Pharmaceutical and Agricultural Significance of *Trichoderma harzianum* Metabolites

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Abstract- *Currently, diseases are mainly managed by chemical pesticides. The use of these chemical pesticides causes environmental problems because they do not undergo biodegradation or degrade very slowly. Therefore, minimizing the use of pesticides has gained importance. To achieve this goal, biological control methods can be effectively combined with other disease control methods. Trichoderma sp. Soil-borne filamentous fungi are effective biocontrol agents against plant pathogens. The present investigation isolated antagonistic organisms and determined the antifungal activity of the antagonistic organisms in HPLC fractions of the mycelial extract. The antifungal activity of HPLC fractions of the mycelial extract were collected in separate vials; thereby, these HPLC fractions of the mycelial extract were analyzed by FT-IR, and many antibiotic compounds were identified. The FT-IR results were then confirmed by GC-MS.*

Keywords- GC-MS, Biocontrol, Trichoderma, FT-IR, HPLC.

I. INTRODUCTION

In recent years, Trichoderma spp. have been widely used in agriculture as biocontrol agents and inoculates to promote plant growth. They are involved in essential activities that ensure the stability and productivity of both agricultural and natural ecosystems. The potential of Trichoderma spp. to produce many volatile (e.g., pyrones, sesquiterpenes) and non-volatile secondary metabolites (e.g., peptaibols) has been reviewed by Reino et al. (2007). Volatile secondary metabolites have been shown to play a key role in the mycoparasitism of Trichoderma and its interaction with plants (Vinale et al., 2008). It is clear that the properties of Trichoderma to inhibit the growth of other fungi are likely due to the combined action of cell-wall degrading enzymes along with the ability of Trichoderma to produce different secondary metabolites. In some countries, there are several commercial formulations available to prevent diseases in crops as well as in economically important forest trees.

1.1 Pharmacological/Biological importance of different groups of secondary metabolites from *Trichoderma:*

Trichoderma spp, have also been reported to produce a plethora of secondary metabolites showing antimicrobial activity (Vinale et al., 2008). The chemical composition of these compounds depends on the strains, and they may be classified as volatile, water-soluble, or water-insoluble compounds (Ghisalberti & Sivasithamparam, 1991). The first demonstration of induced resistance was reported in 1997 (Bigirimana), who described the acquisition of resistance of bean plants towards *Botrytis cinerea* and *Colletotrichum lindemuthianum* after inoculation of the root with the strain T-39 of *Trichoderma harzianum* (Yedidia, Benhamou & Chet, 1999).

Species of Trichoderma have been demonstrated in vitro to act against fungal plant pathogens by producing diffusible volatile antibiotics. Vey, Hoagland & Butt (2001) reported that there are large varieties of volatile secondary metabolites produced by Trichoderma such as ethylene, hydrogen cyanide, aldehydes, and ketones which play an important role in controlling the plant pathogens (Bhagat Someshwar et al., 2014). Many species of Trichoderma are useful biocontrol organisms known to enhance crop yields and control soil-borne pathogens when added to soils. Our recent studies demonstrate that T. harzianum may be able to control the soil-borne pathogen. The aims of this study were to demonstrate that Trichoderma VOCs are a major factor in plant growth promotion and biocontrol. Finally, we characterized the volatile compounds identified by using gas chromatography–mass spectrometry (GC–MS) analysis. In addition, the identification of their biological activities such as anticancer and antiviral activities had been evaluated.

II. MATERIALS AND METHODS

2.1 Isolation and Identification of Antagonist organisms:

Fungal species were isolated from sugarcane rhizosphere soil samples by using potato dextrose agar (PDA) medium. Samples were inoculated over plates by multiple tube dilution technique (MTDT) and the plates were inoculated at 26˚C for 4 days. The fungal colonies were picked up and purified by streaking and incubated at 26˚C for 7-8 days. Conidia forming fungal bodies were selected and based on microscopic observation were identified to be *Trichoderma harzianum* Rifi**.,** *T. viride* Pers., *Aspergillus niger* Tiegh., *A. flavus* Links, *A. fumigates* Fresen., and *A. sulphureus.* Then the cultures were maintained on PDA slants.

Interaction between antagonistic fungi and pathogenic fungi were determined by the method of Dennis &Webster (1971a). The antagonism against used the pathogen (*Colletorichum falcatum*) was studied by dual culture technique. The isolated antagonistic organisms were cultured in nutrient broth, extracted using HPLC grade solvents and analyzed using HPLC. The HPLC analyses of mycelia extract showed few major and peaks. Antifungal activity of HPLC fractions of the mycelial extract were determined by collects the fractions in separate vials. The HPLC fractions of the mycelial extract were identified by FT-IR and the results of FT-IR were compared and confirmed by GC-MS

2.2 HPLC analysis:

From the mycelial extract, 5 µl was injected into the rp-18 octadeclysilyl silica (DDS) column (25 x 1 cm, i.d.) with LC-UV detector (P 3000, Analytical Technologies Limited) and monitored at 254 nm. The flow rate was adjusted to 1.5 µl min^{-1}. The fractionated samples were collected in separate vials.

2.3 Fourier Transform Infrared (FTIR) analysis:

The functional analysis of groups of phyto compounds were carried out by Fourier Transform Infrared Spectroscopy (FTIR). FTIR analysis was performed using Shimadzu 19WL FTIR spectrophotometer available in the common instrumentation centre, Government Arts College, Ariyalur. KBr pellets were made by accurately weighing 2 mg of dried and 300 mg of dried KBr and pressing the mixture under vacuum at 10 for 10 min. Measured wavelength range of 4000-400cm^{-1}. Data collection and processing was performed by GRAMS/386 version 3.02 software.

2.4 Gas chromatography – Mass Spectrum analysis of the culture filtrate:

2.4.1 Extraction of antifungal compounds:

The fungus which showed promising activity against the pathogen was cultured in liquid potato dextrose medium at 24°C in darkness for three weeks. After incubation, the culture was filtered twice through Whatman No.1 filter paper and Seitz filter (G.5). To 100 ml of culture filtrate, 10ml of ethyl acetate was added in a separation funnel (250ml), shaken well for 3 min. and the solvent and aqueous layer were separated. The acetonitrile layer of the culture filtrate was used for further analysis.

2.4.2 Gas chromatography – Mass Spectrometry (GC-MS):

Volatile components were identified by GC-MS using DB 5 - MS capillary standard non-polar column thermo GC - TRACE ULTRA ver: 5.0, THERMO MS DSQ II, with dimensions of 30 mts, ID: 0.25 mm, film: 0.25. The db 5 - ms capillary standard non-polar column detector was used. The carrier gas flow rate was 1 ml per min, split 10:1, and injected volumes were 2µl. The column temperature was maintained initially at 220°C for 2 min (hold), followed by increases up to 300°C at the rate of 5°/min-4 min (hold). The injector temperature was 255 °C, and this temperature was held constant for 36 min. Electron impact (EI) mass scan (m/z) was recorded in the 500 a MU range. Using computer searches on the NIST Ver.2.1 MS data library and comparing the spectrum obtained through GC-MS,the compounds present in the crude sample were identified. This analysis was carried out with the help of the lab technicians in SITRA LAB, Coimbatore.

III. RESULT AND DISCUSSION

3.1 Analysis of secondary metabolites through FT-IR and GC-MS:

3.1.1 FT-IR analysis of HPLC fractions:

The FT-IR analysis of the HPLC purified components of the culture filtrate of antagonistic soil fungi such as *Trichoderma* spp. (*T. harzianum, T. viride*) and *Aspergillus* spp. (*A. niger, A. flavus, A. fumigatus* and *A. sulphureus*) was carried out using Shimadzu FT-IR 1S with an inbuilt library. The analysis revealed the presence of humic acid, ethyl octoate, lineyl acetate,

benzoic acid, pathalic acid, barbituric acid, formic acid, N-methyl-2-pyrolodine, formide, sulfosalicylic acid, adipic acid, tetroconazole, benslide, T-propanol-4, T-acetaldehyde-4, sulfomic acid, tetrahydrogen furan, artesunate, L-valine, Isoamylalcohol, kolin, bleomycin hydrochloride, polyvinylpyrolidene-4, T-carboxymethyl T-butanol, margarine, oleic acid, bismuth vanadate, tetraconazole, alginic acid, sodium thiosulfate, decalin, cyclohexon, molinate, olive oil, lauric acid. Carboxylic acid, ethyl vanillin-4, iminoctadine albesilate, ascorbic acid, 2-glucoside-4, oleamide, allyl hexanoate-4 and p-dimethylamino benzylidenerhodanine.

This study indicated that several compounds that have potential antimicrobial activity and plant growth promoting activity are present in *Trichoderma* spp. and *Aspergillus* spp. The plant growth promoting compounds include sulfosalicylic acid, N-methyl-2-pyrolodine, nicotinamide and humic acid. Some or all the components may be responsible for the antagonistic property of the *Trichoderma* spp. and *Aspergillus* spp. The results of the present study were in conformity with the earlier report by Prakash et al., Niveditha & Tejaswini (2010).

3.1.2 GC–MS Analysis:

We investigate the volatile metabolites excreted by the cultured only from *T. harzianum*, the potential antagonist organism isolated in the present study. The GC-MS data was de-convoluted using the NIST software, and the measured mass spectra were matched to entries in the compound library. When the extract of acetonitrile culture filtrate of *T. harzianum*was subjected to GC-MS analysis to find out the components produced by the fungus, it yielded five prominent peaks with retention time 15.06, 20.54, 25.37, 29.59, and 35.42 min. The peak area biological activity and chemical structure of phyto-compound that were identified is given in Table 1 and Fig. 1. Altogether, 54 compounds were identified, including indazole, furan, naphthanol, pyrazole, triazole, thiazole, quinoline, naphthalene, oxadiazole, thiophene, pyrimidine, pyridin, piperazine, Tetrazole-5-corboxylic acid, 2-phenyl, quinoline, naphthalene, benzimidazole and pyrazole, which were originally characterized, and identified as one of the key bioactive compounds of several species, e.g., *T. harzianum*. The results of the present study also support the work done by Dubey et al. (2011), where the presence of a wide range of secondary metabolites were obtained from the culture filtrate of *T. harzianum.* The results also revealed the presence of saponin, flavonoids, sterol, tannin and phenol, which are reported to have antimicrobial activity. The relatively high antimicrobial activity of *T. harzianum* isolated in the present study could be attributed to the presence of these compounds.

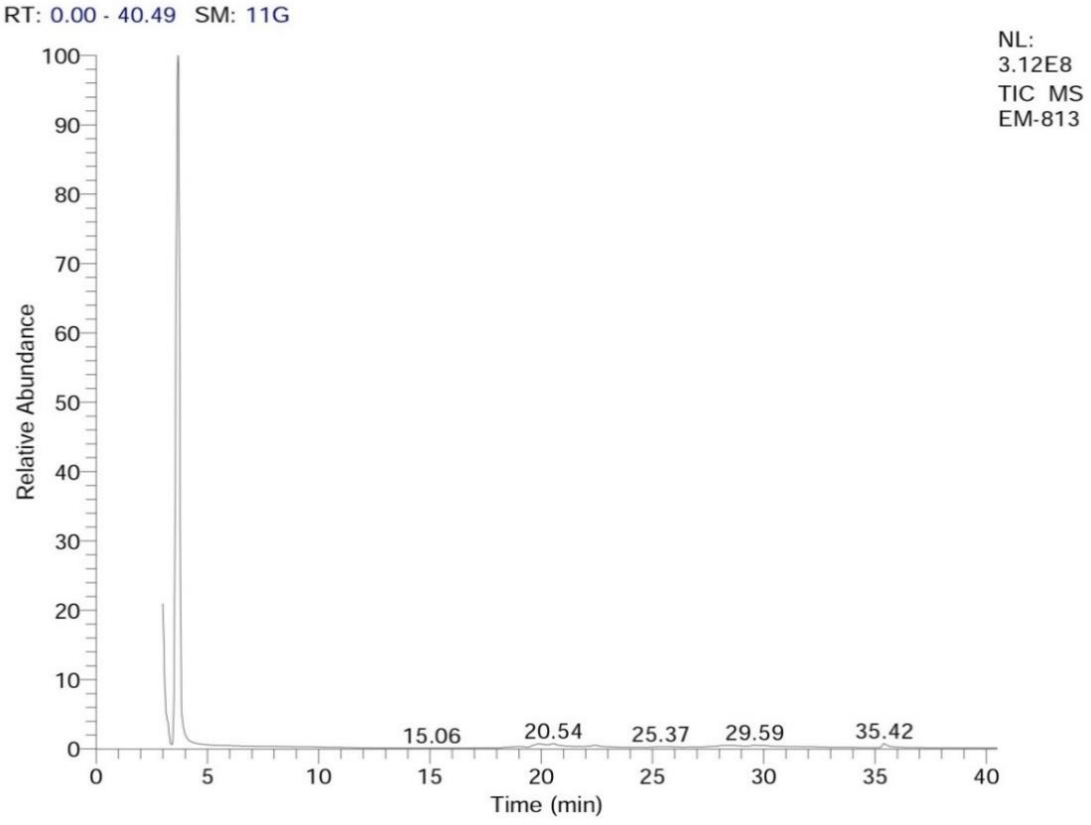

FIGURE 1: Characteristic features of antifungal compound isolated from *Trichoderma harzianum*by GC-MS

TABLE 1
NAME OF SECONDARY METABOLITES IDENTIFIED IN THE MYCELIAL EXTRACT OF *TRICHODERMA HARZIANUMUSING* GC-MS ALONG WITH THEIR ACTIVITY

Name of the Component	Activity
2-Methyl-4-vinyl-6,7-diphenyl-4H-pyrazolo[5,1-c]-1,4-oxazine	Analgesic, Antimicrobial activity, Antipyretic
1-Benzyl-3-methyl-5-(4nitrophenyl) pyrazole	Anti-inflammatory, Antimicrobial activity, Antioxidant
	Antioxidant,
2-Oxo-1-[N(1)-(aminocarbonyl)amino]-4,5-bis(methoxycarbonyl0-6-methyl-2,3 dihydro-1 H-pyrrolo[1,2-b]pyrazole	Anticancer agents, Insecticidal
-Ethyl-6-methyl-5-methylthiopyrazolo[1,5-a]pyrimidine	Antimicrobial activity
2-chloro-4-(2'-thiazolyl)-6-(2''-thienyl)pyrimidine	Anticancer activity, Antimicrobial activity
4-[1-(4-amino-1,2,5-oxadiazol-3-yl)-5-methyl-1h-1,2,3-triazol-4-yl]-1,3-thiazol-2-ylamine	Anti-inflammatory activity, Antimicrobial activity Antitubercular, Antiamoebic, Anthelmintic
1H-Indole,N-ethyl-5,6-dimethoxy-3-methyl-2-(4'methoxyphenyl)	Anti-inflammatory activity Antimicrobial activity
4,6-Dimethoxy-3-methylindolin-2-one	Nematicide, Pesticide, Antimicrobial activity
5-tert-Butyl-7-chloro-1H-indole	Antifungal activity
ethylmethyl-5-oxobeno[f][1,7]naphthyridine-2-carboxylate	Antifungal activity Pesticide degradation
2,2'-Diamino-3'-(methoxycarbonyl)-1,1'-binaphthy	Antifungal activity Antitumor
(R)-isopropyl 2-methoxy-1-naphthyl sulfoxide	Antifungal activity
8-dimethylamino-1-naphthalenecarboxylic acid	Antifungal activity Anti-inflammatory activity
1-methyl-1H-cyclopropa[b]naphthalene	Antibacterial, Pesticide degradation Anti-inflammatory
6,6-dimethoxy-7,8-dihydrocyclohept[fg]acenaphthen-5(6H)-one	Antifungal activity
methyl5-(1,4-naphthoquinon-2-yl)-2-furancarboxylate	Antifungal activity
CIS(3A,9A)-9-phenyl-3,3,3A-triodeuterio-3A,4 dihydronaphtho (2,3-C)furan-1(3H)-one	Antibacterial, Antifungal activity
2-(1-propenyl)naphthanol	Antifungal activity, Pesticide degradation
2-Trichloroacetyl-1,2,3,4 tetrachloroisoquinoline	Antibacterial activity, Anti-tubercular
2,4-Dichloro-3-hydroxy-1H-pyrido[1,2-a]quinolin-1-	Anticancer, Antiproliferative, Antifungal and Antibacterial activities
1à-ethyl-1,2,3,4,6,7,12,12Bá-octahydro-1,3-cycloindolo[2,3-A] quinolizin-3A-methanol	Antimicrobial activity,
	Pesticide
1-Ethyl-6-fluoro-7-[5-(4-methyl-1-piperazinyl)-2,3,4triazabicyclo [3.3.0]oct-2-en-4-yl]-4-oxo-1,4-dihydroquinoline-3-carboxylic acid	Antimicrobial activity,
N-(5,6-dihydrobenzo[h]quinazolin-4-yl)-DL-proline	Antimicrobial activity
	Anticonvulsant agents
1-[3-(dimethylamino)propyl]octahydro-4(1H)-quinolinone	Antimicrobial activity

Quinolin-4-one,1-(3-dimethylaminopropyl)-octahydro-	Antimicrobial activity
3-(2-Hydroxy-1-methyl-2,2-diphenylethyl)-1H-quinoxalin-2-one	Antimicrobial activity
methyl5-(1,4-naphthoquinon-2-yl)-2-furancarboxylate	Antimicrobial activity
2-Acetyl-4-[2-(methoxycarbonyl)ethyl]-3-[(methoxycarbonyl) methyl]pyrrole oxime	Antimicrobial activity,
1-Ethyl-5,6,8-trihydroxy-4-oxo-1,4-dihydro-3-quinolin carboxylic acid	Antimicrobial activity
5,6,8-Trimethoxy-7-methyl-4-phenyl-2(1H)-quinolinone	Antimicrobial activity
6,7-Dimethoxy-3,4-dihydroisoquinoline - N-Oxide	Antimicrobial activity
2-Chloro-3-methyl-5-methoxyquinoline	Antitubercular Activity
1-Hydroxymethyldibenzothiophene	Chemotherapeutic agents, Anti-atherosclerotic agents
Thiophene,2-[(2-fluoro-5-nitrophenylimino)methyl]-3-methyl-	Herbicides/ Pesticides, Anti-atherosclerotic agents
Ethyl2-amino-4-(4-fluorophenyl)-3-thiophenecarboxylate	Insecticide
1-ethyl-3-methyl-2-(2-methylpropylidene)imidazolidine	Antidiabetic Anti-inflammatory[
2-(2'-Furyl)benzimidazole	Anti-inflammatory, Antimicrobial, Antifungal, Antihyperglycemic,
Furan,2-methyl-5-(2-methyl-3-nitrophenyliminomethyl)-	Antimicrobial, Antiviral activity
3-(Ethoxycarbonyl)-2-phenyl-5-[3'-methyl-4' (methoxycarbonyl) buta-1',3'-dienyl] furan	Anti-inflammatory, Antimicrobial, Antifungal, Anticonvulsant activity
(E/Z)-2-(2-nitro-1-phenylethen-1-yl) furan	Antitumor
trans-2,3-Dihydro-5-hydroxy-3-phenylbenzofuran-2 carboxalde hydeN, N-Dimethylhydrazone	pharmacological activities
N,N-dinitroso-piperazine-D8	Antidepressant, Antipsychotic, Tuberculosis,Anthelmenitics
1-(7-Methoxy-benzo[1,3]dioxol-5-ylmethyl)-4-pyridin-3-ylmethyl -piperazine	Asantituberculosis, Anthelmenitics, Antianginals, Anti-Cancer, Analgesic, Antidepressant, Antpsychotic, Antidiabetic, Antihistamines, Hypolipidemic and Flavouring Agent
1,2,6-Trimethyl-4,4-tetramethylene-1,4-dihydropyridine-3,5-dicarbonitrile	anthelmenitics, antianginals, anti-cancer
2-(p-ethylbenzyl)pyridine	Antimicrobial, Biological actvity
2-Bromo-6-(methylthio)-4-(3-pyridyl)pyridine	Antiviral, Anticancer, Antimicrobial, Antidiabetic, Antitubercular
7-ethyl-3-methyl-2-nitro-4-oxo-4,7-dihydrothieno[2,3-b]pyridine-5-carboxylic acid	**Antioxidant** **Antimicrobial activity**
N-carbamoylimino-D5-pyridiniumbetaine	Antimicrobial activity
(5-ethyl-2-imino-4-methyl-6-oxo-3,6-dihydro-1(2h) pyrimidinyl) acetic acid	Antifungal activity
7-(cis-2'-Hydroxy-1'-cyclohexyl)-4-chloro-6-methylpyrrolo[2,3-d]pyrimidine	Anthelmenitics, antifungal activity
2-Acetylamino-4-trifluoromethyl-6,7,8,9,10,11,hexahydro 5,9;7,11-dimethano-5H-[9]annuleno[d]pyrimidine	Anti-inflammatory activity Antimicrobial activity
1-Butyl-6-hydroxy-4-methylhexahydropyrimidin-2-	Antioxidant and Cytotoxic activities

IV. CONCLUSION

The GC-MS analysis of the ethyl acetonitrile extract of *Trichoderma harzianum* has revealed the presence of a diverse range of bioactive volatile compounds, including Triazole, Imidazole, Thiazole, Pyrazole, Indole, Naphthalene, Furan derivatives, Pyrimidine, Thiophene, Quinoline, Pyridine, and Piperazine. These compounds exhibit significant potential for antimicrobial applications, indicating the pharmaceutical relevance of *T. harzianum*. Many of these metabolites can be isolated, purified, and chemically modified to enhance their efficacy against human pathogens. The findings highlight the role of *T. harzianum* as a promising source of natural antimicrobial agents, which could contribute to the development of novel drugs in combating infectious diseases.

In addition to their pharmaceutical applications, the bioactive compounds identified in this study also demonstrate considerable potential in agricultural and environmental sectors. The insecticidal, nematicidal, and pesticidal properties of these compounds provide an opportunity to develop sustainable and eco-friendly pest management strategies. Furthermore, their potential in pesticide degradation and herbicide formulation positions *T. harzianum* as a valuable tool in reducing chemical residues in the environment and promoting sustainable agriculture.

Overall, *T. harzianum* emerges as a versatile microbial resource with applications spanning pharmaceuticals, pest control, and environmental management. The ability to harness its bioactive metabolites paves the way for innovative solutions to address challenges in health, agriculture, and environmental sustainability. Future research should focus on the isolation and structural characterization of these compounds, along with in-depth studies on their mechanisms of action, to unlock their full potential and practical applicability.

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