



Extraction and Identification of Aziridine Derivatives in VOCs from *Pleurotus ostreatus*: Impact on Plant Pathogens [†]

Muhammad Usman ^{1,‡}, Muhammad Akbar ^{1,‡} , Taswar Ahsan ^{2,*}  and Muhammad Hamza ³

¹ Department of Botany, University of Gujrat, Gujrat 50700, Pakistan; mu.usman3214@gmail.com (M.U.); muhammad.akbar@uog.edu.pk (M.A.)

² Institute of Plant Protection, Liaoning Academy of Agricultural Sciences, Shenyang 110161, China

³ Department of Veterinary Science, University of Veterinary & Animal Sciences, Lahore 54000, Pakistan; muhammad357@gmail.com

* Correspondence: taswar.micro@gmail.com or taswarahsan@163.com; Tel.: +86-182-4253-6612

[†] Presented at the 3rd International Electronic Conference on Agronomy, 15–30 October 2023;

Available online: <https://ieag2023.sciforum.net/>.

[‡] These authors contribute equally to this work.

Abstract: *Pleurotus ostreatus* has potent antimicrobial properties. In this study, bioactive compounds were extracted from *P. ostreatus* and screened against bacterial and fungal phytopathogens. In terms of the antibacterial activity, the n-hexane extract of *P. ostreatus* exhibited a significant inhibition zone of 88.55 mm against *Xanthomonas axonopodis*, while the highest antifungal activity of 83% was against *Fusarium oxysporum*. It was observed that the highest level of concentrations, i.e., 25 mg mL⁻¹, caused a 76, 82, 82, 83, and 60% decrease in fungal biomass over the control against the fungal strains, i.e., *A. alternata*, *A. flavus*, *D. australiensis*, *F. oxysporum* and *M. phaseolina*, respectively. GC-MS analysis was performed on the n-hexane extract depicting the presence of 26 compounds. A compound identified as Toluene (Molecular weight = 92) exhibited a peak area of 91% followed by another compound named Cyclopentane, methyl- (Molecular weight = 84) showing a peak area of 56%. A well-known antimicrobial compound Aziridine (Mol. Weight = 99) was identified and showed a maximum hit of 84%, with a peak area of 56%. *P. ostreatus* could be a potent biocontrol antagonist against the plant pathogens.

Keywords: *Pleurotus ostreatus*; Aziridine; antibacterial; antifungal



Citation: Usman, M.; Akbar, M.; Ahsan, T.; Hamza, M. Extraction and Identification of Aziridine Derivatives in VOCs from *Pleurotus ostreatus*: Impact on Plant Pathogens. *Biol. Life Sci. Forum* **2023**, *27*, 37. <https://doi.org/10.3390/IECAG2023-16378>

Academic Editor: Gianni Bellocchi

Published: 29 November 2023



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1. Introduction

Bacteria and fungi are major phytopathogens that affect crop yields worldwide [1]. With a growing global population, a well-organized disease management and control program is essential for ensuring food security and safety [2,3]. Using agrochemicals to control crop diseases impacts the environment and humans [4]. Biocontrol is an eco-friendly solution to this problem [5]. The numbers of tested biocontrol agents (BCAs) and commercial products are not positively connected [3]. Identification of novel BCAs is essential to commercial biocontrol product development and needs a reliable screening procedure [6]. According to research, fungi produce various bioactive volatile organic compounds (VOCs) [7]. Specifically, mushroom-derived compounds exhibit promising potential for biological activity [8,9]. By considering the aforementioned characteristics of macro-fungus, our study investigated the antimicrobial efficacy of *P. ostreatus* against phytopathogens.

2. Experiments

2.1. Pathogens and Antagonist

P. ostreatus was purchased from BioTech, Sahiwal, Pakistan. Bacterial and fungal pathogens were purchased from the Culture Bank of Pakistan, Institute of Agricultural

Sciences Punjab University, located at the main campus in Lahore. The bacterial plant pathogens, *Erwinia carotovora*, *Pseudomonas syringae*, *Ralstonia solanacearum*, and *Xanthomonas axonopodis* were subcultured on malt extract agar, while the fungal pathogens, *Alternaria alternata*, *Aspergillus flavus*, *Drechslera australiensis*, *Fusarium oxysporum*, and *Macrophomina phaseolina* were subcultured on PDA medium. Then, these grown cultures were stored in a refrigerator at 4 °C.

2.2. Extraction of the Bioactive Compounds

P. ostreatus bioactive components were extracted following [10], with minor changes. We used a piston mortar to crush the sun-dried fruiting bodies into powder. 500 g of dry *P. ostreatus* powder was steeped in 1000 mL methanol for 5 days. After filtering using Whatman paper, the extract was evaporated in a rotary evaporator at 45 °C for one hour. The dry methanolic extract was remeasured and re-suspended in 200 mL of distilled water to yield 9 g. We stored it aseptically for future use.

Additional solvent extraction was performed using n-hexane. We added 200 mL of the re-suspended extract to a 500 mL separating funnel and added 200 mL of the n-hexane organic solvent at a 1:1 ratio. We let it sit overnight. The flask had clear layers, which were carefully transferred to an electrically pre-weighted beaker for evaporation. This was performed three times to remove everything. The extract was re-evaporated using a rotary evaporator to pure fractions.

2.3. Gas Chromatography Mass Spectrometry Analysis

The biochemical components of *P. ostreatus* n-hexane extract were evaluated using a GC/MS with column number HP-5MS (30 m × 250 µm × 0.25 µm). GC/MS analysis was conducted on a Thermo GC Trace ultra-version 5.0, Thermo MS DSQ II, using a fused silica column and Elite ZB 5 MS capillary standard nonpolar column (30 m, 0.25 mm, 0.25 µm). The components were separated using helium at 1 mL/min. The Thermo MS DSQ II identified the 1 µL sample extract supplied to the instrument. The oven was set at 260 °C for 38.50 min (mass analyzer). After 40 min of MS detection, the relative amounts of all components were computed.

2.4. Antibacterial Assays

The disk diffusion technique was employed for antibacterial trials. The treatment included dissolving 50 mg of mushroom extract in n-hexane solution and 140 µL of DMSO and adding distilled water to reach 500 µL. To generate the negative control solution, we combined 140 µL of DMSO with 360 µL of distilled water. For the positive control, we dissolved 50 mg of *Penicillin* in 140 µL of DMSO and added distilled water to achieve a volume of 500 µL. All bacteria were cultured on Lysogeny agar.

2.5. Antifungal Assays

Antifungal screening of the isolated compounds was carried out by dilution technique. We prepared Potato Dextrose Broth (PDB) in a conical flask and autoclaved it at 121 °C for 20 min. We mixed 300 mg of the mushroom extract in n-hexane solution; then, we dissolved it in 333 µL of DMSO and add distilled water to reach 1000 µL for the stock solutions. The control solution with DMSO was made similarly. To promote fungal development, mycelial spores of each species were introduced to growth media and incubated at 27 °C for 6 days. Following filtering using pre-weighted filter paper, the filtered material was oven-dried for 2 days at 65 °C to extract the dry fungal biomass.

2.6. Statistical Analysis

For the statistical analysis, two way ANOVA was applied using Fisher's Least Significant Difference (LSD) test to elucidate the treatment means. In ANOVA, two variable factors were analyzed, i.e., Extracts over Control treatments. This analysis was performed utilizing the Minitab-17 statistical software.

3. Results and Discussion

The results showed that *P. ostreatus* has potent antimicrobial properties against bacterial and fungal phytopathogens. This indicated that *P. ostreatus* could be an efficient antagonistic agent. Various species of *basidiomycetes*, including the edible mushroom *P. ostreatus*, have the ability to inhibit the growth of plant pathogens [11].

3.1. Antibacterial Activity of the n-Hexane Extract of *P. ostreatus*

The results showed that the n-hexane extract of *P. ostreatus* showed the highest zone of inhibition of 88.55 mm against *X. axonopodis*. However, *E. carotovora*, was the only pathogenic strain that was not inhibited by this extract (Figure 1). Even the control showed lower bioactivity against *E. carotovora*. This extract also exhibited significant bioactivity against other pathogenic bacteria, such as *P. syringae* and *R. solanacearum*, with inhibition zones of 69.18 mm and 59.79 mm (Figure 1).

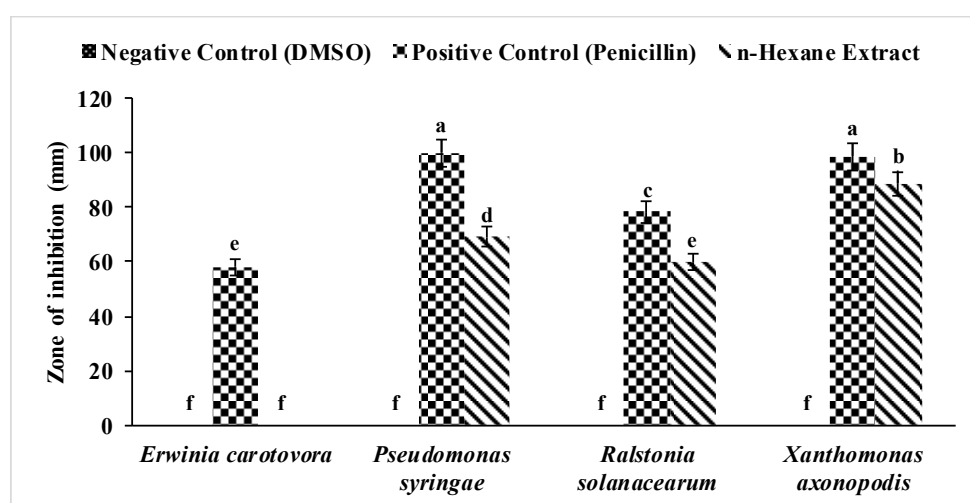


Figure 1. Effect of the n-hexane extract of *P. ostreatus* against *E. carotovora*, *P. syringae*, *R. solanacearum*, and *X. axonopodis*. The vertical bars show the standard error of the means of three replicates. Values with different letters show a significant difference ($p \leq 0.05$) as determined by ANOVA followed by Fisher's LSD Test, using Minitab 17.

The positive control (penicillin) formed significant inhibition zones in all bacterial strains. In *E. carotovora* a 58.02 mm, in *P. syringae* a 99.87 mm, in *R. solanacearum* a 78.34 mm and in *X. axonopodis* a 98.61 mm zone of inhibition was formed. Penicillin is a synthetic antibacterial capsule, which contains a pure compound, which is why it showed maximum activity against all bacterial strains.

The negative control did not show any antibacterial activity against any bacterial strains. This confirmed that the DMSO did not harm any plant pathogenic bacteria. According to this experimental result, this extract was further analyzed by GCMS, and its chemical composition was observed. Heleno et al. [12] studied the antibacterial properties of *P. ostreatus* acidic extract against *Pseudomonas aeruginosa* and found similar results to our experiment. Other information against bacterial strains was not available and was firstly reported in our experiment. n-hexane was significantly active against bacteria. The broad spectrum antibacterial activity of Aziridine, 1-(2-aminoethyl)-, was optimized by a one-variable-at-a-time system coupled with the response surface methodology, which led to a 45% enhancement in antibacterial activity [13].

3.2. Antifungal Activity

The antifungal activity of the n-hexane extract of *P. ostreatus* against *Alternaria alternata*, *Aspergillus flavus*, *Drechslera australiensis*, *Fusarium oxysporum*, and *Macrophomina phaseolina* was examined. It was observed that organic extracts of n-hexane and its

different concentrations increased from 5 to 25 mg mL⁻¹; the fungal growth was inhibited, which resulted in the decrease in the fungal biomass (Figure 2). The maximum growth reduction occurred in the extracts with a 25 mg mL⁻¹ concentration. In these bioassays, the n-hexane extracts showed pronounced inhibitory effects on the tested fungal pathogen. The maximum growth was seen in the control to which no extract was added; as the organic extracts were added the fungal biomass decreased. All the extracts were prepared in DMSO; so, it was used as the control. The DMSO had no activity against any plant pathogenic fungal strains.

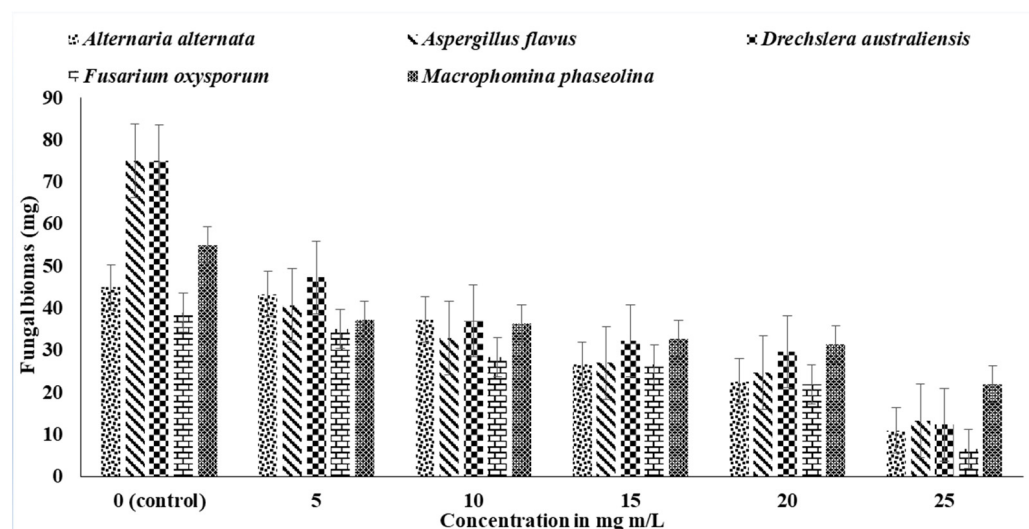


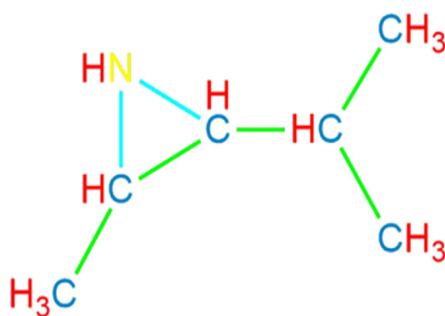
Figure 2. Effect of the n-hexane extract of *P. ostreatus* against *Alternaria alternata*, *Aspergillus flavus*, *Drechslera australiensis*, *Fusarium oxysporum*, and *Macrophomina phaseolina*. Vertical bars show the standard error of the means of three replicates. Values with different letters show a significant difference ($p \leq 0.05$) as determined by ANOVA followed by Fisher's LSD test using Minitab 17.

The maximum antifungal activity was observed in the n-hexane extracts of 25 mg mL⁻¹ concentration on the fungal pathogen *A. flavus* which decreased the fungal biomass by 82% and 81% over the control, respectively. Among all the fungal strains examined in this experiment, the growth of *F. oxysporum* was the most reduced, which confirmed that these extracts were very effective against this strain. In a previous investigation, an antifungal component, obtained from *Pleurotus sajorcaju*, showed bioactivity against *Fusarium* and *Mycosphaerella* species [14]. The isolation of antifungal constituents from *Pleurotus* spp. with activity on these fungi can be fruitful from an industrial point of view [15].

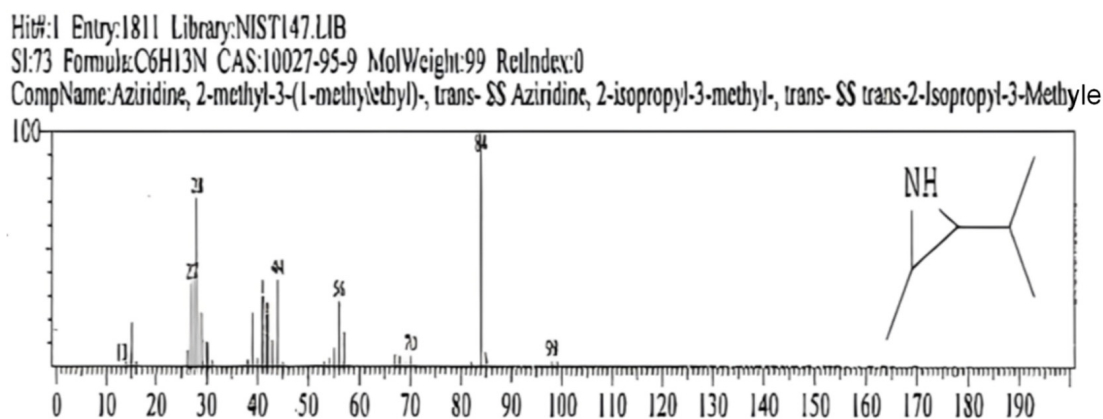
3.3. GC/MS Analysis of the n-Hexane Extract of *P. ostreatus*

A total of 26 compounds were identified in the n-hexane extract of *P. ostreatus* mushroom, as presented in Table S1. Among the 26 compounds, 7 compounds exhibited a higher frequency of occurrence and demonstrated a greater number of successful outcomes. Aziridine, 2-methyl-3-(1-methylethyl)-, trans- Aziridine, 2-isopropyl-3-methyl-, trans 2-Isopropyl-3-Methyl (Mol. Weight = 99) was identified only one time and showed a maximum hit of 84%. The potential structure is depicted in (Figure 3A,B). Santra et al. [13] first reported the Aziridine, 1-(2-aminoethyl)-, from any endophytic source. *Cochliobolus* sp. APS1 possesses industrial importance for the production of bioactive alkaloids with broad spectrum bactericidal action. Kowalczyk et al. [16] reported that Aziridines were widely used as building blocks in the multi-step syntheses of more complex molecules; however, due to the presence of the aziridine ring, their derivatives exhibit significant biological activity. Toluene (Mol. Weight = 92) showed a maximum hit of 91%, and hexane (Mol. Weight = 86) showed a maximum hit of 51%. The other prominent compounds were pentane,

specifically the isomer 3-methylpentane, with a molecular weight of 86, which exhibited a maximum yield of 57%. In the same way, the compounds pentane, 2-methyl-Isohexane, 2-methylpentane, and methyl pentane $(\text{CH}_3)_2\text{CH}(\text{CH}_2)_2\text{CH}_3$, with a molecular weight of 86, were all identified. *P. ostreatus* contains bioactive compounds such as polysaccharides (glucans and chitin), as well as secondary metabolites (phenolic compounds, terpenoids, and lectins). These compounds have been found to possess antibacterial, antiviral, and antifungal properties [17].



(A)



(B)

Figure 3. A typical chemical structure of Aziridine derivatives (A). Chromatogram of Aziridine derivatives (B).

4. Conclusions

This study revealed that the extract derived from *P. ostreatus* exhibits significant antibacterial and antifungal activities against crop pathogens. The GC-MS analysis revealed the presence of aziridine and its derivatives in the extract of *P. ostreatus*. This study could be useful to develop an eco-friendly and sustainable biocontrol agent for crop disease management.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/IECAG2023-16378/s1>, Table S1: GC/MS Analysis of the n-hexane extract of *Pleurotus ostreatus*.

Author Contributions: Conceptualization, M.U. and M.A.; methodology, M.A.; software, M.H.; validation, M.A., T.A. and M.U.; formal analysis, M.U.; investigation, M.U.; resources, M.A.; data curation, M.A. and M.H.; writing—original draft preparation, T.A.; writing—review and editing, M.U. and M.A.; visualization, M.A.; supervision, M.A.; project administration, M.A.; funding acquisition, T.A. All authors have read and agreed to the published version of the manuscript.

Funding: Basic scientific research business expenses from Liaoning Academy of Agricultural Sciences (2022XTCX0502003).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data are available from the corresponding author on reasonable request.

Acknowledgments: A very special thanks to Taswar Ahsan for providing the funds.

Conflicts of Interest: The authors declare no conflicts of interest.

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