

Potential Activity of Fungal Endophyte of *Lasiodiplodia Theobromae* Isolated from *Crateva Religiosa* as a Good Source of Antibacterial and Antifungal Compound

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Abstract—Endophytic microorganisms reside within specific tissues of host plants without causing any apparent symptoms. Fungal endophytes are known to produce a wide range of bioactive compounds with significant therapeutic potential. *Crateva religiosa* is a sacred and traditionally important medicinal plant that has been widely used in various medical systems to treat numerous ailments. This plant serves as a rich reservoir of endophytes capable of synthesizing diverse pharmacologically active metabolites.

The present study focuses on the isolation of the fungal endophyte *Lasiodiplodia theobromae* from different tissues of *C. religiosa*. The crude extracts obtained from this endophytic fungus exhibit antibacterial activity against both Gram-positive and Gram-negative bacteria. Thin Layer Chromatography (TLC) and Gas Chromatography–Mass Spectrometry (GC–MS) were employed to characterize the bioactive compounds produced by the endophyte.

Phytochemical analysis confirmed the presence of alkaloids, flavonoids, tannins, terpenoids, steroids, carbohydrates, and amino acids in the fungal extract. GC–MS profiling of the *Lasiodiplodia* extract revealed several bioactive constituents of medicinal relevance.

Index Terms—*Crateva religiosa*, Endophytes, GC–MS, *Lasiodiplodia theobromae*, Secondary metabolites

I. INTRODUCTION

In a mutually beneficial association with their host plants, endophytes microorganisms that colonize internal plant tissues without causing visible disease symptoms confer protection to the host by producing secondary metabolites. These metabolites help plants withstand adverse environmental conditions. Despite

their potential, the full spectrum of bioactive compounds synthesized by plant-associated endophytes remains inadequately explored (Gunatilaka, 2006).

Studies have shown that endophytes inhabiting plant tissues are rich sources of biologically active substances exhibiting antibacterial, antioxidant, and anticancer properties. Owing to their diverse and potent secondary metabolites, endophytic fungi have garnered considerable attention in recent years (Tan & Zou, 2001; Cui, 2015; Yougen, 2015). They are now recognized as promising sources of natural therapeutic compounds. Among the various microbial endophytes, fungi are the most frequently encountered, as reported by Strobel and Daisy (2003). Endophytes are ubiquitous and inhabit plant tissues without eliciting infection in the host (Bacon and White, 2000).

Most endophytic fungi belong to the phylum Ascomycota, colonizing the intercellular spaces of plants without producing any visible symptoms (Corrêa et al., 2014). These fungi are capable of synthesizing a wide range of bioactive compounds. By accumulating secondary metabolites, endophytic fungi help reduce the damage caused by pathogenic infections in their host plants (Cabezas et al., 2012). The metabolites produced by these fungi have significant applications across food, pharmaceutical, agricultural, and environmental industries (Deshmukh et al., 2015; Suryanarayanan et al., 2009; Kharwar et al., 2011).

The majority of endophytic fungal communities are composed of members from Ascomycota, Basidiomycota, and Zygomycota. In traditional

taxonomy, fungal isolates are primarily identified based on their reproductive structures. However, the classification of non-sporulating fungi remains challenging due to the absence of discernible morphological features (Sun and Guo, 2012).

Crateva religiosa, a member of the Capparaaceae family, is a well-known traditional medicinal plant commonly referred to as the sacred garlic pear or temple plant. Since ancient times, crude extracts derived from various parts of this tree have been widely utilized in traditional medicine. The plant is well documented for its therapeutic applications in Unani, Siddha, and Ayurvedic systems of medicine (Omkar et al., 2006).

The leaves of *C. religiosa*, which are also consumed as vegetables, possess antibacterial compounds and are employed in the treatment of neurological disorders and pain. The bark exhibits diuretic properties and is used for the management of skin diseases, jaundice, and ailments of the urinary system (Goyal et al., 1999; Yu et al., 2005)

In addition to their pleasant fragrance, the flowers of *Crateva religiosa* possess medicinal properties and are used to alleviate inflammation and fever (Kirtikar & Basu, 1993; Sasmal et al., 2007; Shandhar & Kaur, 2011). The roots are employed in the treatment of ailments such as malaria and hypertension (Narendhirakannan & Smeera, 2010; Rathod et al., 2010; Sah & Verma, 2012; Desai et al., 2016). The plant also exhibits notable pharmacological activities, including carminative, laxative, anthelmintic, and diuretic effects. It contains several beneficial phytochemicals, such as alkaloids, glycosides, saponins, and terpenoids.

However, in recent years, research emphasis has increasingly shifted toward endophytic fungi associated with medicinal plants, as these microorganisms may contribute to conserving the biodiversity of important plant species. It is believed that endophytes can produce bioactive compounds similar to those of their host plants, with potential therapeutic applications against complex diseases. Despite this promising potential, relatively few studies have investigated the bioactive metabolites derived from plant-associated endophytic fungi. Furthermore, information regarding the endophytic fungi associated with *Crateva religiosa* remains limited.

Therefore, there is a significant need to explore diverse strategies for drug development and therapeutic

production by utilizing the rich reservoir of bioactive compounds produced by endophytic fungi associated with the medicinally important tree *Crateva religiosa*. The objective of the present study was to isolate the endophytic fungus *Lasiodiplodia theobromae*, culture it in bulk, extract its metabolites, conduct preliminary qualitative analyses to confirm the presence of secondary metabolites and antibacterial activity, and characterize its bioactive compounds using GC-MS profiling.

II. MATERIALS AND METHODS

Sample collection

The medicinally and historically significant plant *Crateva religiosa* was collected from the Civil Lines area and Mahamana Pt. Madan Mohan Malviya Park in the Prayagraj district of Uttar Pradesh, India. The collection site is located at 25°26'09"N and 81°50'47"E.

Surface Sterilization and Inoculation

Fresh leaves and twigs were separated and thoroughly washed under running tap water to remove adhering soil particles and other debris, followed by rinsing with sterile distilled water. Surface sterilization was then carried out under aseptic conditions using 70% ethanol for 2 minutes, followed by treatment with 0.01% mercuric chloride (HgCl₂) for 1 minute, and finally rinsing with sterile distilled water. After sterilization, the samples were blot-dried in an aseptic environment. Using a sterile scalpel, the edges of leaves and twigs were trimmed, and the tissues were cut into segments approximately 0.5 cm in length.

The sterilized leaf and twig explants were placed on sterile Potato Dextrose Agar (PDA) plates and sealed with parafilm. The inoculated plates were incubated at room temperature for 7–10 days to allow endophytic fungal growth and were monitored regularly. Emerging fungal colonies were subcultured to obtain pure isolates for further study.

Morphological Identification of endophytic fungi

Staining techniques were employed to identify the endophytic fungal isolates obtained from *C. religiosa*. Lactophenol cotton blue staining was used to prepare slides of sporulating cultures, which were then examined under a bright-field microscope (Mita et al., 2021). Identification of the fungal endophytes was based on morphological characteristics, including

colony colour, surface texture, margin appearance, hyphal features, spore formation, spore size and texture, along with comparison to standard taxonomic references (Harborne, 1998).

For further investigation, the identified isolates were aseptically subcultured on PDA plates and stored

under refrigeration. For mass cultivation, actively growing fungal cultures were inoculated into 250 mL Erlenmeyer flasks containing 100 mL of sterile Potato Dextrose Broth (PDB). The flasks were incubated at $26 \pm 2^\circ\text{C}$ for 10–20 days to promote mycelial growth and development of fungal mats.

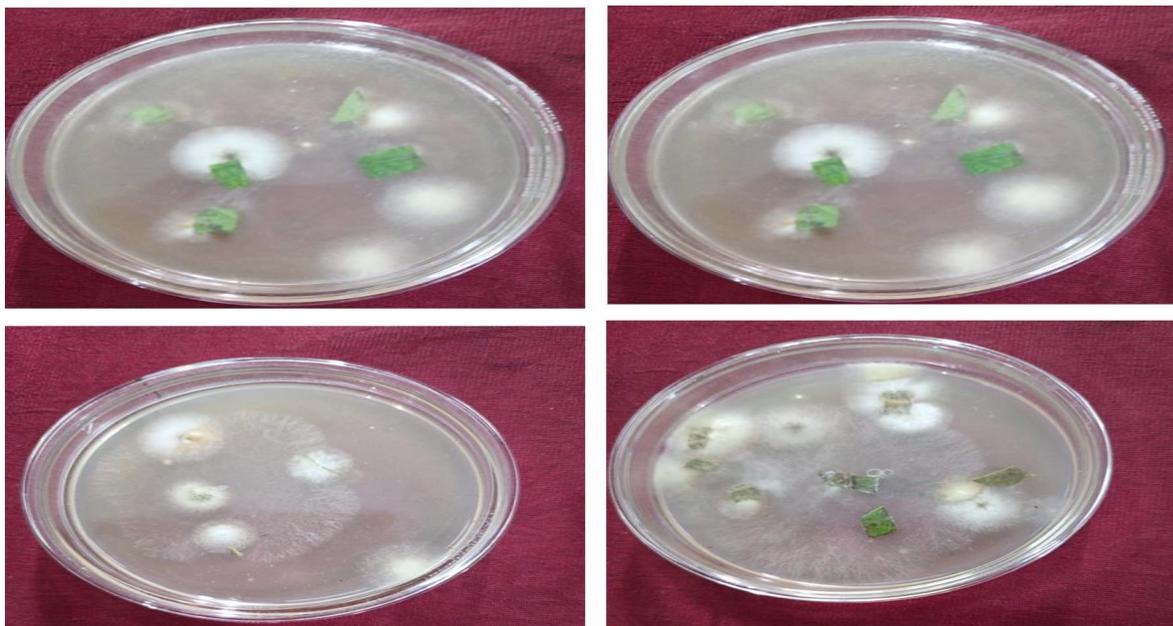


Fig 1: Early growth of endophytic fungus *L. theobromae* treated with Streptomycin PDA Media

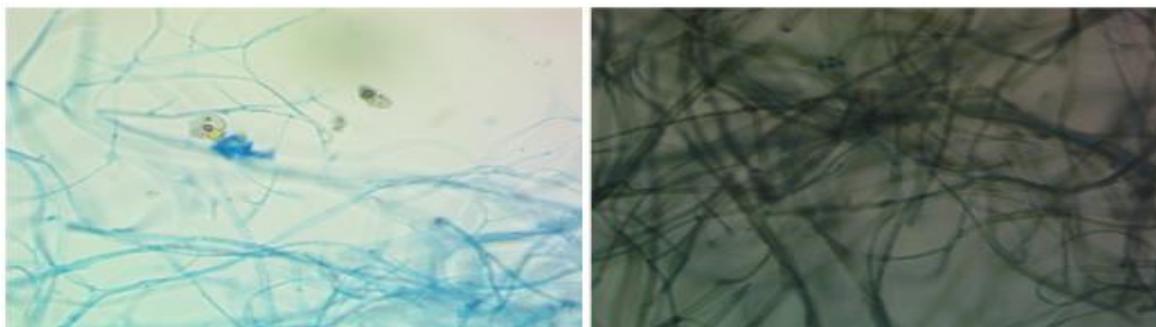


Fig 2: Microscopic picture of endophytic fungus *L. theobromae*

III. PRELIMINARY TESTS FOR SECONDARY METABOLITES

Qualitative screening of secondary metabolites was carried out on the methanolic extracts of *L. theobromae* isolated from *C. religiosa*. Standard procedures were followed to detect the presence of alkaloids, flavonoids, saponins, tannins, carbohydrates, terpenoids, triterpenoids, steroids,

cardiac glycosides, and amino acids (Galindo-Solis et al., 2022).

Thin Layer Chromatography (TLC)

Thin Layer Chromatography (TLC) was conducted to chemically profile and evaluate the crude extracts and their fractions. Preparative TLC plates ($10 \times 25 \text{ cm}^2$) were coated with Merck Silica Gel 60 F254. The fungal extracts were developed on the TLC plates using a solvent mixture of methanol and chloroform in a 7:1 ratio (Kowalska et al., 2022).

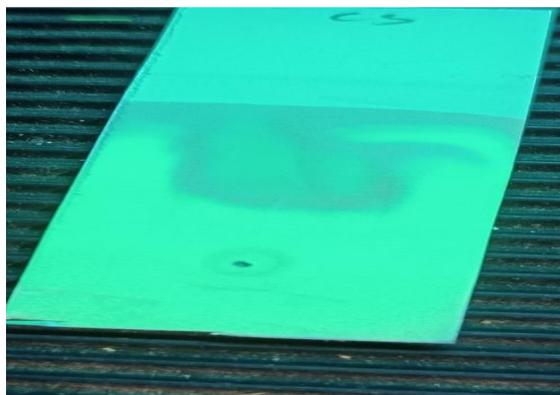


Fig. 4: TLC image of crude extract of fungal endophyte *L. theobromae* from *C. religiosa*

GC–MS Analysis of Crude Fungal Extract

GC–MS analysis of the endophytic fungal extract from *C. religiosa* was carried out using an AOC-20i Auto Injector coupled to a GCMS-TQ8040 system. The instrument was equipped with an Elite-1 fused silica capillary column. Electron ionization (EI) at 70 eV was employed for mass detection. Helium (99.99% purity) served as the carrier gas at a constant flow rate of 1 mL/min. The injection volume was 2 µL with a split ratio of 10:1. The interface and ion source temperatures were maintained at 250°C and 230°C, respectively.

The oven temperature was programmed initially at 110°C (held isothermally for 2 minutes), increased to 200°C, followed by a rise of 50°C per minute up to 280°C, and finally held isothermally at 280°C for 9 minutes. Mass spectra were recorded at 70 eV with a scan interval of 0.5 seconds over a mass range of 45–450 Da. The total run time was 45.67 minutes. The relative percentage composition of each constituent was calculated based on the ratio of its peak area to the total peak area. Mass spectra and chromatograms were

processed using TurboMass software (H. Farhat et al., 2022).

Methanolic preparations of the endophytic fungus *L. theobromae* were subjected to gas chromatography analysis. Important chemicals that correspond to detectable peaks are revealed by the analysis. Based on the compounds' retention duration, area, molecular formula, and molecular weight, we have named them as follows: 2. Nonynoic acid, decamethyl-2,4-Di-tert-butylphenol, dimethyl-cyclopentasiloxane, silanediol, 9, 19 -9,19-Cyclo-9.beta.-lanost-24-en-3.beta.-ol, acetate (7CI,8CI) -Cyclolanost-24-en-3-ol, acetate, (3.beta.) \$\$ Cycloartenol acetate (6CI)\$, (3S,6aR 6bR,8aS,12S,14bR) -4,4,6a,6b,8a,11,12,14b-octamethyl-

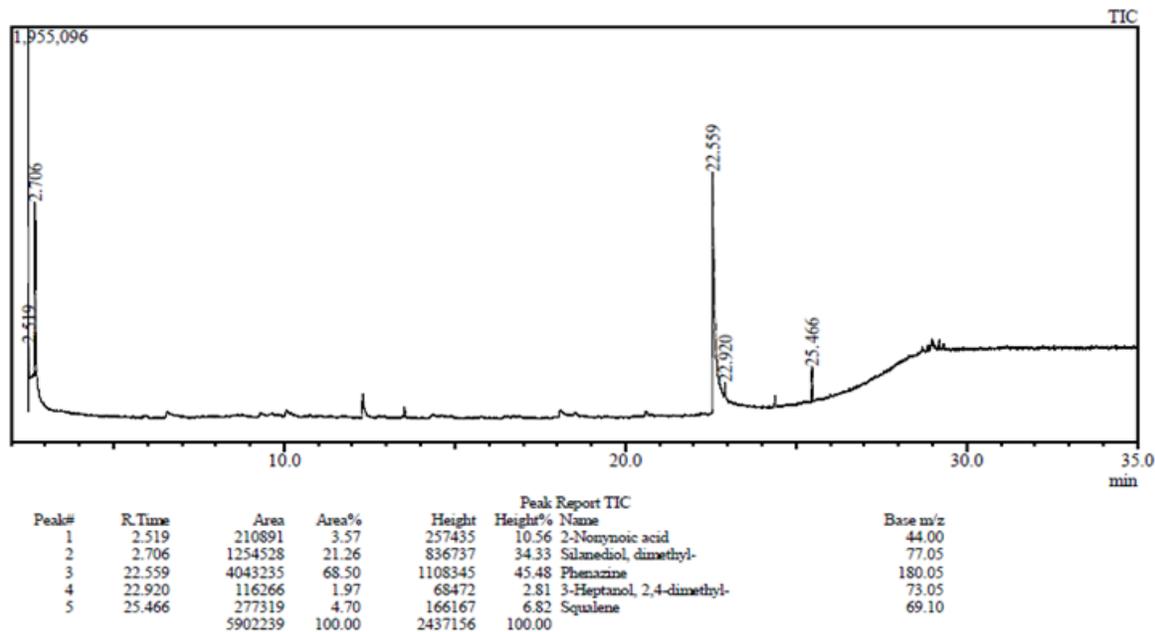
1,2,3,4,4a,5,6,6a,6b,7,8,8a,9,12,12a,12b,13,14,14a,14 b -icosahydricen-3-yl; 1H, 2H, 3H, and 4H -Pyridol [3,4-b]pyrazine, TMS (isomer 2) \$4-(Trimethylsilyl) -1,2,3,4-tetrahydropyridol [3,4-b] pyrazine, (1R,2S,8As) -8-oxo (Yu et al., 2005) -1-carboxymethyl-1,2,5,5-tetramethyl-trans-decalin

Thus, GC–MS analysis of endophytic fungal extracts provides valuable insight into the bioactive compounds produced by these microorganisms, highlighting their potential therapeutic applications. Fungal endophytes are recognized as rich sources of biologically active compounds with diverse industrial and medicinal relevance. In the present study, several bioactive constituents were identified in the methanolic extract of *L. theobromae* using GC–MS. However, further *in vivo* studies are necessary to validate these findings. To the best of our knowledge, this is the first report describing the preliminary screening of secondary metabolites and GC–MS characterization of methanolic extracts from the endophytic fungus *L. theobromae*.

Peak	R.Time	Area	Area%	Height	Height%	Name	Base m/z
1	2.519	219801	5.57	287392	11.33	2-Nonynoic acid	41.05
2	2.736	2002714	50.74	1422076	56.05	Silanediol, dimethyl-	77.05
3	8.338	123918	3.14	64643	2.55	Cyclopentasiloxane, decamethyl-	73.10
4	13.523	100655	2.55	56908	2.24	2,4-Di-tert-butylphenol	191.15
5	28.694	275826	6.99	155694	6.14	(3S,3aR,6R,8aS)-7,7-Dimethyl-8-methyleneoc	189.20
6	28.861	111059	2.81	55309	2.18	Lup-20(29)-en-28-oic acid, 3-hydroxy-, methy	394.35
7	28.980	410412	10.40	157104	6.19	5-Isopropylidene-6-methyldeca-3,6,9-trien-2-o	119.10
8	29.018	363102	9.20	202934	8.00	14,17-Nor-3,21-dioxo-.beta.-amyirin, 17,18-di	189.15

9	29.179	132778	3.36	57202	2.25	(3S,6aR,6bR,8aS,12S,14bR)- 4,4,6a,6b,8a,11,	408.40
10	29.313	206953	5.24	77690	3.06	(3S,6aR,6bR,8aS,12S,14bR)- 4,4,6a,6b,8a,11,	189.20
		3947218	100.00	2536952	100.00		

Table 1: *Crateva* stem (crude extract on PDB)



(B)

Graph 1: GCMS analysis result obtained from crude extract of endophytic fungus of *L. theobromae*

Antimicrobial activity

The antibacterial activity of the crude extracts of *L. theobromae* was assessed using the disc diffusion method against Gram-positive bacteria (*Staphylococcus* 1564) and Gram-negative bacteria (*Escherichia coli* ATCC 25922, *Pseudomonas* ATCC 27853, and *Klebsiella* 4151). Sterile discs were immersed in the fungal extracts for eight hours to allow saturation. Muller Hinton Agar (MHA) medium, petri dishes, and forceps were autoclaved and transferred to a laminar airflow chamber. After

pouring sterile MHA into petri plates and allowing it to solidify, 10 µL of each bacterial culture was spread evenly over the surface.

The extract-soaked discs were then carefully placed on the inoculated MHA plates. Discs soaked in double-distilled water served as the negative control, whereas discs impregnated with streptomycin were used as the positive control. All plates were incubated at 37°C for 24 hours. Antibacterial activity was determined by measuring the Zone of Inhibition surrounding each disc.

Serial No.	Bacterial Strain	Control	50%	40%	30%
		Stock	500 ml	250 ml	125 ml
1	<i>Pseudomonas</i>	-	+	-	+
2	<i>E. Coli.</i>	-	+	-	+
3	<i>Klebsiella</i>	-	+	-	+
4	<i>Staphylococcus</i>	-	+	-	+

Table 1: MIC values of crude extract of endophytic fungus *L. theobromae*

Serial No.	Bacterial Strain	Control	50%	40%	30%
		Stock	500 ml	250 ml	125 ml
1	<i>Pseudomonas</i> ATCC 27853	0.6 cm.	0.2 cm.	0.5 cm.	0.5 cm.
2	<i>E. Coli.</i> ATCC 25922	0.00 cm.	0.5 cm.	0.8 cm.	0.4 cm.

3	Klebsiella 4151	0.2 cm.	0.3 cm.	0.5 cm.	0.2 cm.
4	Staphylococcus 1564	0.7 cm.	0.3 cm.	0.6 cm.	0.7 cm.

Table 2: Sensitivity of crude extract of endophytic fungus *L. theobromae*

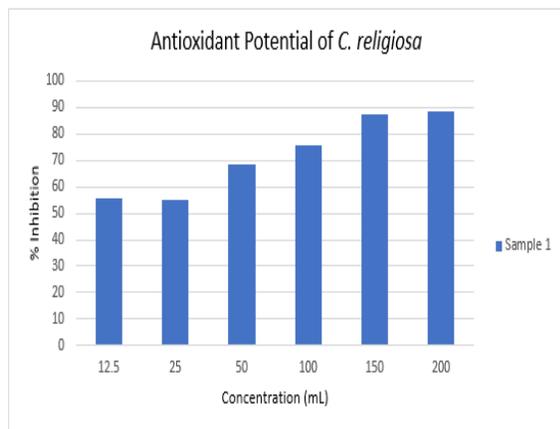
Antioxidant Activity

It is well established that antioxidant compounds can effectively protect cells from damage caused by reactive nitrogen and oxygen species (RNOS). Excessive ROS can lead to DNA damage, cellular degeneration, and the development of cancer (Huang et al., 2007; Seifried et al., 2007). Antioxidants play a

crucial role in counteracting ROS-related disorders, including cancer, atherosclerosis, cardiovascular diseases, hypertension, ischemia–reperfusion injury, diabetes mellitus, and various neurodegenerative conditions. In the present study, methanol was used as the solvent for assessing antioxidant activity through the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay.

Serial no.	Conc. In ml	Methanol in ml	DPPH in ml	<i>C.regillosa</i> crude extract in ml
1	2	0	2	0.052
2	1.5	0.5	2	0.225
3	1	1	2	0.180
4	0.5	1.5	2	0.308
5	0.25	1.75	2	0.280
6	0.125	1.875	2	0.361
7	Control (in ml)	2	2	0.366

Table 3: Antioxidant results of Crude extract of Fungal endophyte *L. theobromae*



Graph 1. of antioxidant activity of fungal extract of *L. theobromae* from *C. religiosa*

IV. RESULTS AND DISCUSSION

Lasiodiplodia theobromae was isolated from multiple sections of *Crateva religiosa* for this study. The fungus was identified morphologically using both low- and high-power compound microscopy, confirming its classification within the Ascomycota. Molecular identification was also performed using universal and specific primers. The accession number for *Lasiodiplodia theobromae* is NFCCI-6136, and the isolated endophytic fungus has been deposited at the

Agharkar Research Institute (ARI), Pune, under this number.

Endophytic fungi are well recognized as prolific sources of novel bioactive compounds and secondary metabolites. According to Fox and Howlett (2008), various endophytic fungi produce new metabolites derived from alkaloids, steroids, flavonoids, and terpenoids, which exhibit biologically beneficial activities, including antibacterial, antifungal, antiviral, anti-inflammatory, and anticancer properties (B. Guo et al., 2008).

Thin layer chromatography (TLC) was employed as an initial method to detect the compounds present in the crude extract of the fungal endophyte, revealing the presence of multiple chemical constituents. The analysis was based on the retention factor (Rf), which served as the primary parameter for the TLC study. As the secondary metabolites extracted from the fungus were insoluble in water, methanol was selected as the most effective solvent for compound separation. The Rf values were calculated as the ratio of the distance traveled by the compound spot to the distance traveled by the solvent front, measured from the origin (Sherma, 2004).

Following inoculation and incubation of *Lasiodiplodia theobromae*, the chemical constituents were further characterized using gas chromatography–mass spectrometry (GC-MS). The GC-MS analysis

identified several compounds along with their corresponding retention times (RTs).

According to Bills and Polishook (1991), fungal endophytes produce a diverse array of secondary metabolites, many of which possess beneficial biological properties. Gas chromatography mass spectrometry (GC-MS) has been widely employed in studies to identify the chemical compounds produced by fungal endophytes (R.R. Hateet, 2020).

Total twenty chemicals were found by GCMS analysis of the *Lasiodiplodia theobromae* extract shown in table in the form of 2-Nonynoic acid, Silanediol, dimethyl-Cyclopentasiloxane, decamethyl-2,4-Di-tert-butylphenol, 9,19-Cyclolanost-24-en-3-ol, acetate, (3.β)- 9,19-Cyclo-9.β.-lanost-24-en-3.β.-ol,acetate (7CI,8CI) Cycloartenol acetate (6CI), (3S,6aR 6bR,8aS,12S,14bR)-4,4,6a,6b,8a,11,12,14b-octamethyl-1,2,3,4,4a,5,6,6a,6b,7,8,8a,9,12,12a,12b,13,14,14a,14b-icosahydricen-3-yl; 1H,2H,3H,4H-Pyridol[3,4-b]pyrazine,TMS (isomer 2) 4-(Trimethylsilyl)-1,2,3,4-tetrahydropyridol[3,4-b]pyrazine (1R,2S,8As)-8-oxo-1-carboxymethyl-1,2,5,5-tetramethyl-trans-decalin. Such observation was also obtained by Abdel-Wareth et al., 2023

Twenty chemical compounds were identified from the GC-MS analysis of the *Lasiodiplodia theobromae* extract, as presented in Table 1. Among them, nonynoic acid also known as non-2-enoic acid or alpha-nonyonic acid is an unsaturated medium-chain fatty acid. Similar compounds, commonly found in mushrooms, are widely used in flavorings, food preservatives, cosmetics, surfactants, pesticide formulations, specialty polymer synthesis, and certain pharmaceuticals. Due to its unsaturated fatty acid structure, nonynoic acid shows potential for drug development and is also employed as a biochemical reagent in life science research (H. G. Rodrigues, 2010).

Silanediols are primarily utilized in conjugate addition reactions to activate nitroalkenes and incorporate CO₂ into cyclic carbonates. Additionally, due to their ability to form stable, moisture-curing networks, silanediols find applications as molecular recognition tools and as bio-inspired enzyme inhibitors in adhesives, sealants, paints, and coatings.

Both organic and inorganic compounds can be analyzed using thin-layer chromatography (TLC), while gases and volatile organic compounds are

typically separated and identified using gas chromatography–mass spectrometry (GC-MS). GC-MS operates using a liquid stationary phase and a gaseous mobile phase, making it a powerful tool in modern analytical chemistry for the effective separation and identification of compounds in complex mixtures (P. Ferranti & M. Gallo, 2016). The technique offers high resolution for low-molecular-weight compounds due to the use of capillary columns. Although TLC is less precise and cannot separate isomers, it is an inexpensive and practical method widely used in laboratories for rapid screening of food, beverage, and plant extracts. In contrast, GC-MS, while more costly, provides greater sensitivity and delivers significantly more detailed information (R. Oprean et al., 1989).

The present study revealed the presence of numerous chemical compounds in the fungal extract that have not been reported in previous investigations. Endophytic fungi are capable of producing unique and novel secondary metabolites, which represent a valuable source of bioactive compounds with anti-inflammatory, antioxidant, anticancer, antibacterial, and antidiabetic properties. Furthermore, the production of natural metabolites from endophytic fungi offers a sustainable approach to drug development, contributing to the conservation of natural resources while expanding the market for plant-derived therapeutic compounds.

ACKNOWLEDGEMENT

The authors gratefully acknowledge Dr. Subhas Biyan for providing the necessary facilities to carry out this experimental work. The authors also thank Patanjali Food and Herbal Park Pvt. Ltd., Laksar Road, Padartha, Haridwar, Uttarakhand (249404), for providing sample testing services.

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