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Inhibitory potential of bioactive extracts from southern Brazil mushrooms on the pathogenic oomycete *Pythium insidiosum*

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ABSTRACT: *Pythium insidiosum* is an important oomycete pathogen of mammals that causes pythiosis, an endemic disease in warm climates that stands out for its unfavorable prognosis, lethality in the affected species, and difficulties in treatment. This study evaluated *in vitro* anti-P. *insidiosum* potential of aqueous, hydroethanolic, and ethanolic extracts of indigenous wild mushrooms from southern Brazil. The extracts were prepared from *Amanita gemmata*, *Amanita muscaria*, *Auricularia auricula*, *Gymnopilus junonius*, *Lactarius deliciosus*, *Laccaria laccata*, *Psilocybe cubensis*, and *Russula xerampelina. In vitro* susceptibility assays employed the microdilution technique according to the M38-A2 protocol CLSI. The hydroethanolic and ethanolic extracts of *R. xerampelina* showed anti-*P. insidiosum* activity at minimum inhibitory concentrations ranging from 1.87–7.50 mg/mL. The other mushroom species extracts showed no inhibitory effects on growth of *P. insidiosum*. This is the first study to evaluate the antimicrobial activity of mushrooms on oomycetes, evidencing the antimicrobial potential of *R. xerampelina* on the pathogen *P. insidiosum.* So, the present study expands new perspectives, since the secondary metabolites produced by mushrooms can be potential targets for the development of new categories of medicines. However, considering the wide biodiversity of Brazilian mushrooms, we suggested that the search for other basidiomycetes species with anti-*P. insidiosum* action needs to be expanded.
Key words: antimicrobial, susceptibility, Oomycota, pythiosis, fungi, basidiomycetes.

Potencial inibitório de extratos bioativos de cogumelos do sul do Brasil sobre o oomiceto patogênico *Pythium insidiosum*

RESUMO: *Pythium insidiosum* é um importante oomiceto patógeno de mamíferos causador da pitiose, uma doença endêmica em climas quentes e que se destaca pelo prognóstico desfavorável, letalidade nas espécies afetadas e dificuldades no tratamento. Este estudo avaliou *in vitro* o potencial anti-*P. insidiosum* dos extratos aquosos, hidroetanólicos e etanólicos de cogumelos silvestres do sul do Brasil. Os extratos foram preparados a partir de *Amanita gemmata, Amanita muscaria*, *Auricularia auricula*, *Gymnopilus junonius*, *Lactarius deliciosus*, *Laccaria laccata*, *Psilocybe cubensis* e *Russula xerampelina*. Os ensaios de suscetibilidade *in vitro* empregaram a técnica de microdiluição em caldo de acordo com o protocolo M38-A2, CLSI. Os extratos hidroetanólico e etanólico de *R. xerampelina* apresentaram atividade anti-*P. insidiosum* em concentrações inibitórias mínimas que variaram de 1,87-7,50 mg/mL. Os extratos das demais espécies de cogumelos não apresentaram efeitos inibitórios sobre o crescimento de *P. insidiosum*. Este é o primeiro estudo a avaliar a atividade antimicrobiana de cogumelos sobre oomicetos, evidenciando o potencial antimicrobiano de *R. xerampelina* sobre o patógeno *P. insidiosum*. Assim, o presente estudo amplia novas perspectivas, uma vez que os metabólitos secundários produzidos pelos cogumelos podem ser alvos potenciais para o desenvolvimento de novas categorias de fármacos. Porém, levando em consideração a ampla biodiversidade de cogumelos brasileiros, sugere-se que a busca por outras espécies de basidiomicetos com atividade anti-*P. insidiosum* precisa ser ampliada.
Palavras-chave: antimicrobiano, suscetibilidade, Oomycota, pitiose, fungos, basidiomicetos.

Pythium insidiosum is an aquatic oomycete and the primary causative agent of pythiosis, a relevant disease with an unfavorable prognosis that affects domestic animals (horses, dogs, cattle, cats, goats, and sheep), wild animals (birds and wild mammals) and humans (YOLANDA & KRAJAEJUN, 2022). In the last decade, other *Pythium* species, including *Pythium aphanidermatum* and *Pythium periculosum*, have been described as affecting mammalian hosts (THONGSUK et al., 2021; MIRAGLIA et al., 2022). The disease has been mainly reported in the Americas, some European countries, Southeast Asia, Oceania, and Africa (YOLANDA & KRAJAEJUN, 2022). In Brazil and Thailand, pythiosis is endemic

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in horses and humans, respectively (SOUTO et al., 2021; YOLANDA & KRAJAEJUN, 2022).

The main clinical manifestations in affected animals are characterized by the development of cutaneous, subcutaneous and gastrointestinal lesions, and in humans, the disease occurs in ocular, subcutaneous and systemic forms (YOLANDA & KRAJAEJUN, 2022). Early detection of the disease and quick treatment are crucial for an effective clinical cure. The currently available treatments for pythiosis in animals and humans include surgery, immunotherapy and different classes of antimicrobial drugs (PEREIRA et al., 2013; YOLANDA & KRAJAEJUN, 2021; PERMPALUNG et al., 2020; YOLANDA & KRAJAEJUN, 2022; MEDHASI et al., 2022).

Nevertheless, treating pythiosis is challenging because conventional drugs are not usually effective, particularly antifungal drugs that act on the ergosterol of the cytoplasmic membrane of fungi, which is absent in oomycetes (MEDHASI et al., 2022). Thus, the perspectives of managing pythiosis, in most cases, are associated with combinations of different forms of treatment (PEREIRA et al., 2013); however, standardized therapeutic protocols are non-existent and the results of currently used treatments are not constantly satisfactory. In this sense, it is crucial to seek new compounds that can contribute for treating pythiosis.

The phylum Basidiomycota is a large group of fungi comprising more than 30,000 species, of which about 15,000 are known. Approximately 2,000 species of mushrooms are used for human consumption and around 700 have medicinal properties (SANTOS & CARVALHO, 2021). The secondary metabolites produced by Agaricomycetes mushrooms, such as terpenoids, flavonoids, tannins, alkaloids, and polysaccharides, are of great importance in the pharmaceutical industry, which may be potential targets for developing novel drug categories. Such bioactive compounds confer several biological activities, including antitumor, anti-inflammatory, antioxidant, immunomodulatory, antiparasitic, antimicrobial (antibacterial, antifungal, antiviral), neuroprotective, and cardioprotective effect (VAMANU, 2018; GEBREYOHANNES et al., 2019; ROSA et al., 2020; SANTOS & CARVALHO, 2021; VOLCÃO et al., 2021a; ABDELSHAFY et al., 2022).

Although, 22 mushroom taxa have been studied for different biological activities (SANTOS & CARVALHO, 2021), no research has been carried out to evaluate the activity of mushroom extracts on pathogenic oomycetes. Thus, this study investigated the potential of different extracts of the macrofungi *Amanita gemmata*, *Amanita muscaria*, *Auricularia auricula*, *Gymnopilus junonius*, *Lactarius deliciosus*, *Laccaria laccata*, *Psilocybe cubensis*, and *Russula xerampelina* on the pathogen oomycete *P. insidiosum*.

The reproductive structure, including pileus and stipe of macrofungi *Amanita gemmata*, *Amanita muscaria*, *Auricularia auricula*, *Gymnopilus junonius*, *Lactarius deliciosus*, *Laccaria laccata*, *Psilocybe cubensis*, and *Russula xerampelina* (SISGEN A487273) were collected in three vegetated areas in the cities of Capão do Leão (31º45'48"S 52º29'02"W) and Pelotas (31º46'19"S 52º20'33"W), located in Rio Grande do Sul State, in the south of Brazil, during autumn, winter and spring. Two areas were characterized by coniferous woodland and small trees, predominately *Pinus* spp. species, and one area contains physiognomic restinga vegetation types, including sandy restinga and peat restinga forests.

After collection, the macrofungi were identified according to macro- and micromorphological characteristics. Subsequently, the mushrooms were aliquoted, packed in brown paper envelopes, and transferred to an oven at 50 ºC for 96 h for basidiocarp dehydration. Subsequently, they were stored in hermetically sealed containers and protected from light and humidity until extract production (VOLCÃO et al., 2021a; VOLCÃO et al., 2021b).

The aqueous, ethanolic and hydroethanolic extracts were produced from the macrofungi dehydrated and triturated by maceration. The aqueous extracts (AQE) were produced for all macrofungi evaluated by diluting each crushed mushroom (25 g) in distilled water (100 mL). The hydroethanolic extracts (HEE) were prepared for *A. auricula*, *L. deliciosus*, *L. laccata*, and *R. xerampelina* by adding the crushed mushrooms (25 g) to 50% ethanol (100 mL). To prepare the ethanolic extracts (EE), 25 g of crushed mushrooms, including *A. gemmata*, *A. auricula*, *L. deliciosus*, *L. laccata*, and *R. xerampelina,* were added to 95% ethanol solution (100 mL). The solutions were then incubated in an ultrasonic bath (SB-5200 DTDN Ultrasonic Cleaner) at 40 ºC for 120 min to extract the compounds present in the macrofungi. The suspensions were filtered on Whatman® filter paper no. 1 to eliminate the particulate matter. The suspensions were stored in Falcon tubes in a freezer at -20 ºC (VOLCÃO et al., 2021a, 2021b). All extracts from mushrooms evaluated were produced by the Laboratório de Biologia, Ecologia e Aplicação de Fungos at Universidade Federal de Pelotas (UFPel).

Eleven *P. insidiosum* isolates (SISGEN A139392), including nine from pythiosis in horses and dogs and two standard strains (CBS702.83

and CBS777.84) belonging to the Laboratório de Micologia (LABMICO), Departamento de Microbiologia and Parasitologia at UFPel, were evaluated. The clinical isolates were identified by their macro and micromorphological characteristics and molecularly confirmed, as previously described by AZEVEDO et al. (2012).

All *P. insidiosum* inocula were obtained using the zoosporogenesis process described by Ianiski et al. (2021). In brief, *P. insidiosum* isolates previously grown on yeast extract agar were transferred to Petri plates containing V8 agar with fragments of sterile grass (*Paspalum notatum*) and incubated at 37 ºC for 3 days. The grass fragments containing *P. insidiosum* mycelium were then transferred to Petri plates containing induction medium (20 mL) and incubated at 37 ºC for 12–24 h. After this period, the plates were incubated at 37 ºC at 3000 rpm for 5 min. The free zoospores in the induction medium were counted in a Neubauer chamber under light microscopy (100 and 400 ×). The induction medium, initially containing 30,000 zoospores/mL for each *P. insidiosum* isolate, underwent a dilution (1:10) in RPMI 1640 broth, adjusted to a pH of 7.0. The susceptibility profile was assessed following the CLSI M38-A2 broth microdilution method (CLSI, 2008).

For testing, the extracts were diluted in RPMI 1640 glucose buffered at pH 7.0 with 0.165 M MOPS in a 1:1 ratio to form the stock solutions. The concentrations of the extracts in the wells ranged from 0.01 to 7.50 mg/mL. Aliquots of 100µL of these dilutions were dispensed sequentially into the microplates, filling the wells belonging to columns numbered one to ten. A volume of 100 µL of the inoculum was dispensed to these columns. Positive (100 µL of RPMI and 100 µL of inoculum) and negative (100 µL of RPMI and 100 µL of the extract) control columns were used for each test. Additionally, a plate containing ethanol + inoculum was prepared as control for all tests. The plates were incubated at 40 rpm shaking at 37 °C for 48 h; all tests were performed in quadruplicate. Readings were performed visually and considered the growth of hyphae. The lowest concentration of the extracts capable of inhibiting *P. insidiosum* growth was identified as the minimum inhibitory concentration (MIC). The concentrations capable of inhibiting 50% and 90% of the isolates were called MIC_{50} and MIC_{90}, respectively. Concentrations above the MIC were used to determine the minimum oomicidal concentration (MOC). For this purpose, 100 µL of the dilution was transferred to tubes containing 900 µL of Sabouraud broth and incubated at 37°C for 48h. The lowest concentration of the extract that did not show hyphae growth was considered the MOC.

Among the different mushroom extracts evaluated, only the HEE and EE of *R. xerampelina* showed anti-*P. insidiosum* activity, with the MIC ranging from 1.87 to 7.50 mg/mL for both extracts. The values obtained in the MIC_{50} and MIC_{90} were 7.50 and 1.87 mg/mL, respectively. The MOC of the HEE and EE was equal to the MIC. The other macrofungal extracts showed no inhibitory effect on *P. insidiosum* at the concentrations evaluated (Table 1).

The severity and lethality of infections caused by *P. insidiosum* in humans and animals and challenges in treating the disease have driven research to expand the therapeutic alternatives and arsenal of antimicrobial compounds with anti-*P. insidiosum* activity (PERMPALUNG et al, 2020; VALENTE et al., 2020; IANISKI et al., 2021; SILVEIRA et al., 2022; MEDHASI et al., 2022; BRAGA et al., 2023). In this sense, this research contributes with these previous studies by demonstrating for the first time the anti-*P. insidiosum* action of the Brazilian mushroom *R. xerampelina*.

Although, interest in food and medicinal effects of mushrooms has grown in Americas in last decades, it is noted that few species of mushrooms from Rio Grande do Sul, the southernmost state in Brazil, have been collected, as well as their biological properties have not been fully evaluated (VOLCÃO et al., 2021a). Interestingly, in this region the pampa biome predominates, a very old natural ecosystem, where there are natural fields, composed of grasses, fragmented and gallery forests. In this biome, some *Pinus* spp. and *Eucalyptus* spp. constitute important associations with different species of basidiomycetes (VOLCÃO et al., 2021a). Thus, research is required to study the diversity and medicinal potential of wild mushrooms in this region of Brazil. In order to fill this gap, the present study sought to evaluate the potential of mushrooms from the pampa biome on an oomycete species.

Few studies have evaluated the antimicrobial activity of *R. xerampelina*. However, VOLCÃO et al. (2021b) studied the antioxidant activity of the HEE of *R. xerampelina* utilized in this study and determined the total flavonoid and phenol contents. They found that the phenolic acid concentration was 3.0 mg GAE/g (microgram of gallic acid equivalent per gram of extract), while the flavonoid concentration was 1098.33 mg CAE/g (microgram of catechin equivalent per gram of extract). Additionally, the authors found that this extract had antimicrobial effects on *Pseudomonas aeruginosa*, with MIC of 2.5 mg/mL, without

Table 1 - *In vitro* susceptibility of *Pythium insidiosum* (n = 11) to aqueous (AQE), hydroethanolic (HEE) and ethanolic extracts (EE) of southern Brazil mushrooms *Amanita gemmata*, *Amanita muscaria*, *Auricularia auricula*, *Gymnopilus junonius*, *Lactarius deliciosus*, *Laccaria laccata*, *Psilocybe cubensis* and *Russula xerampelina*.

----------Number and percentages (%) of isolates in each extract concentration----------						
Mushrooms Extracts	7.50	3.75	1.87	0.93-0.01	MIC50	MIC90
R. xerampelina HEE	5 (45.45%)	4 (36.37%)	2 (18.18%)	+	3.75	1.87
R. xerampelina EE	7 (63.63%)	2 (18.18%)	2 (18.18%)	+	7.50	1.87
R. xerampelina AQE	+	+	+	+	ND	ND
A. gemmata AQE, EE	+	+	+	+	ND	ND
A. muscaria AQE	+	+	+	+	ND	ND
A. auriculata AQE, HEE, EE	+	+	+	+	ND	ND
G. junonius AQE	+	+	+	+	ND	ND
L. deliciosus AQE, HEE, EE	+	+	+	+	ND	ND
L. laccata AQE, HEE, EE	+	+	+	+	ND	ND
P. cubensis AQE	+	+	+	+	ND	ND

MIC: minimum inhibitory concentration; MIC_{50}: minimum concentration for 50% growth inhibition of the isolates; MIC_{90}: minimum concentration for 90% growth inhibition of the isolates; (+) All *P. insidiosum* isolates (n = 11) grew at this concentration. ND: not determined.

cytotoxic effects on Vero cells. It is believed that the probable anti-*P. insidiosum* action of *R. xerampelina* is due to the presence of these bioactive compounds. Nevertheless, further research is required to verify which compounds present antimicrobial activity on this oomycete.

According previous studies, the antimicrobial activity of macrofungi is closely related to the antioxidant effects of bioactive substances, such as phenolic compounds (VAMANU, 2018; ABDELSHAFY et al., 2022). However, the chemical composition of macrofungal extracts and the content of bioactive substances can be influenced by various factors, including solvent type and temperature used for extraction, the structure (pileus, stipe, or both) used for extraction, mushroom maturity and geographical location of the collected mushroom specimen (VOLCÃO et al., 2021b).

Nonetheless, the other species of mushrooms evaluated in this study, except for *R. xerampelina*, did not show any anti-*P. insidiosum* activity. However, a previous study evaluated the antimicrobial action of 35 different macrofungi and reported that only three genera had antibacterial and antifungal activity (GEBREYOHANNES et al., 2019). In this sense, our study evaluated three extracts of eight indigenous wild mushrooms species and we reported one species with anti-*P. insidiosum* activity. It is important to point out that previous studies evaluating the antimicrobial potential of mushroom extracts were carried out on bacteria and fungi (VAMANU, 2018; GEBREYOHANNES et al., 2019; ROSA et al., 2020; VOLCÃO et al., 2021a, 2021b). The differential of our study was the pioneering research into the inhibitory action of basidiomycetes against the mammalian pathogen oomycete. However, considering the wide biodiversity of Brazilian mushrooms, we suggest that the search for other basidiomycetes with potential to inhibit the growth this important fungal-like microorganism needs to be augmented.

This research represents a preliminary and pioneering investigation into the anti-*P. insidiosum* potential of indigenous wild mushrooms from southern Brazil. The antimicrobial activity of ethanolic and hydroethanolic extracts of *R. xerampelina* was demonstrated. Taking into account the wide biodiversity of Brazilian mushrooms, as future perspectives we proposed to expand the search for other species of basidiomycetes with anti-*P. insidisoum* activity. Furthermore, the bioactive compounds produced by Agaricomycetes mushrooms are potential targets for developing novel drug categories.

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DECLARATION OF CONFLICT OF INTEREST

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

AUTHORS' CONTRIBUTIONS

The authors contributed equally to the manuscript.

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