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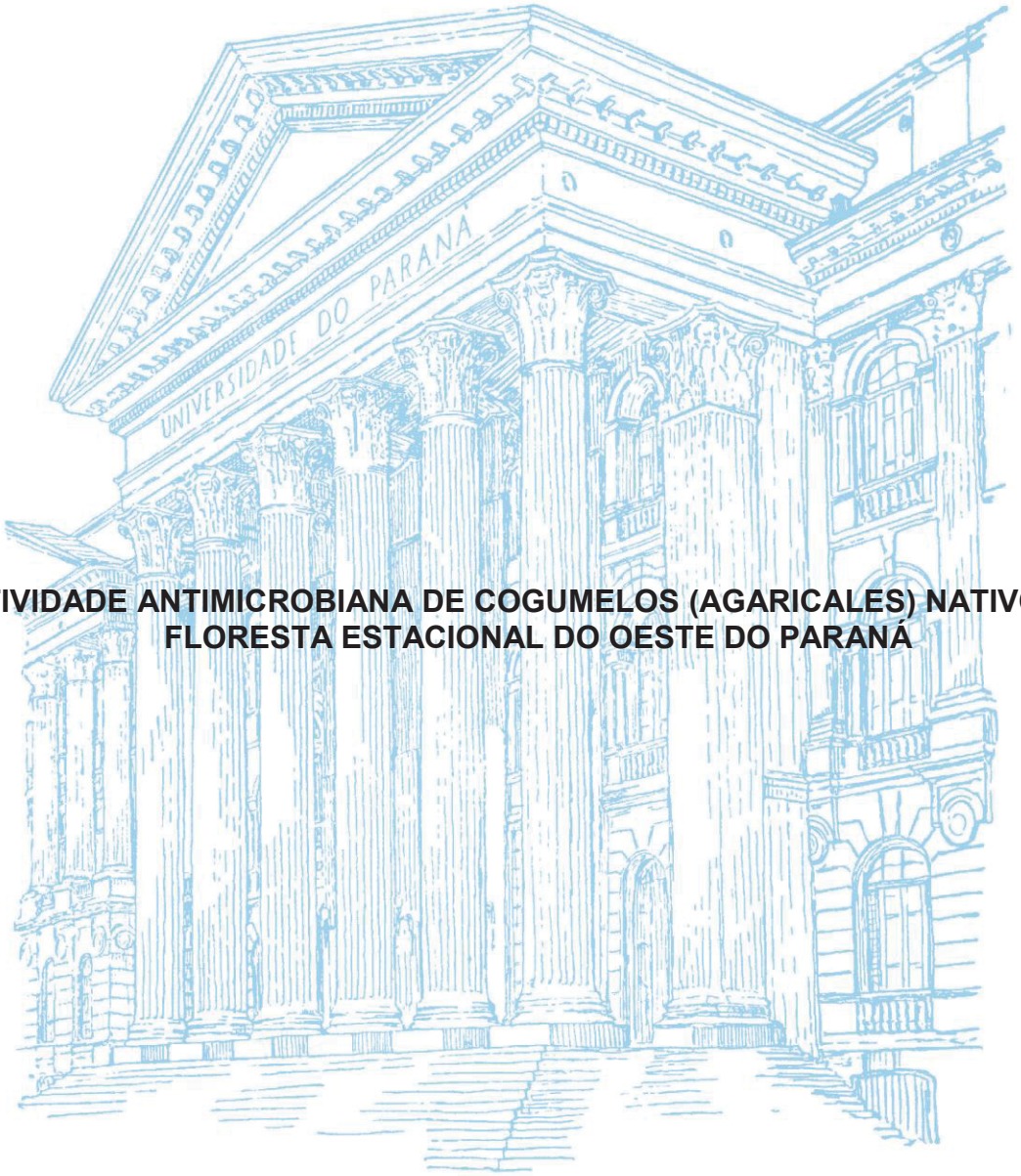

ATIVIDADE ANTIMICROBIANA DE COGUMELOS (AGARICALES) NATIVOS DA FLORESTA ESTACIONAL DO OESTE DO PARANÁ

CURITIBA

2018

MARINA GIOMBELLI ROSENBERGER

ATIVIDADE ANTIMICROBIANA DE COGUMELOS (AGARICALES) NATIVOS DA FLORESTA ESTACIONAL DO OESTE DO PARANÁ

Dissertação apresentada como requisito parcial à obtenção do grau de Mestre em Botânica, no Curso de Pós-Graduação em Botânica, Setor de Ciências Biológicas, da Universidade Federal do Paraná.

Orientador: Prof. Dr. Vagner Gularte Cortez
Coorientadora: Profa. Dra. Roberta Paulert

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UNIVERSIDADE FEDERAL DO PARANÁ

Setor de Ciências Biológicas
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por

MARINA GIOMBELLI ROSENBERGER

Dissertação aprovada como requisito parcial para obtenção do grau de Mestre no Programa de Pós-Graduação em Botânica, pela Comissão formada pelos doutores

Prof. Dr. Vagner Gularte Cortez

Prof. Dr. Victor Pereira Zwiener

Prof. Dr. Roberto Luis Portz

Curitiba, 29 de março de 2018.

Dedico este trabalho aos meus pais,
Ari Osmar Rosenberger e Cleide
Giombelli Rosenberger.

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A Deus, pelo dom da vida e por estar comigo em todos os momentos. Sem Ele nada seria possível.

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"Preferi a ciência ao fino ouro, pois a Sabedoria vale mais que as pérolas e jóia alguma a pode igualar."

(Provérbios 8, 10-11)

RESUMO

Cogumelos são macrofungos do Filo Basidiomycota que representam cerca de 20.000 espécies em todo o mundo. Considerando o fato de que a maior parte das espécies é ainda desconhecida pela ciência, é de grande importância não somente a investigação sobre sua diversidade, mas também a exploração do seu potencial como alimento, fonte de bioprodutos e novos fármacos. Os fungos, em especial os cogumelos, produzem uma ampla diversidade de metabólitos secundários, os quais possuem várias aplicações, entre elas a atividade antimicrobiana. Desta forma, há um interesse crescente em investigar os compostos produzidos por tais fungos, visto que a resistência antimicrobiana é um problema global, e o estudo de novas substâncias farmacológicas e agrícolas é de extrema importância. Devido à importância da investigação de basidiomicetos que possuem substâncias bioativas, torna-se relevante a bioprospecção de espécies nativas brasileiras. Assim, o objetivo deste trabalho foi de testar o potencial antimicrobiano de extratos de basidiomicetos coletados na região Oeste do Paraná, Brasil; além de realizar estudos de caracterização das substâncias com atividade antimicrobiana. Para isso, os extratos metanólicos brutos obtidos a partir dos basidiomas secos de 14 espécies de basidiomicetos coletados em fragmentos de Floresta Estacional Semi-decidual do Oeste do Paraná, foram testados utilizando o teste de difusão em ágar, o teste de diluição em ágar e o teste de bioautografia direta em CCD frente a bactérias (Gram-positivas e Gram-negativas), leveduras e fungos filamentosos. Os resultados obtidos variaram conforme a metodologia utilizada. Para o teste de difusão em ágar observou-se uma inibição maior de bactérias Gram-positivas (*Bacillus cereus* e *Staphylococcus aureus*) sendo que 4 extratos (*Calvatia rugosa*, *Leucoagaricus* sp., *Leucocoprinus venezuelanus* e *Psathyrella* sp.) demonstraram atividade antibacteriana; do que de bactérias Gram-negativas (*Escherichia coli* e *Pseudomonas aeruginosa*) inibidas por apenas 1 extrato (*Leucocoprinus* cf. *brebissonii*), e a levedura (*Candida albicans*) também inibida por apenas 1 extrato (*Psathyrella candolleana*). O mesmo foi observado para o teste de bioautografia direta para os bioautogramas desenvolvidos, as bactérias Gram-positivas foram inibidas por 4 extratos (*Coprinopsis* sp., *Mycena euspeirea*, *Psathyrella* sp. e *Xeromphalina tenuipes*) e as Gram-negativas foram inibidas por 2 extratos (*Simocybe tucumana* e *X. tenuipes*). Já para os bioautogramas não desenvolvidos, 4 extratos (*Coprinopsis* sp., *Marasmius haematocephalus*, *M. eusperirea* e *P. candolleana*) inibiram a bactéria Gram-negativa *P. aeruginosa* e 3 extratos (*Leucocoprinus* cf. *brebissonii*, *L. venezuelanus* e *S. tucumana*) inibiram a bactéria Gram-positiva *S. aureus*. Para o teste de diluição em ágar frente ao fungo fitopatógeno *Fusarium graminearum*, 4 extratos inibiram o crescimento micelial (*C. rugosa*, *Coprinopsis* sp., *S. tucumana* e *X. tenuipes*). Estes resultados confirmam que as espécies de basidiomicetos coletadas em fragmentos de Floresta Estacional Semi-Decidual no Oeste do Paraná produzem substâncias bioativas com propriedades antimicrobianas.

Palavras-chave: atividade antimicrobiana, cogumelos, produtos naturais.

ABSTRACT

Mushrooms are macrofungi of the Phylum Basidiomycota that contain about 20.000 species worldwide. Considering that most species remains unknown to science, it is of great importance not only to investigate their diversity, but also to explore their potential as a food source of bioproducts and new drugs. Fungi, especially mushrooms, produce a wide variety of secondary metabolites, which have various applications, including antimicrobial activity. Thus, there is a growing interest in investigating the compounds produced by such fungi, since antimicrobial resistance is a global problem, and the study of new pharmacological and agricultural substances is of the utmost importance. Due to the importance of the investigation of basidiomycetes that possess bioactive substances, bioprospecting of Brazilian native species becomes highly relevant. Therefore, the aim of this work was to verify the antimicrobial potential of extracts of basidiomycetes collected in the western region of Paraná, Brazil; besides conducting characterization studies of the substances with antimicrobial activity. For this, crude methanolic extracts obtained from dried basidiomata of 14 species collected in fragments of Semideciduous Seasonal Forest of the West of Paraná, were tested using the agar diffusion test, the agar dilution test and the direct bioautography test in TLC against bacteria (Gram-positive and Gram-negative), yeasts and filamentous fungi. Obtained results varied according to the methodology used. For the agar diffusion test a greater inhibition of Gram-positive bacteria was observed (*Bacillus cereus* and *Staphylococcus aureus*) being that 4 extracts (*Calvatia rugosa*, *Leucoagaricus* sp., *Leucocoprinus venezuelanus* and *Psathyrella* sp.) presented antibacterial activity; than Gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) inhibited by only 1 extract (*Leucocoprinus* cf. *brebissonii*), and yeast (*Candida albicans*) also inhibited by only 1 extract (*Psathyrella candolleana*). The same was observed for the direct bioautography test for the developed bioautograms, Gram-positive bacteria were inhibited by 4 extracts (*Coprinopsis* sp., *Mycena euspeirea*, *Psathyrella* sp. and *Xeromphalina tenuipes*) and Gram-negative were inhibited by 2 extracts (*Simocybe tucumana* and *X. tenuipes*). Already for the not developed bioautograms, 4 extracts (*Coprinopsis* sp., *Marasmius haematocephalus*, *M. euspeirea* and *P. candolleana*) inhibited the Gram-negative bacterium *P. aeruginosa* and 3 extracts (*Leucocoprinus* cf. *brebissonii*, *L. venezuelanus* and *S. tucumana*) inhibited Gram-positive bacteria *S. aureus*. For the agar dilution test against the phytopathogenic fungus *Fusarium graminearum*, 4 extracts inhibited the mycelial growth (*C. rugosa*, *Coprinopsis* sp., *S. tucumana* and *X. tenuipes*). These results confirm that the species of basidiomycetes collected in fragments of Semideciduous Seasonal Forest in the West of Paraná produce bioactive substances with antimicrobial properties.

Key-words: antimicrobial activity, mushrooms, natural products.

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LISTA DE ABREVIATURAS E SIGLAS

ABA	- Antibacterial activity
ADF	- Agar diffusion test
ADL	- Agar dilution test
AFA	- Antifungal activity
AMA	- Antimicrobial activity
ATCC	- American Type Culture Collection
AWD	- Agar weel diffusion
BHI	- Brain Heart Infusion
BI	- Bioautography
BR	- Brazil
CB	- Culture broth
CCD	- Cromatografia de camada delgada
CFU	- Colony-forming unit
CL	- Chile
CLSI	- Clinical and Laboratory Standards Institute
DB	- Direct bioautography
DDT	- Disk diffusion test
DMSO	- Dimethylsulfoxide
FB	- Fruiting body
HCP	- Herbário do Campus Palotina
MC	- Mycelia
MDL	- Macrodilution
MH	- Mueller-Hinton
MS	- Moderadately sensitive
PDA	- Potato dextrose agar
R	- Resistant
Rf	- Retention fator
TLC	- Thin-layer chromatography
UV	- Ultraviolet
UY	- Uruguay

SUMÁRIO

1 INTRODUÇÃO

Os fungos constituem um grupo de organismos cosmopolita extremamente diverso, com uma ampla variedade morfológica e metabólica, além de ocuparem vários habitats; são organismos eucariontes, heterotróficos, que se alimentam através da absorção de nutrientes (TEDERSOO et al., 2014). Estes organismos são considerados importantes decompositores da biosfera, sendo que esta atividade é de extrema importância, visto que permite a reciclagem dos nutrientes nos diferentes tipos de ecossistemas. Para isso, possuem um rico complexo enzimático, que quebra as substâncias orgânicas, disponibilizando-as novamente para a biosfera (MOORE et al., 2011).

Os cogumelos, como outros fungos, produzem naturalmente uma grande diversidade de biomoléculas com propriedades nutricionais e medicinais, que são utilizadas em seu ambiente natural como estratégia para sobrevivência (ALVES et al., 2012; AVIN et al., 2012; KLAUS et al., 2015). Devido à síntese destas substâncias bioativas, os cogumelos têm sido estudados nos últimos anos, e desta forma uma ampla variedade de substâncias de origem fúngica têm sido exploradas para uso farmacêutico, tais como antibióticos, agentes hipocolesterolêmicos, antitumorais, antiparasitários, antiviral, antifúngicos, imunoestimulantes, antioxidantes, imunossupressores, hepatoprotetores, entre outros (BRIZUELA et al., 1998; CARVALHO et al., 2007; WASSER, 2011; ALVES et al., 2012).

Entre os basidiomicetos estão os cogumelos (Filo Basidiomycota) que são caracterizados pela produção do basídio, estrutura na qual são produzidos e armazenados os esporos (MOORE et al., 2011), e assim como outros grupos de fungos, também apresentam grande importância ecológica, atuando diretamente na ciclagem de nutrientes (LUNDELL et al., 2010). São considerados ricas fontes de substâncias bioativas ainda inexploradas e com uma ampla variedade de estruturas químicas (HELENO et al., 2013; AJITH, JANARDHANAN, 2015). Assim, compostos isolados a partir destes podem ser úteis na pesquisa de novos produtos farmacêuticos, como agentes antimicrobianos, visto que estes organismos podem ser fontes de antibióticos naturais produzidos principalmente pelo metabolismo secundário (ALVES et al., 2012).

Investigações sobre o potencial antimicrobiano dos basidiomicetos, incluindo extratos de basidiomas e de cultura de micélios possibilitaram isolar e identificar

várias substâncias ativas contra microrganismos, como a pleuromutilina, leianafulveno, pleurotelol, ácido pleurotélico entre muitas outras (BRIZUELA et al.,1998; ROSA et al., 2003; KRUZSELYI et al., 2016). Outro exemplo, a estrobirulina, um produto natural produzido por vários fungos basidiomicetos (como *Oudemansiella mucida* e *Strobilurus tenacellus*), tornou-se importante para controlar fungos causadores de doenças em plantas, sendo que atualmente a estrobilurina comercial é produzida por síntese química (BARTLETT et al., 2002).

Estima-se que o número de espécies de cogumelos na Terra seja de 150.000, sendo atualmente reconhecidas e identificadas aproximadamente 22.000 espécies (ALVES et al., 2012; WASSER, 2011). Dentre os cogumelos desconhecidos e não identificados, assume-se que cerca de 5% seja útil, sugerindo que 7.000 espécies podem ser benéficas para a humanidade (LINDEQUIST et al., 2005; ALVES et al., 2012).

A América do Sul, por abrigar uma grande parcela da biodiversidade global de espécies, representa um potencial reservatório de espécies com possíveis usos (STRASSBURG et al., 2010). Nessa perspectiva, incluem-se também os basidiomicetos, que são favorecidos pelas características ambientais únicas dessa região (MUELLER et al., 2007). Desta forma, a bioprospecção de basidiomicetos na América do Sul é importante para explorar o potencial e as propriedades desses fungos.

Como já mencionado, vários compostos com atividades antioxidantes, antitumoral e antimicrobiana já foram isolados de basidiomicetos. Porém, apesar do enorme potencial e diversidade presente nas regiões tropicais, poucos estudos objetivando a descoberta de novas substâncias produzidas por estes cogumelos nativos foram realizados no Brasil (ROSA et al., 2003; ROSA et al., 2005; CARVALHO et al., 2007, KITZBERGER et al., 2007; DOMINGUES et al., 2011), especialmente com espécies nativas do Oeste do Paraná, onde estudos relacionados à diversidade têm sido realizados.

Assim, o objetivo geral deste trabalho, é testar o potencial antimicrobiano de extratos de cogumelos (Agaricales), coletados na região Oeste do Paraná, Brasil, frente a bactérias e leveduras de importância clínica, e frente a fungos fitopatógenos; além de realizar estudos de caracterização das substâncias com atividade antimicrobiana.

2 CAPÍTULO I - STUDIES OF ANTIMICROBIAL ACTIVITY IN MUSHROOMS (AGARICALES) COLLECTED IN SOUTH AMERICA

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Studies of antimicrobial activity in mushrooms (Agaricales) collected in South America

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Short title: Antimicrobial activity of South American mushrooms

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ABSTRACT: South America harbors much of the global biodiversity, representing a potential reservoir of species with diverse possibilities of use. In this perspective, mushrooms are included due to their natural production of a wide variety of substances, especially ones with antimicrobial activity. In this article, we present a literature review of the studies on antimicrobial activity of mushrooms collected in South America, emphasizing the inhibited bacteria and fungi, the main methodologies used for antimicrobial tests, and some directions

for future research. The review demonstrated that Agar diffusion test was the most prevalent method used in studies regarding South American mushrooms. Given that most studies dealt with specimens collected in Chile (16 species), in Brazil (10 species), and in Uruguay (two species), 27 species among the investigated mushrooms presented antimicrobial activity. Furthermore, most research developed with basidiomycetes in South America aimed only at the screening of antimicrobial agents, while few studies explored the antimicrobial potential of purified secondary metabolites. Thus, it is of great importance to conduct research to isolation of antimicrobial substances in addition to their screening, which can be used to develop new antimicrobial drugs.

KEYWORDS: agarics, antimicrobial bioassay, bioactive mushroom extract, Basidiomycota.

ABBREVIATIONS: ABA, antibacterial activity; **ADF**, agar diffusion test; **ADL**, agar/broth dilution test; **AFA**, antifungal activity; **AMA**, antimicrobial activity; **AWD**, agar well diffusion method; **BI**, bioautography; **BR**, Brazil; **CB**, culture broth; **CL**, Chile; **FB**, fruiting body; **DDT**, disk diffusion test; **MC**, mycelia; **MDL**, macrodilution; **UY**, Uruguay.

2.1 INTRODUCTION

South America, a continent known for its large portion of global biodiversity, comprises a vast reservoir of species with potential uses,[1] among which are the basidiomycete mushrooms, favored by unique environmental features and diverse ecosystems found in this region.[2] However, as yet there is still limited knowledge of the potential and properties of these fungi, thus,

research on the bioprospection of the South American basidiomycete mushrooms can be considered important. In terms of diversity of fungi, researchers have recently estimated that the number of fungal species ranges from 2.2 to 3.8 million in the world.[3] Regarding the mushrooms belonging to the order Agaricales, some 150.000 species are estimated in the world, although only about 22.000 are currently known.[4,5] Among the unknown and unidentified mushrooms, it is assumed that about 5% could be useful, thus suggesting that 7.000 species can be beneficial to mankind.[5,6]

Mushrooms have been widely used based on their nutritional and medicinal properties.[7-9] Also, due to the synthesis of secondary metabolites, mushrooms produce a wide variety of bioactive substances, many of which are exploited for pharmaceutical use, justifying the increasing interest on studying these fungi.[4,10,11]

Among the basidiomycetes, mushrooms are a rich source of bioactive substances, most of them still unexplored, which produce a wide variety of chemical substances that have been useful in the research of new pharmaceutical and agricultural products.[12-14] The basidiomata (i.e. the fruiting bodies of the Basidiomycota) generate substances with recognized immunomodulatory, cardiovascular, liver protective, antifibrotic, anti-inflammatory, antidiabetic, antiviral, antioxidative, antitumor and antimicrobial properties.[5,13] Investigation on the antimicrobial potential of basidiomycetes, including extracts obtained directly from basidiomata or from the mycelial culture, enabled the isolation and identification of substances such as 3-(2-aminophenylthio)-3-hydroxypropanoic acid, which is effective against *Aspergillus fumigatus*, *Candida albicans*, *Escherichia coli*, *Staphylococcus aureus*; among many others, mainly

polysaccharides, glucans, terpenoids, phenolic compounds, lectins, statins, etc.[11,13,15]

Accordingly, the aim of this article is to present a literature review on the studies on antimicrobial activity of mushrooms (Agaricales) collected in South America, emphasizing the antimicrobial activity against bacteria and fungi, as well as the main methods applied in antimicrobial tests, and to provide directions for future studies.

The bibliographic research was performed in the Web of Science and Google Scholar databases, including scientific articles and short communications, without restriction of the date of publication. Searched terms were: 'antibacterial activity', 'antibiotics', 'antifungal activity', 'antimicrobial activity', 'Basidiomycetes', 'fungicides', 'mushrooms' and 'natural products'. An exhaustive literature search was also performed, but only the species of mushrooms collected in South America (even native or not), which presented positive antimicrobial activity, were included here.

2.2 METHODS FOR THE STUDY OF THE ANTIMICROBIAL ACTIVITY OF SOUTH AMERICAN MUSHROOMS

Studies that deal with antimicrobial activity (AMA) of South American mushrooms have adopted a set of methods. However, it must be emphasized that there is not a single standard method for the study of AMA natural products, specifically for mushrooms or even for plants. Hence, some of the most applied methods are briefly described as follows.

- *Agar Diffusion Test* (*ADF*): This test is the most utilized method for studies that deal with South American mushrooms according to our analysis. In it, the microorganism is challenged against a substance of interest, relating the size

of the zone of inhibition regarding the microorganism growth with the concentration of the substance being evaluated.[16] The microorganism activity can be classified according to the size of the inhibition halo as 'sensitive', 'intermediate'/'moderately sensitive' or 'resistant'.[16,17] Some variations of this method are described below.

- *Disk Diffusion Test (DDT)*: The test consists of adding the substance of interest on filter paper discs (6 mm), eventually placing them on the plate already inoculated with the microorganism.[16,17] This is the standard method by the Clinical and Laboratory Standards Institute (CLSI) for antimicrobial susceptibility testing.[17] However, these standards are directed to antimicrobial agents with established parameters (antibiotics), thus they are not applicable to studies of extracts.[18] Nevertheless, DDT can also be applied to test the antimicrobial potential of natural products.

- *Agar Well Diffusion Method (AWD)*: In this test, perforations are made in the agar with a sterile cork borer or a tip in order to form wells, after the removal of the agar. In the wells, the substances are applied to be tested.[16] This assay is largely used to evaluate AMA of microbial agents or plants extracts.[19]

In both methods, after the application of test substances, Petri dishes are incubated and inhibition halos are analyzed after 16-18 hours from the beginning of incubation.[16, 17]

- *Agar/Broth Dilution Test (ADL):* This is another widely used test adopted as the standard method for substance sensitivity analysis.[20] ADL consists of incorporating the antimicrobial agent to agar/broth. Each test plate contains the same concentration of antimicrobial substance. Afterwards, the inoculum is transferred to the surface of the agar. The results are generally analyzed by measuring the radial growth. For evaluating broth in the test plate, the inhibition

of growth is measured by colorful reactions or measured by the optical density and then by comparing the experimental with the control group.[20]

- *Bioautography (BI)*: This assay allows the identification of bioactive substances that demonstrate to have AMA. For this, a thin layer chromatography (TLC) is carried out aiming at the separation of the substances present in the extract. The inoculum is then applied to the TLC plates, which are incubated for 16-18 hours. After incubation, the TLC plates are subjected to a colorimetric analysis for detection of microbial growth.[19, 21]

- *Macrodilution (MDL)*: This tube method considers the ratio between the growth rate of the challenged microorganism in the liquid medium and the compounds concentration. The evaluation is carried out by comparing the turbidity against a biological reference standard.[16]

2.3 SOUTH AMERICAN MUSHROOMS WITH ANTIMICROBIAL PROPERTIES

Antimicrobial activity of (native or not) mushrooms extracts collected in South America has been investigated against Gram-positive bacteria (*Bacillus brevis*, *Bacillus cereus*, *Bacillus subtilis*, *Enterococcus faecalis*, *Listeria monocytogenes*, *Micrococcus luteus*, *Staphylococcus aureus*, *Staphylococcus saprophyticus*, *Streptococcus mutans*, *Streptococcus pyogenes*), Gram-negative bacteria, (*Escherichia coli*, *Pseudomonas aeruginosa*, *Xanthomonas vesicatoria*), filamentous fungi (*Alternaria porri*, *Alternaria solani*, *Aspergillus niger*, *Aspergillus ochraceus*, *Botrytis cinerea*, *Ceratocystis pilifera*, *Cladosporium sphaerospermum*, *Colletotrichum acutatum*, *Fusarium ciliatum*, *Fusarium oxysporum*, *Mucor miehei*, *Paecilomyces variotii*, *Penicillium notatum*, *Rhizoctonia solani*, *Sclerotium rolfsii*, *Ustilago nuda*) and yeasts (*Candida*

albicans, *Candida glabrata*, *Candida krusei*, *Candida tropicalis*, *Nematospora coryli*) (Table 1).

In the present literature review, the studies and respective species whose AMA was evaluated as positive were considered, even though mushrooms from different ecosystems and mushrooms grown on different substrates can produce distinct antimicrobial substances. [22, 23]

In the bibliographic review, 14 papers dealing with the antimicrobial activity of South American mushrooms were found; such analyzed studies were published from 2002 to 2017.[9,10,15,22,24-33]. Among the mushroom species identified in the articles under analysis, 27 presented antibacterial and antifungal activity. Regarding the origin of the mushrooms, most studies were developed with species collected in Chile (16 species)[22,24,25,29], in Brazil (10 species)[10,15,27,28,30,31,33] and in Uruguay (2 species)[9,26,32]. The species *Gymnopilus junonius* (as *G. spectabilis*) was studied in both Chile and Uruguay.

In some cases, however, the species were not native from South America, especially those associated to *Pinus* and *Eucalyptus* trees (e.g. *Hypholoma fasciculare, Inocybe geophylla*) or even commercial strains of edible mushroom species (e.g. *Lentinula edodes*), but they were included in the review by virtue of their properties and of the ease on recollecting them.

The genus *Agaricus* is rich in Neotropical mycobiota,[34] and best known for comprising some of the most cultivated mushroom species, such as 'Portobello' (*A. bisporus*) and 'Almond' (*A. subrufescens*).[35] Besides the well-known culinary importance and its nutraceuticals, AMA has been widely reported to exist in several species over the world.[5,36] Ethyl acetate extract obtained from *Agaricus* cf. *nigrecentulus* (1 mg mL^{-1}) was tested against several pathogenic and non-pathogenic microorganisms and demonstrated activity against *Staphylococcus*

saprophyticus, a Gram-positive bacterium, potentially causing urinary tract infections[37] with an inhibition halo larger than 12 mm.[15]

Ethyl acetate extract of the Brazilian strains of *Agrocybe perfecta*, a native mushroom from Southern and Southeastern Brazil,[38] exhibited activity against pathogenic yeast *Candida krusei* when tested at a concentration of 1 mg mL^{-1} (halo larger than 12 mm), but did not inhibit other species of the genus, as *C. albicans* and *C. glabrata* did[15] In addition to the antifungal activity (AFA), this species also presented trypanocidal, cytotoxic and immunosuppressive activity.[39]

Culture broth of *Collybia butyracea* (another *Pinus*-associated species from North America and currently classified in the genus *Rhodocollybia*)[40] was extracted with ethyl acetate and demonstrated antibacterial activity (ABA) against *B. subtilis* (8 mm), *P. aeruginosa* (7 mm) and *S. aureus* (7 mm). Besides ABA, the extract showed AFA against phytopathogens *Ceratocystis pilifera* (13 mm) and *Rhizoctonia solani* (8mm) when used at the concentration of 100 μg/disc.[24]

Ethyl acetate extract of the culture broth of *Entoloma nubigenum* (misspelled as *E. nubigenum*), a possible endemic agaric from Southern South America,[41] presented AMA against *B. subtilis* (17 mm), *C. pilifera* (7 mm), *Enterococcus faecalis* (9 mm), *Penicillium notatum* (11 mm) and *S. aureus* (9 mm) when used at the concentration of 100 μg/disc. The largest inhibition halo was 17 mm in comparison with *B. subtilis*.[24]

Despite considered toxic, *Gymnopilus spectabilis* (currently known as *G. junonius*) is a common lignicolous mushroom that grows on *Eucalyptus* in South America and is consumed in some countries.[42] This species was tested at a concentration of 100 μl mL^{-1} and showed activity against a variety of microorganisms: *Aspergillus niger* (10-15 mm), *Bacillus brevis* (9-13 mm), *Botrytis cinerea* (8-15 mm), *Fusarium ciliatum* (10 mm), *Fusarium oxysporum* (8-

12 mm), *Mucor miehei* (20-28 mm), *Nematospora coryli* (18-26 mm), *Paecilomyces variotii* (16-22 mm), *P. notatum* (10-16mm), *S. aureus* (10-13 mm), *Streptococcus pyogenes* (10-18 mm).[22,25] When tested by bioautography at a concentration of 4 mg mL^{-1} of purified lectin of *G. junonius*, it inhibited the growth of *A. niger* and *S. aureus*.[26] Extracts (acetone and ethyl acetate) from fruiting bodies of *G. junonius* also demonstrated ABA against methicillin-resistant *S. aureus* strain (ATCC 700699).[9]

The lignicolous temperate mushroom species *Hypholoma fasciculare* and *H. sublateritium* were also tested at a concentration of 100 µl mL^{-1}. The results demonstrated activity against the following bacteria and fungi: *A. niger* (18 mm), *B. brevis* (8-9 mm), *B. subtilis* (8-12 mm), *B. cinerea* (16 mm), *F. oxysporum* (8-10 mm), *M. miehei* (9-15 mm), *N. coryli* (8-12 mm), *P. variotii* (9 mm), *P. notatum* (10 mm) and *S. aureus* (9-13 mm).[22,25]

Culture broth of poisonous mushroom *Inocybe geophylla,* a conifer-associated (ectomycorrhizal) species, was extracted with ethyl acetate and tested at a concentration of 100 µg/disc. It showed activity against *B. subtilis* (14 mm), *E. coli* (7 mm), *F. oxysporum* (6 mm), *P. notatum* (12 mm), *P. aeruginosa* (7 mm), *R. solani* (6 mm), *S. aureus* (7 mm). The largest zone of inhibition was 14 mm against *B. subtilis*.[24] The consumption of this species, when confounded with truly edible species, is associated with cases of accidental poisoning due to production of muscarin.[43]

The mycelial growth broth of Neotropical mushroom *Lentinula boryana* and the widely cultivated *L. edodes* ('shiitake') were filtered and used to test their antimicrobial potential.[10] The filtrate of *L. boryana* inhibited the growth of bacteria *Bacillus cereus* (10-20 mm) and *S. aureus* (10-20 mm), while *L. edodes* inhibited *B. cereus* (10-20 mm), *S. aureus* (20-30 mm) and *Streptococcus mutans*

(10-20 mm).[10] Another study that employed the same method showed that *L. edodes* inhibited the growth of *B. subtilis*.[27] Moreover, the extract of *L. edodes* (obtained from basidiomata by cold maceration in ethanol and partitioned with other solvents) inhibited the growth of *B. cereus* (10-14 mm), *C. albicans* (12 mm), *E. coli* (9 mm) and *Micrococcus luteus* (12-19 mm).[28]

Ethyl acetate extract of the native mushroom species *Leucoagaricus* cf. *cinereus*, *Marasmius* sp. and *Marasmius* cf. *bellus* was tested to a concentration of 1 mg mL^{-1} and presented ABA against *E. coli* (inhibition halo larger than 12mm for all extracts).[15] Methanol and ethyl acetate extracts of the species *Marasmius alliodoratissimus* were tested at 100 μg mL^{-1} and presented AFA against *P. variotti*.[22] Thus, the genus *Marasmius* stands out considering that it is known to produce secondary metabolites of interest, for instance, anti-inflammatory and antimicrobial ones.[15]

Native members of the genus *Mycena* also stand out in the mushroom bioprospection in South America, with at least three species investigated. The extract of *Mycena* sp. was obtained by fermentation and it was later purified to obtain the triterpenoid favolon B, which was tested at concentrations of 0.1; 1.0 and 10.0 μg/disc. It demonstrated activity against *Alternaria porri* (10-26 mm), *Aspergillus ochraceus* (8-20 mm), *B. cinerea* (20-42 mm), *M. miehei* (15-22 mm), *P. variotii* (20-30 mm), *P. notatum* (10-18 mm) and *Ustilago nuda* (9-16 mm).[29] *Mycena chlorinella*, in turn, presented AFA against *P. variotii* and *P. notatum* when ethyl acetate and methanol extracts were used at 100 μg mL^{-1}.[22] Tested at a concentration of 100 μg/disc, the extract of the mycelial growth broth of *Mycena hyalinotricha* inhibited the growth of *B. subtilis* (15 mm), *C. pilifera* (13 mm), *P. notatum* (17 mm), *R. solani* (9 mm) and *S. aureus* (8 mm).[24] *Mycena pura* also

presented ABA and AFA when the ethyl acetate and methanol extracts were tested at 100 μg mL^{-1}.[22]

The ethyl acetate extract of the edible mushroom *Nothopanus hygrophanus* (currently classified in the genus *Neonothopanus*), tested at 1 mg mL^{-1}, inhibited the growth of *Listeria monocytogenes*. *S. aureus* showed inhibition halo larger than 12 mm for two extracts.[15] In addition to AMA, *N. hygrophanus* also presented trypanocidal and antileishmanial activity against *Trypanosoma cruzi* and *Leishmania amazonensis,* respectively.[44]

The edible and supposedly widespread mushroom *Oudemansiella canarii* (possibly belonging to *O. cubensis* in the current taxonomic concept)[45] showed inhibitory activity against *Candida tropicalis*, *C. albicans, C. glabrata* and *C. krusei* (inhibition halo larger than 12 mm for all extracts) when using the agar diffusion test at the concentration of 1 mg mL^{-1}.[15] However, when using the agar dilution test, extract at the concentration of 1 mg mL^{-1} demonstrated AFA against *Alternaria solani* (69.3% inhibition), *Colletotrichum acutatum* (83.3% inhibition) and *Sclerotium rolfsii* (84.0% inhibition), which inhibited mycelial growth and also the germination of sclerotia.[30] Another study shows that the ethyl acetate extract obtained from *O. canarii* broth was tested by bioautography against the phytopathogenic *Cladosporium sphaerospermum*, presenting strong AFA against this phytopathogen (inhibition halo larger than 12 mm in diameter).[31] The crude extract of this specie also showed antitumor and trypanocidal activity.[31]

The broth obtained from mycelial culture of the coprophilous mushroom *Stropharia semiglobata* was extracted with ethyl acetate, and demonstrated ABA against *B. subtilis* (8 mm), *E. coli* (9 mm), *E. faecalis* (9 mm), *P. aeruginosa* (7 mm) e *S. aureus* (8 mm), and AFA against *C. pilifera* (8 mm), *P. notatum* (7 mm) and *R. solani* (7 mm) when used at the concentration of 100 μg/disc. The largest

zone of inhibition was 9 mm against *E. coli* and *E. faecalis*.[24] Other species also presented AMA: the methanolic extract of the mycelium of *Dictyopanus pusillus,* for instance, showed activity against *Xanthomonas vesicatoria* when tested via bioautography.[32]

In addition to these species, other unidentified mushrooms also presented activity against pathogenic bacteria and fungi, namely *Pleurotus* sp.,[33] *Tephrocybe* sp. [22] and *Psathyrella* sp.[24] The species *Anthracophyllum berteroi* and *Marasmiellus alliodorus* presented AMA, but the authors did not report against which organisms they were effective.[22]

To sum up, the total number of microorganisms used for studies with extracts of basidiomycetes in South America is 34, comprising 13 bacteria species (10 Gram-positive and 3 Gram-negative) and 21 fungal species (16 filamentous fungi and 5 yeasts). The bacteria *S. aureus* and the fungus *P. notatum* were the microorganisms inhibited by the largest number of extracts of basidiomycetes (11), followed by *B. subtilis* (10), *P. variotti* (7), *E. coli*, *C. pilifera*, *F. oxysporum* and *R. solani* (5). The remainder microorganisms were inhibited by four or fewer extracts. Extracts of basidiomycetes showed AFA 61 times (52 for filamentous fungi and 9 for yeast) and ABA 44 times (35 for Gram-positive bacteria and 9 for Gram-negative bacteria).

To obtain the fungal extracts, the majority of the studies used only the CB (10 articles)[10,15,24-27,29-31,33] as a source of biomass. Other research used extracts of the CB and the MC[22,32] (2 articles), and few studies used the FB to obtain the extracts[9,28] (2 articles). The preference for using CB is possibly related to the ease of access to this type of material, since the conditions for production of the metabolites can be controlled. In relation to the other sources of biomass, the amount of MC produced *in vitro* is very small when compared to the CB, and the

FB is difficult to find. Furthermore, the fungi can release the metabolites into the culture medium.[46]

2.4 ANTIMICROBIAL SECONDARY METABOLITES IN MUSHROOMS

The antimicrobial properties of mushrooms mainly result from the production of a wide variety of secondary metabolites, such as terpenes (mainly sesquiterpenes), steroids, benzoic acid derivatives, and quinolones. However, primary metabolites may also exhibit such properties, as the oxalic acid, which presents AMA.[5,6] Among the reviewed articles, only three tested isolated metabolites to verify the antimicrobial potential, described as follows[25,26,29].

The first research presented seven compounds that were isolated from basidiomycete mycelial cultures, of which four presented AMA: hepta-4.6-diyn-3-ol and 7-chloro-hepta-4,6-diyn-3-ol isolated from the species *Gymnopilus junonius*; 3.5-dichloro-4-metoxi-benzyl alcohol isolated from *Hypholoma fasciculare*; and a sesquiterpen, marasmal isolated from *H. sublateritium*.[25] In the second, a Lectin was isolated from the extract of basidiomata of *Gymnopilus junonius* by ion exchange chromatography. This substance has demonstrated ABA and AFA, as aforementioned.[26] In addition, in the third study, a triterpenoid was isolated from fermentation broths of *Mycena* sp. strain 96180, called Favolon B. This triterpenoid showed AFA against filamentous fungi, but did not demonstrate activity against yeasts and bacteria.[29]

Even with a number of interesting biological activities, the commercial production of pharmaceuticals from isolated compounds, or even crude extracts, from higher fungi, including mushrooms, is still limited.[13,23,47] Thus, in addition to substance isolation and *in vitro* tests, it is necessary to interact between different

research areas, enabling the commercial production of antifungals and antibiotics where appropriate.

2.5 CONCLUSION

Mushrooms are very important sources for secondary metabolites with numerous applications in biotechnology. The chemical diversity is directly correlated with the species diversity, which is, however, still little known in South America. The low (although growing in recent years) number of specialists in basic fungal taxonomy is a possible cause for the scarce applied studies, especially those dealing with their biological activity. It is noteworthy that in some cases researchers have studied exotic mushrooms species, possibly by virtue of their easier identification.

Screening studies of mushrooms with antimicrobial potential have increased in recent years and, although a few species have effectively been tested, the area of research is promising, especially considering the limited knowledge on the fungal diversity in a continental perspective. To this end, future research should focus on cooperative research involving field mycologists, microbiologists and biochemists to provide integrative results toward correct identification of fungal species and investigation on the antimicrobial properties.

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Table 1. Antimicrobial activity detected in South American mushrooms.

Mushroom	Part used*	Inhibited microorganism	Method**	Country***
Agaricus cf. *nigrecentulus*[15]	CB	*S. saprophyticus*	ADF	BR
Agrocybe perfecta[15]	CB	*C. krusei*	ADF	BR
Anthracophyllum berterii[22]	CB; MC	Active against not specified bacteria and fungi	ADF; MDL	CL
Collybia butyraceae[24]	CB	*B. subtilis*, *C. pilifera*, *P. aeruginosa*, *R. solani*, *S. aureus*	ADF	CL
Dictyopanus pusillus[32]	CB; MC	*Xanthomonas vesicatoria*	BI	UY
Entoloma nubigenum[24]	CB	*B. subtilis*, *C. pilifera*, *E. faecalis*, *P. notatum*, *S. aureus*	ADF	CL
Gymnopilus junonius[9,22,25,26]	CB; FB; MC	*A. niger*, *B. brevis*, *B. cinerea*, *F. ciliatum*, *F. oxysporum*, *M. miehei*, *N. coryli*, *P. variotii*, *P. notatum*, *S. aureus*, *S.*	ADF; MDL; BI	CL/UY

		pyogenes		
Hypholoma fasciculare[22,25]	CB; MC	*A. niger*, *B. brevis*, *B. subtilis*, *B. cinerea*, *F. oxysporum*, *M. miehei*, *N. coryli*, *P. variotii*, *P. notatum*, *S. aureus*	ADF; MDL	CL
Hypholoma sublateritium[22,25]	CB; MC	*A. niger*, *B. brevis*, *B. subtilis*, *B. cinerea*, *F. oxysporum*, *M. miehei*, *N. coryli*, *P. variotii*, *P. notatum*, *S. aureus*	ADF; MDL	CL
Inocybe geophylla[24]	CB	*B. subtilis*, *E. coli*, *F. oxysporum*, *P. notatum*, *P. aeruginosa, R. solani*, *S. aureus*	ADF	CL
Lentinula boryana[10]	CB	*B. cereus*, *S. aureus*	ADF	BR
Lentinula edodes[10,27,28]	CB; FB	*B. cereus*, *B. subtilis*, *C. albicans*, *M. luteus*, *S. aureus*, *S. mutans*	ADF; ADL	BR
Leucoagaricus cf. *cinereus*[15]	CB	*E. coli*	ADF	BR

Marasmiellus alliiodorus[22]	CB; MC	Active against not specified bacteria and fungi	ADF; MDL	CL
Marasmius sp.[15]	CB	*E. coli*	ADF	BR
Marasmius alliodoratissimus[22]	CB; MC	*P. variotii*	ADF; MDL	CL
Marasmius cf. *bellus*[15]	CB	*E. coli*	ADF	BR
Mycena sp.[29]	CB	*A. porri*, *A. ochraceus*, *B. cinerea*, *M. miehei*, *P. variotii, P. notatum*, *U. nuda*	ADF	CL
Mycena chlorinella[22]	CB; MC	*P. variotii*, *P. notatum*	ADF; MDL	CL
Mycena hialinotricha[24]	CB	*B. subtilis*, *C. pilifera, P. notatum*, *R. solani*, *S. aureus*	ADF	CL
Mycena pura[22]	CB; MC	Active against not specified bacteria and fungi	ADF; MDL	CL
Nothopanus hygrophanus[15]	CB	*L. monocytogenes*, *S. aureus*	ADF	BR

Oudemansiella canarii[15,30,31]	CB	*A. solani*, *C. albicans*, *C. glabrata, C. krusei*, *C. tropicalis*, *C. sphaerospermum*, *C. acutatum*, *S. rolfsii*	ADF; ADL; BI	BR
Pleurotus sp.[33]	CB	*B. subtilis*	ADF; ADL	BR
Psathyrella sp.[24]	CB	*B. subtilis*, *C. pilifera*, *E. faecalis*, *F. oxysporum*, *P. notatum*, *R. solani*	ADF	CL
Stropharia semiglobata[24]	CB	*B. subtilis*, *C. pilifera*, *E. coli*, *E. faecalis*, *P. notatum*, *P. aeruginosa*, *R. solani*, *S. aureus*	ADF	CL
Tephrocybe sp.[22]	CB; MC	*B. brevis*, *P. variotii*, *P. notatum*	ADF; MDL	CL

* CB: Culture Broth; FB: Fruiting Body; MC: Mycelia.

** ADF: Agar Diffusion Test; ADL: Agar Dilution Test; BI: Bioautography; MDL: Macrodilution.

*** BR: Brazil; CL: Chile; UY: Uruguay.

APRESENTAÇÃO DOS RESULTADOS

Os resultados obtidos são apresentados na forma de capítulos que correspondem a dois artigos científicos, como dispostos a seguir:

CAPÍTULO II: SCREENING OF ANTIMICROBIAL SUBSTANCES OF SOUTH BRAZILIAN MUSHROOMS (AGARICALES)

Neste artigo foram testados extratos de 14 espécies de basidiomicetos frente à bactérias e leveduras de importância clínica, sendo que os métodos e resultados obtidos são apresentados a seguir.

CAPÍTULO III: ANTIFUNGAL ACTIVITY OF MUSHROOM (AGARICALES) EXTRACTS FOR CONTROL OF *FUSARIUM GRAMINEARUM*

Neste artigo foram testados extratos de 10 espécies de basidiomicetos frente ao fungo fitopatógeno *Fusarium graminearum*, sendo que os métodos e resultados obtidos são apresentados a seguir.

3 CAPÍTULO II – SCREENING OF ANTIMICROBIAL SUBSTANCES FROM MUSHROOMS (AGARICALES) OF SOUTHERN BRAZIL

Artigo será submetido ao periódico International Journal of Medicinal Mushrooms

Screening of Antimicrobial Substances from Mushrooms (Agaricales) of Southern Brazil

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Short title: Screening of antimicrobials of Brazilian mushrooms

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ABSTRACT: Mushrooms are source of bioactive substances due to the synthesis of secondary metabolites, which may be useful in the search of new substances for pharmaceutical use. The aim of this study was to test antimicrobial potential of

extracts of native South Brazilian mushrooms, against bacteria and yeasts. Basidiomata of 14 species of mushrooms were collected, dried and grounded separately into powdered form, and extracted with methanol in the Soxhlet system. Antimicrobial potential of mushroom extracts were tested by agar diffusion (ADF) and direct bioautography (DB) tests against *Bacillus cereus*, *Candida albicans*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. The ADF showed that the extracts of six mushroom species have compounds potentially useful in inhibiting tested microorganisms, showing moderate sensitivity. For DB tests, nine species presented antibacterial substances and the bioautograms revealed ten antimicrobial substances belonging to five fungal species, from which five substances were observed as indicative of terpenes. Among the species that showed antimicrobial activity, it stands out *Simocybe tucumana*, because this is the first record of bioactivity for the genus.

KEY WORDS: antimicrobials, growth inhibition, medicinal mushrooms, methanolic extract, natural products.

ABBREVIATIONS: ADF, agar diffusion test; **ATCC**, American Type Culture Collection; **BHI**, Brain Heart Infusion; **CFU**, Colony-forming unit; **DB**, direct bioautography; **DMSO**, dimethylsulfoxide; **MH**, Mueller-Hinton; **MS**, moderately sensitive; **R**, resistant; **Rf**, retention factor; **TLC**, Thin-layer chromatography; **UV**, ultraviolet.

3.1 INTRODUCTION

Mushrooms (fungi from the Phyllum Basidiomycota) naturally produce numerous substances with bioactive properties such as antitumoral, antidiabetic,

immunomodulators, antioxidant, trypanocides, leishmanicides, anti-inflammatory, antiviral and antimicrobial.[1-4] Among these, research on new antimicrobial substances is highly necessary due to the emergence of resistant bacterial strains and also to the emergence of new opportunistic species.[5-7]

Many substances with antimicrobial activity were obtained from basidiomycetes, but most of performed research with these fungi only evaluated antimicrobial activity of the total/crude extracts, without testing its isolated compounds.[8] One example is the agar diffusion test (ADF), which relates size of the zone of inhibition to concentration of the tested substance, the evaluation being performed by measuring the growth inhibition halo and comparing it with a reference substance such as an antibiotic.[9]

The use of methodologies that verify antimicrobial properties of isolated substances is important for the activity detection profiles of such compounds[8]. The direct bioautography (DB) test combined with thin-layer chromatography (TLC) is widely used to analyze compounds isolated for antimicrobial activity.[10] In this technique, chromatogram containing the separated substances, is introduced into a biodetection process,[8,10] and after subjected to a colorimetric analysis for the detection of microbial growth.[11] Antibiotics naturally produced by mushrooms are mainly secondary metabolites, especially terpenes.[2,12] For the detection of terpene derivatives, TLC plates are developed with sulfuric anisaldehyde and are visualized as purple/pink spots.[13]

South America represents a natural reservoir of species with possible uses, including basidiomycetes. Therefore, screening of basidiomycetes for antimicrobial activity is important in exploring the potential of these organisms that synthesize a wide variety of bioactive substances.[14]

Thus, due to the properties of the mushrooms belonging to Phylum Basidiomycota and the growing interest in the discovery of new antimicrobial substances, the aim of this study was to verify the antimicrobial potential of basidiomycetes extracts; besides to verify the profiles of the bioactive compounds and to identify possible terpenes.

3.2 MATERIALS AND METHODS

Collection and Dehydration of Basidiomata

Basidiomata were collected in Palotina, State of Paraná, South Brazil, from October 2016 to May 2017. After collecting, mushrooms were dehydrated in a chamber with forced air circulation at 40°C until they reached constant weight.[15] After drying, basidiomata were stored in paper bags for further extraction and voucher specimens were preserved at the Herbarium of Campus Palotina (HCP), Universidade Federal do Paraná.

Fourteen mushrooms species were selected to carry out antimicrobial activity tests: *Calvatia rugosa* (FIGURE 1A), *Coprinopsis* sp. (FIGURE 1B), *Crinipellis siparunae* (FIGURE 1C), *Leucoagaricus* sp., *Leucocoprinus* cf. *brebissonii* (FIGURE 1D), *L. venezuelanus*, *Leucopaxillus gracillimus* (FIGURE 1E), *Marasmius haematocephalus*, *Mycena euspeirea* (FIGURE 1F), *Pleurotus opuntiae* (FIGURE 1G), *Psathyrella candolleana* (FIGURE 1H), *Psathyrella* sp., *Simocybe tucumana* (FIGURE 1I), and *Xeromphalina tenuipes*.

Preparation of Extracts

Dried basidiomata were grounded until a fine powder was obtained. Extraction of the material was carried out with methanol, in the Soxhlet extractor,

with duration of approximately 8 hours for each species, totaling five complete cycles in the extractor.[4,16] Methanol present in the extracts was evaporated in a rotary evaporator under vacuum under 45°C. Methanolic crude extracts were placed into glass vials, and stored in the refrigerator.

Agar Diffusion Test (ADF)

Antibacterial activity of the obtained extracts was tested, under four different concentrations, against four bacteria of clinical importance: *Bacillus cereus* ATCC 11778, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and *Staphylococcus aureus* ATCC 25923. For use in the sensitivity tests, bacterial strains were first cultured in Brain Heart Infusion (BHI) broth for 18 hours. Afterward, they were transferred in Petri dishes containing Mueller-Hinton agar (MH) and incubated in a growth chamber at 35°C, from 18 to 24 hours, to obtain active colonies.

In addition, antifungal activity test of four concentrations of the mushrooms extracts against yeast *Candida albicans* ATCC 10231 was also carried out. For this, the strain was cultured in BHI broth for 18 hours at 35°C, and then cultured in MH medium.

ADF was used to investigate antimicrobial activity of the microrganisms. Bacterial inoculum was prepared using direct colony suspension method, where isolated colonies were selected and placed in a saline solution of 0.9%, comparing turbidity to the 0.5 scale of McFarland, which corresponds to the concentration 1×10^8 CFU mL^{-1}.[17]

With a sterile swab, bacterial suspension was inoculated over entire surface of Petri dishes containing MH agar.[18] In each plate, with the aid of a sterile cutter, six wells were made for testing four different concentrations (1.2;

2.5; 5.0; 10.0 mg mL^{-1}), negative and positive control. Crude extracts were previously solubilized with dimethylsulfoxide (DMSO).[19] For positive control of bacteria, it was used commercial antibiotic gentamicin, diluted to a concentration of 0.1 mg mL^{-1},[20] and for yeast *C. albicans* was used nystatin at a concentration of 10 mg mL^{-1}.[9] Negative control was sterile distilled water plus DMSO.

With a micropipette, 40 µL of each extract concentrations were added, positive and negative control in their respective wells. After, plates were incubated at 35°C and reading performed among 16 – 18 hours after incubation, by measuring inhibition halos in millimeters.[9,18]

Bioassays were performed in duplicate with 3 replicates, to calculate the mean. Afterwards, microorganisms were classified as sensitive when diameter of the inhibition halo was greater than positive control or no more than 3 mm less than positive control; moderately sensitive when halo was less than the positive control by more than 3 mm but greater than 2 mm; and resistant when diameter of the halo was equal to or less than 2mm.[9]

Thin-layer Chromatography (TLC)

Separation of the substances present in the crude methanolic extracts was performed through TLC. Crude extract was solubilized with 10 µl of methanol. After, each extract was applied with a capillary in the aluminum chromato-sheets 7×5 cm (silica gel G60 F254, Merck). For mobile phase, a solvent system was used ethyl acetate and hexane (1:1). Plates were developed at a distance of 6 cm, and the solvent was evaporated at room temperature. After drying, stains were observed in ultraviolet (UV) light at 254 nm and revealed with the use of sulfuric anisaldehyde.[8,13,21]

Direct Bioautography (DB)

Chromatographic run was performed as described above. However, for use in the bioautography, the test plate was not developed with sulfuric anisaldehyde.[11,22] Also, methanolic extracts of the mushrooms were applied in the chromatographic plates, submitted to the DB test and in this case, plates were not runned, that is, they were not subjected to the mobile phase.

Bacterial inoculum was prepared using the method direct suspension of the colonies, as described for ADF, and then poured into a sterile Petri dish.

TLC with separate substances and the not runned plates with organic solvent (containing the crude extract only in one point), were submerged in the bacterial suspension where they were held for eight seconds. Later, plates were transferred to another sterile Petri dish containing sterilized moist cotton. Plates were incubated in an oven for 16 – 18 hours under 35°C.[11,21,22] Only the bacteria (*B. cereus*, *E. coli*, *P. aeruginosa,* and *S. aureus*) were tested.

For visualization of antibacterial activity, after incubation period, bioautograms were sprayed with a solution of nitro-tetrazolium blue chloride, re-incubated for 3-4 hours at 35°C.[11,22] The zones of inhibition were observed and measured for the calculation of the retention factor (Rf - ratio between the distance traveled by the substance and the distance traveled by the mobile phase).[11]

For each extract, five TLC plates were developed. One of these plates, reference chromatogram, was revealed with sulfuric anisaldehyde to identify terpenes. Other four plates were used for DB tests (one plate for each bacteria).

3.3 RESULTS

Agar Diffusion Test

Among the 14 mushroom species evaluated by the ADF, only 6 species showed inhibitory effects. Results obtained with ADF against tested microorganisms are shown in Table 1.

Three mushroom extracts inhibited the growth of *B*. *cereus*, namely: *Calvatia rugosa*, *Leucoagaricus* sp. and *Psathyrella* sp. Major inhibition halo of *B*. *cereus* was observed with *C*. *rugosa* extract and the concentration of 10.0 mg mL^{-1} was most active.

The second most effective extract against *B*. *cereus*, was obtained from *Psathyrella* sp. under concentration of 10.0 mg mL^{-1}, followed by the extract of *Leucoagaricus* sp. which showed higher antibacterial activity in the concentrations of 5.0 and 2.5 mg mL^{-1}.

For the yeast *C*. *albicans,* only the extract of *P*. *candolleana* inhibited its growth, exhibiting antimicrobial activity under the concentration of 10.0 mg mL^{-1} (FIGURE 2A).

Extract of the mushroom *Leucocoprinus* cf. *brebissonii* was the only one able to inhibit *E*. *coli*, whose larger inhibition halo was observed under concentration of 10.0 mg mL^{-1} (FIGURE 2B).

Two mushroom extracts inhibited growth of *P*. *aeruginosa*: *Leucocoprinus* cf. *brebissonii* and *C*. *siparunae*. The larger inhibition halo observed for this bacteria was obtained by the extract of *Leucocoprinus* cf. *brebissonii* at the concentrations of 5.0 and 10.0 mg mL^{-1} (FIGURE 2C). Second largest inhibition halo *P*. *aeruginosa* was observed by the extract of *C*. *siparunae* under 10.0 mg mL^{-1} concentration of (FIGURE 2D).

The results for to the ADF against *S. aureus* demonstrated that only four mushroom extracts inhibited its growth. The larger inhibition halo was observed for the extract of *L. venezuelanus,* under the concentration of 10.0 mg mL^{-1} (FIGURE 2E), followed by the extract of *C. siparunae* at the concentrations of 10.0 and 5.0 mg mL^{-1}.

When the extract of mushroom *X. tenuipes* was tested against *S. aureus*, the largest inhibition halo was observed at the concentrations of 5.0 and 10.0 mg mL^{-1} (FIGURE 2F). The extract of *Leucocoprinus* cf. *brebissonii* inhibited *S. aureus* under concentrations of 1.2; 5.0 and 10.0 mg mL^{-1}.

Direct Bioautography - Thin-layer Chromatography (TLC-DB)

The solvent mixture (ethyl acetate and hexane 1:1) used as mobile phase was able to separate substances present in the methanolic crude extracts of the species, except for the *Leucoagaricus* sp. extract, which showed no visible substances.

Bioautographs performed against *B. cereus* revealed that only the extract of *M. euspeirea* presented antimicrobial activity. The substance responsible for such activity had retention factor (Rf) of 0.56 (FIGURE 3B). However, unlike to isolated substances, crude extract of *M. euspeirea* did not presented antimicrobial activity against *B. cereus*, when applied at one point on the chromatographic plate.

For *E. coli*, the bioautographic tests did not detected any substance or extract with antimicrobial action thus, no inhibition halo was observed.

To verify the antibiotic activity against *S. aureus,* bioautograms showed that extracts of *Coprinopsis* sp. (FIGURE 4B), *Psathyrella* sp., *M. euspeirea* and *X. tenuipes* showed substances with inhibitory activity (Rf of 0.56; 0.54; 0.22,

0.26, 0.56; and 0.51, respectively). The extract of *M. euspeirea* presented three regions of bacterial growth inhibition. The substance isolated from *Coprinopsis* sp. extract inhibited the bacterial growth as showed in the FIGURE 4B.

On the other hand, in the bioautograms not runned against *S. aureus* was observed that, in addition to extracts of *Coprinopsis* sp., *Psathyrella* sp. and *M. euspeirea*, extracts of *Leucocoprinus* cf. *brebissonii*, *L. venezuelanus* and *S. tucumana* showed inhibition halo, revealing that crude extracts of these mushrooms presented antibacterial activity against this bacteria. However, for crude extract of *X. tenuipes* no antibiotic activity was observed.

Within the developed and tested bioautograms against *P. aeruginosa*, only extracts of *S. tucumana* (FIGURE 5B) and *X. tenuipes* presented substances with antibacterial activity. For not runned bioautograms of *P. aeruginosa*, it was observed that extracts of *Coprinopsis* sp., *M. haematocephalus*, *M. euspeirea* and *P. candolleana* presented inhibition halos, revealing that crude extracts of the mushrooms showed antibacterial activity. On the other hand, bioautograms not developed of *S. tucumana* and *X. tenuipes* not showed inhibition halos.

3. 4 DISCUSSION

Species of *Calvatia* are widely studied from the biotechnological point of view, presenting medicinal properties, as antitumor activity and anticancer, antiviral, antibacterial and antifungal.[23] Ethanolic extract of the basidiomata of *Calvatia excipuliformis* demonstrated activity against bacteria *B. subtilis*, *E. coli*, *Micrococcus luteus*, *P. aeruginosa* and *Staphylococcus epidermidis*.[24] Ethanolic extract of *Calvatia* sp. inhibited growth of *E. coli* and *S. aureus*, and the aqueous extract inhibited only *S. aureus*.[25] The filtrate of *Calvatia craniiformis* showed

activity against *P. aeruginosa* and *S. aureus*.[26] These results corroborate with what was observed here.

The culture broth of *Crinipellis schevczenkovi* presented activity against *B. subtilis*, *E. coli* and *S. aureus* by ADF,[7] as was verified for *C. siparunae* extract here. On the other hand, of the four diterpenes isolated from *C. stipitaria*, none of them presented activity against bacteria *E. coli*, *B. subtilis*, *Bacillus pumilus* and *S. aureus*.[27]

Antibacterial potential of the species of *Leucoagaricus* has been investigated. In a study with *L. gongylphorus*, culture broth inhibited the growth of *E. coli*, but did not present activity versus *B. subtilis*, *P. aeruginosa*, *Salmonella typhimurium* and *S. aureus*.[28] Ethanolic extract of *L. leucothites* inhibited the growth of several bacteria, as *B. cereus*, *E. coli*, *Listeria monocytogenes*, *P. aeruginosa*, *Proteus vulgaris*, *S. aureus*, *Salmonella enteritidis*, *Yersinia enterocolitica*, and *Shigella sonnei*.[29] Extracts of *L. pudicus* also inhibited a wide variety of species of bacteria: being: *B. subtilis*, *E. coli*, *Enterobacter aerogenes*, *S. typhimurium*, *S. aureus* and *Staphylococcus epidermidis*.[30] We observed antimicrobial activity of the extract of the *Leucoagaricus* sp. only against *B. cereus*.

Ethyl acetate extracts of *Leucocoprinus* cf. *longistriatus* and *Leucocoprinus* sp., were tested against *B. cereus*, *B. subtilis*, *Enterococcus faecalis*, *E. coli*, *L. monocytogenes*, *P. aeruginosa*, *S. typhimurium*, *S. aureus*, *S. epidermidis*, *S. saprophyticus*, *Streptococcus pyogenes*, and *S. pneumoniae*, but did not presented inhibitory activity on these bacteria.[31] We verified antibacterial action of the methanolic extracts of *Leucocoprinus* sp. and *Leucocoprinus* cf. *brebissonii*.

Several species of *Mycena* have already been tested for antimicrobial potential, as for example *Mycena* cf. *alcalina*, *M. aurantiomarginata*, *M. leucogala*, *M. maculata*,[32] *M. pura*,[33,34] *M. hialinotricha*[35] among others. It is importance to notice that the first report of antibacterial activity related to *M. euspeirea* was obtained in this study. When the crude extract substances were separated by TLC, the active substance (Rf 0.56) seems to be a terpene (FIGURE 3A). Besides the antibacterial activity, another interesting feature of *M. euspeirea* is bioluminescence, which evidence the rich secondary metabolism of this species.[36]

Antimicrobial activity studies also have been done using extracts from *Psathryrella* species. Extracts (both aqueous and ethanolic) obtained from the basidiomata of the *Psathyrella* sp. showed activity against *E. coli* and *S. aureus*.[25] The extract of the *Psathyrella* sp. inhibited the growth of *B. subtilis* (19 mm) and *Escherichia faecalis* (16 mm), but did not inhibited the growth of *E. coli*, *P. aeruginosa* and *S. aureus*.[35] Chloroform extract of *P. candolleana* inhibited the growth of *Moraxella catarrhalis* and *Streptococcus pneumoniae*, on the other hand, the extract of *P. piluliformis* did not presented activity against any tested bacteria.[37] Culture filtrate of *P. atroumbonata* showed inhibitory activity against *E. coli* and *S. aureus*.[38]

Here DB test against *S. aureus* showed that *Psathyrella* sp. extract has a bioactive substance at Rf 0.54. Thus, as this spot was of visible by rosy coloration after the sulfuric anisaldehyde reagent application, the substance may be indicative of belonging to the terpene group. In addition, the antimicrobial activity of the extracts of *Psathyrella* sp. and *P. candolleana* was noted in the ADF.

Methanolic extract of *S. tucumana* also presented a substance with antibacterial potential and it can be an indicative of terpene (FIGURE 5A). The

report of antibacterial activity of *S. tucumana* seems to be the first for the genus *Simocybe*.

Ethyl acetate extract of *Xeromphalina tenuipes* showed no inhibitory activity against the tested bacteria according to previous study.[31] Different from the data observed for substances isolated from *Xeromphalina* sp. which presented antibacterial and antifungal activity and were characterized as sesquiterpenes.[39] We observed that the extract of *X. tenuipes* also presented a substance with antibacterial potential (Rf 0.51), being indicative of terpene too. Antibacterial activity of *X. tenuipes* was also observed in the agar diffusion test.

Antifungal activity of basidiomycetes extracts against the yeast *C. albicans* also has been investigated. Methanolic extract of *P. atroumbonata* did not presentrf inhibitory activity when tested against *C. albicans*.[40] The extracts of *Irpex lacteus* and *Oudemansiella canarii* inhibited growth of this yeast.[31] Here, the extract of *P. candolleana* showed activity against *Candida albicans.*

We observed that even with inhibition halos in the agar diffusion test, the microorganisms were classified as resistant for most of the extracts used, since this halo had averages equal to or lower than 2 mm. However, some extracts inhibited the growth of microorganisms by more than 2 mm and were considered moderately sensitive. *Bacillus cereus* showed moderate sensitivity for extracts of *Calvatia rugosa*, *Psathyrella* sp. and *Leucoagaricus* sp. *Pseudomonas aeruginosa* was classified as moderately sensitive to the extract *Leucocoprinus* cf. *brebissonii*; and *S. aureus* to the extract of *L. venezuelanus*. *Candida albicans* presented moderate sensitivity against extract of *P. candolleana*. None of the microorganisms were classified as sensitive to the extracts tested.

When compared to inhibition halos of the extracts with positive control (gentamicin and nystatin), we verified that extracts presented smaller halos,

indicating discrete antibacterial activity. This fact may be associated with the amount of substances present, since it is the crude extract was used. Another reason may be associated with environmental and nutritional factors, since these conditions may alter the metabolism of the mushrooms, leading to variations in the production of their secondary metabolites. Thus, mushrooms from different ecosystems and mushrooms grown on different substrates can produce distinct antimicrobial substances.[12,34]

3.5 CONCLUSION

Based on results obtained with ADF it can be assumed that of the extracts of the 14 species of mushrooms, six of them have compounds potentially useful in inhibiting the tested microorganisms, since they presented moderate sensitivity.

In the other hand, when using the bioautography test, we observed that nine crude extracts presented antimicrobial substances against tested bacteria; and ten substances (belonging to five mushrooms species) with antimicrobial properties were observed. Among these substances, five of them are indicative of terpenes.

Thus, the bioprospection of basidiomycetes aiming at antimicrobial potential is important for the search for new substances. However, the identification of the bioactive compounds and the study of mechanisms of actions are further necessary. Given the extent of Brazilian mycobiota, it should be expected that with the progress of the present research new and promising substances with antimicrobial properties can be discovered.

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Table 1. Averages of the inhibition halo (mm) of basidiomata methanolic extracts against microorganisms of clinical importance.

	10.0 mg mL^{-1}	5.0 mg mL^{-1}	2.5 mg mL^{-1}	1.2 mg mL^{-1}
B. cereus				
Calvatia rugosa	6.3 (MS*)	2.0 (R)	0.0 (R)	1.3 (R)
Leucoagaricus sp.	1.8 (R)	2.7 (MS)	2.7 (MS)	0.5 (R)
Psathyrella sp.	2.8 (MS)	1.8 (R)	2.0 (R)	1.2 (R)
Gentamicin	11.0			
C. albicans				
Psathyrella candolleana	3.8 (MS)	0.0 (R)	0.0 (R)	0.0 (R)
Nystatin	7.0			

E. coli

Leucocoprinus brebissonii	cf.	2.0 (R)	1.5 (R)	1.0 (R)	1.0 (R)
Gentamicin		10.0			

P. aeruginosa

Crinipellis siparunae		2.0 (R)	1.5 (R)	0.0 (R)	0.0 (R)
Leucocoprinus brebissonii	cf.	3.0 (MS)	3.0 (MS)	1.0 (R)	0.0 (R)
Gentamicin		7.0			

S. aureus

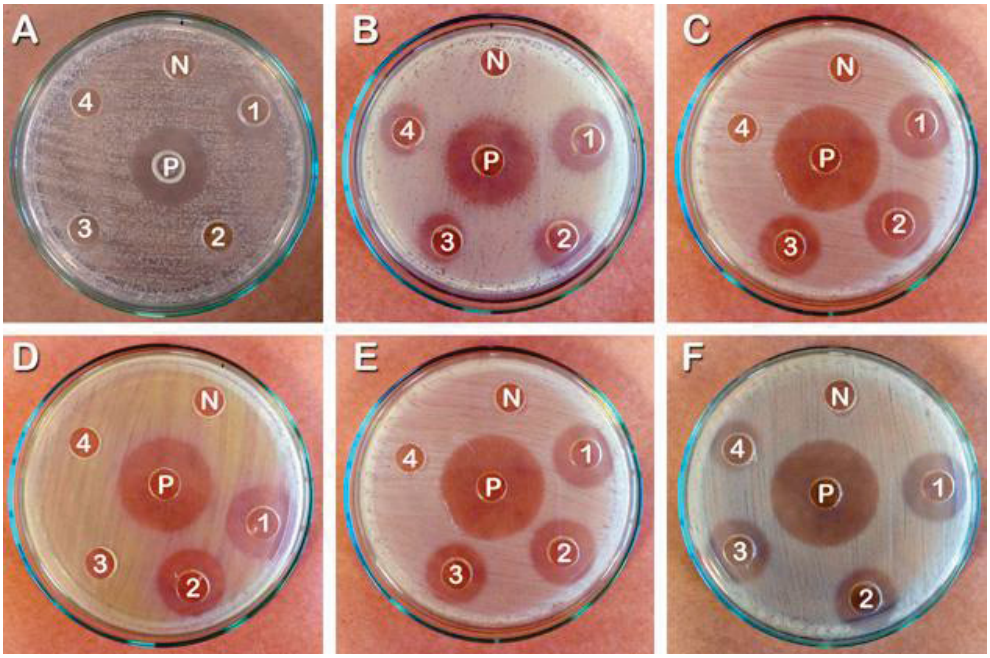
Crinipellis siparunae		2.0 (R)	2.0 (R)	0.0 (R)	0.0 (R)
Leucocoprinus brebissonii	cf.	1.0 (R)	1.0 (R)	0.0 (R)	0.5 (R)
Leucocoprinus venezuelanus		2.1 (MS)	1.8 (R)	1.0 (R)	0.0 (R)
Xeromphalina tenuipes		1.7 (R)	1.7 (R)	1.2 (R)	1.0 (R)
Gentamicin		11.0			

*MS: moderately sensitive; R: resistant.

FIGURE 1 – Basidiomata of mushrooms tested for antimicrobial activity.

A. *Calvatia rugosa*; **B.** *Coprinopsis* sp.; **C.** *Crinipellis siparunae*; **D.** *Leucocoprinus* cf. *brebissonni*; **E.** *Leucopaxillus gracillimus*; **F.** *Mycena euspeirea*; **G.** *Pleurotus opuntiae*; **H.** *Psathyrella candolleana*; **I.** *Simocybe tucumana*.

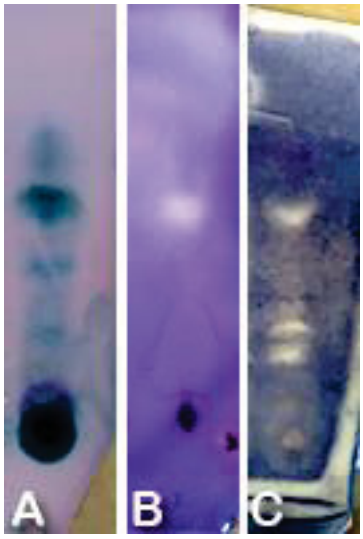
FIGURE 2 – Agar diffusion test of the methanolic extracts of basidiomycetes against microorganisms.



P: positive control; N: negative control; 1: 10,0 mg mL^{-1}; 2: 5,0 mg mL^{-1}; 3: 2,5 mg mL^{-1}; 4: 1,2 mg mL^{-1}.

A. Extract of *P. candolleana* against *C. albicans*; **B.** Extract of *Leucocoprinus* cf. *brebissonii* against *E. coli*; **C.** Extract of *Leucocoprinus* cf. *brebissonii* against *P. aeruginosa*; **D.** Extract of *C. siparuanae* against *P. aeruginosa*; **E.** Extract of *L. venezuelanus* against *S. aureus*; **F.** Extract of *X. tenuipes* against *S. aureus*.

FIGURE 3 – Antimicrobial activity evaluation of *Mycena euspeirea* extract.

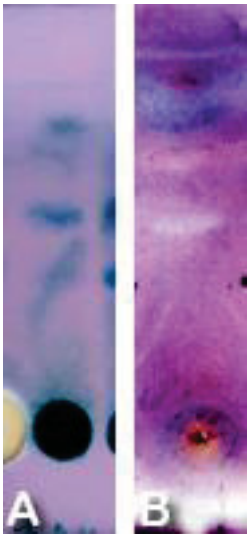


A. Thin-layer chromatography revealed with sulfuric anisaldehyde; **B**. Bioautogram against *B. cereus*; **C**. bioautogram against *S. aureus*.

FIGURE 4 – Antimicrobial activity evaluation of *Coprinopsis* sp. extract.

A. Thin-layer chromatography revealed with sulfuric anisaldehyde; **B**. Bioautogram against *S. aureus*.

FIGURE 5 – Antimicrobial activity evaluation of *Simocybe tucumana* extract.



A. Thin-layer chromatography revealed with sulfuric anisaldehyde; **B**. Bioautogram against *P. aeruginosa*.

4 CAPÍTULO III – ANTIFUNGAL ACTIVITY OF MUSHROOM (AGARICALES) EXTRACTS FOR CONTROL OF *Fusarium graminearum*

Artigo será submetido ao periódico Pesquisa Agropecuária Brasileira

Antifungal activity of mushroom (Agaricales) extracts for control of *Fusarium graminearum*

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Abstract - Mushrooms produce a wide variety of bioactive secondary metabolites that may be useful to control phytopathogenic bacteria and fungi. Aiming to verify *in vitro* the antifungal potential against *Fusarium graminearum,* methanolic extracts from the basidiomata of ten mushroom species were obtained. Antifungal activity was evaluated through agar dilution test at a concentration of 1.0 mg ml^{-1}. Data obtained from the third to the fifteenth day of mycelial growth were submitted to analysis of variance, and the means were compared by the Tukey test at 5% of probability. Among ten evaluated extracts, six significantly inhibited the growth of *F. graminearum*, namely: *Calvatia rugosa*, *Coprinopsis* sp., *Leucocoprinus* cf. *brebissonii*, *Leucopaxillus gracillimus*, *Simocybe tucumana* and *Xeromphalina tenuipes*. The results indicate that these four mushroom species produce substances with antifungal activity. On the other hand, the extract of *Pleurotus opuntiae* stimulated mycelial growth of the fungus. When

comparing the effect of the extracts with the fungicide Cercobin®, only the *Simocybe tucumana* obtained similar results, proving the antifungal potential of this species.

Index terms: alternative control, Basidiomycota, fusariosis, natural products, phytopathogen.

Atividade antifúngica de extratos de cogumelos (Agaricales) no controle de *Fusarium graminearum*

Resumo - Os cogumelos produzem uma ampla variedade de metabólitos secundários bioativos que podem ser úteis no controle de bactérias e fungos fitopatogênicos. O objetivo deste trabalho foi testar *in vitro* o potencial antifúngico de extratos metanólicos de basidiomas de dez espécies de cogumelos, frente ao fitopatógeno *Fusarium graminearum*. A atividade antifúngica dos extratos foi avaliada pelo teste de diluição em ágar na concentração de 1,0 mg ml^{-1}. Os dados obtidos, do terceiro ao décimo quinto dia de crescimento micelial, foram submetidos à análise de variância, sendo que as médias foram comparadas pelo teste de Tukey a 5% de probabilidade. Entre os dez extratos avaliados, seis inibiram significativamente o crescimento de *F. graminearum* sendo: *Calvatia rugosa*, *Coprinopsis* sp., *Leucocoprinus* cf. *brebissonii*, *Leucopaxillus gracillimus*, *Simocybe tucumana* e *Xeromphalina tenuipes*. Os resultados indicam que as quatro espécies de cogumelos produzem substâncias com atividade antifúngica. Entretanto, o extrato de *Pleurotus opuntiae* estimulou o crescimento micelial do fungo. Quando o efeito dos extratos dos cogumelos foi comparado com o fungicida Cercobin®, somente a espécie *Simocybe tucumana* obteve resultados similares, comprovando o potencial antifúngico desta espécie.

Termos para indexação: Basidiomycota, controle alternativo, fitopatógeno, fusariose, produtos naturais.

4.1 Introduction

Several fungi are widely known as responsible for causing diseases in plants, especially members of the genera *Alternaria*, *Aspergillus*, *Botrytis*, *Fusarium*, *Penicillium* and *Rhizopus* (Aqueveque et al., 2016). Among these, members of *Fusarium* are considered phytopathogens of major importance in agriculture, as they affect several agricultural crops (Ma et al., 2013). Cob fusariosis is one of the most important diseases of wheat crops in the world and is caused mainly by *Fusarium graminearum*, although other species of the genus may cause such disease (Ferrigo et al., 2016). Besides reducing grain production, the fungus produces toxic secondary metabolites (mycotoxins), which can contaminate agricultural products, rendering infected grains unsuitable for human and animal consumption (Alves et al., 2013; Ma et al., 2013).

Excessive use of agrochemicals to control diseases in plants, besides presenting a series of risks of contamination to the environment, can also harm the health of producers and consumers (Domingues et al., 2011; Aqueveque et al., 2016). Thus, must be emphasized the importance of research the use of alternative methods in agriculture that are efficient and generate the minimum environmental impact, such as the use of natural products (Ribas et al., 2016). In addition, resistant phytopathogens may arise to the chemical substances used in the field (Aqueveque et al., 2016), being necessary the search for new substances with antimicrobial properties.

Among the most important sources of bioactive metabolites, the fungi that belong to the phylum Basidiomycota, known as mushrooms, are considered highly promising, since various compounds were isolated from these organisms, presenting various biological functions, including antifungal activity (Barneche et al., 2016). As for example, the metabolite Favolon B, obtained from the fermentation of mycelial cultures of basidiomycete *Mycena* sp., which presents antifungal activity against species: *Alternaria porri*, *Aspergillus ochraceus*,

Botrytis cinerea, *Mucor miehei*, *Paecilomyces variotii*, *Penicillium notatum* and *Ustilago nuda* (Aqueveque et al., 2005).

Another example, the strobilurin, a natural product produced by a variety of basidiomycete fungi, such as the species *Oudemansiella mucida* and *Strobilurus tenacellus*. This natural product has become important for controlling a variety of disease-causing fungi in plants, and is currently produced by chemical synthesis for commercialization (Bartlett et al., 2002).

Based on the search for alternative and efficient products to control the growth of microorganisms, this work aimed to test, *in vitro*, the antifungal potential of methanolic extracts from mushrooms collected in the Western of Paraná, southern Brazil, against the phytopathogen *F. graminearum*.

4.2 Materials and Methods

Basidiomata (i.e., the macroscopic spore-producing bodies of agaricoid basidiomycetes) were collected in fragments of the Semideciduous Seasonal Forest, in Palotina, in the West of Paraná, from October 2016 to May 2017. After collecting, the mushrooms were dehydrated in a chamber with forced air circulation at 40°C until they reached constant weight (Carvalho et al., 2012). After drying, the basidiomata were stored in paper bags for further extraction.

Ten species of mushrooms were selected to perform antimicrobial activity tests, namely: *Calvatia rugosa*, *Coprinopsis* sp., *Leucoagaricus* sp., *Leucocoprinus* cf. *brebissonii*, *Leucopaxillus gracillimus*, *Pleurotus opuntiae*, *Psathyrella* sp., *Psathyrella candolleana*, *Simocybe tucumana* and *Xeromphalina tenuipes*.

Dried basidiomata were ground until a fine powder was obtained and the extraction was carried out with methanol using Soxhlet extractor. Extraction was carried out for

approximately 8 hours for each species, completing five cycles in the extractor (Figueiredo & Silva, 2014; Ajith & Janardhanan, 2015). Methanol present in the extracts was evaporated in a rotary evaporator under vacuum at 45°C. Crude extracts were placed in glass jars, and stored at 4°C.

Samples of the fungus *F. graminearum* were kindly supplied by the Universidade Estadual de Maringá (UEM) and stored in Petri dishes containing potato dextrose agar (PDA). For the experiment, the fungus was transferred onto a new plate containing PDA medium and incubated at 28°C for seven days.

The obtained mushroom extracts were tested against the phytopathogen *F. graminearum*. The inhibition of micelial growth test (agar dilution test) was used to evaluate the antifungal activity, which consists of incorporating the dissolved extract to the agar; thus, each plate contains a different concentration of the agent (CLSI, 2012).

Culture medium used in the tests was also the PDA, prepared according to the manufacturer's instructions, and the extract of basidiomycetes was added at the end concentration of 1 mg ml^{-1} before autoclaving the medium. The crude extract was previously solubilized with dimethylsulfoxide (DMSO), as it is a non-toxic solvent (Klaus et al., 2015). As a negative control, was used only the PDA medium plus DMSO, without the addition of the basidiomycete extract. For the positive control, the culture medium plus the fungicide methyl thiophanate (Cercobin®) at a concentration of 1 mg ml^{-1} (Garcia et al., 2008; Silva et al., 2014).

After agar solidification, a mycelial disc (10 mm diameter) was transferred to the center of the culture medium surface. Petri dishes were kept at a controlled temperature of 28°C (D'addazio et al., 2016). The evaluation of the mycelial growth was performed every 48 hours for 15 days or until the fungus completed the entire diameter of the Petri dish, and the

colony diameter (cm) was measured in perpendicularly opposite directions. For each extract, five replicates were performed.

The data were submitted to analysis of variance. The means between the different treatments were compared by the Tukey test at 5% probability. Statistical analyzes were performed in the Sisvar 5.6 Program of the Universidade Federal de Lavras.

4.3 Results and Discussion

Extracts of the mushroom species *Psathyrella* sp. and *P. candolleana* did not presented significant differences in relation to the negative control, indicating that the extracts of these species did not influence the growth of *F. graminearum*. In contrast to our observations in this study, the extract of *Psathyrella* sp. was reported as active against *Fusarium oxysporum* (Reinoso et al., 2013). These differences between the results may be related to the method of obtaining the extract and also the species used, which certainly are different each other.

For extract of *P. opuntiae* (Table 1) it was observed that from the ninth evaluation day extract started to significantly stimulate the mycelial growth of the fungus *F. graminearum*. As well as reported for the mushroom extract *Agaricus blazei* against *Botrytis cinerea*, where it was verified that the extract had a stimulating effect both on the germination of the conidia and on the mycelial growth (Camili et al., 2009). This fact was also verified when the aqueous extract of coriander (*Coriandrum sativum*) was used in front of the fungus *F. verticillioides*; a significant increase in the conidia production was observed (Barros et al., 2013). The stimulus of mycelial growth or in the production and germination of conidia, may be related to the presence of substances, which favor the fungi growth and development, such as carbohydrates, proteins, vitamins and even chemical elements.

Extract of the species *Calvatia rugosa* significantly inhibited the growth of *F. graminearum* from the fifth day of evaluation (Table 2; Figure 1), with percentages of inhibition varying between 6.2% and 36.4%. Members of the genus *Calvatia* has been studied due to its biological properties, such as antitumor, anticancer, antiviral, antibacterial and antifungal activities (Coetzee & Van Wyk, 2009). Methanolic extract of *Calvatia fragilis* presented antifungal activity against *Candida albicans* and *Candida maltosa*, besides showing antibacterial activity against *B. subtilis*, *Micrococcus flavus* and *Staphylococcus aureus* (Al-Fatimi et al., 2013).

From the fifth day of evaluation, treatment containing *Coprinopsis* sp. extract showed significantly lower growth than the negative control (Table 2). The highest percentage of inhibition was observed on the fifth day of evaluation (38.6%). Methanolic extract of *Coprinopsis atramentaria* also showed antifungal activity against *Aspergillus fumigatus*, *Aspergillus niger*, *Aspergillus ochraceus*, *Aspergillus versicolor*, *Penicillium funiculosum*, *Penicillium ochrochloron*, *Penicillium verrucosum* and *Trichoderma viride* (Heleno et al., 2014).

For the extract obtained from *Leucoagaricus* sp. basidiomata (Table 2), a significant inhibition of fungal growth was observed when measurements were performed between the fifth and ninth day of mycelial growth, with percentages of inhibition varying from 6.8% to 16.8%.

For the treatment containing the extract of *Leucocoprinus* cf. *brebissonii* we observed that from the ninth day of evaluation the mycelial growth of phytopathogenic *F. graminearum* was significantly inhibited (Table 2), with percentages of inhibition varying from 7.5 to 9.6%. Extract of *L. fragilissimus* showed antifungal activity against Colletotrichum coffeanum (Yaling et al., 2014).

When *Leucopaxillus gracillimus* extract was added to the culture medium we observed that the mycelial growth of *F. graminearum* was significantly inhibited mainly in the last days of evaluation (Table 2). Extract of *L. albissimus* showed antimicrobial activity against Penicillium inflatum and Streptomyces galilaeus (Alves et al., 2013). Even as L. giganteus which presented activity against several fungi, such as *Alternaria solani*, *Aspergillus solani*, *A. niger*, *Colletotrichum graminicola*, *Fusarium solani* and *F. oxysporum* (Feleke & Doshi, 2017). The antifungal activity of *L. gracillimus* observed here seems to be the first report for the species.

For the extract of *Xeromphalina tenuipes* it was observed that fungal mycelial growth was significantly lower in comparison to the negative control, exhibiting up to 24.3% inhibition (Table 2). On the third and on the thirteenth day no significant difference was observed, being that the phytopathogen *F. graminearum* already occupied the entire Petri dish for the negative control and for the treatment.

Antimicrobial activity of *Xeromphalina* sp. has already been described, two isolated substances, xeromphalinones 1 and 2, presented antifungal activity against *Mucor miehei*, *Nematospora coryli*, *Penicillium notatum* and *Paecilomyces variotii*, besides showing antibacterial activity against *Bacillus brevis*, *Bacillus subtilis*, *Enterobacter dissolvens* and *Micrococcus luteus* (Liermann et al., 2010). Xeromphalinone 4 presented activity only against the fungus *N. coryli* (Liermann et al., 2010).

Extract of *Simocybe tucumana* (Table 3, Figure 2) significantly inhibited mycelial growth of *F. graminearum* during all evaluated days. Antifungal activity is reported for the first time to members of this genus.

When comparing the effect of the extracts with the Cercobin® fungicide, used as a positive control, only the *S. tucumana* species obtained significantly similar results (Table 3),

and on the fifth day of evaluation it was more efficient than the fungicide, proving a strong antifungal potential.

The use of extracts of basidiomycetes to inhibit the mycelial growth of fungi of the genus *Fusarium* has already been described in the literature. Extract of the basidiomycete *Inocybe geophylla* inhibited the growth of the fungus *Fusarium oxysporum* (Reinoso et al. 2013). Extracts of species *Pycnoporus sanguineus* and *Lentinus crinitus*, also showed inhibition effect on mycelial growth, germination of conidia of the phytopathogen *Fusarium* sp. (Figueiredo & Silva, 2014). Evidencing that extracts of basidiomycetes contain substances that inhibit mycelial growth of fungi of the genus *Fusarium*.

Besides the antifungal activity observed here, i*n vivo* studies are required to prove the efficacy of *C. rugosa*, *Coprinopsis* sp., *Leucocoprinus* cf. *brebissonii*, *L. gracillimus*, *S. tucumana* and *X. tenuipes* extracts on fusariosis. In addition, characterization and isolation studies of antifungal substances should be conducted, as well as studies on the mechanism of action of these substances.

4.4 Conclusions

1. Methanolic extracts of *Calvatia rugosa*, *Coprinopsis* sp., *Leucocoprinus* cf. *brebissonii*, *Leucopaxillus gracillimus*, *Simocybe tucumana* and *Xeromphalina tenuipes* showed direct fungitoxic action inhibiting the mycelial growth of *Fusarium graminearum*.

2. Methanolic extract of *Pleurotus opuntiae* stimulated the mycelial growth of *Fusarium graminearum*.

3. Extract of *Leucopaxillus gracillimus* showed antifungal activity against *Fusarium graminearum*, and seems to be the first report of this type of activity for this species.

4. Extract of *Simocybe tucumana* showed significantly inhibition of the mycelia growth of *Fusarium graminearum* and it seems to be the first report demonstrating the antifungal activity of this genus.

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Table 1. Methanolic extract effect of *Pleurotus opuntiae* on the mycelial growth of *Fusarium graminearum*.

	3° Day	5° Day	7° Day	9° Day	11° Day	13° Day	15° Day
P. opuntiae	1.80[(1)]b	3.18 b	4.32 a	5.58 a	7.02 a	8.48 a	8.80 a
Negative control	3.02 a	3.68 a	4.30 a	4.88 b	5.56 b	6.12 b	6.90 b
Positive control[(2)]	1.76 b	2.36 c	2.88 b	3.32 c	3.76 c	4.22 c	4.54 c
CV[(3)] (%)	16.78	6.56	8.05	7.73	4.66	5.51	5.28

Means among the different treatments were compared among themselves within the same column by the Tukey 5% test.

[(1)] Mean diameter of the mycelium in cm. [(2)] Cercobin fungicide as positive control. [(3)] Coefficient of variation.

Table 2. Percentage inhibition of mycelial growth of *Fusarium graminearum* when using the extracts of mushrooms in the 15 days of evaluation.

	3°	5°	7°	9°	11°	13°	15°
Calvatia rugosa	ns[1]	26,9[2]	36,4	31,2	17,7	8,0	6,2
Coprinopsis sp.	ns	38,6	38,3	25,1	12,7	6,4	4,7
Leucoagaricus sp.	ns	14,5	16,8	6,8	-[3]	-	-
Leucocoprinus cf. *brebissonii*	ns	ns	ns	9,6	9,7	8,4	7,5
Leucopaxillus gracillimus	11,6	ns	ns	8,0	8,2	4,9	3,3
Xeromphalina tenuipes	ns	13,7	24,3	17,9	7,1	-	-

[1] Not statistically significant. [2] Percentage inhibition of mycelial growth of *F. graminearum*. [3] Mycelial growth of *F. graminearum* already occupied the entire Petri dish.

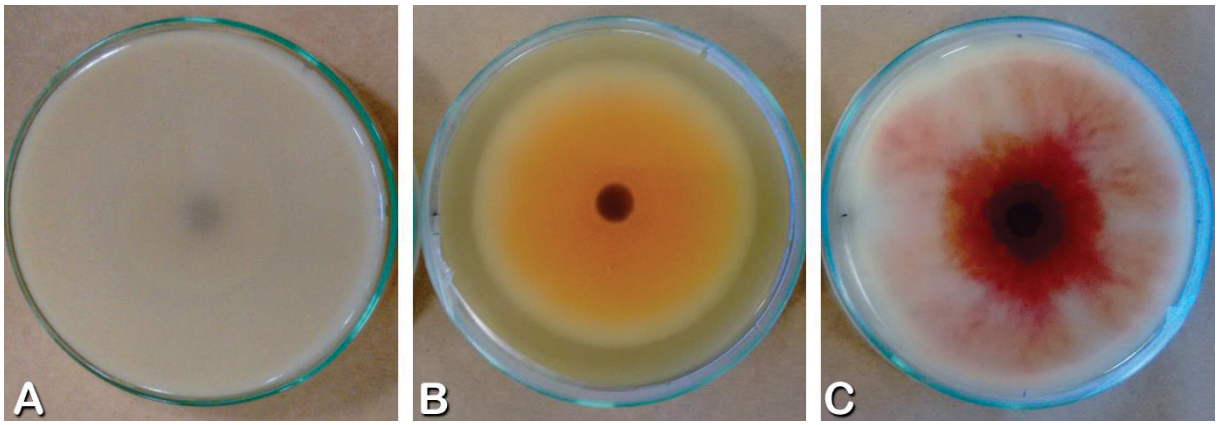
Table 3. Methanolic extract effect of *Simocybe tucumana* on the mycelial growth of *Fusarium graminearum*.

	3° Day	5° Day	7° Day	9° Day	11° Day	13° Day	15° Day
S. tucumana	1.52[1]b	1.98 c	2.72 b	3.54 b	4.12 b	4.58 b	4.96 b
Negative control	3.02 a	3.88 a	4.50 a	4.96 a	5.72 a	6.76 a	8.02 a
Positive control[2]	1.76 b	2.36 b	2.88 b	3.32 b	3.76 b	4.22 b	4.54 b
CV (%)	14.82	6.22	7.92	7.63	6.62	6.00	8.38

Means among the different treatments were compared among themselves within the same column by the Tukey 5% test.

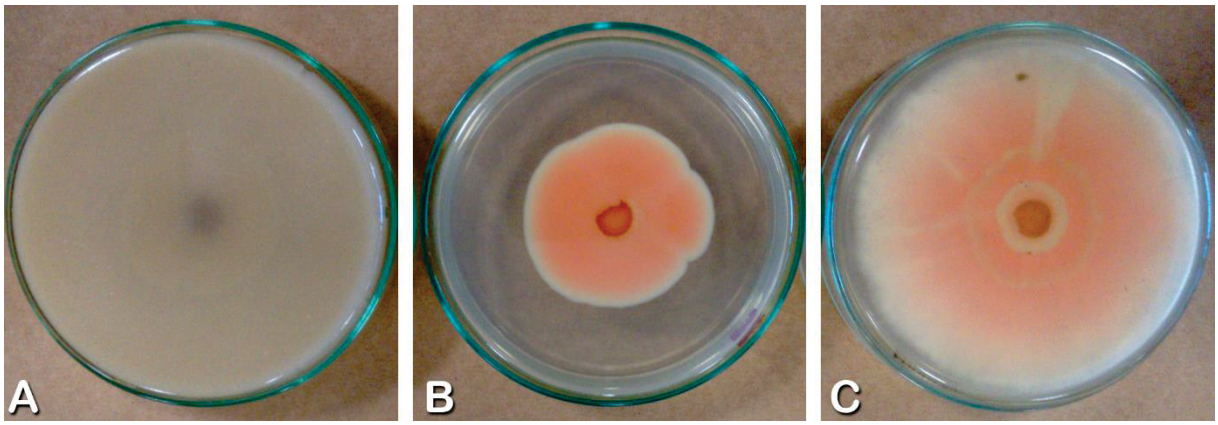
(1) Mean diameter of the mycelium in cm. (2) Cercobin fungicide as positive control. (3) Coefficient of variation.

Figure 1. *Fusarium graminearum* mycelial growth inhibition test, after fifteen days of incubation.



A. Positive control; **B.** Methanolic extract of *Calvatia rugosa*; **C.** Negative control.

Figure 2. *Fusarium graminearum* mycelial growth inhibition test, after fifteen days of incubation.



A. Positive control; **B.** Methanolic extract of *Simocybe tucumana*; **C.** Negative control.

CONSIDERAÇÕES FINAIS

O desenvolvimento do presente estudo possibilitou analisar o potencial antimicrobiano de extratos de cogumelos (Agaricales) nativos da Floresta Estacional Semidecidual do Oeste do Paraná, contribuindo para o conhecimento das propriedades antimicrobianas das espécies estudadas.

Com os resultados obtidos foi possível observar que, das catorze espécies coletadas e estudadas, seis apresentaram atividade moderada frente a levedura e/ou as bactérias testadas, quando utilizado o método de difusão em ágar. Já para o teste de bioautografia direta, foi observado que nove extratos brutos apresentaram atividade antibacteriana; e dez substâncias com propriedades antimicrobianas foram observadas (pertencendo a cinco espécies de cogumelos), sendo que cinco delas são indicativas de terpenos. Quando os extratos foram testados pelo método de diluição em ágar, quatro apresentaram ação fungitóxica direta inibindo significativamente o crescimento micelial deste fungo.

Assim, o objetivo deste trabalho foi alcançado, já que foi possível confirmar que espécies coletadas nesta região sintetizam substâncias antimicrobianas, reforçando desta forma, que o estudo das propriedades biológicas dos basidiomicetos visando o potencial antimicrobiano é importante para a busca de novas substâncias úteis para o controle de microrganismos.

No entanto, além das espécies utilizadas neste trabalho, a bioprospecção de outros basidiomicetos nativos da Floresta Estacional Semidecidual do Oeste do Paraná deve ser continuada, visto que estudos taxonômicos e de biodiversidade estão sendo desenvolvidos e outras espécies podem apresentar propriedades antibacterianas e antifúngicas.

Os estudos futuros sobre a atividade antimicrobiana de basidiomicetos, devem considerar a utilização de metodologias para o isolamento das espécies *in vitro*. Assim, será possível preservar o material vivo e realizar o cultivo destas espécies em laboratório, com a finalidade de obter as substâncias de interesse e seu subsequente estudo químico.

Para as espécies já abordadas neste estudo, devem ser adotados métodos que possibilitem o isolamento, a identificação e a caracterização das substâncias bioativas, e também realizar estudos para verificar o mecanismo de ação da substância contra os microrganismos.

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ANEXO 1 – NORMAS PARA SUBMISSÃO DO PERIÓDICO INTERNATIONAL JOURNAL OF MEDICINAL MUSHROOMS

Capítulos I e II

AUTHOR INSTRUCTIONS

Submission of Review Article: Manuscripts are to be sent in duplicate to:
Mrs. Katherina Tsukor
Institute of Evolution, University of Haifa, International Centre of Cryptogamic Plants and Fungi, Mt. Carmel, Haifa 31905, Israel. Telephone: 972-4-8-249-218, Fax: 972-4-8-288-197, E-mail: spwasser@research.haifa.ac.il

Receipt is acknowledged. The editor will inform authors of the editor's decision and of any action to be taken on the manuscript as soon as possible. Proof corrections must be made within 48 hours and should be limited to *typographical* errors

Articles must be concise, clear, and fluent. English should be checked by a native English speaker, spelling should conform consistently to the American form.

Print the *entire* manuscript double spaced (including references, tables, and figure legends); 26 lines per page of 210-297 mm. Leave margins of 55 mm (left) and 15 to 25 mm (right). Type in lowercase with uppercase only where required by grammar or convention (initials, symbols, acronyms, formulae, abbreviations). Do not break words at the end of a line. Organize the manuscript as follows: title page, abstract, key words, text, acknowledgments, references, tables, legends to figures, figures.

Manuscript Style: Manuscripts should be written with 12 pt. Times New Roman, double spaced, with pages numbered consecutively throughout. DOC or RTF are preferred formats. The manuscript should contain the following parts:
Title page: A full title (preferably not exceeding 80 characters), authors, affiliations, short title (less than 40 characters), and corresponding author information.
Abstract: Try to keep within 250 words.
Text: The main text body should be divided into relevant headings and subheadings.
Tables: Tables should be used only when they can present information more effectively than can be done in running text. Avoid any arrangement that unduly increases the depth of a table, column heads should be as brief as possible, use abbreviations liberally. Lines of data should not be numbered nor run numbers given unless those numbers are needed for reference in the text.
Illustrations: Figures should be numbered in series, and all legends should be typed double spaced and placed at the end of the text file. All figures should be called out in the text in numerical order. Figures should be supplied in a separate file or in individual files. Symbols used in figures (open or closed circles, triangles, squares, etc.) and lettering of labels should be sized for optimum reproduction but should not exceed the size of the journal pages (size requirements can be obtained by emailing journals@begellhouse.com). Color reproduction of figures is possible at the author's expense, rates will be provided upon request.

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References: Number references to the literature or other bibliographic notes consecutively in the text, avoiding repetition by using the number that corresponds to the original reference. Reference numbers are typed as superscripts without parentheses following either the author name(s) or the sentence or clause containing the referenced material. *Place numbers after all punctuation with no space between. International Journal of Medicinal Mushrooms* uses the **Vancouver Style** for all references. This style is available in all commonly-used reference management programs and style manuals. For detailed instructions on using Vancouver Style, visit www.icmje.org. No manuscript will be accepted for publication unless the references are correctly formatted, as

follows. List names of all authors, "et al." is no longer allowed. Bibliographic references to classified documents and reports or to unpublished material not generally available to the scientific public should not be used.

Following are samples of reference formatting.

Journal Articles
Parkin DM, Clayton D, Black RJ, Masuyer E, Friedl HP, Ivanov E. Childhood leukaemia in Europe after Chernobyl: 5 year follow-up. Br J Cancer. 1996;73(8):1006-1012.

Book
Ringsven MK, Bond D. Gerontology and Leadership Skills for Nurses. 2 ed. Albany, NY: Delmar, 1996.

Chapter in edited work
Phillips SJ, Whisnant JP. Hypertension and stroke. In: Laragh JH, Brenner BM, editors. Hypertension: Pathophysiology, Diagnosis, and Management. 2 ed. New York: Raven, 1995, p. 93-114.

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ANEXO 2 – NORMAS PARA SUBMISSÃO DO PERIÓDICO PESQUISA AGROPECUÁRIA BRASILEIRA

Capítulo III

Diretrizes para Autores

Escopo e política editorial
A revista Pesquisa Agropecuária Brasileira (PAB) é uma publicação mensal da Embrapa, que edita e publica trabalhos técnico-científicos originais, em inglês, resultantes de pesquisas de interesse agropecuário. A principal forma de contribuição é o Artigo, mas a PAB também publica Notas Científicas e Revisões a convite do Editor. As submissões de artigos científicos, notas científicas e revisões (a convite do editor) devem ser encaminhadas via eletrônica e em inglês, a partir do dia primeiro de março de 2018.

Forma e preparação de manuscritos
Os trabalhos enviados à PAB devem ser inéditos (não terem dados – tabelas e figuras – publicadas parcial ou integralmente em nenhum outro veículo de divulgação técnico-científica, como boletins institucionais, anais de eventos, comunicados técnicos, notas científicas etc.) e não podem ter sido encaminhados simultaneamente a outro periódico científico ou técnico. Dados publicados na forma de resumos, com mais de 250 palavras, não devem ser incluídos no trabalho.
- São considerados, para publicação, os seguintes tipos de trabalho: Artigos Científicos, Notas Científicas e Artigos de Revisão, este último a convite do Editor.
- Os trabalhos publicados na PAB são agrupados em áreas técnicas, cujas principais são: Entomologia, Fisiologia Vegetal, Fitopatologia, Fitotecnia, Fruticultura, Genética, Microbiologia, Nutrição Mineral, Solos e Zootecnia.
- O texto deve ser digitado no editor de texto Microsoft Word, em espaço duplo, fonte Times New Roman, corpo 12, folha formato A4, com margens de 2,5 cm e com páginas e linhas numeradas.

Organização do Artigo Científico
A ordenação do artigo deve ser feita da seguinte forma:
- Artigos em inglês - Título, autoria, endereços institucionais e eletrônicos, Abstract, Index terms, título em português, Resumo, Termos para indexação, Introduction, Materials and Methods, Results and Discussion, Conclusions, Acknowledgements, References, tables, figures.
- O título, o resumo e os termos para indexação devem ser vertidos fielmente para o inglês, no caso de artigos redigidos em espanhol.
- O artigo científico deve ter, no máximo, 20 páginas, incluindo-se as ilustrações (tabelas e figuras), que devem ser limitadas a seis, sempre que possível.

Título
- Deve representar o conteúdo e o objetivo do trabalho e ter no máximo 15 palavras, incluindo-se os artigos, as preposições e as conjunções.
- Deve ser grafado em letras minúsculas, exceto a letra inicial, e em negrito.
- Deve ser iniciado com palavras chaves e não com palavras como “efeito” ou “influência”.
- Não deve conter nome científico, exceto de espécies pouco conhecidas; neste caso, apresentar somente o nome binário.
- Não deve conter subtítulo, abreviações, fórmulas e símbolos.
- As palavras do título devem facilitar a recuperação do artigo por índices desenvolvidos por bases de dados que catalogam a literatura.

Nomes dos autores
- Grafar os nomes dos autores com letra inicial maiúscula, por extenso, separados por vírgula; os dois últimos são separados pela conjunção “and”.
- O último sobrenome de cada autor deve ser seguido de um número em algarismo arábico, em forma de expoente, entre parênteses, correspondente à chamada de endereço do autor.

Endereço dos autores

- São apresentados abaixo dos nomes dos autores, o nome e o endereço postal completos da instituição e o endereço eletrônico dos autores, indicados pelo número em algarismo arábico, entre parênteses, em forma de expoente.
- Devem ser agrupados pelo endereço da instituição.
- Os endereços eletrônicos de autores da mesma instituição devem ser separados por vírgula.

Abstract/Resumo
- O termo Abstract, ou Resumo, deve ser grafado em letras minúsculas, exceto a letra inicial, na margem esquerda, e separado do texto por travessão.
- Deve conter, no máximo, 200 palavras, incluindo números, preposições, conjunções e artigos.
- Deve ser elaborado em frases curtas e conter o objetivo, o material e os métodos, os resultados e a conclusão.
- Não deve conter citações bibliográficas nem abreviaturas.
- O final do texto deve conter a principal conclusão, com o verbo no presente do indicativo.

Termos para indexação
- A expressão Termos para indexação, seguida de dois-pontos, deve ser grafada em letras minúsculas, exceto a letra inicial.
- Os termos devem ser separados por vírgula e iniciados com letra minúscula.
- Devem ser no mínimo três e no máximo seis, considerando-se que um termo pode possuir duas ou mais palavras.
- Não devem conter palavras que componham o título.
- Devem conter o nome científico (só o nome binário) da espécie estudada.
- Devem, preferencialmente, ser termos contidos no AGROVOC: Multilingual Agricultural Thesaurus ou no Índice de Assuntos da base SciELO .

Introdução
- A palavra Introdução deve ser centralizada e grafada com letras minúsculas, exceto a letra inicial, e em negrito.
- Deve apresentar a justificativa para a realização do trabalho, situar a importância do problema científico a ser solucionado e estabelecer sua relação com outros trabalhos publicados sobre o assunto.
- O último parágrafo deve expressar o objetivo de forma coerente com o descrito no início do Resumo.

Material e Métodos
- A expressão Material e Métodos deve ser centralizada e grafada em negrito; os termos Material e Métodos devem ser grafados com letras minúsculas, exceto as letras iniciais.
- Deve ser organizado, de preferência, em ordem cronológica.
- Deve apresentar a descrição do local, a data e o delineamento do experimento, e indicar os tratamentos, o número de repetições e o tamanho da unidade experimental.
- Deve conter a descrição detalhada dos tratamentos e variáveis.
- Deve-se evitar o uso de abreviações ou as siglas.
- Os materiais e os métodos devem ser descritos de modo que outro pesquisador possa repetir o experimento.
- Devem ser evitados detalhes supérfluos e extensas descrições de técnicas de uso corrente.
- Deve conter informação sobre os métodos estatísticos e as transformações de dados.
- Deve-se evitar o uso de subtítulos; quando indispensáveis, grafá-los em negrito, com letras minúsculas, exceto a letra inicial, na margem esquerda da página.

Resultados e Discussão
- A expressão Resultados e Discussão deve ser centralizada e grafada em negrito, com letras minúsculas, exceto a letra inicial.
- Todos os dados apresentados em tabelas ou figuras devem ser discutidos.
- As tabelas e figuras são citadas seqüencialmente.
- Os dados das tabelas e figuras não devem ser repetidos no texto, mas discutidos em relação aos apresentados por outros autores.
- Evitar o uso de nomes de variáveis e tratamentos abreviados.
- Dados não apresentados não podem ser discutidos.
- Não deve conter afirmações que não possam ser sustentadas pelos dados obtidos no próprio trabalho ou por outros trabalhos citados.
- As chamadas às tabelas ou às figuras devem ser feitas no final da primeira oração do texto em questão; se as demais sentenças do parágrafo referirem-se à mesma tabela ou figura, não é necessária nova chamada.
- Não apresentar os mesmos dados em tabelas e em figuras.
- As novas descobertas devem ser confrontadas com o conhecimento anteriormente obtido.

Conclusões

- O termo Conclusões deve ser centralizado e grafado em negrito, com letras minúsculas, exceto a letra inicial.
- Devem ser apresentadas em frases curtas, sem comentários adicionais, com o verbo no presente do indicativo.
- Devem ser elaboradas com base no objetivo do trabalho.
- Não podem consistir no resumo dos resultados.
- Devem apresentar as novas descobertas da pesquisa.
- Devem ser numeradas e no máximo cinco.

Agradecimentos

- A palavra Agradecimentos deve ser centralizada e grafada em negrito, com letras minúsculas, exceto a letra inicial.
- Devem ser breves e diretos, iniciando-se com “Ao, Aos, À ou Às” (pessoas ou instituições).
- Devem conter o motivo do agradecimento.

Referências

- A palavra Referências deve ser centralizada e grafada em negrito, com letras minúsculas, exceto a letra inicial.
- Devem ser de fontes atuais e de periódicos: pelo menos 70% das referências devem ser dos últimos 10 anos e 70% de artigos de periódicos.
- Devem ser normalizadas de acordo com a NBR 6023 da ABNT, com as adaptações descritas a seguir.
- Devem ser apresentadas em ordem alfabética dos nomes dos autores, separados por ponto-e-vírgula, sem numeração.
- Devem apresentar os nomes de todos os autores da obra.
- Devem conter os títulos das obras ou dos periódicos grafados em negrito.
- Devem conter somente a obra consultada, no caso de citação de citação.
- Todas as referências devem registrar uma data de publicação, mesmo que aproximada.
- Devem ser trinta, no máximo.

Exemplos:

- Artigos de Anais de Eventos (aceitos apenas trabalhos completos)

AHRENS, S. A fauna silvestre e o manejo sustentável de ecossistemas florestais. In: SIMPÓSIO LATINO-AMERICANO SOBRE MANEJO FLORESTAL, 3., 2004, Santa Maria. Anais.Santa Maria: UFSM, Programa de Pós-Graduação em Engenharia Florestal, 2004. p.153-162.

- Artigos de periódicos

SANTOS, M.A. dos; NICOLÁS, M.F.; HUNGRIA, M. Identificação de QTL associados à simbiose entre Bradyrhizobium japonicum, B. elkanii e soja. Pesquisa Agropecuária Brasileira, v.41, p.67-75, 2006.

- Capítulos de livros

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