL, Wang LB, Zhao DX, Chen L. Effects of extracts from sporoderm-broken spores of Ganoderma lucidum on HeLa cells. Cell Bio Toxicol. 2000;16(3):201-206.

13. Fu YJ, Liu W, Zu YG, Shi XG, Liu ZG, Schwarz G, Efferth T. Breaking the spores of the fungus Ganoderma lucidum by supercritical CO_2. Food Chem. 2009;112(1):71-76.

14. Min BS, Nakamura N, Miyashiro H, Bae KW, Hattori M. Triterpenes from the spores of Ganoderma lucidum and their inhibitory activity against HIV-1 protease. Chem Pharm Bull. 1998;46(10):1607-1612.

15. Dong Q, Wang Y, Shi L, Yao J, Li J, Ma F, Ding K. A novel water-solubleb-D-glucan isolated from the spores of

Ganoderma lucidum . Carbohyd Res. 2012;353:100-105.

16. Liu X, Xu SP, Wang JH, Yuan JP, Guo LX, Li X, Huang XN. Characterization of Ganoderma spore lipid by stable carbon isotope analysis: implications for authentication. Anal Bioanal Chem. 2007;388(3):723-31.

17. Chen TQ, Wu JG, Wu YB, Wang H, Mao FH, Wu JZ. Supercritical fluid CO_2 extraction, simultaneous determination of total sterols in the spore lipids of Ganoderma lucidum by GC-MS/SIM methods. Chem Nat Cpd. 2012;48(4):657-658.

18. Gao P, Hirano T, Chen Z, Yasuhara T, Nakata Y, Sugimoto A. Isolation and identification of C-19 fatty acids with anti-tumor activity from the spores of Ganoderma lucidum (reishi mushroom). Fitoterapia. 2012;83(3):490-499.

19. Min BS, Gao JJ, Hattori M, Lee HK, Kim YH. Anticomplement activity of terpenoids from the spores of Ganoderma lucidum. Planta Med. 2001;67(9):811-814.

20. Boh B. Ganoderma lucidum: a potential for biotechnological production of anti-cancer and immunomodulatory drugs. Recent Pat Anticancer Drug Discov. 2013;8(3):255-87.

21. Chen TQ, Wu YB, Wu JG, Wang HY, Mao FH, Wu JZ. Fatty acids, essential oils, and squalene in the spore lipids of Ganoderma lucidum by GC-MS and GC-FID. Chem Nat Cpd. 2013;49(1):143-144.

22. Gao JJ, Nakamura N, Min BS, Hirakawa A, Zuo F, Hattori M. Quantitative determination of bitter principles in specimens of Ganoderma lucidum using high-performance liquid chromatography and its application to the evaluation of Ganoderma products. Chem Pharm Bull. 2004;52(6):688-695.

23. Min BS, Gao JJ, Nakamura N, Hattori M. Triterpenes from the spores of Ganoderma lucidum and their cytotoxicity against Meth-A and LLC tumor cells. Chem Pharm Bull. 2000;48(7):1026-1033.

24. Russo A, Cardile V, Piovano M, Caggia S, Espinoza CL, Garbarino JA. Pro-apoptotic activity of ergosterol peroxide and (22E)-ergosta-7,22-dien-5 alpha-hydroxy-3,6-dione in human prostate cancer cells. Chem Biol Interact. 2010;184(3):352-358.

25. Yuan JP, Wang JH, Liu X, Kuang HC, Huang XN. Determination of ergosterol in Ganoderma spore lipid from the germinating spores of Ganoderma lucidum by high-performance liquid chromatography. J Agr Food Chem. 2006;54(17):6172-6176.

26. Yuan JP, Wang JH, Liu X. Distribution of free and esterified ergosterols in the medicinal fungus Ganoderma lucidum. Appl. Microbiol. Biotechnol. 2007;77(1):159-165.

27. Fukuzawa M, Yamaguchi R, Hide I, Chen Z, Hirai Y, Sugimoto A, Yasuhara T, Nakata Y. Possible involvement of long chain fatty acids in the spores of Ganoderma lucidum (Reishi Houshi) to its anti-tumor activity. Bio Pharm Bull. 2008;31(10):1933-

1937.

28. Weng Y, Xiang L, Matsuura A, Zhang Y, Huang Q, Qi J. Ganodermasides A and B, two novel anti-aging ergosterols from spores of a medicinal mushroom Ganoderma lucidum on yeast via UTH1 gene. Bioorg Med Chem. 2010;18(3):999-1002.

29. Weng Y, Lu J, Xiang L, Matsuura A, Zhang Y, Huang Q, Qi J. Ganodermasides C and D, two new anti-aging ergosterols from spores of the medicinal mushroom Ganoderma lucidum. Biosci Biotechnol Biochem. 2011;75(4):800-803.

30. Huang Z, Fang F, Wong CW. Ganoderma lucidum spore lipid induces peroxisome proliferator-activated receptor alpha activity. J Food Biochem. 2011;35(5):1508-1513.

31. Gao Y, Deng XG, Li N, Luo GW, Chung PCK. The Comparative Protective Effects of Ganoderma Spores Lipid and Fish Oil on N-Methyl-N-Nitrosourea-Induced Photoreceptor Cell Lesion in Rats. J Evid Based Complementary Altern Med. 2011:1-10.

32. Zhao W, Jiang X, Deng W, Lai Y, Wu M, Zhang Z. Antioxidant activities of Ganoderma lucidum polysaccharides and their role on DNA damage in mice induced by cobalt-60 gamma-irradiation. Food Chem Toxicol. 2012;50(2):303-309.

33. Jin H, Jin F, Jin JX, Xu J, Tao TT, Liu J, Huang HJ. Protective effects of Ganoderma lucidum spore on cadmium hepatotoxicity in mice. Food Chem Toxicol. 2013;52:171-175.

34. Zhu X, Chen X, Xie J, Wang P, Su W. Mechanochemical-assisted extraction and antioxidant activity of polysaccharides from Ganoderma lucidum spores. Intern J Food Technol. 2012;47(5):927-932.

35. Heleno SA, Barros L, Martins A, Queiroz MJRP, Santos-Buelga C, Ferreira ICFR. Fruiting body, spores and in vitro produced mycelium of Ganoderma lucidum from Northeast Portugal: A comparative study of the antioxidant potential of phenolic and polysaccharidic extracts. Food Res Intern. 2012;46(1):135-140.

36. Zhu K, Lu M, Wang S, Zhang S, Sun D. Influence of Ganoderma lucidum spores on the levels of neuropeptide Y and somatostatin in brains of seizure rats. Neural Regen Res. 2008;3(5):486-489.

37. Zhou Y, Qu ZQ, Zeng YS, Lin Y, Li YK, Chung P, Wong R, Hagg U. Neuroprotective effect of preadministration with Ganoderma lucidum spore on rat hippocampus. Exp Toxicol Pathol. 2012;64(7):673-680.

38. Bao XF, Zhen Y, Ruan L, Fang JN. Purification, characterization, and modification of T lymphocyte-stimulating polysaccharide from spores of Ganoderma lucidum. Chem Pharm Bull. 2002;50(5):623-629.

39. Chan WK, Lam DTW, Law HKW, Wong WT, Koo MWL, Lau ASY, Lau YL, Chan, GCF. Ganoderma lucidum mycelium and spore extracts as natural adjuvants for immunotherapy. J Altern and Complement Med. 2005;11(6):1047-1057.

40. Ma C, Guan SH, Yang M, Liu X, Guo DA. Differential protein expression in mouse splenic mononuclear cells treated with polysaccharides from spores of Ganoderma lucidum. Phytomedicine. 2008;15(4):268-276.

41. Hsu PY, Chen JL, Chen HY, Yang SH. Extract of sporoderm-broken germinating spores of Ganoderma lucidum activates human polymorphonuclear neutrophils via the P38 mitogen-activated protein kinase pathway. Chang Gung Med J. 2012;35(2):140-147.

42. Guo L, Xie J, Ruan Y, Zhou L, Zhu H, Yun X, Jiang Y, Lu L, Chen K, Min Z, Wen Y, Gu J. Characterization and immunostimulatory activity of a polysaccharide from the spores of Ganoderma lucidum. Int Immunopharmcol. 2009;9(10):1175-1182.

43. Chang CJ, Chen YYM, Lu CC, Lin CS, Martel J, Tsai SH, Ko YF, Huang TT, Ojcius DM, Young JD, Lai HC. Ganoderma lucidum stimulates NK cell cytotoxicity by inducing NKG2D/NCR activation and secretion of perforin and granulysin. Innate immun. 2014;20(3):301-311.

44. Xie YZ, Li SZ, Yee A, La Pierre DP, Deng Z, Lee DY, Wu QP, Chen Q, Li C, Zhang Z, Guo J, Jiang Z, Yang BB. Ganoderma lucidum inhibits tumour cell proliferation and induces tumour cell death. Enzyme Microb Tech. 2005;40(1):177-185.

45. Wicks SM, Tong R, Wang CZ, O'Connor M, Karrison T, Li S, Moss J, Yuan CS. Safety and tolerability of Ganoderma lucidum in healthy subjects: A double-blind randomized placebo-controlled trial. Am J Chin Med. 2007;35(3):407-414.

46. Zhao H, Zhang Q, Zhao L, Huang X, Wang J, Kang X. Spore Powder of Ganoderma lucidum Improves Cancer-Related Fatigue in Breast Cancer Patients Undergoing Endocrine Therapy: A Pilot Clinical Trial. J Evid Based Complementary Altern Med. 2012:1-8.

47. Yan B, Meng X, Shi J, Qin Z, Wei P, Lao L. Ganoderma lucidum Spore Induced CA72-4 Elevation in Gastrointestinal Cancer: A Five-Case Report. Integr Cancer Ther. 2014;13(2):161-166.

48. Wu QP, Xie YZ, Li SZ, La Pierre DP, Deng Z, Chen Q, Li C, Zhang Z, Guo J, Wong CKA, Lee DY, Yee A, Yang BB. Tumour cell adhesion and integrin expression affected by Ganoderma lucidum. Enzyme Microb Tech. 2006;40(1):32-41.

49. Sliva D, Labarrere C, Slivova V, Sedlak M, Lloyd FP, Ho NWY. Ganoderma lucidum suppresses motility of highly invasive breast and prostate cancer cells. Biochem Biophy Res Commun. 2002;298(4):603-612.

Figures capitations

Figure 1. Number of *G. lucidum* spores` patents from 1995 to 2013

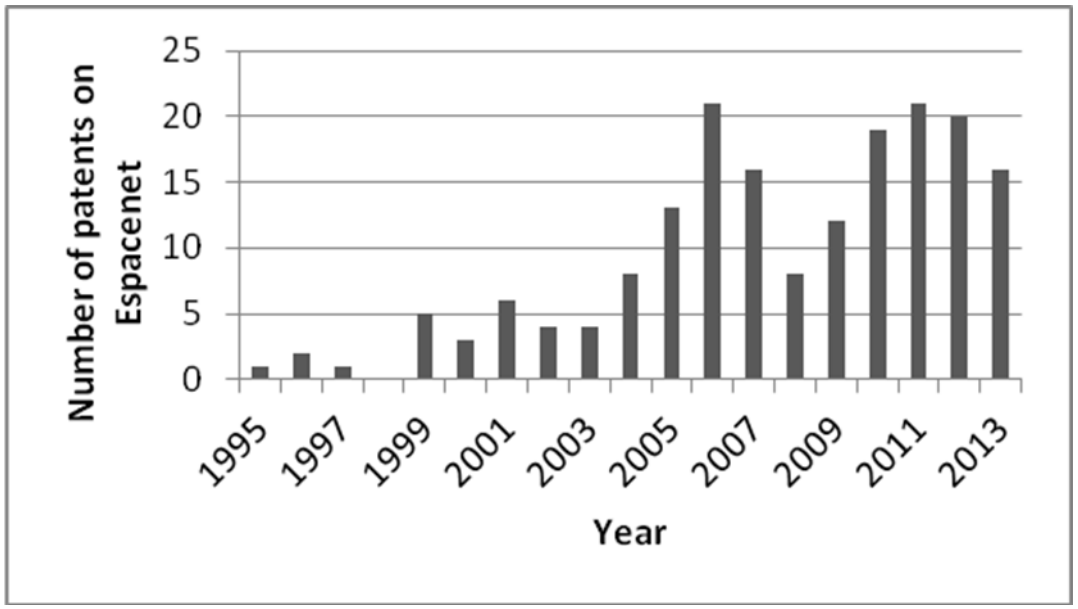


Figure 2. Structure of lanosterol and ganoderic acid A

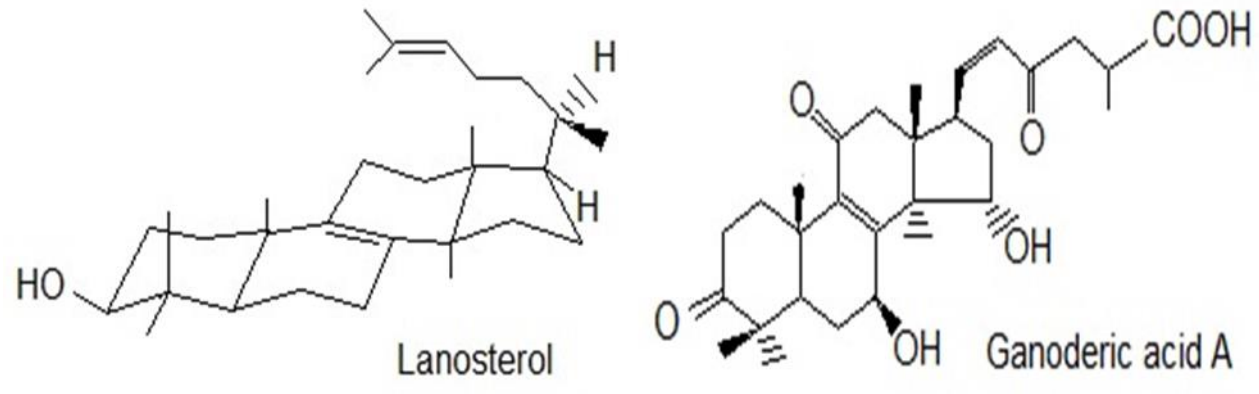
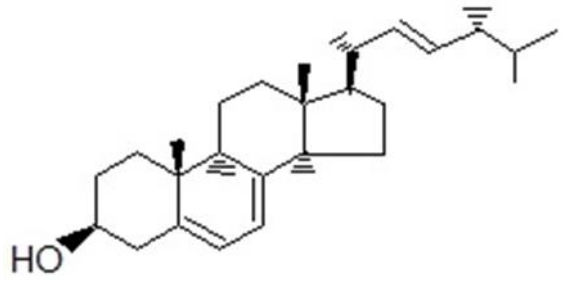


Figure 3. Structure of an ergosterol molecule



CHAPTER - II

EXTRACTS OF C*ordyceps sinensis* INCREASE THE RESISTENCE TO OXIDATIVE STRESS IN C*aernohabitis elegans* AND EXHIBIT ANTITUMOR ACTIVITY AGAINST HUMAN NEUROBLASTOMA AND ADRENOCORTICAL CARCINOMA CELLS

EXTRACTS OF *Cordyceps sinensis* INCREASE THE RESISTENCE TO OXIDATIVE STRESS IN *Caernohabitis elegans* AND EXHIBIT ANTITUMOR ACTIVITY AGAINST HUMAN NEUROBLASTOMA AND ADRENOCORTICAL CARCINOMA CELLS

L.Y. Bissoqui[1]· L. F. dos Santos[2] · A. Gollo[1] · S.H. da Silva[3]· A.L.L. da Silva[1] · S.J.R Bonatto[3] · R. Rubel[4] · C.R. Soccol[1]*

[1] Bioprocess Engineering and Biotechnology Department, Federal University of Paraná, CEP 81531-980, Curitiba, Paraná, Brazil. *E-mail: soccol@ufpr.br

[2] Department of Pharmacy, Midwest State University of Paraná, Guarapuava, Paraná, Brazil.

[3] Instituto de Pesquisa Pelé Pequeno Princípe, Curitiba, Paraná, Brazil.

[4] Clinical Hospital Complex, Federal University of Paraná, Curitiba, Paraná, Brazil.

Abstract

The food and pharmaceutical industries are constantly in search of biocompounds from natural sources due to their biological activity and low toxicity effects. Mushrooms are rich natural sources of bioactive compounds and also extensively studied for their medicinal properties. In the present study, hot aqueous extracts of biomass from *Cordyceps sinensis* (Cs) produced by submerged fermentation was analyzed for their chemical composition, antioxidant and antitumoral activity. The extracts were found to contain high amounts of carbohydrates, mostly of the non-reducing type, phenolic compounds, protein and/or polysaccharide-protein complexes, all of which probably contributed to the activities evaluated in this research. In a whole organism, *Caernohabitis elegans*, extracts from stationary phase increased resistance to oxidative stress in 72%, which resulted in a higher survival of the worm against 5-hydroxy-1,4-naphthalenedione (juglone), an oxidative agent used as positive control. Futhermore, the extracts exhibited moderate antitumor activity ranging from 19.1 to 31% and from 19.2 to 38.4% inhibition, to the carcinogenic growth of neuroblastoma (IMR-32) and adrenocortical carcinoma (H295R) cells, respectively. This is the first trial study which addressed the effects of mushroom extracts of *C. sinensis* against human adrenocortical carcinoma cells (H295R). All these findings suggest that a water extract of the mushrooms biomass evaluated could be a promising source of natural biocompounds to develop innovative functional foods, nutraceuticals and cosmeceuticals which could protect the health cells against oxidative stress and prevent or combat tumor cells.

Keywords: *Juglone; Oxidative stress; Caernohabitis elegans; neuroblastoma; adrenocortical carcinoma.*

1. INTRODUCTION

Mushrooms are macrofungi widely used in culinary and in Eastern medicine. Recently, the mushrooms have received the attention of food and pharmaceutical industries due to their potential beneficial effects on human health, as well as on the prevention and treatment of diseases (BARROS et al., 2008; GIAVASIS, 2014). It has been estimated that there are about 150 thousand species of mushrooms distributed worldwide. Concerning all these species, approximately 10% or 15 thousand species are known to science (HAWKSWORTH, 2001; MUELLER, 2007).

Due to its high nutritional and medicinal value, the mushrooms have been considered as a promising natural source of antioxidant and antitumor biocompounds. Polysaccharides, proteins, phenolics and triterpenes are the main biocompounds in mushrooms that exhibit antioxidant and/or antitumor activities (CARNEIRO et al., 2013; SIU et al., 2014; ZHANG et al., 2016).

Oxidative stress is caused by an imbalance between the production of free radicals and reactive metabolites, so-called oxidants or reactive oxygen species (ROS), and their elimination by antioxidants in a cell or an organism. This imbalance causes damage to biomolecules and important cells. As a result, it demonstrates a negative impact on the human being. In addition, it is believed that a diet rich in antioxidant compounds is responsible for maintaining good health and prevents various diseases including cancer (DURACKOVA, 2010).

Antioxidant and antitumoral activities of extracts from *Cordyceps sinensis* has been studied and established as a natural source of biocompounds that exhibit great biological properties (JAYAKUMAR, 2009; XIAOPING et al., 2009; PATEL and GOYAL, 2012; YAN et al., 2014). Moreover, there are no reports that assess the effect of extracts from these species on human adrenocortical carcinoma (ACC) and neuroblastoma (NB) cancers.

Adrenocortical carcinoma (ACC) is a rare aggressive malignant tumor with poor prognosis. Primary treatment consists of surgery. On top of that, this treatment has high rates of recurrence and metastasis (BERTAGNA and ORTH, 1981; NG and LIBERTINO, 2003).

Neuroblastoma (NB) is an extracranial solid tumor which occurs mainly in childhood and it is responsible for approximately 15% of all pediatric tumors. In addition, this cancer has a poor prognosis in children above 1 year of age (KOWALCZYK et al., 2009; RACIBORSKA et al., 2015).

Apart from their biological properties, *C. sinensis* mycelia can be obtained from submerged fermentation which consists of an interesting method to produce high amount of biomass under strict control of the process. Moreover, submerged fermentation shows excellent rates of biomass recovery that enables the research on bioactive compounds, as well as it develops on cheap substrates.

Finally, prospecting natural compounds which have antioxidant and antitumor activity that can be produced by mushrooms seems to be essential for the development of new pharmaceutical applications and development of functional foods. Thus, this study evaluated the protection "*in vivo*" that mycelial extracts from *C. sinensis* confer against oxidative stress in *Caenorhabditis elegans* and the antitumor activity against human neuroblastoma (IMR32) and adrenocortical carcinoma (H295R) cells. The present results and data might provide new insights to develop novel food bioingredients based on water extracts from mushrooms that have antioxidant properties and also could act as an adjuvant to prevent or combat cancer.

2. MATERIAL AND METHODS

2.1 MUSHROOM STRAIN AND SUBMERGED FERMENTATION

Strains of *Cordyceps sinensis* (Cs) was acquired from the collection of the Bioprocesses and Biotechnology Laboratory / Federal University of Parana – Paraná - Brazil. These strains were cultivated on Potato Dextrose-Agar (PDA) medium at 28°C for 10 days and then stored at 4°C in a refrigerator. Subculture was made at each three months to maintain the strains active. All experiments were carried out using the 10-day-old mycelium. The inoculum was performed using three agar blocks (0.7 cm with a self-designed cutter) placed in 250 mL Erlenmeyer flasks containing 50 mL medium (pH 5.5) and incubated in shaker at 28°C ± 2°C, 120 rpm, for 10 days. The culture medium consisted of the following components ($g.L^{-1}$): glucose 20, yeast extract 3, K_2HPO_4 0.6 and $MgSO_4$ 0.3

(FAN et al., 2007). The fermentation medium was inoculated using 5 mL of homogenized inoculum for 50 mL medium and kept at the same conditions described above. The fermentation time was 11 days for *C. sinensis* and then mycelia was recovered daily from the liquid medium by filtration, washed with distilled water and dried at 45°C during 24 h to obtain the yield of biomass ($g.L^{-1}$).

2.2 EXTRACTION OF THE BIOACTIVE COMPOUNDS

In order to isolate the water-soluble bioactive compounds from the three mushroom species, dried mycelial mass was suspended in distilled water (1:20 w/v) and homogenized. The solution was heated for 4 h at 90°C to extract heat-stable mycelial compounds, followed by centrifugation (3000 g / 20 min) and the supernatant was freeze-dried. The freeze-dried powders were stored in freezer (-20^{o} C) until use.

2.3 CHEMICAL ANALYTICAL PROCEDURES

The reducing and total sugars of the extracts were determined by using the dinitrosalicylic (MILLER, 1959) and phenol sulfuric (DUBOIS, 1956) methods, respectively, and expressed as glucose equivalents. Proteins were measured by a colorimetric assay (LOWRY, 1951). Total phenolic compounds were measured according to the spectrophotometric method of Folin-Ciocalteau (SINGLETON and ROSSI, 1965) using gallic acid as a reference standard. Results were expressed in µg of gallic acid equivalent per mg of dry biomass (µg GAE.mg^{-1} dry biomass).

Reducing sugars were evaluated daily to establish a consumption kinetic to compare with the biomass growth kinetics. All the other chemical analytical procedures were performed on exponential and stationary phase of mycelium growth at 4th and 7th day of culture for *C. sinensis.*

2.4 EVALUATION OF ANTIOXIDANT PROPERTIES

2.4.1 Scavenging ability on 2,2 Diphenyl-2- Picrylhydrazyl radical (DPPH)

Scavenging activity of the free radicals by bio-compounds of mushrooms was measured in terms of hydrogen atom donating, using the stable radical DPPH method described by Brand-Williams et al. (1995). Aliquots of 1 mL of the various concentrations of mushroom extracts were added to 4 mL of 0.004% methanol solution of DPPH. After 30 minutes of reaction at room temperature, the absorbance was read against a blank (i.e. methanol was used as blank) at 517 nm. Inhibition of free radical by DPPH was expressed in percentage (%) by the following equation (1):

$$I\% = (A_{blank} - A_{sample} / A_{blank}) \times 100 \qquad \text{Eq. (1)}$$

Where A_{blank} is the absorbance of the control reaction and A_{sample} is the absorbance of the test extract. The extract concentration producing 50% inhibition (EC_{50}) was calculated from the graph of the DPPH scavenging effect against the extract concentration. Ascorbic acid, butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) were used as standards. Tests were carried out in triplicate.

2.4.2 ABTS assay

The ABTS assay was carried out using a method based on the original and classic method developed by Miller (1993) with some modifications according to Li et al. (2012). The ABTS radical should be pre-formed by reacting equal volumes of 1.1 $mg.mL^{-1}$ aqueous ABTS and 0.68 $mg.mL^{-1}$ potassium persulfate ($K_2S_2O_8$), and then stored in the dark for 6 h at room temperature. Then ABTS•+ solutions (50 µL) were added to samples of different concentrations (200 µL, 0.25–8.0 $mg.mL^{-1}$). These solutions were gently mixed and incubated in the dark for 30 min at room temperature. Then the absorbances of the resulting solutions were measured at 734 nm. The scavenging capability of test compounds was calculated using the following equation (2):

$$\text{ABTS•+ scavenging activity (\%)} = (1 - (\lambda_{734}\text{-S}/\lambda_{734}\text{-C})) \text{ X } 100 \qquad \text{Eq. (2)}$$

where λ_{734}-C is the absorbance of a control with no radical scavenger and λ_{734}-S is the absorbance of the remaining ABTS in the presence of scavenger.

2.4.3 Phosphomolybdenum method

Total antioxidant capacity assay is a spectrophotometric method for the quantitative determination of antioxidant capacity, through the formation of phosphomolybdenum complex. The assay is based on the reduction of Mo (VI) to Mo (V) by the sample analyte and subsequent formation of a green phosphate Mo (V) complex at acidic pH. Total antioxidant capacity can be calculated by the method described by Prieto et al. (1999). 0.1 mL of sample solution was added to a tube containing 1 mL of reagent (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tube was capped and incubated in a boiling water bath at 95 °C for 90 min. After cooling the sample to room temperature, the absorbance of the aqueous solution was measured at 695 nm against blank in UV spectrophotometer. A typical blank solution contained 1 mL of reagent solution and the appropriate volume of the same solvent used for the sample and it was incubated under same conditions as the rest of the samples. The antioxidant capacities were expressed as equivalents of ascorbic acid by the following formula (3):

Acid ascorbic concentration: $y = 236.48(ABS) – 16.131$ ($R^2 = 0.9998$) Eq. (3)

2.5 EVALUATION OF TOXICITY AND “IN VIVO” ANTIOXIDANT PROPERTIES OF EXTRACTS IN *C. ELEGANS*

2.5.1 *C. elegans* strain and culture condition

C. elegans wild-type strain N2 (Bristol) and *E. coli* strain OP_{50} were generous gifts from Dr. Marcelo Mori (Federal University of Sao Paulo, Brazil). The stocks were maintained at 20°C on *Escherichia coli* OP_{50}/NGM (nematode growth media) plates as previously described (BRENNER, 1974). Synchronous L1 populations were obtained by isolating embryos from gravid hermaphrodites using bleaching solution according to standard procedures (SOLIS and PETRASCHECK, 2011).

2.5.2 Toxicity assessment and antioxidant activity in C. elegans

Toxicity assessment with wild-type *C. elegans* was performed with complete S-medium in 96-well cell culture plates (SOLIS and PETRASCHECK, 2011). On the day of experiments, appropriate aliquots of the aqueous extract solutions were added to S-medium containing between 15 and 20 synchronized first-stage larvae (L2). Final concentrations of the extract were added in the range of 0.78–25.0 $mg.mL^{-1}$. As a negative control experiment, nematodes were exposed to complete S-medium with water. The animals were incubated for 24 h at 25°C and the LC_{50} values, representing 50% lethality at a given concentration, were calculated. The evaluation of protective effect of the hot aqueous extract against oxidative stress was done keeping the extract concentration fixed (10 $mg.mL^{-1}$) and varying the juglone concentration (50, 100, 150, 200, 300 µM). The animals were incubated for 24 h at 25°C and the LC_{50} values, representing 50% lethality at a given concentration, were calculated.

2.6 ANTITUMOR ACTIVITY

The IMR-32 human neuroblastoma (TUMILOWICZ et al., 1970) and H295R human adrenocortical carcinoma cells (GAZDAR et al. 1990; RAINEY et al., 1994) were obtained from the cell bank of the Instituto de Pesquisa Pelé Pequeno Príncipe – Curitiba – Paraná - Brazil (IPPPP). The cells were cultived in 25 cm^2 culture flasks in Dubelcco´s Modified Eagle Medium (DMEM; Gibco). The medium was supplemented with 5% fetal bovine serum (Gibco). The cells were grown in a CO_2 (5%) incubator that was humidified at 37°C. The MTT (Thiazolyl Blue Tetrazolium Bromide) assay was performed according to the method described by Mosmann (1983). IMR-32 and H295R cells were seeded in a 96-well culture plate (10^5 cells/well) and incubated for a period of 24 h to stabilize. The cells were then treated with different hot water extract concentrations (100, 250, 500 and 1000 µg/mL) of *Cordyceps sinensis*, *Ganoderma lucidum* and *Pleurotus ostreatus.* Next, the treatment medium was discarded, and the plate was incubated at 37°C for 4 h with MTT diluted in phosphate buffered saline. The MTT was then discarded, and dimethyl sulfoxide (DMSO) was added to dissolve

the formazan. The optical density was measured at 550 nm using a spectrophotometer plate reader. The inhibition rate was calculated according to the formula below (4) (TONG et al., 2009):

Growth inhibition ratio (%) = (1- $ABS_{experimental\ groups}/ABS_{control\ group}$) x 100 Eq. (4)

Statistical analysis

All the analyses were performed in triplicate. The data were expressed as mean ± standard deviations and one-way analysis of variance (ANOVA) and Tukey or Duncan test were carried out to assess for any significant differences between the means. Differences between mean at the 5% ($p<0.05$) were considered significant.

3. RESULTS AND DISCUSSION

Biomolecules such as polysaccharides, polysaccharides-protein complexes, proteins and peptides, phenolic compounds, lipids and triterpenes from *Cordyceps sinensis* is an attractive source of natural compounds for the development of functional foods and pharmacological products because of their notable bioactivities (BISHOP et al., 2015; SHASHIDHAR et al., 2013; CORRÊA et al., 2016). One way to produce mushroom biomass and an extract with these biomolecules in large quantities, rapidly and under controlled condition is by submerged fermentation and aqueous extraction. Thus, exploring the bioactivities of a hot water mycelial extract of *Cordyceps sinensis* it was found that it promoted a resistance “in vivo” against oxidative stress in *Caenorhabditis elegans* as well as antitumor actitivity against human neuroblastoma (IMR32) and adrenocortical carcinoma (H295R) cells.

3.1 GROWTH KINETICS OF THE MUSHROOMS AND MYCELIAL EXTRACT COMPOSITION

Fig. 1 shows the growth curve of *C. sinensis* (CS) in submerged culture. Maximum production of biomass was 10.75±0.12 $g.L^{-1}$ obtained after 7 days of cultivation in the beginning of stationary growth phase for CS. After that, the analysis of residual reducing sugars showed depletion of glucose and a decline in dry weight owed to autolysis of the fungi (late stationary growth phase).

To evaluate the main chemical components as well as the antioxidant activity, mycelia obtained at two times of cultivation were collected, one at exponential phase of cultivation and another at stationary phase. Table 1 shows the chemical composition of the CS aqueous extract obtained from mycelial biomass in a submerged fermentation process. The extracts presented high amounts of carbohydrates, mostly of the non-reducing type. The evaluation of extracts showed the presence of protein and total phenolic compounds were higher at stationary phase than at exponential phase.

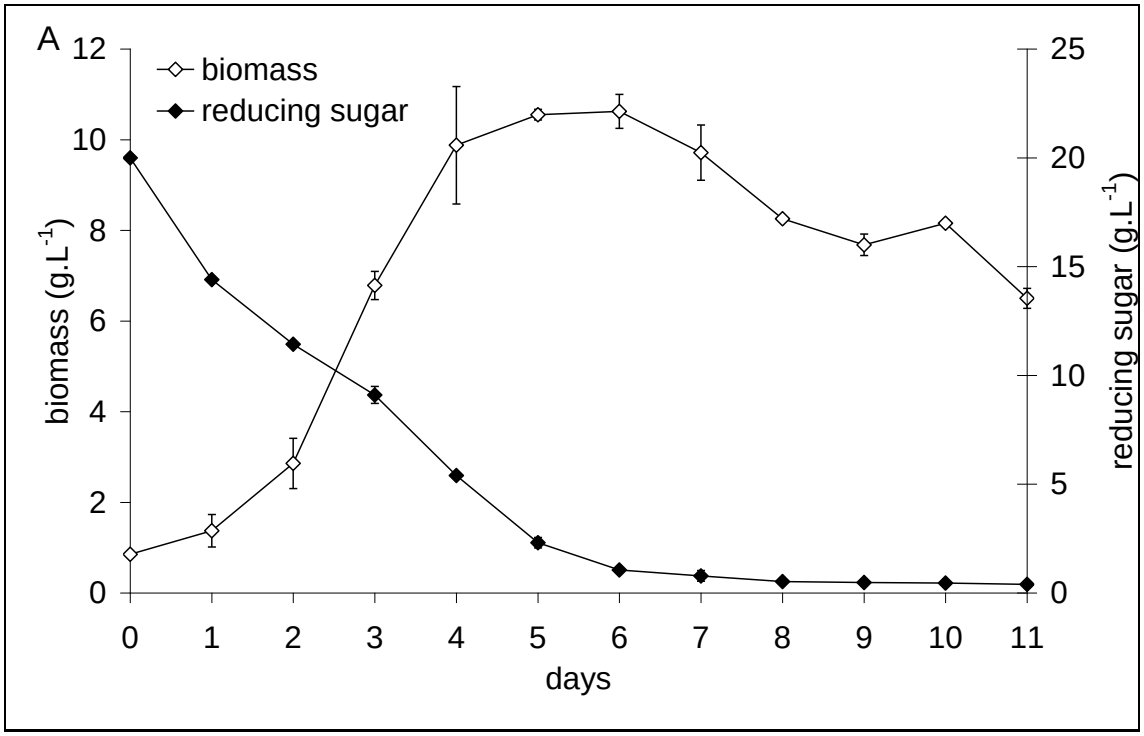


Fig. 1. Kinetics of production of mycelia and consumption of sugar by *Cordyceps sinensis* (A) at 28°C ± 2◦C on rotatory shaker at 120 rpm.

Table 1. Chemical composition of *Cordyceps sinensis* aqueous extract collected in different period of cultivation in submerged fermentation.

Day of culture	Total carbohydrates (µg/mg extract)	Reducing carbohydrates (µg/mg extract)	Non-reducing carbohydrates (µg/mg extract)	Protein (µg/mg extract)	Total phenolics (µg/mg extract)[1]
4th (CS4)	66±5	10±2	56	29±12	2.9±0.8
7 th (CS7)	111±3	15±2	96	84±4	3.7±0.4

[1] µg of gallic acid equivalent – GAE.

3.2 ANTIOXIDANT ACTIVITY AND *IN VIVO* RESISTANCE AGAINST OXIDATIVE STRESS

Antioxidant activities *in vitro* were measured and the results are summarized in Table 2. DPPH radical and ABTS radical cation assays were used for evaluating the free radical scavenging properties and the results were expressed in EC_{50}(in $mg.mL^{-1}$), which assessing the least concentration of extract capable of inhibiting 50% of radicals present in the solution. For evaluating the total antioxidant capacity the phosphomolibdenum method was used.

The EC_{50} values obtained using the ABTS assay were better than those obtained using the DPPH method for both, extracts and standards. The results obtained with the two methods are pointing to the same directions. The free radical scavenging capacity of the mycelial crude extract of stationary phase tested was higher than that of the exponential phase.

The results showed that extracts had an interesting DPPH and ABTS radical scavenging activity, however, this effect was lower than that of ascorbic acid, BHA and BHT. Among the standards, the highest EC_{50} for DPPH and ABTS scavenging activity were 15.2±0.8 and 6.0±0.1 $\mu g.mL^{-1}$, respectively, for BHA compound.

Table 2. Antioxidant activity of hot aqueous extracts from mycelial biomass of *Cordyceps sinensis* obtained by submerged fermentation.

	DPPH (EC_{50}: $mg.mL^{-1}$)	**ABTS (EC_{50}: $mg.mL^{-1}$)**	**Phosphomolybdenum ($\mu g.mg\ extract^{-1}$)**
BHA EC_{50} ($\mu g.mL^{-1}$)	15.2 ± 0.83[1]	6.0 ± 0.3	-
BHT EC_{50} ($\mu g.mL^{-1}$)	151.5 ± 5.7	10.7 ± 0.9	-
Ascorbic acid EC_{50} ($\mu g.mL^{-1}$)	21.7 ± 2.5	6.9 ± 0.2	-
CS4 ($mg.mL^{-1}$)	8.9 ± 0.3	1.7 ± 0.1	4.5 ± 0.3
CS8 ($mg.mL^{-1}$)	7.9 ±0.7	1.2 ± 0.02	5.4 ± 0.3

[1] Standard deviation of the mean.

The organism *C. elegans* was used as model to determine the toxicity of the extracts once this worm represents a suitable model for easy and fast preliminary studies of pharmaceutical compounds (DENGG and MEEL, 2004). Previous studies have indicated the possible protective effects of biomolecules on a whole organism (KAMPKÖTTER et al., 2007). In the *in vivo* assay system, we firstly was performed a safety evaluation in nematodes exposed to

mushrooms aqueous extract. No lethality was observed after worms exposure at the extract concentration range 0.78 – 25.0 $mg.mL^{-1}$ for 24 h. The animals used as control were exposed to the vehicle (sterile water) in the same conditions. This result suggests that exposure to the aqueous extract at the examined concentrations did not affect the survival and development of nematodes.

To evaluate the potential stress resistance effect of the crude hot aqueous extracts on wild-type *C. elegans* N_2 under oxidative stress, the LC_{50} (letal concentration to 50% of population) was calculated. Worms at L1/L2 stage were exposed to 5-hydroxy-1,4-naphthoquinone ("juglone," a generator of free radicals in worms) at concentration ranging at 50.0 – 300 µM and crude extracts (10 $mg.mL^{-1}$) together for 24 h. The values obtained of LC_{50} are shown in Table 3.

Table 3. Lethal concentration of juglone on wild-type *Caernohabditis elegans* N2.

	Toxicity and Oxidative stress resistance		
	LC50	**Intervalo de confiança 95% (µM)**	**TSK trim (%)**
Juglone	79.81	71.49 – 89.10	7,41
Ascorbic acid + Juglone	769.92	745.10 – 795.58	0
CS4 + Juglone	125.91	113.06 - 140.21	0
CS7 + Juglone	137.78	124.95 - 151.94	0

Juglone treatment significantly decreased the survival rate in *C. elegans* (Table 3, LC_{50} compared to the other treatments). Juglone is a pro-oxidant that can induce oxidative stress through various mechanism, such as superoxide (O2−•), hydroxyl radical (•OH) and hydrogen peroxide (H_2O_2) formation, triggering toxic effects by protein modifications, lipid oxidation and nuclear DNA damage (KAMPKOTTER et al., 2007).

Table 3 indicated that antioxidant compounds such as acid ascorbic (used as control in the experiment) and extract protection of *C. elegans* against juglone induced oxidative stress. This is very interesting because works have been shown the relationship between oxidative stress and the aging process (CASTRO et al., 2004).

Furthermore, the survival of *C. elegans* increased in 9.42% when exposed to extracts from stationary phase CS7, in relation to extracts from exponential phase CS4. These results may be explained by the fact that a lot of compounds

exhibiting antioxidant activity produced by mushrooms are secondary metabolites (ZHONG and XIAO, 2009).

The extracts antioxidant capacity is predominantly ascribed to molecules such as nonenzymatic free radical scavengers and/or also to increasing antioxidant enzymes levels such as superoxide desmutase (SOD) that scavenges toxic environmental and metabolic by-products such as reactive oxygen species (KIM et al., 2008). Results of this study suggested that hot aqueous extracts from mycelial biomass of *C. sinensis* in submerged fermentation, possesse potential stress alleviating properties which modulate oxidative stress response in *C. elegans* and increase survival.

In the evaluation of antioxidant activity, hot aqueous extract from stationary phase was better than those of the exponential phase. Then, the aqueous extract from stationary phase was selected to assess antitumor activity in human neuroblastoma (IMR32) and adrenocortical carcinoma cells (H295R).

3.3 ANTITUMOR ACTIVITY

As shown in Table 4, hot aqueous extracts from stationary phase of *C. sinensis* exhibited moderate antitumor activity ranging from 19.1 to 31% and from 19.2 to 38.4% inhibition, in different concentrations to the carcinogenic growth of IMR-32 and H295R cells, respectively.

Although there was no significant statistical difference for different concentration, the best percentual of inhibition of IMR-32 and H295R was 31.0% and 38.4% in 24 h at 500 $\mu g.mL^{-1}$, respectively.

Lee et al. (2009) reported that the maximum inhibitory effect on apoptosis of SK-N-SH neuroblastoma cells (64.6%) was observed when intrapolysaccharides (IPS) from *Cordyceps takaomontana* were added at a concentration of 2 $mg.ml^{-1}$, for 72 h. In fact, there is no report about the effect of hot water extract from *C. sinensis* mycelial biomass against IMR32, however, previous studies have demonstrated that biocompounds produced by the genus *Cordyceps*, such as polysaccharide-peptide complexes and cordycepin, also known as 3-deoxyadenosine, may induce apoptosis in neuroblastoma SK-N-SH cell line (LI et al., 2015; OH et al., 2008; YANG et al., 2012).

Baik et al. (2013) reported that cordycepin at concentrations greater than 200 µM inhibited the human neuroblastoma SK-N-BE(2)-C cell proliferation (<50%) and suggested that cordycepin induces upregulation of hST8Sia I gene expression through NF-kB activation.

The search for new compounds with antitumor capacity is a constant challenge especially for adrenocortical carcinoma (AC). AC is a rare cancer that has a poor response to cytotoxic treatment and mitotane® is the only drug approved for the treatment of adrenocortical carcinoma (FASSNACHT et al., 2012). In this study, the hot aqueous extracts of *Cordyceps sinensis* exhibited moderate antitumor activity ranging from 19.2 to 38.4% inhibition of the carcinogenic growth H295R cells (Table 4).

To the best of our knowledge, this is the first study which addressed the effects of mushroom extracts of *C. sinensis* against human adrenocortical carcinoma cells (H295R).

Together, these findings are important due to the lack of chemotherapeutic agents to some forms of malignant cancer. Mushroom products are set to create a revolution in therapeutic strategies in curbing various forms of cancers.

Table 4. The inhibition ratio of H295R and IMR32 cells by hot aqueous extracts of *Cordyceps sinensis* at different concentrations. Data were presented as mean ± SDM (N = 3).

Treatments (µg/mL)	**IMR32**	**H295R**
100	24.5 a[1] ± 3.9	19.2 a[1] ± 10.7
250	21.6 a ± 1.4	20.3 a ± 1.4
500	31.0 a ± 9.6	38.4 a ± 5.5
1000	19.1 a ± 6.9	31.8 a ± 6.9
CV(%)	**15.3**	**14.6**

[1] Treatments followed by same lower case letters in the same column do not differ significantly (P<0.05) by Duncan´s test.

4. CONCLUSION

The hot water extract of *Cordyceps sinensis* mycelial biomass produced by submerged fermentation showed beneficial *in vitro* and *in vivo* antioxidant effect and *in vitro* antitumor activities. The extracts promoted a protection against oxidative stress in *C. elegans* which resulted in a higher survival of the worm, moreover inhibited the growth of tumor cells IMR32 (neuroblastoma) and H295R (adrenocortical carcinoma), common types of cancers in babies and infants. The extracts were found to contain high amounts of carbohydrates, mostly of the non-reducing type, phenolic compounds, protein and/or polysaccharide-protein complexes, all of which probably contributed to the evaluated activities. Since the extracts were found to show an interesting anti-tumor effect, the extract purification and fractionation are in progress. These findings suggest that a water extract of the *Cordyceps sinensis* biomass could be a promising source of natural biocompounds for development of innovative functional foods and nutraceuticals which could protect the health cells against oxidative stress and prevent or combat tumor cells.

REFERENCES

1. Baik J-S, Kim K-S, Moon H-I, An H-K, Park S-J, Kim C-H, et al. Cordycepin-mediated transcriptional regulation of human GD3 synthase (hST8Sia I) in human neuroblastoma SK-N-BE (2)-C cells. gene. 2014;17:20.
2. Barros L, Cruz T, Baptista P, Estevinho LM, Ferreira IC. Wild and commercial mushrooms as source of nutrients and nutraceuticals. Food Chem Toxicol. 2008;46(8):2742-7.
3. Bertagna C, Orth DN. Clinical and laboratory findings and results of therapy in 58 patients with adrenocortical tumors admitted to a single medical center (1951 to 1978). The American journal of medicine. 1981;71(5):855-75.
4. Brand-Williams W, Cuvelier M-E, Berset C. Use of a free radical method to evaluate antioxidant activity. LWT-Food science and Technology. 1995;28(1):25-30.
5. Brenner S. The genetics of Caenorhabditis elegans. Genetics. 1974;77(1):71-94.
6. Carneiro AA, Ferreira IC, Dueñas M, Barros L, Da Silva R, Gomes E, et al. Chemical composition and antioxidant activity of dried powder formulations of Agaricus blazei and Lentinus edodes. Food chemistry. 2013;138(4):2168-73.
7. Chien R-C, Yen M-T, Tseng Y-H, Mau J-L. Chemical characteristics and anti-proliferation activities of Ganoderma tsugae polysaccharides. Carbohydrate polymers. 2015;128:90-8.
8. de Castro E, de Castro SH, Johnson TE. Isolation of long-lived mutants in Caenorhabditis elegans using selection for resistance to juglone. Free Radical Biology and Medicine. 2004;37(2):139-45.

9. Dengg M, van Meel JC. Caenorhabditis elegans as model system for rapid toxicity assessment of pharmaceutical compounds. Journal of pharmacological and toxicological methods. 2004;50(3):209-14.
10. DuBois M, Gilles KA, Hamilton JK, Rebers Pt, Smith F. Colorimetric method for determination of sugars and related substances. Analytical chemistry. 1956;28(3):350-6.
11. Durackova Z. Some current insights into oxidative stress. Physiological Research. 2010;59(4):459.
12. Fan L, Soccol AT, Pandey A, Soccol CR. Effect of nutritional and environmental conditions on the production of exo-polysaccharide of Agaricus brasiliensis by submerged fermentation and its antitumor activity. LWT-Food Science and Technology. 2007;40(1):30-5.
13. Fan L, Soccol AT, Pandey A, Soccol CR. Effect of nutritional and environmental conditions on the production of exo-polysaccharide of Agaricus brasiliensis by submerged fermentation and its antitumor activity. LWT-Food Science and Technology. 2007;40(1):30-5.
14. Fassnacht M, Terzolo M, Allolio B, Baudin E, Haak H, Berruti A, et al. Combination chemotherapy in advanced adrenocortical carcinoma. New England Journal of Medicine. 2012;366(23):2189-97.
15. Gazdar AF, Oie HK, Shackleton CH, Chen T, Triche TJ, Myers CE, et al. Establishment and characterization of a human adrenocortical carcinoma cell line that expresses multiple pathways of steroid biosynthesis. Cancer Research. 1990;50(17):5488-96.
16. Giavasis I. Bioactive fungal polysaccharides as potential functional ingredients in food and nutraceuticals. Curr Opin Biotechnol. 2014;26:162-73.
17. Hawksworth DL. Mushrooms: the extent of the unexplored potential. International Journal of Medicinal Mushrooms. 2001;3(4).
18. Jayakumar T, Thomas P, Geraldine P. In-vitro antioxidant activities of an ethanolic extract of the oyster mushroom, Pleurotus ostreatus. Innovative Food Science & Emerging Technologies. 2009;10(2):228-34.
19. Kampkötter A, Nkwonkam CG, Zurawski RF, Timpel C, Chovolou Y, Wätjen W, et al. Investigations of protective effects of the flavonoids quercetin and rutin on stress resistance in the model organism Caenorhabditis elegans. Toxicology. 2007;234(1):113-23.
20. Kampkötter A, Timpel C, Zurawski RF, Ruhl S, Chovolou Y, Proksch P, et al. Increase of stress resistance and lifespan of Caenorhabditis elegans by quercetin. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology. 2008;149(2):314-23.
21. Kobayashi H, Motoyoshi N, Itagaki T, Tabata K, Suzuki T, Inokuchi N. The inhibition of human tumor cell proliferation by RNase Pol, a member of the RNase T1 family, from Pleurotus ostreatus. Bioscience, biotechnology, and biochemistry. 2013;77(7):1486-91.
22. Kobayashi H, Motoyoshi N, Itagaki T, Tabata K, Suzuki T, Inokuchi N. The inhibition of human tumor cell proliferation by RNase Pol, a member of the RNase T1 family, from Pleurotus ostreatus. Bioscience, biotechnology, and biochemistry. 2013;77(7):1486-91.
23. Kowalczyk A, Gil M, Horwacik I, Odrowąż Ż, Kozbor D, Rokita H. The GD2-specific 14G2a monoclonal antibody induces apoptosis and enhances cytotoxicity of chemotherapeutic drugs in IMR-32 human neuroblastoma cells. Cancer letters. 2009;281(2):171-82.
24. Li WJ, Cheng XL, Liu J, Lin RC, Wang GL, Du SS, et al. Phenolic compounds

and antioxidant activities of Liriope muscari. Molecules. 2012;17(2):1797-808.
25. Li Y, Li R, Zhu S, Zhou R, Wang L, Du J, et al. Cordycepin induces apoptosis and autophagy in human neuroblastoma SK-N-SH and BE (2)-M17 cells. Oncology letters. 2015;9(6):2541-7.
26. Miller G. Modified DNS method for reducing sugars. Anal Chem. 1959;31(3):426-8.
27. MILLER NJ, RICE-EVANS C, DAVIES MJ. A new method for measuring antioxidant activity. Portland Press Limited; 1993.
28. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. Journal of immunological methods. 1983;65(1-2):55-63.
29. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. Journal of immunological methods. 1983;65(1-2):55-63.
30. Mueller GM, Schmit JP, Leacock PR, Buyck B, Cifuentes J, Desjardin DE, et al. Global diversity and distribution of macrofungi. Biodiversity and conservation. 2007;16(1):37-48.
31. Ng L, Libertino JM. Adrenocortical carcinoma: diagnosis, evaluation and treatment. The Journal of urology. 2003;169(1):5-11.
32. Oh JY, Baek YM, Kim SW, Hwang HJ, Hwang HS, Lee SH, et al. Apoptosis of human hepatocarcinoma (HepG2) and neuroblastoma (SKN-SH) cells induced by polysaccharides-peptide complexes produced by submerged mycelial culture of an entomopathogenic fungus Cordyceps sphecocephala. J Microbiol Biotechnol. 2008;18(3):512-9.
33. Patel S, Goyal A. Recent developments in mushrooms as anti-cancer therapeutics: a review. 3 Biotech. 2012;2(1):1-15.
34. Pinweha S, Wanikiat P, Sanvarinda Y, Supavilai P. The signaling cascades of Ganoderma lucidum extracts in stimulating non-amyloidogenic protein secretion in human neuroblastoma SH-SY5Y cell lines. Neuroscience letters. 2008;448(1):62-6.
35. Prieto P, Pineda M, Aguilar M. Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: specific application to the determination of vitamin E. Analytical biochemistry. 1999;269(2):337-41.
36. Raciborska A, Bilska K, Węcławek-Tompol J, Ussowicz M, Pogorzała M, Janowska J, et al. Solid Cancers in the Premature and the Newborn: Report of Three National Referral Centers. Pediatrics & Neonatology. 2015.
37. Rainey WE, Bird IM, Mason JI. The NCI-H295 cell line: a pluripotent model for human adrenocortical studies. Molecular and cellular endocrinology. 1994;100(1):45-50.
38. Schlumberger S, Kristan KČ, Ota K, Frangež R, Molgó J, Sepčić K, et al. Permeability characteristics of cell-membrane pores induced by ostreolysin A/pleurotolysin B, binary pore-forming proteins from the oyster mushroom. FEBS letters. 2014;588(1):35-40.
39. Singleton V, Rossi JA. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. American journal of Enology and Viticulture. 1965;16(3):144-58.
40. Siu K-C, Chen X, Wu J-Y. Constituents actually responsible for the antioxidant activities of crude polysaccharides isolated from mushrooms. Journal of Functional Foods. 2014;11:548-56.
41. Solis GM, Petrascheck M. Measuring Caenorhabditis elegans life span in 96 well microtiter plates. JoVE (Journal of Visualized Experiments). 2011(49):e2496-e.
42. Solis GM, Petrascheck M. Measuring Caenorhabditis elegans life span in 96 well

microtiter plates. JoVE (Journal of Visualized Experiments). 2011(49):e2496-e.
43. Tumilowicz JJ, Nichols WW, Cholon JJ, Greene AE. Definition of a continuous human cell line derived from neuroblastoma. Cancer research. 1970;30(8):2110-8.
44. XiaoPing C, Yan C, ShuiBing L, YouGuo C, JianYun L, LanPing L. Free radical scavenging of Ganoderma lucidum polysaccharides and its effect on antioxidant enzymes and immunity activities in cervical carcinoma rats. Carbohydrate Polymers. 2009;77(2):389-93.
45. Yan J-K, Wang W-Q, Wu J-Y. Recent advances in Cordyceps sinensis polysaccharides: Mycelial fermentation, isolation, structure, and bioactivities: A review. Journal of Functional Foods. 2014;6:33-47.
46. Yang C, Kao Y, Huang K, Wang C, Lin L. Cordyceps militaris and mycelial fermentation induced apoptosis and autophagy of human glioblastoma cells. Cell death & disease. 2012;3(11):e431.
47. Zhang J, Meng G, Zhai G, Yang Y, Zhao H, Jia L. Extraction, characterization and antioxidant activity of polysaccharides of spent mushroom compost of Ganoderma lucidum. International journal of biological macromolecules. 2016;82:432-9.

CHAPTER - III

ANTITUMOR ACTIVITY OF PALLADIUM NANOPARTICLES SYNTHESIZED USING POLYSACCHARIDES FROM *Cordyceps sinensis*

Antitumor activity against adrenocortical carcinoma human cell line H295R of palladium nanoparticles synthesized using polysaccharides from *Cordyceps sinensis*

L.Y. Bissoqui[1], V.O.A.Tanobe[1], L. F..A.Santos[2], A. L. L. da Silva[1], S.H.Silva[3], A. Gollo[1], S.J.R. Bonatto[3], R. Rubel[4], ·C.R. Soccol[1]*

[1]Bioprocess Engineering and Biotechnology Department, Federal University of Paraná, CEP 81531-980, Curitiba, Paraná, Brazil. *E-mail: soccol@ufpr.br

[2]Department of Pharmacy, Midwest State University of Paraná,Guarapuava, Paraná, Brazil.

[3]Instituto de Pesquisa Pelé Pequeno Príncipe, Curitiba, Paraná, Brazil.

[4] Clinical Hospital Complex, Federal University of Paraná, Curitiba, Paraná, Brazil.

Abstract

A green synthesis of palladium nanoparticles (PdNPs) using polysaccharides from *Cordyceps sinensis* (Cs) was reported in the present study. The polysaccharides were used as “green” capping and reducing agents for palladium nanoparticle synthesis (PdNPs). The characterization of the polysaccharides conjugated with PdNPs was done by UV-Visible Spectroscopy, Transmission Electron Microscopy (TEM), X-ray diffraction (XRD) and Fourier Transform Infrared Spectroscopy (FTIR). The PdNPs formation was confirmed by UV–VIS spectrophotometry and spherical shaped PdNPs with size range of 1–10 nm were observed in TEM analysis. The crystalline nature of PdNPs was confirmed by XRD pattern. Furthermore, the antitumoral activity of prepared PdNPs was evaluated for the first time on adrenocortical carcinoma human cell line H295R. Palladium nanoparticles conjugated with F2A polysaccharide fraction (F2A-PdNPs) increased the inhibition of tumor cell growth significantly ($p<0.05$) from 12 to 26%, when compared with control, which represents on average approximately an increase around 100% on the cytotoxicity of F2A polysaccharide fraction. Therefore, the results showed that polysaccharides from *C. sinensis* may be utilized as an effective non-toxic reducing and capping agent for PdNPs green synthesis and our results suggest a potential increase in the antitumoral effect of PdNPs that could be useful in biomedical applications.

Keywords: *Cordyceps sinensis;* palladium; nanotechnology; nanoparticles; green synthesis; H295R; antitumor.

1. INTRODUCTION

Palladium is a noble metal that has attracted attention due the vast possibilities and applications of palladium chemistry in the areas of biosensors (Wang et al., 2016), catalysis (Hamasaki et al., 2016) and medicine (Fanelli et al., 2016). In the last decades, the interest in nanoparticles has received considerable attention because their structure and properties differ significantly from non-nanoscale materials.

There are several nanoparticles synthesized for numerous biological applications, however the interest in noble metals such as palladium nanoparticles (PdNPs) in medicine is increasing because they may form a series of complex compounds with antitumor activities (Medici et al., 2015). Recently, PdNPs were reported as particles with antimicrobial potential (Maninkandam et al., 2016).

Green synthesis of nanoparticles is a relatively new field that involves safer, less toxic synthesis routes and energy efficiency. In this way, due their non-toxicity and water solubility many polysaccharides are recognized as “green” capping/reducing agents for nanoparticle synthesis (Duan et al., 2015). Polysaccharides have hydroxyl groups and a reducing end in the molecules that plays a key role on bioreduction of precursor salts (Mata et al., 2009).

In this context, polysaccharides from *Cordyceps sinensis*, a medicinal mushroom in Tradicional Chinese Medicine (TCM) from ancient times, could be a green and non-toxic agent to synthesize palladium nanoparticles. Moreover, polysaccharides from *C. sinensis* are bioactive molecules that exhibit several activities such as antioxidant, antitumor and immunomodulation properties (Yan et al., 2013). These polysaccharides can be extracted from cultivated mycelia (intrapolysaccharides) in submerged fermentation or from mycelial fermentation broth (exopolysaccharides).

There are few researches published on palladium nanoparticles in the medicine field compared to the great number of articles on green synthesis, sensors, technological applications and none of them using polysaccharides from *C. sinensis*.

In this study, for the first time, three fractions of polysaccharides from *C. sinensis* cultivated in submerged fermentation system were evaluated for

palladium nanoparticles green synthesis. The physical properties of these PdNPs were characterized through Ultraviolet–visible spectroscopy (UV–VIS spectra), Fourier transform infrared spectroscopy (FTIR), X-ray diffraction (XRD), and transmission electron microscopy (TEM). In addition, PdNPs were investigated for their antitumor activity against adrenocortical carcinoma human cell line H295R for the first time.

2. MATERIAL AND METHODS

2.1 MUSHROOM STRAIN AND SUBMERGED FERMENTATION

The strain of *C. sinensis* (Cs) was acquired from the collection of Bioprocesses and Biotechnology Laboratory/Federal University of Parana – Paraná-Brazil. The strain was cultivated on Potato Dextrose-Agar (PDA) plate at 25°C for 10 days and then stored at 4°C in a refrigerator. Subculture was made at each three months to maintain the strain active. All experiments were carried out using the 10-day-old mycelium to inoculate the flask medium. The inoculum was prepared with three agar blocks (1.3 cm with a self-designed cutter) in 250 mL Erlenmeyer flasks containing 50 mL of medium (pH 5.5) and incubated in shaker at 25°C ± 2°C, 120 rpm, for 7 days. The culture medium consisted of the following components ($g.L^{-1}$): glucose 20, yeast extract 3, K_2HPO_4 0.6 and $MgSO_4$ 0.3 (Dos Santos, 2013). Batch fermentation conditions of *C. sinensis* was carried out at 400 rpm, 25°C, initial pH 5.5 and an air flow rate of 1.5 vvm (vessel volumes per minute) controlled automatically in a fermenter (14-L, Bioflo, Germany) for 7 days using the same liquid culture medium composition.

2.2 ISOLATION AND FRACTIONATION OF *C. sinensis* POLYSACCHARIDES

C. sinensis polysaccharides were isolated by sequential extractions with solvents and fractioned by water solubility according to Chen et al. (2014) with modifications. In brief, mycelial biomass was freeze-dried and a sequence of extractions was made using hot water (90ºC for 4h) and hot alkali solution (90ºC for 1h, NaOH 1M) as shown in Fig.1. The supernatants of hot water soluble fraction and hot alkali soluble fraction were obtained after centrifugation (5,000g

for 10 min) and subjected to ethanol precipitation (1:3 v/v - supernatant liquid: 95% ethanol), stirred vigorously, kept at 4 °C overnight and centrifuged at 10,000g for 15 min to obtain F1 and F2 fraction.

The exopolysaccharide (EPS) crude fraction consisted of polysaccharides from the fermentation broth of *C. sinensis* which were concentrated under reduced pressure and mixed with 95% ethanol (1:3 v/v - supernatant liquid: 95% ethanol), stirred vigorously, kept at 4 °C overnight and centrifuged at 10,000g for 15 min to obtain the EPS fraction.

F1, F2 and EPS fractions were ressuspended in distilled H_2O (10 $mg.mL^{-1}$), treated with 15% trichloroacetic acid (TCA) for 1 h (1:1 v/v), dialysed (cut off 10-12 kDa) and lyophilized. Then, F1, F2 and EPS1 were subjected to further fractionation by water solubility which resulted in F1A, F2A and EPS2 fractions (soluble fractions) (i.e. supernatant) and F1B, F2B and EPS3 (insoluble fractions) (i.e. precipitate fractions). The insoluble fractions were discarded.

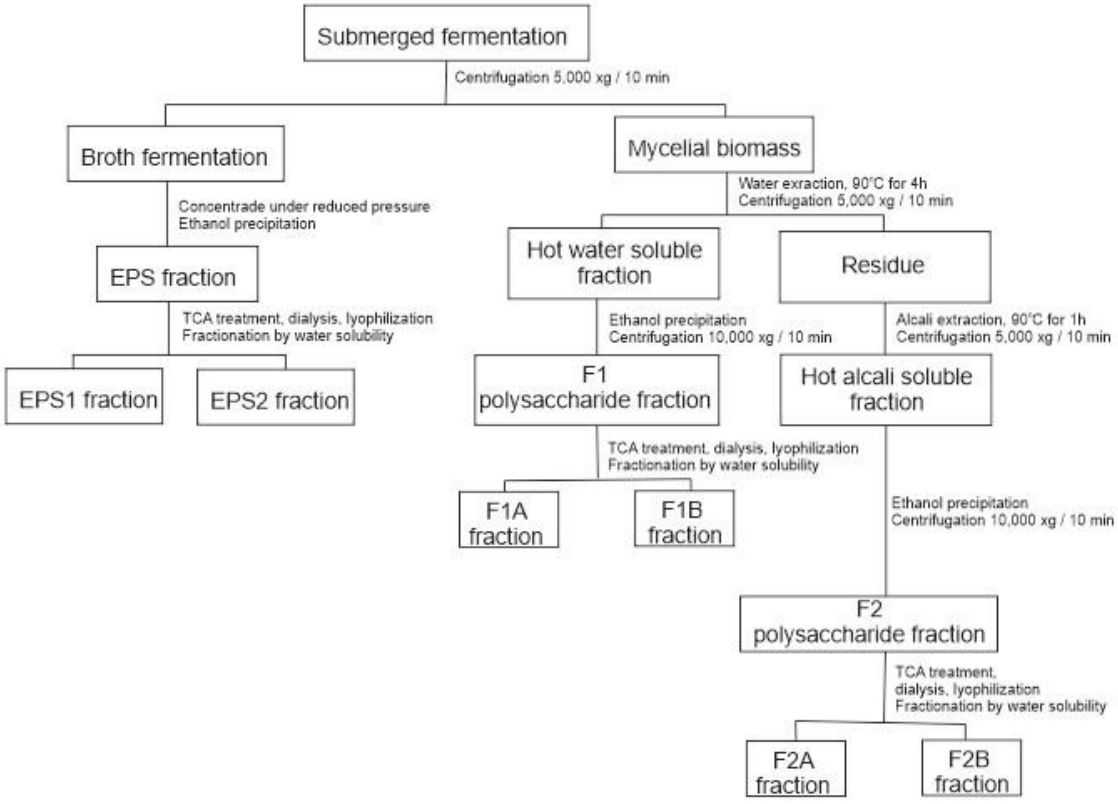


Fig. 1 Scheme of *Cordyceps sinensis* polyssacharides production, isolation and fractionation.

2.3 HIGH-PERFORMANCE SIZE EXCLUSION CHROMATOGRAPHY (HPSEC)

The polysaccharides fraction (F1A, F2A and EPS2) were solubilized (1mg.mL^{-1}) in the mobile phase containing ultra pure water and NaN_3 (0.2 g.L^{-1}). HPSEC was carried out using a Shimadzu equipment using a RI detector with isocratic elution at room temperature. The chromatographic separation was achieved with an Ultrahydrogel (Waters) 120 (7.8 x 300 mm) column with a flow rate of 0.4 mL.min^{-1}. Samples were injected manually with a Rheodyne 7725i injector (50 µL sample loop).

2.4 SYNTHESIS OF PALLADIUM NANOPARTICLES

The palladium nanoparticles were synthetized according Nadaroglu et al., (2016) and Sen et al., (2013). The source of palladium was palladium chloride ($PdCl_2$, 99.99% Sigma) in distilled water. Typical reaction mixtures contained 100 mL of polysaccharides (F1A, F2A and EPS2 fractions) 0.05 w/v in 2 mL of palladium chloride solution (1 mM) in water bath at 80 °C for 30 min. After completion of the reaction, the solution was dyalized (cut-off <10-12 kDa), freeze-dried and stored.

2.5 CHARACTERIZATION OF PALLADIUM NANOPARTICLES

The following procedures and equipment were used to characterize the nanoparticles using standard protocols:

i) UV–Visible spectroscopy analysis was carried out on a VIS1601PC spectrophotometer (Shimadzu) over wavelengths from 200 to 800 nm at a resolution of 1 nm;

ii) Transmission electron microscopy (TEM)

Images were obtained on a JEOL JEM 1200 EX-II Microscope (using CCD Gatan – Bioscan and Orius SC1000B camera) at 200 keV. Samples for TEM were prepared by placing one drop of the suspension on carbon coated copper grids and allowing water to completely evaporate.

iii) Degree of crystallinity by X-Ray Diffraction (XRD): Wide-angle X-ray scattering patterns of the samples were obtained in the reflection mode with a SHIMADZU XRD 700 MAXIMA diffractometer and Ni-filtered copper radiation (CuKα, λ = 1,5418 Å). The samples were scanned in the 2Θ angle range of 10–90°, and the generator was operated at 40 kV and 20 mA.

iv) Functional Groups by Fourier transform infrared spectroscopy (FTIR): The polymers obtained from different methods were characterized by FTIR spectroscopy in a VERTEX 70 equipment (Bruker), with the accessory DRIFTS (diffuse reflectance) with 64 scans, 4 cm^{-1} resolution, without the elimination of atmospheric compensation. The samples were previously freeze-dried, then ground and mixed to homogeneity in spectroscopic KBr and placed in DRIFTS accessories for the acquisition of the spectra.

2.6 ANTITUMORAL ACTIVITY

The test of the antiproliferative activity of tumor cells used the following media and solutions: Nutrient Mixture (DMEM) - $NaHCO_3$, antibiotic solution (Gibco®), fetal bovine serum (Gibco®); Trypsin (Sigma-Aldrich), MTT solution 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium hydrobromic, Sigma-Aldrich) and phosphate buffered saline (PBS buffer).The antitumoral activity of the extracts was tested on adrenocortical carcinoma human cell line H295R acquired from the collection of Instituto de Pesquisa Pelé Pequeno Príncipe – Paraná – Brazil. After thawing, the cells were transferred to a cell culture bottle with Nutrient Mixture culture medium (DMEM) with 10% fetal bovine serum containing antibiotic solution (10 $U.mL^{-1}$ streptomycin and 20 $U.mL^{-1}$ penicillin), followed by an incubation period of 24h at 37 °C and in a CO_2 (5%) chamber that was humidified. For the cell suspension, the cells were treated by adding 4 mL trypsin solution for 5 min, followed by the addition of 4 mL of complete culture medium, thus forming the cell suspension. The standardization of cancer cells in the ELISA plate was checked by counting in a Neubauer chamber using 180 µL of the culture medium and 1 x 10^6 cancer cells in each well. A column was reserved for

the blank containing only the culture medium and another column was reserved for the negative control (cancer cells without addition of compounds). After incubating the ELISA plate for 24 hours, 20 µL of the treatments with different concentrations were added in 180 µL of fresh culture medium. After more 24h of incubation, the treatments were removed and 100 µL MTT (3.33 $g.mL^{-1}$) was added in each well for 3 hours. After removal of the MTT 100 µL dimethyl sulfoxide (DMSO) was added. The readings occurred at 550 nm on microplate reader. All operations occurred under aseptic conditions.

Statistical analysis

All the analyses were performed in triplicates. The data were expressed as mean ± standard deviations and analyzed by analysis of variance (ANOVA) and means compared by Scott-Knott's test using the SOC software (Embrapa, 1990). Differences between mean at the 5% ($p<0.05$) were considered significant.

3. RESULTS AND DISCUSSION

3.1 SYNTHESIS AND CHARACTERIZATION OF PALLADIUM NANOPARTICLES

Polysaccharide fractions obtained from *C. sinensis* mycelial biomass by hot water and alkali extractions and from the fermented broth followed by precipitation with ethanol were analyzed by HPSEC. The analysis of F1A, F2A and EPS2 fractions showed heterogeneous elution profiles (Figure 2) and for this reason it was not possible to determine their molecular weights.

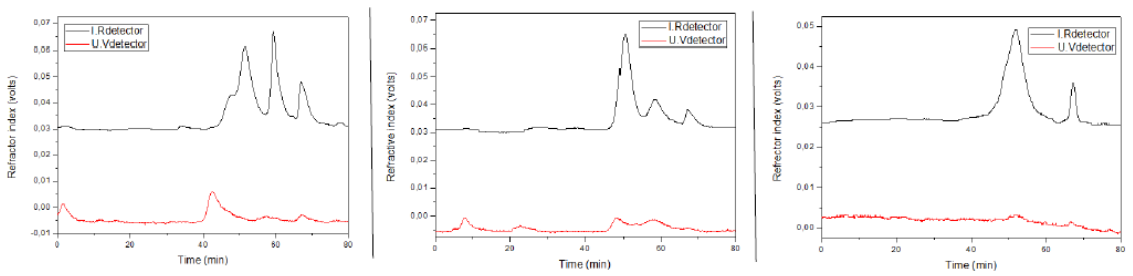


Fig 2. High-performance size exclusion chromatography elution profiles of polysaccharide fractions (A) F1A, (B) F2A and (C) EPS2 from *Cordyceps sinensis*.

The F1A, F2A and EPS2 polysaccharide fractions were used to produce palladium nanoparticles (PdNPs) after bioreduction of palladium ions in a green synthesis method. Fig. 3 shows the UV-Vis spectra of $PdCl_2$ solution and palladium colloidal suspensions in deionized water in the 350-700 nm range. When Pd ion is reduced to Pd atom the color of the solution turned brownish-yellow into dark brown, indicating the generation of PdNPs-polysaccharide conjugates. The distinct band at around 420 nm indicated the existence of Pd ion (II), the peak was completely removed after 10 min of reaction. Similar visual observation has been reported for other biological materials such as Banana peel, *Chlorella vulgaris* and *Moringa oleifera* extract (Arsiah et al., 2017; Bankar et al., 2010; Surendra et al., 2016).

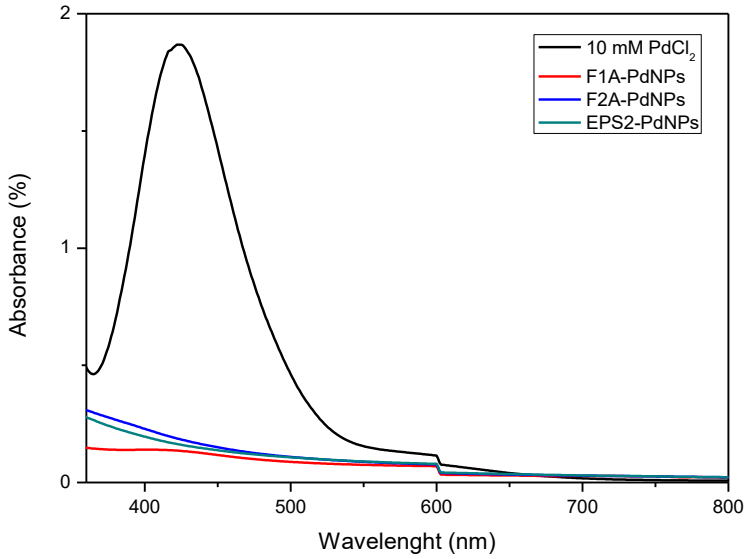


Fig 3. UV-VIS spectra of palladium nanoparticles (PdNPs) prepared with different polyssacharide fractions from *Cordyceps sinensis* at 80 °C for 30 min.

TEM images confirmed the formation of palladium nanoparticles showing the morphology and size of the palladium nanoparticles synthesized with different types of polyssacharides from *C. sinensis* (Figure 4). PdNPs were well dispersed and relatively sparse, spherical and display a regular contour mostly ranging from 2 to 10 nm in size. Arsiah et al. (2016) synthesized particles within the size range

of 5 to 20 nm using *Chlorella vulgaris* aqueous extracts. On the other hand, Maninkandan et al. (2016) synthesized PdNPs ranging from 50 to 150 nm in size using *Prunus yedoensis* tree leaf extract. The difference in size between the PdNPs may be explained among other factors by the concentration of the $PdCl_2$ solution, once higher concentration of $PdCl_2$ formed smaller PdNPs. Temperature, pH and reaction time may also influence the size of the nanoparticles (Nadaroglu et al., 2016; Yang et al., 2010).

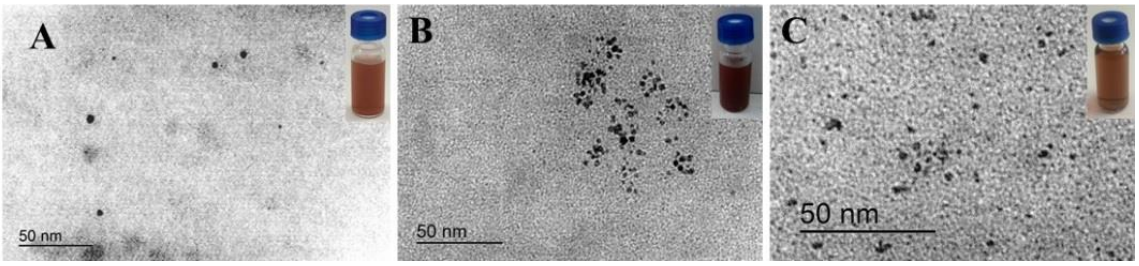


Fig 4. Transmission electron microscopy (TEM) images of palladium nanoparticles (PdNPs). (A) F1A fraction, (B) F2A fraction and (C) EPS2 fraction from *Cordyceps sinensis*.

XRD analysis of thin films of the synthesized PdNPs-polysaccharide conjugates are shown in Fig. 5. It is possible to observe a common peak for all samples at 2θ of 86.9° which was indexed as (222) Bragg reflections of a face centered cubic PdNPs planes matched with Standard JCPDS data (89-4897). This observation is consistent with previous reports confirming PdNPs formation (Shanti et al., 2015; Yang et al., 2010; Arsiah et al., 2017). However, a new intense peak appears only in EPS2-PdNPs diffraction at 2θ of 33° not has been reported this plane. Other diffraction peaks at 2θ values 40°, 46°, 68° and 82° commonly indexed as reflections arising from (111), (200), (220), (311) planes of face centered cubic lattice of PdNPs were not observed.

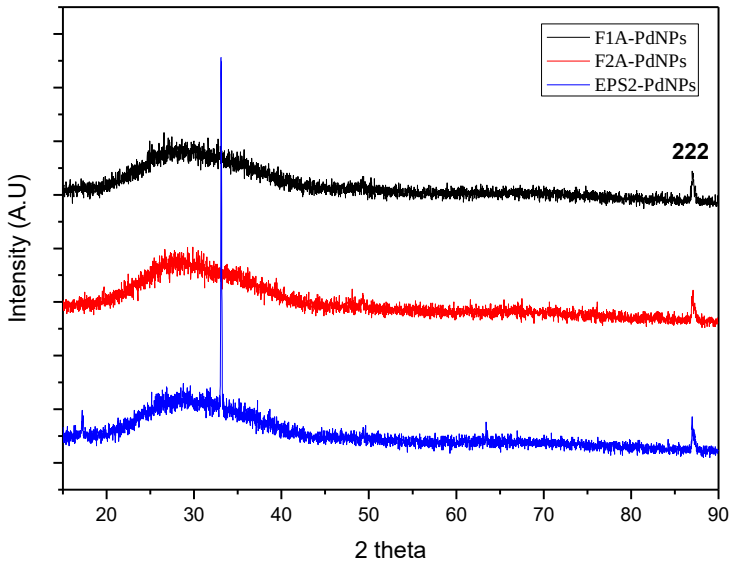


Fig 5. X-ray diffraction pattern of synthesized palladium nanoparticles of palladium nanoparticles (PdNPs). (A) F1A fraction, (B) F2A fraction and (C) EPS2 fraction from *Cordyceps sinensis*.

The FTIR spectrum of polysaccharides and PdNPs-polysaccharide conjugates are shown in Fig 6a and b, respectively. The FTIR spectrum of samples isolated from *C. sinensis* showed characteristic bands for polysaccharides, a broad band at 3000–3500 cm^{-1} is characteristic of O-H stretching frequency and at 2920, 1074 and 890 are assigned to aliphatic C-H stretching, the presence of β-linkages in the glucosidic chain and α-linked glycosyl residues of the main chain, respectivelly. In case of PdNPs-polysaccharide conjugates, the broad band at 3000 – 3500 cm^{-1} were shifts to 3400 $cm^{-1}$and became narrower and less intense after palladium nanoparticle synthesis (Fig. 6b). New bands were observed at 820 and 1384 for all PdNPs-polysaccharide fractions and F1A-PdNPs fraction presented new bands at 505 and 723 in FTIR analysis indicating a complex nature of interaction with biological material. The results indicated that polyols, carboxyl, amide and hydroxyl groups in polysaccharide fractions may have participated in the synthesis of palladium nanoparticles (Arsiah et al., 2017; Bankar et al., 2010; Manikandan et al., 2016).

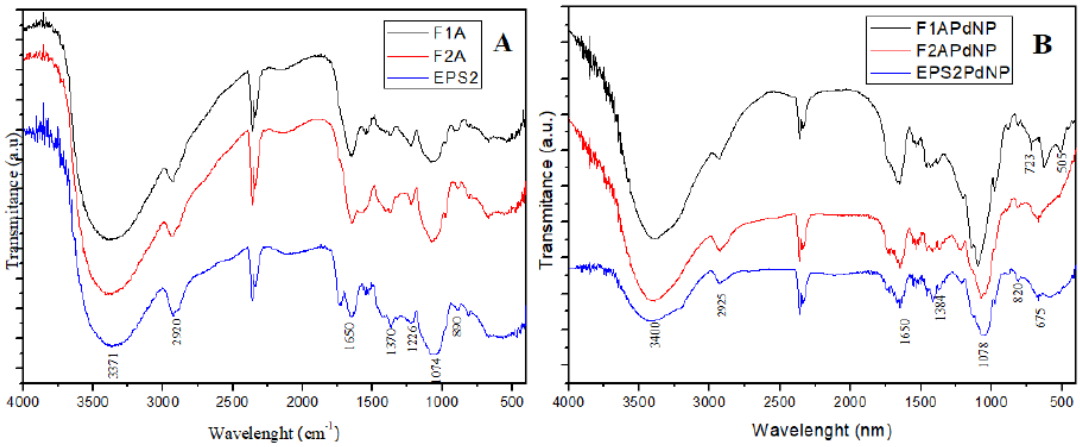


Fig 6. Fourier transform infrared (FTIR) spectra of (A) polysaccharide fractions and (B) palladium nanoparticles (PdNPs) conjugates with different polysaccharide fractions from *Cordyceps sinensis*.

3.2 ANTITUMORAL ACTIVITY USING CELL VIABILITY ASSAYS BY MTT

In vitro cytotoxicity of polysaccharide fractions (F1A, F2A and EPS2) and conjugated polysaccharides palladium nanoparticles was evaluated against human adrenocortical tumor cell line H295R at different concentrations (Figure 7). In the present study, only F2A polysaccharide fraction was able to inhibit tumor cell growth significantly ($p<0.05$) from 9.4 to 13.7% at concentrations ranging from 50 to 800µg.mL^{-1}, when compared with control, however, it is notable that the increase of concentration of the polysaccharide fractions did not affect the cell proliferation. On the other hand, palladium nanoparticles conjugated with F2A polysaccharide fraction (F2A-PdNPs) increased the inhibition of tumor cell growth significantly ($p<0.05$) from 12 to 26%, when compared with control, which represents on average approximately an increase of around 100% on the cytotoxicity of the F2A fraction.

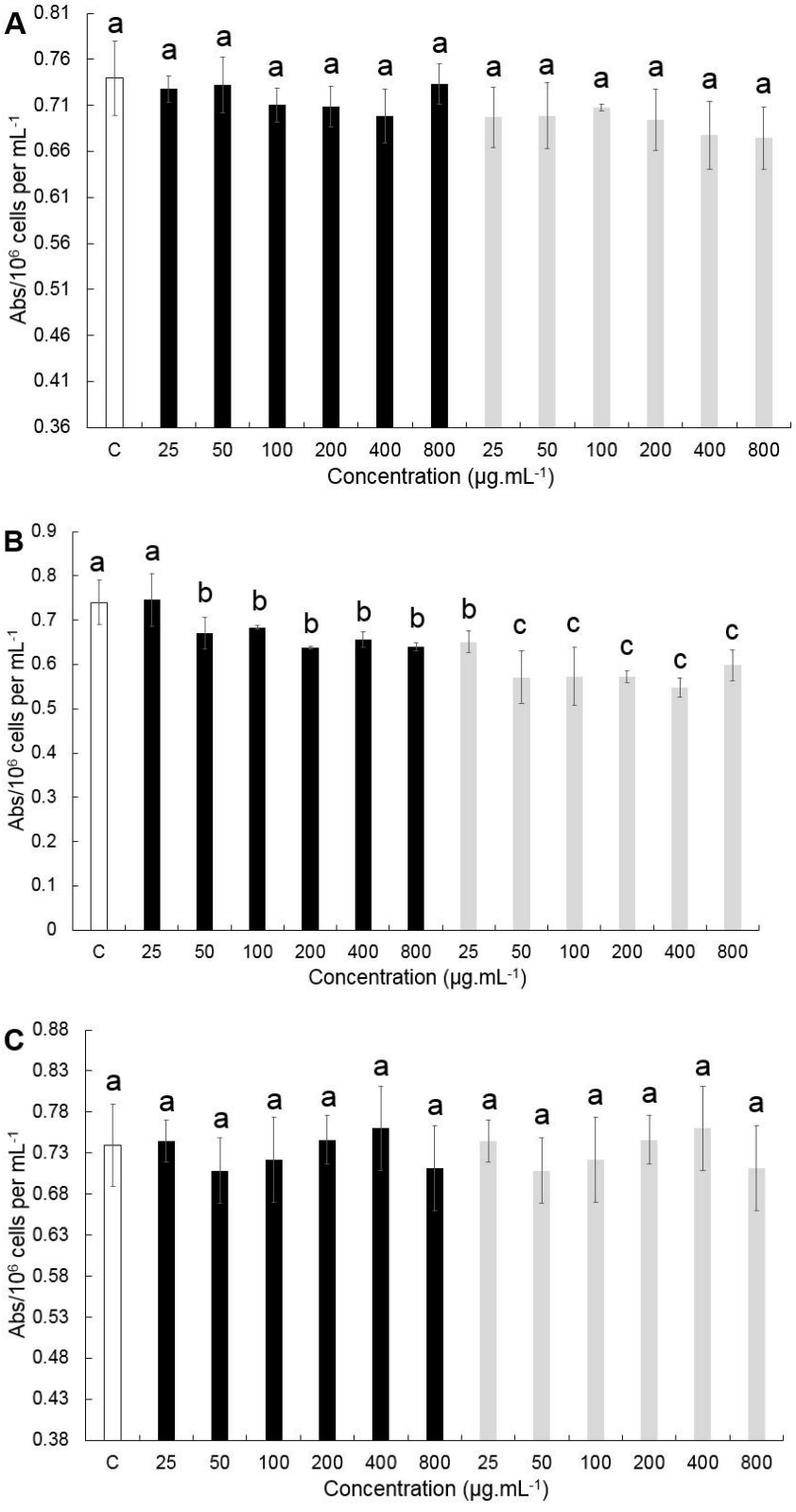


Fig 7. The effect of polysaccharide fractions (■) and conjugated polysaccharides-palladium nanoparticles (■) on viability of human adrenocortical tumor (cell line H295R) at different concentrations. (A) F1A fraction; (B) F2A fraction and (C) EPS2A fraction from *Cordyceps sinensis.* Results were presented by mean ± standard error of the mean. The means expressed

are result of three replicates. (C) Control. Bars followed by the same letter are not different at $p<0.05$ by Scott Knott's test.

In the same way, Zhang et al. (2016) reported a synergistic effect of trichostatin A (TSA), an antifungal antibiotic, and PdNPs on HeLa cell cytotoxicity. HeLa cells treated with TSA or PdNPs at a concentration of 50 nM had a 25 – 30% and 20 – 25% of decrease in cell viability compared to the untreated control after 24h, respectively. However, the viability of cells co-incubated with TSA and PdNPs decreased by 75%.

The improved cytotoxic effects of palladium nanoparticles may be by the fact that palladium nanoparticles may enter cells and mediate a variety of catalysed reactions causing damage to cellular components or promoting unnatural reactions inside the cell (Yusop et al., 2011). Clavadetscher et al. (2017) reported an *in vivo* application of cancer-targeting palladium nanoparticles that catalyzed two anticancer drugs by intracellular activation on brain cancer (glioblastoma) which enhanced the therapeutic effect of the drugs.

Shanti et al. (2015) have reported the PdNP dose-dependent cytotoxicity in HeLa cells with IC_{50} value PdNPs at 15±0.5 µg/mL in 48h. More investigations are necessary to identify the possible mechanism involved in the palladium anticancer activity.

In fact, to our knowledge, this is the first report on cytotoxic effects of green synthesized palladium nanoparticles using *C. sinensis* polysaccharides against human adrenocortical tumor cell line H295R. Furthermore, a green synthesis process may produce biocompatible PdNPs, environmentally-friendly, non-toxic and low-cost process that may be useful to improve the activity and promote new combinations to treat cancer and reduce the side effects of chemotherapy.

CONCLUSION

In the present investigation, for the first time, palladium nanoparticles were synthesized using polysaccharides from *C. sinensis* mushroom as reducing and capping agent. An inexpensive, nontoxic and rapid green synthesis method was employed. The characterization with UV–VIS spectroscopy, Fourier transmission infrared spectroscopy (FTIR), transmission electron microscopic (TEM), X-ray

diffraction (XRD) confirmed the formation of nanoparticles. Moreover, palladium nanoparticles increased around in 100% the cytotoxicity of F2A polysaccharide fraction on human adrenocortical tumor. In general, this research may contribute for the development of creative applications in medicine or pharmacology for the treatment of cancer, such as the promotion of unnatural reactions capable of modifying the chemistry within cells.

Acknowledgments

The authors would like to thank CAPES and CNPq for providing financial support. We are grateful to Electronic Microscopy Center, LORXI, Chemistry and Biochemistry departments of UFPR and Instituto Pelé Pequeno Príncipe for analysis support.

REFERENCES

ARSIYA, F.; SAYADI, M. H.; SOBHANI, S. Green synthesis of palladium nanoparticles using Chlorella vulgaris. **Materials Letters,** v. 186, p. 113-115, 2017. ISSN 0167-577X.

BANKAR, A. et al. Banana peel extract mediated novel route for the synthesis of palladium nanoparticles. **Materials Letters,** v. 64, n. 18, p. 1951-1953, 2010. ISSN 0167-577X.

CHEN, L. et al. Cell wall structure of mushroom sclerotium (Pleurotus tuber regium): Part 1. Fractionation and characterization of soluble cell wall polysaccharides. **Food Hydrocolloids,** v. 36, p. 189-195, 2014. ISSN 0268-005X.

CLAVADETSCHER, J. et al. In-Cell Dual Drug Synthesis by Cancer-Targeting Palladium Catalysts. **Angewandte Chemie,** v. 129, n. 24, p. 6968-6972, 2017. ISSN 1521-3757.

DOS SANTOS, L. F. et al. Hypolipidemic and antiatherosclerotic potential of Pleurotus ostreatus, cultived by submerged fermentation in the high-fat diet fed rats. **Biotechnology and Bioprocess Engineering: BBE,** v. 18, n. 1, p. 201, 2013. ISSN 1226-8372.

DUAN, H.; WANG, D.; LI, Y. Green chemistry for nanoparticle synthesis. **Chemical Society Reviews,** v. 44, n. 16, p. 5778-5792, 2015.

EMBRAPA. NúcleoTecnológicoNúcleo Tecnológico para Informática. SOC-Software Científico. Campinas, 1990.

FANELLI, M. et al. New trends in platinum and palladium complexes as antineoplastic agents. **Coordination Chemistry Reviews,** v. 310, p. 41-79, 2016. ISSN 0010-8545.

HAMASAKI, T.; KAKIUCHI, F.; KOCHI, T. Chain-walking Cycloisomerization of 1, n-Dienes Catalyzed by Pyridine–Oxazoline Palladium Catalysts and Its Application to Asymmetric Synthesis. **Chemistry Letters,** v. 45, n. 3, p. 297-299, 2016. ISSN 1348-0715.

MANIKANDAN, V. et al. Synthesis and antimicrobial activity of palladium nanoparticles from Prunus× yedoensis leaf extract. **Materials Letters,** v. 185, p. 335-338, 2016. ISSN 0167-577X.

MATA, Y. et al. Gold (III) biosorption and bioreduction with the brown alga Fucus vesiculosus. **Journal of hazardous materials,** v. 166, n. 2, p. 612-618, 2009. ISSN 0304-3894.

MEDICI, S. et al. Noble metals in medicine: Latest advances. **Coordination Chemistry Reviews,** v. 284, p. 329-350, 2015. ISSN 0010-8545.

NADAROGLU, H. et al. Green synthesis and characterisation of platinum nanoparticles using quail egg yolk. **Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy,** v. 172, p. 43-47, 2017. ISSN 1386-1425.

SEN, I. K. et al. Green synthesis of silver nanoparticles using glucan from mushroom and study of antibacterial activity. **International journal of biological macromolecules,** v. 62, p. 439-449, 2013. ISSN 0141-8130.

SHANTHI, K. et al. Cytotoxic Effect of Palladium Nanoparticles Synthesized From Syzygiumaromaticum Aqueous Extracts and Induction of Apoptosis in Cervical Carcinoma. **Proceedings of the National Academy of Sciences, India Section B: Biological Sciences**, p. 1-12, 2015. ISSN 0369-8211.

SURENDRA, T. et al. RSM optimized Moringa oleifera peel extract for green synthesis of M. oleifera capped palladium nanoparticles with antibacterial and hemolytic property. **Journal of Photochemistry and Photobiology B: Biology,** v. 162, p. 550-557, 2016. ISSN 1011-1344.

WANG, Q. et al. Triple-enzyme mimetic activity of nickel–palladium hollow nanoparticles and their application in colorimetric biosensing of glucose. **Chemical Communications,** v. 52, n. 31, p. 5410-5413, 2016.

YAN, J.-K.; WANG, W.-Q.; WU, J.-Y. Recent advances in Cordyceps sinensis polysaccharides: Mycelial fermentation, isolation, structure, and bioactivities: A review. **Journal of Functional Foods,** v. 6, p. 33-47, 2014. ISSN 1756-4646.

YANG, X. et al. Green synthesis of palladium nanoparticles using broth of Cinnamomum camphora leaf. **Journal of Nanoparticle Research,** v. 12, n. 5, p. 1589-1598, 2010. ISSN 1388-0764.

YUSOP, R. M. et al. Palladium-mediated intracellular chemistry. **Nature Chemistry,** v. 3, n. 3, p. 239-243, 2011. ISSN 1755-4330.

ZHANG, X.-F. et al. Trichostatin a enhances the apoptotic potential of palladium nanoparticles in human cervical cancer cells. **International journal of molecular sciences,** v. 17, n. 8, p. 1354, 2016.

CHAPTER - IV

GREEN SYNTHESIS OF SILVER NANOPARTICLES USING POLYSACCHARIDES FROM *Cordyceps sinensis* AND EVALUATION OF ITS IMMUNOMODULATORY ACTIVITY ON MACROPHAGES

Green synthesis of silver nanoparticles using polysaccharides from *Cordyceps sinensis* and evaluation of their immunomodulatory activity on macrophages

L.Y. Bissoqui[1], V.O.A Tanobe[1], L.F.A.Santos[2], A. L. L. da Silva[1], S.H.Silva[3], A. Gollo[1], S.J.R. Bonatto[3], R. Rubel[4], C.R. Soccol[1]*

[1] Bioprocess Engineering and Biotechnology Department, Federal University of Paraná, CEP 81531-980, Curitiba, Paraná, Brazil. *E-mail: soccol@ufpr.br

[2] Department of Pharmacy, Midwest State University of Paraná, Guarapuava, Paraná, Brazil.

[3] Instituto Pelé Pequeno Princípe, Curitiba, Paraná, Brazil.

[4] Clinical Hospital Complex, Federal University of Paraná, Curitiba, Paraná, Brazil.

Abstract

In this study, the green synthesis of silver nanoparticles (AgNPs) using polysaccharides from *Cordyceps sinensis*, a medicinal mushroom in Tradicional Chinese Medicine (TCM) was reported. The polysaccharides were used as “green” capping and reducing agents for silver nanoparticle synthesis (AgNPs). Characterization of the polysaccharides conjugated with AgNPs was done by UV-Visible Spectroscopy, Transmission Electron Microscopy (TEM), X-ray diffraction (XRD), Dynamic light scattering (DLS) and Fourier transform infrared spectroscopy (FTIR). Furthermore, the conjugated polysaccharides-AgNPs demonstrated immunosupressant activity in macrophages cell line RAW 264.7. A decrease effect on phagocytic, lysosomal volume and adhesion capacities was observed ($p<0.05$) which is a critical factor in determining inflammatory immune response. Therefore, the current study describes a simple, efficient, and green method of synthesis of silver nanoparticles and the results suggest a potential anti-inflamatory effect that could be applicable to diverse medical devices.

Keywords: *Cordyceps sinensis;* silver; nanotechnology; nanoparticles; immunomodulation; macrophages.

1. INTRODUCTION

Nanotechnology is one of the most active areas of modern research. The interest in nanoparticles have received considerable attention because their structure and properties differ significantly from non-nanoscale materials.

Silver nanoparticles have attracted attention due the vast possibilities and applications of silver chemistry in the areas of electronics (Zhang et al., 2017), environment (Devi and Ahmaruzzaman et al., 2017) and biomedicine (Azeez et al., 2017).

Various synthesis methods have been developed to prepare AgNPs such as physical, chemical reduction and microwave irradiation (Bae et al., 2002; Yin et al., 2004; Suber et al., 2005). However, recently, a facile and alternative way to produce nanoparticles that involves safer methods, less toxic reagents and low cost is through the use of microorganisms, biomolecules or extracts from plants, fungi and bacteria, which is called green synthesis.

In this way, due to their non-toxicity and water solubility, many polysaccharides are recognized as “green” capping/reducing agents for nanoparticle synthesis (Duan et al., 2015). Polysaccharides have hydroxyl groups and a reducing end in the molecules that plays a key role on bioreduction of precursors salts (Mata et al., 2009).

Moreover, polysaccharides from *C. sinensis*, a medicinal mushroom in Tradicional Chinese Medicine (TCM) for ancient times, could be a green and non-toxic agent to synthesize silver nanoparticles. In addition, polysaccharides from *C. sinensis* are bioactive molecules that exhibit several activities such as antioxidant, antitumor and immunomodulation properties (Yan et al., 2013). These polysaccharides can be extracted from cultivated mycelia (intrapolysaccharides) in submerged fermentation or from mycelial fermentation broth (exopolysaccharides).

There are few researches published in the literature on immunomodulatory activity of silver nanoparticles in the medicine field compared to the great number of articles on antibacterial activity. Besides that, an important activity of silver nanoparticles is the potential synergistic effect that occurs when these particles are combined with others compounds (Biasi-Garbin et al., 2015).

In this present study, three fractions of polysaccharides from *C. sinensis* cultivated in submerged fermentation system were evaluated for green synthesis of silver nanoparticles and immunomodulatory evaluation. The physical properties of the AgNPs were characterized throught ultraviolet–visible spectroscopy (UV–VIS spectra), Dynamic light scattering (DLS), Fourier transform infrared spectroscopy (FTIR), X-ray diffraction (XRD), and Transmission electron microscopy (TEM).

2. MATERIAL AND METHODS

2.1 MUSHROOM STRAIN AND SUBMERGED FERMENTATION

The strain of *Cordyceps sinensis* (Cs) was acquired from the collection of the Bioprocesses and Biotechnology Laboratory / Federal University of Parana – Paraná - Brazil. The strain was cultivated on Potato Dextrose-Agar (PDA) plate at 25°C for 10 days and then stored at 4°C in a refrigerator. Subculture was made at each three months to maintain the strain active. All experiments were carried out using the 10-day-old mycelium to inoculate the flask medium. The inoculum was prepared with three agar blocks (1.3 cm with a self-designed cutter) in 250 mL Erlenmeyer flasks containing 50 mL of medium (pH 5.5) and incubated in shaker at 25°C ± 2°C, 120 rpm, for 7 days. The culture medium consisted of the following components ($g.L^{-1}$): glucose 20, yeast extract 3, K_2HPO_4 0.6 and $MgSO_4$ 0.3 (Dos Santos, 2013). Batch fermentation of *C. sinensis* was carried out at 400 rpm, 25°C, initial pH 5.5 and an air flow rate of 1.5 vvm (vessel volumes per minute) controlled automatically in a fermenter (14-L, Bioflo, German) for 7 days using the same liquid culture medium composition.

2.2 ISOLATION AND FRACTIONATION OF *Cordyceps sinensis* POLYSACCHARIDES

Cordyceps sinensis polyssacharides were isolated by sequential extractions with solvents and fractioned by water solubility according to Chen et al. (2014) with modifications. In brief, mycelial biomass was freeze-dried and a sequence of extractions using hot water (90ºC for 4h) and hot alkali solution (90ºC

for 1h, NaOH 1M) was made as shown in Fig.1. The supernatants of hot water soluble fraction and hot alkali soluble fraction were obtained after centrifugation (5,000g for 10 min) and subjected to ethanol precipitation (1:3 v/v - supernatant liquid: 95% ethanol), stirred vigorously, kept at 4 °C overnight and centrifuged at 10,000g for 15 min to obtain the F1 and F2 fractions.

The exopolysaccharide (EPS) crude fraction consisted of polyssacharides from the fermentation broth of *C. sinensis* which was concentrated under reduced pressure and mixed with 95% ethanol (1:3 v/v - supernatant liquid: 95% ethanol), stirred vigorously, kept at 4 °C overnight and centrifuged at 10,000g for 15 min to obtain the EPS fraction.

F1, F2 and EPS fractions were ressuspended in distilled H_2O (10 $mg.mL^{-1}$), treated with 15% trichloroacetic acid (TCA) for 1 h (1:1 v/v), dialysed (cut off 10 kDa) and lyophilized. Then, F1, F2 and EPS1 were subjected to further fractionation by water solubility which resulted in F1A, F2A and EPS2 fractions (soluble fractions) (i.e. supernatant) and F1B, F2B and EPS3 (insoluble fractions) (i.e. precipitate fractions). The insoluble fractions were discarded.

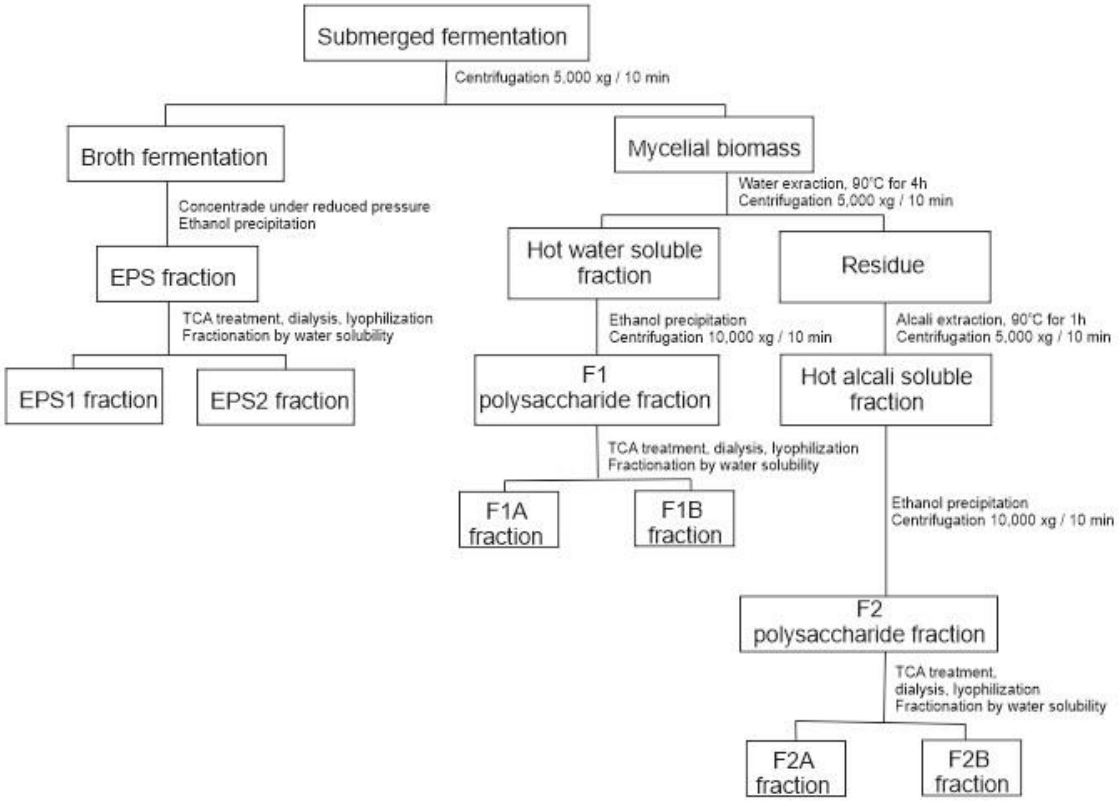


Fig. 1 Scheme of *Cordyceps sinensis* polysaccharides production, isolation and fractionation.

2.3 HIGH-PERFORMANCE SIZE EXCLUSION CHROMATOGRAPHY (HPSEC)

The polysaccharide fractions (F1A, F2A and EPS2) were solubilized (1 $mg.mL^{-1}$) in the mobile phase containing ultrapure water and NaN_3 (0.2 $g.L^{-1}$). HPSEC was carried out using a Shimadzu equipment using a RI detector with isocratic elution at room temperature. The chromatographic separation was achieved with an Ultrahydrogel (Waters) 120 (7.8 x 300 mm) column with a flow rate of 0.4 $mL.min^{-1}$. Samples were injected manually with a Rheodyne 7725i injector (50 µL sample loop).

2.4 MORPHOLOGY BY SCANNING ELECTRONIC MICROSCOPY (SEM)

The morphology of the polysaccharide fractions was examined by scanning electronic microscopy (SEM) Teskan mod Vega 3LMO (Oxford Instruments), operating at 15 keV. Before examination, the samples were sputter-coated with a thin layer of gold in a vacuum chamber

2.5 SYNTHESIS OF SILVER NANOPARTICLES

The synthesis of AgNPs with polyssacharides from *C. sinensis* followed a reported procedure according Chen et al. (2016). The $AgNO_3$ solution (1 mM) was mixed with an equal volume of polyssacharides (F1A, F2A and EPS2) at 1.0 $mg.mL^{-1}$. The reaction mixture was incubated with constant stirring for 120 min at 100°C. After completion of the reaction, the solution was dyalized (cut-off <10-12 kDa), freeze-dried and stored.

2.6 CHARACTERIZATION OF SILVER NANOPARTICLES

The following procedures and equipment were used to characterize the nanoparticles using standard protocols:

i) UV–Visible spectroscopy analysis was carried out using a VIS1601PC spectrophotometer (Shimadzu) over wavelengths from 200 to 800 nm at a resolution of 1 nm.

ii) Transmission electron microscopy (TEM)

Images were obtained on a JEOL JEM 1200 EX-II Microscope (using CCD Gatan – Bioscan and Orius SC1000B camera) at 200 keV. Samples for TEM were prepared by placing one drop of the suspension on carbon coated copper grids and allowing water to completely evaporate.

iii) Degree of crystallinity by X-Ray Diffraction (XRD): Wide-angle X-ray scattering patterns of the samples were obtained in the reflection mode with a SHIMADZU XRD 700 MAXIMA diffractometer and Ni-filtered copper radiation (CuKα, λ = 1,5418 Å). The samples were scanned in the 2° range of 10–90°, and the generator was operated at 40 kV and 20 mA.

iv) Functional Groups by Fourier transform infrared spectroscopy (FTIR): The polymers obtained from different methods were characterized by FTIR spectroscopy in a VERTEX 70 equipment (Bruker), with the accessory DRIFTS (diffuse reflectance) with 64 scans, 4 cm^{-1} resolution, without the elimination of atmospheric compensation. The samples were previously freeze-dried, then ground and mixed to homogeneity in spectroscopic KBr and placed in DRIFTS accessories for the acquisition of the spectra.

v) Dynamic light scattering (DLS)

The diameters of the nanoparticles were analyzed by dynamic light scattering (DLS) on a NanoDLS (Brookhaven Instruments, Holtsville, NY, USA). Each sample was dispersed in water and poured into cuvette with 4 mL.

2.7 IMMUNOMODULATORY ACTIVITY

Macrophages activity was analyzed using phagocytic and adhesion capacity, lysosomal volume and ion production (superoxide anion, hydrogen

peroxide) of the macrophage cell line RAW 264.7. Macrophages cell solution containing 1 x 10^6 cel.mL^{-1} was counted using trypan blue solution (1%). Then the macrophages were resuspended in Phosphate-buffered saline (PBS) (pH = 7.4) or for culture in DMEN medium (Sigma, St. Louis, MO, USA), supplemented with 10% fetal bovine serum containing antibiotic solution (10 U.mL^{-1} streptomycin and 20 U.mL^{-1} penicillin). For the assays, 100 µL of macrophages solution was added to the 96-well plates (Biofil®) and were exposed and divided into the following experimental groups: control (C) representing cells without treatment, polysaccharide fractions (F1A, F2A and EPS2) and the conjugated polysaccharides-AgNPs (F1A-AgNPs, F2A-AgNPs, EPS2-AgNPs) at concentrations 25, 50, 100, 200, 400 and 800 µg.mL^{-1}.

2.7.1 Phagocytic capacity

Aliquots of peritoneal macrophages (1 x 10^6 cells.mL^{-1}) were added to the wells of a 96-well flat bottomed culture plate (Biofil®) and phacocytic capacity was evaluated according Piper et al. (1995) modified by Bonatto et al. (2004). 10 µL of neutral-red stained zymosan (10^8 particles.mL^{-1}) was added to each well. After the incubation (37 °C for 30 minutes) the macrophages were fixed with Baker formol-calcium (4% formaldehyde, 2% sodium chloride and 1% calcium acetate) for 30 minutes. The absorbance of each well was read on a plate reader at 595 nm and the results were expressed as absorbance (per 1 x 10^6 cells.mL^{-1}). Zymosan activates the TOLL-like receptors of these cells, causing the internalization of the reagent particles, shown by the different absorbance values with the presence of the dye in the solution (Underhill, 2003).

2.7.2 Adhesion capacity of macrophages

Adhesion capacity of macrophages was evaluated as described by Rosen and Gordon (1987) with modifications. Cells adhered to the plate were fixed with 50% methanol for 10 minutes and after the supernatant was discarded. Then 0.2% Giemsa solution was added and the plate was incubated for 40 minutes at room temperature. Then the wells were carefully washed three times with PBS

and the dye adhered to the cells was solubilized with 50% methanol for 30 minutes. The absorbance was read at 595nm and adhesion capacity was expressed as absorbance (per 1 x 10^6 cells.mL^{-1}).

2.7.3 Lysosomal volume

The uptake of the cationic dye neutral red, which concentrates in macrophages, was used to assess the volume of the macrophages according Pipe *et al.* (1995) modified by Bonatto et al. (2004). In each well 20 µL of 2% neutral red was added to 100 µL of macrophages per microplate well and incubated for 30 min. After discarding the supernatant, neutral red was solubilized adding 100 µL of extraction solution (0.1 mL of 10% acetic acid plus 40% ethanol solution). The absorbance was read at 595 nm, and lysosomal volume was expressed as absorbance (per 1 x 10^6 cells.mL^{-1}).

2.7.4 Superoxide anion production ($O_2^{\bullet -}$)

Superoxide anion production was estimated by the nitro blue tetrazolium (NBT – Sigma) reduction assay according Choi et al. (2006). Macrophages (100 µL) were incubated at 37ºC in the presence of 0.2% NBT and Zimozan. After 30 minutes the supernatant was discarded, and the macrophages were fixed by adding 100 µL of methanol (50%) for 10 min. After, the supernatant was discarded, and the plate was dried. Then 120 µL of KOH (2M) and 140 µL of dimethyl sulfoxide were added to the wells. After 30 min the reduction of NBT resulted in the formation of blue formazan. The absorbance was read at 595 nm and the results were expressed as absorbance (per 1 x 10^6 cells.mL^{-1}).

2.7.5 Hydrogen peroxide production (H_2O_2)

Hydrogen peroxide production was based on the horseradish peroxidase-dependent conversion of phenol red into a colored compound by H_2O_2 (Pick and Keisari, 1980). Macrophages (100 µL) were incubated in the presence of glucose

(5 mM), phenol red solution (0.56 mM), and horseradish peroxidase (8.5 $U.mL^{-1}$) in the dark for 30 min at 37ºC. Then, 10µL of 1M NaOH was added to the wells to stop the reaction. The absorbance was read at 620 nm and the results were expressed as absorbance (per 1 x 10^6 $cells.mL^{-1}$).

Statistical analysis

All the analyses were performed in triplicate. The data were expressed as mean ± standard deviations and analyzed by analysis of variance (ANOVA) and means compared by Scott-Knott's test using the SOC software (Embrapa, 1990). Differences between means at the 5% of confidence interval ($p<0.05$) were considered significant.

3. RESULTS AND DISCUSSION

3.1 POLYSACCHARIDE PRODUCTION AND CHARACTERIZATION

Polysaccharide fractions obtained from *C. sinensis* mycelial biomass by hot water and alkali extractions and fro the fermented broth followed by precipitation with ethanol were analyzed by SEM and HPSEC. SEM images shows the morphology of the fractions and HPSEC analysis revealed heterogeneous elution profiles (Figure 2) and for this reason, it was not possible to determine their molecular weights.

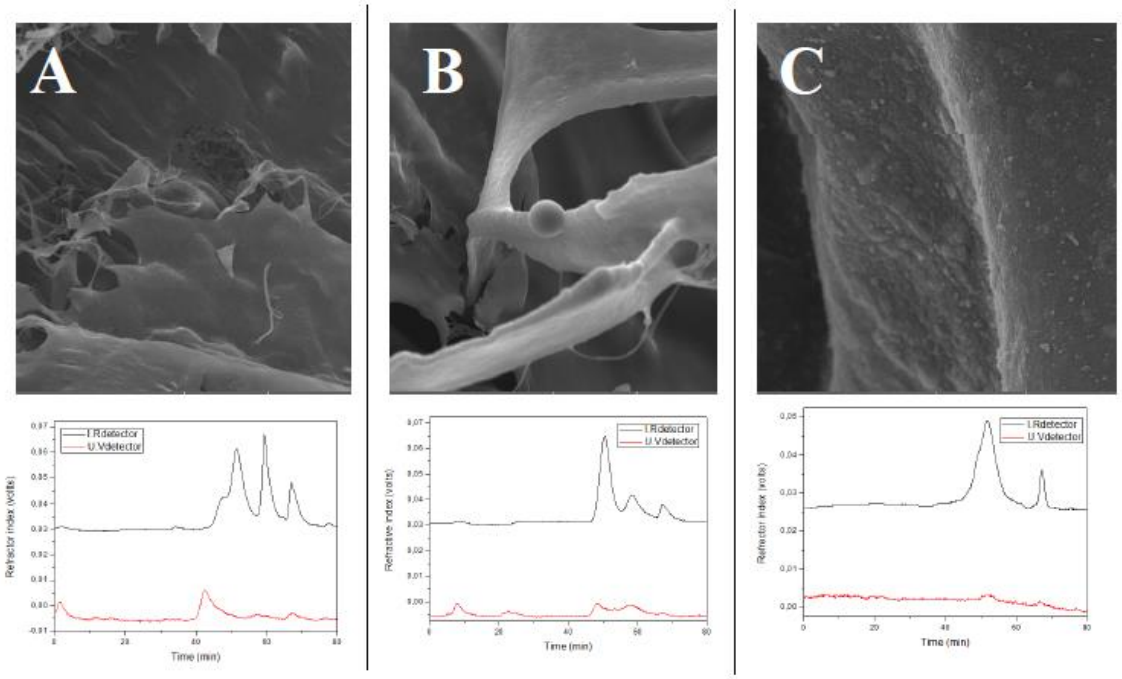


Fig 2. Scanning electron microscopy (SEM) image at 7000x magnification of morphology and structure of polysaccharide fractions (A) F1A, (B) F2A and (C) EPS2 from *Cordyceps sinensis* and their high-performance size exclusion chromatography (HPSEC) elution patterns.

3.2 SYNTHESIS AND CHARACTERIZATION OF POLYSACCHARIDES-CONJUGATED WITH AgNPs

Polysaccharides isolated from *C. sinensis* were used for the shynthesis of AgNPs-polysaccharides conjugates and the prepared solution showed an ultraviolet-absorption spectrum at 450 nm, which is a typical absorption band of AgNPs. Figure 3 shows the UV-Vis spectra of $AgNO_3$ solution after bioreduction by F1A, F2A and EPS2 polysaccharide fractions solution in ultrapure water in the 300-800 nm range. When the Ag ion is reduced to Ag atom in a solution, the colorless solution is turned into yellowish brown. Similar observation has been reported for other biological materials such as *Carissa carandas* fruits and starch (Anupama and Madhumitha, 2016; Mohanty et al., 2012).

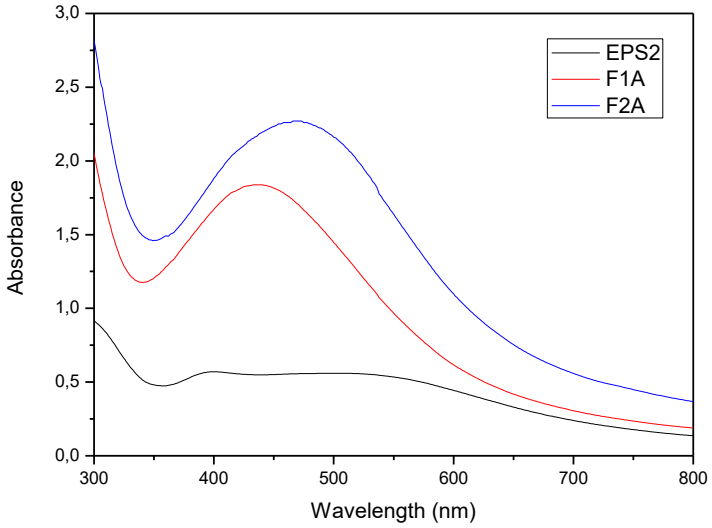


Fig 3. UV-VIS spectra of AgNPs prepared with different polysaccharide fractions from *Cordyceps sinensis* at 100 °C for 120 min.

Fig. 4 shows the TEM images of the morphology and size of the silver nanoparticles synthesized with different types of polyssacharide fractions from *C. sinensis*. AgNPs were well dispersed and relatively sparse, spherical and display a regular contour.

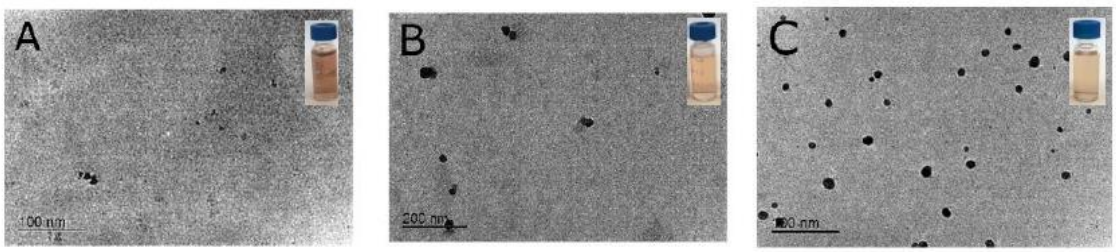


Fig 4. Transmission electron microscope (TEM) images at 50000x magnification of AgNPs conjugates with (A) F1A, (B) F2A and (C) EPS2A polysaccharide fractions from *Cordyceps sinensis*.

The distribution of size of the silver nanoparticles by DLS is shown in Fig. 5. The size of the nanoparticles ranged from 80 to 130 nm, 55 to 130 and 60 to 115 nm, respectively, for F1A, F2A and EPS2 polysaccharide fractions, suggesting the influence of the type and characteristic of the polysaccharides involved in the process of particle formation.

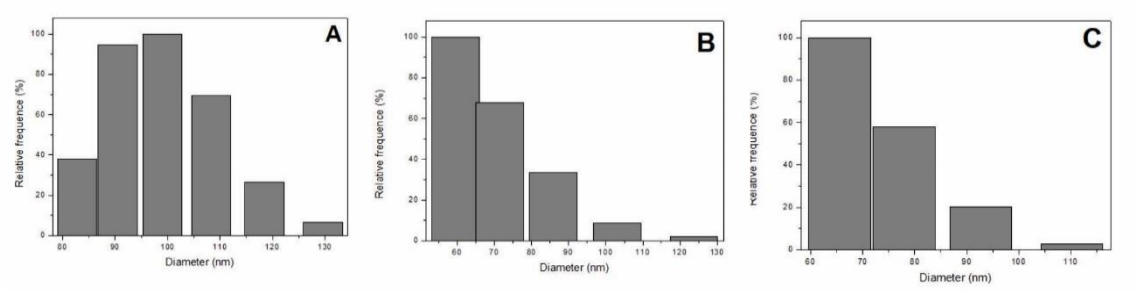


Fig 5. Distribution of silver nanoparticles determined by dynamic light scattering (DLS) at 90° using different polysaccharide fractions: (A) fraction F1A; (B) fraction F2A and (C) fraction EPS2 from *Cordyceps sinensis*.

XRD analysis of thin films of the synthesized AgNPs-polysaccharide conjugates are shown in Fig. 6. The three peaks at 2Θ values of 38.00, 44.14 and 80.00 degree corresponded to (111), (200) and (222) planes of silver standard powder diffractions card of Joint Committee on Powder Diffractions Standards (JCPDS), silver file N° 04-0783. The XRD study confirms the crystalline nature and indicates that the resultant particles are silver nanoparticles. This result is consistent with previous reports confirming AgNPs formation (Ma et al., 2017; Sen et al., 2013; Wang L. et al., 2017). However new peaks appeared at 17.80 and 32.15 in F1A-AgNPs and F2A-AgNPs and at 24.00 and 57.00 in EPS2-AgNPs.

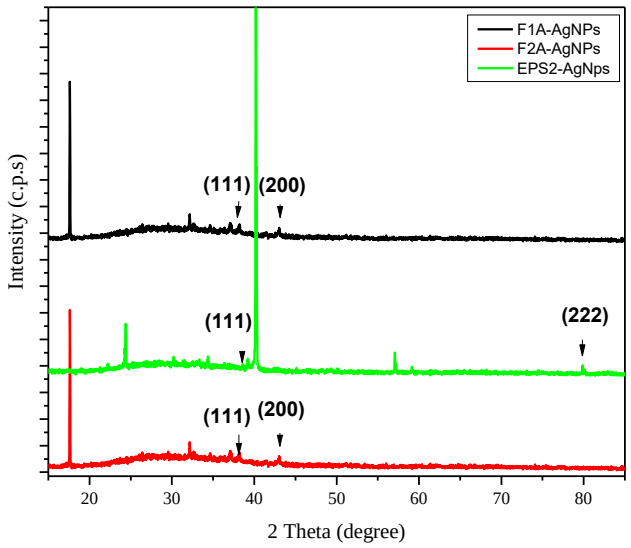


Fig 6. X-ray diffraction pattern of synthesized silver nanoparticles in different polysaccharide fractions conjugated with AgNPs (FA1, FA2 and EPSA2).

The FTIR spectrum of polysaccharides and AgNPs-polysaccharides conjugates are shown in Fig 7A and B, respectively. The FTIR spectrum of samples isolated from *C. sinensis* showed characteristic bands for polysaccharides, a broad band at 3000–3500 cm^{-1} is characteristic of O-H stretching frequency and the bands at 2920, 1074 and 890 are assigned to aliphatic C-H stretching, the presence of β-linkages in the glucosidic chain and α-linked glycosyl residues of the main chain, respectively. In case of AgNPs-polysaccharides conjugates the broad band at 3000 – 3500 cm^{-1} were shifts to 3440 cm^{-1} (Fig. 7B). The C=N absorption band at 1386 cm^{-1} overlaps with the residual NO_3^-, and the broad band at 990–1100 cm^{-1} in conjugated polysaccharides-AgNPs represents the C-OH bending vibrations due to proteins (Ghaseminezhad et al., 2012). New bands were observed at 530 and 600 for F1A-AgNPs and at 550 for F2A-AgNPs in FTIR analysis indicating a complex nature of interaction with biological material. The FTIR spectrum reveals that the stabilization of AgNPs could be due to the O-H, C-H, and C-O functional groups of polysaccharide fractions (F1A, F2A and EPS2).

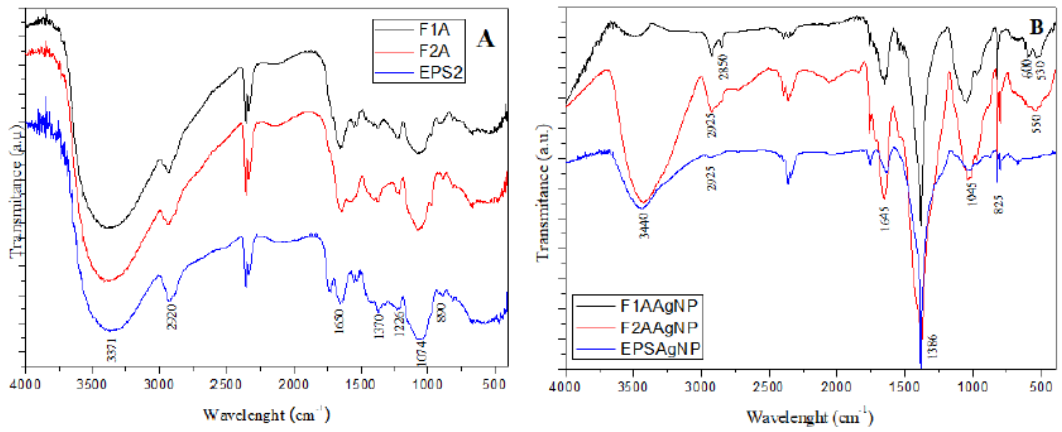
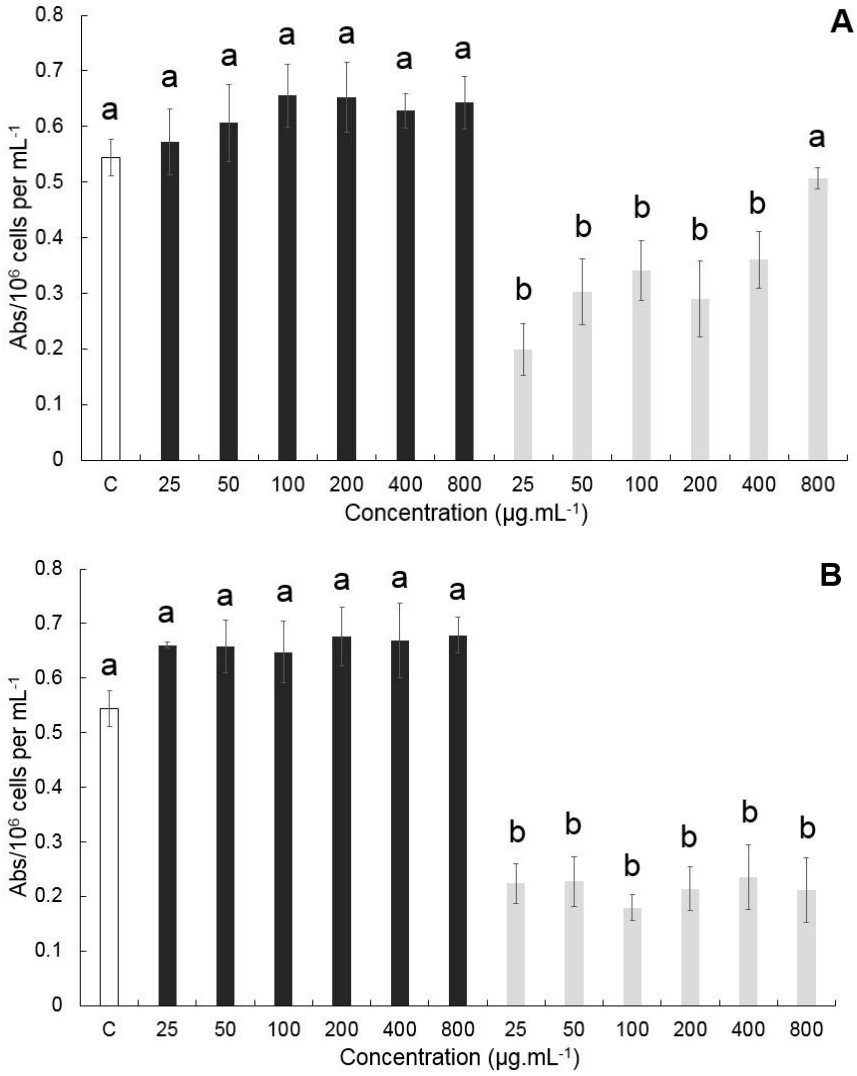


Fig 7. Fourier transform infrared (FTIR) spectra of (A) polysaccharide fractions and (B) AgNPs-polysaccharides from *Cordyceps sinensis* conjugates.

3.3 IMMUNOMODULATORY ACTIVITY

Macrophages were assessed by metabolic activity (Hydrogen peroxide and superoxide anion) and morphological indicators, as lysosomal volume, phagocytic and adhesion capacity (Fig 8-12).

The nanoparticles internalization by phagocytic cells occurs due to different mechanisms that may be divided into phagocytosis (larger than 100 nm), pinocytosis (smaller than 100 nm) and receptor-mediated endocytosis (particle coated with a specific functional group) (Yen et al., 2009; Zhao et al., 2011). The phagocytosis analysis of the macrophages had no significant differences for F1A, F2A and EPS2 fractions tested (Figure 8). However, in the presence of the silver nanoparticles the phagocytosis showed a significant reduction ($p<0.05$) for all fraction ranging from 31 to 65 %.



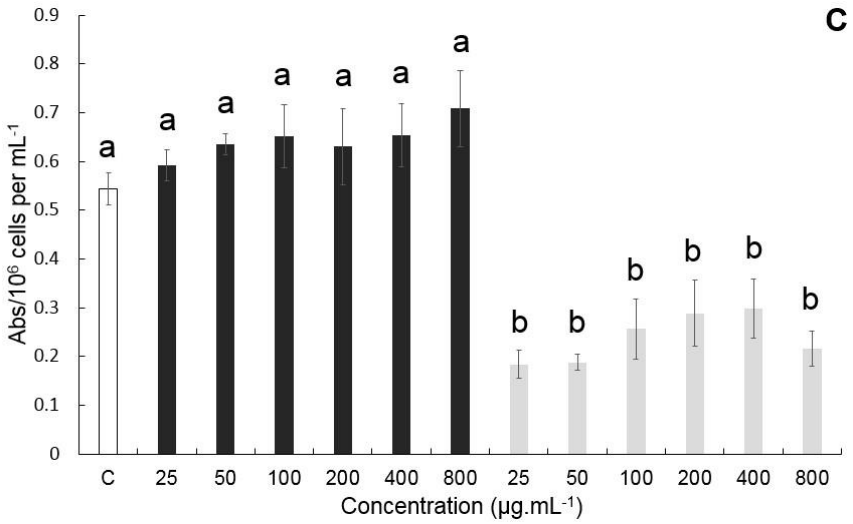


Fig 8. Phagocytosis activity analysis of macrophages (cell line RAW 267) treated with polysaccharide fractions (■) and polysaccharides-AgNPs conjugates (▒). (A) F1A fraction; (B) F2A fraction and (C) EPS2A fraction from *Cordyceps sinensis*. Results were presented by mean ± standard error of the mean. The means expressed are results of three replicates. (C) Control. Bars followed by the same letter are not different at $p<0.05$ by Scott Knott's test.

Lysossomal retention analysis showed that neither F1A, F2A and EPS2 polysaccharide fractions were different from the control ($p>0.05$) as shown in Figure 9. On the other hand polysaccharides-AgNPs conjugates exhibited a significant reduction ranging from 32 to 62% in the lysosomal retention ($p<0.05$).

Current studies have found that AgNPs were found in lysosomes as agglomerates and high levels of Ag ions are released in the presence of reactive oxygen species (ROS) which may explain the toxicity mechanism on mammalian cells, such as mouse macrophages cells (Arai et al., 2015; Hsiao et al., 2015).

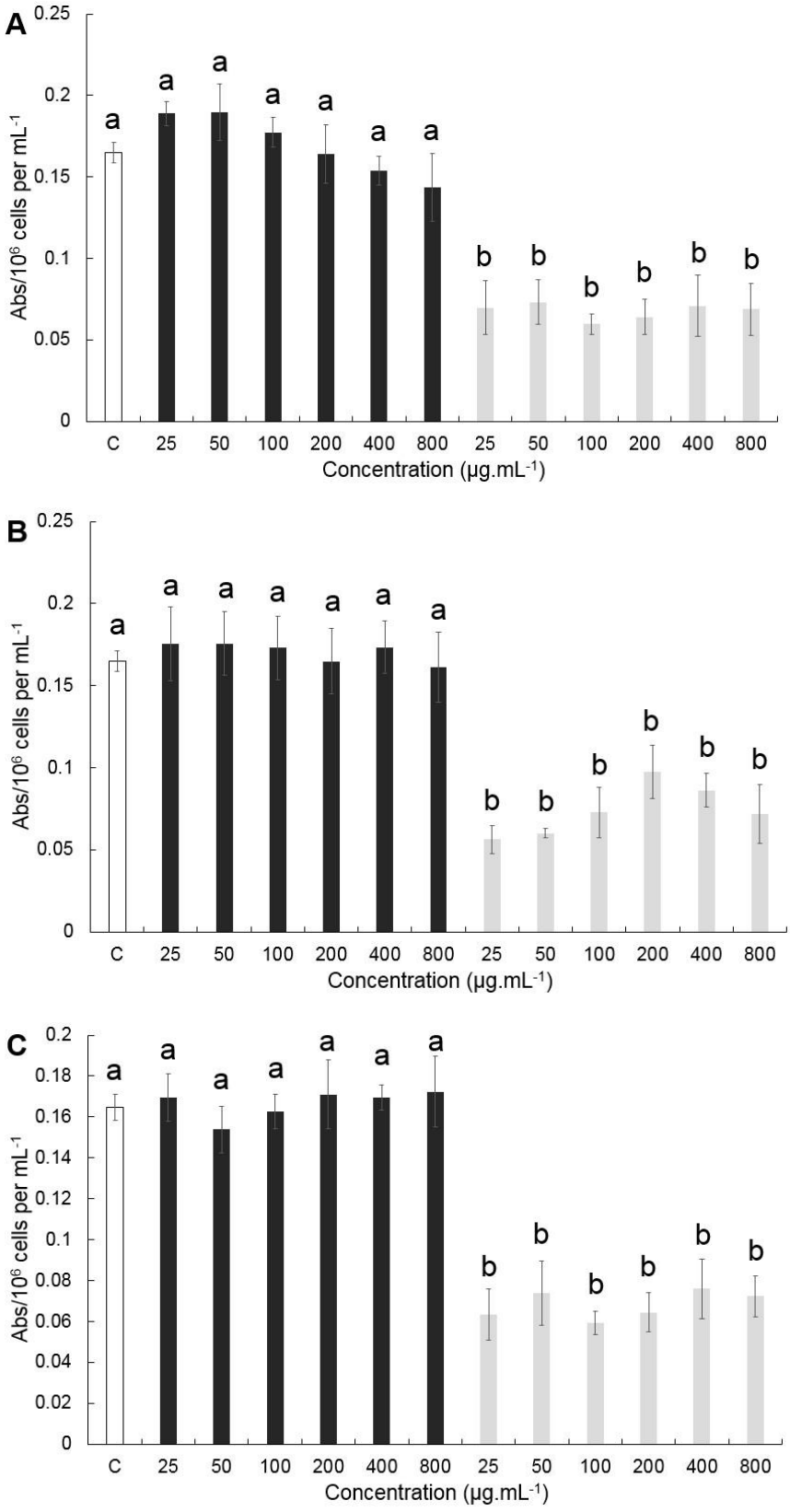


Fig 9. Lysossomal retention of macrophages (cell line RAW 267) treated with polysaccharide fractions (■) and polysaccharides-AgNPs conjugates (■). (A) F1A fraction; (B) F2A fraction and (C) EPS2A fraction from *Cordyceps sinensis*. Results were presented by mean ± standard error

of the mean. The means expressed are result of three replicates. (C) Control. Bars followed by the same letter are not different at $p<0.05$ by Scott Knott's test.

Figure 10 shows the macrophages adhesion activity when treated with polysaccharide fractions and polysaccharides-AgNPs conjugates. Only F1A was not significant ($p>0.05$) at any concentration, while F2A and EPS2 were different from the control ($p<0.005$) at any concentration and in concentration of 400 and 800 $\mu g.mL^{-1}$, respectively. On the other hand polysaccharides-AgNPs conjugates exhibited a significant reduction ranging from 18 to 45% in the macrophages adhesion activity ($p<0.05$).

Macrophages are an important source of inflammatory cytokines that induce the expression of endothelial selectins, vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) on the endothelium that bind β1and β2 integrins expressed on leukocytes. The absence of IL6 in macrophages reduced significantly the induction of VCAM-1 and P-selectin expression (Zhang et al., 2011). Yen et al. (2009) reported that interleukin-1 (IL-1), interleukin-6 (IL-6) and tumor necrosis factor (TNF-α) pro-inflammatory gene expression were down-regulated for long term AgNPs exposure (24 to 72h) in J774 A1 macrophages for different sizes (2 to 4, 5 to 7 and 20 to 40 nm) and concentration (1 and 10 $\mu g.mL^{-1}$) which may explain the reduction in the adhesion capacity of macrophages in our results.

Previous studies have demonstrated that silver nanoparticles triggered macrophages to release interleukin-8 (IL-8), an important pro-inflammatory cytokine that stimulates the migration of immune cells and expression of adhesion molecules by endothelial cells, however this influence may be related to size, concentration and cell type (Bachand et al., 2012; Park et al., 2011).

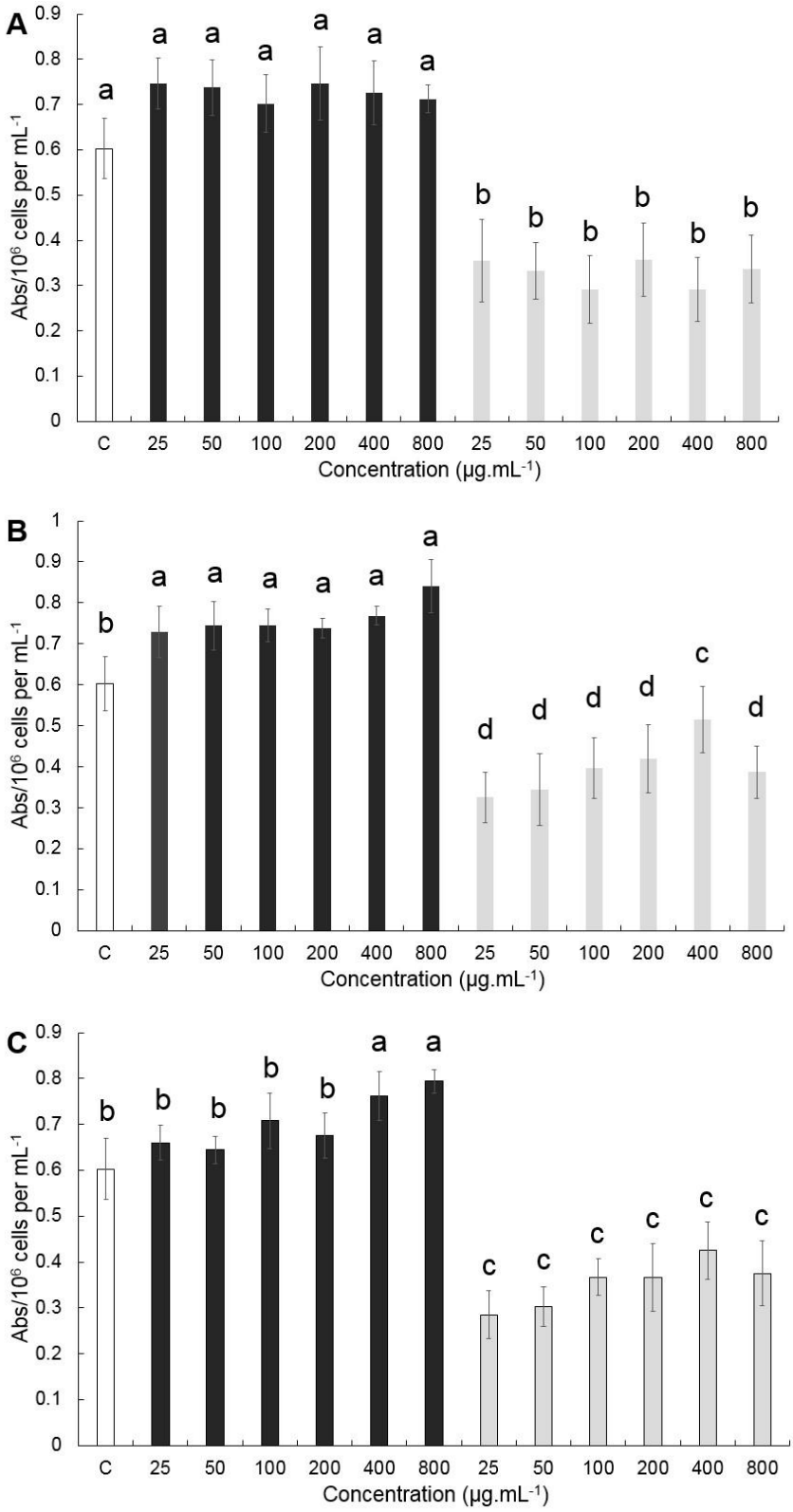
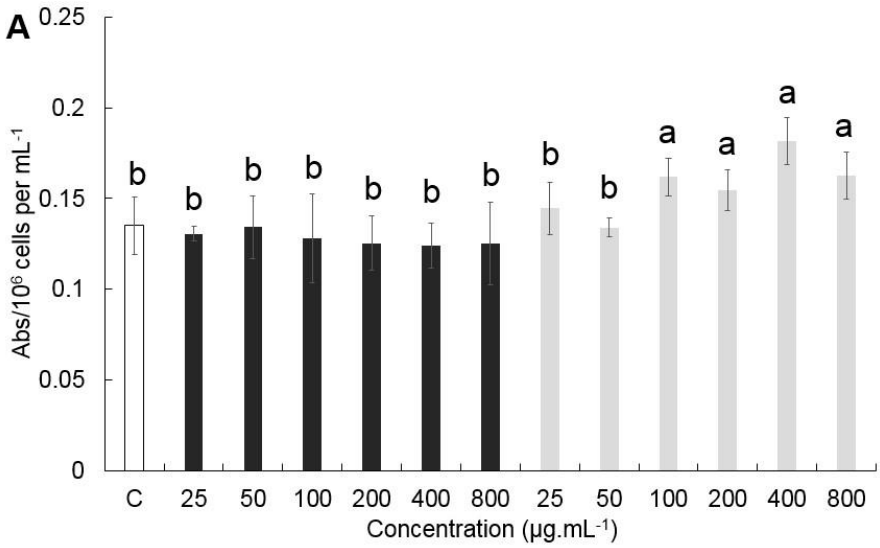


Fig 10. Macrophages adhesion activity (cell line RAW 267) treated with polysaccharide fractions (■) and polysaccharides-AgNPs conjugates (■). (A) F1A fraction; (B) F2A fraction and (C) EPS2A fraction from *Cordyceps sinensis*. Results were presented by mean ± standard error of the mean. The means expressed are results of three replicates. (C) Control. Bars followed by the same letter are not different at $p<0.05$ by Scott Knott's test.

Hydrogen peroxide (H_2O_2) and superoxide anion ($O_2^{\cdot-}$) are important reactive oxygen species (ROS) involved in the inflammation response and have been reported as one of the mechanisms of AgNPs toxicity (Chairuangkitti et al., 2013; Wang E. et al., 2017).

Hydrogen peroxide production assay results showed that polysaccharide fractions (F1A, F2A and EPS2) were not different in comparison to the control at any concentration ($p>0.05$) as seen in Figure 11. However, when cells (RAW 264.7) were treated with polysaccharides-AgNPs conjugates it was possible to identify a significant difference in the results ($p<0.05$). F1A-AgNPs, F2A-AgNPs and EPS2-AgNPs increased the concentration of the hydrogen peroxide in comparison to the control at concentration of 100, 200 and 400 $\mu g.mL^{-1}$, respectively. An interesting reduction occurred at the concentration of 800 $\mu g.mL^{-1}$ for F2A-AgNPs (Figure 11) and may be related whit the initiation of cytotoxic effects (Kloet et al., 2016).



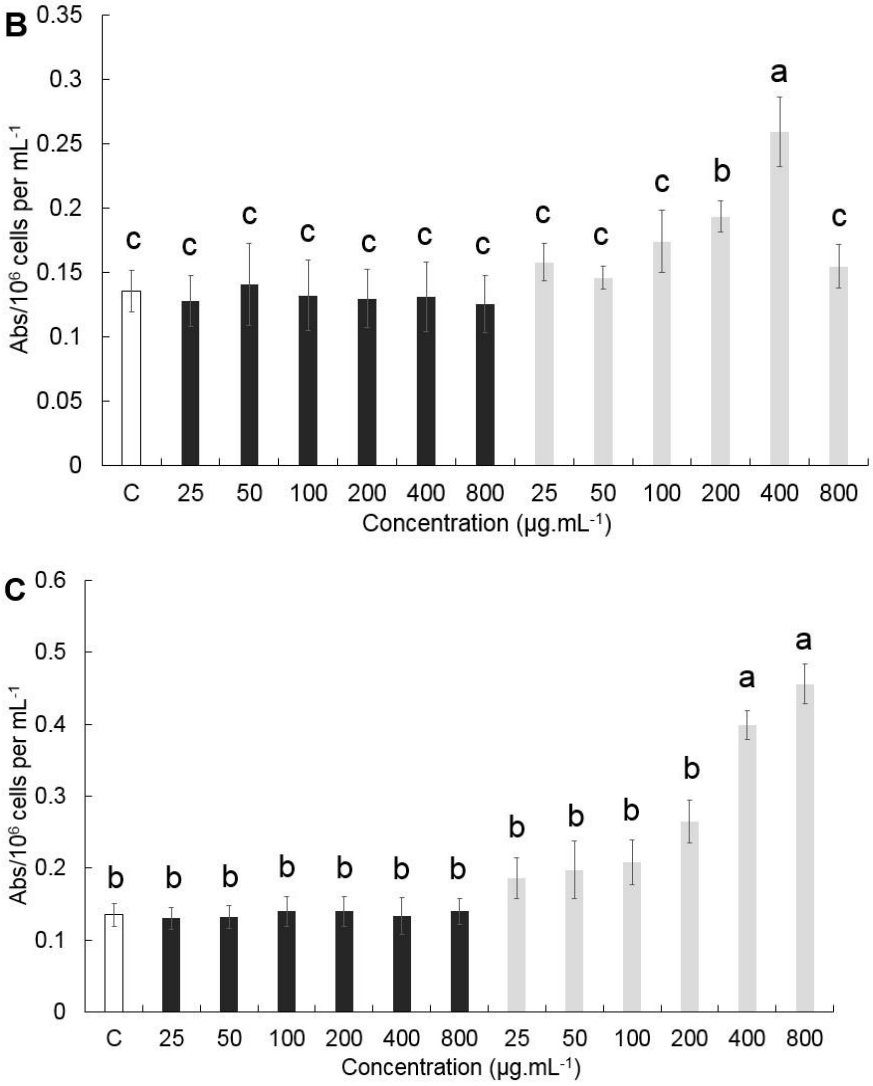
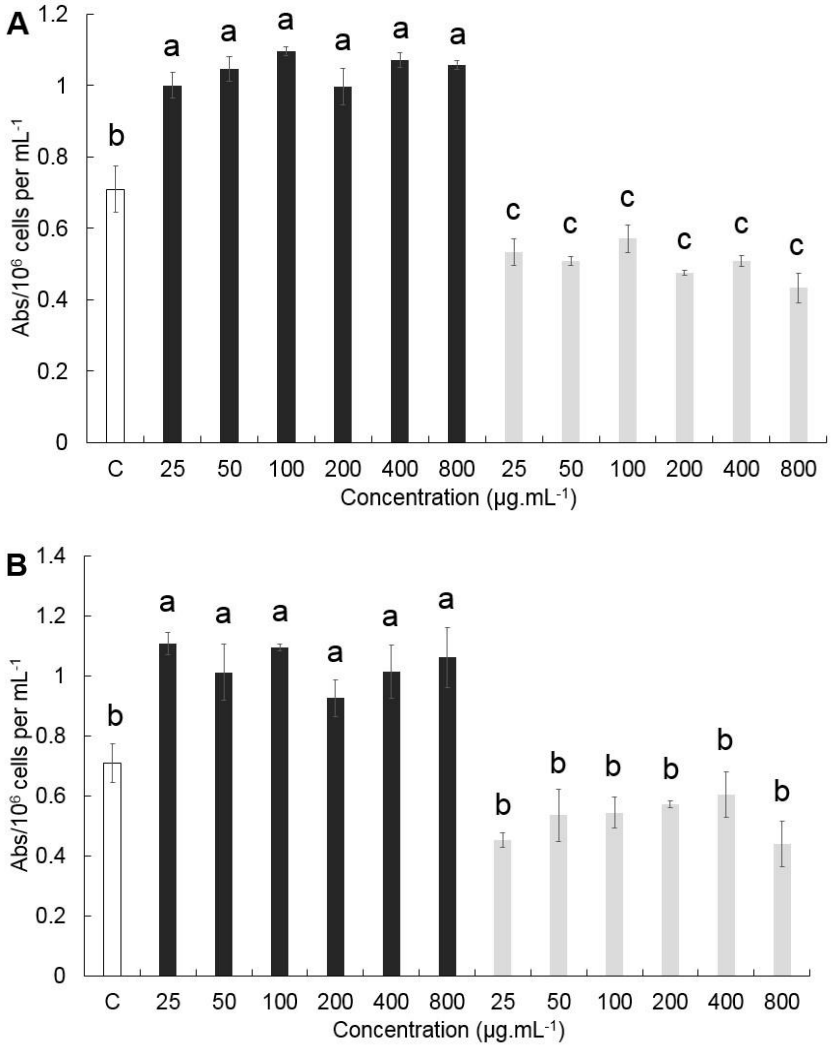


Fig 11. Macrophages hydrogen peroxide production (cell line RAW 267) treated with polysaccharide fractions (■) and polysaccharides-AgNPs conjugates (■). (A) F1A fraction; (B) F2A fraction and (C) EPS2A fraction from *Cordyceps sinensis*. Results were presented by mean ± standard error of the mean. The means expressed are results of three replicates. (C) Control. Bars followed by the same letter are not different at $p<0.05$ by Scott Knott's test.

The analysis of the superoxide anion production by the macrophages resulted in significant results when cells were treated with polysaccharides fractions and polysaccharides-AgNPs conjugates (Figure 12).

All polysaccharide fractions increased significantly the production of superoxide anion ($p<0.05$), however, polysaccharides-AgNPs (F2A and EPS2) were not significant in comparison with the control ($p>0.05$). For F1A-AgNPs a significant decrease was observed for any concentration evaluated (Figure 12).

Kloet et al. (2016) reported a decrease in ROS production when cells (RAW 264.7) were treated with silver nanoparticles (50 nm) at 10 to 200 µg.mL^{-1} for 24h. The authors suggested that probably cytotoxic processes initiated or progressed faster than ROS generation, which could explain our results.



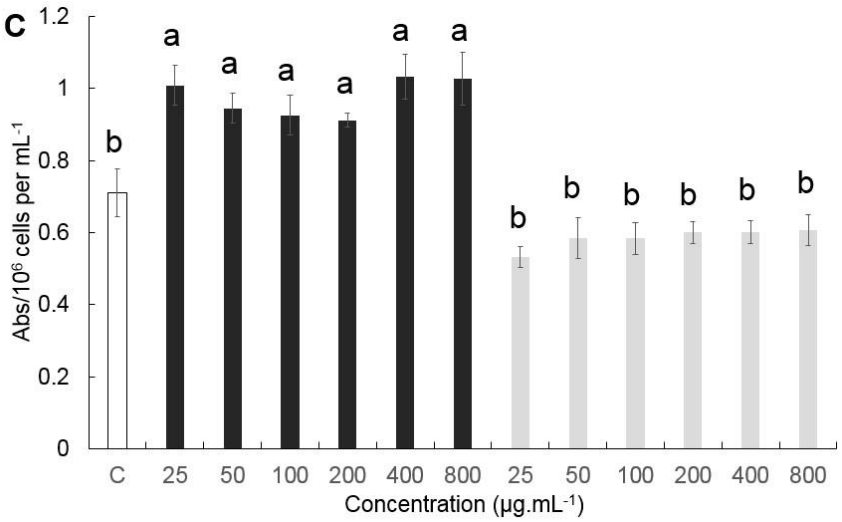


Fig 12. Macrophages superoxide anion production (cell line RAW 267) treated with polysaccharide fractions (■) and polysaccharides-AgNPs conjugates (▒). (A) F1A fraction; (B) F2A fraction and (C) EPS2A fraction. Results were presented by mean ± standard error of the mean. The means expressed are results of three replicates. (C) Control. Bars followed by the same letter are not different at $p<0.05$ by Scott Knott's test.

In fact, the cytotoxicity of nanoparticles seems to be mainly related to their size, concentration and nanoparticle surface, besides the interaction with different cellular types (Kloet et al., 2016; Park et al., 2011; Yen et al., 2009). However, AgNPs of 70 nm did not caused a cytotoxic response neither an increase of IL-8, a pro-inflammatory cytokine, or ROS when human macrophages cell line U937 were treated at the concentration of 50 $\mu g.mL^{-1}$ (Park et al., 2011). Jang et al, (2016) reported that silver nanoparticles (10 – 50 nm) were not toxic to normal immune system (RAW 264.7) at 500 $\mu g.mL^{-1}$ after 48h.

In summary, the immumodulatory effect of AgNPs-polysaccharide conjugates on macrophages was evaluated by the metabolic activity and morphological indicators. A decrease effect on phagocytic, lysosomal volume and adhesion capacities were observed which is a critical factor in determining inflammatory immune response. In this current study, superoxide anion production appeared to have been inhibited when exposed to AgNPs, which could be related to the beginning of a cytotoxic process. Despite the complexity in determining toxicity of AgNPs, the *in vitro* or *in vivo* models are yet a challenge that needs further evaluation.

CONCLUSION

In conclusion, it has been demonstrated that the polysaccharides from *Cordyceps sinensis*, as capping and reducing agent, are capable of producing silver nanoparticles by the bio-reduction of aqueous Ag^+ ions. This green chemistry approach toward the synthesis of silver nanoparticles has many advantages, such as ease in process scale-up. These eco-friendly immunosuppressant nanoparticles could be applicable to diverse medical devices which makes this method potentially interesting for the large-scale synthesis of other nanomaterials. The characterization with UV–VIS spectroscopy, Fourier transmission infrared spectroscopy (FTIR), transmission electron microscopy (TEM), X-ray diffraction (XRD) confirmed the formation of nanoparticles. Moreover, a decrease effect on phagocytic capacity, lysosomal volume and adhesion capacity were observed which is a critical factor in determining inflammatory immune response.

Acknowledgments

The authors would like to thank CAPES and CNPq for providing financial support. We are grateful to Electronic Microscopy Center, LORXI, Chemistry and Biochemistry departments of UFPR and Instituto Pelé Pequeno Príncipe for analysis support.

REFERENCES

ANUPAMA, N.; MADHUMITHA, G. Green synthesis and catalytic application of silver nanoparticles using Carissa carandas fruits. **Inorganic and Nano-Metal Chemistry,** v. 47, n. 1, p. 116-120, 2017a. ISSN 2470-1556.

ARAI, Y.; MIYAYAMA, T.; HIRANO, S. Difference in the toxicity mechanism between ion and nanoparticle forms of silver in the mouse lung and in macrophages. **Toxicology,** v. 328, p. 84-92, 2015. ISSN 0300-483X.

AZEEZ, M. A. et al. Biomedical applications of cocoa bean extract-mediated silver nanoparticles as antimicrobial, larvicidal and anticoagulant agents. **Journal of Cluster Science,** v. 1, n. 28, p. 149-164, 2016. ISSN 1040-7278.

BACHAND, G. D. et al. Cytotoxicity and inflammation in human alveolar epithelial cells following exposure to occupational levels of gold and silver nanoparticles. **Journal**

of Nanoparticle Research, v. 14, n. 10, p. 1-10, 2012. ISSN 1388-0764.

BAE, C. H.; NAM, S. H.; PARK, S. M. Formation of silver nanoparticles by laser ablation of a silver target in NaCl solution. **Applied Surface Science,** v. 197, p. 628-634, 2002. ISSN 0169-4332.

BONATTO, S. J. et al. Lifelong exposure to dietary fish oil alters macrophage responses in Walker 256 tumor-bearing rats. **Cellular immunology,** v. 231, n. 1, p. 56-62, 2004. ISSN 0008-8749.

CHAIRUANGKITTI, P. et al. Silver nanoparticles induce toxicity in A549 cells via ROS-dependent and ROS-independent pathways. **Toxicology in vitro,** v. 27, n. 1, p. 330-338, 2013. ISSN 0887-2333.

CHEN, L. et al. Cell wall structure of mushroom sclerotium (Pleurotus tuber regium): Part 1. Fractionation and characterization of soluble cell wall polysaccharides. **Food Hydrocolloids,** v. 36, p. 189-195, 2014. ISSN 0268-005X.

CHEN, X.; YAN, J.-K.; WU, J.-Y. Characterization and antibacterial activity of silver nanoparticles prepared with a fungal exopolysaccharide in water. **Food Hydrocolloids,** v. 53, p. 69-74, 2016. ISSN 0268-005X.

DEVI, T. B.; AHMARUZZAMAN, M. Bio-inspired facile and green fabrication of Au@ Ag@ AgCl core–double shells nanoparticles and their potential applications for elimination of toxic emerging pollutants: A green and efficient approach for wastewater treatment. **Chemical Engineering Journal,** v. 317, p. 726-741, 2017. ISSN 1385-8947.

DOS SANTOS, L. F. et al. Hypolipidemic and antiatherosclerotic potential of Pleurotus ostreatus, cultived by submerged fermentation in the high-fat diet fed rats. **Biotechnology and Bioprocess Engineering: BBE,** v. 18, n. 1, p. 201, 2013. ISSN 1226-8372.

DUAN, H.; WANG, D.; LI, Y. Green chemistry for nanoparticle synthesis. **Chemical Society Reviews,** v. 44, n. 16, p. 5778-5792, 2015.

EMBRAPA. Núcleo Tecnológico para Informática. SOC-Software Científico. Campinas, 1990.

GHASEMINEZHAD, S. M.; HAMEDI, S.; SHOJAOSADATI, S. A. Green synthesis of silver nanoparticles by a novel method: comparative study of their properties. **Carbohydrate polymers,** v. 89, n. 2, p. 467-472, 2012. ISSN 0144-8617.

HSIAO, I.-L. et al. Trojan-horse mechanism in the cellular uptake of silver nanoparticles verified by direct intra-and extracellular silver speciation analysis. **Environmental science & technology,** v. 49, n. 6, p. 3813-3821, 2015. ISSN 0013-936X.

KLOET, S. K. **In vitro assays for hazard identification of nanoparticles**. 2016. Wageningen University

MA, Y. et al. Antibacterial evaluation of sliver nanoparticles synthesized by polysaccharides from Astragalus membranaceus roots. **Biomedicine &**

Pharmacotherapy, v. 89, p. 351-357, 2017. ISSN 0753-3322.

MATA, Y. et al. Gold (III) biosorption and bioreduction with the brown alga Fucus vesiculosus. **Journal of hazardous materials,** v. 166, n. 2, p. 612-618, 2009. ISSN 0304-3894.

MOHANTY, S. et al. An investigation on the antibacterial, cytotoxic, and antibiofilm efficacy of starch-stabilized silver nanoparticles. **Nanomedicine: Nanotechnology, Biology and Medicine,** v. 8, n. 6, p. 916-924, 2012. ISSN 1549-9634.

PARK, J. et al. Size dependent macrophage responses and toxicological effects of Ag nanoparticles. **Chemical communications,** v. 47, n. 15, p. 4382-4384, 2011.

PERUGINI BIASI-GARBIN, R. et al. Effect of eugenol against Streptococcus agalactiae and synergistic interaction with biologically produced silver nanoparticles. **Evidence-Based Complementary and Alternative Medicine,** v. 2015, 2015. ISSN 1741-427X.

PICK, E.; KEISARI, Y. A simple colorimetric method for the measurement of hydrogen peroxide produced by cells in culture. **Journal of immunological methods,** v. 38, n. 1-2, p. 161-170, 1980. ISSN 0022-1759.

PIPE, R.; COLES, J.; FARLEY, S. Assays for measuring immune response in the mussel Mytilus edulis. **Tech Fish Immunol,** v. 4, p. 93-100, 1995.

ROSEN, H.; GORDON, S. Monoclonal antibody to the murine type 3 complement receptor inhibits adhesion of myelomonocytic cells in vitro and inflammatory cell recruitment in vivo. **Journal of Experimental Medicine,** v. 166, n. 6, p. 1685-1701, 1987. ISSN 0022-1007.

SEN, I. K. et al. Green synthesis of silver nanoparticles using glucan from mushroom and study of antibacterial activity. **International journal of biological macromolecules,** v. 62, p. 439-449, 2013. ISSN 0141-8130.

SIM CHOI, H. et al. A quantitative nitroblue tetrazolium assay for determining intracellular superoxide anion production in phagocytic cells. **Journal of Immunoassay and Immunochemistry,** v. 27, n. 1, p. 31-44, 2006. ISSN 1532-1819.

SUBER, L. et al. Preparation and the mechanisms of formation of silver particles of different morphologies in homogeneous solutions. **Journal of Colloid and Interface Science,** v. 288, n. 2, p. 489-495, 2005. ISSN 0021-9797.

WANG, E. et al. Silver nanoparticle induced toxicity to human sperm by increasing ROS (reactive oxygen species) production and DNA damage. **Environmental Toxicology and Pharmacology,** v. 52, p. 193-199, 2017. ISSN 1382-6689.

WANG, L. et al. Characterization of silver nanoparticles biosynthesized using crude polysaccharides of Psidium guajava L. leaf and their bioactivities. **Materials Letters**, 2017. ISSN 0167-577X.

YAN, J.-K.; WANG, W.-Q.; WU, J.-Y. Recent advances in Cordyceps sinensis

polysaccharides: Mycelial fermentation, isolation, structure, and bioactivities: A review. **Journal of Functional Foods,** v. 6, p. 33-47, 2014. ISSN 1756-4646.

YEN, H. J.; HSU, S. H.; TSAI, C. L. Cytotoxicity and immunological response of gold and silver nanoparticles of different sizes. **Small,** v. 5, n. 13, p. 1553-1561, 2009. ISSN 1613-6829.

YIN, H. et al. Large-scale and size-controlled synthesis of silver nanoparticles under microwave irradiation. **Materials chemistry and Physics,** v. 83, n. 1, p. 66-70, 2004. ISSN 0254-0584.

ZHANG, J. et al. Regulation of endothelial cell adhesion molecule expression by mast cells, macrophages, and neutrophils. **PloS one,** v. 6, n. 1, p. e14525, 2011. ISSN 1932-6203.

ZHANG, X.-Y. et al. Seeds triggered massive synthesis and multi-step room temperature post-processing of silver nanoink—application for paper electronics. **RSC Advances,** v. 7, n. 1, p. 8-19, 2017.

ZHAO, F. et al. Cellular uptake, intracellular trafficking, and cytotoxicity of nanomaterials. **Small,** v. 7, n. 10, p. 1322-1337, 2011. ISSN 1613-6829.

CONCLUSÕES

Foi possível sintetizar e caracterizar nanopartículas de prata e paládio utilizando apenas polissacarídeos obtidos de *Cordyceps sinensis* como agente redutor e estabilizante das nanopartículas;

Nanopartículas de prata conjugadas com frações de polissacarídeos (F1A-AgNPs, F2A-AgNPs e EPS2-AgNPs) demonstraram potencial atividade imunossupressora em macrófagos (RAW 264.7), visto que foi observado um efeito de diminuição significativa ($p<0,05$) nas capacidades de fagocitose, retenção lisossomal e adesão de macrófagos, que por sua vez é um fator crítico na determinação da resposta imune inflamatória;

Nanopartículas de paládio conjugadas com a fração F2A de polissacarídeos (F2A-PdNPs) inibiram significativamente ($p<0,05$) o crescimento de células tumorais (H295R) de 12 para 26%, quando comparadas com o controle, o que representa em média um aumento em torno de 100% da citotoxicidade da fração de polissacarídeos F2A;

Os resultados mostraram que os polissacarídeos de *C. sinensis* avaliados podem ser utilizados como um agente de redução e estabilizantes eficazes, não tóxicos para a síntese verde de nanopartículas, que por sua vez podem ser aplicáveis à criação e inovação em diversos dispositivos médicos.

CONSIDERAÇÕES PARA TRABALHOS FUTUROS

1. Purificar e caracterizar os polissacarídeos empregados como agentes redutores e estabilizantes na síntese de nanopartículas metálicas.

2. Selecionar microorganismos capazes de metabolizar solução de sais de metais para produção biológica de nanopartículas metálicas em cultivo de fermentação submersa.

3. Otimizar as condições de fermentação e a síntese de nanopartículas metálicas.

4. Avaliar a toxicidade das nanopartículas metálicas *in vitro* e *in vivo* em diferentes modelos e organismos.

5. Desenvolver e avaliar a estabilidade de formulações para aplicações biomédicas.

6. Desenvolver nanoestruturas com sistemas de entrega alvo dirigidas e/ou liberação controlada e prolongada.

REFERÊNCIAS

ABE, I. Enzymatic synthesis of cyclic triterpenes. **Nat Prod Rep,** v. 24, n. 6, p. 1311-31, Dec 2007. ISSN 0265-0568. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/18033581 >.

ALLEN, R. et al. Sustained Akt/PKB activation and transient attenuation of c-jun N-terminal kinase in the inhibition of apoptosis by IGF-1 in vascular smooth muscle cells. **Apoptosis,** v. 10, n. 3, p. 525-535, MAY 2005 2005. ISSN 1360-8185.

AMIL USMANI, M. et al. Current Trend in the Application of Nanoparticles for Waste Water Treatment and Purification: A Review. **Current Organic Synthesis,** v. 14, n. 2, p. 206-226, 2017. ISSN 1570-1794.

ANUPAMA, N.; MADHUMITHA, G. Green synthesis and catalytic application of silver nanoparticles using Carissa carandas fruits. **Inorganic and Nano-Metal Chemistry,** v. 47, n. 1, p. 116-120, 2017. ISSN 2470-1556.

ARAI, Y.; MIYAYAMA, T.; HIRANO, S. Difference in the toxicity mechanism between ion and nanoparticle forms of silver in the mouse lung and in macrophages. **Toxicology,** v. 328, p. 84-92, 2015. ISSN 0300-483X.

ARSIYA, F.; SAYADI, M. H.; SOBHANI, S. Green synthesis of palladium nanoparticles using Chlorella vulgaris. **Materials Letters,** v. 186, p. 113-115, 2017. ISSN 0167-577X.

AZEEZ, M. A. et al. Biomedical applications of cocoa bean extract-mediated silver nanoparticles as antimicrobial, larvicidal and anticoagulant agents. **Journal of Cluster Science,** v. 1, n. 28, p. 149-164, 2016. ISSN 1040-7278.

BACHAND, G. D. et al. Cytotoxicity and inflammation in human alveolar epithelial cells following exposure to occupational levels of gold and silver nanoparticles. **Journal of Nanoparticle Research,** v. 14, n. 10, p. 1-10, 2012. ISSN 1388-0764.

BAE, C. H.; NAM, S. H.; PARK, S. M. Formation of silver nanoparticles by laser ablation of a silver target in NaCl solution. **Applied Surface Science,** v. 197, p. 628-634, 2002. ISSN 0169-4332.

BAIK, J.-S. et al. Cordycepin-mediated transcriptional regulation of human GD3 synthase (hST8Sia I) in human neuroblastoma SK-N-BE (2)-C cells. **gene,** v. 17, p. 20, 2014.

BANKAR, A. et al. Banana peel extract mediated novel route for the synthesis of palladium nanoparticles. **Materials Letters,** v. 64, n. 18, p. 1951-1953, 2010. ISSN 0167-577X.

BAO, X. et al. Chemical modifications of the (1 -> 3)-alpha-D-glucan from spores of Ganoderma lucidum and investigation of their physicochemical properties and immunological activity. **Carbohydrate Research,** v. 336, n. 2, p. 127-140, NOV 8 2001 2001. ISSN 0008-6215.

BAO, X.; FANG, J.; LI, X. Structural characterization and immunomodulating activity of a complex glucan from spores of Ganoderma lucidum. **Bioscience Biotechnology and Biochemistry,** v. 65, n. 11, p. 2384-2391, NOV 2001 2001. ISSN 0916-8451.

BAO, X. et al. Structural and immunological studies of a major polysaccharide from spores of Ganoderma lucidum (Fr.) Karst. **Carbohydrate Research,** v. 332, n. 1, p. 67-74, MAY 8 2001 2001. ISSN 0008-6215.

_____. Purification, characterization, and modification of T lymphocyte-stimulating polysaccharide from spores of Ganoderma lucidum. **Chemical & Pharmaceutical Bulletin,** v. 50, n. 5, p. 623-629, MAY 2002 2002. ISSN 0009-2363.

BARNES, P. M. et al. Complementary and alternative medicine use among adults: United States, 2002. **Adv Data**, n. 343, p. 1-19, May 2004. ISSN 0147-3956. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/15188733 >.

BARROS, L. et al. Wild and commercial mushrooms as source of nutrients and nutraceuticals. **Food Chem Toxicol,** v. 46, n. 8, p. 2742-7, Aug 2008. ISSN 0278-6915. Disponível em: < https://www.ncbi.nlm.nih.gov/pubmed/18538460 >.

BERTAGNA, C.; ORTH, D. N. Clinical and laboratory findings and results of therapy in 58 patients with adrenocortical tumors admitted to a single medical center (1951 to 1978). **The American journal of medicine,** v. 71, n. 5, p. 855-875, 1981. ISSN 0002-9343.

BOECK, C. et al. NMDA preconditioning protects against seizures and hippocampal neurotoxicity induced by quinolinic acid in mice. **Epilepsia,** v. 45, n. 7, p. 745-750, JUL 2004 2004. ISSN 0013-9580.

BOH, B. Ganoderma lucidum: a potential for biotechnological production of anti-cancer and immunomodulatory drugs. **Recent Pat Anticancer Drug Discov,** v. 8, n. 3, p. 255-87, Sep 2013. ISSN 2212-3970. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/23227951 >.

BONATTO, S. J. et al. Lifelong exposure to dietary fish oil alters macrophage responses in Walker 256 tumor-bearing rats. **Cellular immunology,** v. 231, n. 1, p. 56-62, 2004. ISSN 0008-8749.

BRAND-WILLIAMS, W.; CUVELIER, M.-E.; BERSET, C. Use of a free radical method to evaluate antioxidant activity. **LWT-Food science and Technology,** v. 28, n. 1, p. 25-30, 1995. ISSN 0023-6438.

BRENNER, S. The genetics of Caenorhabditis elegans. **Genetics,** v. 77, n. 1, p. 71-94, 1974. ISSN 0016-6731.

CAMOUGRAND, N. et al. The product of the UTH1 gene, required for Bax-induced cell death in yeast, is involved in the response to rapamycin. **Molecular Microbiology,** v. 47, n. 2, p. 495-506, JAN 2003 2003. ISSN 0950-382X.

CARNEIRO, A. A. et al. Chemical composition and antioxidant activity of dried powder formulations of Agaricus blazei and Lentinus edodes. **Food chemistry,** v. 138, n. 4, p.

2168-2173, 2013. ISSN 0308-8146.

CHA, S.; PECK, A.; HUMPHREYS-BEHER, M. Progress in understanding autolmmune exocrinopathy using then on-obese diabetic mouse: An update. **Critical Reviews in Oral Biology & Medicine,** v. 13, n. 1, p. 5-16, 2002 2002. ISSN 1045-4411.

CHAIRUANGKITTI, P. et al. Silver nanoparticles induce toxicity in A549 cells via ROS-dependent and ROS-independent pathways. **Toxicology in vitro,** v. 27, n. 1, p. 330-338, 2013. ISSN 0887-2333.

CHALOUPKA, K.; MALAM, Y.; SEIFALIAN, A. M. Nanosilver as a new generation of nanoproduct in biomedical applications. **Trends in biotechnology,** v. 28, n. 11, p. 580-588, 2010. ISSN 0167-7799.

CHAN, W. et al. Ganoderma lucidum mycelium and spore extracts as natural adjuvants for immunotherapy. **Journal of Alternative and Complementary Medicine,** v. 11, n. 6, p. 1047-1057, DEC 2005 2005. ISSN 1075-5535.

CHANG, Y. H. et al. Gandoderma lucidum extract promotes immune responses in normal BALB/c mice In vivo. **In Vivo,** v. 23, n. 5, p. 755-9, 2009 Sep-Oct 2009. ISSN 0258-851X. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/19779111 >.

CHEN, L. et al. Cell wall structure of mushroom sclerotium (Pleurotus tuber regium): Part 1. Fractionation and characterization of soluble cell wall polysaccharides. **Food Hydrocolloids,** v. 36, p. 189-195, 2014. ISSN 0268-005X.

CHEN, T. et al. Supercritical fluid CO2 extraction, simultaneous determination of total sterols in the spore lipids of Ganoderma lucidum by GC-MS/SIM methods. **Chemistry of Natural Compounds,** v. 48, n. 4, p. 657-658, SEP 2012 2012. ISSN 0009-3130.

_____. Fatty acids, essential oils, and squalene in the spore lipids of Ganoderma lucidum by GC-MS and GC-FID. **Chemistry of Natural Compounds,** v. 49, n. 1, p. 143-144, MAR 2013 2013. ISSN 0009-3130.

CHEN, X.; YAN, J.-K.; WU, J.-Y. Characterization and antibacterial activity of silver nanoparticles prepared with a fungal exopolysaccharide in water. **Food Hydrocolloids,** v. 53, p. 69-74, 2016. ISSN 0268-005X.

CHIEN, R.-C. et al. Chemical characteristics and anti-proliferation activities of Ganoderma tsugae polysaccharides. **Carbohydrate polymers,** v. 128, p. 90-98, 2015. ISSN 0144-8617.

CLAVADETSCHER, J. et al. In-Cell Dual Drug Synthesis by Cancer-Targeting Palladium Catalysts. **Angewandte Chemie,** v. 129, n. 24, p. 6968-6972, 2017. ISSN 1521-3757.

CUI, Y. et al. Use of complementary and alternative medicine by chinese women with breast cancer. **Breast Cancer Res Treat,** v. 85, n. 3, p. 263-70, Jun 2004. ISSN 0167-6806. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/15111765 >.

CUSTANCE, O.; PEREZ, R.; MORITA, S. Atomic force microscopy as a tool for atom manipulation. **Nature nanotechnology,** v. 4, n. 12, p. 803-810, 2009. ISSN 1748-3387.

DE CASTRO, E.; DE CASTRO, S. H.; JOHNSON, T. E. Isolation of long-lived mutants in Caenorhabditis elegans using selection for resistance to juglone. **Free Radical Biology and Medicine,** v. 37, n. 2, p. 139-145, 2004. ISSN 0891-5849.

DE LA MONTE, S. M.; WANDS, J. R. Review of insulin and insulin-like growth factor expression, signaling, and malfunction in the central nervous system: relevance to Alzheimer's disease. **J Alzheimers Dis,** v. 7, n. 1, p. 45-61, Feb 2005. ISSN 1387-2877. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/15750214 >.

DENGG, M.; VAN MEEL, J. C. Caenorhabditis elegans as model system for rapid toxicity assessment of pharmaceutical compounds. **Journal of pharmacological and toxicological methods,** v. 50, n. 3, p. 209-214, 2004. ISSN 1056-8719.

DEVI, T. B.; AHMARUZZAMAN, M. Bio-inspired facile and green fabrication of Au@ Ag@ AgCl core–double shells nanoparticles and their potential applications for elimination of toxic emerging pollutants: A green and efficient approach for wastewater treatment. **Chemical Engineering Journal,** v. 317, p. 726-741, 2017. ISSN 1385-8947.

DONG, Q. et al. A novel water-soluble beta-D-glucan isolated from the spores of Ganoderma lucidum. **Carbohydrate Research,** v. 353, p. 100-105, MAY 15 2012 2012. ISSN 0008-6215.

DOROTHY, M. **The perception of *Ganoderma lucidum* in Chinese and Western culture** Mycologist. 18 2004.

DOS SANTOS, L. F. et al. Hypolipidemic and antiatherosclerotic potential of Pleurotus ostreatus, cultived by submerged fermentation in the high-fat diet fed rats. **Biotechnology and Bioprocess Engineering: BBE,** v. 18, n. 1, p. 201, 2013. ISSN 1226-8372.

DUAN, H.; WANG, D.; LI, Y. Green chemistry for nanoparticle synthesis. **Chemical Society Reviews,** v. 44, n. 16, p. 5778-5792, 2015.

DUBOIS, M. et al. Colorimetric method for determination of sugars and related substances. **Analytical chemistry,** v. 28, n. 3, p. 350-356, 1956. ISSN 0003-2700.

DURACKOVA, Z. Some current insights into oxidative stress. **Physiological Research,** v. 59, n. 4, p. 459, 2010. ISSN 0862-8408.

ERNST, E. Prevalence of use of complementary/alternative medicine: a systematic review. **Bull World Health Organ,** v. 78, n. 2, p. 252-7, 2000. ISSN 0042-9686. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/10743298 >.

ERNST, E.; RESCH, K. L.; WHITE, A. R. Complementary medicine. What physicians think of it: a meta-analysis. **Arch Intern Med,** v. 155, n. 22, p. 2405-8, 1995 Dec 11-25 1995. ISSN 0003-9926. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/7503598 >.

FAN, L. et al. Effect of nutritional and environmental conditions on the production of exo-polysaccharide of Agaricus brasiliensis by submerged fermentation and its antitumor activity. **LWT-Food Science and Technology,** v. 40, n. 1, p. 30-35, 2007. ISSN 0023-6438.

FANELLI, M. et al. New trends in platinum and palladium complexes as antineoplastic agents. **Coordination Chemistry Reviews,** v. 310, p. 41-79, 2016. ISSN 0010-8545.

FASSNACHT, M. et al. Combination chemotherapy in advanced adrenocortical carcinoma. **New England Journal of Medicine,** v. 366, n. 23, p. 2189-2197, 2012. ISSN 0028-4793.

FEYNMAN, R. P. There's plenty of room at the bottom. **Engineering and science,** v. 23, n. 5, p. 22-36, 1960. ISSN 0013-7812.

FU, Y. et al. Breaking the spores of the fungus Ganoderma lucidum by supercritical CO2. **Food Chemistry,** v. 112, n. 1, p. 71-76, JAN 1 2009 2009. ISSN 0308-8146.

FUKUZAWA, M. et al. Possible involvement of long chain fatty acids in the spores of Ganoderma lucidum (Reishi Houshi) to its anti-tumor activity. **Biological & Pharmaceutical Bulletin,** v. 31, n. 10, p. 1933-1937, OCT 2008 2008. ISSN 0918-6158.

GAO, J. et al. Quantitative determination of bitter principles in specimens of Ganoderma lucidum using high-performance liquid chromatography and its application to the evaluation of Ganoderma products. **Chemical & Pharmaceutical Bulletin,** v. 52, n. 6, p. 688-695, JUN 2004 2004. ISSN 0009-2363.

GAO, P. et al. Isolation and identification of C-19 fatty acids with anti-tumor activity from the spores of Ganoderma lucidum (reishi mushroom). **Fitoterapia,** v. 83, n. 3, p. 490-499, APR 2012 2012. ISSN 0367-326X.

GAO, Y. et al. The Comparative Protective Effects of Ganoderma Spores Lipid and Fish Oil on N-Methyl-N-Nitrosourea-Induced Photoreceptor Cell Lesion in Rats. **Evidence-Based Complementary and Alternative Medicine**, p. 1-10, 2011 2011. ISSN 1741-427X.

_____. A phase I/II study of *Ganoderma lucidum* (Curt.: Fr.) P. Karst. extract (Ganopoly) in patientas with advanced cancer. **International Journal of Medicinal Mushrooms,** v. 4, p. 7, 2002.

GAZDAR, A. F. et al. Establishment and characterization of a human adrenocortical carcinoma cell line that expresses multiple pathways of steroid biosynthesis. **Cancer Research,** v. 50, n. 17, p. 5488-5496, 1990. ISSN 0008-5472.

GEMS, D.; PARTRIDGE, L.; JULIUS, D. Genetics of Longevity in Model Organisms: Debates and Paradigm Shifts. **Annual Review of Physiology, Vol 75,** v. 75, p. 621-644, 2013 2013. ISSN 0066-4278.

GHASEMINEZHAD, S. M.; HAMEDI, S.; SHOJAOSADATI, S. A. Green synthesis of silver nanoparticles by a novel method: comparative study of their properties.

Carbohydrate polymers, v. 89, n. 2, p. 467-472, 2012. ISSN 0144-8617.

GIAVASIS, I. Bioactive fungal polysaccharides as potential functional ingredients in food and nutraceuticals. **Curr Opin Biotechnol,** v. 26, p. 162-73, Apr 2014. ISSN 1879-0429. Disponível em: < https://www.ncbi.nlm.nih.gov/pubmed/24518400 >.

GUO, L. et al. Characterization and immunostimulatory activity of a polysaccharide from the spores of Ganoderma lucidum. **International Immunopharmacology,** v. 9, n. 10, p. 1175-1182, SEP 2009 2009. ISSN 1567-5769.

HAMASAKI, T.; KAKIUCHI, F.; KOCHI, T. Chain-walking Cycloisomerization of 1, n-Dienes Catalyzed by Pyridine–Oxazoline Palladium Catalysts and Its Application to Asymmetric Synthesis. **Chemistry Letters,** v. 45, n. 3, p. 297-299, 2016. ISSN 1348-0715.

HAWKSWORTH, D. L. Mushrooms: the extent of the unexplored potential. **International Journal of Medicinal Mushrooms,** v. 3, n. 4, 2001. ISSN 1521-9437.

HEDAYATNASAB, Z.; ABNISA, F.; DAUD, W. M. A. W. Review on magnetic nanoparticles for magnetic nanofluid hyperthermia application. **Materials & Design,** v. 123, p. 174-196, 2017. ISSN 0264-1275.

HELENO, S. et al. Fruiting body, spores and in vitro produced mycelium of Ganoderma lucidum from Northeast Portugal: A comparative study of the antioxidant potential of phenolic and polysaccharidic extracts. **Food Research International,** v. 46, n. 1, p. 135-140, APR 2012 2012. ISSN 0963-9969.

HIRAI, K.; SEKI, T. Cerebrospinal fluid somatostatin levels in febrile seizures and epilepsy in children. **Neuropeptides,** v. 34, n. 1, p. 18-24, FEB 2000 2000. ISSN 0143-4179.

HO, Y. W.; NAWAWI, A. **Germination Studies of *Ganoderma boninense* Spores from Oil P.alms in Malaysia**: Pertanika. 9**:** 151-154 p. 1986.

HSIAO, I.-L. et al. Trojan-horse mechanism in the cellular uptake of silver nanoparticles verified by direct intra-and extracellular silver speciation analysis. **Environmental science & technology,** v. 49, n. 6, p. 3813-3821, 2015. ISSN 0013-936X.

HSU, P. Y. et al. Extract of sporoderm-broken germinating spores of ganoderma lucidum activates human polymorphonuclear neutrophils via the P38 mitogen-activated protein kinase pathway. **Chang Gung Med J,** v. 35, n. 2, p. 140-7, 2012 Mar-Apr 2012. ISSN 2309-835X. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/22537928 >.

HSU, W. et al. Berberine induces apoptosis in SW620 human colonic carcinoma cells through generation of reactive oxygen species and activation of JNK/p38 MAPK and FasL. **Archives of Toxicology,** v. 81, n. 10, p. 719-728, OCT 2007 2007. ISSN 0340-5761.

HUANG, Z.; FANG; WONG, C.-W. **GANODERMA LUCIDUM SPORE LIPID INDUCES PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR**

ALPHA ACTIVITY. Journal of Food Biochemistry Volume 35, Issue 5. Journal of Food Biochemistry. 35**:** 1508-1513 p. 2011.

ISO (International Standards Organization) (2015) TS 80004-1:2015. https://www.iso.org/obp/ui/#iso:std:iso:ts:80004:-1:ed-2:v1:en

ISO. ISO/TS 80004–5:2011– nanotechnologies – vocabulary – part 5: nano/bio interface (2011). https://www.iso.org/iso/iso_catalogue/catalogue_tc/catalogue

JAIN, P. K. et al. Noble metals on the nanoscale: optical and photothermal properties and some applications in imaging, sensing, biology, and medicine. **Accounts of chemical research,** v. 41, n. 12, p. 1578-1586, 2008. ISSN 0001-4842.

JAYAKUMAR, T.; THOMAS, P.; GERALDINE, P. In-vitro antioxidant activities of an ethanolic extract of the oyster mushroom, Pleurotus ostreatus. **Innovative Food Science & Emerging Technologies,** v. 10, n. 2, p. 228-234, 2009. ISSN 1466-8564.

JI, D. et al. Antiaging Effect of Cordyceps sinensis Extract. **Phytotherapy Research,** v. 23, n. 1, p. 116-122, JAN 2009 2009. ISSN 0951-418X.

JIN, H. et al. Protective effects of Ganoderma lucidum spore on cadmium hepatotoxicity in mice. **Food and Chemical Toxicology,** v. 52, p. 171-175, FEB 2013 2013. ISSN 0278-6915.

KALAC, P. Chemical composition and nutritional value of European species of wild growing mushrooms: A review. **Food Chemistry,** v. 113, n. 1, p. 9-16, MAR 1 2009 2009. ISSN 0308-8146.

KAMPKÖTTER, A. et al. Investigations of protective effects of the flavonoids quercetin and rutin on stress resistance in the model organism Caenorhabditis elegans. **Toxicology,** v. 234, n. 1, p. 113-123, 2007. ISSN 0300-483X.

_____. Increase of stress resistance and lifespan of Caenorhabditis elegans by quercetin. **Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology,** v. 149, n. 2, p. 314-323, 2008. ISSN 1096-4959.

KASPRZYK, I. et al. Hourly predictive artificial neural network and multivariate regression trees models of Ganoderma spore concentrations in Rzeszow and Szczecin (Poland). **Science of the Total Environment,** v. 409, n. 5, p. 949-956, FEB 1 2011 2011. ISSN 0048-9697.

KAUSHIK, M.; MOORES, A. nanocelluloses as versatile supports for metal nanoparticles and their applications in catalysis. **Green Chemistry,** v. 18, n. 3, p. 622-637, 2016.

KAY, L.; SHAPIRA, P. Developing nanotechnology in Latin America. **Journal of Nanoparticle Research,** v. 11, n. 2, p. 259-278, 2009. ISSN 1388-0764.

KIM, M.-Y. et al. Phenolic compound concentration and antioxidant activities of edible and medicinal mushrooms from Korea. **Journal of Agricultural and Food Chemistry,**

v. 56, n. 16, p. 7265-7270, 2008. ISSN 0021-8561.

KINO, K. et al. Isolation and characterization of a new immunomodulatory protein, ling zhi-8 (LZ-8), from Ganoderma lucidium. **J Biol Chem,** v. 264, n. 1, p. 472-8, Jan 1989. ISSN 0021-9258. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/2909532 >.

KLOET, S. K. **In vitro assays for hazard identification of nanoparticles**. 2016. Wageningen University

KOBAYASHI, H. et al. The inhibition of human tumor cell proliferation by RNase Pol, a member of the RNase T1 family, from Pleurotus ostreatus. **Bioscience, biotechnology, and biochemistry,** v. 77, n. 7, p. 1486-1491, 2013. ISSN 1347-6947.

KOMODA, Y. et al. Ganoderic acid and its derivatives as cholesterol synthesis inhibitors. **Chem Pharm Bull (Tokyo),** v. 37, n. 2, p. 531-3, Feb 1989. ISSN 0009-2363. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/2743504 >.

KOWALCZYK, A. et al. The GD2-specific 14G2a monoclonal antibody induces apoptosis and enhances cytotoxicity of chemotherapeutic drugs in IMR-32 human neuroblastoma cells. **Cancer letters,** v. 281, n. 2, p. 171-182, 2009. ISSN 0304-3835.

KOZARSKI, M. et al. Antioxidative and immunomodulating activities of polysaccharide extracts of the medicinal mushrooms Agaricus bisporus, Agaricus brasiliensis, Ganoderma lucidum and Phellinus linteus. **Food Chemistry,** v. 129, n. 4, p. 1667-1675, DEC 15 2011 2011. ISSN 0308-8146.

KRENS, S.; SPAINK, H.; SNAAR-JAGALSKA, B. Functions of the MAPK family in vertebrate-development. **Febs Letters,** v. 580, n. 21, p. 4984-4990, SEP 18 2006 2006. ISSN 0014-5793.

KUO, C.; HSIEH, C.; LIN, W. Proteomic response of LAP-activated RAW 264.7 macrophages to the anti-inflammatory property of fungal ergosterol. **Food Chemistry,** v. 126, n. 1, p. 207-212, MAY 1 2011 2011. ISSN 0308-8146.

LEI, L. S.; LIN, Z. B. Effect of Ganoderma polysaccharides on T cell subpopulations and production of interleukin 2 in mixed lymphocyte response. **Yao Xue Xue Bao,** v. 27, n. 5, p. 331-5, 1992. ISSN 0513-4870. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/1442052 >.

LI, W. J. et al. Phenolic compounds and antioxidant activities of Liriope muscari. **Molecules,** v. 17, n. 2, p. 1797-1808, 2012.

LI, Y. et al. Cordycepin induces apoptosis and autophagy in human neuroblastoma SK-N-SH and BE (2)-M17 cells. **Oncology letters,** v. 9, n. 6, p. 2541-2547, 2015. ISSN 1792-1074.

LIANG, Y. et al. An Abnormal Elevation of Serum CA72-4 by Ganoderma Lucidum Spore Powder. **Annals of Clinical and Laboratory Science,** v. 43, n. 3, p. 337-340, SUM 2013 2013. ISSN 0091-7370.

LIN, Z. B. Cellular and molecular mechanisms of immuno-modulation by Ganoderma lucidum. **J Pharmacol Sci,** v. 99, n. 2, p. 144-53, Oct 2005. ISSN 1347-8613. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/16230843 >.

LIU, G. T. **Pharmacology and Clinical Application of the spores of Ganoderma lucidum (Curt.: Fr.) P. Karst. and Mycelium of Ganoderma capense (Lloyd) Teng. (Aphyllophromycitideae)**: International Journal of Medicinal Mushrooms. 1**:** 217-222 p. 1999.

LIU, X. et al. Characterization of ganoderma spore lipid by stable carbon isotope analysis: implications for authentication. **Analytical and Bioanalytical Chemistry,** v. 388, n. 3, p. 723-731, JUN 2007 2007. ISSN 1618-2642.

_____. Antitumor activity of the sporoderm-broken germinating spores of Ganoderma lucidum. **Cancer Letters,** v. 182, n. 2, p. 155-161, AUG 28 2002 2002. ISSN 0304-3835.

LIU, Y. et al. Sensitive and selective liquid chromatography-tandem mass spectrometry method for the determination of five ganoderic acids in Ganoderma lucidum and its related species. **Journal of Pharmaceutical and Biomedical Analysis,** v. 54, n. 4, p. 717-721, MAR 25 2011 2011. ISSN 0731-7085.

LU, Q. et al. Ganoderma lucidum extracts inhibit growth and induce actin polymerization in bladder cancer cells in vitro. **Cancer Letters,** v. 216, n. 1, p. 9-20, DEC 8 2004 2004. ISSN 0304-3835.

MA, C. et al. Differential protein expression in mouse splenic mononuclear cells treated with polysaccharides from spores of Ganoderma lucidum. **Phytomedicine,** v. 15, n. 4, p. 268-276, APR 2008 2008. ISSN 0944-7113.

MA, J. et al. Breaking and characteristics of Ganoderma lucidum spores by high speed entrifugal shearing pulverizer. **Journal of Wuhan University of Technology-Materials Science Edition,** v. 22, n. 4, p. 617-621, DEC 2007 2007. ISSN 1000-2413.

MA, Y. et al. Antibacterial evaluation of sliver nanoparticles synthesized by polysaccharides from Astragalus membranaceus roots. **Biomedicine & Pharmacotherapy,** v. 89, p. 351-357, 2017. ISSN 0753-3322.

MAFUNÉ, F. et al. Full physical preparation of size-selected gold nanoparticles in solution: laser ablation and laser-induced size control. **The Journal of Physical Chemistry B,** v. 106, n. 31, p. 7575-7577, 2002. ISSN 1520-6106.

MAINE, E. et al. The emergence of the nanobiotechnology industry. **Nature nanotechnology,** v. 9, n. 1, p. 2-5, 2014. ISSN 1748-3387.

MANIKANDAN, V. et al. Synthesis and antimicrobial activity of palladium nanoparticles from Prunus× yedoensis leaf extract. **Materials Letters,** v. 185, p. 335-338, 2016. ISSN 0167-577X.

MATA, Y. et al. Gold (III) biosorption and bioreduction with the brown alga Fucus vesiculosus. **Journal of hazardous materials,** v. 166, n. 2, p. 612-618, 2009. ISSN 0304-

3894.

MEDICI, S. et al. Noble metals in medicine: Latest advances. **Coordination Chemistry Reviews,** v. 284, p. 329-350, 2015. ISSN 0010-8545.

MILLER, G. Modified DNS method for reducing sugars. **Anal. Chem,** v. 31, n. 3, p. 426-428, 1959.

MILLER, N. J.; RICE-EVANS, C.; DAVIES, M. J. **A new method for measuring antioxidant activity**: Portland Press Limited 1993.

MIMS, C. W.; SEABURY, F. **Ultrastructure of tube formation and basidiopore development in *Ganoderma lucidum***: Mycologia. 81**:** 754-764 p. 1989.

MIN, B. et al. Anticomplement activity of terpenoids from the spores of Ganoderma lucidum. **Planta Medica,** v. 67, n. 9, p. 811-814, DEC 2001 2001. ISSN 0032-0943.

_____. Triterpenes from the spores of Ganoderma lucidum and their cytotoxicity against Meth-A and LLC tumor cells. **Chemical & Pharmaceutical Bulletin,** v. 48, n. 7, p. 1026-1033, JUL 2000 2000. ISSN 0009-2363.

_____. **Triterpenes from the spores of Ganoderma lucidum and their inhibitory activity against HIV-1 protease**: Chemical & Pharmaceutical Bulletin. 46**:** 1607-1612 p. 1998.

MOHANTY, S. et al. An investigation on the antibacterial, cytotoxic, and antibiofilm efficacy of starch-stabilized silver nanoparticles. **Nanomedicine: Nanotechnology, Biology and Medicine,** v. 8, n. 6, p. 916-924, 2012. ISSN 1549-9634.

MOLLNES, T. et al. Complement analysis in the 21st century. **Molecular Immunology,** v. 44, n. 16, p. 3838-3849, SEP 2007 2007. ISSN 0161-5890.

MOSMANN, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. **Journal of immunological methods,** v. 65, n. 1-2, p. 55-63, 1983. ISSN 0022-1759.

MUELLER, G. M. et al. Global diversity and distribution of macrofungi. **Biodiversity and conservation,** v. 16, n. 1, p. 37-48, 2007. ISSN 0960-3115.

NADAROGLU, H. et al. Green synthesis and characterisation of platinum nanoparticles using quail egg yolk. **Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy,** v. 172, p. 43-47, 2017. ISSN 1386-1425.

NATSUKI, J.; NATSUKI, T.; HASHIMOTO, Y. A review of silver nanoparticles: synthesis methods, properties and applications. **Inter J Mat Sci Appl,** v. 4, p. 325-332, 2015.

NG, L.; LIBERTINO, J. M. Adrenocortical carcinoma: diagnosis, evaluation and treatment. **The Journal of urology,** v. 169, n. 1, p. 5-11, 2003. ISSN 0022-5347.

NSF (National Science Foundation) (2001) Societal implications of nanoscience and nanotechnology. Arlington, VA (Also published by Kluwer Academic Publishing, 2001). See NSF website: http://www.nsf.gov/crssprgm/nano/reports/nsfnnireports.jsp

OH, J. Y. et al. Apoptosis of human hepatocarcinoma (HepG2) and neuroblastoma (SKN-SH) cells induced by polysaccharides-peptide complexes produced by submerged mycelial culture of an entomopathogenic fungus Cordyceps sphecocephala. **J Microbiol Biotechnol,** v. 18, n. 3, p. 512-9, 2008.

PARK, J. et al. Size dependent macrophage responses and toxicological effects of Ag nanoparticles. **Chemical communications,** v. 47, n. 15, p. 4382-4384, 2011.

PARK, Y. et al. Polysaccharides and phytochemicals: a natural reservoir for the green synthesis of gold and silver nanoparticles. **IET nanobiotechnology,** v. 5, n. 3, p. 69-78, 2011. ISSN 1751-875X.

PATEL, S.; GOYAL, A. Recent developments in mushrooms as anti-cancer therapeutics: a review. **3 Biotech,** v. 2, n. 1, p. 1-15, 2012. ISSN 2190-572X.

PERUGINI BIASI-GARBIN, R. et al. Effect of eugenol against Streptococcus agalactiae and synergistic interaction with biologically produced silver nanoparticles. **Evidence-Based Complementary and Alternative Medicine,** v. 2015, 2015. ISSN 1741-427X.

PHIELER, J. et al. The role of the complement system in metabolic organs and metabolic diseases. **Semin Immunol,** v. 25, n. 1, p. 47-53, Feb 2013. ISSN 1096-3618. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/23684628 >.

PICK, E.; KEISARI, Y. A simple colorimetric method for the measurement of hydrogen peroxide produced by cells in culture. **Journal of immunological methods,** v. 38, n. 1-2, p. 161-170, 1980. ISSN 0022-1759.

PINWEHA, S. et al. The signaling cascades of Ganoderma lucidum extracts in stimulating non-amyloidogenic protein secretion in human neuroblastoma SH-SY5Y cell lines. **Neuroscience letters,** v. 448, n. 1, p. 62-66, 2008. ISSN 0304-3940.

PIPE, R.; COLES, J.; FARLEY, S. Assays for measuring immune response in the mussel Mytilus edulis. **Tech Fish Immunol,** v. 4, p. 93-100, 1995.

PRIETO, P.; PINEDA, M.; AGUILAR, M. Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: specific application to the determination of vitamin E. **Analytical biochemistry,** v. 269, n. 2, p. 337-341, 1999. ISSN 0003-2697.

QI, G. et al. Effects of Ganoderma lucidum spores on sialoadenitis of nonobese diabetic mice. **Chinese Medical Journal,** v. 122, n. 5, p. 556-560, MAR 5 2009 2009. ISSN 0366-6999.

RACIBORSKA, A. et al. Solid Cancers in the Premature and the Newborn: Report of Three National Referral Centers. **Pediatrics & Neonatology**, 2015. ISSN 1875-9572.

RAI, M.; YADAV, A.; GADE, A. Silver nanoparticles as a new generation of antimicrobials. **Biotechnology advances,** v. 27, n. 1, p. 76-83, 2009. ISSN 0734-9750.

RAINEY, W. E.; BIRD, I. M.; MASON, J. I. The NCI-H295 cell line: a pluripotent model for human adrenocortical studies. **Molecular and cellular endocrinology,** v. 100, n. 1, p. 45-50, 1994. ISSN 0303-7207.

RAVEENDRAN, P.; FU, J.; WALLEN, S. L. Completely "green" synthesis and stabilization of metal nanoparticles. **Journal of the American Chemical Society,** v. 125, n. 46, p. 13940-13941, 2003. ISSN 0002-7863.

REDDY, J.; RAO, M. Lipid metabolism and liver inflammation. II. Fatty liver disease and fatty acid oxidation. **American Journal of Physiology-Gastrointestinal and Liver Physiology,** v. 290, n. 5, p. G852-G858, MAY 2006 2006. ISSN 0193-1857.

RIVERA, J.; LOYA, A.; CEBALLOS, R. **Use of Herbal Medicines and Implications for Conventional Drug Therapy Medical Sciences** Alternative and integrative medicine. 2**:** 1-6 p. 2013.

ROCO, M. C. **The long view of nanotechnology development: the National Nanotechnology Initiative at 10 years**: Springer 2011.

ROSEN, H.; GORDON, S. Monoclonal antibody to the murine type 3 complement receptor inhibits adhesion of myelomonocytic cells in vitro and inflammatory cell recruitment in vivo. **Journal of Experimental Medicine,** v. 166, n. 6, p. 1685-1701, 1987. ISSN 0022-1007.

RUSSELL, R.; PATERSON, M. Ganoderma - A therapeutic fungal biofactory. **Phytochemistry,** v. 67, n. 18, p. 1985-2001, SEP 2006 2006. ISSN 0031-9422.

RUSSO, A. et al. Pro-apoptotic activity of ergosterol peroxide and (22E)-ergosta-7,22-dien-5 alpha-hydroxy-3,6-dione in human prostate cancer cells. **Chemico-Biological Interactions,** v. 184, n. 3, p. 352-358, MAR 30 2010 2010. ISSN 0009-2797.

SAFAVI, A.; BANAZADEH, A.; SEDAGHATI, F. Synthesis of palladium nanoparticles on organically modified silica: Application to design of a solid-state electrochemiluminescence sensor for highly sensitive determination of imipramine. **Analytica chimica acta,** v. 796, p. 115-121, 2013. ISSN 0003-2670.

SATARUG, S. et al. Cadmium, environmental exposure, and health outcomes. **Environ Health Perspect,** v. 118, n. 2, p. 182-90, Feb 2010. ISSN 1552-9924. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/20123617 >.

SCHETT, G.; ZWERINA, J.; FIRESTEIN, G. The p38 mitogen-activated protein kinase (MAPK) pathway in rheumatoid arthritis. **Annals of the Rheumatic Diseases,** v. 67, n. 7, p. 909-916, JUL 2008 2008. ISSN 0003-4967.

SCHLUMBERGER, S. et al. Permeability characteristics of cell-membrane pores induced by ostreolysin A/pleurotolysin B, binary pore-forming proteins from the oyster mushroom. **FEBS letters,** v. 588, n. 1, p. 35-40, 2014. ISSN 1873-3468.

SEELEN, M. et al. Functional analysis of the classical, alternative, and MBL pathways of the complement system: standardization and validation of a simple ELISA. **Journal of Immunological Methods,** v. 296, n. 1-2, p. 187-198, JAN 2005 2005. ISSN 0022-1759.

SEN, I. K. et al. Green synthesis of silver nanoparticles using glucan from mushroom and study of antibacterial activity. **International journal of biological macromolecules,** v. 62, p. 439-449, 2013. ISSN 0141-8130.

SEO, H. W. et al. Steroids and triterpenes from the fruit bodies of Ganoderma lucidum and their anti-complement activity. **Arch Pharm Res,** v. 32, n. 11, p. 1573-9, Nov 2009. ISSN 0253-6269. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/20091270 >.

SHANTHI, K. et al. Cytotoxic Effect of Palladium Nanoparticles Synthesized From Syzygiumaromaticum Aqueous Extracts and Induction of Apoptosis in Cervical Carcinoma. **Proceedings of the National Academy of Sciences, India Section B: Biological Sciences**, p. 1-12, 2015. ISSN 0369-8211.

SHARMILA, G. et al. Green synthesis, characterization and antibacterial efficacy of palladium nanoparticles synthesized using Filicium decipiens leaf extract. **Journal of Molecular Structure,** v. 1138, p. 35-40, 2017. ISSN 0022-2860.

SHI, M.; ZHANG, Z.; YANG, Y. Antioxidant and immunoregulatory activity of Ganoderma lucidum polysaccharide (GLP). **Carbohydr Polym,** v. 95, n. 1, p. 200-6, Jun 2013. ISSN 1879-1344. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/23618260 >.

SIM CHOI, H. et al. A quantitative nitroblue tetrazolium assay for determining intracellular superoxide anion production in phagocytic cells. **Journal of Immunoassay and Immunochemistry,** v. 27, n. 1, p. 31-44, 2006. ISSN 1532-1819.

SINGLETON, V.; ROSSI, J. A. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. **American journal of Enology and Viticulture,** v. 16, n. 3, p. 144-158, 1965. ISSN 0002-9254.

SIU, K.-C.; CHEN, X.; WU, J.-Y. Constituents actually responsible for the antioxidant activities of crude polysaccharides isolated from mushrooms. **Journal of Functional Foods,** v. 11, p. 548-556, 2014. ISSN 1756-4646.

SLIVA, D. et al. Ganoderma lucidum suppresses motility of highly invasive breast and prostate cancer cells. **Biochemical and Biophysical Research Communications,** v. 298, n. 4, p. 603-612, NOV 8 2002 2002. ISSN 0006-291X.

_____. Biologic activity of spores and dried powder from Ganoderma lucidum for the inhibition of highly invasive human breast and prostate cancer cells. **Journal of Alternative and Complementary Medicine,** v. 9, n. 4, p. 491-497, AUG 2003 2003. ISSN 1075-5535.

SOLIS, G. M.; PETRASCHECK, M. Measuring Caenorhabditis elegans life span in 96 well microtiter plates. **JoVE (Journal of Visualized Experiments)**, n. 49, p. e2496-

e2496, 2011. ISSN 1940-087X.

STEELE, J. F. et al. Synthetic plant virology for nanobiotechnology and nanomedicine. **Wiley Interdisciplinary Reviews: Nanomedicine and Nanobiotechnology**, 2017. ISSN 1939-0041.

SUBER, L. et al. Preparation and the mechanisms of formation of silver particles of different morphologies in homogeneous solutions. **Journal of Colloid and Interface Science,** v. 288, n. 2, p. 489-495, 2005. ISSN 0021-9797.

SUN, K. et al. Anti-Aging Effects of Hesperidin on Saccharomyces cerevisiae via Inhibition of Reactive Oxygen Species and UTH1 Gene Expression. **Bioscience Biotechnology and Biochemistry,** v. 76, n. 4, p. 640-645, APR 2012 2012. ISSN 0916-8451.

SURENDRA, T. et al. RSM optimized Moringa oleifera peel extract for green synthesis of M. oleifera capped palladium nanoparticles with antibacterial and hemolytic property. **Journal of Photochemistry and Photobiology B: Biology,** v. 162, p. 550-557, 2016. ISSN 1011-1344.

TANG, W. et al. A randomized, double-blind and placebo-controlled study of a Ganoderma lucidum polysaccharide extract in neurasthenia. **Journal of Medicinal Food,** v. 8, n. 1, p. 53-58, SPR 2005 2005. ISSN 1096-620X.

THAKKAR, K. N.; MHATRE, S. S.; PARIKH, R. Y. Biological synthesis of metallic nanoparticles. **Nanomedicine: Nanotechnology, Biology and Medicine,** v. 6, n. 2, p. 257-262, 2010. ISSN 1549-9634.

TILBURT, J. C.; KAPTCHUK, T. J. Herbal medicine research and global health: an ethical analysis. **Bull World Health Organ,** v. 86, n. 8, p. 594-9, Aug 2008. ISSN 1564-0604. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/18797616 >.

TUMILOWICZ, J. J. et al. Definition of a continuous human cell line derived from neuroblastoma. **Cancer research,** v. 30, n. 8, p. 2110-2118, 1970. ISSN 0008-5472.

VIRKUTYTE, J.; VARMA, R. S. Green synthesis of metal nanoparticles: biodegradable polymers and enzymes in stabilization and surface functionalization. **Chemical Science,** v. 2, n. 5, p. 837-846, 2011.

WACHTEL-GALOR, S.; TOMLINSON, B.; BENZIE, I. F. Ganoderma lucidum ("Lingzhi"), a Chinese medicinal mushroom: biomarker responses in a controlled human supplementation study. **Br J Nutr,** v. 91, n. 2, p. 263-9, Feb 2004. ISSN 0007-1145. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/14756912 >.

WANG, E. et al. Silver nanoparticle induced toxicity to human sperm by increasing ROS (reactive oxygen species) production and DNA damage. **Environmental Toxicology and Pharmacology,** v. 52, p. 193-199, 2017. ISSN 1382-6689.

WANG, J. et al. Active lipids of Ganoderma lucidum spores-induced apoptosis in human leukemia THP-1 cells via MAPK and PI3K pathways. **Journal of Ethnopharmacology,**

v. 139, n. 2, p. 582-589, JAN 31 2012 2012. ISSN 0378-8741.

WANG, L. et al. Characterization of silver nanoparticles biosynthesized using crude polysaccharides of Psidium guajava L. leaf and their bioactivities. **Materials Letters**, 2017. ISSN 0167-577X.

WANG, Q. et al. Triple-enzyme mimetic activity of nickel–palladium hollow nanoparticles and their application in colorimetric biosensing of glucose. **Chemical Communications,** v. 52, n. 31, p. 5410-5413, 2016.

WANG, S. et al. Intervention Effects of Ganoderma Lucidum Spores on Epileptiform Discharge Hippocampal Neurons and Expression of Neurotrophin-4 and N-Cadherin. **Plos One,** v. 8, n. 4, APR 24 2013 2013. ISSN 1932-6203.

WANG, X. et al. A study of Ganoderma lucidum spores by FTIR microspectroscopy. **Spectrochimica Acta Part a-Molecular and Biomolecular Spectroscopy,** v. 91, p. 285-289, JUN 2012 2012. ISSN 1386-1425.

WENDT, K. U. et al. Enzyme Mechanisms for Polycyclic Triterpene Formation. **Angew Chem Int Ed Engl,** v. 39, n. 16, p. 2812-2833, Aug 2000. ISSN 1521-3773. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/11027983 >.

WENG, Y. et al. Ganodermasides A and B, two novel anti-aging ergosterols from spores of a medicinal mushroom Ganoderma lucidum on yeast via UTH1 gene. **Bioorganic & Medicinal Chemistry,** v. 18, n. 3, p. 999-1002, FEB 1 2010 2010. ISSN 0968-0896.

WICKS, S. et al. Safety and tolerability of Ganoderma lucidum in healthy subjects: A double-blind randomized placebo-controlled trial. **American Journal of Chinese Medicine,** v. 35, n. 3, p. 407-414, 2007 2007. ISSN 0192-415X.

WU, G. S. et al. Anti-cancer properties of triterpenoids isolated from Ganoderma lucidum - a review. **Expert Opin Investig Drugs,** v. 22, n. 8, p. 981-92, Aug 2013. ISSN 1744-7658. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/23790022 >.

WU, J. et al. Comparison of Chemical Composition of Spore Lipids Extracted from Two Species of Ganoderma. **Chemistry of Natural Compounds,** v. 49, n. 4, p. 736-737, SEP 2013 2013. ISSN 0009-3130.

WU, Q. et al. Tumour cell adhesion and integrin expression affected by Ganoderma lucidum. **Enzyme and Microbial Technology,** v. 40, n. 1, p. 32-41, DEC 6 2006 2006. ISSN 0141-0229.

WU, Q. P. et al. Ergosterol peroxide isolated from Ganoderma lucidum abolishes microRNA miR-378-mediated tumor cells on chemoresistance. **PLoS One,** v. 7, n. 8, p. e44579, 2012. ISSN 1932-6203. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/22952996 >.

WU, T. S.; SHI, L. S.; KUO, S. C. Cytotoxicity of Ganoderma lucidum triterpenes. **J Nat Prod,** v. 64, n. 8, p. 1121-2, Aug 2001. ISSN 0163-3864. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/11520245 >.

X, L.; WANG, J.; YUAN, J. Pharmacological and anti-tumor activities of Ganoderma spores processed by top-down approaches. **Journal of Nanoscience and Nanotechnology,** v. 5, n. 12, p. 2001-2013, DEC 2005 2005. ISSN 1533-4880.

XIAOPING, C. et al. Free radical scavenging of Ganoderma lucidum polysaccharides and its effect on antioxidant enzymes and immunity activities in cervical carcinoma rats. **Carbohydrate Polymers,** v. 77, n. 2, p. 389-393, 2009. ISSN 0144-8617.

XIE, Y. et al. Ganoderma lucidum inhibits tumour cell proliferation and induces tumour cell death. **Enzyme and Microbial Technology,** v. 40, n. 1, p. 177-185, DEC 6 2006 2006. ISSN 0141-0229.

XING, Y. et al. Localized Synthesis of Conductive Copper–Tetracyanoquinodimethane Nanostructures in Ultrasmall Microchambers for Nanoelectronics. **ACS Applied Materials & Interfaces,** v. 9, n. 20, p. 17271-17278, 2017. ISSN 1944-8244.

YAN, B. et al. Ganoderma lucidum Spore Induced CA72-4 Elevation in Gastrointestinal Cancer: A Five-Case Report. **Integr Cancer Ther,** v. 13, n. 2, p. 161-6, Mar 2014. ISSN 1552-695X. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/24282100 >.

YAN, J.-K.; WANG, W.-Q.; WU, J.-Y. Recent advances in Cordyceps sinensis polysaccharides: Mycelial fermentation, isolation, structure, and bioactivities: A review. **Journal of Functional Foods,** v. 6, p. 33-47, 2014. ISSN 1756-4646.

YAN, Z. et al. Fast analysis of triterpenoids in Ganoderma lucidum spores by ultra-performance liquid chromatography coupled with triple quadrupole mass spectrometry. **Biomedical Chromatography,** v. 27, n. 11, p. 1560-1567, NOV 2013 2013. ISSN 0269-3879.

YANG, C. et al. Cordyceps militaris and mycelial fermentation induced apoptosis and autophagy of human glioblastoma cells. **Cell death & disease,** v. 3, n. 11, p. e431, 2012.

YANG, G. et al. **Ganoderma spore lipid inhibits N-methyl-N-nitrosourea-induced retinalphotoreceptor apoptosis in vivo**: Experimental Eye Research. 90**:** 397-404 p. 2010.

YANG, X. et al. Green synthesis of palladium nanoparticles using broth of Cinnamomum camphora leaf. **Journal of Nanoparticle Research,** v. 12, n. 5, p. 1589-1598, 2010. ISSN 1388-0764.

YEH, C. et al. Polysaccharides PS-G and Protein LZ-8 from Reishi (Ganoderma lucidum) Exhibit Diverse Functions in Regulating Murine Macrophages and T Lymphocytes. **Journal of Agricultural and Food Chemistry,** v. 58, n. 15, p. 8535-8544, AUG 11 2010 2010. ISSN 0021-8561.

YEN, H. J.; HSU, S. H.; TSAI, C. L. Cytotoxicity and immunological response of gold and silver nanoparticles of different sizes. **Small,** v. 5, n. 13, p. 1553-1561, 2009. ISSN 1613-6829.

YIN, H. et al. Large-scale and size-controlled synthesis of silver nanoparticles under

microwave irradiation. **Materials chemistry and Physics,** v. 83, n. 1, p. 66-70, 2004. ISSN 0254-0584.

YUAN, J.; WANG, J.; LIU, X. Distribution of free and esterified ergosterols in the medicinal fungus Ganoderma lucidum. **Applied Microbiology and Biotechnology,** v. 77, n. 1, p. 159-165, NOV 2007 2007. ISSN 0175-7598.

YUAN, J. et al. Determination of ergosterol in Ganoderma spore lipid from the germinating spores of Ganoderma lucidum by high-performance liquid chromatography. **Journal of Agricultural and Food Chemistry,** v. 54, n. 17, p. 6172-6176, AUG 23 2006 2006. ISSN 0021-8561.

YUE, G. et al. Comparative Studies on the Immunomodulatory and Antitumor Activities of the Different Parts of Fruiting Body of Ganoderma lucidum and Ganoderma Spores. **Phytotherapy Research,** v. 22, n. 10, p. 1282-1291, OCT 2008 2008. ISSN 0951-418X.

YUE, Q. X. et al. Effects of triterpenes from Ganoderma lucidum on protein expression profile of HeLa cells. **Phytomedicine,** v. 17, n. 8-9, p. 606-13, Jul 2010. ISSN 1618-095X. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/20092987 >.

YUKUYAMA, M. et al. Nanoemulsion: process selection and application in cosmetics–a review. **International journal of cosmetic science,** v. 38, n. 1, p. 13-24, 2016. ISSN 1468-2494.

YUSOP, R. M. et al. Palladium-mediated intracellular chemistry. **Nature Chemistry,** v. 3, n. 3, p. 239-243, 2011. ISSN 1755-4330.

ZHANG, J. et al. Regulation of endothelial cell adhesion molecule expression by mast cells, macrophages, and neutrophils. **PloS one,** v. 6, n. 1, p. e14525, 2011. ISSN 1932-6203.

_____. Extraction, characterization and antioxidant activity of polysaccharides of spent mushroom compost of Ganoderma lucidum. **International journal of biological macromolecules,** v. 82, p. 432-439, 2016. ISSN 0141-8130.

ZHANG, J. P. et al. Lipid extract from completely sporoderm-broken germinating Ganoderma sinensis spores elicits potent antitumor immune responses in human macrophages. **Phytother Res,** v. 23, n. 6, p. 844-50, Jun 2009. ISSN 1099-1573. Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/19117333 >.

ZHANG, S.; WANG, S. Effects of Ganoderma lucidum spore powder on astrocyte expression and glutamine synthetase activity in the hippocampal region of epileptic rats. **Neural Regeneration Research,** v. 3, n. 12, p. 1304-1307, DEC 2008 2008. ISSN 1673-5374.

ZHANG, W.; ZHONG, J. Effect of oxygen concentration in gas phase on sporulation and individual ganoderic acids accumulation in liquid static culture of Ganoderma lucidum. **Journal of Bioscience and Bioengineering,** v. 109, n. 1, p. 37-40, JAN 2010 2010. ISSN 1389-1723.

ZHANG, X.-F. et al. Trichostatin a enhances the apoptotic potential of palladium

nanoparticles in human cervical cancer cells. **International journal of molecular sciences,** v. 17, n. 8, p. 1354, 2016.

ZHANG, X.-Y. et al. Seeds triggered massive synthesis and multi-step room temperature post-processing of silver nanoink—application for paper electronics. **RSC Advances,** v. 7, n. 1, p. 8-19, 2017.

ZHAO, F. et al. Cellular uptake, intracellular trafficking, and cytotoxicity of nanomaterials. **Small,** v. 7, n. 10, p. 1322-1337, 2011. ISSN 1613-6829.

ZHAO, H. et al. Spore Powder of Ganoderma lucidum Improves Cancer-Related Fatigue in Breast Cancer Patients Undergoing Endocrine Therapy: A Pilot Clinical Trial. **Evidence-Based Complementary and Alternative Medicine**, 2012 2012. ISSN 1741-427X.

ZHAO, S. et al. Effect of Ganoderma lucidum spore on expression of insulin-like growth factor-1, nuclear factor-kappa B, and neuronal apoptosis in the epileptic rat brain. **Neural Regeneration Research,** v. 3, n. 8, p. 858-862, AUG 2008 2008. ISSN 1673-5374.

ZHAO, S.; ZHANG, S.; WANG, S. Ganoderma lucidum spore powder modulates Bcl-2 and Bax expression in the hippocampus and cerebral cortex, and improves learning and memory in pentylenetetrazole-kindled rats. **Neural Regeneration Research,** v. 6, n. 30, p. 2347-2351, OCT 2011 2011. ISSN 1673-5374.

ZHAO, W. et al. Antioxidant activities of Ganoderma lucidum polysaccharides and their role on DNA damage in mice induced by cobalt-60 gamma-irradiation. **Food and Chemical Toxicology,** v. 50, n. 2, p. 303-309, FEB 2012 2012. ISSN 0278-6915.

ZHONG, J.-J.; XIAO, J.-H. Secondary metabolites from higher fungi: discovery, bioactivity, and bioproduction. In: (Ed.). **Biotechnology in China I**: Springer, 2009. p.79-150.

ZHOU, K.; PINCUS, Z.; SLACK, F. Longevity and stress in Caenorhabditis elegans. **Aging-Us,** v. 3, n. 8, p. 733-753, AUG 2011 2011. ISSN 1945-4589.

ZHOU, Y. et al. Neuroprotective effect of preadministration with Ganoderma lucidum spore on rat hippocampus. **Experimental and Toxicologic Pathology,** v. 64, n. 7-8, p. 673-680, NOV 2012 2012. ISSN 0940-2993.

ZHU, H. et al. Effects of extracts from sporoderm-broken spores of Ganoderma lucidum on HeLa cells. **Cell Biology and Toxicology,** v. 16, n. 3, p. 201-206, 2000 2000. ISSN 0742-2091.

ZHU, K. et al. Influence of Ganoderma lucidum spores on the levels of neuropeptide Y and somatostatin in brains of seizure rats. **Neural Regeneration Research,** v. 3, n. 5, p. 486-489, MAY 2008 2008. ISSN 1673-5374.

ZHU, X. et al. **Mechanochemical-assisted extraction and antioxidant activity of polysaccharides from *Ganoderma lucidum* spores**: International Journal of Food and Technology. 47**:** 927-932 p. 2012.