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Characterization of bioactive compounds by TLC, FTIR and UV-Vis spectrum of mycelia extract of *Trichoderma harzianum*

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Abstract

The genus *Trichoderma* includes significant agents for biological control. The mycelia extract obtained from the fermentation broth of *Trichoderma harzianum* was separated and the primary fractions were preliminary characterized using Thin Layer Chromatography (TLC), Fourier Transform Infrared Spectroscopy (FT-IR) and UV-visible Spectrophotometer (UV-vis). Consequently, we examined the spectral regions for characterization, assessed the interference of the prepared sample solutions regarding their resolution and evaluated the capacity to distinguish between *Trichoderma harzianum*. The active fraction is subjected to bioassay-guided purification through the thin-layer chromatography (TLC) technique following its extraction with five bioactive compounds. The genetic diversity observed was consistent with the findings from FTIR spectroscopy. The application of FTIR in the mid-infrared range of 449.75–3957.60 cm−1, using samples prepared with 50% alcohol facilitated the characterization and differentiation of *Trichoderma harzianum* based on their genetic diversity. The UV-vis spectrum exhibited distinct peaks, indicating the existence of secondary metabolites. From this study, methodology presents a rapid and efficient approach for the characterization and differentiation of *Trichoderma* samples. The TLC techniques are applicable to a variety of plant extracts and can also be utilized with different fungal species, whether (aerobic or anaerobic) as well as fungi can be used as test organisms if culture conditions are modified to fit the growth requirements of the species.

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Introduction

Trichoderma is recognized as a significant and extensively utilized fungal biocontrol agent. Fungi are significant pathogens in plants, capable of reducing agricultural yields by as much as 90% (Manimegalai *et al.,* 2011). The biological efficacy of this genus is attributed to the variety and range of metabolites it produces (Jogaiah *et al.,* 2018). Numerous biopesticides derived from various *Trichoderma* species are commercially accessible. Several strains of *Trichoderma* have demonstrated the ability to colonize plant roots thereby inducing systemic resistance (ISR) and preparing the host for a robust defensive response against subsequent pathogen attacks (Woo *et al.,* 2006; Reglinski *et al.,* 2012). Additionally, different species of *Trichoderma* have been investigated for their capacity to produce lipids (Boregowda *et al.,* 2020).

Biocontrol organisms promote resistance in host plants by triggering defense responses through the interaction with specific receptor signal molecules, which are conveyed by compounds referred to as elicitors. Elicitors, whether biotic or abiotic, attach to designated receptors and stimulate the expression of defense genes in host plants. Researches indicated that surfactants with a high degree of ethoxylation enhance the permeability of water-soluble compounds and increase the water content of the cuticle (Joshi *et al.,* 2019). Investigations into *Trichoderma* and the formulation of its membrane elicitors contribute to our understanding of host-pathogen interactions and the mechanisms associated with pathogen-associated molecular patterns (PAMPs).

The contemporary bioautography technique has reaffirmed the role of standard thin-layer chromatography (TLC) as an effective screening model for the separation of potentially significant target compounds. This approach facilitates bioactive screening, enables parallel comparisons among samples, and allows for semi-quantitative analysis. As one of the most straightforward methods available for this purpose, it can produce reproducible results with minimal equipment requirements (Choma and Grzelak, 2011). Furthermore, the inherent simplicity of bioautography lies in the ability to monitor each phase of the extraction, fractionation, and separation processes as the active compounds are purified. The use of thin-layer chromatography (TLC) combined with an in-situ bioassay along with the comparison of Rf values against reference chromatograms facilitates the identification of biologically active components within the intricate matrix (Smith *et al.,* 2023).

Fourier transform infrared spectroscopy (FTIR) serves as an alternative method for the characterization and identification of various microorganisms with a particular emphasis on fungi. This technique relies on obtaining a distinct spectral signature for each microorganism following cultivation under specific conditions. The resulting spectral signature primarily reflects the composition of proteins, lipids, nucleic acids and carbohydrates present in the biomass (Lecellier *et al.,* 2015). The vibrational spectra of different fungal species exhibit numerous common signals and encompass a wide array of variables, necessitating the integration of multivariate statistical analysis methods for effective comparison. The reproducibility of FTIR in fungal identification, it is essential to standardize every aspect of the analytical protocol including culture conditions, sample preparation and spectral acquisition parameters (Rony *et al.,* 2021).

UV constitutes a minor fraction of the total solar radiation that reaches the Earth's surface accounting for approximately 0.33% of the visible radiation; however, it exhibited a diverse array of biological effects (Raquel *et al.,* 2024). Fungi are particularly recognized for their ability to synthesize melanin under challenging conditions, functioning as a natural sunscreen that provides protection against ultraviolet radiation. The presence of melanin is essential for the survival of fungi in environments characterized by high levels of UV exposure (Elsayis *et al.,* 2022). For characterization of bioactive compounds, we performed TLC, FT-IR and UV-visible spectrum

analyses of mycelia extract from *Trichoderma harzianum*.

Materials and methods

Extraction method

The process of extracting secondary metabolites from *T. harzianum* was carried out utilizing a modified technique as described in (Wassima *et al.,* 2023). Following 14-days cultivation of the fungus in a liquid PDB medium under fermentation conditions, the mycelia were isolated from the broth via vacuum filtration. Subsequently, the mycelia were dried and subjected to extraction using Soxhlet apparatus with n-butanol and ethyl acetate for duration of 2 hours. The resulting extract was then concentrated using a rotary evaporator and stored for future investigation.

Thin layer chromatography

Sample application

Draw a line lightly with a pencil about 1.5 - 2.0 cm from the bottom. If the thin layer is too soft to draw a pencil line place a scale at the bottom and spot at a distance of 1 cm. The samples were spotted using capillary tubes at 1.5 cm distance between them. For TLC prepared, the sample was applied as a band across the layer rather than as a spot.

Solvent preparation

Saponins

The saponins were separated by using chloroform, glacial acetic acid, methanol and (64:34:12:8) solvent mixture.

Phenols

The phenols were separated by using chloroform and methanol (27:3:8) solvent mixture.

Sterols

The sterols were separated by using acetone, glacial acetic acid, methanol and water (64: 34: 12: 8) solvent mixture.

Flavonoids

Flavonoids was separated by using butanol, acetic acid and water (4:1:5) solvent mixture.

Amino acids

Amino acid were separated by using ethanol, water, butanol, acetic acid (9:6:8:8) solvent mixture.

Plate development

The chromatographic tank was filled with developing solvent to a depth of 1.5 cm and equilibrated for about 5hrs. The thin layer plate was placed gently in the tank and allowed to stand for about 60 min. Care was taken so that the spots did not touch the solvent directly, and the capillary action caused the solvent to ascend as in paper chromatography and the separation of compounds took place. As the solvents front reached about 1.2 cm from the top of the plate, the plate was removed, solvent front was marked with a pencil immediately and allowed to air dry placing the plate upside down.

Component detection

Several methods were available to detect the separated compounds. Different types of spraying reagents were used to detect different components.

Saponins

The presence of saponin in the developed chromatograms was detected by iodine vapours. A positive reaction indicated by the formation of yellow colour spot.

Phenols

The presence of phenol in the developed chromatograms was detected by spraying the folin-ciocalteu's reagent. The plates were then heated at 80°C for 10 min. A positive reaction was indicated by the formation of blue colour spot.

Sterols

The presence of sterol in the developed chromatogram was detected by spraying the folin – ciocalteu's reagent. The plates were then reacted at 80°C for 10 min. A positive reaction was indicated by the formation of bluecolour spot.

Flavonoids

The presence of flavonoid in the developed chromatogram was detected by the formation of yellow colour spot.

Amino acids

Spray with 0.1% pink or purple hydrin in acetone and heat the plates for 15 min at 100 to 110°C. The formation of pink was indicated the presence of amino acid.

Determination of Rf value

The R_f values of the various compounds were calculated using the following formula. After incubation period, the results were observed and the diameter of the inhibition zone was measured around the isolates.

R_f={Distance travelled by solute (measured to center of the spot)}/Distance travelled by solvent

FT-IR analysis

The functional groups of bioactive compounds were carried out by Fourier transform infrared spectroscopy (FTIR). A small quantity of solid sample product was collected, ground adequately and was pressed to tablet by KBr method. Infrared spectrum of sample product was recorded in the range of 4000-400 cm^{-1} using Perking Elmer Rx1 Infrared scanner.

UV-visible spectrophotometer

The fractional sample was dissolved in acetone nitrate and then detected its UV absorbance values with Lambda 35 ultra violet scanner.

Results and discussion

The flavonoids and phenolic compounds extracts are correlated with the proliferation of *Trichoderma* species (Surekha *et al.,* 2013). The accumulation of phenolic compounds in response to *Trichoderma* has been associated with biochemical mechanisms that enhance plant defense against diseases. Additionally, the heightened production of phenols and flavonoids directly influences antioxidant activity by functioning as free radical scavengers and aiding in the formation of cell walls thereby providing protection to plants against biotic stressors (Hashem *et al.,* 2016). Flavonoids also act as intrinsic regulators of auxin transport and play a role in developmental processes (Mona *et al.,* 2017). Furthermore, polyphenolic compounds are acknowledged for their pharmacological benefits, which include antioxidative, hepatoprotective, antibacterial, anti-inflammatory, anticancer and potential antiviral effects (Wassima *et al.,* 2023). Tannins and alkaloids, as polyphenolic substances, exhibit astringent, diuretic, anti-inflammatory, antiseptic, antioxidant, and hemostatic properties, and they are also utilized in the treatment of gastric and duodenal cancers. The presence of alkaloids and tannins in *Trichoderma* species was confirmed by Omomowo *et al.* (2020), while reported the existence of tannins but not alkaloids in *T. harzianum*. According to Sriwati *et al.* (2019), both *T. harzianum* and *T. virens* contain only alkaloid compounds.

Table 1. Isolation of bioactive compounds from *T. harzianum* by TLC

Bioactive compounds	Spot observation	Results	R_f value
Flavonoids	Yellow	Present	0.7 ± 0.1
Phenols	Blue	Present	0.8 ± 0.1
Saponins	Yellow	Present	0.9 ± 0.1
Amino acids	Pink	Present	0.6 ± 0.2
Sterols	Dark blue	Present	0.8 ± 0.1

The values are expressed in terms of (Mean ± Standard deviation)

From an ecological perspective, the accumulation of alkaloids represents a significant chemical defense mechanism that plants employ to cope with environmental pressures, including those posed by endophytes, pathogens and herbivores.

In the present study, the mycelium of *T. harzianum* was harvested from PDA broth. The TLC of the extract of *T. harzianum* was identified the bioactive compounds such as saponins, flavonoids, sterols, amino acids and phenols. The R_f value of bioactive compounds was recorded in the given by (Table 1 and Fig. 1). The detection of multiple bioactive biomolecules in *T. harzianum* was accomplished through thin-layer chromatography. Application of various reagents resulted in a diverse array of colors, indicating the presence of different compounds, particularly carbonylated structures. The TLC profile reveals intriguing chemical compositions that contain

bioactive compounds associated with abiotic activities found in the ethyl acetate and n-butanol extracts.

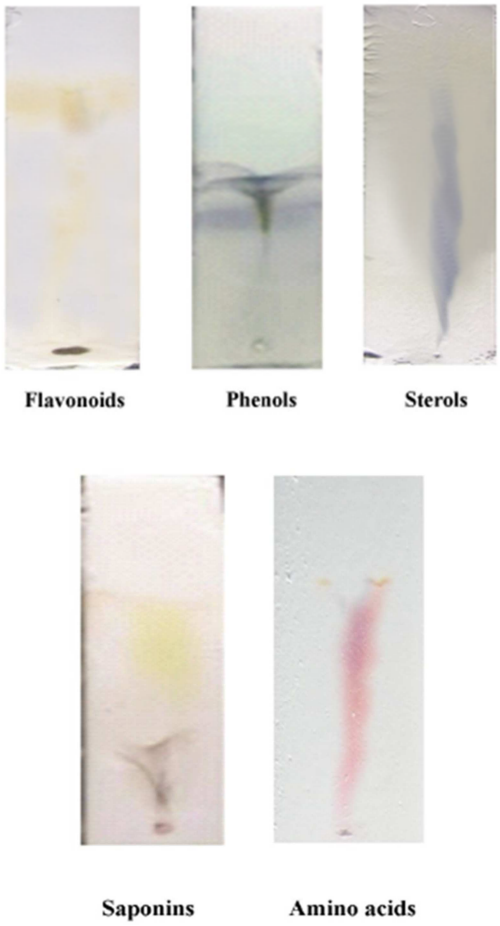

Fig. 1. Separation of bioactive compounds from *T. harzianum* by using TLC

T. harzianum is most frequently saprobic soil inhabitant belongs to ascomycota. Fungus cell wall comprised of chitin-a β-(1,4)-linked N-acetylglucosamine and β-(1,3) glucan, both are together embedded in the amorphous fraction of α-glucans, galactomannans and other carbohydrate polymers (Shoaib *et al.,* 2013). All six polysaccharides demonstrated a pronounced vibrational peak within the range of 3200–3400 cm^{-1}, which corresponds to the O–H stretching vibration associated with hydrogen bonding. Furthermore, these polysaccharides exhibited C–H vibrations at 2920–2940 cm^{-1}, indicative of the presence of acyclic saturated hydrocarbons (Kandasamy *et al.,* 2021). In the present study, the chemical functional group characteristics of the purified compounds were determined by FTIR analysis (Fig. 2). *T. harzianum* revealed the broad band of highest peak were recorded at the Sulfur compounds, S=O stretching vibrations, sulfoxidis (1057.04 cm^{-1}) followed by Amines, secondary, N-H stretching vibrations, Imines (=N-H); one band (3412.10 cm^{-1}) and Hydrocarbon chromophore, C-H Stretching, alkane (2953.68 cm^{-1}). The results obtained indicated the presence of the following functional groups: sulfur, amines and alkane-stretching compounds.

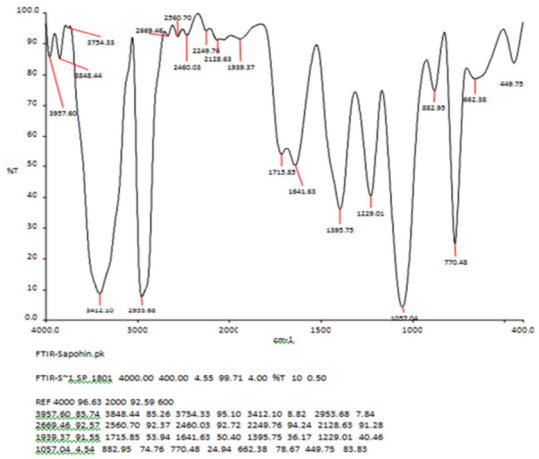

Fig. 2. FT - IR spectrum derived from TLC of the mycelial extract of *T. harzianum*

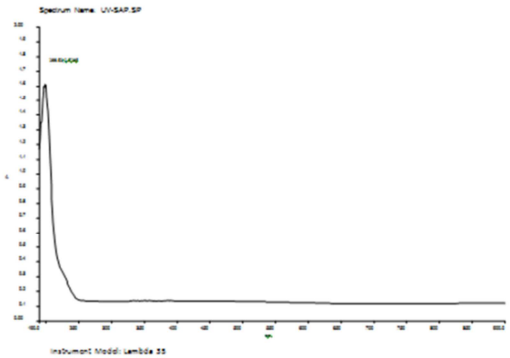

Fig. 3. UV spectrum of derived from TLC of the mycelial extract of *T. harzianum*

The findings from UV-Visible spectroscopy indicated the existence of various compounds, each exhibiting distinct peaks corresponding to their absorption maxima. The PDB extracted with chloroform displayed absorption peaks at wavelengths of 235,

246 and 271 nm. In contrast, the ethyl acetate extracts revealed absorption peaks within the range of 248 nm to 278 nm. Notably, these peaks do not suggest the presence of pigments. Only substances that absorb visible light (400 to 700 nm) are likely to exhibit color (Narendrababu and Shishupala, 2017). In the present study, the findings from UV-Visible spectroscopy indicated the existence of multiple compounds, each exhibiting distinct peaks corresponding to their absorption maxima. The ultraviolet spectrum exhibited a pronounced absorption peak at 199.5 nm corresponding to the carbonyl (C=O) functional group of the ketone (Fig. 3).

Conclusion

It can be concluded that, the mycelia extract obtained from the *Trichoderma harzianum* were preliminary characterized using TLC, FT-IR and (UV-vis) Spectrophotometer. A variety of TLC, FT-IR and (UV-vis) spectrum of bioactive compounds in *T. harzianum* extracts that provide beneficial effects. These results revealed that the fungus *T. harzianum* could be a potential source of polysaccharides with unique structures and bioactivity. *T. harzianum* biosynthesizes biopotent products and has varied nutritional, industrial and medical applications. *T. harzianum* possesses the advantage of large-scale production of diverse bioactive metabolites and potential drug leads. They are widely used in agriculture as biofungicides and bioremediation agents. The current research demonstrated the scientific endorsement and evidence supporting the application of *Trichoderma harzianum* as an optimal eco-friendly and cost-effective alternative fungicide for managing all fungal pathogens in place of utilizing harmful chemicals.

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