**Production and Applications of Secondary Metabolites extracted from *Trichoderma viride***

**ABSTRACT**

Aims: To investigate the production, extraction, and characterization of bioactive metabolites from *Trichoderma viride*, to evaluate their antimicrobial and anticancer potential. This study aims to validate the applicability of *Trichoderma viride* metabolites as therapeutic and agricultural agents.

**Study design:** Experimental *in -vitro* study involving extraction, biochemical analysis, and therapeutic applications.

**Place and Duration of Study:** Conducted at Centre for Bioscience and Nanoscience Research during the period from May to July 2025.

**Methodology**: *Trichoderma viride*, a filamentous fungus, was cultured using MGYP media for metabolite production. Metabolites were extracted using solvent extraction methods and subjected to Thin Layer Chromatography (TLC), Column Chromatography, and UV-Visible Spectroscopy. TLC revealed distinct Rf values indicating the presence of bioactive compounds. Fraction 4, isolated via column chromatography, showed a distinct absorbance peak under UV-visible analysis, suggesting chromophoric group presence. Antibacterial activity was assessed using agar well diffusion against *Staphylococcus aureus* and *Klebsiella pneumoniae*. Anticancer potential was analyzed using the MTT assay on MCF-7 breast cancer cell lines.

**Results**: TLC confirmed multiple bioactive metabolites. Among the separated fractions, Fraction 4 showed the highest activity. UV-visible spectroscopy indicated potent chromophoric constituents. Antibacterial testing revealed clear inhibition zones against both *Staphylococcus aureus* and *Klebsiella pneumoniae*. The MTT assay revealed dose-dependent cytotoxicity, with decreasing MCF-7 cell viability at increasing concentrations of the extract. The results demonstrate that *Trichoderma viride* metabolites possess both antimicrobial and anticancer properties.

**Conclusion**: *Trichoderma viride* serves as a promising source of natural compounds with potential biomedical and agricultural applications. Its metabolites exhibit significant antibacterial and anticancer activity and may support sustainable therapeutic and bio control innovations.

*Keywords: UV-Visible spectroscopy; antibacterial activity; anticancer activity; Thin layer chromatography; column chromatography.*

1. **INTRODUCTION**

Trichoderma species, filamentous fungi commonly found in soil, decaying wood, and plant roots, are recognized for producing white mycelium that turns green upon conidia formation. Among them, Trichoderma viride is particularly notable for its antagonistic activity against plant pathogenic fungi, serving as an effective biocontrol agent for soil-borne diseases (Awad et al., 2018). A central mechanism in biological control is **antibiosis**, wherein organisms synthesize and release chemically diverse secondary metabolites with cytotoxic properties that suppress or inhibit pathogen growth. This mechanism plays a pivotal role in the biocontrol efficacy of Trichoderma species and other beneficial microbes like plant growth-promoting bacteria (Mukhopadhyay and Kumar, 2020).

Trichoderma species are prolific producers of such metabolites, including enzymes, antibiotics, vitamins, polysaccharides, and organic acids. These compounds perform multiple ecological functions ranging from nutrient competition and iron chelation to induction of plant resistance and promotion of plant growth (Vinale and Sivasithamparam, 2020). Trichoderma viride generates a wide array of bioactive compounds such as Azaphilones, Viridins, nitrogen-containing heterocycles, and volatile terpenes that significantly enhance its biocontrol capabilities. It also produces Trichodermamides, which are associated with antibacterial, anticancer, and antifungal activities (Sood et al., 2020).The ability of *Trichoderma viride* to produce structurally diverse and pharmacologically significant secondary metabolites has been well established through various studies, which highlight its potential applications beyond agriculture, including in therapeutic development (Awad et al., 2018; Sood et al., 2020).

In addition to secondary metabolites, Trichoderma produces numerous hydrolytic enzymes and proteases, including exo- and endochitinases, xylanases, glucanases, lipases, and various peptidases. These enzymes degrade pathogen cell walls, further reinforcing Trichoderma’s antifungal arsenal (Esparza-Reynoso et al., 2021). The genus is extensively studied and widely applied in sustainable agriculture due to its diverse modes of action, which include competition for space and nutrients, secretion of cell wall-degrading enzymes, production of antifungal secondary metabolites, and mycoparasitism (Mukhopadhyay and Kumar, 2020; Herrera et al., 2020). It is found naturally in the soil, rhizosphere, and even endophytically within plant tissues. Trichoderma species are essential allies in eco-friendly crop production. Their integrated functions suppressing pathogens, stimulating plant defenses, and enhancing growth make them indispensable in modern agricultural practices. Moreover, Trichoderma can activate induced systemic resistance (ISR) in host plants, amplifying their ability to defend against a broad spectrum of pathogens (Esparza-Reynoso et al., 2021).This study aims to explore the production, extraction, and characterization of bioactive metabolites derived from Trichoderma viride, and to evaluate their antimicrobial and anticancer potential. Through chromatographic and spectroscopic analyses, followed by biological assays, the study investigates the therapeutic relevance of Trichoderma viride metabolites and highlights their application in biomedical and agricultural domains.

1. **MATERIALS AND METHODS**
	1. **Collection and sub culturing of fungi**

The fungi *Trichoderma viride* was collected from Centre for Bioscience and Nanoscience Research. The fungi was sub cultured to Malt agar media (Himedia, India), which was prepared by dissolving 45 g in 1000mL of distilled water and sterilized under autoclave at 121°C for 15minutes. After sterilization, the media was poured on to sterilised plate and allowed for solidification. To the solidified media fungi was inoculated using wire loop and incubated at 29 to 30°C for 5 to 10 days.

* 1. **Preparation of Production Media**

The isolated fungi were sub cultured in MGYP broth (Himedia, Mumbai, India). The media was prepared by dissolving - Yeast extract: 3.0 g, Malt extract: 3.0 g, Glucose: 5.0 g, Peptone 5g in 1000mL of distilled water and sterilized under autoclave at 121°C for 15 minutes. Sterilized media was cooled to room temperature under aseptic condition and the fungal sample was inoculated, followed by incubated at 30°C for 5 to 7 days (Shanmuga et al., 2021).

* 1. **Extraction of Secondary Metabolites**

The cell - free extract was collected after centrifugation at 10000 rpm for 10 minutes, the final extract was undergone for precipitation with an equal amount of the ethyl acetate solution. After adding the solutions, which were vigorously mixed for 2 hours and the aqueous layer was transferred to another flask and used for further study (Shawky et al., 2019).

* 1. **Characterization of the metabolite**
		1. **Column Chromatography**

Chromatography is a technique used to separate, identify, and purify components of a mixture for both quantitative and qualitative analysis. In this process, molecules are separated based on their interaction with a stationary adsorbent phase. In the present study, DEAE Sephadex A50 (Himedia, India) was used as the stationary phase in the column. After preparing the column with the stationary phase, the mobile phase was added and incubated for 15 min. The sample was then eluted with distilled water, and fractions were collected slowly to obtain pure compounds. The eluted compounds were identified by measuring the optical density (OD) at 280 nm using a spectrophotometer, as described by Berg et al., (2013).

* + 1. **UV-Visible Analysis**

UV-visible spectrophotometric analysis was performed on the extract using a UV-visible spectrophotometer (Labtronics LT291) with a slit width of 100 nm and 0.5 absorbance. The extract was analyzed under both visible and UV light across a wavelength range of 200–800 nm to assess the presence of chromophoric groups. Prior to analysis, the extract was centrifuged at 3000 rpm for 10 minutes, and the supernatant was used for further examination, following the procedure outlined by Dwivedi et al. (2023).

* + 1. **Thin Layer Chromatography**

The aqueous extract was applied as a spot using capillary tubes 1 cm above the base of the thin-layer plate. After air-drying, the plate was placed in a beaker containing a solvent system composed of n-butanol: methanol: acetic acid: water in a ratio of 2:1:1:1. The samples were allowed to migrate upward by capillary action. Once the solvent front reached an appropriate height, the plate was removed, air-dried, and incubated in an iodine chamber for 5–10 minutes for spot visualization. The retention factor (Rf) values were then calculated to identify the separated compounds, using methods similar to those reported by Azerang et al., (2019).

* 1. **Antibacterial Activity**

The agar well diffusion method was employed to assess the antibacterial activity of the sample. Nutrient broth (Himedia, India) was used to subculture the bacterial strains, which were incubated at 37°C for 24 hours. Subsequently, 45µL of *Staphylococcus aureus* and *Klebsiella pneumoniae* cultures were spread evenly on nutrient agar plates (prepared using 28g of nutrient agar dissolved in 1000mL distilled water and sterilized at 121°C for 15 minutes). Wells were created using a sterile cork borer, and the sample was introduced into each well along with a positive control (Streptomycin antibiotic disc). The plates were incubated at 37°C for 24 h (Devagoud and Simon, 2024). The antibacterial potential was determined by measuring the diameter of the inhibition zones surrounding each well (Jesteena *et al.,* 2018).

* 1. **Anticancer Activity (MTT Assay)**

MCF-7 human breast cancer cells (NCCS, Pune, India) were subcultured in Dulbecco’s Modified Eagle Medium (DMEM, Himedia, Mumbai), enriched with sodium carbonate, glucose, and 10% bovine serum albumin (BSA). After seeding into T-flasks, the cells were incubated at 37°C in a CO₂ incubator, maintaining a pH of 7.0–7.5 and 70–80% humidity, for 24 to 72 h. For the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay) assay, the cells were transferred to 96-well plates and incubated for 24 hours. Various concentrations of the fungal extract were added to the wells, with DMSO used as the blank and untreated cells as the control. After 24 hours of treatment, the medium was removed and the wells were gently rinsed with DMSO and trypsin. Then, 20µL of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, Himedia, India) dye was added to each well, followed by incubation at 37°C for another 24 h. The formation of purple formazan crystals indicated metabolically active cells, and absorbance was measured at 570nm using a 96-well ELISA reader (Roboniks, India). The percentage of viable cells (Control OD- Treated OD/Control OD X100) was calculated to determine the cytotoxic effect of the sample (Jacob et al.,2023).

1. results and discussion
	1. Isolation and extraction of secondary metabolite from *Trichoderma viride*

The filamentous fungus *Trichoderma viride* was successfully isolated and subcultured on malt extract agar (MEA) to promote active fungal growth. MEA is a nutrient-rich medium frequently employed for cultivating fungi due to its high carbohydrate content, which promotes sporulation and biomass development (Herrera *et al.,* 2020). The fungi demonstrated optimal growth within 5 to 10 days at an incubation temperature of 29–30°C, forming a distinct green sporulating colony characteristic of *T. viride* (Mukhopadhyay & Kumar, 2020).Following successful isolation, the fungi were subcultured into MGYP broth (Malt Extract–Glucose–Yeast Extract–Peptone), which is commonly utilized to enhance secondary metabolite production. MGYP is known to provide the essential nutrients required for the biosynthesis of fungal metabolites, such as peptaibols, polyketides, and terpenoids (Sood et al., 2020). Incubation at 30°C for 5 to 7 days facilitated the production of bioactive compounds in the culture medium.

After the incubation period, the fungal broth was subjected to centrifugation to obtain a cell-free extract. Ethyl acetate was used as the organic solvent for extracting extracellular secondary metabolites. Ethyl acetate is widely preferred due to its moderate polarity and efficiency in isolating a broad range of bioactive compounds (Awad et al., 2018). After thorough mixing, phase separation yielded an organic phase containing the extracted fungal metabolites. These extracted secondary metabolites are known to include enzymes, antibiotics, and low molecular weight compounds that exhibit bioactivity against various pathogens. Previous studies have shown that *Trichoderma viride* produces non-volatile and volatile secondary metabolites with antimicrobial, antifungal, and plant growth-promoting activities (Esparza-Reynoso et al., 2021; Vinale & Sivasithamparam, 2020). The successful recovery of these compounds from the culture extract forms the basis for subsequent biological activity testing, such as antibacterial and anticancer assays. The optimized conditions for fungal growth and metabolite extraction reported here are consistent with the standard approaches documented in fungal biotechnology for maximizing bioactive compound yield (Azerang et al., 2019). The obtained extract will be further subjected to biological screening to determine its efficacy in various biomedical applications.

* 1. **Characterization of the metabolite**

The culture filtrate obtained from *Trichoderma viride* was subjected to column chromatography to isolate and fractionate the secondary metabolites based on their physicochemical properties. This method allowed for the separation of the crude extract into distinct fractions, enhancing the resolution and identification of individual compounds. Column chromatography is a widely used technique in natural product research, especially in fungal metabolite separation, as it facilitates the enrichment of bioactive components from complex mixtures (Azerang et al., 2019). In this study, multiple fractions were collected, each representing compounds with varying polarities. Among the collected fractions, Fraction 4 appeared notably prominent, exhibiting an intense color and clearer separation during elution. This visual distinction hinted at a higher concentration of target metabolites. The clarity and separation efficiency achieved through the column suggest that the applied gradient and adsorbent matrix were suitable for effective purification. Similar findings have been reported by Jacob, (2023), who also achieved distinct separation of antimicrobial compounds from fungal species using chromatographic techniques. The isolation of such fractions is a critical step toward the chemical and biological characterization of fungal secondary metabolites.

Following chromatographic separation, the individual fractions were analyzed using UV-visible spectrophotometry to determine the presence of chromophoric compounds. Fraction 4 exhibited the highest absorbance value of 0.766, indicating a dense presence of conjugated systems or light-absorbing functional groups. These include aromatic rings and phenolic structures, which are known to contribute to antimicrobial and antioxidant activity. The spectroscopic analysis provides preliminary insight into the structural complexity and potential biological relevance of the isolated metabolites (Dwivedi et al., 2023).The absorption spectrum of Fraction 4 closely aligns with previously characterized secondary metabolites from *Trichoderma* species. A study by Lakhdari et al. (2023) demonstrated similar peak characteristics in *Trichoderma harzianum* extracts, attributing them to phenolic compounds and other aromatic derivatives. The significant UV absorbance observed in this study suggests that the *Trichoderma viride* extract contains a comparable profile of bioactive compounds. These results support the hypothesis that *Trichoderma viride* is a promising source of therapeutically valuable metabolites, and Fraction 4 may be prioritized for further FTIR, GC-MS, and biological activity studies.

The broth containing *Trichoderma viride* was centrifuged, and the resulting supernatant was extracted with ethyl acetate to isolate secondary metabolites. The organic phase was allowed to undergo solvent partitioning, after which the aqueous layer was carefully separated for further analysis. Thin layer chromatography (TLC) was employed as a preliminary screening method to assess the chemical diversity and polarity of the extracted compounds. The sample was spotted onto a silica gel TLC plate and developed in a suitable solvent system. The compound migrated with a retention factor (Rf) value of 0.6226, suggesting moderate polarity and efficient separation of a metabolite from the crude extract. The Rf value of 0.6226 is of particular interest, as it indicates the presence of mid-polar bioactive compounds typically associated with therapeutic properties such as antimicrobial, antioxidant, or anticancer effects. Classes of compounds like flavonoids, alkaloids, and terpenoids commonly exhibit Rf values in this range, and their separation through TLC provides a rapid, cost-effective method of preliminary screening (Lakhdari et al., 2023). The Rf value observed in the *T. viride* extract aligns well with earlier studies on *Trichoderma harzianum*, where similar retention behavior was recorded for fractions exhibiting strong biological activity. This similarity implies that *T. viride* may harbor structurally and functionally analogous secondary metabolites, highlighting its potential as a promising fungal source in natural product-based therapeutic research.

* 1. **Antibacterial Activity**

The antibacterial activity of the *Trichoderma viride* extract was assessed using the agar well diffusion method against two human pathogenic bacteria: *Staphylococcus aureus* and *Klebsiella pneumoniae*. After 24 hours of incubation at 37°C, clear zones of inhibition were observed around the wells, indicating effective antimicrobial action. The diameter of the inhibition zone produced by the crude extract was 10 mm against *S. aureus* and 0 mm against *K. pneumoniae*, whereas the column-purified extract showed improved activity, producing zones of 12 mm and 16 mm, respectively. In contrast, the positive control (streptomycin disc) exhibited 28 mm inhibition against *S. aureus* and 29 mm against *K. pneumoniae*. These findings indicate a significant enhancement in antibacterial potency after chromatographic purification, especially in the case of *K. pneumoniae*, where no activity was observed in the crude form.



**Fig.1. Antibacterial activity of the extracted metabolite**

Our observations are consistent with previous findings reported by Stracquadanio et al., (2020), where extracts from *Trichoderma asperellum* and *Trichoderma atroviride* demonstrated broad-spectrum antimicrobial properties. The increased activity observed in the purified column fraction of *Trichoderma viride* highlights the value of selective extraction and fractionation in enriching active metabolites. This enhancement can be attributed to the concentration and isolation of bioactive compounds during the purification process, which eliminates interfering or inactive substances present in the crude extract. Consequently, this study supports the effectiveness of chromatographic techniques in optimizing the therapeutic potential of fungal secondary metabolites and further establishes *Trichoderma* species as promising candidates for the development of natural antimicrobial agents.

* 1. **Anticancer activity**

The anticancer potential of the *Trichoderma viride* extract was evaluated against MCF-7 human breast cancer cell lines using the MTT assay. The cells were exposed to increasing concentrations of the crude extract (2, 4, 6, 8, and 10µg/mL) for 24 hours, and the resulting cell viability percentages were observed to be 90.76%, 88.28%, 84.49%, 81.02%, and 74.42%, respectively, with a control absorbance value of 0.606 indicating 100% viability. The results clearly indicate a dose-dependent cytotoxic effect, where higher concentrations of the fungal extract resulted in progressively reduced cancer cell viability. This decline in viability confirms the presence of bioactive secondary metabolites in the fungal extract that are capable of interfering with cancer cell metabolic processes, ultimately leading to reduced proliferation.



**Fig.2. Graphical representation showing the anticancer activity of the extracted metabolite**

These findings are consistent with previous studies that have highlighted the anticancer efficacy of fungal-derived metabolites. For instance, Staropoli et al., (2023) demonstrated that several species of *Trichoderma*, particularly *T. harzianum* and *T. longibrachiatum*, exhibited potent anticancer effects due to the presence of peptaibols, trichothecenes, and polyketides—classes of compounds known for their cytotoxic and apoptotic activities. Furthermore, Kumar et al., (2020) noted that fungal metabolites could induce oxidative stress and mitochondrial dysfunction in cancer cells, contributing to apoptosis. In the context of the present study, the decreasing trend in MCF-7 cell viability implies that *T. viride* may produce similar cytotoxic compounds, which interact with cellular components to trigger programmed cell death or inhibit proliferation. Therefore, this data underscores the relevance of *Trichodermaviride* as a promising candidate in natural product-based cancer therapeutics, particularly due to its ability to deliver bioactive compounds in a cost-effective and eco-friendly manner.

1. **CONCLUSION**

This study highlights the bioactive potential of *Trichoderma viride* through the production, extraction, and characterization of its secondary metabolites. Cultivation in MGYP broth, followed by TLC, column chromatography, and UV–visible spectroscopy, facilitated the isolation of metabolite-rich fractions, with Fraction 4 exhibiting the highest absorbance (0.766), suggesting the presence of chromophoric compounds such as flavonoids and phenolics. Antibacterial assays demonstrated significant inhibitory effects, particularly in the purified column fractions, where *Staphylococcus aureus* and *Klebsiella pneumoniae* exhibited enhanced susceptibility compared to the crude extract. These results indicate that purification significantly enriches the concentration of bioactive constituents, thereby improving antimicrobial efficacy. Additionally, the MTT assay on MCF-7 breast cancer cells revealed a clear dose-dependent cytotoxic effect, with cell viability decreasing from 90.76% to 74.42% across increasing extract concentrations. This confirms the anticancer potential of *Trichoderma viride* metabolites and supports their application in the development of fungal-derived chemotherapeutic agents. Overall, *Trichoderma viride* emerges as a promising source of natural antimicrobial and anticancer compounds. Its compatibility with scalable fermentation and solvent-based extraction techniques positions it as a sustainable microbial platform for eco-friendly innovations in agriculture, pharmaceuticals, and biomedicine.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) herebydeclare that no generative AI technologies such as large language models (chatgpt, copilot, etc) and text-to-image generators have been used during writing or editing of this manuscript

**CONFLICTS OF INTEREST**

 The authors declare that there is no conflict of interest.

COMPETING INTERESTS:

Authors have declared that they have no known competing financial interests OR non-financial interests OR personal relationships that could have appeared to influence the work reported in this paper.

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