Project closing report

Antimicrobial compounds from basidiomycetous macrofungi for plant protection

Grant number: OTKA PD 134467

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The research project, which began on the 1st of Sept 2020, is aimed at the detection, isolation and identification of antimicrobial agents from macrobasidiomycetous taxa within the *Boletaceae* and *Pleurotaceae* families. Additionally, the project seeks to determine the microbiological properties of these extracts and compounds with different chromatographic and microbiological methods.

One of the biggest challenges in plant protection today is the resistance to antibiotics and pesticides in plant pathogens including both bacterial and fungal species, causing significant economic and health damage and risks to humans and livestock. Traditional mycological research often focuses on individual mushroom species or single components within them, but the present study offers a more comprehensive examination of detailed patterns of bioactive components in the examined mushroom species. During the research, different analytical and microbiological methods have been employed and developed, moreover, "*in planta"* experiments have also been initiated.

In the first year, research of microbiologically useful agents from basidiomycetous macrofungi was started and several species from the *Pleurotus* genus, including *P. citrinopileatus*, *P. eryngii*, and *P. ostreatus* were successfully collected for experiments. Unfortunately, adverse weather conditions limited the collection of samples (*Leccinum duriusculum*) from the *Boletaceae* family.

The collected fruiting bodies of cultivated mushrooms were processed, which included cleaning and cutting, followed by freeze-drying. However, due to the limited capacity of the freeze-dryer, only 100-200 g of fresh samples could be lyophilized simultaneously. The tandem extraction of the powdered samples with solvents of different strengths followed. The methanolic and *n*-hexane extracts proved the best, but the methanol extract was easier to work with. Thin-layer chromatography (TLC) developments were performed using different eluents to explore the chemical differences between the samples under optimal resolution. High-performance TLC (HPTLC) was also employed, requiring modification of the eluent ratios for better separation.

Due to logistical delays caused by the COVID-19 pandemic, the microdilution assays of the extracts had not begun by the end of the first year. I planned to take further steps for the clean-up and fractionation of the tandem-solvent extracts with SPE (solid-phase extraction). The tools and materials arrived at the end of September 2021, so the SPE clean-up experiments began, and as a result, a new method was developed to separate the interfering compounds from the important ones.

The detection and examination of antifungal activity were of particular importance to us, and one of the most effective tools for mapping different plant and mushroom bioactive components is the combination of thin-layer chromatography with direct bioautography (TLC-DB). It is suitable for effect-directed detection of molecules with varying bioactivities from a complex matrix. The development of the antifungal effect detection method has resulted in the creation of a manuscript." (**DOI: 10.1021/acs.jafc.1c03676**).

In the second year of the research, from November 2021 onward, I supervised a master's thesis project, which focused on *in vitro* and *in planta* experiments related to the research project. The student's thesis, entitled "*Extracts of basidiomycetous mushrooms, as potential effective biopesticides*", explored the potential cytotoxicity of mushroom extracts on plants and their effectiveness against various plant pathogens, including viruses, bacteria and microscopic fungi.

Preliminary results from these experiments demonstrated a substantial reduction in tobacco mosaic virus (TMV) infection in tobacco plants, prompting a randomised repetition of the experiment. We also measured membrane leakage level based on electroconductivity, an indicator of plant health.

Direct bioautography of antifungal effect was carried out with extracts of three different *Pleurotus* mushrooms, and two components were discovered, which had antibacterial and antifungal activity against *Rhodococcus fascians*, *Xanthomonas euvesicatoria*, *Fusarium avenaceum*, and *Bipolaris sorokiniana*. These results were presented at the 25th International Symposium for HPTLC (Ljubljana, Slovenia) in July 2022.

As a result of last year's SPE experiments, we have developed a procedure that makes it easier to isolate and examine the minor components we want to investigate. A scientific poster was presented at the 33rd International Symposium on Chromatography (Budapest, Hungary) based on these results.

For the separation of the diverse active components in large quantities from the extracts, the development of a flash chromatography method has been started. It also could selectively isolate a greater amount of the important compounds. Microdilution assays have commenced with extracts and flash fractions from various *Pleurotus* species. The chromatographic and microbiological experiments also started with *Leccinum duriusculum* species. Unfortunately, the drought made mushroom picking difficult in the second year.

In the third year, the supervised undergraduate student defended her thesis successfully in May 2023. The extracts of *Pleurotus* spp. inhibited *Tobacco mosaic virus* (TMV) infection: a significant difference in the number of lesions between fungal extract treated and untreated leaf sections was observed (on average 20% difference). Furthermore, conductivity measurements (membrane leakage assay) of treated samples were significantly different from the untreated controls. The multiple treatment reduced the number of lesions by 49.6±9.21%. This reduction was more significant than by the single treatment, suggesting that repeated treatments have a more effective antiviral effect. Later, a virus strain of major agricultural concern was included in the *in planta* experiments, tomato brown rugose fruit virus (ToBRFV). In this case, the virus was also inhibited by different mushroom fractions (mainly fractions of the *L. duriusculum* and *P. citrinopileatus* were active). The virus infection experiments could be carried out intermittently, the plants were grown from seed, in a sufficient number and replicates for statistical evaluation. Healthy plants and the right condition were required, which could not be ensured in all experiments, so time delays were also given here. The determination and isolation of the active compound(s) are in progress, the manuscript is already half-finished and will be submitted as soon as the missing data have been collected.

Our studies also determined microbial parameters, as the extracts and fractions were tested against several plant pathogens. All examined extracts of mushrooms inhibited the growth of *Xanthomonas campestris* pv. *phaseoli*, whereas only *P. ostreatus* and *P. eryngii* inhibited the growth of *Ralstonia solanacearum*. The MIC values of the extracts from different *Pleurotus* species showed that they were effective against plant pathogenic fungi (MIC-value against *Botrytis cinerea* 0.28 g/ml for all tested *Pleurotus* species).

A novel two-step dual-layer solid-phase extraction method was developed to separate antioxidant and antibacterial components from the extract of *P. citrinopileatus* using a costum-made cartridge, which is composed of C_{18} and silica gel. This method offers many advantages, including a significant decrease in fatty acid content - primarily linoleic acid - in the examined samples, increased concentration and detectability of bioactive minor compounds, and more effective separation of bioactive components into eluates. Further research aims to evaluate the wider applicability of this method for other mushroom species and optimize its performance for other non-basidiomycetous mushroom species. Optimization steps will focus on determining the necessary ratios of eluents, determining the maximum sample capacity and exploring various conditioning strategies for the dual-layer cartridge.

Another flash chromatography method, built upon the previous SPE results, was proven useful in separating mushroom components based on chemical polarity. This method has allowed for the isolation of a great number of antioxidant compounds. In our manuscript detailing the patterns and quantitative ratios of antioxidant components in *Cyclocybe cylindracea* and *Leccinum duriusculum*, the HPTLC-DPPH and microplate-DPPH methods have also been compared (**DOI: 10.1007/s00764-023-00271-y**). The antimicrobial fractions of the examined *Pleurotus* species (*P. citrinopileatus, P. eryngii, P. ostreatus*) were successfully separated, and we purified the active components, which were analysed through high-resolution mass spectrometry (HR-MS/MS); these isolates will be further analysed by nuclear magnetic resonance (NMR) spectroscopy.

In the case of *Leccinum durisculum*, two antifungal components were identified using effect-directed screening and HPTLC-MS analysis. These agents showed activity against *Fusarium graminearum* and *Bipolaris sorokiniana*. The antifungal activity of fractionated compounds was examined following preparative flash chromatographic separation and the active compounds were characterized through derivatization techniques. One was an alkaloid, while the other had not formed a derivative during any chemical procedure, except a reaction with vanillin-sulfuric acid. However, vanillin-sulfuric acid is a common derivatizing compound not suitable for determining a class of compounds. Fortunately, both could be ionised, so these antifungal components underwent HPTLC-MS determination. This result was presented in a poster at the 13th Balaton Symposium in Siófok. The preparation and submission of a manuscript reporting these results are currently underway.

Thanks to favourable weather conditions in 2023, various quantities of species belonging to the *Boletaceae* family were successfully collected, including the Rotting Bolete (*Caloboletus radicans*), False Yellow Bolete (*Neoboletus xanthopus*), Suede Bolete (*Xerocomus subtomentosus*), Iodine Bolete (*Hemileccinum impolitum*), and Devil's Bolete (*Rubroboletus satanas*). The lyophilizing and extracting of these specimens were started in progress for the experiments.

A request for an extension of the research project was submitted due to delays caused by the COVID-19 pandemic and financial constraints. Following the approval of the extension, our manuscript on the SPE method was published in the January 2024 issue of Talanta One (**DOI: 10.1016/j.talo.2024.100304**). During this period, the isolation and purification of active compounds from different extracts and fractions of the *Pleurotus* species and *Leccinum duriusculum* was continued. Additionally, HPLC-MS analysis and isolation efforts were conducted on various *Pleurotus* samples. Here, some components failed to ionize properly, necessitating adjustment of the methodology.

We began chromatographic experiments on mushroom samples collected in 2023, initially focusing on three taxa. Among these, the extract of *Rubroboletus satanas* underwent flash chromatographic separation using a dual-eluent system. This system, which involved utilizing two different eluent mixtures on a single cartridge, produced results similar to those achieved through the SPE method, though further refinement is required. The preliminary findings were presented in a poster at the 26th International Symposium for High-Performance Thin-layer Chromatography in Budapest.

The isolation of sufficient quantities of compounds for NMR structure determination is still ongoing, particularly for species from the *Pleurotacea* and *Boletaceae* families. A preparative flash chromatography method has been developed to aid in the isolation process, however these plans were postponed due to relocation and other previously mentioned reasons.

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Budapest, 27 September 2024