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Secondary Metabolite Profiling of the Endophytic Fungus *Trichoderma longibrachiatum* L2D2 Isolated from *Anaphalis contorta*

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

In the last few decades, research on endophytic fungi has been increasing due to its applications in agriculture, medicine, and the pharmaceutical industry. Many studies indicate that fungal endophytes produce various types of bioactive compounds. In this study, the endophytic fungus *Trichoderma longibrachiatum* L2D2 was isolated from the leaf of the medicinal plant *Anaphalis contorta* and secondary metabolites were collected. The crude extract was analyzed for the presence of seven phytochemicals, and it was observed that alkaloids, flavonoids, phenolics, steroids, tannins, and terpenoids were produced. It was also found that the endophyte extract had a total flavonoid content of 126.07 (µg of quercetin equivalent/mg) and a total phenolic content of 43.97 (µg of gallic acid equivalent/mg). The Gas Chromatography–Mass Spectrometry (GC-MS) analysis reveals the presence of the major compound Dithiooxamide, which has a retention time (RT) of 50.030%, and the Liquid Chromatography–Mass Spectrometry (LC-MS) study shows the

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most abundant compounds as Hexanal (RT of 3.687 min) in positive ionization and Rosamultin (RT of 24.387 min) in negative ionization. Fourier-Transform Infrared (FTIR) Spectroscopy analysis also shows the presence of alcohol, aldehyde, aliphatic ether, aliphatic primary amine, alkene, alkyl halide, amine, aromatic amine, aromatic compound, carboxylic acid, ketone, phenol, and sulfoxide as functional groups. Further studies, like the purification of bioactive compounds and their biological activities, will reveal the applicability of *T. longibrachiatum* L2D2 in industries.

Keywords: Endophytic fungi; bioactive; secondary metabolite; alkaloids; GC-MS; FTIR.

1. INTRODUCTION

Biologically active compounds are derived from microbes, plants, and animals in their natural state. Berries of *Juniperus excelsa* are a rich source of diterpenes that has strong cytotoxic activity [1]. As observed by [2], flower extracts of *Althaea officinalis* contains strong antioxdant compouds that inhibits upto 96.4%. Fungi are the most extensively researched category among microbes in terms of the synthesis of novel compounds [3]. There has been a growing global health apprehension in recent decades regarding the ineffectiveness of numerous antibiotics against drug-resistant microorganisms [4]. The classification 'endophytic fungi' refers to those fungal groups that establish symbiotic associations with photosynthetic plant tissue without inducing any harm to the host plant [5]. Once isolated, the endophytic fungi can be sustained and cultured continuously to generate secondary metabolites within a short time period. Secondary metabolites are non-essential compounds with a low molecular weight that do not contribute to growth and development of the organism but play a critical role in cell signalling and defence mechanisms [6]. Plants have a diverse range of microorganisms, which can be found both inside and externally in their tissues. These microorganisms include bacteria, fungi, archaea, algae, and protists. The presence of these organisms has a significant impact on the survival and biodiversity of plants, as well as the overall functioning of the ecosystem [7]. The most prevalent types of symbiotic partnerships that plants form are mycorrhizal and endophytic relationships. These relationships play an important role in enhancing the growth and disease resistance of plants [8]. According to [9], microorganisms are responsible for producing over 20,000 bioactive metabolites. The endophytes isolated from medicinal plants create potent fungicidal, bactericidal, and cytotoxic chemicals. Additionally, they synthesize enzymes that are used in many activities, including the biotransformation and degradation of organic molecules [10]. Endophytes produce bioactive chemicals that the host plant employs to defend itself against harmful infections. Endophytes can be cultured using synthetic culture media to extract bioactive substances, particularly for healthcare purposes [11]. The unique characteristics and specialized environment of endophytes make them a fascinating area of study for the identification of innovative pharmaceuticals to tackle the increasing prevalence of life-threatening illnesses. [12] claim that endophytic fungi are actively engaged in the production of bioactive metabolites, immunosuppressants, anticancer compounds, and biocontrol agents. A multitude of endangered plants have been heavily and without discrimination collected for their inherent chemicals that possess medical benefits. In order to address this problem, thorough examinations were conducted on the endophytic fungi that are linked with these plants. Endophytic fungi are the least studied group of fungi, and their investigation might lead to the discovery of novel compounds.

The research undertaken by [13] examined the ability of endophytic fungi to create growth-promoting chemicals that are comparable to those generated by their host plants, though in greater magnitudes. The investigation carried out by [14] showed that host plants without fungal endophytes were incapable of withstanding extreme temperature variations, drought, high salt levels, and pathogen infestations. Fungal identification can be accomplished through the application of molecular analysis techniques, including the utilization of the internal transcribed spacer (ITS) section of rDNA, restriction fragment length polymorphism (RFLP), randomly amplified polymorphic DNA (RAPD), and several other molecular markers [15]. The ITS region is widely regarded as the primary barcode marker for fungi. It has three parts: the ITS1 coding area, which is between the 18S and 5.8S ribosomal RNA (rRNA); the 5.8S rRNA; and the ITS2 coding area, which is between the 5.8S and 28S rRNA [16]. Several studies have shown that secondary metabolites produced by endophytic

fungi include alkaloids, benzopyranones, chinones, cytochalasines, depsipeptides, enniatines, flavonoids, furandiones, isocumarines, peptides, polyketones, phenols, quinols, terpenoids, tetralones, and xanthones [17,18]. It has been shown that these compounds are effective against bacteria, fungi, viruses, cancer, free radicals, malaria, insects, inflammation, and atherosclerosis, which makes them promising therapeutic agents for treating a range of ailments, including cancer, malaria, neurological disorders, cardiovascular issues, and autoimmune disorders [19]. In recent decades, there has been a worldwide increase in the market for microbial inoculants that have the capacity to function as biological control. Endophytic fungi derived from medicinal plants have demonstrated to be a plentiful reservoir of bioactive compounds that possess antibacterial, antioxidant, and anticancer properties [20]. Thus, endophytic communities occurring in the tissues of living plants are potential reservoirs of novel natural compounds for exploitation by the pharmaceutical sector. Ethno-medicine has long been crucial in the treatment of diseases, with around 80% of individuals in underdeveloped nations relying on traditional medicinal plants for healthcare [21]. *Anaphalis contorta* is a perennial herb that has traditionally been employed in medicinal practices to heal cuts and wounds, stimulate appetite, induce relaxation, and invigorate the body [22]. The antibacterial effects of the essential oil derived from the leaves of *A. contorta* have been documented by [23]. Analysis of the oil has revealed the presence of β-caryophyllene, γ-curcumene, δ-cadinene, labda-7,14-dien-13-ol, epi-α-cadinol, bulnesol, α-cadinol, β-bisabolol, and labda-8,14-dien-13-ol [24]. This investigation involved the identification of the remaining endophytes and the attempt to discover the bioactive compounds produced by the isolates. In this study, the endophytic fungus *Trichoderma longibrachiatum* L2D2 was isolated from *Anaphalis contorta* and analyzed the secondary metabolites by Gas Chromatography–Mass Spectrometry, Liquid Chromatography–Mass Spectrometry and Fourier-Transform Infrared Spectroscopy.

2. MATERIALS AND METHODS

2.1 Isolation and Identification of Fungal Endophyte

The plant, *Anaphalis contorta* was collected in sterile plastic bags from Phangrei Hill in Ukhrul District, Manipur, and processed for isolation in the laboratory within 24 hours of collection. Isolation was conducted following the protocol given by [25] using 70% ethanol and 4% sodium hypochlorite. Leaf part was used for the isolation and deposited in National Fungal Culture Collection of India (NFCC), Pune, India. The isolate was identified using morphological characters, and confirmed by ITS-rDNA gene sequencing and deposited in GenBank.

2.2 Secondary Metabolite Production

The mycelial plug (0.5 cm diameter) of the endophytic fungus was cut off from a 7 days old culture and inoculated on 500 mL of Potato Dextrose Broth (PDB) for 15 days using a rotory shaker. After filtering through muslin cloth and filter paper (Whatman No. 2), the filtrate was extracted three times with an equal amount of ethyl acetate. The mixture was then dried at 40°C to vaporize the ethyl acetate. The solid mass obtained after drying is the crude ethyl acetate extract of the endophyte, which is stored at 4°C for further analysis [26].

2.3 Biochemical Analysis

The crude extract of *T. longibrachiatum* L2D2 was analyzed for qualitative production of alkaloids, flavonoids, terpenoids, tannins, saponins, steroids, and phenols, and further Total Phenolic Content (TPC) and Total Flavonoid Content (TPC) were evaluated [27,28].

2.3.1 Test for alkaloids

The extract was dissolved in a solution of 2N hydrochloric acid (HCl) and treated with Mayer's reagent. The formation of a precipitate (cream-coloured) indicated the presence of alkaloids.

2.3.2 Test for flavonoids

The crude extract solution (1 mL) was mixed with a 20% sodium hydroxide (NaOH) solution. The colour change of the mixture from yellow to colourless after the addition of dilute hydrochloric acid showed the presence of flavonoids.

2.3.3 Test for phenols

To the fungal extract solution, 5% ferric chloride ($FeCl_3$) was added, and the appearance of a green colour indicated the presence of phenols.

2.3.4 Test for tannins

The fungal extract solution was mixed with an alcoholic $FeCl_3$ reagent. The observation of a bluish-black colour, that disappears with the

addition of dilute H_2SO_4, followed by the formation of a yellowish-brown precipitate, indicated the presence of tannins.

2.3.5 Test for terpenoids

The fungal extract solution was mixed with chloroform and then added with concentrated H_2SO_4. The formation of a reddish-brown precipitate indicated the presence of terpenoids.

2.3.6 Test for saponins

The dried crude extract was mixed with water and shaken. The formation of intense foam suggested the presence of saponins.

2.3.7 Test for steroids

The crude extract was mixed with acetic anhydride and subsequently added with H_2SO_4. The colour change from violet to blue or green in samples indicated the presence of steroids.

2.3.8 Total Phenolic Content (TPC)

The quantification of phenolic compounds in the ethyl acetate extract was conducted by employing the Folin-Ciocalteu method. The extracts of varying concentrations were combined with 10% Folin-Ciocalteu solution and $NaHCO_3$ and incubated at 45°C for 30 minutes. The absorbance was taken at a wavelength of 765 nm, and calibration curves were constructed using different concentrations of gallic acid (100 to 500 µg/mL) as standard.

2.3.9 Total Flavonoid Content (TFC)

The quantification of the total flavonoid content was conducted using the colorimetric technique. The endophyte extract was diluted with deionized water, added to a 5% sodium nitrite solution, and then incubated for 6 minutes at room temperature. Further, a solution of 10% aluminium chloride was added to the mixture and incubated for 5 minutes with a mixed sodium hydroxide solution. The solution was diluted with distilled water and incubated at 25 ºC for 30 minutes, and the absorbance was recorded at 510 nm. The flavonoid concentration was determined by employing a standard curve (5 to 100 µg/mL) of quercetin.

2.4 Gas Chromatography–Mass Spectrometry (GC-MS) Analysis

The crude ethyl acetate extract of *T. longibrachiatum* L2D2 was analyzed with GC-MS to detect the presence of volatile compounds using Clarus 680C GC and Clarus 600C MS. A mixture of 5% diphenyl and 95% dimethyl polysiloxane was used as the stationary phase, and helium gas (99.99% purity) was employed as the mobile phase. The overall duration of the run was roughly 39 minutes [29]. The peaks that appeared in the chromatogram were selected based on maximum area percentages and identified by using library search software, National Institute of Standard and Technology-2014 (NIST-2014).

2.5 Liquid Chromatography–Mass Spectrometry (LC-MS) Analysis

The presence of non-volatile compounds in the ethyl acetate extract of *T. longibrachiatum* L2D2 was analyzed with LC-MS using HPLC 1260 Infinity coupled with Aligent 6410 MS. The mobile phase consists of two components: 0.1% formic acid dissolved in water and 0.1% formic acid dissolved in acetonitrile. The nitrogen gas was used for ionization with a nebulizing and drying flow rate of 1.5 L/minute and 10 L/minute, respectively. Both positive and negative ionization modes were performed [30]. The peaks with higher abundance in the chromatogram were identified by using the CHEMnetBASE and MassBank databases.

2.6 Fourier-Transform Infrared Spectroscopy (FTIR) Analysis

The crude extract of *T. longibrachiatum* L2D2 was analyzed with FTIR for the presence of various functional groups using the IRAffinity-1S - FTIR spectrophotometer. The scanning range of 400–4000 cm^{-1} and a resolution of 1 cm^{-1} were employed. The peaks were identified using standard references [31,32].

3. RESULTS AND DISCUSSION

3.1 Isolation and Identification of Fungal Endophyte

Gene sequencing showed 100% similarity with the known stains of *Trichoderma longibrachiatum*. The NFCCI and GenBank accession numbers obtained were “NFCCI 4725” and “OR357716,” respectively (Table 1, Fig. 1). The isolate *T. longibrachiatum* L2D2 was observed to have a fast growth rate that covers the entire 90 mm petriplate in 3 days of incubation, sporulate after day 4, produce a mint odour, and have light yellow pigmentation. In various studies, several stains of *T.*

longibrachiatum were isolated as endophytic fungi that possess biological activities. *Trichoderma longibrachiatum* WKA55 was isolated from peanut seeds and found to inhibit spore germination of three mycotoxinogenic fungi while at the same time increasing seed germination and the vigor index of peanut seeds [33]. In another study, *T. longibrachiatum* MD33, isolated from the medicinal plant *Dendrobium nobile*, produced the natural bioactive compound dendrobine, and its secondary metabolite showed potent antibacterial activity against *Bacillus subtilis*, *B. mycoides*, and *Staphylococcus* sp. [34,35] isolated *Trichoderma longibrachiatum* EF5 from rice and found it to exhibit potential biocontrol activity against the rot pathogen *Macrophomina phaseolina* with 58% inhibition.

3.2 Secondary Metabolite Production

The crude ethyl acetate extract of *T. longibrachiatum* L2D2 yields 634 mg (approximately) of dry metabolite in 1000 mL of PDB (Fig. 2). Endophytic fungi have been known as a novel source of bioactive secondary metabolites that possess diverse biological activities.

Fig. 1. Sequence allignment report for *T. longibrachiatum* L2D2 with the closest genetic neighbour strain *Trichoderma longibrachiatum* strain CEN1281 in NCBI GenBank database

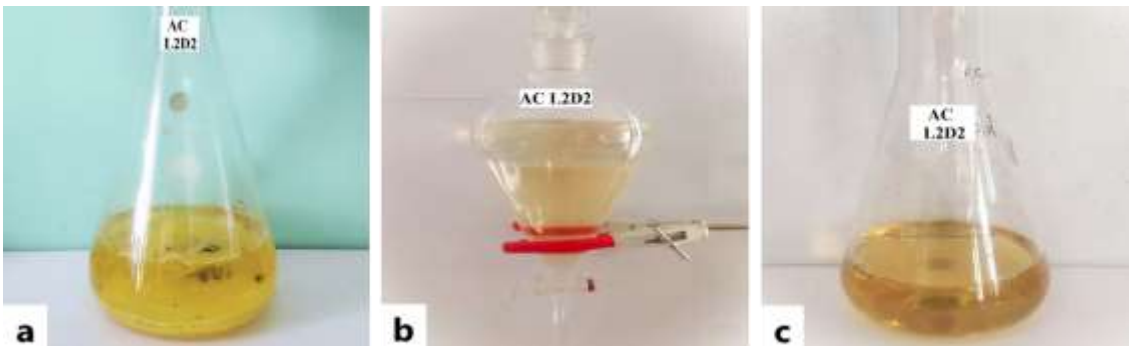

Fig. 2. Secondary metabolite extraction of *T. longibrachiatum* L2D2, (a) culture in potato dextrose broth, (b) extraction using separating funnel, (c) crude ethyl acetate extract before drying

Table 1. The BLAST analysis report for *T. longibrachiatum* L2D2 showing closely related five taxa available in GenBank database

Gene Bank Accession No.	Description	Max score	Query cover	Query coverage (%)	E value	Identity (%)
OM515078	*Trichoderma longibrachiatum* strain CEN1281	1045	1045	100	0.0	100.00
KJ174214	*Trichoderma longibrachiatum* isolate ISHAM-ITS_ID MITS2528	1045	1045	100	0.0	100.00
FJ459964	*Trichoderma longibrachiatum* isolate T50	1045	1045	100	0.0	100.00
KF889068	*Trichoderma longibrachiatum* isolate 124L	1038	1038	99	0.0	100.00
OM515079	*Trichoderma longibrachiatum* strain CEN1562	1040	1040	100	0.0	99.83

Table 2. Biochemical analysis of the secondary metabolite produce by *T. longibrachiatum* L2D2

Endophyte	Biochemical analysis								
	Qualitative							Quantitative	
	AL	FL	PH	SA	ST	TA	TE	TFC (µg of quercetin equivalent/ mg of endophyte extract)	TPC (µg of gallic acid equivalent/ mg of endophyte extract)
Trichoderma longibrachiatum L2D2	+	+	+	-	+	+	+	126.07±1.12	43.97±0.71

AL-Alkaloids, FL-Flavonoids, PH-Phenolics, SA-Saponins, ST-Steroids, TA- Tannins, TE- Terpenoids
'+' indicates presence, '-' indicates absence

3.3 Biochemical Analysis

The ethyl acetate extract of *T. longibrachiatum* L2D2 reveals the presence of all the tested biochemicals except Saponin. Quantitative analysis has shown a higher total flavonoid content of 126.07 (µg of quercetin equivalent/mg of endophyte extract) and a total phenolic content of 43.97 (µg of gallic acid equivalent/mg of endophyte extract) (Table 2). Alkaloids are of great importance due to their biological activities and medicinal properties. Vincristine and vinblastine are major alkaloids that have been used as anticancer agents [36]. The famous drug, Quinine, is an alkaloid isolated from the bark of *Cinchona ledgeriana* that possesses strong anti-malarial properties against *Plasmodium vivax* [37]. Endophytic fungi are known to produce a wide range of alkaloids. In a study, Diketopiperazine alkaloids were obtained from the endophytic fungus *Aspergillus* sp. isolated from *Sterculia apetala* and were found to exhibit antidiabetic activity against α-glucosidase and the PTP1B enzyme [38]. The alkaloid Amoenamide obtained from the secondary metabolite of the endophytic fungus *Fusarium sambucinum* residing in *Nicotiana tabacum* has shown antimicrobial and insecticidal activities [39]. In a recent study conducted by [40], an alkaloid, Penicimine A, was extracted from the endophytic fungus *Penicillium expansum* isolated from *Plantago depressa* and showed anti-inflammatory activity against LPS-induced nitric oxide with an IC_{50} value of 25.65 µM. Flavonoid compounds are known to show a variety of pharmacological activities, namely, anti-inflammatory, antioxidant, antiviral, antimicrobial, anticancer, cardioprotective, and neuroprotective. In a study, the flavonoid Chrysin was isolated from the endophytic fungi *Alternaria alternata*, *Colletotrichum capsici*, and *C. taiwanense*, which showed significant cytotoxic activity against human liver carcinoma cells (HepG2) [41]. The phenolic compounds had been demonstrated to have anticancer, antimicrobial, and antioxidant activities. The phenolic compounds tyrosol and p-hydroxyphenylacetamide were isolated from the endophytic fungus *Coriolopsis rigida* associated with the medicinal plant *Cochlospermum regium* and found to show strong antioxidant properties with an EC_{50} of 0.33 mg/mL [42]. In another study, the phenolic compound 4-(2,4,7-trioxa-bicyclo[4.1.0]heptan-3-yl) phenol was extracted from the endophyte *Pestalotiopsis mangiferae* associated with *Mangifera indica* and reported to exhibit potential antibacterial and antifungal activity against *Bacillus subtilis*, *Klebsiella pneumoniae*, *Escherichia coli*, *Micrococcus luteus*, *Pseudomonas aeruginosa*, and *Candida albicans* [43]. [44] have isolated seven new phenolic compounds, viz., p-hydroxyphenyllactic acid, p-hydroxybenzoic acid, p-hydroxybenzaldehyde, phenyllactic acid, n-butyl-3,4-dihydroxybenzoate, n-hexyl-3,4-dihydroxybenzoate, and n-octyl-3,4-dihydroxybenzoate, from the ethyl acetate extract of endophyte *Camarops* sp. associated with *Alibertia macrophylla*. Compounds belonging to steroids are well known for their cytotoxic, antimicrobial, anti-inflammatory, and immunosuppressive properties. Three steroidal compounds, norcyclocitrinol A, erythro-11α-hydroxyneocyclocitrinol, and pesudocyclocitrinol A, were isolated from the broth culture of the endophytic fungus *Penicillium chrysogenum* collected from the stem of *Huperzia serrata* and have shown moderate cytotoxic activity [45]. [46] extracted the steroid Aspergilolide from the endophytic fungus *Aspergillus* sp. isolated from *Paeonia ostia*, which showed potential anticancer activity against five human cancer cell lines. Tannins are also an important source of antioxidant, anti-inflammatory, anti-diabetic, cardioprotective, and antimicrobial agents. Several endophytic fungi were reported to produce tannins, viz., *Aspergillus nidulans* from *Passifora incarnate* [47]; *Alternaria alternata*, *Thielaviopsis basicola*, *Geotrichium albida*, and *Penicillium frequentans* from *Pinus roxburghii* [48]; *Xylaria feejeensis* from *Avicennia marina* [49]; and *Penicillium roqueforti* from *Solanum surattense* [50]. Terpenoids also possess a wide range of biological activities, including anticancer, antimicrobial, anti-inflammatory, antioxidant, and antiallergic. The terpenoids Harziane and Cadinane, extracted from the endophyte *Trichoderma asperellum* of the red alga *Gracilaria verrucosa*, have shown potent inhibition of phytoplankton [51]. [52] isolated five terpenoids, Tricycloalternarenes A–E that showed anticancer activity. This study indicates that the secondary metabolite produced by the endophytic fungus *T. longibrachiatum* L2D2 might possess significant biological activities.

3.4 GC-MS Analysis

From the GC-MS data, 15 peaks of volatile compounds with higher area percentages were identified, namely: benserazide, Benserazide, Ethanedithioamide, N-hexyl acrylate, L-azetidine-2-carboxylic acid, Phenylethyl alcohol, Dodecane, 1-fluoro-, Tritetracontane,

Benzeneethanol, 4-hydroxy-, Hexadecane, Photocitral B, 18-Norabietane, Dotriacontane, 7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione, Diisobutyl phthalate and Eicosane, 1-iodo. The major compound obtained was Dithiooxamide (50.030%), followed by Diisobutyl phthalate (14.455%) and Benserazide (9.502%) (Table 3, Fig. 3). In a study, two dithiooxamide derivatives, 1-Morpholinomethyl-3(1′ -N-dithiooxamide)iminoisatin and 1-diphenylaminomethyl-3-1′-N-dithiooxamide)iminoisatin, have shown antibacterial and anticancer activities [51]. Diisobutyl phthalate has been reported to show antifungal activity against *Rhizoctonia solani*, which causes the spot disease of tobacco leaf [52]. [53] observed the tumour suppression activity of Benserazide by targeting hexokinase 2. The GC-MS analysis of the essential oil of *Origanum syriacum* have identified the presence of γ-terpinene (26.7%), thymol (26.6%) and carvacrol (22.9%) as major components and the oil also showed strong insecticidal activity against *Rhyzopertha dominica* [54].

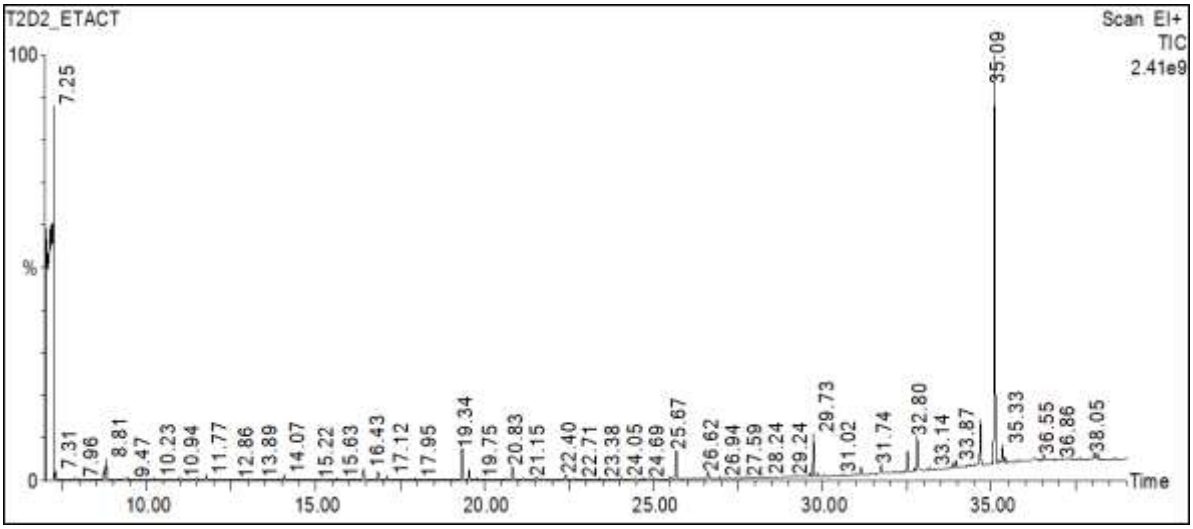

Fig. 3. GC-MS chromatogram of the ethyl acetate extract of secondary metabolite produced by *T. longibrachiatum* L2D2 reveals the presence of volatile compounds

Table 3. Volatile compounds detected by GC-MS analysis of ethyl acetate extract of *T. longibrachiatum* L2D2

Retention Time (min)	Compound Name	Area %	Molecular Weight	Molecular formula
7.023	Benserazide	9.502	257	$C_{10}H_{15}N_3O_5$
7.248	Dithiooxamide	50.030	120	$C_2H_4N_2S_2$
8.808	N-hexyl acrylate	0.655	156	$C_9H_{16}O_2$
16.427	L-azetidine-2-carboxylic acid	0.650	101	$C_4H_7NO_2$
19.338	Phenylethyl alcohol	1.397	122	$C_8H_{10}O$
20.834	Dodecane, 1-fluoro-	0.528	188	$C_{12}H_{25}F$
25.671	Tritetracontane	1.204	604	$C_{43}H_{88}$
26.622	Benzeneethanol, 4-hydroxy-	0.470	138	$C_8H_{10}O_2$
29.733	Hexadecane	1.475	226	$C_{16}H_{34}$
31.739	Photocitral B	0.494	152	$C_{10}H_{16}O$
32.514	18-Norabietane	0.778	262	$C_{19}H_{34}$
32.799	Dotriacontane	1.291	450	$C_{32}H_{66}$
34.670	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	1.762	276	$C_{17}H_{24}O_3$
35.095	Diisobutyl phthalate	14.455	278	$C_{16}H_{22}O_4$
35.330	Eicosane, 1-iodo	0.602	408	$C_{20}H_{41}I$

3.5 LC-MS Analysis

The LC-MS analysis of ethyl acetate extracts of *T. longibrachiatum* L2D2 has revealed the presence of various non-volatile compounds. Based on higher abundance, 11 peaks were identified in the positive mode, viz., Pyrogallol, Hordenine, Hexanal, Fisetin, Resveratrol, Hydroxycaffeic acid, Stachydrine, Equol, Lotaustralin, Betalamic acid and Glepidotin B. From negative mode 9 most abundant peaks were identified namely Hippuric acid, Eugenol, Tricinonoic acid, Hordatine A, Vanilloyl-glycine, Norambreinolide, Rosamultin, L-histidinol, and Kaempferide. In the case of positive ionization, the most prominent peak was found to be Hexanal (RT of 3.687 min), and for negative ionization, Rosamultin (RT of 24.387 min) was the most abundant (Table 4, Fig. 4). [55] observed that the Hexanal vapour can be used as an effective control measure for post-harvest pathogens of banana viz., *Colletotrichum gloeosporioides* and *Lasiodiplodia theobromae* which results in a reduction of anthracnose and stem-end rot diseases upto 75.2% and 80.2%, respectively. [56] reported that Rosamultin extracted from *Potentilla anserine* possesses antioxidant as well as nephroprotective properties, which reduce urinary protein excretion, kidney index, blood urea nitrogen level, and ameliorate histopathological damage and fibrosis of renal tissue. In another study, [57] isolated Rosamultin from *Rosa rugose*, which showed a potent inhibitory effect against HIV-1 protease upto 53% at a concentration of 100 µM.

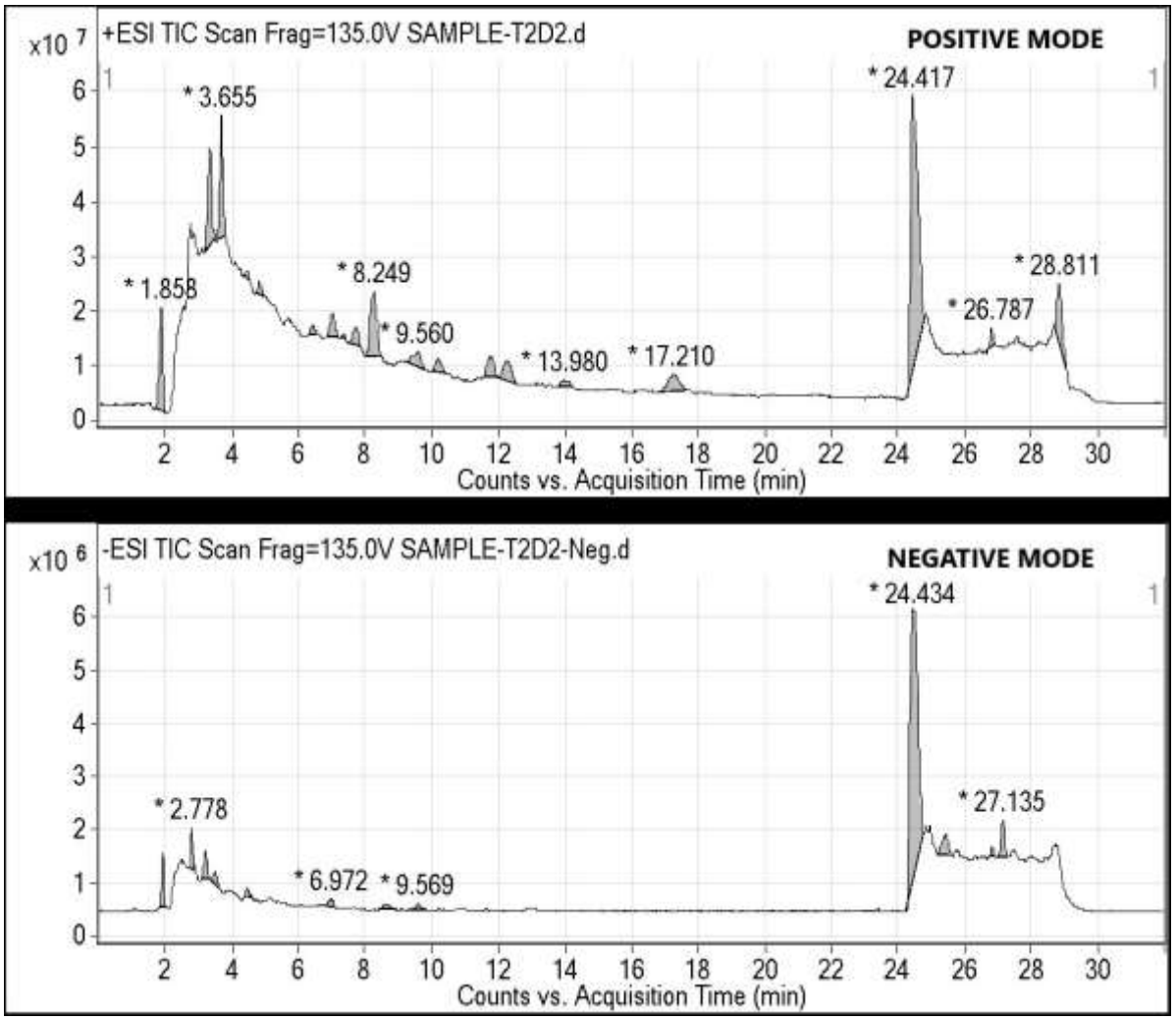

Fig. 4. GC-MS chromatogram of the ethyl acetate extract of secondary metabolite produced by *T. longibrachiatum* L2D2 reveals the presence of volatile compounds

Table 4. LC-MS peak identification of major compounds obtained from ethyl acetate extracts of *T. longibrachiatum* L2D2

Positive Ionization Mode					
Retention time (min)	**Charge-to-mass (m/z)**	**Compound name**	**Abundance**	**Molecular Weight**	**Molecular Formula**
1.843	127.0	Pyrogallol	187640.4	126	$C_6H_6O_3$
3.383	166.1	Hordenine	1286919.3	165	$C_{10}H_{15}NO$
3.687	100.1	Hexanal	2493423	100	$C_6H_{12}O$
4.453	171.1	Fisetin	86888.6	286	$C_{15}H_{10}O_6$
4.823	227.1	Resveratrol	506430.6	228	$C_{14}H_{12}O_3$
7.184	197.1	Hydroxycaffeic acid	275533.7	196	$C_9H_{10}O_4$
7.651	144.1	Stachydrine	113972.8	143	$C_7H_{13}NO_2$
9.562	243.1	Equol	71201.5	242	$C_{15}H_{14}O_3$
12.268	261.1	Lotaustralin	176056.1	261	$C_{11}H_{19}NO_6$
13.974	211.1	Betalamic acid	61836.7	211	$C_9H_9NO_5$
24.523	453.4	Glepidotin B	239459.3	340	$C_{20}H_{20}O_5$
Negative Ionization Mode					
Retention time (min)	**Charge-to-mass (m/z)**	**Compound name**	**Abundance**	**Molecular Weight**	**Molecular Formula**
1.896	179.2	Hippuric acid	7670.4	179	$C_9H_9NO_3$
3.176	164.1	Eugenol	26217.5	164	$C_{10}H_{12}O_2$
3.483	241.2	Tricinonoic acid	19310.4	252	$C_{15}H_{24}O_3$
4.438	275.2	Hordatine A	18046	550	$C_{28}H_{38}N_8O_4$
8.691	225.1	Vanilloyl-glycine	4875.8	225	$C_{10}H_{11}NO_5$
9.568	249.2	Norambreinolide	6744.4	250	$C_{16}H_{26}O_2$
24.387	511.4	Rosamultin	107593.2	650	$C_{36}H_{58}O_{10}$
25.358	279.3	L-histidinol	21921.3	141	$C_6H_{11}N_3O$
27.193	299.3	Kaempferide	51948.6	300	$C_{16}H_{12}O_6$

3.6 FTIR Analysis

The FTIR analysis of the ethyl acetate extract of *T. longibrachiatum* L2D2 has shown the presence of 13 functional groups, viz., alcohol, aldehyde, aliphatic ether, aliphatic primary amine, alkene, alkyl halide, amine, aromatic amine, aromatic compound, carboxylic acid, ketone, phenol, and sulfoxide (Table 5, Fig. 5). These functional groups are important for the formation of biochemicals, which are important in bioactivities. Alkaloids contain functional groups such as hydroxyl, amine, alcohol, carbonyl, nitrile, ester, and amide [57,58]. Terpenoids contain hydroxyl, carboxylic acid, ketone, and aldehyde [59-62].

Table 5. Interpretation of FTIR spectrum of ethyl acetate extracts of *T. longibrachiatum* L2D2

Functional group	Peak frequency (cm^{-1})
Alcohol (O-H stretching)	1419.61; 1078.21
Aldehyde (C-H stretching)	1697.36
Aliphatic ether (C-O stretching)	1097.50
Aliphatic primary amine (N-H stretching)	3267.41
Alkene (C=C stretching)	2956.87; 1471.69; 1463.97; 920.05; 719.45; 688.59
Alkyl halide (C-F stretching)	1296.16; 1259.52; 1016.49; 798.53 659.66; 640.37; 545.85
Amine (N-H bending)	1558.48; 1541.12; 1508.33
Aromatic amine (C-N stretching)	1199.72
Aromatic compound (C-H bending)	1869.02; 1843.95; 1830.45
Carboxylic acid (O-H stretching)	2916.37; 2848.86
Ketone (C=O stretching)	1683.86
Phenol (O-H bending)	1311.59
Sulfoxide (S=O stretching)	1049.28

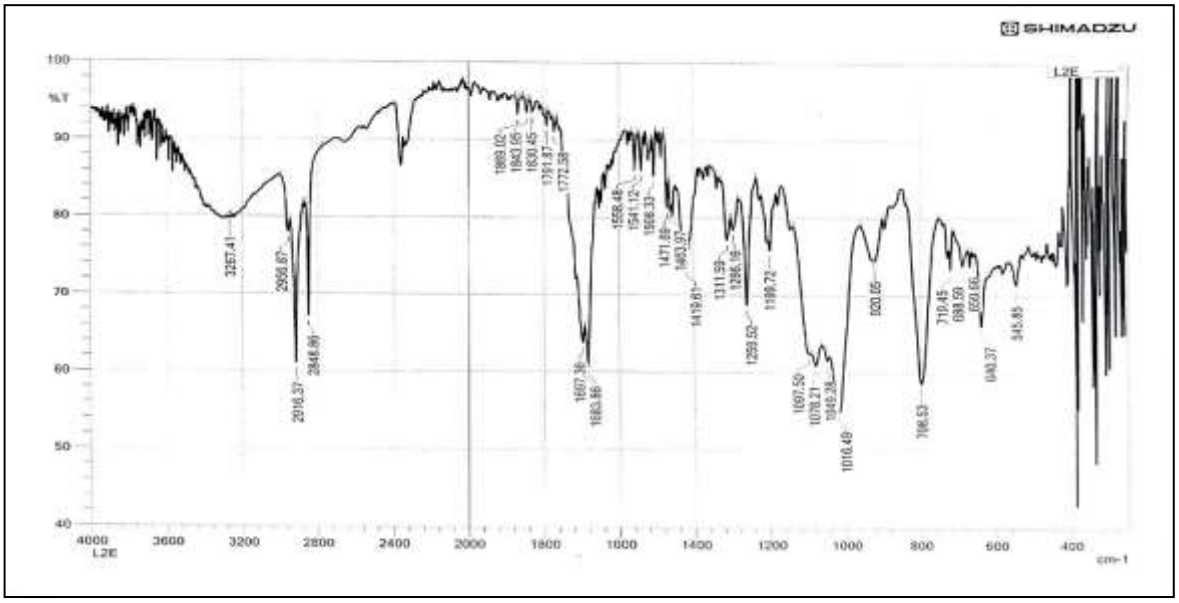

Fig. 5. FTIR spectrum chromatogram of ethyl acetate extracts of *T. longibrachiatum* L2D2. X-axis represents frequency (cm^{-1}) and Y-axis represents Transmittance (%)

4. CONCLUSION

From the above study, it can be concluded that medicinal plants are associated with mycoflora inside the tissue and produce several types of compounds as secondary metabolites. Endophytic fungi release biochemicals that are similar to phytochemicals produced by the plant. The isolate *T. longibrachiatum* L2D2 produces six biochemicals out of seven, which might play an important role in plant defence against biotic and abiotic factors. GC-MS and LC-MS analysis reveal that the ethyl acetate extract of *T. longibrachiatum* L2D2 contains numerous bioactive compounds, and FTIR analysis shows the major functional groups present in the compounds. Further analysis, like the purification of the compounds from the crude extract and the evaluation of biological activities, are important research areas that will provide its potential application in the medicinal and pharmaceutical industries.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Topçu G, Erenler R, Çakmak O, Johansson CB, Çelik C, Chai HB, Pezzuto JM. Diterpenes from the berries of *Juniperus excelsa*. Phytochemistry. 1999;50(7):1195-1199. Available:https://doi.org/10.1016/S0031-9422(98)00675-X
2. Elmastas M, Ozturk L, Gokce I, Erenler R, Aboul-Enein HY. Determination of antioxidant activity of marshmallow flower (*Althaea officinalis* L.). Analytical Letters. 2004;37(9):1859-1869. Available:https://doi.org/10.1081/AL-120039431
3. Sanchez S, Demain AL. Bioactive products from fungi. Food bioactives: Extraction and biotechnology applications. 2017;59-87. Available:https://doi.org/10.1007/978-3-319-51639-4_3
4. Prestinaci F, Pezzotti P, Pantosti A. Antimicrobial resistance: A global multifaceted phenomenon. Pathogens and Global Health. 2015;109:309-318. Available:https://doi.org/10.1179/2047773215Y.0000000030
5. Amirita A, Sindhu P, Swetha J, Vasanthi NS, Kannan KP. Enumeration of endophytic fungi from medicinal plants and screening of extracellular enzymes. World J Sci Technol. 2012;2:13-19.
6. Jha Y, Mohamed HI. Plant secondary metabolites as a tool to investigate biotic

stress tolerance in plants: A review. Gesunde Pflanzen. 2022;74:771-790. Available:https://doi.org/10.1007/s10343-022-00669-4

7. Perreault R, Laforest-Lapointe I. Plant-microbe interactions in the phyllosphere: Facing challenges of the anthropocene. The ISME Journal. 2022;16:339-345. Available:https://doi.org/10.1038/s41396-021-01109-3
8. Khare E, Mishra J, Arora NK. Multifaceted interactions between endophytes and plant: Developments and prospects. Frontiers in Microbiology. 2018;9:2732. Available:https://doi.org/10.3389/fmicb.2018.02732
9. Berdy J. Bioactive microbial metabolites. The Journal of Antibiotics. 2005;58:1-26. Available:https://doi.org/10.1038/ja.2005.1
10. Sudha V, Govindaraj R, Baskar K, Al-Dhabi NA, Duraipandiyan V. Biological properties of endophytic fungi. Brazilian Archives of Biology and Technology. 2016;59:e16150436. Available:https://doi.org/10.1590/1678-4324-2016150436
11. Pimentel MR, Molina G, Dionísio AP, Maróstica Junior MR, Pastore GM. The use of endophytes to obtain bioactive compounds and their application in biotransformation process. Biotechnology Research International; 2011. Available:https://doi.org/10.4061/2011/576286
12. Njoroge HW. Screening of *Streptomyces* isolates from mau forest comple for antimicrobial activity against selected plant pathogens (Doctoral dissertation, Egerton University); 2017. Available:http://41.89.96.81:8080/xmlui/handle/123456789/2095
13. Zhao J, Zhou L, Wang J, Shan T, Zhong L, et al. Endophytic fungi for producing bioactive compounds originally from their host plants. Curr Res, Technol Educ Trop Appl Microbiol Microbial Biotechnol. 2010; 1:567-576.
14. Saikkonen K, Saari S, Helander M. Defensive mutualism between plants and endophytic fungi?. Fungal Diversity. 2010; 41:101-113. Available:https://doi.org/10.1007/s13225-010-0023-7
15. Shamim M, Kumar P, Kumar RR, Kumar M, Kumar RR, et al. Assessing fungal biodiversity using molecular markers. Molecular Markers in Mycology: Diagnostics and Marker Developments. 2017;305-333. Available:https://doi.org/10.1007/978-3-319-34106-4_15
16. Bellemain E, Carlsen T, Brochmann C, Coissac E, Taberlet P, et al. ITS as an environmental DNA barcode for fungi: An in silico approach reveals potential PCR biases. BMC Microbiology. 2010;10: 1-9. Available:https://doi.org/10.1186/1471-2180-10-189
17. Nisa H, Kamili AN, Nawchoo IA, Shafi S, Shameem N, et al. Fungal endophytes as prolific source of phytochemicals and other bioactive natural products: A review. Microbial Pathogenesis. 2015;82:50-59. Available:https://doi.org/10.1016/j.micpath.2015.04.001
18. Aharwal RP, Kumar S, Sandhu SS. Endophytic mycoflora as a source of biotherapeutic compounds for disease treatment. Journal of Applied Pharmaceutical Science. 2016;6:242-254. Available:https://doi.org/10.7324/JAPS.2016.601034
19. Manganyi MC, Ateba CN. Untapped potentials of endophytic fungi: A review of novel bioactive compounds with biological applications. Microorganisms. 2020;8: 1934. Available:https://doi.org/10.3390/microorganisms8121934
20. Rana KL, Kour D, Kaur T, Devi R, Negi C, et al. Endophytic fungi from medicinal plants: biodiversity and biotechnological applications. In Microbial endophytes 273-305. Woodhead Publishing; 2020. Available:https://doi.org/10.1016/B978-0-12-819654-0.00011-9
21. Bhandari BS ed. Ethnomedicinal plant use and practice in traditional medicine. IGI Global; 2020.
22. Joshi RK. Essential oil of flowers of *Anaphalis contorta*, an aromatic and medicinal plant from India. Natural Product Communications. 2013;8:1934578X1300800224. Available:https://doi.org/10.1177/1934578X13008002
23. Rawat K, Prasad K, Bisht G. Phytochemical analysis and antioxidant activity essential oil of *Anaphalis contorta* from Uttrakhand Himalayas. J Anal Pharm Res. 2017;6:00172. Available:https://doi.org/10.15406/japlr.2017.06.00172

24. Pande C, Joshi RK, Sammal SS. Chemical Composition of the Essential Oil of *Anaphalis contorta* Hook f. Journal of Essential Oil Research. 2008;20:444-445. Available:https://doi.org/10.1080/10412905.2008.9700054
25. Hallmann J, Berg G, Schulz B. Isolation procedures for endophytic microorganisms. In Microbial root endophytes: 299-319. Berlin, Heidelberg: Springer Berlin Heidelberg; 2006.
26. Sharma D, Pramanik A, Agrawal PK. Evaluation of bioactive secondary metabolites from endophytic fungus *Pestalotiopsis neglecta* BAB-5510 isolated from leaves of *Cupressus torulosa* D. Don. 3 Biotech. 2016;6:210. Available:https://doi.org/10.1007/s13205-016-0518-3
27. Devi NN, Prabakaran JJ, Wahab F. Phytochemical analysis and enzyme analysis of endophytic fungi from *Centella asiatica*. Asian Pacific Journal of Tropical Biomedicine. 2012;2:S1280-S1284. Available:https://doi.org/10.1016/S2221-1691(12)60400-6
28. Kumar V, Prasher IB. Phytochemical Analysis and Antioxidant Activity of Endophytic Fungi Isolated from *Dillenia indica* Linn. Applied Biochemistry and Biotechnology. 2023;1-18. Available:https://doi.org/10.1007/s12010-023-04498-7
29. Bhattacharyya R, Boruah J, Medhi K, Borkataki S. Phytochemical Analysis of Leaves of *Xylosma longifolia* Clos.: A Plant of Ethnomedicinal Importance. Int. J. Pharm. Sci. Res. 2020;11:2065-2074. Available:http://dx.doi.org/10.13040/IJPSR.0975-8232.11(5).2065-74
30. Shaker KH, Zohair MM, Hassan AZ, Sweelam HTM, Ashour WE. LC–MS/MS and GC–MS based phytochemical perspectives and antimicrobial effects of endophytic fungus *Chaetomium ovatoascomatis* isolated from *Euphorbia milii*. Archives of Microbiology. 2022;204:661. Available:https://doi.org/10.1007/s00203-022-03262-5
31. Talari ACS, Martinez MAG, Movasaghi Z, Rehman S, Rehman IU. Advances in Fourier transform infrared (FTIR) spectroscopy of biological tissues. Applied Spectroscopy Reviews. 2017;52:456-506. Available:https://doi.org/10.1080/05704928.2016.1230863
32. Nandiyanto ABD, Oktiani R, Ragadhita R. How to read and interpret FTIR spectroscope of organic material. Indonesian Journal of Science and Technology. 2019;4:97-118.
33. Al-Askar AA, Rashad EM, Moussa Z, Ghoneem KM, Mostafa AA, et al. A novel endophytic *Trichoderma longibrachiatum* WKA55 with biologically active metabolites for promoting germination and reducing mycotoxinogenic fungi of peanut. Frontiers in Microbiology. 2022;13:772417. Available:https://doi.org/10.3389/fmicb.2022.772417
34. Sarsaiya S, Jain A, Fan X, Jia Q, Xu Q, et al. New insights into detection of a dendrobine compound from a novel endophytic *Trichoderma longibrachiatum* strain and its toxicity against phytopathogenic bacteria. Frontiers in Microbiology. 2020;11:337. Available:https://doi.org/10.3389/fmicb.2020.00337
35. Sridharan AP, Sugitha T, Karthikeyan G, Nakkeeran S, Sivakumar U. Metabolites of *Trichoderma longibrachiatum* EF5 inhibits soil borne pathogen, *Macrophomina phaseolina* by triggering amino sugar metabolism. Microbial Pathogenesis. 2021;150:104714. Available:https://doi.org/10.1016/j.micpath.2020.104714
36. Alam MM, Naeem M, Khan MMA, Uddin M. Vincristine and vinblastine anticancer catharanthus alkaloids: Pharmacological applications and strategies for yield improvement. *Catharanthus roseus*: Current Research and Future Prospects. 2017;277-307. Available:https://doi.org/10.1007/978-3-319-51620-2_11
37. Parveen S, Maurya N, Meena A, Luqman S. Cinchonine: A Versatile Pharmacological Agent Derived from Natural Cinchona Alkaloids. Current Topics in Medicinal Chemistry; 2023. Available:https://doi.org/10.2174/0115680266270796231109171808
38. Ye G, Huang C, Li J, Chen T, Tang J, et al. Isolation, structural characterization and antidiabetic activity of new diketopiperazine Alkaloids from mangrove endophytic fungus *Aspergillus* sp. 16-5c. Marine Drugs. 2021;19:402. Available:https://doi.org/10.3390/md19070402

39. Zhang P, Yuan XL, Du YM, Zhang HB, Shen GM, et al. Angularly prenylated indole alkaloids with antimicrobial and insecticidal activities from an endophytic fungus *Fusarium sambucinum* TE-6L. Journal of Agricultural and Food Chemistry. 2019;67:11994-12001. Available:https://doi.org/10.1021/acs.jafc.9b05827
40. Yao S, Zhang R, Wang J, Gu L, Hu Z, et al. A new diketopiperazine-type alkaloid from the endophytic fungus *Penicillium expansum*. Natural Product Research. 2023;37:3716-3721. Available:https://doi.org/10.1080/14786419.2022.2099391
41. Seetharaman P, Gnanasekar S, Chandrasekaran R, Chandrakasan G, Kadarkarai M, et al. Isolation and characterization of anticancer flavone chrysin (5, 7-dihydroxy flavone)-producing endophytic fungi from *Passiflora incarnata* L. leaves. Annals of Microbiology. 2017;67:321-331. Available:https://doi.org/10.1007/s13213-017-1263-5
42. Dantas SBS, Moraes GKA, Araujo AR, Chapla VM. Phenolic compounds and bioactive extract produced by endophytic fungus *Coriolopsis rigida*. Natural Product Research. 2023;37:2037-2042. Available:https://doi.org/10.1080/14786419.2022.2115492
43. Subban K, Subramani R, Johnpaul M. A novel antibacterial and antifungal phenolic compound from the endophytic fungus *Pestalotiopsis mangiferae*. Natural Product Research. 2013;27:1445-1449. Available:https://doi.org/10.1080/14786419.2012.722091
44. Gubiani JR, Habeck TR, Chapla VM, Silva GH, Bolzani VS, et al. One strain-many compounds (OSMAC) method for production of phenolic compounds using *Camarops* sp., an endophytic fungus from *Alibertia macrophylla* (Rubiaceae). Química Nova. 2016;39:1221-1224. Available:https://doi.org/10.21577/0100-4042.20160169
45. Ying YM, Zheng ZZ, Zhang LW, Shan WG, Wang JW, et al. Rare C25 steroids produced by *Penicillium chrysogenum* P1X, a fungal endophyte of *Huperzia serrata*. Helvetica Chimica Acta. 2014;97:95-101. Available:https://doi.org/10.1002/hlca.201300153
46. Zhang PL, Wang G, Xu FQ, Liu JS, Wang JT, et al. Aspergilolide, a steroid lactone produced by an endophytic fungus *Aspergillus* sp. MBL1612 isolated from *Paeonia ostii*. Natural Product Research. 2019;33:2133-2138. Available:https://doi.org/10.1080/14786419.2018.1488706
47. da Silva MHR, Cueva-Yesquén LG, Júnior SB, Garcia VL, Sartoratto A, et al. Endophytic fungi from *Passiflora incarnata*: an antioxidant compound source. Archives of Microbiology. 2020;202:2779-2789. Available:https://doi.org/10.1007/s00203-020-02001-y
48. Bhardwaj A, Sharma D, Jadon N, Agrawal PK. Antimicrobial and phytochemical screening of endophytic fungi isolated from spikes of *Pinus roxburghii*. Arch Clin Microbial. 2015;6:1-9.
49. Sopalun K, Laosripaiboon W, Wachirachaikarn, Iamtham S. Biological potential and chemical composition of bioactive compounds from endophytic fungi associated with thai mangrove plants. South African Journal of Botany. 2021;141:66-76. Available:https://doi.org/10.1016/j.sajb.2021.04.031
50. Ikram M, Ali N, Jan G, Hamayun M, Jan FG, et al. Novel antimicrobial and antioxidative activity by endophytic *Penicillium roqueforti* and *Trichoderma reesei* isolated from *Solanum surattense*. Acta Physiologiae Plantarum. 2019;41:1-11. Available:https://doi.org/10.1007/s11738-019-2957-z
51. Song YP, Miao FP, Liang XR, Yin XL, Ji NY. Harziane and cadinane terpenoids from the alga-endophytic fungus *Trichoderma asperellum* A-YMD-9-2. Phytochemistry Letters. 2019;32:38-41. Available:https://doi.org/10.1016/j.phytol.2019.05.001
52. Yuan L, Zhao PJ, Ma J, Li GH, Shen YM. Tricycloalternarenes A–E: Five new mixed terpenoids from the endophytic fungal strain *Alternaria alternata* Ly83. Helvetica Chimica Acta. 2008;91:1588-1594. Available:https://doi.org/10.1002/hlca.200890172
53. Abdulghani AJ, Abbas NM. Synthesis characterization and biological Activity study of New Schiff and mannich bases and some metal complexes derived from

isatin and dithiooxamide. Bioinorganic Chemistry and Applications; 2011.
Available:https://doi.org/10.1155/2011/706262

54. Karan T, Simsek S, Yildiz I, Erenler R. Chemical composition and insecticidal activity of *Origanum syriacum* L. essential oil against *Sitophilus oryzae* and *Rhyzopertha dominica*. International Journal of Secondary Metabolite. 2018; 5(2):87-93.
Available:https://doi.org/10.21448/ijsm.404114

55. Ahsan T, Chen J, Zhao X, Irfan M, Wu Y. Extraction and identification of bioactive compounds (eicosane and dibutyl phthalate) produced by *Streptomyces* strain KX852460 for the biological control of *Rhizoctonia solani* AG-3 strain KX852461 to control target spot disease in tobacco leaf. AMB Express. 2017;7:1-9.
Available:https://doi.org/10.1186/s13568-017-0351-z

56. Li W, Zheng M, Wu S, Gao S, Yang M, et al. Benserazide, a dopadecarboxylase inhibitor, suppresses tumor growth by targeting hexokinase 2. Journal of Experimental & Clinical Cancer Research. 2017;36:1-12.
Available:https://doi.org/10.1186/s13046-017-0530-4

57. Dhakshinamoorthy D, Sundaresan S, Iyadurai A, Subramanian KS, Janavi GJ, et al. Hexanal vapor induced resistance against major postharvest pathogens of banana (*Musa acuminata* L.). The Plant Pathology Journal. 2020;36:133.
Available:https://doi.org/10.5423/PPJ.OA.03.2019.0072

58. He L, Liu N, Wang K, Zhang L, Li D, et al. Rosamultin from *Potentilla anserine* L. exhibits nephroprotection and antioxidant activity by regulating the reactive oxygen species/C/EBP homologous protein signaling pathway. Phytotherapy Research. 2021;35:6343-6358.
Available:https://doi.org/10.1002/ptr.7285

59. Park JC, Kim SC, Choi MR, Song SH, Yoo EJ, et al. Anti-HIV protease activity from rosa family plant extracts and rosamultin from *Rosa rugosa*. Journal of Medicinal Food. 2005;8:107-109.
Available:https://doi.org/10.1089/jmf.2005.8.107

60. Kukula-Koch WA, Widelski J. Alkaloids. In Pharmacognosy 163-198. Academic Press; 2017.
Available:https://doi.org/10.1016/B978-0-12-802104-0.00009-3

61. Tahara A, Nagashima H. Recent topics of iridium-catalyzed hydrosilylation of tertiary amides to silylhemiaminals. Tetrahedron Letters. 2020;61:151423.
Available:https://doi.org/10.1016/j.tetlet.2019.151423

62. Ludwiczuk A, Skalicka-Woźniak K, Georgiev MI. Terpenoids. In *Pharmacognosy* 233-266. Academic Press; 2017.
Available:https://doi.org/10.1016/B978-0-12-802104-0.00011-1

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