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# GC-MS Profiling and Antifungal Activity of Secondary Metabolites from Endophytic Fungi Isolated from the Velamen Roots of *Epidendrum radicans*

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## ABSTRACT

**Endophytic fungi, residing within plant tissues have beneficial roles in promoting plant health and resistance to various stresses. This study investigates the antifungal properties of twelve endophytic fungi isolated from the velamen roots of the epiphytic orchid *Epidendrum radicans*, against the plant pathogenic fungus *Pythium myriotylum*. Dual culture and well diffusion methods were used to test the antifungal activity of nine fungal species against *P. myriotylum*. Six out of nine fungal species demonstrated inhibitory activity against *P. myriotylum*. *Diaporthe eucalyptorum* exhibited the highest zone of inhibition (5.67 mm ± 0.07) and the least Zone of inhibition was exhibited by *Xylaria aristata* (1.36 mm ± 0.04 in dual culture method. On the other hand, the highest Zone of inhibition in Well diffusion method was exhibited by *Trichoderma atroviridae* (8.13 mm ± 0.85) and the least by *T. asperellum* (2.38 mm ± 0.48). The antifungal efficacy of these endophytes is attributed to the presence of bioactive secondary metabolites, analyzed through GC-MS. The exact mechanisms of antifungal activity exhibited by endophytic fungi need to be elucidated. The bioactive secondary metabolites responsible for antifungal activity require further characterization and purification. Our findings confirm the presence of various volatile compounds across six fungal species, suggesting their potential application as eco-friendly fungicides in agriculture. The study also highlights the importance of endophytic fungi in sustainable agriculture. Future studies can build upon the findings of this research and explore the potential applications of endophytic fungi in sustainable agriculture and pharmaceutical industries.**

**Keywords:** Endophytic fungi, antifungal, Well diffusion method, dual culture method, GC-MS, secondary metabolites

## INTRODUCTION

Endophytic fungi, are harmless microbes that are very common, found associated with a variety of plant tissues (Deshmukh *et al*., 2018). The endophytic fungi exhibit multifaceted complex interactions in plants (Lu *et al*., 2021; Mattoo and Nonzom., 2021) including phyto-protective roles against predators and pathogens (Zang *et al*.,2006). Endophytes assist their host plants in several ways such as improved nutrient uptake, tolerance to biotic and abiotic stressors, and generation of secondary metabolites that may find use in medicines (Patil *et al*., 2016; Enyi *et al*., 2024). They are also found to be important source of bioactive metabolites (Vaz *et al*.,2009).

*Pythium myriotylum* is a soil-borne fungal pathogen that has a broad host range. It causes soft rot disease in a wide range of economically important plants (Le *et al*., 2014). The impact of this pathogen is more pronounced in tropical countries including India. Through irrigated water, it can spread over large areas and can pose threat to economically important crops.

Deepthi and Ray (2019) identified endophytic fungi and algal species that play a nutritional role in the orchids. The current study is based on the antifungal activity of the fungal endophytic species isolated from the velamen roots of epiphytic orchids *Epidendrum radicans* by Deepthi and Ray (2021). Based on observations and prior research, the authors postulated that the production of certain putative secondary metabolites by the isolated and identified endophytic fungi confers antimicrobial potential. The current study aims to identify the endophytic fungi with antifungal potentials and explores the secondary metabolites produced by them.

## MATERIALS AND METHODS

The present investigation was carried out using lyophilized samples of endophytic fungi, isolated from the velamen roots of the epiphytic orchid *Epidendrum radicans.* The samples were sub cultured on PDA (2% Bactoagar) at 26 ± 2°C for 10 days.

**Screening of anti-fungal activity of the endophytic fungal strains**

The antifungal activity of the nine isolated endophytic strains were studied through dual culture method, where they were treated against a potential plant pathogenic fungus *P. myriotylum.* Pure cultures of *P. myriotylum* were obtained from the Laboratory of Microbiology, School of Biosciences, Mahatma Gandhi University, Kottayam. Four days old fungal culture grown on potato dextrose agar (PDA) was used for inoculation of isolated endophytic fungal strain on PDA plates. Two wells were made on plane PDA plate using well cutter. One well was inoculated with *P. myriotylum* disc and the other with fungal endophyte. The inoculated plates were incubated at room temperature (25 ± 2°C) for 2 days. The zone of inhibition (ZOI) was observed, measured, recorded and compared with *P. myriotylum* as the control. The experiment was conducted in triplicates and the average values were calculated.

**Confirmation of the anti-fungal activity of isolated endophytic fungal strains**

The antifungal activity of the isolated fungal endophytic strains against *P. myriotylum* were confirmed through well diffusion method (Dissanayake.,2014), using crude fungal extracts. Five discs (1mm diameter) of seven days old fungal colony on PDA were inoculated in to 500 mL Erlenmeyer flask containing 200 mL potato dextrose broth (PDB) medium and incubated at 28°C in a shaker at 150 rpm for 21 days. 5 wells were made on 4 days old cultures of *P. myriotylum* on PDA using sterilized well cutter. To each well added 100µL of crude fungal extract. $10^{th}$ well was kept as control with 100µL of methanol. Plates were incubated for 2 days at room temperature (25 ± 2°C). The zone of inhibition (ZOI) was observed, measured, recorded and compared with control. The experiment was carried out in triplicates and the average values were calculated.

**Extraction and identification of secondary metabolites**

After 21 days of incubation of fungal species on PDB, the cell contents were released by sonication. Total extraction of culture media was done following Haque e*t al*., (2005). Cell-free supernatants were harvested by centrifugation at 7000 rpm for 10 minutes and $P^H$ of the supernatant was adjusted to 2.8 with 1N HCl and aqueous phase was extracted three times with double volume ethyl acetate by vigorous shaking for 20 minutes. Ethyl acetate fraction was separated. All obtained extracts were dried under reduced pressure at 40°C. The solid residue was made up to 1 mL with methanol, centrifuged at 8000 rpm for 10 minutes and filtered through 0.22µ membrane filters and kept at -20°C for further analysis.

**GC-MS analysis**

The crude extracts of each fungal species were subjected to GC-MS analysis at School of Bio sciences, Mahatma Gandhi University, Kottayam using a Shimadzu GC-MS QP 2010S series chromatograph equipped with a Shimadzu ion trap mass spectrometer. GC-MS analysis is a powerful tool in revealing the chemical profile of crude extracts, thereby providing a better understanding of their potential as natural fungicides (Arivoli *et al*., 2019).

Samples were separated on a column of Shimadzu Rxi-5Sil MS model (size 30mx 250µm x 0.25mm). The mobile phase was methanol-water (linear gradient from 30% to 100% methanol over 30 min) at a flow rate of 1mL / $min^{-1}$ at 300-325°C. 50µL volume of extract was injected and Helium was used as the carrier gas. The eluted compounds were monitored using ion mass spectrometer and PDA detector. Identification of the compounds were done based on absorbance at nm over 10 to 25 minutes (total analysis time 35 minutes). The structure of particular compounds was putatively identified and evaluated by comparing the molecular masses (m/z values) of the eluted compounds following NIST 11 Library.

**Statistical analysis**

The results of dual culture and well diffusion methods were represented as mean ± standard deviation (SD). Differences between mean values were tested for significance with one-way analysis of variance (ANOVA) followed by Duncan's multiple-range test (DMRT) using SPSS 16.0 software.

**RESULT AND DISCUSSION**

**Antifungal activity of isolated fungi**

**Dual culture method**

Nine different species of fungi used in the present investigation exhibited inhibitory activity against

the potential plant pathogen *P. myriotylum,* in dual culture method (Fig.1). The highest zone of inhibition (ZOI) was noted in *Diaporthe eucalyptorum* (5.67 ± 0.07 mm) and the lowest in *Xylaria aristata* (1.36 ± 0.04 mm) after 48 hrs of incubation (Table 1).

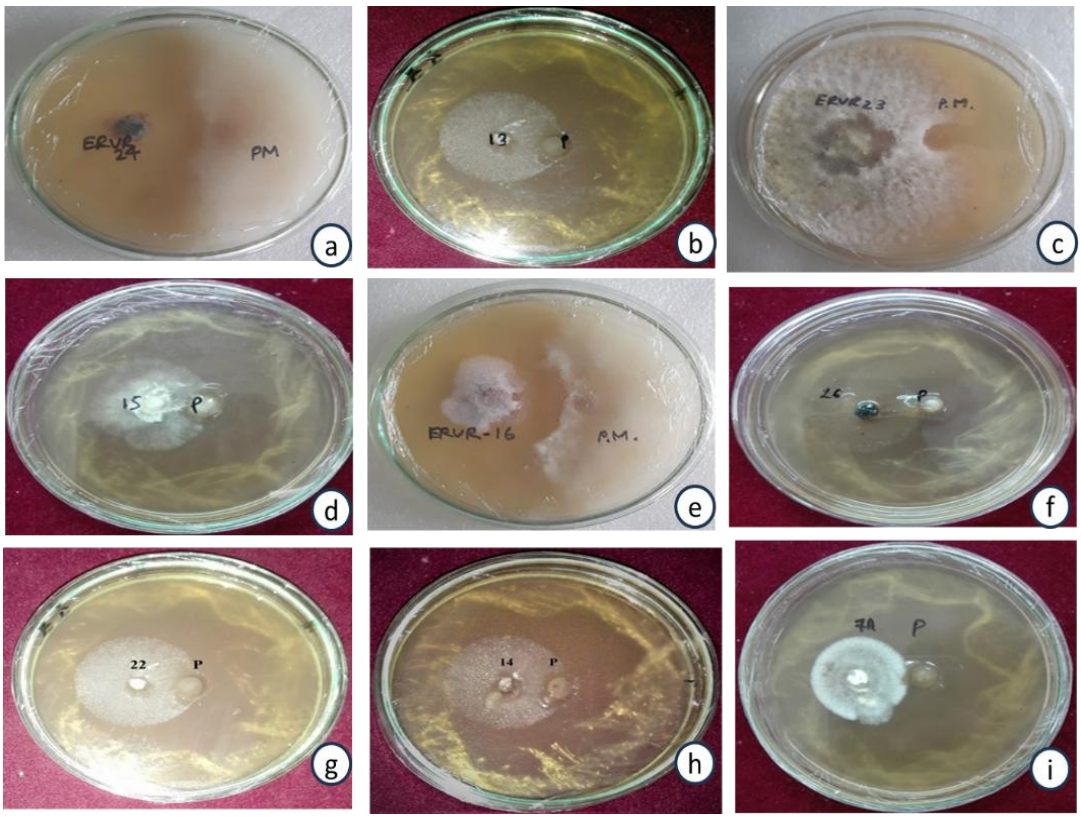


**Figure 1:** Anti-fungal activity of endophytic fungal strains isolated from the velamen roots of *E. radicans* against plant fungal pathogen *Pythium myriotylum* by dual culture method. a, *Diaporthe eucalyptorum*; b, *Diaporthe longicolla*; c, *Endomelanconiopsis endophytica*; d, *Nigrospora sphaerica*; e, *Trichoderma asperellum*; f, *Trichoderma atroviridae;* g, *Trichoderma harzianum*; h, *Trichoderma viride*; I, *Xylaria aristate.*

**Table 1:** Zone of inhibition produced by endophytic fungal strains on plant fungal pathogen *Pythium myriotylum*

| Fungal Endophyte | GenBak Accession Number (Deepthi and Ray 2021) | Zone of inhibition (mm) | |
|---|---|---|---|
| | | Dual culture method | Well diffusion method |
| Control | | - | 1.88 ± 0.48 a |
| *Diaporthe eucalyptorum* | MK182372.1 | 5.67 ± 0.07 | 3.00 ± 0.91 ab |
| *Diaporthe longicolla* | MK182365.1 | 4.86 ± 0.06 | 4.75 ± 1.26 cd |
| *Endomelanconiopsis endophytica* | MK182373.1 | 2.27 ± 0.06 | 3.88 ± 1.55bc |
| *Nigrospora sphaerica* | MK182363.1 | 3.23 ± 0.14 | 3.63 ± 1.11bc |
| *Trichoderma asperellum* | MK182360.1 | 2.09 ± 0.23 | 2.38 ± 0.48 ab |
| *Trichoderma atroviridae* | MK182382.1 | 3.11 ± 0.15 | 8.13 ± 0.85 e |
| *Trichoderma harzianum* | MK182367.1 | 1.53 ± 0.08 | 5.00 ± 1.68 cd |
| *Trichoderma viride* | MK182364.1 | 2.00 ± 0.09 | 6.50 ± 0.91 d |
| *Xylaria aristata* | MK142801.1 | 1.36± 0.04 | 2.50±0.71 ab |

All data were mean ± SD; Values with the different letter in the same column are significantly different after one way ANOVA and Duncan's Multiple Range Test (DMRT) at p< 0.001significance level

**Well diffusion method**

Crude extracts of the six different fungal species were found to exhibit statistically significant inhibition against the plant pathogen *P. myriotylum* by well diffusion method **(Fig. 2).** Crude extract from the species *Trichoderma atroviridae* showed highest Zone of inhibition against *P. myriotylum* (8.13 ± 0.85 mm) followed by *T. viride* (6.50 ± 0.91 mm). Minimum ZOI was exhibited by *Trichoderma asperellum* (2.38 ± 0.48 mm). Fungal endophytes *Diaporthe longicolla*, *Endomelanconiopsis endophytica*, *Trichoderma atroviridae, Trichoderma harzianum* and *Trichoderma viride* showed statistically significant ZOI against *P. myriotylum* (Table 1).

**GC- MS analysis of secondary metabolites**

Crude ethyl acetate extract mixed with GC grade methanol (2mL) was used for GC-MS analysis. Out of the crude extracts from nine fungal species examined, presence of volatile compounds was detected only in six species such as *D. eucalyptorum*, *D. longicolla*, *E. endophytica*, *T. asperellum*, *T. atroviridae* and *T. viride*.

The chromatogram predicted the presence of three volatile compounds in *D. eucalyptorum,* ten volatile compounds in *D. longicolla*, four volatile compounds in *E. endophytica,* five volatile compounds in *T. asperellum* and seven volatile compounds each in *T. atroviridae* and *T. viride*

based on retention time, molecular weight and molecular formula **(Table 2)**. The GC-MS mass spectrum was interpreted through the data base of National Institute of Standards and Technology (NIST). The mass spectrum of the unknown components was compared with those of known components available in the NIST database so as to ascertain the name, molecular weight and structure of the sample components.

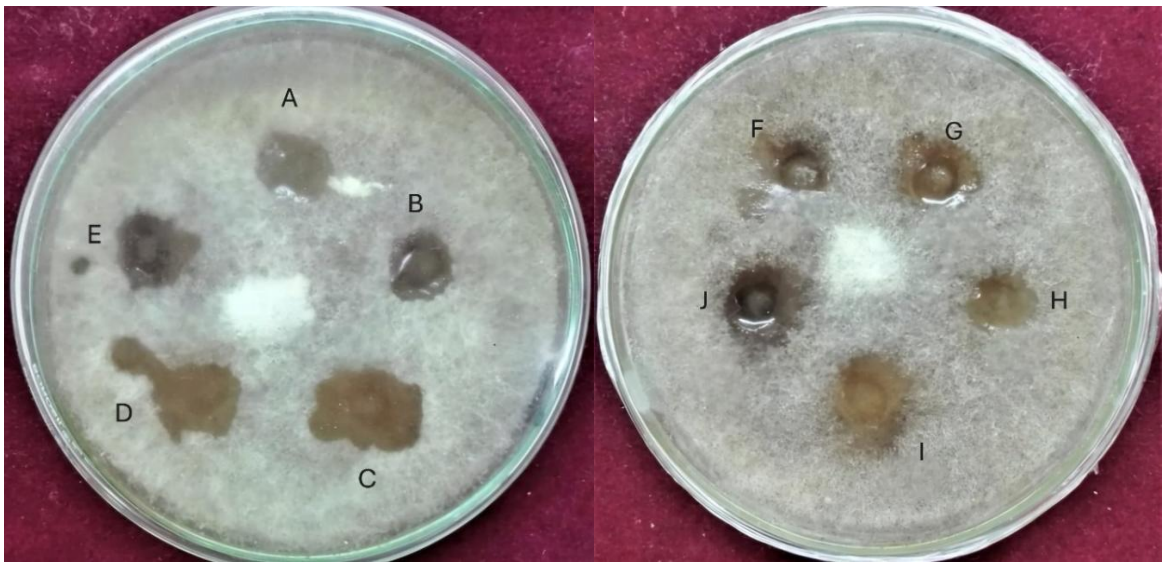


**Figure 2:** Anti-fungal activity of crude extract of endophytic fungal strains isolated from the velamen roots of *E.radicans* against plant fungal pathogen *Pythium myriotylum* by well diffusion method. A. Control B. *Xylaria aristata*; C. *Trichoderma atroviridae*; D. *Diaporthe eucalyptorum*; E. *Nigrospora sphaerica;* F. *Trichoderma viride* G. *Diaporthe longicolla* H. *Trichoderma asperellum*; I. *Endomelanconiopsis endophytica*; J. *Trichoderma harzianum.*

**TABLE 2:** Compounds identified in the methanolic crude extract of endophytic fungal species associated with velamen roots of *Epidendrum radicans* through GC-MS analysis

| Fungal species | Compound name | Chemical formula | Retention time (in min) | Molecular weight |
|---|---|---|---|---|
| *Diaporthe eucalyptorum* | 1-Tetradecanol | $C_{14}H_{30}O$ | 12.573 | 214 |
| | n-Nonadecanol-1 | $C_{19}H_{40}O$ | 15.704 | 284 |
| | 1- Hexa decanol | $C_{16}H_{34}O$ | 18. 345 | 242 |

| Fungal species | Compound name | Chemical formula | Retention time (in min) | Molecular weight |
|---|---|---|---|---|
| *Diaporthe longicolla* | 2,3-Pyridinedicarboxylic acid | $C_7H_5NO_4$ | 7.605 | 167 |
| | 5-(Hydroxymethyl)-2-furancarboxylic acid | $C_6H_6O_4$ | 10.119 | 142 |
| | Alpha-methyl-benzeneethanol | $C_9H_{12}O$ | 14.342 | 136 |
| | Alpha-bisabolol | $C_{15}H_{26}O$ | 15.004 | 222 |
| | Trans-6-(p-tolyl)-2-methyl-2-heptanol | $C_{15}H_{22}O$ | 15.440 | 218 |
| | p-Formophenetidide | $C_9H_{11}NO_2$ | 15.726 | 165 |
| | n-Hexadecanoic acid | $C_{16}H_{32}O_2$ | 17.884 | 256 |
| | Bicyclo[9.3.1]pentadeca-3,7-dien-12-ol, 4,8,12,15,15-pentamethyl-, [1R-(1R*,3E,7E,11R*,12R*)]- | $C_{20}H_{34}O$ | 20.967 | 290 |
| | Ursodeoxycholic acid | $C_{24}H_{40}O_4$ | 23.087 | 392 |
| | 1,3,6,10-Cyclotetradecatetraene, 3,7,11-trimethyl-14-(1-methylethyl) | $C_{20}H_{32}$ | 23.365 | 272 |
| *Endomelanconiopsis endophytica* | Alpha-methyl-benzene ethanol | C9H12O | 14.333 | 136 |
| | 13Beta-methyl-13-vinyl-podocarp-7-ene-3beta-ol | C20H32O | 23.092 | 288 |
| | Bicyclo[9.3.1]pentadeca-3,7-dien-12-ol, 4,8,12,15,15-pentamethyl-, [1R-(1R*,3E,7E,11R)] | $C_{20}H_{34}O$ | 23.361 | 290 |
| | 2'-carboxy-2,5-dimethyl-benzophenone | $C_{16}H_{14}O_3$ | 25.316 | 254 |
| *Nigrospora sphaerica* | Nil | - | - | - |
| *Trichoderma asperellum* | Niacin | $C_6H_5NO_2$ | 7.599 | 123 |
| | 2,4-Hexadienedioic acid | C6H6O4 | 10.121 | 142 |
| | 2,6 Bis (1,1-dimethylethyl-phenol) | $C_{14}H_{22}O$ | 11.492 | 206 |
| | Alpha-methyl-benzene ethanol | $C_9H_{12}O$ | 14.326 | 136 |
| | 1,3,6,10-Cyclotetradecatetraene, 3,7,11-trimethyl-14-(1-methylethyl) | $C_{20}H_{32}$ | 23.360 | 272 |
| *Trichoderma atroviridae* | 2,3-Dihydro-thiophene | $C_4H_6S$ | 7.558 | 86 |
| | 5-(Hydroxymethyl)-2-furancarboxylic acid | $C_6H_6O_4$ | 10.124 | 142 |
| | Methylphenyl-ethyl ester-carbamic acid | $C_{10}H_{13}NO_2$ | 12.618 | 179 |
| | Alpha-bisabolol | $C_{15}H_{26}O$ | 15.002 | 222 |
| | p-Formophenetidide | $C_9H_{11}NO_2$ | 15.719 | 165 |
| | N-[3-(Benzylloxy)phenyl]-N-methylacetamide | $C_{16}H_{17}NO_2$ | 20.113 | 255 |
| | 1,3,6,10-Cyclotetradecatetraene, 3,7,11-trimethyl-14-(1-methylethyl) | $C_{20}H_{32}$ | 23.365 | 272 |
| *Trichoderma harzianum* | Nil | - | - | - |

| Fungal species | Compound name | Chemical formula | Retention time (in min) | Molecular weight |
|---|---|---|---|---|
| *Trichoderma viride* | Hexahydrofuro[3,2-b] furan-3,6-diol | $C_6H_{10}O_4$ | 7.556 | 146 |
| | Quinoline-3-carboxylic acid, 1,4,5,6,7,8-hexahydro-2-methyl-5-oxo-4-(2-thienyl)-, 1-methylpropyl | $C_{19}H_{23}NO_3S$ | 13.467 | 345 |
| | 1-(p-Ethoxyphenyl)-3-phenyl-2-thiourea | $C_{15}H_{16}N_2OS$ | 13.555 | 272 |
| | Alpha-methyl-benzene ethanol | $C_9H_{12}O$ | 14.339 | 136 |
| | 3-Methyl-5-nitrosotropone | $C_8H_7NO_3$ | 15.717 | 165 |
| | Bicyclo[9.3.1]pentadeca-3,7-dien-12-ol, 4,8,12,15,15-pentamethyl-, [1R-(1R*,3E,7E,11R)] | $C_{20}H_{34}O$ | 23.364 | 290 |
| | Bis (2-ethylhexyl)phthalate | $C_{24}H_{38}O_4$ | 23.5 | 390 |
| *Xylaria aristata* | Nil | - | - | |

## DISCUSSION

The findings indicate that all the nine species of endophytic fungi exhibited inhibitory activity against the target pathogen, as determined by the dual culture method. These findings are in concordance with the previous works carried out by Zhao *et al*., 2022; and Yu *et al*., 2018. The highest ZOI was observed for *D. eucalyptorum* at 5.67 ± 0.07 mm, while the lowest ZOI was noted for *X. aristata* at 1.36 ± 0.04 mm, both measured after 48 hours of incubation similar to the results obtained by Kumar *et al*., (2012). According to Manganyi and Ateba (2020), the observed antifungal activity of the tested endophytic fungi may be attributed to the presence of bioactive compounds such as tannins, alkaloids, saponins, and terpenes. According to a review published by Hilario and Goncalves (2022), many species of *Diaporthe* are found to possess antifungal and antimicrobial properties. The present study reveals the antifungal potential of *D. eucalyptorum* and *D. longicolla* against the plant pathogen *P. myriotylum.*

Several studies conducted across the world has proved the anticancer, antidiabetic, insecticidal, antimicrobial and immunosuppressive properties of endophytic fungi (Strobel and Daisy, 2003.). Investigating the antifungal properties of various endophytic microbes has become an increasingly important area of research (Deshmukh *et al*., 2018; Paul *et al*., 2012). *P. myriotylum* enjoys cosmopolitan distribution especially in tropical and subtropical regions and are found to cause severe economic losses in agricultural, horticultural and greenhouse soilless cultivation systems (Wang *et al*., 2003; Perneel *et al*., 2006; Vitale *et al*.,2018).

Fungi are known to produce a diverse array of volatile organic compounds that play crucial roles in their ecological interactions (Hashem *et al.*, 2023; Manganyi and Ateba, 2020; Deshmukh *et al*.,2018). The GC-MS data highlights the presence of a wide array of bioactive volatile compounds in the methanolic crude extract of endophytic fungi isolated from the velamen roots of *Epidendrum radicans* **(Figure 3)**. 1-Tera decanol ,1-Hexadecanol and n-Nonadecanol-1 was exclusively found in *D. eucalyptorum*. 2,3-Pyridinedicarboxylic acid was present only in *D. longicolla.* 5-(Hydroxymethyl)-2-furancarboxylic acid was seen both in *D. longicolla and T. atroviridae.* Alpha-methyl-benzene ethanol was widely present in fungal species such as *D. longicolla, E. endophytica, T. asperellum and T. viride.* The secondary metabolite Alpha-bisabolol were found both in *D. longicolla and T. atroviridae.* Trans-6-(p-tolyl)-2-methyl-2-heptanol was exclusively seen in *D. longicolla.* p-Formophenetidide were identified both in *T. atroviridae and D. longicolla.* n-Hexadecanoic acid and Ursodeoxycholic acid were present only in *D. longicolla.* The secondary metabolite Bicyclo [9.3.1] pentadeca-3,7-dien-12-ol,4,8,12, 15,15-pentamethyl-, [1R-(1R*,3E,7E,11R*,12R*)] was widely represented by the fungal strains *D. longicolla, E.*

*endophytica and T. viride.* 1,3,6,10-Cyclotetradecatetraene, 3,7,11-trimethyl-14-(1-methylethyl) was present in three species of fungi such as *D. longicolla, T. asperellum and T. atroviridae. The compound* Alpha-methyl-benzene ethanol was represented by *E. endophytica, T. asperellum and T. viride.* 13 Beta-methyl-13-vinyl-podocarp-7-ene-3 beta-ol and 2'-carboxy-2,5-dimethyl-benzophenone was exclusively presented in *Endomelanconiopsis endophytica.* Bicyclo[9.3.1] pentadeca-3,7-dien-12-ol, 4,8,12,15,15-pentamethyl-, [1R-(1R*,3E,7E,11R)] was seen in *E. endophytica, and T. viride.* Niacin, 2,4-Hexadienedioic acid and 2,6 Bis (1,1-dimethylethyl-phenol) was present only in *T. asperellum.* 2,3-Dihydro-thiophene, 5-(Hydroxymethyl)-2-furancarboxylic acid and Methylphenyl-ethyl ester-carbamic acid and N-[3-(Benzylloxy)phenyl]-N-methylacetamide were seen only in *T. atroviridae.* The fungal strain *T. viride* was an exclusive source of secondary metabolites such as Hexahydrofuro[3,2-b] furan-3,6-diol, Quinoline-3-carboxylic acid, 1,4,5,6,7,8-hexahydro-2-methyl-5-oxo-4-(2-thienyl)-, 1-methylpropyl, 1-(p-Ethoxyphenyl)-3-phenyl-2-thiourea, 3-Methyl-5-nitrosotropone and Bis (2-ethylhexyl) phthalate. No significant antifungal compounds were identified from species such as *N. sphaerica, T. harzianum and X. aristata.* The antifungal activity of the compounds that have been identified in the present investigation can be attributed to their ability to disrupt cell membrane integrity (Aderiye and Olusola, 2015) or by interfering with essential cellular processes, such as nutrient uptake or DNA synthesis or enzyme activity (Ivanov *et al*.,2022; Avis, 2007).

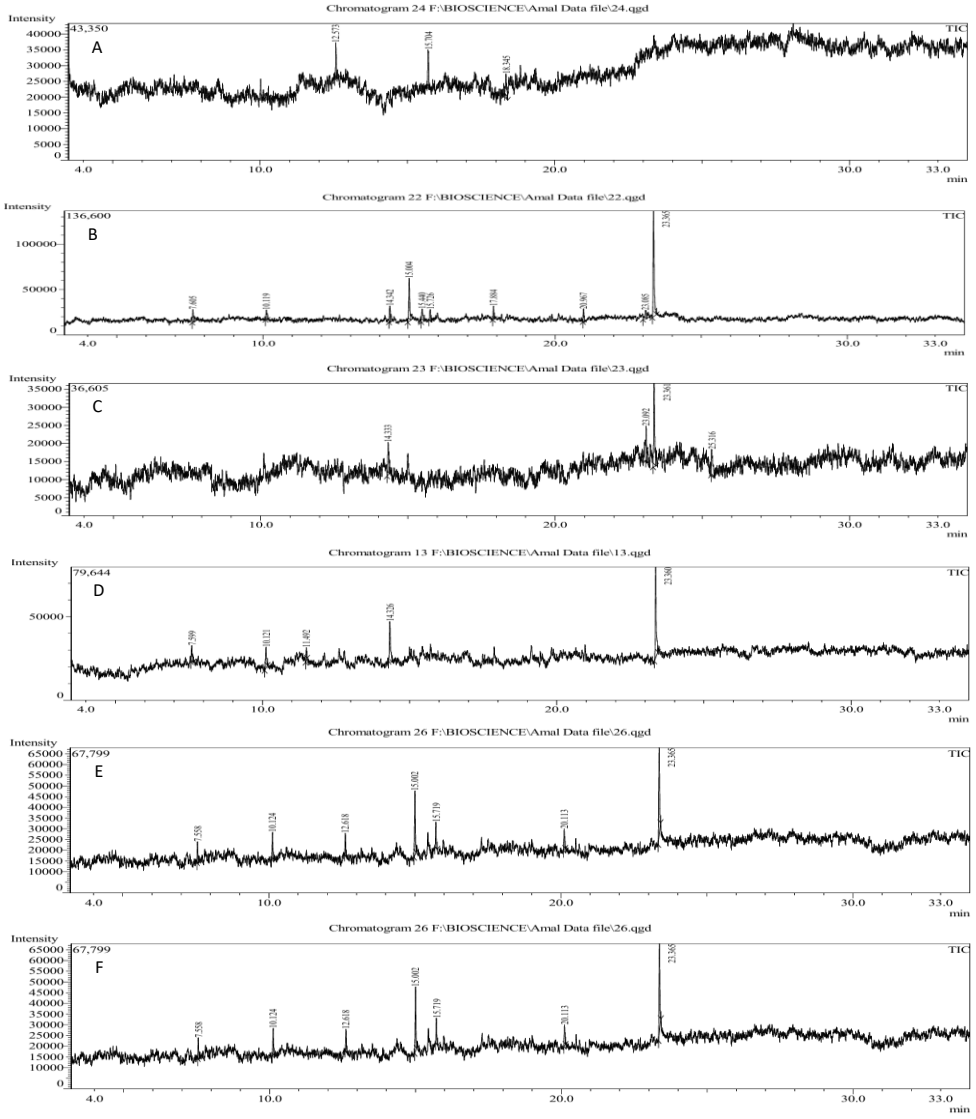


**Figure 3:** GC-MS Chromatogram of the Bio active Compounds identified from A, *Diaporthe eucalyptorum;* B, *Diaporthe longicolla;* C, *Endomelanconiopsis endophytica;* D, *Trichoderma asperellum;* E, *Trichoderma atroviridae;* F, *Trichoderma viride*

The present findings are consistent with previous studies that have reported the production of a wide range of volatile organic compounds by endophytic fungi, including *Trichoderma* species. (Tabarestani *et al*., 2016; Gualtieri *et al*., 2022). The presence of antifungal volatile compounds had been previously testified from methanolic extract of *Trichoderma* spp. (Mulatu *et al*., (2022) and ethyl acetate extract of *E. endophytica* (Pushpavathi and Krishnamurthy, 2023).

A few recent studies have ascertained the antifungal activity of the volatile compounds that were detected in the present study (Hawar *et al*., 2023; Sornakili *et al*., 2020; Santra and Banerjee, 2022; Farhat, 2022). These findings highlight the potential of the endophytic fungi explored in the present study to be used as a source of green chemicals with enormous applications in agriculture, such as the development of eco-friendly, plant-based fungicides.

## CONCLUSION

The present investigation revealed the occurrence of several volatile compounds in the crude fungal extracts through GC-MS analysis. Furthermore, the findings contribute to understanding the chemical diversity of fungal volatiles, which can play a crucial role in fungal interactions, communication and defense mechanisms. The identification of these compounds can aid in exploring their potential roles in biological control and biofuel production. Future studies should focus on elucidating the functional roles of these volatiles and their biosynthetic pathways to harness their full potential. As we continue to unravel the secrets of these fascinating organisms, we open the door to a world of possibilities that could benefit agriculture, medicine, and beyond.

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## DISCLAIMER (ARTIFICAL INTELLIGENCE)

Author(s) here by declare that NO generative AI technologies such as lab language models

(ChatGPT, COPILOT etc) and text to image generators have been used during writing or editing of this manuscript.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

## AUTHOR CONTRIBUTIONS

PK prepared initial draft of the manuscript and managed the literature search. ASD associated with the laboratory experiments, interpreted GC-MS data, did the statistical analysis and final correction of the manuscript. CPG collected the sample for analysis and conducted the laboratory studies.

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