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Deciphering the antimicrobial potential of secondary metabolites isolated from *Trichoderma harzenium*

RAJESHWARI VITTAL[1], SMITHA SAHADEVAN[2], KAVITHA GULADAHALLI MANJUNATHA[3], ABHIRAMI SOMASEKHARAN[4], AKHILA DHARNAPPA SANNEJAL*

[1,2,3,4,5]Nitte (Deemed to be University), Nitte University Centre for Science Education and Research (NUCSER), Paneer Campus, Deralakatte, Mangalore-575018, India

[1] Corresponding author: akhila@nitte.edu.in*

ABSTRACT

Background & Objectives: *The genus Trichoderma is a filamentous fungi belonging to the phylum Ascomycota, and is known for thriving in diverse environments such as soil, plant surfaces, and decomposing organic materials. One of the most important characteristics of Trichoderma is its ability to produce a diverse range of secondary metabolites. This study aimed to isolate and characterize Trichoderma species from rhizosphere soil samples collected from the different parts of Dakshina Kannada.*

Methods: *Fifty agricultural soil samples were collected in sterile zip-lock bags. Soil samples were serially diluted and spread on Trichoderma selective agar. Obtained colonies were confirmed by lactophenol cotton blue staining and molecular method. The secondary metabolite of Trichoderma was extracted using ethyl acetate in varying concentrations using the solvent extraction method. The obtained crude extracts were screened against various Gram-negative and Gram-positive bacteria.*

Results: *Out of 50 samples, thirteen were identified as positive based on microscopic analysis using lactophenol cotton blue staining. Phenotypically identified isolates were used for molecular confirmation. Among thirteen isolates, three were confirmed as T. harizenum using PCR and Internal Transcribe space (ITS) sequencing. The obtained crude extracts were screened against Staphylococcus aureus, Bacillus subtilis, Pseudomonas aeruginosa, Klebsiella pneumoniae, Escherechia coli, Vibrio parahemolyticus, Aeromonas hydrophila and Salmonella. Significant antimicrobial activity was observed against Salmonella with minimum inhibitory concentration (MIC) values of 31.25µg/mL and 15.62µg/mL, followed by Vibrio parahaemolyticus having a MIC value of 62.5µg/mL for the extracts. Phytochemical analysis of the crude extracts resulted in the presence of terpenoids, alkaloids, and saponins. Further analysis of the compounds was determined using FTIR spectroscopy by identifying the functional groups based on the peaks generated.*

Conclusion: *The present study highlights the antimicrobial potency of secondary metabolites from T. harizenum. Due to their wide range of advantageous characteristics, including their application in agriculture, industry, and environmental management, Trichoderma spp. continue to be a subject of extensive research.*

Keywords: *Antibacterial activity; Minimum inhibitory concentration; Trichoderma spp.; Polymerase chain reaction*

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1. Introduction

Trichoderma species is a diverse group of filamentous fungi, are prolific inhabitants of soil and plant environments and are widely recognized for their ability to alter their structure in response to adverse conditions (Frąc et al., 2018). These fungi are ubiquitous and significantly impact human existence, including biotechnology, phytopathology, medicine, and biodiversity conservation (Cai and Druzhinina, 2021).

They occur in plant litter, soil organic debris, and as endophytes within plant tissues. Due to their ability to thrive in various conditions, they are the most commonly culturable fungi, isolated from the soil (Hermosa et al.,2014; Mukherjee et al., 2013). Many endophytes are known to biosynthesize beneficial secondary metabolites that may help the host defend itself and survive against harmful bacteria (Ratnaweera et al., 2015). Secondary metabolites (SMs) are chemical substances produced by particular biosynthetic pathways that are not required for usual fungal growth and development in the laboratory; however, they give the host a competitive advantage (Keller et al.,2005). One of the most distinguishing characteristics of fungi is their vast metabolic adaptability, which is reflected by the production of a wide variety of secondary metabolites in many species. These metabolites (SMs) produced by fungi exhibit antimicrobial properties that serve as biocontrol agents and as prolific producers of diverse chemical compounds including polyketides (Schuster and Schmoll, 2010). Resistance to antibiotics is an increasing problem globally, and as a result, researchers are focusing more on developing promising antibacterial agents against pathogenic bacteria. Consequently, there is an urgent need for novel antibacterial agents. *Trichoderma*, with their extensive metabolic capabilities and genetic diversity, present a promising source of new antibacterial compounds (Guo et al.,2022). This study focuses on isolating *Trichoderma* species and evaluating the antibacterial activity of their extracted secondary metabolites against pathogens.

2. Methodology

2.1 Sample collection

Fifty soil samples were collected from different parts of Mangalore (figure.1). The samples were collected and stored in the refrigerator at 4°C until further use.

Figure 1. Sample collection sites

2.2. Isolation of *Trichoderma* spp. from soil

1g of soil sample was weighed and serially diluted in 9 mL of 0.85% sterile NaCl. From each dilution, 100 µL was plated onto sterile *Trichoderma* selective media (HiMedia M1836) under aseptic conditions. For all the samples, triplicates were maintained. The plates were then incubated at 30˚C in a fungal incubator for 4-7 days. The growth on the plate was monitored regularly.

2.3. Identification of *Trichoderma* spp.

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The isolated colonies were sub-cultured onto *Trichoderma* selective media to obtain pure culture. The presumptive identification of the fungus was carried out based on the morphological examination of the lactophenol cotton blue stained smear under a microscope at 40x magnification for detailed examination.

2.4. DNA Extraction

The DNA extraction followed the method given by Kadu et al (2019) with slight modification. The fungal mycelium grown in the culture broth was separated using a sterile muslin cloth. For the extraction of DNA, 250 mg of fungal mycelia were crushed in 500 µL of lysis buffer (50 mM Tris HCl, 50 mM EDTA (pH 8.2), 3% SDS) in a sterile centrifuge tube followed by adding 500 µL of lysis buffer to the same tube. The mixture was then vortexed for 5 minutes and incubated at 65˚C for 1 hour in a water bath. After incubation, 500 µL of Chloroform: Isoamyl alcohol (24:1) was added, vortexed briefly, and centrifuged at 14,000 rpm for 10 minutes at 4˚C. The aqueous layer was transferred to a new tube, mixed with 500 µL of Phenol: Chloroform: Isoamyl alcohol (25:24:1), inverted, and centrifuged. The aqueous phase was collected, mixed with chilled absolute ethanol, and incubated at -20°C for 30 minutes to precipitate the DNA. After centrifugation, the pellet was washed with 70% ethanol and centrifuged at 14000 rpm for 3 minutes at 4˚C, then the supernatant was discarded. The pellet was dried, resuspended in TE buffer, and stored at -20°C. Further DNA was confirmed as *Trichoderma* species using genus-specific primers by polymerase chain reaction (PCR). The details of the primer used in this study are given in Table 1. The obtained PCR product was subjected to sequencing.

Table 1: Gene-specific primers for *Trichoderma* species

Primer	Primer Sequence (5'-3')	Product size	Reference
TGF	GAACCCCTCCGGGGGGHC	110 bp	Zhou et al.,2021
TGR	TGTGCAAACTACTGCGCAKGA		

2.5. Extraction of secondary metabolites

2.5.1. Preparation of the culture for extraction

Trichoderma culture was filtered through a sterile muslin cloth. The obtained liquid phase was used for the extraction of secondary metabolites by mixing with ethyl acetate in a 1:2 ratio (w/v). These mixtures were incubated in a shaker incubator at 30°C for 2 hour at 120 rpm, followed by a 30-minute stationary phase in a 500 mL separating funnel. The ethyl acetate layer containing the secondary metabolites was separated using a funnel and subjected to a rotary vacuum evaporator. The temperature was adjusted as per the boiling point of the solvent. The obtained powder extract was dissolved in 5% Dimethyl sulfoxide (DMSO) and stored at 4°C for further use.

2.5.2. Phytochemical analysis

Phytochemical analysis of the *Trichoderma* extracts was carried out to determine the presence of alkaloids, anthraquinones, flavonoids, saponins, tannins, and terpenoids.

2.5.2.1. Test for alkaloids

0.4 g of fungal extract was stirred with 8 mL of 1% HCl, warmed, and filtered. A 2 mL of the filtrate was treated separately with Mayer's reagent. The presence of turbidity or precipitation indicated the existence of alkaloids (Siddiqui and Ali, 1997).

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2.5.2.2. Test for saponins

20 mg of extract was boiled in 20 mL of distilled water in a water bath for five minutes and filtered. 10 mL of the filtrate was mixed with 5 ml of distilled water and shaken vigorously to form froth. 3 drops of olive oil were then added to the froth, shaken vigorously, and observed for emulsion development (Siddiqui and Ali, 1997).

2.5.2.3. Test for terpenoids

5 mL of fungal extract was mixed with 2 mL chloroform, followed by 3 mL of concentrated H_2SO_4. The presence of terpenoids was confirmed by a reddish-brown coloration at the interface (Siddiqui and Ali, 1997).

2.5.2.4. Test for Flavonoids

50mg of extract was suspended in 100 mL of distilled water and filtrate. To 10mL of the filtrate, 5mL of diluted ammonia solution was added, followed by a few drops of concentrated H_2SO_4. The appearance of yellow color confirmed the presence of flavonoids (Sofowara, 1993).

2.5.2.5. Test for tannins

50 mg of extract was boiled in 20 mL of distilled water and filtered. A few drops of 0.1% $FeCl_3$ were added to the filtrate. The presence of tannin was indicated by the brownish-green or blue-black coloration (Sofowara, 1993).

2.5.2.6. Test for anthraquinones

200 mg of the extract was boiled with 6 mL of 1% HCl and filtered. The filtrate was mixed with 5 mL of benzene and filtered again. To this, 2mL of 10% ammonia solution was added, and the mixture was vigorously shaken. The presence of free hydroxyl anthraquinones was indicated by pink, violet, or red colour (Trease and Evans, 1989).

2.6. Screening of Antibacterial activity

2.6.1. Antibacterial activity by Disc diffusion assay

In vitro, the antibacterial activity of ethyl acetate extract of *Trichoderma* was evaluated using the Kirby-Bauer disc diffusion assay. The test bacterial cultures were inoculated onto nutrient broth and incubated at 37˚C in the shaker incubator for 18 hours. The bacterial strains were adjusted to 0.5 McFarland standard (1.5×10^6) and swabbed onto sterile Mueller Hinton agar plates. 20 uL of each extract having a concentration of 10 mg/mL were added to the sterile disc (6mm) and allowed to dry for 10 minutes. The discs were then placed onto the media containing the test organisms. Ciprofloxacin (5mcg) was used as a positive control, while ethyl acetate and 5% DMSO served as negative controls. The plates were then incubated at 37℃ for 24 hours in the static incubator. The antibacterial activity was evaluated by measuring the diameter of the zone of inhibition. The experiment was performed in duplicates. *Staphylococcus aureus* (ATCC25923), *Pseudomonas aeruginosa* (ATCC15692), *Klebsiella pneumoniae* (ATCC700603), *Escherechia coli* (ATCC25922), *Bacillus subtilis* (ATCC6051), *Vibrio parahemolyticus* (ATCC1780), *Aeromonas hydrophila* (ATCC7966), *Salmonella* (ATCC35664) were used for the assay.

2.6.2. Determination of Minimum inhibitory concentration (MIC)

The MIC, which is the lowest concentration at which the extract inhibits microbial growth was determined using the microdilution method in a 96-well plate. Test microorganisms were inoculated into Muller Hinton broth (MHB), and the OD was adjusted to 0.5 McFarland

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standard. The plates were incubated at 37℃ for 24 hours. After incubation, the concentration at which there was no visible growth was considered as MIC of that extract.

2.6.3. Determination of Minimum bactericidal concentration (MBC)

MBC determines the lowest level of the extract to kill the microorganisms. To determine MBC, a dilution representing the MIC of the extract was plated on MHB and incubated at 37˚C for 24 hours to determine MBC. The tube that showed no turbidity was considered as MBC, representing that there was 99% inhibitory activity of the tested bacteria. FTIR spectroscopy was performed for each sample to characterize the type of compound present. The samples were subjected to FTIR analysis, which resulted from the vibrations caused by the IR radiation passing through the sample. Based on the functional groups a disturbance is produced resulting in sharp peaks in the spectrum.

3. Results

3.1. Isolation of *Trichoderma* spp. from soil

A total of three *Trichoderma* strains were isolated from fifty soil samples. The presumed colonies were used for the microscopic examination using the lactophenol cotton blue staining method. The colonies typically exhibited a white cottony texture with the presence of conidia. Out of 50 samples, thirteen isolates were phenotypically confirmed by microscopic analysis. It was observed that there were regularly branching conidiophores in pairs (figure. 2). These conidiophores bear conidia that are typically ellipsoidal.

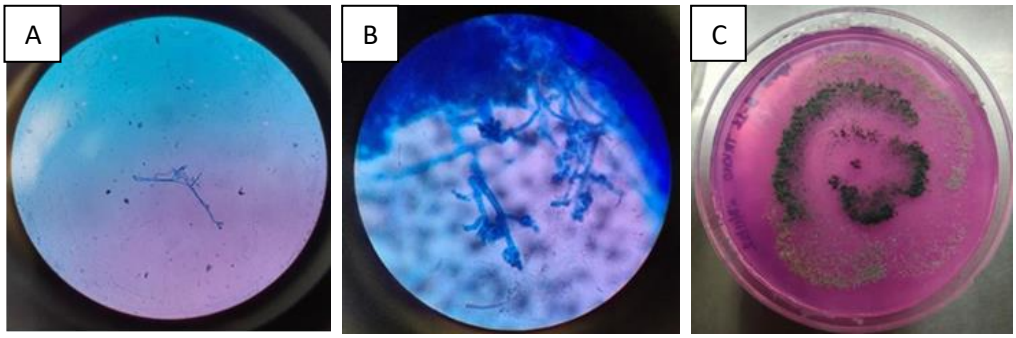

Figure 2. Microscopic examination of *Trichoderma* spp (A & B) and growth on selective agar (C)

3.2. Molecular Characterization

The phenotypically positive isolates were subjected to PCR. A purified DNA concentration was utilized as the template for PCR amplification using genus-specific primer (TGF/TGR) thereby confirming the identification as belonging to the genera *Trichoderma* (figure. 3). Out of thirteen isolates, three isolates showed positive for *Trichoderma* species.

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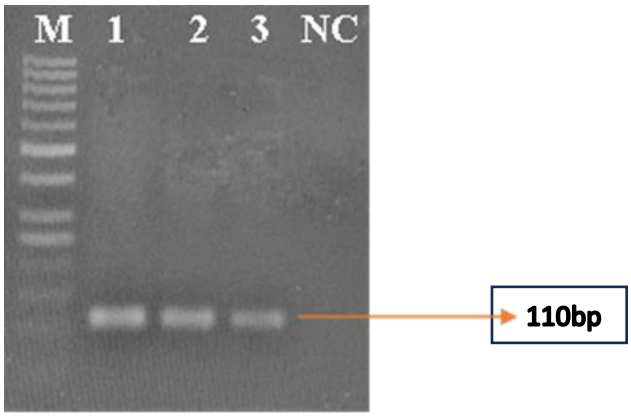

Figure 3. Agarose gel image showing PCR amplification of ITS region. Lane M- 50bp marker, Lane 1: Positive control, Lane 2-3: Positive isolates, Lane 4: Negative control.

3.3. Sequencing

All three isolates were sequenced by the automated Sanger technique. The sequence reads were aligned with reference sequences retrieved from NCBI GenBank for species confirmation. The multiple sequence alignment was carried out by Multalin software (http://multalin.toulouse.inra.fr/multalin/). The alignment analysis showed more than 98% sequence identity to the reference sequence of *Trichoderma harzenium* thus confirming the identity of the isolates as *Trichoderma harzenium.*

3.4. Phytochemical analysis

Phytochemical analyses of one of the *Trichoderma harzenium* extracts were carried out to determine the presence of alkaloids, anthraquinones, flavonoids, saponins, tannins, and terpenoids. The extract showed positive for alkaloids, terpenoids, and saponins.

Table 2. Table showing the result of phytochemical analysis

Test	Observation	Result
Alkaloids	Turbidity or precipitation shows the presence of alkaloids.	Positive
Terpenoids	A reddish-brown coloration of the interface shows the presence of terpenoids.	Positive
Anthraquinones	A pink, violet, or red color shows the presence of free hydroxyl anthraquinones.	Negative
Flavonoids	Yellow coloration confirms the presence of flavonoids.	Negative
Saponins	Emulsion development shows the presence of saponins.	Positive
Tannins	Brownish green or a blue-black coloration shows the presence of tannins.	Negative

3.5. Screening of Antimicrobial activity

3.5.1. Disc diffusion assay

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The ethyl acetate extracts of *T. harzenium* were evaluated for their inhibitory effects against various bacteria using disc diffusion and agar well diffusion assays. The extract showed inhibition against *E. coli* and *Salmonella* with a zone of 15 and 23 mm respectively. It showed the least activity against *B. subtilis* and *V. parahaemolyticus* (figure. 4).

3.5.2. Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC)

The MIC was carried out for extracts in a series of two-fold dilutions. The extract showed the highest antibacterial activity against *Salmonella* (31.25 µg/mL), *Vibrio parahaemolyticus* (62.5 µg/mL), *B. subtilis* (500 µg/mL). Although strong activity was observed against *Salmonella*, there was growth observed when MBC was performed.

3.5.3. Characterization by FTIR spectroscopy

Crude extracts obtained from the *Trichoderma* were analyzed through the FT-IR spectrum for the peak locations. For the crude extracts of T. *harzenium* peak positions of FT-IR spectra were assigned in the wavelength between the range of 500–4000 cm^{-1}. The peak at 3690 cm^{-1} and 3487.52 cm-^{1} corresponds to O-H stretching that is assigned to alcohol. The frequencies of 2998.45 cm^{-1} and 2917.52 cm^{-1} represent medium C-H stretching corresponding to alkane. Whereas the wavelength of 1314.26 cm^{-1} indicates the presence of medium O-H bending representing phenol.

In the observed IR spectrum for the second crude extract S2, the frequencies of 3321.97 cm^{-1} and 1438.49 were assigned to the carboxylic acid. The IR frequency of 1637.14 cm^{-1} corresponds to the C=C stretching of alkenes. The frequencies of 1320.89 cm^{-1} indicate the strong appearance of S=O stretching of sulfones and 1080.89 cm^{-1} corresponds to strong C-O stretching of aliphatic ether. The peak at 865.20 cm^{-1} represents strong C-H bending.

4. Discussion

Trichoderma species are widely recognized for their potential to inhibit or control plant diseases as well as several other pathogens, which makes them effective biocontrol agents. This is achieved through a variety of strategies, including competition for resources, and the production of enzymes and secondary metabolites that prevent the growth of harmful pathogens. A total of 50 soil samples were screened from different parts of Mangalore for the isolation of fungi. Out of thirteen phenotypically confirmed isolates, only three were confirmed as positive through PCR. However, in comparison to other microorganisms, its population density might be rather low (Baazeem et al., 2021). The quantity and makeup of *Trichoderma* communities in the soil can be influenced by a variety of elements, such as soil type, agricultural techniques, plant species, and environmental circumstances. Due to their low quantity, *Trichoderma* colonies are challenging to separate and distinguish from the numerous other fungi and bacteria found in soil samples. Previous studies revealed the production of several antibiotics from *Trichoderma* such as gliotoxin, gliovirin as they are known to inhibit the plant pathogens effectively (Khan et al., 2020). In this study soil-derived *Trichoderma* was isolated for the screening of its antimicrobial activity by secondary metabolites. The obtained *Trichoderma* isolate was subjected to extraction of secondary metabolites using an organic solvent ethyl acetate. Several research on the efficiency of the extraction techniques revealed that ethyl acetate extraction produced stronger antibacterial activity than n-hexane and methanol (Saeed et al., 2012). It was evident through many studies that, as compared to water extraction, the use of organic solvents invariably results in better efficiency in the extraction of antimicrobial compounds (Forfang et al., 2017). The intriguing fungal family known as *Trichoderma* has received a lot of attention in the fields of agriculture and biocontrol. In this

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study the secondary metabolites were screened against gram-negative and gram-positive bacteria. Although the antibacterial assay and minimum inhibitory concentration performed showed strong inhibitory activity against *Salmonella*, the MBC showed negative results, and the least activity was observed against *B. subtilis*. As per the study conducted on the biological activity of secondary metabolite extracted from *Trichoderma*, there was inhibitory action reported against *B. subtilis* and *S. aureus* (Narendran and Kathiresan, 2016). The phytochemical analysis revealed the presence of alkaloids, terpenoids, and saponins. In some of the reports on phytochemical screening, the extracted secondary metabolites are entirely dependent on the strain hence the compound may vary with differences in the strain. Also, it depends on the location it grows, nutrient availability, stress conditions, etc. Additionally, it is crucial to consider appropriate controls and compare the results with standard antibiotics or positive controls to validate the antibacterial activity of the secondary metabolites. Before attempting the chemical identification of the compounds responsible for such activities, it is necessary to have a better understanding of the antimicrobial activities specific to a species or genotype. Due to the increase in drug resistance to harmful pathogens, *Trichoderma* has gained extensive attention because of its natural availability. Therefore, it is necessary to explore the compounds produced by *Trichoderma* more since the applications are restricted to the agricultural field and many other fields of the pharmaceutical industry.

5. Conclusion

Trichoderma is a diverse and advantageous group of fungi that produce secondary metabolites with tremendous potential for use in various fields, including agriculture and pharmaceuticals. The study demonstrated that the secondary metabolites isolated from *T. harzianum* possess significant antibacterial properties, inhibiting Gram-positive and Gram-negative bacteria. The phytochemical analysis showed the presence of terpenoids, alkaloids, and saponins, whereas the FTIR analysis reported the appearance of various peaks that revealed the functional groups of the compounds. These findings suggest that the secondary metabolites generated from *Trichoderma* have important applications in many industries, particularly as alternative or supplementary antimicrobial agents. In light of rising antibiotic resistance, these compounds present a promising path for creating new antimicrobial agents. However, the efficiency of these metabolites can differ depending on the target bacteria. These organic compounds could provide alternative or supplementary methods for treating bacterial infections.

Conflict of Interest

The authors have no conflicts of interest regarding this investigation.

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References

Baazeem, A., Almanea, A., Manikandan, P., Alorabi, M., Vijayaraghavan, P., Abdel-Hadi, A., 2021. In vitro antibacterial, antifungal, nematocidal and growth promoting activities of *Trichoderma hamatum* FB10 and its secondary metabolites. Journal of Fungi 7, 331.

Cai, F., Druzhinina, I.S., 2021. In honor of John Bissett: authoritative guidelines on molecular identification of *Trichoderma*. Fungal Diversity 107, 1–69.

 https://doi.org/10.5281/zenodo.13643275

Forfang, K., Zimmermann, B., Kosa, G., Kohler, A., Shapaval, V., 2017. FTIR spectroscopy for evaluation and monitoring of lipid extraction efficiency for oleaginous fungi. PloS one, 12, e0170611.

Frąc, M., Hannula, SE., Bełka, M., Jędryczka, M., 2018. Fungal biodiversity and their role in soil health. Frontiers in microbiology. 9, 10-3389.

Guo, S., Zhang, Z., Guo, L., 2022. Antibacterial molecules from marine microorganisms against aquatic pathogens: a concise review. Marine Drugs.20, 230.

Hermosa, R., Cardoza, R.E., Rubio, M.B., Gutiérrez, S., Monte, E., 2014. Secondary metabolism and antimicrobial metabolites of *Trichoderma*. In Biotechnology and biology of *Trichoderma,* Elsevier.125-137.

Kadu, T. P., Gade, R. M., Rathod, J. P., Paraskar, S., Malghane, B., Shedmake, A., 2019. Rapid and Efficient Procedure for Genomic DNA Extraction from Trichoderma spp. International Journal of Current Microbiology and Applied Sciences. 8, 993-996.

Keller, NP., Turner, G., Bennett, J.W., 2005. Fungal secondary metabolism—from biochemistry to genomics. Nature Reviews Microbiology.3, 937-47.

Khan, R. A. A., Najeeb, S., Mao, Z., Ling, J., Yang, Y., Li, Y., Xie, B., 2020. Bioactive secondary metabolites from *Trichoderma* spp. against phytopathogenic bacteria and root-knot nematode. Microorganisms, 8, 401.

Mukherjee, P.K., Horwitz, B.A., Herrera-Estrella, A., Schmoll, M., Kenerley, C.M., 2013 *Trichoderma* research in the genome era. Annual review of phytopathology. 51,105-29.

Narendran, R., Kathiresan, K., 2016. Antimicrobial activity of crude extracts from Mangrove-derived *Trichoderma* species against human and fish pathogens. Biocatalysis and agricultural biotechnology. 6, 189-194.

Ratnaweera, P. B., de Silva, E. D., Williams, D. E., Andersen, R. J., 2015. Antimicrobial activities of endophytic fungi obtained from the arid zone invasive plant *Opuntia dillenii* and the isolation of equisetin, from endophytic *Fusarium* sp. BMC complementary and alternative medicine, 15, 1-7.

Saeed, N., Khan, M.R., Shabbir, M., 2012. Antioxidant activity, total phenolic and total flavonoid contents of whole plant extracts *Torilis leptophylla L*. BMC complementary and alternative medicine. 12,1-2.

Schuster, A., Schmoll, M., 2010. Biology and biotechnology of *Trichoderma*. Applied microbiology and biotechnology. 87,787-99.

Siddiqui, A.A., Ali, M., 1997. Practical pharmaceutical chemistry.1st Ed., CBS Publishers and Distributors, New Delhi: 126-131.

Sofowara, A.E., 1993. Medicinal plants and traditional medicine in Africa. Ibadan, Nigeria: Spectrum books Ltd., 289-2

Trease, G.E., Evans, W.C., 1989. Pharmacognsy. London: Brailliar Tiridel Can Macmillian Publishers, 60-75. 11

Zhou, Y., Wang, J., Yang, L., Guo, L., He, S., Zhou, W., Huang, J., 2021. Development of species/genus specific primers for identification of three Trichoderma species and for detection of Trichoderma genus.

 https://doi.org/10.5281/zenodo.13643275