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Structural And Phytochemical Characterization of Bioactive Components of the Endophytic Fungi (*Trichoderma harzianum*) Extracts

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ABSTRACT: This research aims to investigate the antioxidant activity of the crude extract of endophytic fungi (*T. harzianum*) and to test for the presence of phytochemicals. Fungi isolated from the leaf and stem of endemic medicinal plant were extracted with ethyl acetate. The fungi extract was then investigated for its phytochemicals, antioxidants and active compounds through LC-MS. Some of the phytochemicals present in abundance include saponins, alkaloids, flavonoids, phenols, and steroids, with tanins and cardiac glycosides also present in reasonable amounts. Those compounds identified by LC-MS with antioxidant properties include inosine diphosphate, vigabatrin, isoamylnitrite, proline, trihexyphenidyl-N-oxide, N-methyl gabapentin, penbutolol, dextromoramide M2, solanidine, aceclidine, desethyleneciproflaxin, sapropterin and kinetin.

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Microorganisms have been the other major source of new bioactive metabolites, and almost 75% of the current antimicrobials originate from them. (Segaran and Sathiavelu, 2019) These microorganisms are isolated from the natural habitat, mostly from the soil. There is evidence that only a fraction of microbes can be isolated using the current isolation and culture techniques, and less than 1% of isolated bacteria and 5% fungal species could be characterized. There is a need to explore alternative microbial habitats for isolating novel microbes with chemical and functional diversity.(Peters *et al*., 2020) The necessity of new therapeutic and medically useful compounds continues to increase in demand to resolve complexities facing increasing antibiotic resistance, which is at a great lag. (Rahman *et al*., 2020) Endophytes from an untapped diverse habitat are a significant source of new bioactive molecules that have attracted the attention of many investigators and may perhaps provide solutions to several unanswered questions. Endophytes are microorganisms residing in plant tissues without causing any apparent symptoms.(Ahmad *et al*., 2020) They possess the advantage of large-scale production of diverse bioactive metabolites and potential drug leads, which is not always possible in plants. They are widely used in agriculture as biofungicides and bioremediation agents. They protect the host plant for their entire life cycle and have the ability to act as biocontrol agents. (Adeleke and Babalola, 2021; Segaran and Sathiavelu, 2019; Sheeba *et al.* 2019) Endophytes protect the host plant through antibiosis, parasitism and competition mechanisms in the biocontrol process. The improper and excessive use of agrochemicals makes phytopathogens insensitive and leads to the development of resistant fungal pathogens.(Segaran and Sathiavelu, 2019) Chemical fungicides are expensive and have many negative impacts on the environment. The use of endophytes as biocontrol agents is effective in controlling plant disease and achieving sustainable agriculture. Endophytes have antagonistic activity against disease-causing phytopathogens and are capable of producing antimicrobial, insecticidal, antioxidant,

antitumor and antiviral metabolites. (Peters *et al*., 2020) A large number of novel natural products with various biological activities have been isolated from endophytes, including alkaloids such as perfumoid, phomoenamide, joxysporidinone, alantrypinene, alantryleunone, (−)-4,6′-anhydrooxysporidinone and (−)-6-deoxyoxyoxysporidinone, which are the few alkaloid compounds of endophytic fungi. Nidurufin, sterigmatocystin, averantin, 11a-methoxycurvularin 4,11b-methoxycurvularin, tenellone H, phomopene and 1-chloro-2,4-dihydroxy-5-methoxy-7-methylanthraquinone are endophytic fungal compounds reported to have cytotoxic activity in the past decade. Many of these metabolites act as potential therapeutic agents against cancer and infectious diseases.(Harwoko *et al*., 2021) Bioactive compounds classified as alkaloids, terpenoids, steroids, quinones, phenylpropanoids, phenols and lactones.

MATERIALS AND METHODS

Materials: Plant Endophytes, Dichloromethane, ethanol, n-hexane, distilled water, ethyl acetate and methanol. The materials as well as the solvents were obtained commercially and are of analytical grade and therefore used as obtained

Collection and Treatment of Samples: Leaf and stem samples of endemic medicinal plant samples were washed thoroughly under running tap water to remove debris and washed with double distilled deionized water. The samples were thereafter cut into small pieces and sterilized by sequential washes in different concentrations of sterilizing agent **(**70% ethanol, 2% sodium hypochlorite, 70% alcohol), rinsed with distilled water and allowed to air dry under sterile conditions. The efficiency of surface sterilizations was checked by the imprint (Biological) method (Schulz *et al.* 1993), and samples washed with double-distilled deionized water were inoculated in broth media as a control to check growth in liquid medium. Most endophytes generally start sporulation after a few weeks either in darkness or in daylight.

Isolation of Endophytes: The dissected tissues were inoculated onto potato dextrose agar (PDA) with 50 mg/L chloramphenicol to avoid bacterial contamination. Endophytic fungi usually begin to produce hyphal filaments after 5–6 days of incubation at 30 °C. The hyphal tips that appeared were carefully transferred to new sterile PDA plates (Chiranjeevi *et al*., 2021) to obtain pure cultures of the growing fungi.

Preparation of Fungal Extracts: The procedure of Bhardwaj *et al.* (2015) was adopted in the preparation of the fungal extract with slight modifications. Briefly, pure endophytic fungal cultures on potato dextrose agar (PDA) media, normally in the log phase, were transferred into a 1 L Erlenmeyer flask containing 300 mL of potato dextrose broth (PDB) media. The endophytic fungal cultures were incubated at room temperature for three weeks. After fermentation, the media and fungal matrix were separated by filtration to extract extracellular and intracellular metabolites. Ethyl acetate (250 mL) was added to each conical flask, mixed thoroughly and kept overnight to ensure that the fungal cells died. The mixture was placed in an ultraturrax for 10 min to disrupt the cells with the upper layer of solvent containing the extracted compounds separated using a separating funnel. The extract was concentrated under vacuum to yield the crude metabolite. (Bharadwaj *et al*., 2020)

DPPH Radical Scavenging Activity: The free radical scavenging capacity of the fungal extracts was determined using the DPPH (1,1-diphenyl-2-picrylhydrazyl) assay as described by Braca et al. (2001) with slight modification. The tested sample was mixed with 95% methanol to prepare the stock solution (5 mg/mL). One milliliter of DPPH (0.25 mM) in methanol was placed in tubes, and a 2.0 mL solution of various concentrations of plant extract was added. The reaction mixture was then allowed to stand at room temperature in a dark chamber for 30 mins. The change in color from deep violet to light yellow was then measured at 518 nm on a spectrophotometer. Ascorbic acid was used as a reference standard and dissolved in distilled water to make the stock solution with the same concentration (5 mg/mL). Ninety-five percent methanol served as blank (Braca *et al.*, 2001). The percentage scavenging activity (SA) of the DPPH free radical was measured using equation 1.

$$\% \text{ SA} = \frac{\text{A}_C - \text{A}_S}{\text{A}_C} \; x \; 100 \quad \ldots\ldots\ldots 1$$

Where Ac = absorbance of control; As = absorbance of sample

*Qualitative Phytochemical Screening of T. Harzianum (TH) Crude Extracts***:** Preliminary phytochemical analysis of the crude extracts of fungi was carried out for the presence of the following metabolites: alkaloids, flavonoids, tannins, phenols, saponins, terpenoids and carbohydrates using standard methods with modification as reported by Devi *et al*. 2012 and Bhardwaj *et al*. 2015.

The results were graded as present (+) or nil (-) based on the intensity of the color produced from the reactions (Bharadwaj *et al*., 2020; Devi *et al*., 2012; Sheeba *et al.,* 2019).

Structural Characterization of the Extract (LC/MS): Liquid chromatography-mass spectrometry (LC/MS) analysis was carried out at the Chemical Purification Analysis and Screening Core Facility, University of South Florida, USA.

RESULTS AND DISCUSSIONS

Phytochemical analysis was carried out on the extract of *T. harzianum*. The isolated extract showed significant phytochemical constituents. In this analysis, terpenoids, alkaloids, proteins, amino acids, steroids, phenols, flavonoids, cardiac glycosides, tannins and saponins were screened. Phytochemicals such as saponins, tannins, alkaloids, flavonoids, phenols, steroids and cardiac glycosides were present in large amounts in the *T. harzianum* extract (Table 1).

Table 1: Results of the Phytochemical Test Ethyl Acetate extract of *Trichoderma harzianum*

S/N	Test	Extract
1	Saponins	+++
2	Tannins	++
3	Alkaloid	+++
4	Amino acid	-
5	Proteins	-
6	Flavonoid	+++
7	Terpenes	_
8	Phenols	+++
9	Steroid	+++
10	Cardiac glycosides	++

LC/MS analysis was carried out on the ethyl acetate extract of *T. harzianum.* The LC/MS data were deconvoluted using ESI software, and the measured mass spectra were matched to entries in the compound library. According to these criteria, over 50 antifungal metabolites were identified from the *T. harzianum* sample. The volatile and nonvolatile compounds detected in the cultured sample constitute members of the compound classes of alkanes, alcohols, ketones, pyrones, furans, monoterpenes, amino acids, peptides and sesquiterpenes. The compositions of all compounds are presented in Table 2, and numerous minor peaks were found. Primarily, hydrocarbons, fatty acids, alcohols and benzene derivatives were identified from the *T. harzianum* extract. Some of the compounds identified have been reported to show antioxidant properties, and research has shown that these compounds have been used in the treatment of ailments (Ahmad *et al*., 2020). Those compounds identified with antioxidant properties include inosine diphosphate, vigabatrin, isoamylnitrite, proline, trihexyphenidyl-N-oxide, N-methyl gabapentin, penbutolol, dextromoramide M2, solanidine, aceclidine, desethyleneciproflaxin, sapropterin and kinetin. The structures of some of the compounds identified are presented in Figure 1.

Table 2: Volatile and Non-Volatile LC-MS Analysis of Ethyl Acetate extract of *Trichoderma Harzianum*

Retention Time	Mass	Name	Molecular Formula
1.238	323.1216	N-Acetyl-8-O-methylNeuraminic acid	C_{12} H_{21} NO_9
1.238	341.1332	His Ala Asp	$C_{13}H_{19}N_5O_6$
1.25	296.1625	Lactone of PGF-MUM	$H_{24}O_5$
1.265	314.1832	Pergolide	$C_{19}H_{26}N_2S$
1.266	325.1374	Acarbose (M8)	$C_{12}H_{23}NO_9$
1.27	219.0742	O-Succinyl-L-homoserine	$C_8H_{13}NO_6$
1.277	249.0853	S-Acetyl dihydrolipoamide	$ClOHIYNO_2S_2$
1.302	276.0965	fluorophenyl) butyric acid	$H_{14}F_2O_2$
1.303	147.054	O-Acetyl serine	$C_5H_9NO_4$
1.303	162.0532	2-Hydroxyadipic acid	C_6HlCO_5
1.304	258.0846	3-Methyluridine	$ClOH_{14}N_2O_6$
1.305	144.0425	Alylmalonic acid	$C_6H_8O_4$
1.305	108.0213	Quinone	$C_6H_4O_2$
1.308	255.0987	Nicotinamide riboside	$C_{11}H_{15}N_2O_5$
1.31	126.0318	Hydroxy hydroquinone	$C_6H_6O_3$
1.325	251.1009	Muramic acid	$C_9H_{17}NO_7$
1.329	215.0797	KINETIN	$ClOH_9N_5O$
1.342	241.1185	Sapropterin	C_9NSO_3
1.4	305.117	Desethyleneciprofloxacin	$C_{15}H_{16}FN_3O_3$
1.413	257.103		$C_5H_9NO_2$
1.419	115.0635	Proline	
1.423	117.0793	isoamyl nitrite	C_5NO_2
1.441	107.0372	Nitroso benzene	C_6H_5NO
1.447	137.0477	p-Aminobenzoic acid	C_7H_7NCI
1.486	103.1	2-Amino-3-methyl-1-butane	$C_5H_{13}NO$
1.541	129.0793	Vigabatrin	$C_6H_{11}NO_2$
1.687	107.0372	Nitrosobenzene	C_6H_5NO
1.751	129.0791	Vigabatrin	C_6 NO_2

2.29	129.0427	Pyroglutamic acid	$C_5H_7NO_3$
5.381	268.1052	N-Seryltyrosine	$C_{12}H_{16}N_2O_5$
6.387	154.063	3-hydroxy-phenylglycol	C_8O_3

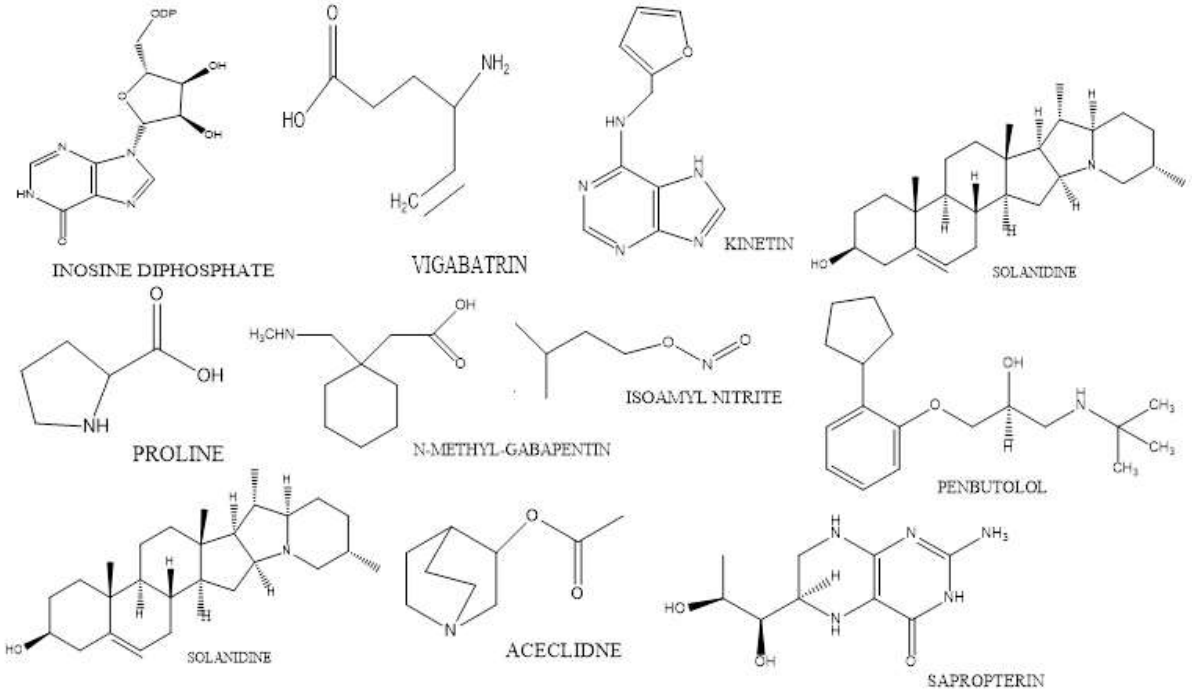

Fig 1: Structures of Volatile and Nonvolatile Compounds Identified in the Ethyl Acetate Extract of *Trichoderma Harzianum* with Antioxidant Properties

Antioxidant Activity of Trichoderma Harzianum: Table 4 presents the percentage DPPH inhibition of the ethyl acetate extract of *Trichoderma Harzianum*. The Table revealed that the antioxidant activities of the extract are concentration dependent. The activities of the extract decrease initially from 0.01 to 0.03 nM and increase steadily with subsequent increase in concentration. The highest and lowest activities of the extract were obtained at 0.01 and 0.05 nM respectively (Table 4). In comparison with the standard, ascorbic acid, it is noted that the extract recorded higher activities than the standard (Table 4).

Table 4: UV spectroscopy results of Trichoderma Harzianum

Concentration (mM)	TH Absorbance	Ascorbic acid
0.01	0.013478	0.013157
0.02	0.008154	0.008144
0.03	0.006986	0.003772
0.04	0.029253	0.034052
0.05	0.034276	0.004

This result shows that at a concentration of 0.05 mM, the antioxidant activity of *Trichoderma Harzianum* was high. From Figure 1, it is evident that the antioxidant activities of *Trichoderma* harzanium were far higher than that of the standard (ascorbic acid) at a concentration of 0.05 mM. The high activity can be linked to the presence of numerous volatile and nonvolatile metabolites, as shown in the phytochemical and LC-MS analyses (Vitti, *et al*., 2016; Lombardi, *et al*., 2020; Saravanakumar, *et al*., 2021). These compounds have been reported to be potent against various microorganisms.

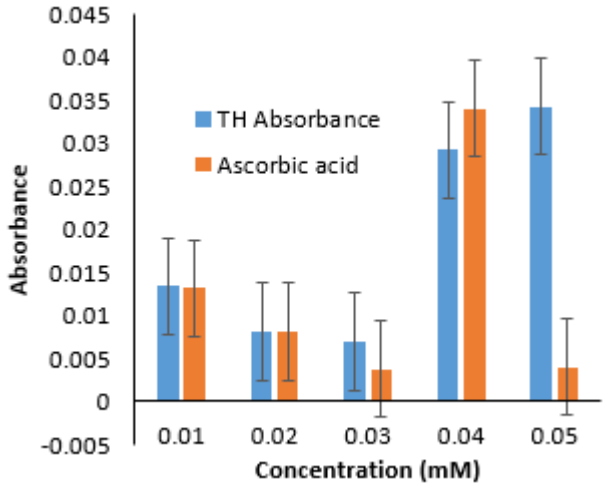

Fig 2: Graphical Representation of the Antioxidant Properties of *T. Herzanium* Extract

Conclusion: Endophytic fungal extracts isolated from *Trichoderma harzianum*, endemic plants of almost all climatic zones, exhibit strong antioxidant activity (significantly higher than the ascorbic acid used as the standard at 0.05 mM) due to the bioactive natural

compounds present therein. Volatile and nonvolatile compounds detected by LC-MS analysis provide information for developing new pharmacological agents that can act as antioxidants. These active compounds can be used as sources of natural antioxidants and replace extraction from actual plants.

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