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Secondary Metabolite Profiles: Trichoderma, *Aspergillus flavus* Glocladium and Penicillium as Biocontrol Agents

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ABSTRACT

A fungus is utilized as a biocontrol agent to suppress plant diseases by employing an antagonistic mechanism, wherein it releases enzymes that degrade the cell walls of pathogens and inhibit their growth. The antibiosis mechanism is initiated by biocontrol fungi through the production of secondary molecules or metabolites. The objective of the research was to examine the secondary metabolite composition of the biocontrol fungi *Trichoderma spp*., Aspergillus flavus, and *Gliocladium sp*. and *Penicillium sp*ecies The implementation approach involved the use of qualitative phytochemical testing and HPLC analysis. The outcomes of the investigation into the metabolite profiles of *Trichoderma spp*., Aspergillus flavus, and *Gliocladium sp*. were observed. and *Penicillium sp*ecies The presence of alkaloids, phenolics, and flavonoids was detected in the samples. The results of the High-Performance Liquid Chromatography (HPLC) analysis indicated the presence of 10-11 compounds, as evidenced by the peaks observed in the chromatogram. These compounds are presumed to be associated with the phenolic and alkaloid groups.

Keywords: Biocontrol, Fungi, HPLC, Phytochemistry, Secondary Metabolites

1. INTRODUCTION

A notable biocontrol agent is a fungus that effectively manages plant diseases or pathogens through antagonistic mechanisms. This process involves the secretion of enzymes that break down the cell walls of pathogens, as well as the production of antimicrobial compounds. The antibiosis mechanism is characterized by the synthesis of specific molecules or secondary metabolites by biocontrol fungi, which exhibit antimicrobial properties against targeted microorganisms. Numerous bacterial and fungal strains are capable of generating antibiotics and secondary metabolites that possess potential anti phytopathogenic effects. The efficacy of biocontrol agents is directly correlated with the variety of compounds they produce (Nguyen et al., 2017).

Examples of biocontrol agents that demonstrate the ability to inhibit plant pathogens include Gliocladium, which exhibits an inhibitory capacity of 74.79%, and Trichoderma, which shows an inhibitory effect of 73.19% against the pathogen Fusarium oxysporum (Hikmahwati et al., 2021, 2022). Additionally, Trichoderma atroviride has been reported to inhibit Fusarium oxysporum f. sp. lycopersici by 92.11% (Nofal et al., 2021), while T. harzianum offers disease protection of up to 82.9% against Plasmopara viticola (Kamble et al., 2021). One of the mechanisms through which these biocontrol agents exert their inhibitory effects is through antibiosis, facilitated by the production of secondary metabolite compounds.

Microbial secondary metabolites are low molecular weight compounds produced through metabolic processes involving biosynthetic pathways. These compounds are not essential for the growth of the microbe, but they play an important role in defence and competition mechanisms in nature (Hazarika et al., 2022).

Antifungal secondary metabolites can be classified into two principal groups: polyketides and nonribosomal peptides (NRPs). NRPs can be classified into smaller dipeptides, cyclic peptides and larger lipopeptides. Lipopeptide groups, including surfactin, iturin, and fengycin, function as active surfactant agents and exhibit antimicrobial activity. Secondary metabolites can also be classified into the following groups: alkaloids, flavonoids, steroids, terpenoids and saponins, all of which possess antimicrobial properties (Nurulita et al., 2020).

Fungi, such as Trichoderma, Penicillium, and Gliocladium, possess the ability to synthesize antimicrobial compounds. Trichoderma produces a variety of secondary metabolites, including non-ribosomal peptide synthetases (NRPSs) compounds such as peptaibiotics, siderophores, and gliotoxin and gliotoxin-like diketopiperazines, as well as polyketides, terpenes, pyrones, and isocyanate metabolites. These compounds, such as harzianolide, 6-PP, peptaibols, trichokonins, and harzianic acid, act as plant soil in the form of 6-pentyl-pyrone (6-PP). Additionally, Trichoderma synthesizes polyketide synthases (PKSs), terpenoids, and total phenols. (Zeilinger et al., 2016; Pascale et al., 2017; Pereira-Dias et al., 2023; Pradhan et al., 2023). The ethyl acetate filtrate culture derived from Trichoderma viride exhibited inhibitory activity of 51.9% and 63% against the pathogen Curvularia lunata, as reported by Yassin et al. (2021). Additionally, Penicillium produces terpenoid secondary metabolites with an inhibitory power of 33.78% against the pathogen Candida albicans, as observed by Nurulita et al. (2020b). As indicated by Abdelaziz et al. (2022), the production of ethyl acetate is attributed to Aspergillus flavus, A. fumigatus, and A. nidulans, resulting in an inhibitory effect of 84.37% against the pathogen Fusarium oxysporum. Furthermore, the synthesis of Albupeptins A-D2 is observed in the Gliocladium album, exhibiting

suppressive properties against Phytophthora infestans (Pereira-Dias et al., 2023).

The metabolomic analysis of antagonistic fungi may be conducted using either the high-performance liquid chromatography (HPLC) method (Warsito, 2018) or the Liquid Chromatography-Mass Spectrometry (LC-MS) analysis (Theowidavitya et al., 2019). The HPLC method allows the content of secondary metabolites to be identified, as indicated by the resulting chromatogram pattern.

In light of the explanations mentioned earlier, an analysis was undertaken to ascertain the secondary metabolite profile of the biocontrol fungi Trichoderma, Aspergillus flavus, Glocladium and Penicillium. The secondary metabolite profile of these biocontrol fungi was determined through qualitative tests on phytochemicals and by HPLC.

2. MATERIAL AND METHODS

2.1 Research Time and Place

This research was conducted in the Plant Disease Laboratory, Department of Protection, Faculty of Agriculture, Biochemistry Laboratory, Department of Chemistry, Faculty of Mathematics and Natural Sciences, Hasanuddi University and Makassar Health Office Laboratory. This research was conducted in June-September 2024.

2.2 Propagation of fungi on Potato Dextro Agar (PDA) medium

Biocontrol fungi were obtained from the collection of the Plant Disease Laboratory, Department of Protection, Faculty of Agriculture, Hasanuddin University, then multiplied on PDA media for 7 days at room temperature and then used for further testing

2.3 Extraction of Secondary Metabolite Compounds

Fungi were cultivated for a duration of 7 to 14 days in Potato Dextrose Broth (PDB) media. Following this incubation period, the fungal colonies were isolated from the filtrate culture through filtration using Whatman GF/C filter paper in conjunction with a Buchner funnel. A total of 1 liter of the filtrate culture was subjected to two extractions with 500 mL of ethyl acetate, maintaining a solvent-to-media ratio of 2:1. The resulting ethyl acetate extract was treated with anhydrous sodium sulfate (Na2SO4) until saturation was achieved, after which it was concentrated using a rotary evaporator at ambient temperature. The residue obtained from this evaporation process was subsequently re-dissolved in methanol, yielding a crude ethyl acetate extract from the ethyl acetate layer. This extract is intended for content analysis and pathogen testing and can be preserved at -20°C before utilization.

2.4 Terpenoid Test

A terpenoid test was conducted following the methodology outlined by Aristyawan et al. (2024). The ethyl acetate extract was transferred to a test tube, followed by the addition of chloroform. Subsequently, the chloroform portion was transferred with a pipette, Lieberman-Burchard reagent was added, and the mixture was allowed to evaporate. A red-brown colouration is indicative of a positive result for terpenoids, whereas a green-blue colouration is indicative of a positive result for steroids.

2.5 Alkaloid Test

The alkaloid test was performed according to the procedure described by Sukmawaty et al. (2021), utilizing an ethyl acetate extract. The extract was placed into a test tube, and ammoniated chloroform was added. The resulting mixture was then pipetted, and sulfuric acid was introduced. The acid layer was carefully separated and transferred onto a dropper plate, where it was subsequently treated with Dragendorff's reagent and Mayer's reagent. The existence of alkaloids is manifested by a shift in color to orange following the introduction of the Dragendorff reagent, and a shift in color to white following the addition of Mayer's reagent.

2.6 Phenolic Test

A phenolic test was conducted following the methodology proposed by Rohmawati and Harahap (2017). The ethyl acetate extract was dripped onto a dropper plate, and an iron (III) chloride solution was subsequently added. A positive result was indicated by a change in the solution's colour to blue-black.

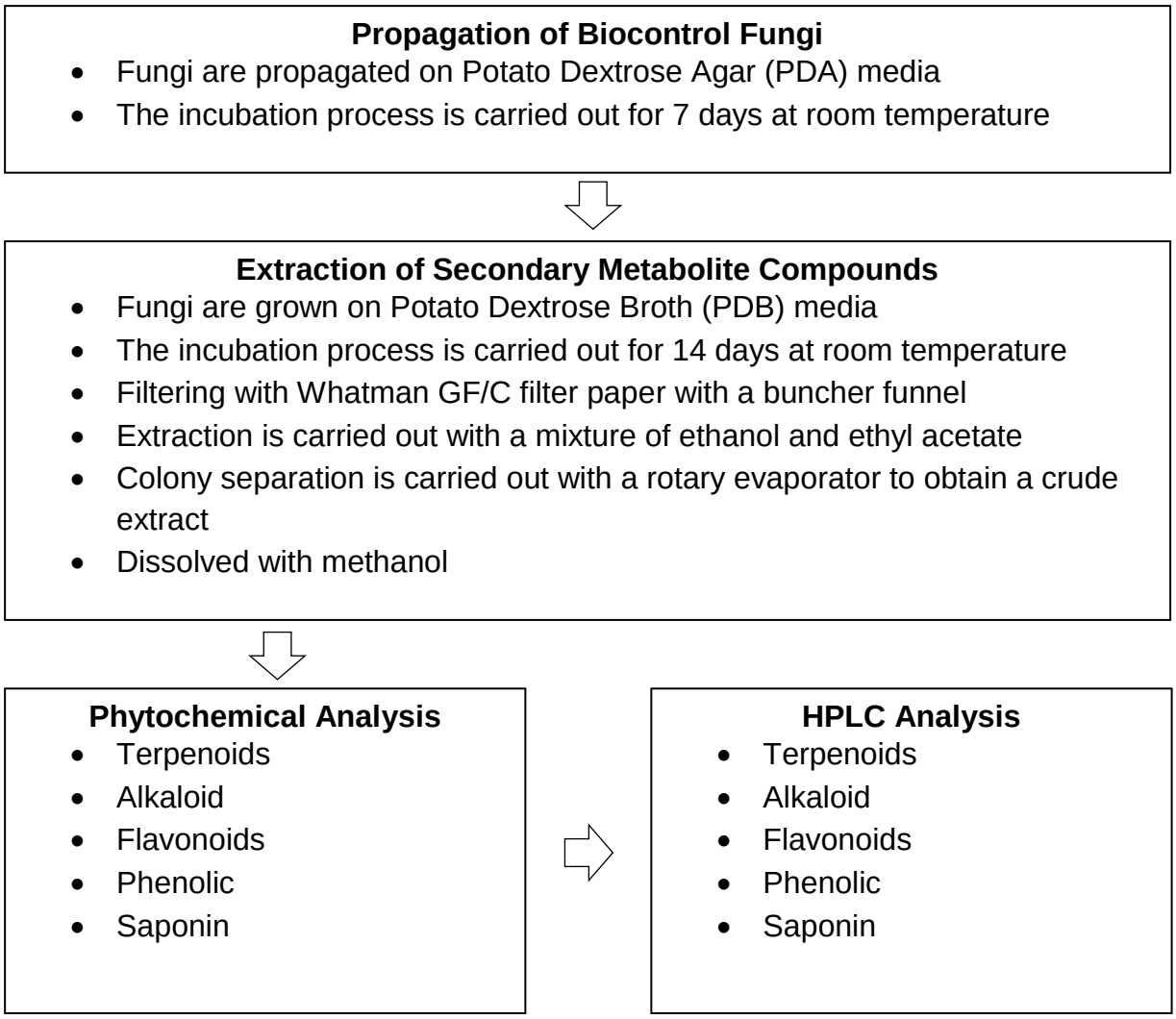

Figure 1. Research flow diagram

2.7 Flavonoid Test

The Rohmawati and Harahap (2017) method was employed for the flavonoid test. The ethyl acetate extract was transferred to a test tube and 70% ethanol was added, after which the mixture was heated. The aforementioned mixture was then combined with a magnesium metal plate and a single drop of concentrated hydrochloric acid. A change in the solution's colour to pink is indicative of a positive result. In addition to the aforementioned method, an alternative approach involves dissolving the ethyl acetate extract in a 70% ethanol solution and heating it in a water bath, followed by the addition of a few drops of 10% NaOH. The presence of a yellow colouration is indicative of the presence of flavonoids.

2.8 Saponin Test

The saponin test was conducted following the methodology outlined by Aristyawan et al. (2024). Initially, ethyl acetate extract was combined with water and subsequently heated to boiling. The mixture was then subjected to vigorous shaking, and the presence of stable foam for approximately 10 minutes indicated

that the ethyl acetate extract contained saponins.

2.9 HPLC Analysis

The HPLC analysis commences with the dissolution of the ethyl acetate extract with methanol, with an injection volume of 5 µL, in the C18 Thermo Scientific column. The injection time is 20 minutes (19.92 minutes), with an eluent Aquadest: Acetonitrile ratio of 20:80, utilising a Gradient. The instrumentation employed is as follows: The HPLC-Vanquish Flex Duo, a Thermo Scientific brand, was used with a wavelength of 210 nm, channel UV_2, a dilution factor of 1, and a sample weight of 1. Overall, the research implementation procedure is described in the flow diagram in Figure 1.

3. RESULTS AND DISCUSSION

3.1 Phytochemical Test (Terpenoids, Alkaloids, Phenolics, Flavonoids, and Saponins)

The qualitative phytochemical analysis of secondary metabolites derived from the filtrate culture of fungal isolates revealed the presence of alkaloid and phenolic groups across all isolates, whereas flavonoid groups were exclusively identified in Penicillium isolates. As noted by Nurulita et al. (2020), *Penicillium sp*. is known to contain terpenoids, while Trichoderma asperellum has been reported to produce a diverse array of compounds, including alkaloids, flavonoids, phenols, saponins, tannins, and terpenoids. Mycotoxins belonging to the terpenoid category, specifically trichodermin and trichothecenes, are metabolites synthesized by *Trichoderma spp*. as part of their antagonistic strategies (Chua et al., 2022). Infrared photometer spectral analysis indicated that the secondary metabolites of Trichoderma sp. fungi comprise six distinct types of chemical compounds (Pamekas, 2020). Furthermore, phytochemical testing of the ethyl acetate extract from the endophytic fungus Aspergillus sp. demonstrated the presence of flavonoid, alkaloid, terpenoid, and tannin compounds (Sukmawaty et al., 2021). The absence of certain compound groups in the samples suggests that the growth conditions in PDB media may not have been optimal.

Based on the presence of phytochemical compounds in the form of alkaloids, phenolics and flavonoids. This shows that the metabolite profile of the tested isolates has the potential to be antimicrobial. Alkaloids have a mechanism of inhibiting various cellular processes in microorganisms, including DNA and protein synthesis. Phenolics and flavonoids can inhibit microbial growth through mechanisms such as cell membrane damage or inactivation of important enzymes. Alkaloids, Saponins, Tannins, Phenolics, Flavonoids and Terpenoids from the acetyl acetate extract of the Kasturi Mango plant have antibacterial properties (Suhendar et al., 2019).

Table 1. Phytochemical Test Results

Sample Code	Alkaloid	Flavonoids	Phenolics	Terpenoids	Saponin
Trichoderma spp.	+	-	+	-	-
Gliocladium sp.	+	-	+	-	-
Aspergillus flavus	+	-	+	-	-
*Penicillium sp*p.	+	+	+	-	-

Antifungal agents exhibit a range of inhibitory mechanisms targeting fungal cells. These agents function by neutralizing enzymes that facilitate fungal invasion and colonization, compromising the integrity of fungal cell membranes, and inhibiting enzymatic systems critical for the development of hyphal tips. Additionally, they interfere with the synthesis of nucleic acids and proteins (Rohmawati & Harahap, 2017).

Alkaloids, recognized for their antimicrobial properties, inhibit esterase activity as well as DNA and RNA polymerases, disrupt cellular respiration, and contribute to DNA intercalation (Rohmawati & Harahap, 2017). Flavonoids, which represent the most extensive category of polyphenolic compounds, operate by denaturing proteins, thereby enhancing cell membrane permeability. This denaturation leads to disturbances in cellular structure, altering the composition of protein components. The phenolic compounds present in flavonoids can denature cellular proteins and reduce the thickness of cell walls, resulting in lysis of fungal cell walls, hindering fungal growth, and potentially leading to cell death. (Rohmawati & Harahap, 2017).

Saponins constitute a group of compounds that can inhibit or kill pathogenic fungi. This is achieved by reducing the surface tension of the sterol membrane of the cell wall, thereby increasing its permeability. The increased permeability draws more concentrated intracellular fluid out of the cell, resulting in the release of nutrients, metabolic substances, enzymes, and proteins within the cell. This ultimately leads to the death of pathogenic fungi (Rohmawati & Harahap, 2017).

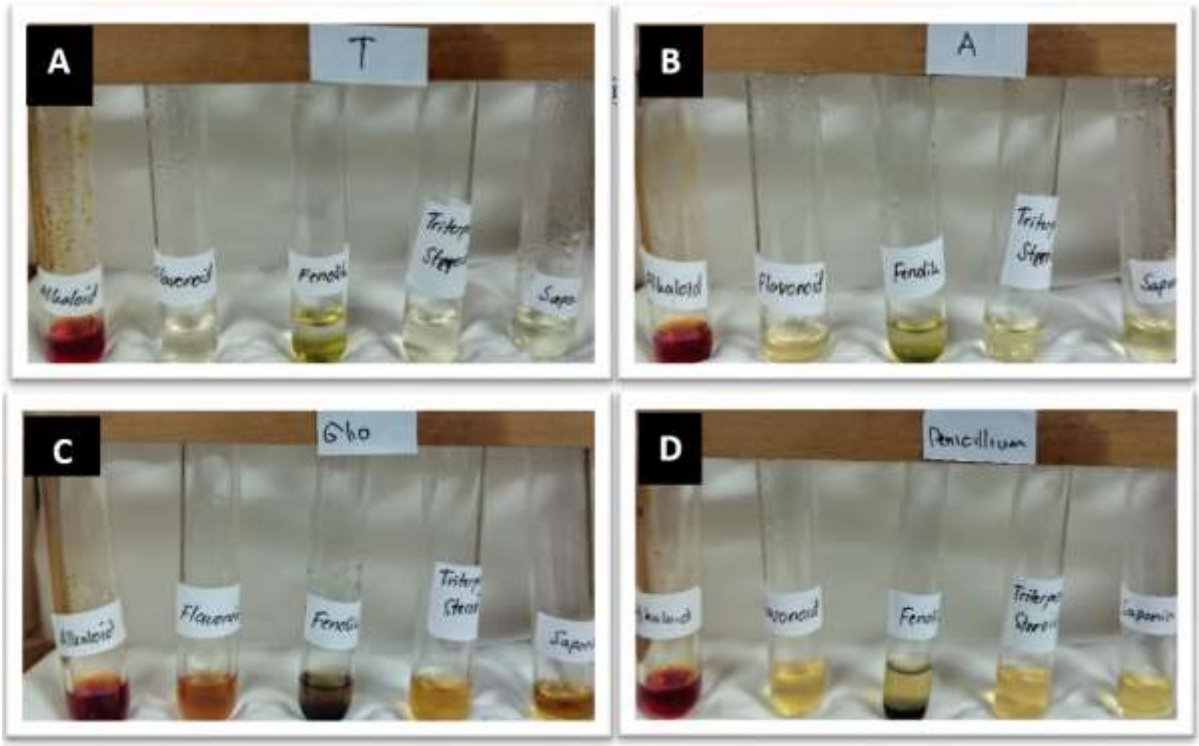

Figure 2. Phytochemical Test Results (**A.** *Trichoderma spp*., **B.** *Aspergillus flavus*, **C.** *Gliocladium sp*., **D.** *Penicilium sp*.).

A variety of compounds, including steroids, xanthones, phenols, isocoumarins, and their derivatives (e.g., perylene, quinones, furandiones, terpenoids, depsipeptides, and cytochalasins), have been identified in endophytic fungi (Nisa et al., 2015; Chua et al., 2022). The *A. flavus* extract was found to contain 20 active compounds, including fatty acids, fatty acid esters, tetrahydrofurans, and sterols (Sharaf et al., 2022).

3.2 HPLC Analysis Results

Chromatography is a physical separation technique for a mixture of chemical substances (analytes) based on the differences in migration/distribution of each component of the mixture. The mixture is separated in a stationary phase under the influence of a mobile phase, which can be either a gas or liquid. The

stationary phase can be either a liquid or solid.

HPLC analysis on 4 isolates, namely *Trichoderma spp*., *A. flavus*, *Gliocladium sp*. and Penicilium sp. showed the presence of 10-11 compounds based on the peaks produced in the chromatogram. This indicates that there are different types and amounts of compounds. The results of HPLC analysis on polyphenol compounds contained 19 compounds that triggered increased plant antioxidants and antibacterials (Hajlaoui et al., 2022).

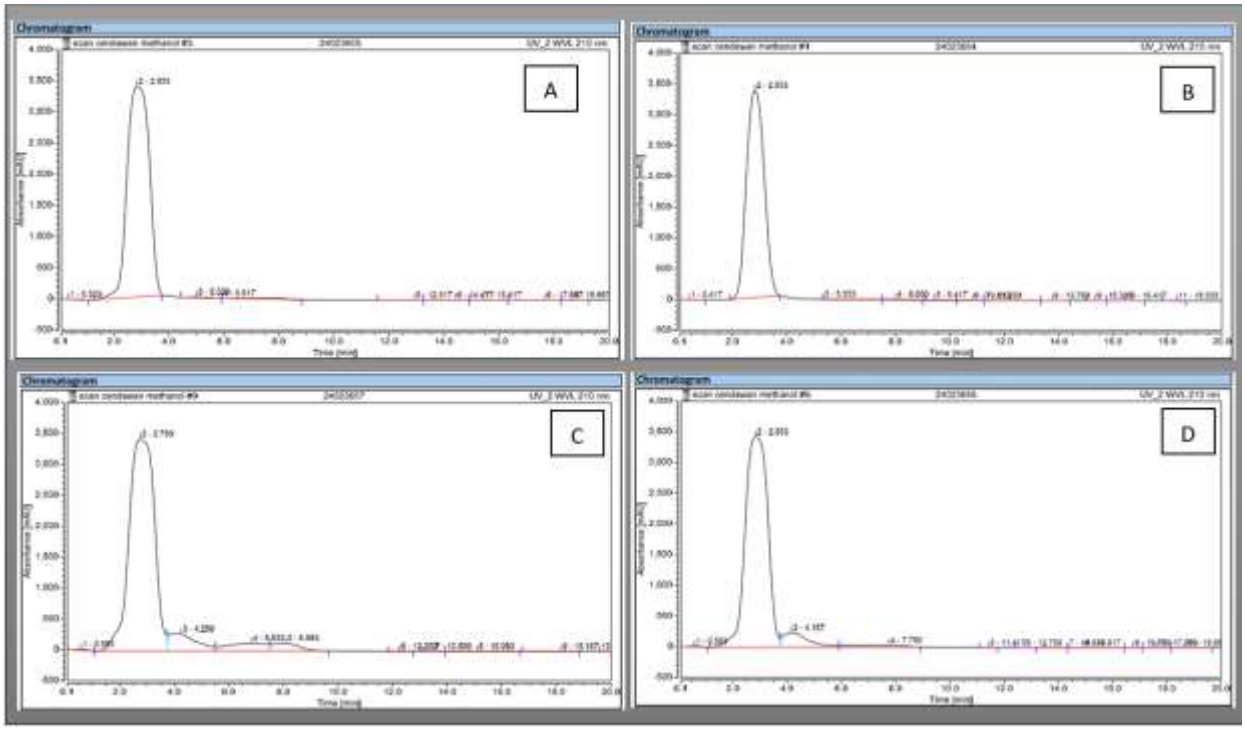

Figure 3. HPLC Analysis Results Chromatogram (**A.** *Trichoderma spp*., **B.** *A. flavus*, **C.** *Gliocladium sp*., **D.** *Penicilium sp*.).

The chromatographic analysis of the ethyl acetate extract derived from four distinct mushroom species reveals a unique curve for each, characterized by a prominent peak within the range of 3371.321 to 3430.109 mAU (million Absorbance Units). This distinctive curve is observed at retention times of 2.750 and 2.833 mAU * min (million Absorbance Units per minute). The consistency of this unique curve across the samples suggests the presence of similar compounds, specifically phenolic, alkaloid, or isoprenoid compounds. This finding aligns with the flavonoid components identified in the HPLC analysis of Trichoderma longibrachiatum, which includes quercetin 3-O-glucoside, quercetin 4'-O-glucoside, quercetin, and kaempferol (Abdelrahman et al., 2016).

Phytochemical assessments and HPLC results indicate the presence of antifungal compounds belonging to the phenolic, alkaloid, or isoprenoid classes. Furthermore, endophytic fungi associated with Azadirachta indica exhibit a range of compounds from the glucosinolate group, phenolic acids, aromatic aldehydes, diterpenoids, iridoids, and polyketides, as evidenced by HPLC analysis. Notable compounds identified include Glucobrassicin, Ferulic acid, 4-methoxybenzaldehyde, 12-Hydroxy-16-scalaren, 12-O-deacetyl-12-episcalarin, Ixoside, Citreodrimene F, and Cytosporin D, all of which demonstrate antifungal properties (Ujam Nonye Treasure et al., 2020).

The compounds from *Trichoderma spp*., *Penicillium sp*. and *A. flavus* have a retention time (Rt) of 2,833 minutes, while

those from *Gliocladium sp*. have an Rt of 2,750 minutes. The discrepancy in Rt values suggests the presence of a greater number of electronegative atoms in Trichoderma, Penicillium and *A. flavus* in comparison to the compounds derived from Gliocladium. The interaction between more polar compounds and the polar stationary phase is stronger, resulting in a shorter retention time in the normal phase column. Conversely, in the reverse phase column, less polar compounds are retained for a longer period, exhibiting a longer retention time. The presence of functional groups in the hydrocarbon structure affects the overall polarity of the molecule (McMurry, 2012). In *Penicillium sp*., fungal strains LBKURCC29 and LBKURCC30 exhibited disparate patterns in their respective extracts, potentially indicative of compositional and dominant peak pattern variations. However, the ethyl acetate extracts of both *Penicillium sp*. strains exhibited a similar range of compounds. The observed compounds were predominantly semi-polar, with a limited presence of polar compounds (Nurulita et al., 2020).

4. CONCLUSION

The research findings indicated that the metabolite profile of *Trichoderma spp*., *A. flavus*, *Gliocladium sp*. and *Penicillium sp*. exhibited qualitative characteristics consistent with the presence of alkaloids, phenolics and flavonoids. The HPLC analysis revealed the presence of 10-11 compounds, as indicated by the peaks observed in the chromatogram. However, the highest peak was identified as potentially belonging to the phenolic and alkaloid groups.

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