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ALEX GRAÇA CONTATO

BIOLOGICAL PROPERTIES OF BASIDIOMYCETES ISOLATED FROM BRAZILIAN ATLANTIC FOREST

Maringá
Fevereiro - 2018

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Dissertação apresentada ao Programa de Pós-Graduação em Ciências Biológicas (área de concentração - Biologia Celular e Molecular), da Universidade Estadual de Maringá para a obtenção do grau de Mestre em Ciências Biológicas.

Orientadora: Prof.ª Dr.ª Cristina Giatti Marques de Souza
Coorientadora: Prof.ª Dr.ª Anacharis Babeto de Sá-Nakanishi

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Aprovado em: 21/02/2018

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BIOGRAFIA

Alex Graça Contato nasceu em Arapongas/PR em 17 de junho de 1994. Filho de Anésio Contato e Antonia da Graça Contato. Possui graduação em Bioquímica pela Universidade Estadual de Maringá (2016), com experiência em Bioquímica de Microrganismos, atuando principalmente nos seguintes temas: atividade antioxidante, atividade antimicrobiana, citotoxicidade e basidiomicetos. No entanto, também já atuou na área de Microbiologia Médica, em especial, com protozoários do gênero *Leishmania* e com ascomicetos. Aprovado para realizar o doutorado no Programa de Pós-Graduação em Bioquímica da Faculdade de Medicina de Ribeirão Preto da Universidade de São Paulo.

À minha família,
na sua perfeita composição.

"Quanto mais eu estudo a natureza, mais me maravilho com a obra do Criador."

Louis Pasteur (1822-1895)

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Um agradecimento também a todos os meus familiares, amigos e colegas de laboratório. Sem a força e orações de todos, não seria o que sou hoje e muito menos chegado a este momento.

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APRESENTAÇÃO

Este trabalho foi realizado no Laboratório de Bioquímica e Fisiologia de Microrganismos, no Laboratório de Metabolismo Hepático e no Laboratório de Inflamação, ambos da Universidade Estadual de Maringá. A presente dissertação está apresentada na forma de um artigo científico.

Em consonância com as normas do Programa de Pós-Gradução em Ciências Biológicas (Biologia Celular e Molecular), o artigo foi redigido de acordo com o periódico **"FEMS Microbiology Ecology"**.

Alex Graça Contato, Tatiane Brugnari, Ana Paula Ames Sibin, Ana Júlia dos Reis Buzzo, Anacharis Babeto de Sá-Nakanishi, Lívia Bracht, Ciomar Aparecida Bersani-Amado, Rosane Marina Peralta & Cristina Giatti Marques de Souza. **Biological Properties of Basidiomycetes isolated from Brazilian Atlantic Forest.**

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LISTA DE ABREVIATURAS

AAPH 2,2′-Azobis(2-metillpropionamidina) dihidroclorido

ABTS [(2,2-azino-bis(3-etilbenzotiazolina-6-ácido sulfônico))]

ADP Adenina Dinucleotídeo

BD Batata Dextrose

BDA Ágar Batata Dextrose

BHT Hidroxitolueno butilado

CE Concentração Efetiva

CBM Concentração Bactericida Mínima

CFM Concentração Fungicida Mínima

CIM Concentração Inibitória Mínima

DMSO Dimetil Sulfóxido

DPPH (1,1-diphenyl-2-picrilhydrazyl)

EAG Equivalentes de ácido gálico

EGTA Ácido egtázico

EROS Espécies Reativas de Oxigênio

FAD Flavina Adenina Dinucleotídeo

FRAP Poder Antioxidante de Redução Férrica

MEM Meio Essencial Mínimo

MH Müller Hinton

MTT Brometo de 3-(4,5-Dimetilltiazol-2-il)-2,5-difeniltetrazólio

NAD^+ Niconamida Adenina Dinucleotídeo Oxidada

NADH Niconamida Adenina Dinucleotídeo Reduzida

ORAC Capacidade de Absorbância do Radical Oxigênio

PBS Tampão fosfato-salino

PMSF Fenilmetilsulfonil fluoreto

RC Controle respiratório

TEAC Atividade antioxidante equivalente ao Trolox

TPTZ (2,4,6-tripiridil-*s*-diazina)

Trolox (6-hidroxi-2,5,7,8-tetrametilcromo-2-ácido carboxílico)

UFC Unidades Formadoras de Colônias

RESUMO GERAL

INTRODUÇÃO

A oxidação é um processo vital aos organismos vivos para a geração de energia a partir dos combustíveis biológicos. Contudo, pode ser que ocorra o efeito denominado de estresse oxidativo, ou seja, a produção em excesso das espécies reativas do oxigênio (EROS) e outros radicais, afetando assim, a estrutura e a organização celular. O organismo possui mecanismos de proteção, todavia, um desequilíbrio na produção de EROS pode afetar as funções fisiológicas e resultar em doenças sérias, como por exemplo: câncer, artrite reumatóide, aterosclerose e processos degenerativos associados à idade.

Estudos comprovam que os cogumelos, nome popular dado às frutificações de alguns tipos de fungos (das divisões Basidiomycota e Ascomycota), são capazes de produzir compostos antioxidantes, antimicrobianos e antitumorais. Sua eficácia está no fato de sintetizar diversos metabólitos secundários, tais como os compostos fenólicos, um grupo de substâncias distribuídas com abundância entre os vegetais e cogumelos, que participam do controle de algumas enfermidades devido ao seu poderio antioxidante. As duas formas, basidioma e micélio são amplamente estudados para usos na indústria alimentícia, inclusive no que diz respeito a alimento funcional, mas com relação às propriedades biológicas e à capacidade antioxidante do micélio, pouco tem sido descrito na literatura.

Estima-se que o número de espécies de cogumelos é maior que 100.000 e que apenas 15-20% são conhecidas. Considerando o número de espécies descritas, são poucas as que ainda foram investigadas quanto ao seu conteúdo em metabólitos secundários, atividade antioxidante, antimicrobiana e demais propriedades, tais como citotoxicidade. No Brasil, existe em torno de 20.000 espécies de basidiomicetos, a maioria localizada em regiões tropicais e sub-tropicais. Devido às quantidades de biomas presentes neste país, é possível a realização de pesquisas na área de bioprospecção de novos microrganismos, em especial, os basidiomicetos.

A Mata Atlântica brasileira é um dos biomas mais diversificados existente no planeta, sendo até mesmo reconhecida por possuir uma maior variedade por área do que a própria Floresta Amazônica. Estende-se de regiões tropicais a sub-tropicais e estima-se que sua fauna e flora correspondam de 1-8% do total de espécies mundiais, além

disso, possuem uma enorme diversidade de microrganismos. Estudos indicam que os fungos exercem um papel essencial na conservação deste tipo de floresta.

OBJETIVOS

Desta forma este trabalho possui como natureza inovadora descrever a capacidade antioxidante, antimicrobiana e de citotoxicidade de alguns dos isolados de basidiomicetos. O micélio foi utilizado para obtenção dos extratos. Os fungos foram escolhidos de acordo com a escassez de relatos literários sobre os mesmos. No entanto, isolados de espécies de gêneros conhecidos (*Pleurotus* e *Phellinus*) foram também avaliadas para efeito de comparação.

MATERIAIS E MÉTODOS

Foram avaliados oito isolados de basidiomicetos, gentilmente cedidos pela Embrapa Florestas (Colombo, PR, Brasil): *Flaviporus venustus* EF30, *Hydnopolyporus fimbriatus* EF41 e EF44, *Inonotus splitgerberi* EF46, *Oudemansiella canarii* EF72, *Perenniporia* sp. EF79, *Phellinus linteus* EF81 e *Pleurotus albidus* EF84.

As bactérias e leveduras utilizadas foram cedidas pelo Departamento de Ciências Básicas da Saúde (DBS) da Universidade Estadual de Maringá (UEM) para os testes antimicrobianos.

Ratos Wistar machos (~200 g) alimentados *ad libitum* com uma dieta padrão do laboratório (Nuvilab®, Colombo, Brazil) foram utilizados nos experimentos. Todos os experimentos foram aprovados pela Comissão de Ética no Uso de Animais da Universidade Estadual de Maringá (CEUA/UEM) sob o protocolo nº 7669090317.

As propriedades biológicas analisadas foram: capacidade antioxidante, atividade antimicrobiana e citotoxicidade, e cada uma delas foi avaliada por diferentes metodologias. As atividades antioxidantes foram determinadas pelos métodos do ABTS, TEAC, FRAP e ORAC; a antimicrobiana pela técnica de disco-difusão, CIM, CBM e CFM; enquanto que a avaliação da citotoxicidade foi pelos métodos de respiração mitocondrial e citotoxicidade em macrófagos peritoneais murinos.

RESULTADOS E DISCUSSÃO

Todos os cogumelos apresentaram destaques, no entanto, *H. fimbriatus* e *F. venustus* foram os que apresentaram os melhores valores nos ensaios antioxidantes; *F. venustus*, *P. linteus* e *P. albidus* resultaram em atividade antimicrobiana; enquanto *I. splitgerberi* e *P. albidus* apresentaram resultados que comprovam suas citotoxicidades.

Entre os oito basidiomicetos cujos micélios foram avaliados neste estudo, *H. fimbriatus* destacou-se com bons resultados em todos os testes de atividade antioxidante e citotoxicidade. Este fungo, conhecido por ser um destruidor de madeiras e produtor de celulases, já foi descrito na literatura por possuir atividade antimicrobiana, mas não atividade antioxidante e de citotoxicidade, como foi verificado neste estudo. Os dois isolados testados apresentaram diferenças de resultados com destaque para o isolado EF41 o que reforça a importância de estudos de bioprospecção.

CONCLUSÕES

Os dados obtidos mostram que os micélios avaliados também podem ser uma importante fonte de antioxidantes. O cogumelo *H. fimbriatus* foi o que apresentou os resultados mais significativos nos testes antioxidantes. Três basidiomicetos apresentaram atividade antimicrobiana, indicando um potencial para novos estudos. Os resultados apresentados sugerem que *I. spligerberi* possui uma atividade desacopladora, mesmo na menor concentração testada, dissipando o gradiente eletroquímico mitocondrial. Por outro lado, *P. albidus* exerceu efeito somente sobre a atividade da succinato-oxidase não influenciando a eficiência respiratória mitocondrial. Portanto, ambos interferem negativamente na respiração mitocondrial, inferindo assim, suas citotoxicidades. A partir dos dados obtidos, pode-se ressaltar a importância de pesquisas de bioprospecção em biomas pouco explorados, como por exemplo, a Mata Atlântica.

Palavras-chave: antioxidante; antimicrobiano; citotoxicidade; cogumelos; Mata Atlântica; diversidade brasileira.

ABSTRACT

INTRODUCTION

Oxidation is a vital process for living organisms to generate energy from biological fuels. However, it may be that the so-called oxidative stress effect, that is, excess production of reactive oxygen species (ROS) and other radicals, thus affecting cell structure and organization. The organism has mechanisms of protection however an imbalance in the production of ROS can affect the physiological functions and result in serious diseases, such as cancer, rheumatoid arthritis, atherosclerosis and age-related degenerative processes.

Studies have shown that mushrooms, a popular name given to the fruiting of some types of fungi (from the Basidiomycota and Ascomycota divisions), are capable of producing antioxidant, antimicrobial and antitumor compounds. Its efficacy is in the fact of producing several secondary metabolites, such as phenolic compounds, a group of substances distributed with abundance between vegetables and mushrooms that participate in the control of some diseases due to its antioxidant power. The two forms, basidioma and mycelium are widely studied for uses in the food industry, including with regard to functional food, but with respect to the biological properties and the antioxidant capacity of the mycelium, little has been described in the literature.

It is estimated that the number of species of mushrooms is greater than 100,000 and that only 15-20% are known. Considering the number of species described, few are still investigated for their content in secondary metabolites, antioxidant activity, antimicrobial and other properties, such as cytotoxicity. In Brazil, there are around 20,000 species of basidiomycetes, most of them located in tropical and subtropical regions. Due to the quantities of biomes present in this country, it is possible to carry out research in the area of bioprospecting of new microorganisms, especially basidiomycetes.

The Brazilian Atlantic Forest is one of the most diversified biomes on the planet, and is even recognized for having a greater variety by area than the Amazon Forest itself. It extends from tropical to subtropical regions and it is estimated that its fauna and flora correspond to 1-8% of the total world species, besides have an enormous diversity of microorganisms. Studies indicate that fungi play an essential role in the conservation of this type of forest.

AIMS

In this way, this work has the innovative nature to describe for the first time the antioxidant, antimicrobial and cytotoxicity capacity of some of the isolates of basidiomycetes. To obtain the extracts the mycelium was used. The fungi were chosen according to the scarcity of literary reports about them. However, isolates of species of known genera (*Pleurotus* and *Phellinus*) were also evaluated for comparison.

MATERIALS AND METHODS

Eight isolates of basidiomycetes, kindly provided by Embrapa Florestas (Colombo, PR, Brazil) were evaluated: *Flaviporus venustus* EF30, *Hydnopolyporus fimbriatus* EF41 and EF44, *Inonotus splitgerberi* EF46, *Oudemansiella canarii* EF72, *Perenniporia* sp. EF79, *Phellinus linteus* EF81 and *Pleurotus albidus* EF84.

The bacteria and yeasts used were provided by the Department of basic Health Sciences (DBS) of the State University of Maringá (UEM) for the antimicrobial assay.

Male Wistar rats (~200 g) fed *ad libitum* with a standard laboratory diet (Nuvilab®, Colombo, Brazil) were used in the experiments. All experiments were approved by the Committee on Ethics in the Use of Animals of the State University of Maringá (CEUA/UEM) under the protocol nº 7669090317.

The biological properties analyzed were antioxidant capacity, antimicrobial activity and cytotoxicity, and each of them was evaluated by different methodologies. The antioxidant activities were analysed by ABTS, TEAC, FRAP and ORAC methods; the antimicrobial by the disc-diffusion technique, MIC, MBC and MFC; while evaluation of cytotoxicity was by mitochondrial respiration and cytotoxicity in murine peritoneal macrophages.

RESULTS AND DISCUSSION

All the mushrooms presented highlights, however, *H. fimbriatus* and *F. venustus* presented the best values in the antioxidant methods; *F. venustus*, *P. linteus* and *P. albidus* resulted in antimicrobial activity; while *I. splitgerberi* and *P. albidus* presented results that prove their cytotoxicity.

Among the eight basidiomycetes whose mycelium were evaluated in this study, *H. fimbriatus* stood out with good results in all tests of antioxidant activity and cytotoxicity. This fungus, known to be a wood destroyer and producer of cellulases, has already been described in the literature as having antimicrobial activity, but not antioxidant activity and cytotoxicity, as were in this study. The two isolates tested showed differences of results with emphasis on the isolate EF41, which reinforces the importance of bioprospecting studies.

CONCLUSIONS

The data obtained show that the mycelium evaluated can also be an important source of antioxidants. The *H. fimbriatus* was the one that presented the most significant results in the antioxidant assays. Three basidiomycetes presented antimicrobial activity, indicating a potential for further studies. The results presented suggest that *I. spligerberi* has a decoupling activity, even at the lowest concentration tested, dissipating the mitochondrial electrochemical gradient. On the other hand, *P. albidus* exerted only effect on succinate-oxidase activity without influencing mitochondrial respiratory efficiency. Therefore, both interfere negatively in mitochondrial respiration, thus inferring their cytotoxicities. From the data obtained, it is possible to emphasize the importance of bioprospecting research in little explored biomes, such as the Atlantic Forest.

Keywords: antioxidant; antimicrobial; cytotoxicity; mushrooms; Atlantic Forest; Brazilian diversity.

Biological Properties of Basidiomycetes isolated from Brazilian Atlantic Forest

Alex Graça Contato[1,*], Tatiane Brugnari[1], Ana Paula Ames Sibin[2], Ana Julia dos Reis Buzzo[1], Anacharis Babeto de Sá-Nakanishi[2], Lívia Bracht[2], Ciomar Aparecida Bersani-Amado[3], Rosane Marina Peralta[1] and Cristina Giatti Marques de Souza[1]

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Once sentence summary: Basidiomycetes isolated from Brazilian Atlantic Forest were studied in relation its biological properties: antioxidant activity, antimicrobial and cytotoxicity by the first time.

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ABSTRACT: There are different varieties of mushrooms not yet studied spread all over the planet, especially biomes where little is known about the existing microorganisms. Considering the number of species described, there is still little research regarding its content in secondary metabolites, antioxidant activity, antimicrobial and cytotoxicity. The objective of this study was to evaluate the biological properties of eight basidiomycetes isolated from the Brazilian Atlantic Forest, using their mycelium. The fungi studied were *Flaviporus venustus* EF30, *Hydnopolyporus fimbriatus* EF41 and EF44, *Inonotus splitgerberi* EF46, *Oudemansiella canarii* EF72, *Perenniporia* sp. EF79, *Phellinus linteus* EF81 and *Pleurotus albidus* EF84. All the mushrooms presented highlights, however, *H. fimbriatus* and *F. venustus* presented the best values in the antioxidant trials; *F. venustus*, *P. linteus* and *P. albidus* resulted in antimicrobial activity; while *I. splitgerberi* and *P. albidus* presented results that prove their cytotoxicity.

Keywords: antioxidant activity; antimicrobial; cytotoxicity; mushrooms; Atlantic Forest; Brazilian diversity.

INTRODUCTION

Oxidation is a vital process for living organisms to generate energy from biological fuels. However, it may be that the so-called oxidative stress effect, that is, excess production of reactive oxygen species (ROS) and other radicals, thus affecting cell structure and organization (Reczek and Chandel 2015). The organism has mechanisms of protection, however, an imbalance in the production of ROS can affect the physiological functions and result in serious diseases, such as cancer, rheumatoid arthritis, atherosclerosis and age-related degenerative processes (Kaludercic, Dushwal and Di Lisa 2014).

Studies have shown that mushrooms, a popular name given to the fruiting of some types of fungi (from the Basidiomycota and Ascomycota divisions) (Nagano and Nagahama 2012), are capable of producing antioxidant, antimicrobial and antitumor compounds (Ivanova et al. 2014). Its efficacy is in the fact of producing several secondary metabolites, such as phenolic compounds, a group of substances distributed with abundance between vegetables and mushrooms that participate in the control of some diseases due to its antioxidant power (Chen et al. 2018; Corrêa et al. 2015). The production of basidioma of several mushrooms is well known and standardized, being practiced by producers around the planet. However, there is also the biomass production of some species under submerged conditions, practiced in recent years for use as food, nutraceutical preparations (Giavasis 2014), anti-inflammatory agents (Eusayed et al. 2014) source of protein or lipid in feeds, extraction of aromas and other metabolites such as enzymes or polysaccharides (Ren, Perera and Hemar 2012). The two forms, basidioma and mycelium are widely studied for uses in the food industry, including with regard to functional food (Corrêa et al. 2016), but with respect to the biological

properties and the antioxidant capacity of the mycelium, little has been described in the literature.

It is estimated that the number of species of mushrooms is greater than 100,000 and that only 15-20% are known. Considering the number of species described, few are still investigated for their content in secondary metabolites, antioxidant activity, antimicrobial and other properties, such as cytotoxicity. In Brazil, there are around 20,000 species of basidiomycetes, most of them located in tropical and subtropical regions (Santos-Silva, Aprile and Scudeller 2005). Due to the quantities of biomes present in this country, it is possible to carry out research in the area of bioprospecting of new microorganisms (Menezes et al. 2010; Nascimento et al. 2015), especially basidiomycetes. In this context, Embrapa Florestas (Colombo-PR), yielded a variety of isolates from of the collection cultures obtained through a bioprospecting program in the Brazilian Atlantic Forest, and many of these isolates have not yet been investigated.

The Brazilian Atlantic Forest is one of the most diversified biomes on the planet, and is even recognized for having a greater variety by area than the Amazon Forest itself (Joly, Metzger and Tabarelli 2014). It extends from tropical to subtropical regions and it is estimated that its fauna and flora correspond to 1-8% of the total world species (Ribeiro et al. 2009), besides have an enormous diversity of microorganisms (Ávila et al 2017; Dall'Agnol et al. 2017). Studies indicate that fungi play an essential role in the conservation of this type of forest (Halme, Holec and Heilmann-Clausen 2017).

In this way, this work has the innovative nature to describe for the first time: (I) antioxidant capacity; (II) antimicrobial activity and; (III) cytotoxicity of some isolates of basidiomycetes, but using mycelium to obtain extracts. The fungi were chosen according to the scarcity of literary reports about them. However, isolates of species of known genera (*Pleurotus* and *Phellinus*) were also evaluated for comparison.

MATERIALS AND METHODS

Biological materials

Eight isolated of basidiomycetes, kindly provided by Embrapa Florestas (Colombo, PR, Brazil) were evaluated: *Flaviporus venustus* EF30, *Hydnopolyporus fimbriatus* EF41 and EF44, *Inonotus splitgerberi* EF46, *Oudemansiella canarii* EF72, *Perenniporia* sp. EF79, *Phellinus linteus* EF81 and *Pleurotus albidus* EF84.

The bacteria and yeasts for microbiological assays were carried out by the Department of Basic Health Sciences (DBS) of the State University of Maringá (UEM). The bacteria were: *Bacillus cereus* INCGS 000003, *Bacillus subtilis* ATCC 6051, *Escherichia coli* ATCC 25922, *Klesbisiella pneumoniae* ATCC 700603, *Pseudomonas aeruginosa* ATCC 15442, *Salmonella enterica* ATCC 13076 and *Staphylococcus aureus* ATCC 25923. While yeasts were: *Candida albicans* ATCC 10231 and *Saccharomyces cerevisiae* (Sigma, St. Louis, MO, USA).

Male Wistar rats (~200 g) fed *ad libitum* with a standard laboratory diet (Nuvilab®, Colombo, Brazil) kept in a light-dark cycle were used in the experiments. According to the protocols, they were fasted 18 h prior to all experiments. All experiments were approved by the Committee on Ethics in the Use of Animals of the State University of Maringá (CEUA/UEM) under the protocol nº 7669090317.

Maintenance of fungi and culture medium

The fungi were removed from the Castellani medium and reactivated in agar-wheat bran extract medium (2% agar and 2% wheat bran). To obtain the biomass, a

Petri dish was scraped with the aid of a spatula and the mycelium transferred to Erlenmeyer flasks containing liquid medium of wheat bran extract. The Erlenmeyer flasks (125 mL) containing 25 mL of medium were shaken for five days at 28 °C at 120 rpm for the production of biomass. After this period, the biomass produced was transferred to 250 mL Erlenmeyer flasks, containing 50 mL of the same medium, where they kept for another five days at 28 ºC and shaking at 120 rpm. All materials used were sterilized at 121 °C and 1 atm for 20 min prior to use and handled under aseptic conditions.

Preparation of biomass and extracts

The biomass obtained from the culture medium through filtration was washed with distilled water and placed to lyophilize. The aqueous extraction was performed in a ratio of 1 g of mycelium to 25 mL of sterile water. The material was stirred for one hour at 120 rpm and at 28 °C. The material was vacuum filtered and the residue subjected to the same procedure twice. The filtrates were pooled and subjected to lyophilize. The lyophilized extracts were stored in a freezer at -20 °C until use. The procedure was performed in triplicate.

Total phenolic compounds

The total phenolic compounds in the aqueous extracts were estimated using the Folin-Ciocalteu reagent, according to the method of Singleton and Rossi (1965) with some modifications. To 1 mL of the appropriately diluted extract (1000 µg) were mixed 150 µL of Na_2CO_3 (1.9 M) and 50 µL of Folin-Ciocalteu reagent (1 N). The mixture

was kept in the dark at room temperature for 60 min. After, the absorbance was read at 725 nm against a blank. The phenolic content was expressed in micrograms of gallic acid equivalents (GAE) per milligram of extract.

Antioxidant activity by the scavenging of the ABTS$^{\cdot+}$ radical

The scavenging activity of the free radical of the aqueous extracts was measured using ABTS [(2,2-Azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid)] based on the method described by Carvajal et al. (2012).

Trolox Equivalent Antioxidant Capacity (TEAC)

The same methodology described in the previous item was used. Trolox at the concentrations of 100, 200, 300, 400, 500, 600 and 700 μM prepared in ethanol was used as the standard solution. The assay was performed in triplicate and the results were expressed in μM Trolox equivalents per mg extract.

Ferric Reducing Antioxidant Power (FRAP)

It was performed according to Benzie and Strain (1996) with some modifications. The stock solutions included are 300 mM acetate buffer (3.1 g of $C_2H_3NaO_2.3H_2O$ and 16 mL of $C_2H_4O_2$), pH 3.6, 10 mM TPTZ (2,4,6-tripyridyl-*s*-diazine) in 40 mM HCl and 20 mM ferric chloride solution ($FeCl_3.6H_2O$). The FRAP solution was prepared by mixing 25 mL of acetate buffer, 2.5 mL of the TPTZ solution and 2.5 mL of the ferric chloride solution. For the reaction, 450 μL of FRAP solution

prepared in the day, 45 µL of distilled water and 30 µL of the sample were used. It was waited 30 min at 37 °C in the dark and centrifuged for 5 min at 3500 rpm. Reading of the colored product (iron complex and tripidyltriazine) was performed at 595 nm. The standard curve was linear between 10 and 150 µM Trolox. All readings were performed in triplicate and the results expressed in µM of Trolox equivalents per mg extract.

Oxygen Radical Absorbance Capacity (ORAC)

The methodology followed was that of Min, McClung and Chen (2011) with modifications. The reconstitution fraction in DMSO, Trolox standard, fluorescein and AAPH were diluted with 75 mM phosphate buffer, pH 7.0. Aliquots of 25 µL of each sample and standard were added to a Greiner Black 96-well plate. Then, 150 µl of fluorescein solution (final concentration of 67.95 nM) was added and the plate preincubated at 37 °C for 20 min in the microplate reader. After rapid addition of 25 µl of AAPH (final concentration of 37.71 mM), the plate was immediately transferred to the microplate reader, shaken for 10 s, and the fluorescence was measured over a period of 2 min for 70 min at 37 °C. The ORAC values were calculated using a linear regression equation between a series of Trolox standards (6.25 to 50 µM) and a network area to the decay curve of the fluorescence. The area under the curve was calculated according to Davalos et al. (2004), with excitation at 485 nm and emission at 520 nm.

Antimicrobial assay

The microtube containing the microorganism of interest was thawed and collected which was scored by depletion on Müeller Hinton (MH) agar plate or potato

dextrose (PDA). The plates were incubated for 24 h. An aliquot of 10 µL of each fungal mycelial extract at concentration of 100 mg/mL was pipetted onto each filter paper disc. The disks soaked with extract were distributed on the plate keeping 2 cm away from the edge of the plate and 2 cm between the disks. The plates were incubated for 24 h and then the size of the inhibition halos were measured, if any. All analyzes were performed in triplicate and the antibiotic Gentamicin and the antifungal Fluconazole were used as controls.

Minimal Inhibitory Concentration (MIC), Minimal Bactericidal Concentration (MBC) and Minimal Fungicidal Concentration (MFC)

The antimicrobial activity was determined by the Minimum Inhibitory Concentration (MIC) and performed based on the microdilution methodology in wells. Various concentrations of the extracts were prepared by serial dilution in specific broth medium of each microorganism, Müller Hilton (MH) broth for bacteria and dextrose potato (PD) broth for fungi. For standardization of the inoculum, three to five colonies of each microorganism were collected and suspended in 3 mL of 0.85% physiological saline. The turbidity of the suspension was then compared visually with the standard 0.5 of the McFarland scale, which is equivalent to 10^8 CFU/mL for bacteria and 10^6 CFU/mL for fungi. In each well containing 100 µL of sample was added 100 µL of inoculum. Control of the culture medium, control of bacterial and fungal growth, and negative control of each extract were also performed. As a positive control, the antibiotic Gentamicin and the antifungal Fluconazole were used. The 96-well plates were incubated for 24 h in an incubator and the optical reading performed on a

microplate reader at 630 nm. The MIC was given by the lower concentration of extract that inhibits the growth of the microorganism.

The Minimal Bactericidal Concentration (MBC) and Minimum Fungicidal Concentration (MFC) were determined based on the methodology of Santurio et al. (2007), where from the wells in which there was no visible microbial growth, an aliquot of 10 µL was taken and sown in surface of the Müeller Hilton agar or PDA, respectively, and incubated for 24 h. MBC and MFC were defined as the lowest concentration of the extract capable of causing the death of the inoculum.

Isolation of mitochondria

In previously anesthetized rats, the liver was removed, fragmented and suspended in isolation medium (0.2 M mannitol, 75 mM sucrose, 2.0 mM Tris-HCl pH 7.4, 0.2 mM EGTA, 0.1 mM PMSF and 50 mg% albumin). Mitochondria were isolated by sedimentation, according to Voss, Campello and Bacila (1961) and maintained at 0 to 4 °C.

Determination of mitochondrial oxygen consumption, ADP/O ratio and

respi ratory control (RC)

The intact mitochondria were incubated in the oxygraph chamber in buffered medium (250 mM mannitol, 10 mM KCl, 10 mM Tris-HCl pH 7.4, 0.2 mM EGTA, 5 mM potassium phosphate and 50 mg% albumin). The extract of the fungus was added in the incubation medium at concentrations of 15.6, 125, 500 and 1000 μg/ml. The substrates α-ketoglutarate (10 mM), succinate (10 mM) and ADP (125 μM) were used.

Oxygen consumption rates were expressed as nmol min^{-1} mg $protein^{-1}$. The respiratory control (RC) and ADP/O ratio were calculated according to Chance and Willians (1955). The protein content was determined according to Lowry et al. (1951) using bovine albumin as standard.

Enzymatic activity associated with the membrane

Mitochondria ruptured by freeze-thaw were used as sources for determination of enzymatic activities associated with the membranes. The NADH-oxidase and succinate-oxidase activities were measured polarographically using a medium containing 20 mM Tris-HCl (pH 7.4). The reactions were initiated by addition of the substrates: NADH (10 mM) or succinate (10 mM) (SIMÕES et al. 2017).

Cytotoxicity in peritoneal macrophages

It was performed according to Noleto et al. (2004) with modifications. Murine peritoneal macrophages were collected by the injection of ice-cold PBS into the peritoneal cavity. Cells were plated in 96-well plates with minimal essential medium (MEM, Invitrogen, USA) added with 10% fetal bovine serum at a concentration of $5x10^5$ cells/mL. After 3 h of incubation at 37 °C and 5% CO_2, the unbound cells were

removed by two washes with PBS at 37 °C. The adhered macrophages were incubated for 24 h with the aqueous extracts at different concentrations (1.25 - 100 mg/mL). The cytotoxicity evaluation was performed against the MTT reagent at 495 nm.

Statistical analysis

The experimental results were expressed by the mean ± standard deviation of three independent extractions, except for cases of mitochondrial respiration, where data were expressed as mean ± standard error. The determination of significance was evaluated through the analysis of one variance (ANOVA), post hoc Tukey test of the program GraphPad Prisma® 5.0 (GraphPad Software, San Diego, USA). The level of significance used was 5% ($P < 0.05$). Calculations to obtain EC_{50} by the ABTS method were performed using the same graphing program by linear regression and non-linear regression. In the latter case, two different options were used. The cytotoxicity data in peritoneal macrophages were evaluated by the program Scientist® 3.0 (MicroMath Research, Salt Lake City, UT, USA), using Stineman interpolation.

RESULTS AND DISCUSSION

Extraction yield and total phenolics content

The yield and total soluble phenolic content in the aqueous extracts are shown in Table 1. The average yield was 11.69 ± 3.95 μg/mg and the extract containing the highest amount of phenolic compounds was the *H. fimbriatus* EF41 isolate (25.85 ± 0.45 μg/mg extract), followed by *P. albidus* EF84 (16.67 ± 1.34 μg/mg), *H. fimbriatus* EF44 (14.58 ± 1.05 μg/mg) and *O. canarii* EF72, which presented 12.86 ± 1.18 μg/mg

extract. The others presented smaller amounts.

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Antioxidant activity evaluated by the ABTS method (CE_{50})

The Table 1 shows the results of these analyzes. It can be observed that there are significant differences ($P < 0.05$) between the results obtained for the EC_{50} values in the three types of analysis for some fungi. The graphs for these analyze show that depending on the extract the data are also coherent when using non-linear analysis (Supplementary material).

In relation to EC_{50} values, the lowest concentrations that cause inhibition of ABTS radicals were found for extracts of *H. fimbriatus* EF41 (0.119 mg/mL) followed by *O. canarii* EF72 (0.181 mg/mL), *F. venustus* EF30 (0.193 mg/mL) and *H. fimbriatus* EF44 (0.215 mg/mL).

TEAC, FRAP and ORAC

In TEAC, it was verified that, unlike the other methods made in this study, *H. fimbriatus* extract was not the best (Table 2). The extract of *F. venustus* isolate presented the best result in this assay, even with a value much higher than those found for the other extracts (6,300 μM Trolox equivalents/mg extract). The fungi *H. fimbriatus* EF41 and *O. canarii* EF72 showed the values of 3,460 μM/mg and 2,880 μM/mg, respectively.

The FRAP assay measures the antioxidant power of iron reduction (Koehnlein et al. 2016). At low pH, when the iron-tripyridyltriazine complex (Fe^{+3}-TPTZ) is reduced to Fe^{+2} form and an intense blue coloration is developed. The reaction is not specific (Benzie and Strain 1996). The best results were obtained with the extracts of *H. fimbriatus,* which presented much better values than the other extracts tested: 162.27 μM/mg (isolate EF41) and 102.98 μM/mg for the extract of the isolate EF44 (Table 2).

323 Below these values the best result was for *I. splitgerberi* EF46 (24.47 μM/mg).

The ORAC method evaluates the scavenging capacity of an antioxidant against the formation of a peroxyl radical (ROO·) (Ou et al. 2002). Among the eight basidiomycetes evaluated by this methodology, the high value (1,135.24 ± 17.61 μM/mg extract) was obtained for the *I. splitgerberi* extract, followed by *H. fimbriatus* EF44 > *P. albidus* EF84 > *F. venustus* EF30 > *H. fimbriatus* EF41 > *O. canarii* EF72 ≥ *P. linteus* EF81 > *Perenniporia* sp. EF79 (Table 2).

The evaluation of the same extracts using the radical ABTS (TEAC method) showed higher antioxidant activities when compared to those obtained by the ORAC, reaching a ratio of up to 7.63 fold higher in the extract of *F. venustus* EF30. The smallest rations were for extracts of *I. splitgerberi* (1.37 times) and *P. albidus* EF84 (1.39 times) (Table 2).

Antimicrobial activity

Antimicrobial resistance has increased in recent years and the demand for new molecules has become an alternative for the production of new drugs. In this study, some extracts presented antimicrobial activity. However, in the antimicrobial assay, three results are significant: inhibition of *S. aureus* by *P. linteus* EF81 (MIC of 50 mg/mL and MBC of 75 mg/mL), *P. aeruginosa* by *P. albidus* EF84 and *B. cereus* by *F. venustus* EF46, both with MIC of 100 mg/mL and MBC > 100 mg/mL (Fig. 1). The

other microorganisms were not inhibited by any of the extracts tested.

346 3 **Mitochondrial Respiration**
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Two different substrates were used as electron donors: succinate (FAD dependent) and α-ketoglutarate (NAD^+ dependent). The breathing directed by oxidation of these substrates was determined in the absence of exogenous ADP (basal state), presence of exogenous ADP (state III), and after the exogenous ADP was added (state IV) in the absence and presence of various extracts concentrations. As the results of *I. spligerberi* EF46 and *P. albidus* EF84 were the most significant for this analysis (Fig. 2 and 3), resulting in cytotoxicity, are the ones that were given the most attention. The results of the other basidiomycetes can be visualized in Supplementary material. In relation with the effects of the aqueous extracts on the oxidation of succinate and NADH in ruptured mitochondria are in Fig. 4.

As can be observed in Fig. 3A and 3C, in the presence of succinate, the aqueous extract of *P. albidus* did not present alterations in the respiratory parameters evaluated. In contrast, the extract of *I. splitgerberi* increased oxygen consumption in the basal, III and IV states (Fig. 2A). However, the activity of succinate-oxidase (Fig. 4D) tended to increase in the presence of *I. splitgerberi*, although it did not present significant difference. On the other hand, *P. albidus* extract decreased the succinate-oxidase activity at the highest concentrations (Fig. 4H). This effect of the *P. albidus* extract on succinate-oxidase activity and the absence of effect on intact mitochondria can be justified by the fact that the succinate-oxidase assay uses ruptured mitochondria, so the extract may have had greater access to the complexes of the respiratory chain.

In respect to the effects of extracts on oxygen uptake, phosphorylation rate and respiratory control in the presence of α-ketoglutarate as a substrate, the *P. albidus* fungus was able to reduce the phosphorylation rate only at the highest concentration (Fig. 3B and 3D), whereas other parameters were not modified. In contrast, the extract of *I. splitgerberi* increased the basal, III and IV states (Fig. 2B). These changes were

reflected in the reduction of RC. Additionally, this extract reduced the ADP/O ratio only at the highest concentration (Fig. 2D). To evaluate the effects of the extract on the activity of the mitochondrial electron transport chain, the oxidation of NADH in ruptured mitochondria was evaluated. It can be observed in Fig. 4D that *I. splitgerberi* tended to stimulate this activity with increasing concentration.

Cytotoxicity in peritoneal macrophages

Table 3 shows that the two strains of *H. fimbriatus* studied were the ones with the lowest cytotoxicity for peritoneal macrophages, EF41 (81.87 ± 1.37 mg/mL extract) and EF44 (55.65 ± 1.67 mg/mL), whereas *O. canarii* (5.77 ± 0.67 mg/mL) and *F. venustus* (10.82 ± 1.24 mg/mL) were cytotoxic according to this analysis.

DISCUSSION

Among the eight basidiomycetes whose mycelium was evaluated in this study, *H. fimbriatus* stood out with good results in all tests of antioxidant activity and cytotoxicity. The antioxidant capacity of extracts obtained from different parts of plants and mushrooms has been associated with the presence of phenolic compounds such as phenolic acids and flavonoids. The fungus *H. fimbriatus*, known to be a wood destroyer

(López and Garcia 2011) and producer of cellulases, has already been described in the literature as having antimicrobial activity (Ranadive et al. 2013), but not antioxidant activity and cytotoxicity, as were in this study. The two isolates tested showed differences of results with emphasis on the isolate EF41, which reinforces the importance of bioprospecting studies.

398 Studies of antioxidant activity in *P. albidus* are scarce, where Gambato et al.
399 (2016) obtained phenolic compounds from ethanolic extraction using the mushroom.
400 The results found in the present study are similar to those of Sudha et al. (2012) that
401 reported the value of 8.77 ± 0.22 mg/g of phenolic compounds in aqueous extract of *P.*
402 *eous*. Values of 11.1 ± 0.25 mg/g and 7.73 ± 0.23 mg/g of aqueous extracts from *P.*
403 *ostreatus* and *P. ferulae*, respectively, were found by Tsai et al. (2009).

404 *O. canarii*, a species widely present in the America (Petersen, Desjardin and
405 Krüger 2008), also cultivated on lignocellulosic substrates (Ruegger et al. 2001),
406 already has been mentioned with antifungal effect (Rosa et al. 2005) and antimicrobial
407 (Rosa et al. 2003), but its antioxidant activity is a new study.

408 In this study, the radical ABTS was used and performed the analysis of the data
409 by linear regression and non-linear regression according to the proposed in the study by
410 Chen, Berti and Froldi (2013). The Table 2 shows the EC_{50} results obtained through
411 these analyzes and in Supplementary material it is possible to verify the greatest trend,
412 whether linear or not, followed by the obtained data. All extracts showed a good
413 linearity between the analyzed parameters (r^2 near or above 0.9), but some showed a
414 tendency to form a sigmoid curve, such as *H. fimbriatus* and *O. canarii*. Differently,
415 some extracts did not present this profile, meaning that simple linear regression is the
416 best way to find EC_{50} for the antioxidants present in the extract. For those extracts that
417 presented a slight tendency to curve formation, the analysis of four parameters was
418 more adequate than that of five parameters.

419 The study of the evaluation of the antioxidant activity of orange juice and milk
420 performed by Zulueta, Esteve and Frígola (2009) showed that the greatest antioxidant
421 activities were obtained by the ORAC method instead of the TEAC, the opposite found
422 in this work. Some authors believe that ORAC is a better method because it evaluates

423 the antioxidant activity of other compounds, in addition to phenolic compounds, which
424 is not observed with TEAC (Silva et al. 2007).

425 The *P. linteus* mushroom has been used for centuries in traditional medicine in
426 China, Korea and Japan for the treatment of various cancers (Kang et al. 2013), diseases
427 of gastrointestinal tract dysfunctions (Sliva 2010), diarrhea (Zhu, Kim and Chen 2008),
428 bleedings (Suabjakyong et al. 2015), allergies (Song et al. 2011) and diabetes (Hsieh,
429 Wu and Wu 2013). It has also been mentioned with antimicrobial effect (Ranadive et al.
430 2013), antioxidant and antifungal in Mexico (Ayala-Zavala et al. 2012), thus confirming
431 its results with *S. aureus*. This Gram-positive bacterium, on the other hand, may be an
432 optional anaerobic microorganism, and although it is normally in the form of a
433 commensal with humans, it may also be the etiological agent of several infections
434 associated with human health (Plata, Rosato and Wegrzyn 2009). Over the years, this
435 bacterium has acquired resistance to several existing antimicrobials (Lowy 2003), hence
436 the importance of the discovery of new antimicrobials that are able to inhibit its growth.

437 The results of *P. albidus* against *P. aeruginosa* and the *F. venustus* against *B.*
438 *cereus* are important data, since no records of the antimicrobial activity of these
439 mushrooms were found. The bacterium *P. aeruginosa* is classified as Gram-negative
440 and is one of the main causes of opportunistic infections (Gellatly and Hancock 2013)
441 and, like *S. aureus*, its resistance to several antimicrobials has already been reported
442 (Mesaros et al. 2007), confirming again the importance of these results.

443 The data obtained in mitochondrial respiration clearly show that the aqueous
444 extract of *I. splitgerberi* increases the rate of mitochondrial respiration. This increase in
445 intact mitochondria seems to be associated with an increase in the activity of the
446 enzymes linked to the internal mitochondrial membrane that make up the complexes I,
447 II and IV, as evidenced in the experiments with ruptured mitochondria.

448 It is interesting to note, however, that although there was a marked increase in
449 IV state respiration in intact mitochondria, RC values decreased only with α-
450 ketoglutarate substrate. Decoupling agents, such as dinitrophenol and the anti-
451 inflammatory drugs diclofenac and acetylsalicylic acid, stimulate the rate of IV-state
452 respiration and decreased RC (Petrescu and Tarba, 1997). In the case of these
453 substances, however, it is not observed an increase in the velocity of the mitochondrial
454 respiration state III, as it was observed in the present study. It is assumed that, at least in
455 the presence of the α-ketoglutarate substrate, the extract of *I. splitgerberi* is acting as a
456 decoupling agent for oxidative phosphorylation, as indicated by the stimulation of the
457 basal and IV states, as well as a decrease in RC. This can be confirmed, at least in part,
458 by the change in the energy transduction efficiency observed by the reduction of the
459 ADP/O ratio.

460 Unlike *P. albidus* extract, a fungus whose genus is known for its cytotoxic
461 properties inhibited the transfer of electrons from the mitochondrial respiratory chain at
462 higher concentrations (500 and 1000 mg/mL) as observed in experiments with ruptured
463 mitochondria. This inhibition did not reflect changes in respiration of intact
464 mitochondria (basal state, state III and IV), although it compromised energy efficiency,
465 as observed by reduction of the ADP/O ratio. The data obtained in mitochondrial
466 respiration corroborate the conclusion that the aqueous extract of *I. splitgerberi* impairs
467 the energetic metabolism, probably because it acts as a decoupler of the oxidative
468 phosphorylation. The decoupling action is indicated, more precisely by the stimulating
469 effect of the basal and IV states, as well as a decrease in RC. Unlike, *P. albidus* extract,
470 a genus known for its cytotoxic properties, it had practically no effect on the oxygen
471 consumption of isolated mitochondria at the concentrations evaluated, except for the

reduction of the ADP/O ratio at the highest concentration tested in the presence of α-ketoglutarate as substrate.

The only fungus used, not yet mentioned, was *Perenniporia* sp., known to inhabit hardwood trees (Decock, Mosebo and Yombiyeni 2011) and conifers (Feng et al. 2012). The main studies on the same, relate to its association with other species of mushrooms on the African continent (Decock 2011), however, no reports of its antioxidant activity in the literature.

CONCLUSIONS

The data obtained show that the mycelium evaluated can also be an important source of antioxidants. The *H. fimbriatus* was the one that presented the most significant results for the antioxidant assays. Three basidiomycetes presented antimicrobial activity, indicating a potential for further studies. The results presented suggest that *I. spligerberi* has a decoupling activity, even at the lowest concentration tested, dissipating the mitochondrial electrochemical gradient. On the other hand, *P. albidus* exerted only effect on succinate-oxidase activity without influencing mitochondrial respiratory efficiency. Therefore, both interfere negatively in mitochondrial respiration, thus inferring their cytotoxicities. From the data obtained, it is possible to emphasize the importance of bioprospecting research in little explored biomes, such as the Atlantic Forest.

494 4 **ACKOWLEDGMENTS**
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Table 1

Yield, total phenolic content and EC_{50} (mg/mL) evaluated by the ABTS method in the mycelium extracts from isolated basidiomycetes.

Isolated	Yield (%)	Total Phenolics (GAE)*	GraphPad[1]	GraphPad 4P[2]	GraphPad 5P[3]
F. venustus EF30	14.45 ± 1.55	8.60 ± 0.79	$0.193^{b,d}$	$0.113^{f,g}$	$0.363^{j,k}$
H. fimbriatus EF41	15.30 ± 1.10	25.85 ± 0.45	$0.119^{b,c}$	$0.096^{f,g}$	$0.099^{i,k}$
H. fimbriatus EF44	8.75 ± 1.45	14.58 ± 1.05	$0.215^{a,c}$	$0.170^{e,h}$	$0.216^{i,l}$
I. splitgerberi EF46	3.85 ± 0.05	4.47 ± 0.30	$0.246^{b,c}$	$0.209^{f,h}$	$0.240^{i,l}$
O. canarii EF72	9.85 ± 0.45	12.86 ± 1.18	$0.181^{b,d}$	$0.132^{f,h}$	$0.232^{j,l}$
Perenniporia sp EF79	11.35 ± 0.05	4.18 ± 0.37	$0.360^{b,d}$	$0.340^{f,h}$	$\sim 0.773^{j,l}$
P. linteus EF81	9.85 ± 0.05	6.38 ± 0.30	$0.399^{b,d}$	$0.316^{f,h}$	$\sim 0.686^{j,l}$
P. albidus EF84	17.85 ± 2.05	16.67 ± 1.34	$0.363^{a,c}$	$0.309^{e,g}$	$0.462^{i,k}$

*Results expressed in μg of gallic acid equivalents per mg of extract; [1]GraphPad linear regression; [2]GraphPad log (inhibitor) *vs.* normalized response model (variable slope); [3]GraphPad regression model five parameters; [a]EC_{50} does not differ statistically (P> 0.05) in relation to EC_{50} of GraphPad 4P; [b]EC_{50} differs statistically (P <0.05) from the EC_{50} of GraphPad 4P; [c]EC_{50} does not differ statistically (P> 0.05) in relation to EC_{50} of GraphPad 5P; [d]EC_{50} differs statistically (P <0.05) from the EC_{50} of GraphPad 5P; [e]EC_{50} does not differ statistically (P> 0.05) from the EC_{50} of GraphPad; [f]EC_{50} differs statistically (P <0.05) from the EC_{50} of GraphPad; [g]EC_{50} does not differ statistically (P> 0.05) in relation to EC_{50} of GraphPad 5P; [h]EC_{50} differs statistically (P <0.05) from the EC_{50} of GraphPad 5P; [i]EC_{50} does not differ statistically (P> 0.05) in relation to EC_{50} of GraphPad; [j]EC_{50} differs statistically (P <0.05) from EC_{50} of GraphPad; [k]EC_{50} does not differ statistically (P> 0.05) in relation to EC_{50} of GraphPad 4P; [l]EC_{50} differs statistically (P <0.05) from EC_{50} of GraphPad 4P.

Table 2

Antioxidant activity with FRAC, TEAC and ORAC methods and comparison between the TEAC and the ORAC.

Isolated	FRAP (μM/mg extract)	TEAC* (μM TE/mg extract)	ORAC* (μM TE/mg extract)	TEAC/ ORAC
F. venustus EF30	8.39 ± 0.60	6,300 ± 165	825.91 ± 9.00	7.63
H. fimbriatus EF41	162.27 ± 2.34	3,460 ± 96	595.63 ± 9.31	5.81
H. fimbriatus EF44	102.98 ± 5.10	1,840 ± 67	905.48 ± 23.32	2.03
I. splitgerberi EF46	24.57 ± 2.80	1,560 ± 58	1,135.24 ± 17.61	1.37
O. canarii EF72	16.94 ± 4.43	2,880 ± 109	421.42 ± 49.68	6.80
Perenniporia sp. EF79	17.28 ± 4.31	1,020 ± 51	251.49 ± 18.03	4.05
P. linteus EF81	11.18 ± 1.22	917 ± 23	394.03 ± 20.71	2.30
P. albidus EF84	19.76 ± 1.35	1,120 ± 15	860.80 ± 6.99	1.39

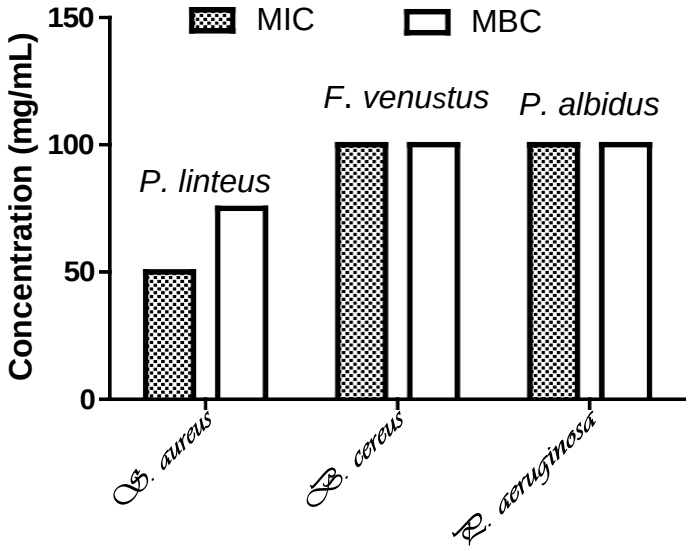
*Results (mean ± standard deviation; n=3) expressed in μM of Trolox equivalents (TE) per mg extract.

Table 3.

CC_{50} calculated by the cytotoxicity in peritoneal macrophages method.

Isolated	CC_{50} (mg/mL extract)	Isolated	CC_{50} (mg/mL extract)
F. venustus EF30	10.82 ± 1.24	*O. canarii* EF72	5.77 ± 0.67
H. fimbriatus EF41	81.87 ± 1.37	*Perenniporia* sp. EF79	44.87 ± 2.46
H. fimbriatus EF44	55.65 ± 1.67	*P. linteus* EF81	29.55 ± 2.04
I. splitgerberi EF46	48.94 ± 1.63	*P. albidus* EF84	50.78 ± 1.73

*Results (mean ± standard deviation; n=3) expressed as mg/mL extract. CC_{50}= concentration that killed 50% of cells.



699

700 **Fig. 1**. Antimicrobial activity of mycelium extracts from isolated basidiomycetes.

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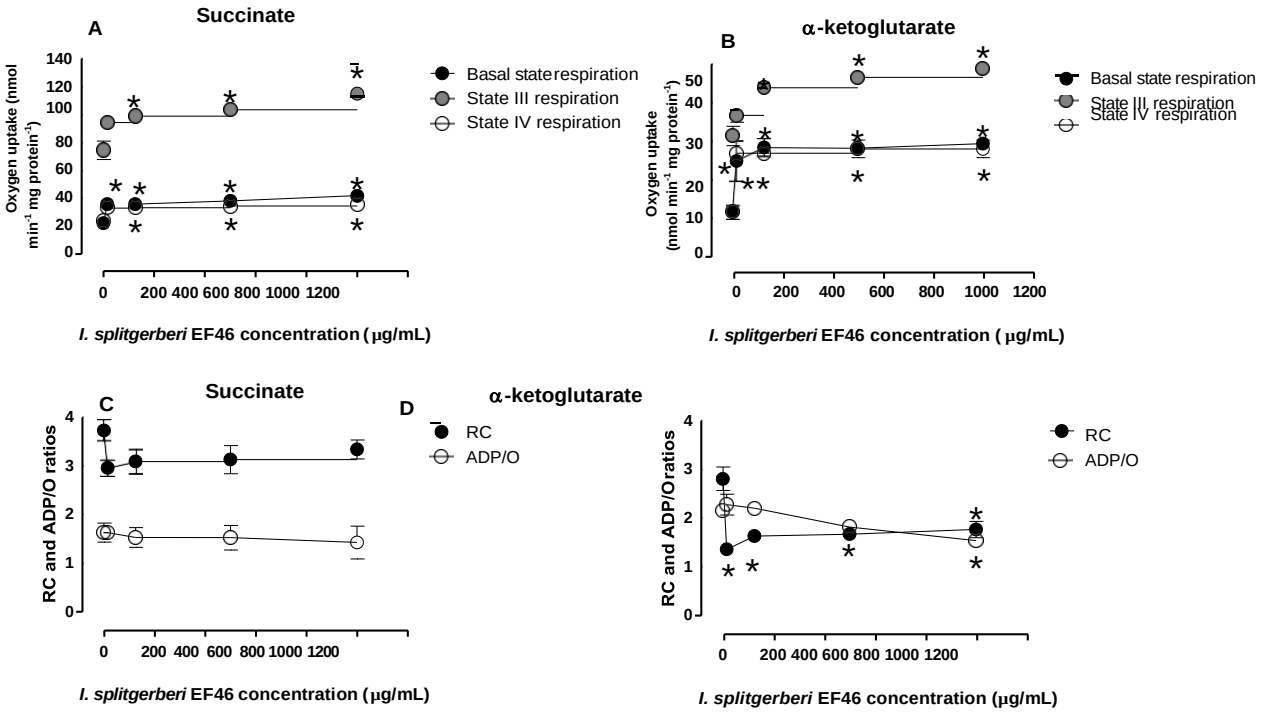
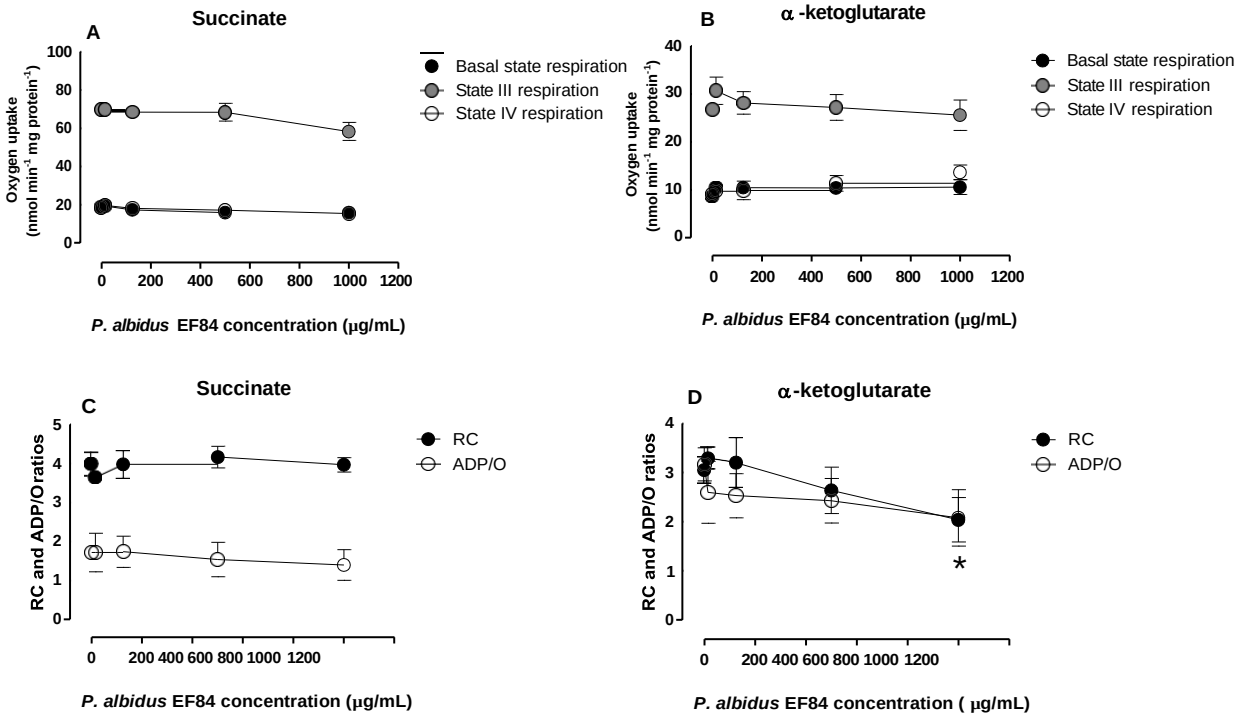


Fig. 2. Effect of *I. splitgerberi* EF46 on the respiratory activity of mitochondria isolated from liver with substrate succinate (**A** and **C**) and α-ketoglutarate (**B** and **D**). ***** indicates statistical difference compared to control condition, (ANOVA) post hoc Tukey test ($P < 0.05$).



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730 **Fig. 3.** Effect of *P. albidus* EF84 on the respiratory activity of mitochondria isolated from liver with
731 substrate succinate (**A** and **C**) and α-ketoglutarate (**B** and **D**). ***** indicates statistical difference
732 compared to control condition, (ANOVA) post hoc Tukey test ($P < 0.05$).

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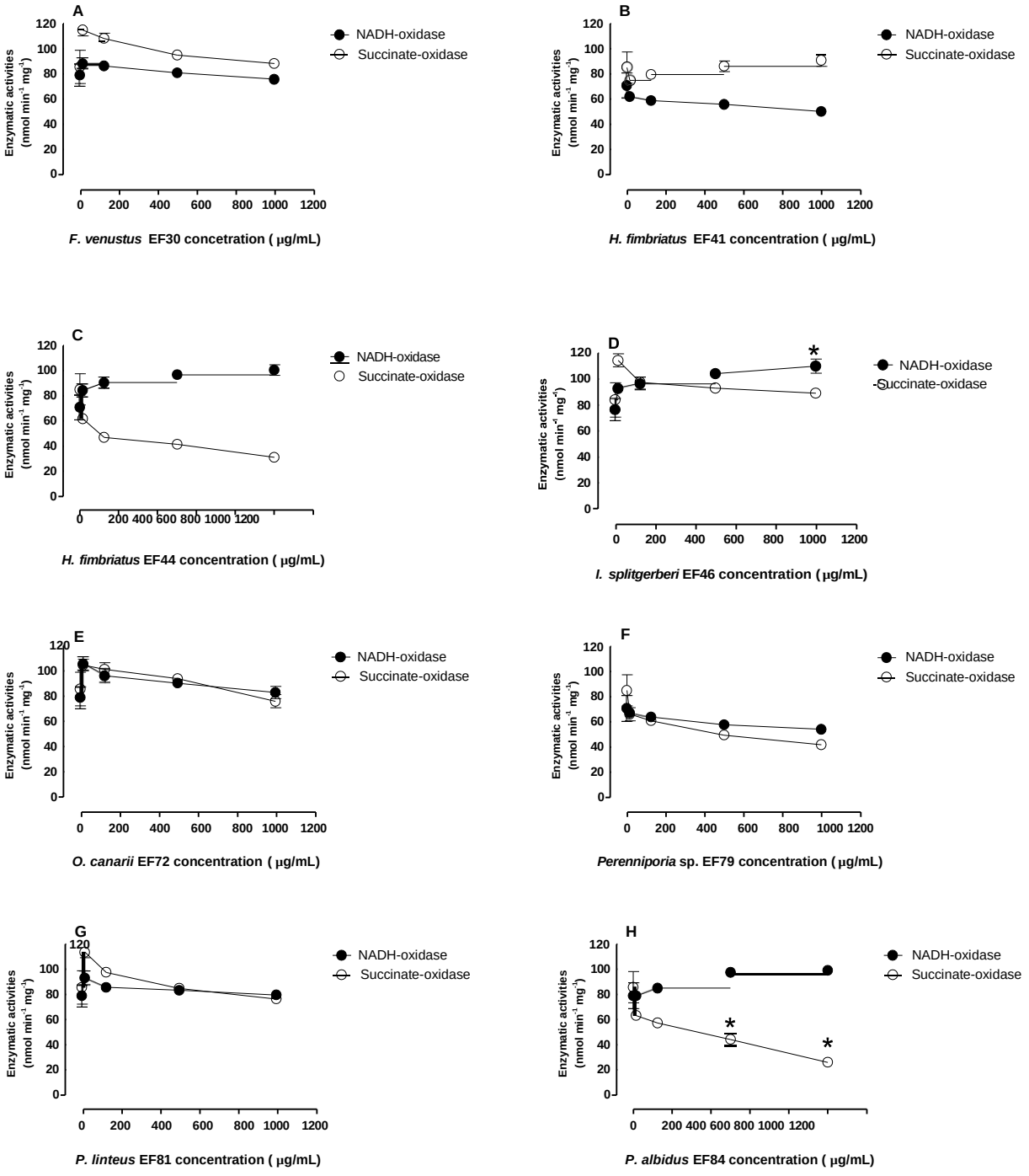

744

745 **Fig. 4.** Effects of the aqueous extracts on the oxidation of succinate and NADH in ruptured
746 mitochondria. **A:** *F. venustus* EF30; **B:** *H. fimbriatus* EF41; **C:** *H. fimbriatus* EF44; **D:** *I.*
747 *splitgerberi* EF46; **E:** *O. canarii* EF72; **F:** *Perenniporia* sp. EF79; **G:** *P. linteus* EF81; **H:** *P.*
748 *albidus* EF84. ***** indicates statistical difference compared to control, (ANOVA) post hoc Tukey
749 test ($P < 0.05$).

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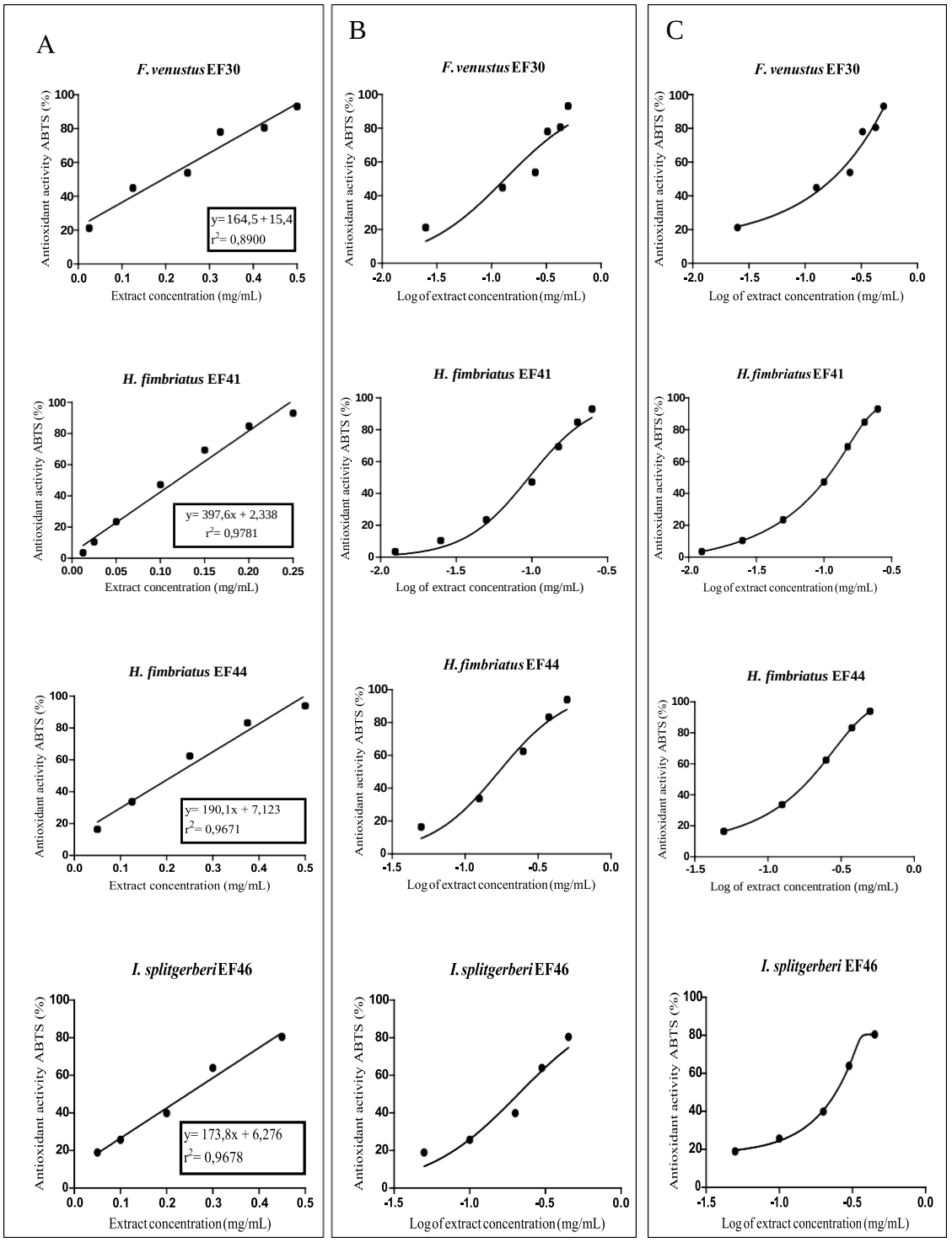
A
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C
F. venustus EF30
H. fimbriatus EF41
H. fimbriatus EF44
I. splitgerberi EF46
Antioxidant activity ABTS (%)
Extract concentration (mg/mL)
Log of extract concentration (mg/mL)
y=164,5+15,4
r²= 0,8900
y= 397,6x + 2,338
r²= 0,9781
y= 190,1x + 7,123
r²= 0,9671
y= 173,8x + 6,276
r²= 0,9678

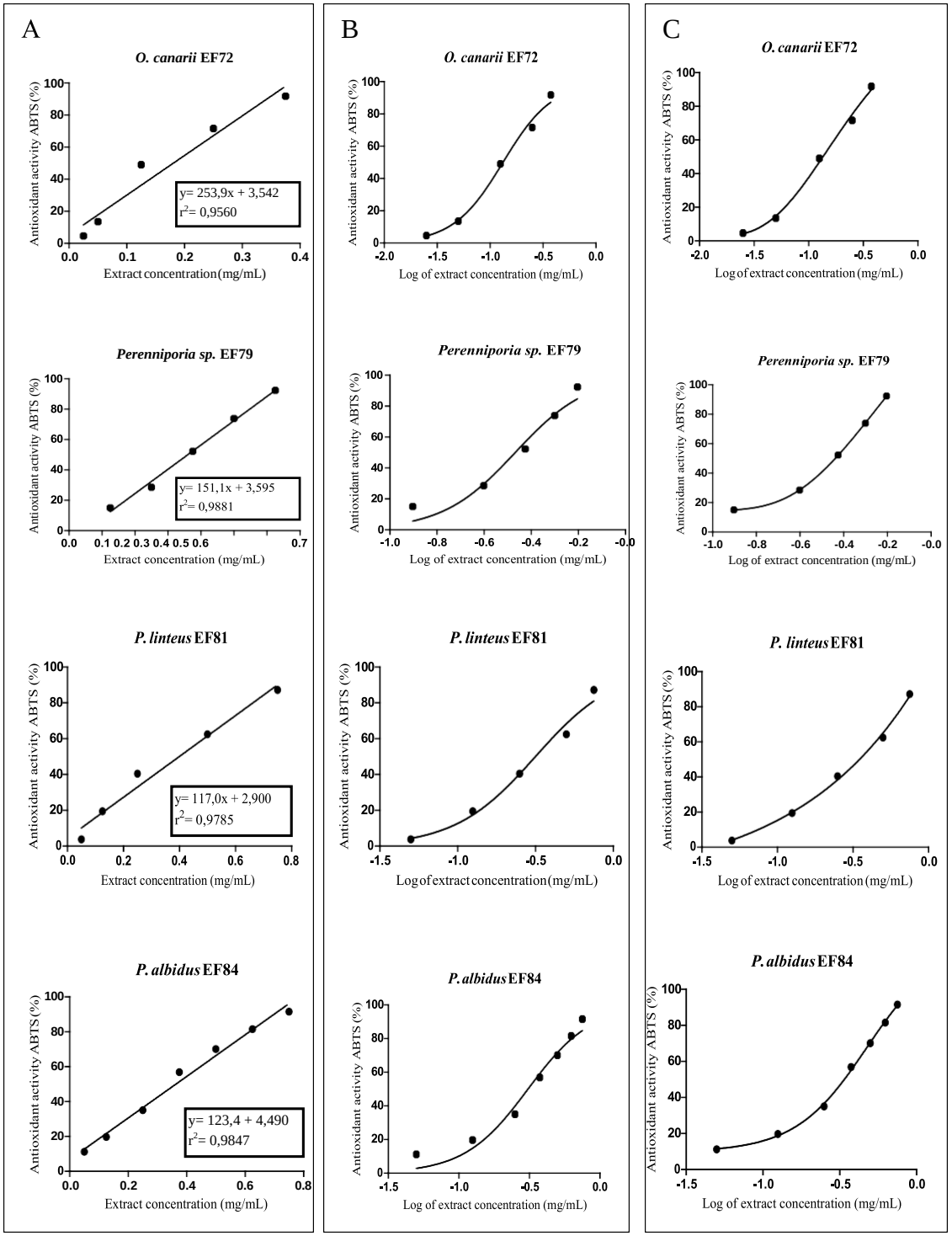
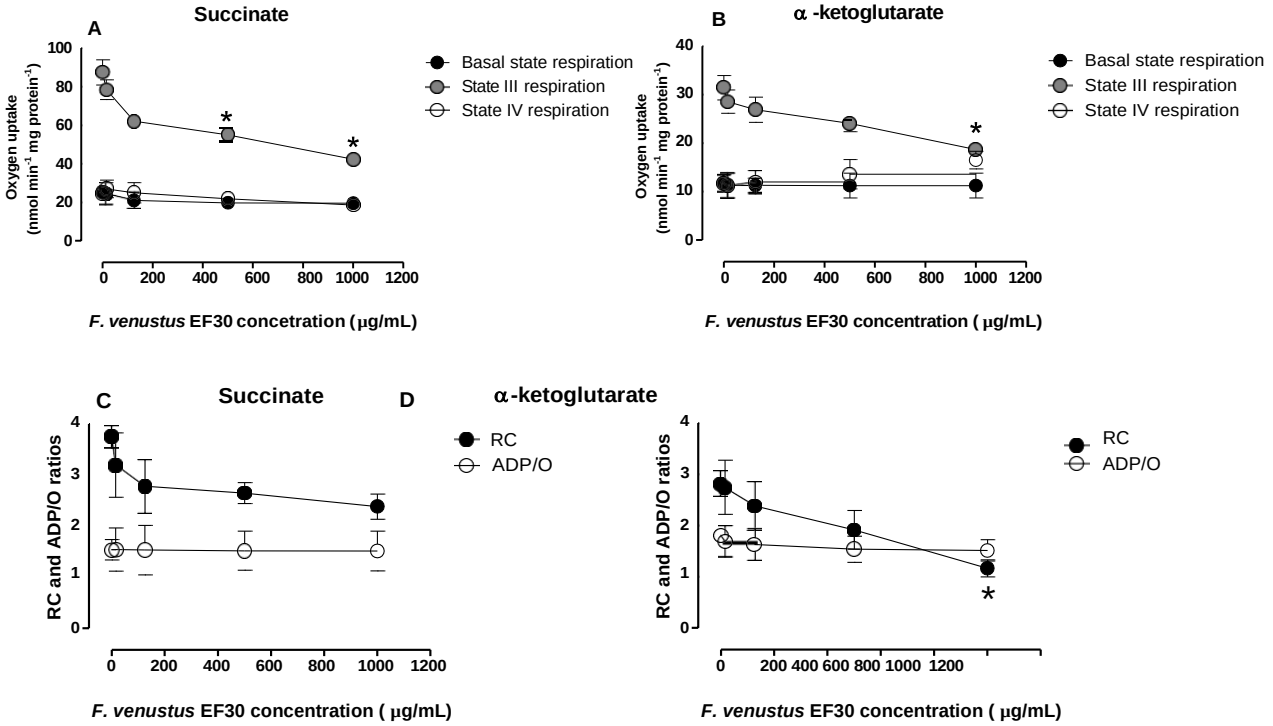


Fig. S1. The EC_{50} graphs for the eight different isolates calculated from linear regression or not. **A:** linear regression; **B:** log non-linear regression (inhibitor) versus normalized response; **C:** non-linear asymmetric regression (five-parameter).



770

771 **Fig. S2.** Effect of *F. venustus* EF30 on the respiratory activity of mitochondria isolated from liver
772 with substrate succinate (**A** and **C**) and α-ketoglutarate (**B** and **D**). ***** indicates statistical difference
773 compared to control condition, (ANOVA) post hoc Tukey test ($P < 0.05$).

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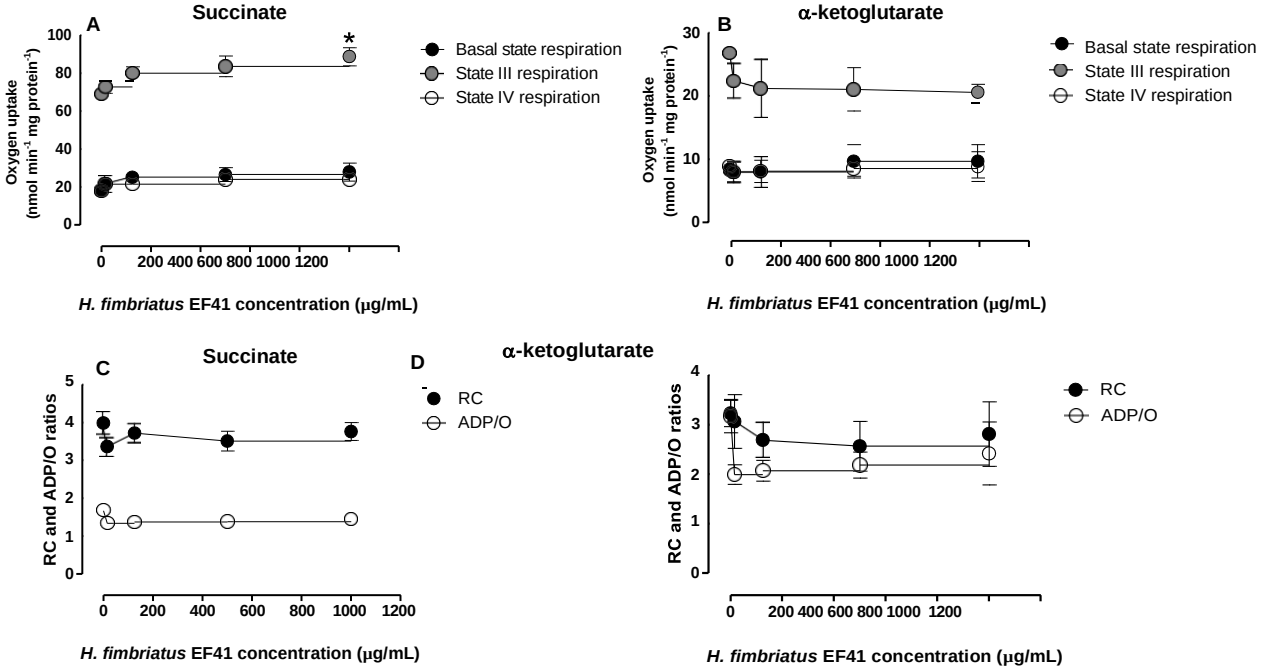
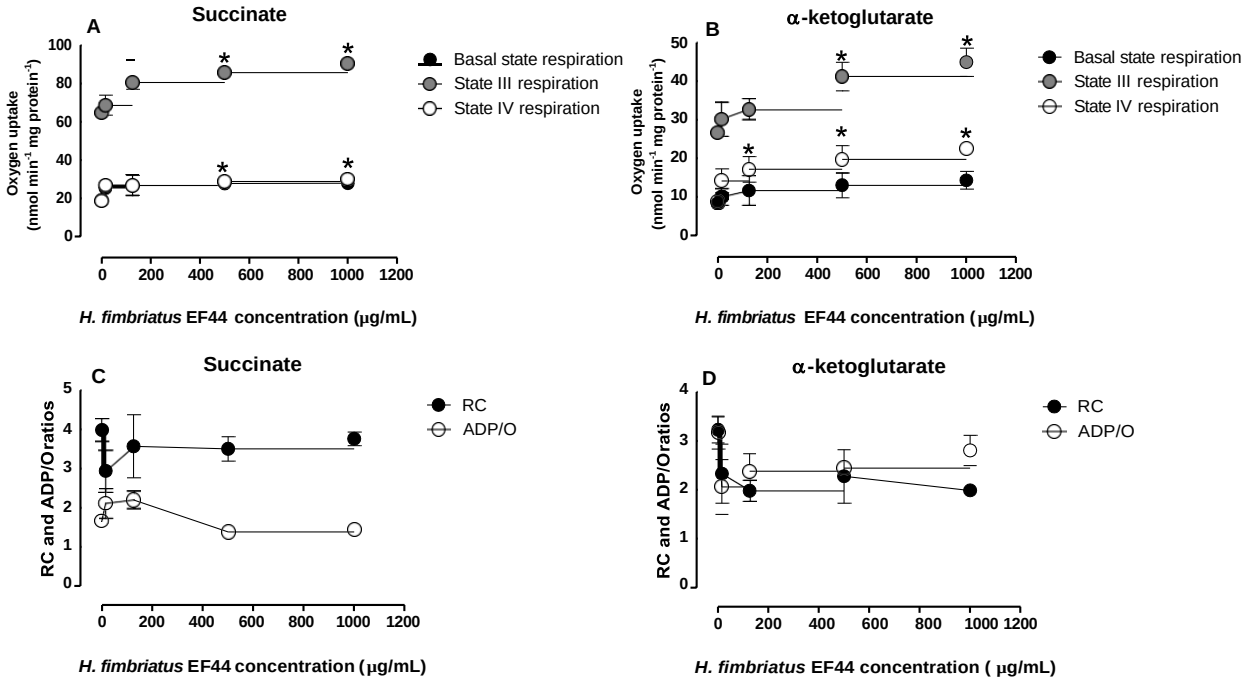


Fig. S3. Effect of *H. fimbriatus* EF41 on the respiratory activity of mitochondria isolated from liver with substrate succinate (**A** and **C**) and α-ketoglutarate (**B** and **D**). ***** indicates statistical difference compared to control condition, (ANOVA) post hoc Tukey test ($P < 0.05$).



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800 **Fig. S4.** Effect of *H. fimbriatus* EF44 on the respiratory activity of mitochondria isolated from liver
801 with substrate succinate (**A** and **C**) and α-ketoglutarate (**B** and **D**). ***** indicates statistical difference
802 compared to control condition, (ANOVA) post hoc Tukey test ($P < 0.05$).

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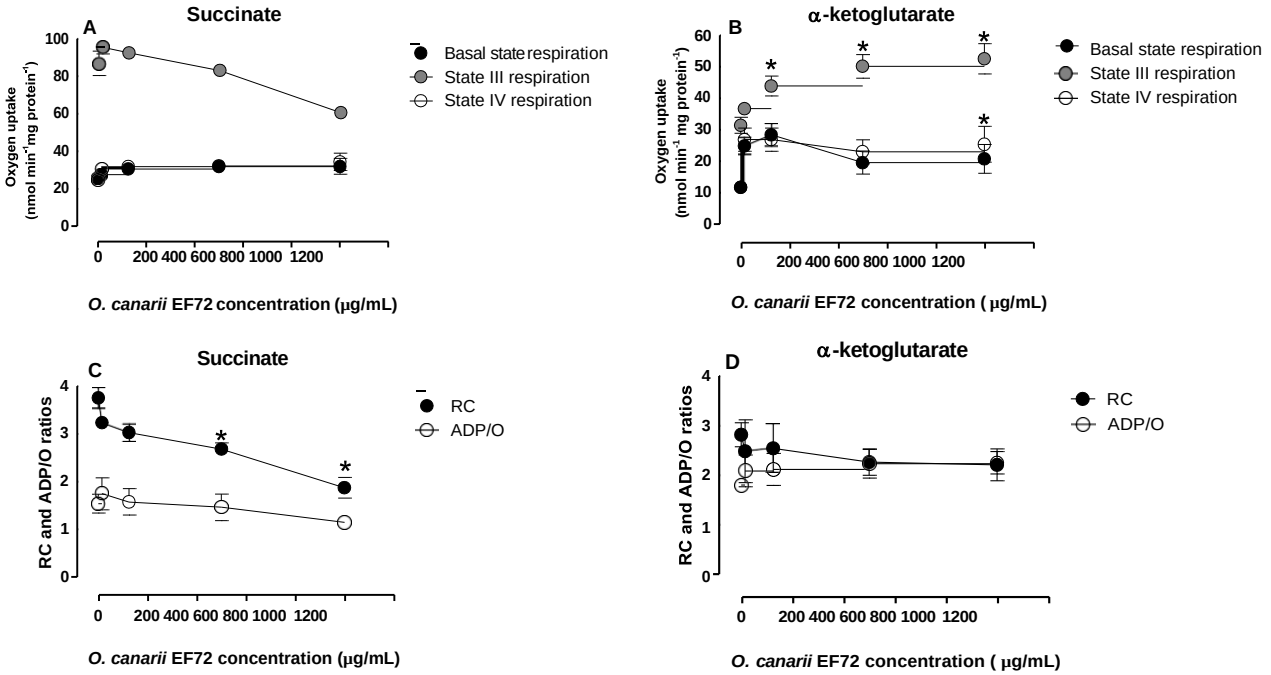

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815 **Fig. S5.** Effect of *O. canarii* EF72 on the respiratory activity of mitochondria isolated from liver
816 with substrate succinate (**A** and **C**) and α-ketoglutarate (**B** and **D**). ***** indicates statistical difference
817 compared to control condition, (ANOVA) post hoc Tukey test ($P < 0.05$).

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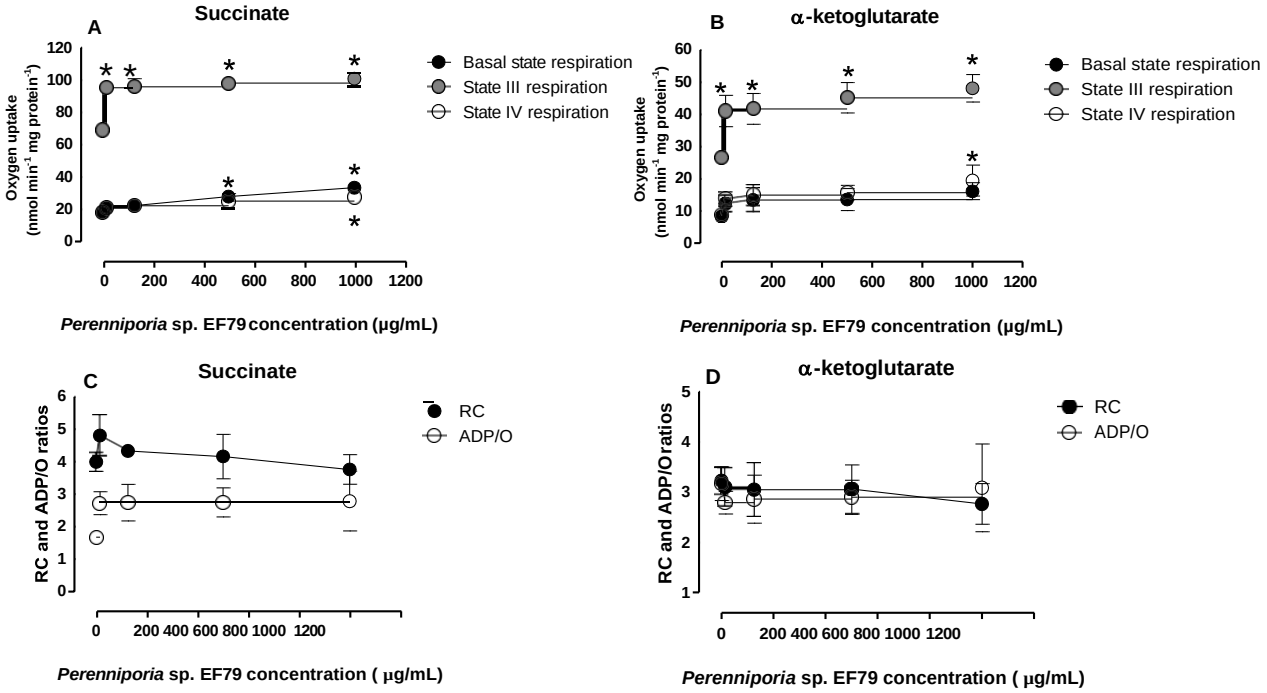
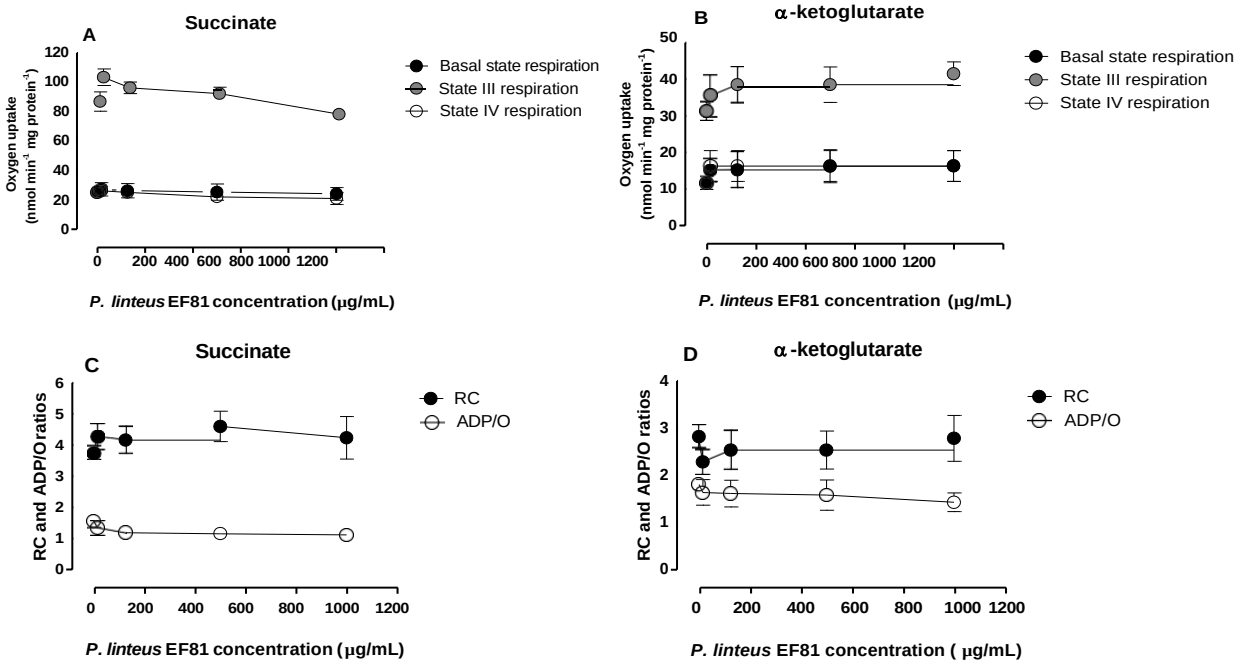


Fig. S6. Effect of *Perenniporia* sp. EF79 on the respiratory activity of mitochondria isolated from liver with substrate succinate (**A** and **C**) and α-ketoglutarate (**B** and **D**). ***** indicates statistical difference compared to control condition, (ANOVA) post hoc Tukey test ($P < 0.05$).



844
845 **Fig. S7.** Effect of *P. linteus* EF81 on the respiratory activity of mitochondria isolated from liver with
846 substrate succinate (**A** and **C**) and α-ketoglutarate (**B** and **D**).

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CERTIFICADO

Certificamos que a proposta intitulada "ATIVIDADE BIOLÓGICA DOS EXTRATOS OBTIDOS DA BIOMASSA DE BASIDIOMICETOS ISOLADOS DA MATA ATLÂNTICA DO ESTADO DO PARANÁ", protocolada sob o CEUA nº 7669090317, sob a responsabilidade de **Anacharis Babeto de Sá Nakanishi** *e equipe; Alex Graça Contato; Cristina Giatii Marques de Souza* - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais da Universidade Estadual de Maringá (CEUA/UEM) na reunião de 12/06/2017.

We certify that the proposal "Biological activity of the extracts obtained from the biomass of basidiomicets isolated of state Parana at atlantic mata", utilizing 162 Heterogenics rats (162 males), protocol number CEUA 7669090317, under the responsibility of **Anacharis Babeto de Sá Nakanishi** *and team; Alex Graça Contato; Cristina Giatii Marques de Souza* - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the State University of Maringá (CEUA/UEM) in the meeting of 06/12/2017.

Finalidade da Proposta: Pesquisa

Vigência da Proposta: de 06/2017 a 10/2018 Área: Dbq-Bioquímica

Origem:	Biotério Central da UEM						
Espécie:	Ratos heterogênicos	sexo: Machos		idade:	2 a 2 meses	N:	162
Linhagem: Wistar				Peso:	200 a 250 g		

Resumo: Existem diferentes variedades de cogumelos comestíveis, cada um com seu sabor, cor, odor e textura. Sua utilização se dá, principalmente, na culinária, como fonte de antioxidantes para outras propriedades terapêuticas. O processo oxidativo é essencial aos organismos vivos para a produção de energia a partir dos combustíveis biológicos. No entanto, a produção excessiva de espécies reativas de oxigênio (EROs) e outros radicais, os quais são continuamente produzidos “in vivo”, podem resultar em estresse oxidativo, afetando estruturas celulares e causando morte celular. Os alimentos são fonte natural de compostos antioxidantes, substâncias capazes de inibir a oxidação de outras moléculas pela remoção de radicais livres, e desempenham papel importante como fatores de proteção à saúde. Compostos fenólicos são metabólitos secundários largamente distribuídos entre os vegetais e cogumelos. Alguns estudos evidenciam a capacidade de cogumelos de produzir substâncias antioxidantes, antimicrobianas e antitumorais. Considerando o número de espécies descritas, ainda são poucas as que foram investigadas quanto ao seu conteúdo em metabólitos secundários, atividade antioxidante, antimicrobiana e demais propriedades. O objetivo deste projeto é avaliar a capacidade antioxidante, antimicrobiana e de citotoxicidade, de nove isolados de basidiomicetos do Laboratório de Microrganismos do DBQ/UEM, cedidos pela Embrapa, fazendo o uso de seu micélio como material primário de estudo. Adicionalmente este projeto pretende avaliar o efeitos de uma curva de concentração do extrato aquoso dos diferentes basidiomicetos sobre a atividade de mitocôndrias isoladas de fígado de rato Os fungos estudados serão Flaviporus venustus EF30, Hydnopolyporusvenustus EF41 e EF44, Inonotus splitgerberi EF46, Lentinula boryana EF48, Oudemansiella canarii EF72, Perenniporia sp. EF79, Phellinus linteus EF81 e Pleurotus albidus EF84.

Local do experimento: Laboratório de Metabolismo Hepático

Maringá, 12 de junho de 2017

Profa. Dra. Tatiana Carlesso dos Santos
Coordenadora em Exercício da CEUA/UEM
Universidade Estadual de Maringá

Vice-Coordenador da CEUA/UEM
Universidade Estadual de Maringá

CEUA N 7669090317