




Antifungal activity of *Lactarius deliciosus* and *Laccaria laccata* extracts against phytopathogenic fungi

Lisiane Martins Volcão^{1*} , Paula Florêncio Ramires² , Ronan Adller Tavella² , Rodrigo de Lima Brum² , Daniela Fernandes Ramos² , Eduardo Bernardi³ , Flávio Manoel Rodrigues da Silva Júnior² 

1 Universidade Federal da Fronteira Sul, SC-484, Km 02 - Fronteira Sul, Chapecó - SC, 89815-899, Brasil

2 Universidade Federal do Rio Grande, Km 8 Avenida Itália Carreiros, Rio Grande - RS, 96203-900, Brasil

3 Universidade Federal de Pelotas, Rua Gomes Carneiro, 1 - Centro - CEP 96010-610 - Pelotas, RS, Brasil

*Corresponding Author: lisivolcao@hotmail.com

Received 01 August 2023.

Accepted 09 January 2024.

Published 24 January 2024.

Abstract - Urban expansion, coupled with lack of environmental control, has created major challenges in the relationship humans/environment. One of these challenges is the safe and efficient control of pests in agriculture. New products have been researched for use in controlling phytopathogenic organisms. Thus, this study aimed to produce mushroom basidiomycetes extracts collected in the southern region of the state of Rio Grande do Sul, Brazil, as well as a preliminary chemical evaluation of these extracts and the analysis of their antifungal potential against phytopathogens. The extracts produced from basidiomycetes mushrooms, *Lactarius deliciosus* and *Laccaria laccata*, collected in their natural environment, were processed to produce 95% aqueous and ethanolic extracts. The composition of phenols and total flavonoids was evaluated, as well as the antifungal potential of these extracts against three species of phytopathogenic fungi, *Fusarium solani*, *Monilinia fructicola* and *Penicillium citrinum*. The aqueous extract of *L. deliciosus* showed the highest amount of total phenols in the study, while the 95% ethanolic extract of *L. deliciosus* demonstrated the highest rate of total flavonoids. As for antifungal activity, ethanol extracts showed better effects than aqueous extracts against the three phytopathogens evaluated.

Keywords: Mushrooms. Antimicrobial activity. Fruit pathogens. Sustainability.

Atividade antifúngica dos extratos de *Lactarius deliciosus* e *Laccaria laccata* contra fungos fitopatogênicos

Resumo - A expansão urbana, somada à falta de controle ambiental, vem criando grandes desafios na relação humanos/ambiente. Um destes desafios é o controle seguro e eficiente de pestes na agricultura. Novos produtos tem sido pesquisado para o controle de organismos fitopatogênicos. Com isso, este estudo objetivou a produção de extratos de cogumelos basidiomicetos coletados na região Sul do estado do Rio Grande do Sul, Brasil, bem como uma avaliação química preliminar destes extratos

e a análise do seu potencial antifúngico contra fitopatógenos. Os extratos produzidos a partir de cogumelos basidiomicetos, *Lactarius deliciosus* e *Laccaria laccata*, coletados em seu ambiente natural foram processados para a produção de extratos aquosos e etanólicos a 95%. Foi avaliada a composição de fenóis e flavanóides totais, bem como o potencial antifúngico destes extratos contra três espécies de fungos fitopatogênicos, *Fusarium solani*, *Monilinia fructicola* e *Penicillium citrinum*. O extrato aquoso de *L. deliciosus* apresentou a maior quantidade de fenóis totais do estudo, enquanto o extrato etanólico a 95% de *L. deliciosus* demonstrou a maior taxa de flavanóides totais. Quanto a atividade antifúngica, os extratos etanólicos apresentaram melhor efeito do que os extratos aquosos contra os três fitopatógenos avaliados.

Palavras-chave: Cogumelos. Atividade antimicrobiana. Patógenos de frutos. Sustentabilidade.

Actividad antifúngica de los extractos de *Lactarius deliciosus* y *Laccaria laccata* contra hongos fitopatógeno

Resumen - La expansión urbana, sumada a la falta de control ambiental, viene generando grandes desafíos en la relación hombre/medio ambiente. Uno de estos desafíos es el control seguro y eficiente de plagas en la agricultura. Se han investigado nuevos productos para el control de organismos fitopatógenos. Así, este estudio tuvo como objetivo producir extractos de hongos basidiomicetos recolectados en la región sur del estado de Rio Grande do Sul, Brasil, así como una evaluación química preliminar de estos extractos y el análisis de su potencial antifúngico contra fitopatógenos. Los extractos producidos a partir de hongos basidiomicetos, *Lactarius deliciosus* y *Laccaria laccata*, recolectados en su ambiente natural, fueron procesados para producir extractos acuosos y etanólicos al 95%. Se evaluó la composición de fenoles totales y flavonoides, así como el potencial antifúngico de estos extractos frente a tres especies de hongos fitopatógenos, *Fusarium solani*, *Monilinia fructicola* y *Penicillium citrinum*. El extracto acuoso de *L. deliciosus* mostró la mayor cantidad de fenoles totales en el estudio, mientras que el extracto etanólico al 95% de *L. deliciosus* mostró la mayor tasa de flavonoides totales. En cuanto a la actividad antifúngica, los extractos etanólicos mostraron mejor efecto que los extractos acuosos frente a los tres fitopatógenos evaluados.

Palabras clave: Hongos. actividad antimicrobiana. Patógenos de frutas. Sostenibilidad.

Introduction

Land use for agriculture purpose intensified social and environmental changes during the 20th century. In Brazil, agriculture has undergone important transformations since the 1960s (Vieira Filho and Gasquez 2016). Currently, Brazil is considered a world potency in food production, as well as an important agricultural exporter (IBGE 2019).

In the current model, a relatively large area is required for agricultural production. In addition, several challenges arise in the relationship between humans and the environment when we evaluate

this topic. Among these challenges, we find the safe and effective control of organisms, such as arthropods and microorganisms, which generate significant losses in the yield of agricultural crops (Yorinori and Sartorato 2011).

The conventional control of microorganisms harmful to crops is carried out through preventive and corrective applications of chemical products, the use of which has been increasing since the last century (Lambropoulou *et al.* 2015). Therefore, it is important to note that the indiscriminate and incorrect use of these products can cause damage to the environment, human health and the plants itself (Richter *et al.* 2015). Even taking into account the benefits arising from the use of pesticides, such as the increase in crop yields and the reduction in the prices of agricultural products, the detection of their residues in the environment and in food has raised questions about their safety (Akoto *et al.* 2016; Fang *et al.* 2017).

Faced with this problem, natural products have been researched as an alternative in the control of phytopathogenic organisms, such as extracts of plants and mushrooms (Arruda *et al.* 2012; Degenkolb and Vilcinkas 2016; Shali *et al.* 2018). Understanding that plants and mushrooms co-evolve with insects and microorganisms, we observe that these organisms become sources of insecticidal and antimicrobial substances produced for their own defense (Bahram and Netherway 2022).

Thus, the objective of the present study was the production of extracts of basidiomycetes mushrooms collected in the southern region of the state of Rio Grande do Sul, Brazil, as well as a preliminary chemical evaluation of the extracts and the analysis of their antifungal potential against three phytopathogens.

Material and Methods

Study sites and collection samples

For the study, two species of ectomycorrhizal basidiomycetes mushrooms were collected in the south of Rio Grande do Sul, Brazil (Figure 1), during the fall and winter of 2016. The study area is located in the Universidade Federal de Pelotas (31° 48'54" S, 52° 25'48"), comprising humid areas, forest composed of *Pinus* sp. and semideciduous climate (Wolf *et al.* 2016). The mushrooms *Lactarius deliciosus* and *Laccaria laccata* (Figure 2) were collected and identified with the aid of an identification guide (Wright and Albertó 2002). Immediately after collection, the material was taken to the Laboratory of Biology, Ecology and Application of Fungi, belonging to the Biology Institute of the Universidade Federal de Pelotas for processing.

Figure 1. Map of the sampling area located at the Capão do Leão Campus (UFPel), Rio Grande do Sul, Brazil.

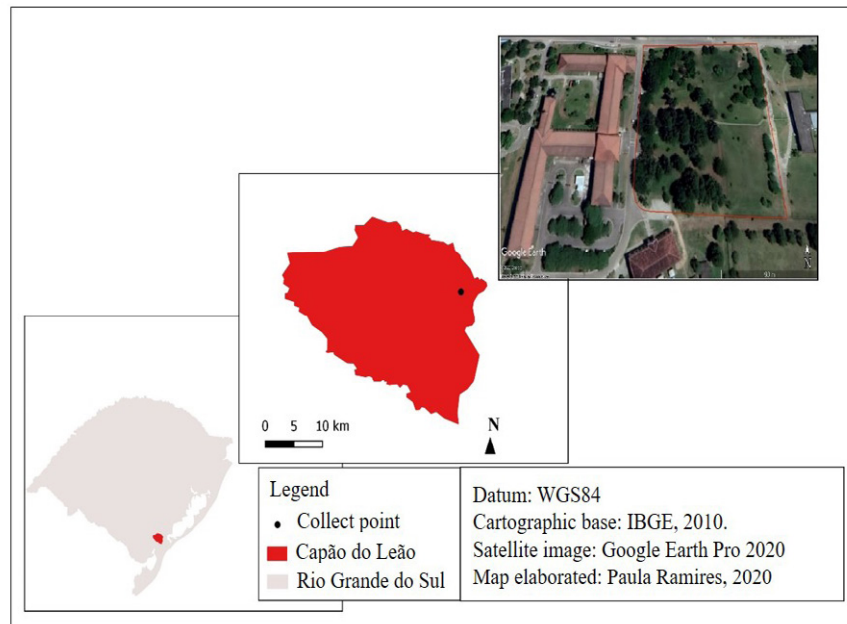


Figure 2. Species of basidiomycetes mushrooms, *Laccaria laccata* (upper part) and *Lactarius deliciosus* (lower part), used in the study.

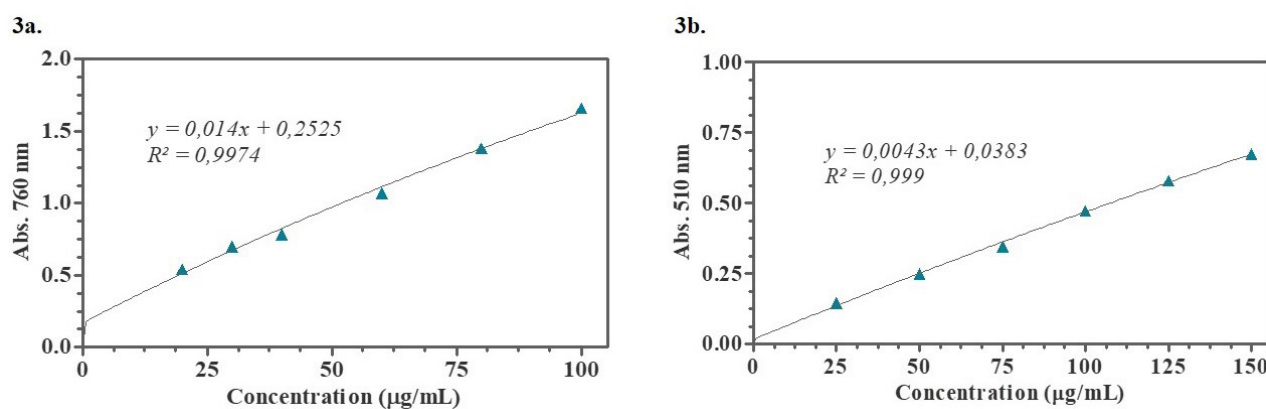


Extract production and chemical analysis

The fruiting bodies of the mushrooms were dried in an oven at 50° C for 5 days. After this period, the material was stored in sterile flasks, hermetically sealed and protected from light until the extracts were produced. The extraction and chemical analysis took place at the Instituto Federal de Educação, Ciência e Tecnologia do Rio Grande do Sul - Campus Sertão (IFRS / Campus Sertão), in the Experimentation and Analytical Studies Laboratory. Extracts were produced for both species of mushrooms using two different solvents, deionized water and ethanol in a concentration of 95% (EtOH95%). The dried fruiting bodies were macerated, and 25g of each species was added to their respective solvent (100 ml of MiliQ water or 100 ml of 95% EtOH). The solutions were submitted to an ultrasound process (SB - 5200 DTDN Ultrasonic Cleaner) for 120 min at 40° C (Roesler et al. 2007). At the end of the procedure, the mixtures were filtered on filter paper (Whatman® No. 1) in order to eliminate particulate material. The solutions were kept in an ultra-freezer (- 80 ° C) until chemical analysis.

The ethanolic (EtOH95%) and aqueous (AQ) extracts were analysed for the phenols and flavonoids total content. For the colorimetric reaction of total phenols content, a mixture of methanolic extraction (1:20) was added to an aqueous solution of Folin-Ciocalteu (10%) and CaCO₃ (7.5%). The final solution was incubated in ultrasound (SB - 5200 DTDN Ultrasonic Cleaner) for a period of 5 min at 50° C (ROESLER et al., 2007). For the reaction of total flavonoids content, the sample was diluted in deionized water (1:10), followed by the addition of NaNO₂, AlCl₃ and NaOH, and incubated in an ultrasonic bath at 10° C for 30 min (Zhishen et al. 1999). The calibration curve for total phenols was performed using gallic acid (Figure 3a), the absorbance of the solution being measured at $\lambda = 760$ nm (UV spectrophotometer - 1800 Shimadzu), and the results expressed as μg of gallic acid equivalents per mg of sample (μg GAE/mg of sample). The curve for total flavonoids (Figure 3b) was performed using catechin, and the absorbance read at $\lambda = 510$ nm. The results were expressed as μg of catechin equivalents per mg of sample (μg CE/mg of sample).

Figure 3. Standard curve for total phenols (3a) performed with gallic acid, and standard curve for flavonoids (3b) performed with catechin.



Antifungal activity

Before the evaluation of antifungal activity, the extracts AQ and EtOH95% were lyophilized under vacuum, and later solubilized in aqueous solution (10 mg / mL) containing DMSO (5%) + Tween 80 (0.1%). The Broth Microdilution Test (CLSI 2015) was carried out for the analysis, with the purpose of establishing the minimum concentration of the extract capable of inhibiting fungal growth (Minimum Inhibitory Concentration - MIC). The target species were: *Fusarium solani* IOC 2163, *Monilinia fructicola* IOC 4630 and *Penicillium citrinum* IOC 4150, all strains were kindly provided by Fundação Oswaldo Cruz (FIOCRUZ). Spore suspensions in 0.85% (v / v) saline from cultures previously incubated in Medium Agar Potato Dextrose (BDA - Sigma®) for 7 days were used for the test. The fungal spores were counted with the aid of a Neubauer Chamber, and subsequently exposed to serial dilutions of the extracts (2.5 - 0.625 mg/mL) performed in RPMI-1640 medium in microtiter plates. For the colorimetric reaction, 10 µl of resazurin (0.002%) was added to all wells of the plate as an indicator of cell viability. Subsequently, the plates were incubated for 24 h at 36 °C ± 1 °C, the experiment being carried out in triplicate, with positive controls of fungal growth and sterility controls of the culture medium and extracts (Monteiro et al. 2012).

Data analysis

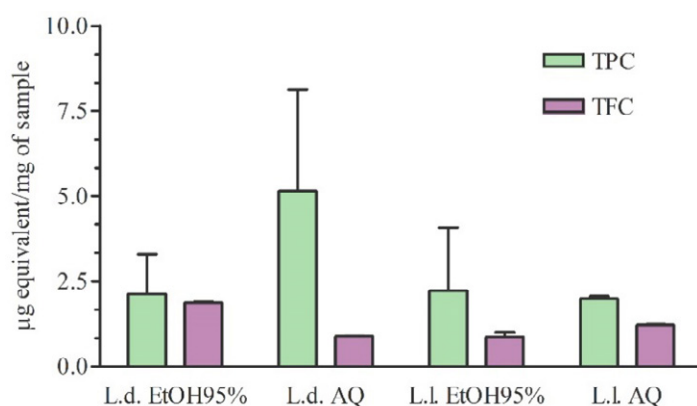
The data on the chemical composition of the extracts were analysed using two-way analysis of variance (ANOVA) using the statistical program GraphPad Prism 4. The results were expressed as means and standard deviation.

Results and discussion

According to the data obtained in the present study, it was possible to detect the presence of 2.13 and 5.16 µg of GAE/mg of the total phenols in the extract EtOH95% and AQ of *L. deliciosus*, respectively. While, for the *L. laccata* extract, we detected the presence of 2.22 and 1.99 µg of GAE/mg of the total phenols sample, in the extract EtOH95% and AQ, respectively. For flavonoids, we observed a value of 1.86 and 0.89 µg of CE/mg of sample of total flavonoids in the extracts EtOH95% and AQ of *L. deliciosus*, respectively. The values of *L. laccata* extract were 0.86 µg CE/mg of sample for extract EtOH95% and 1.22 µg CE/mg of sample of total flavonoids for extract AQ. Based on the results, we observed a considerable variability in the amount of phenols and flavonoids extracted in the two species of mushrooms (Figure 4). Some factors are determinant to the compounds that will be detected, both in plants and in mushrooms, mainly with regard to the geographic location of the specimen collection. Climatic stress conditions can directly influence the amount of secondary metabolites produced by mushrooms (Halbwachs and Simmel 2018).

Another decisive factor in the difference in the content of phenolic compounds is the solvent used for the extraction process. Aqueous extracts tend to have a higher amount of phenols when compared to other types of solvent (Wang and Xu 2014; Elbatrawy et al. 2015), as we can see for the *L. deliciosus* mushroom. Aroso et al. (2017) also analyzed the phenolic composition, in the case of a plant species, of extracts produced with solvents of different polarity, and observed that the greatest amount of phenols occurred in hydroalcoholic extracts, containing 50% water and 50% ethanol.

Figura 4. Content of phenols (μg of gallic acid equivalents per mg of sample) and total flavonoids (μg of catechin equivalents per mg of sample) in the 95% (EtOH95%) and aqueous (AQ) ethanol extracts of *Lactarius deliciosus* mushrooms (L.d.) *Laccaria laccata* (L.l.).



In the study of Volcão et al. (2019), the potential of aqueous extracts of different mushrooms against some bacterial species was demonstrated. It was possible to observe activity in bacteria with different characteristics, and no cell toxicity in vitro at the concentration tested. In the present study, we can demonstrate that 95% EtOH extracts were more efficient than AQ extracts in the three species of fungal phytopathogens tested (Table 1). The *F. solani* species complex has phytogetic potential in several agricultural crops, such as peas, peaches, strawberries and 100 other crops (Šišić et al. 2018; Villarino et al. 2019; Zhu et al. 2019). Meanwhile, *M. fructicola* is a stone fruit phytopathogen commonly found in Australia, South Africa and the Americas, and listed as a European quarantine organism (European and Mediterranean Plant Protection Organization 1992; Larena et al. 2005). The introduction into the country of species of this genus is extremely important, since it has already been demonstrated that *Monilinia* spp. isolated from imported fruits are able to adapt to peach-producing regions (Pereira et al. 2019). *Penicillium citrinum*, in turn, is a fungus isolated from citrus fruits and known to cause damage after harvest (Coutinho et al. 2020; Mincuzzi et al. 2020). Furthermore, it is of fundamental importance in the health area, as they are producers of citrinine, mycotoxin less harmful than ochratoxin, but also with nephrotoxic and hepatotoxic potential (Flajs and Peraica 2009; Freire et al. 2017).

Although each phytopathogen tested has its own characteristics that can influence the action of the extracts, the fungi have a cell wall composed mainly of chitin and β -glucans (Free 2013). These components become targets of most fungicidal substances, with the effectiveness of phenolic compounds being observed to alter the morphology of fungal hyphae, which included cytoplasmic disorganization and loss of protoplasmic content (M'Piga et al. 1997). In a study, Mohamed et al. (2017) demonstrated a positive correlation between the amount of phenols and flavonoids, in plant extracts, with the antifungal activity in *Fusarium oxysporum*. The same authors also demonstrated that the ability of the *Pulicaria incisa* extract to prevent mycelial growth and hinder the activity of enzymes important for the establishment of infection in the plant.

Tabela 1. Antifungal activity of ethanolic 95% (EtOH95%) and aqueous (AQ) extracts from *Lactarius deliciosus* and *Laccaria laccata* mushrooms in *Fusarium solani*, *Monilinia fructicola* and *Penicillium citrinum*.

| Target organism | Inhibitory effect (mg/mL) | | | |
|-----------------------------|-----------------------------|-------|-------------------------|-------|
| | <i>Lactarius deliciosus</i> | | <i>Laccaria laccata</i> | |
| | EtOH95% | AQ | EtOH95% | AQ |
| <i>Fusarium solani</i> | 1,25 | > 2,5 | ≤ 0,625 | > 2,5 |
| <i>Monilinia fructicola</i> | 1,25 | > 2,5 | > 2,5 | > 2,5 |
| <i>Penicillium citrinum</i> | 2,5 | > 2,5 | 2,5 | > 2,5 |

Other compounds found in mushrooms, in addition to phenols, have been researched for the control of phytopathogenic microorganisms, such as the polysaccharides chitin and chitosan. It is estimated that these compounds have the ability *in vivo* to induce plant resistance against invading organisms (Pusztahelyi 2018; Coutinho et al. 2020). The variety of compounds that can be extracted from plants and mushrooms, such as phenols, flavonoids, alkaloids, lipids, carbohydrates, demonstrate the countless possibilities of their application for the development of biopesticides and biofertilizers that cause the least impact on the environment and in human and animal health.

Conclusion

It was observed the presence of phenols and flavonoids total content in ethanolic and aqueous extracts produced from wild mushrooms collected in the southern region of Rio Grande do Sul, Brazil. In addition, under controlled conditions, we demonstrate the potential of using ethanol extracts (95%) from *Laccaria laccata* and *Lactarius deliciosus* to control three species of phytopathogens. Generally, the antimicrobial activity of extracts has been correlated with the content of total phenols, however, other compounds may be the cause of this activity. The importance of basidiomycetes mushrooms in food and alternative medicine is unquestionable, and the data from the present study demonstrate that these organisms can contribute to the development of sustainable agriculture, mainly by reducing agricultural inputs.

Authors' participation: LMV - Conceptualization, partnership establishment, data collection, image analysis and statistics, article writing; PFR – assistance in analyzing maps and images; RAT - Formal analysis and english correction; RLB - assistance in methodology and development of experiments; DFR – Resources, assistance in analyzing results and correcting the article; EB - Resources, estabelecimento parceria, co-orientador; FMRSJ – Resources, conceptualization, establishment of partnership, advisor and supervisor of project execution.

Ethical approval or research licenses: Does not apply to the study carried out.

Data availability: Manuscript developed from the first author's doctoral thesis. The data is available in the Library Administration System of Universidade Federal do Rio Grande – (FURG) via the link: <https://argo.furg.br/?BDTD13722>

Funding: To coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brazil (CAPES) – Financing Code 001.

Conflict of Interest: the authors declare that there is no conflict of interest.

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