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# ISOLATION AND PRODUCTION OF SECONDARY METABOLITES FROM ENDOPHYTIC FUNGI

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## ABSTRACT

Endophytic parasites are found in plant tissues. They colonize host plant tissues inter- and intracellular spaces without causing disease. They have a mutually beneficial relationship. Endophytic fungi were isolated from healthy plant of *Heliotropism indicum*, a medicinal plant found in India which is used in folk medicine. Among five endophytic fungi isolated, the fungal isolates showed activity against two tested phytopathogens (*Xanthomonas sp*) in preliminary screening, and the best results were obtained with *Aspergillus sp* (EF9). After fermentation in liquid media, the *Aspergillus sp* (EF9) demonstrated antibacterial activity in Potato Dextrose Broth. In the next step, ethyl acetate extract from the supernatant of broth culture were prepared and both exhibited antibacterial activity against phytopathogens (*Xanthomonas sp*). The best result was observed with 70 mg/mL. This study is the first report about the antibacterial activity of endophytic fungi residing in *H. indicum* roots, in which the fungus *Aspergillus sp* (EF9)demonstrated the ability to produce bioactive agents with antibacterial potential, and may provide a new lead in the pursuit of new biological sources of drug candidates in agricultural industry.

**Keywords:** *Heliotropium indicum*, endophytic fungi, Blight, *Lablab purpureus*, GCMS.

## INTRODUCTION

Endophytic fungi inhabit their hosts inner tissues and plant cells without causing obvious symptoms or damage. They engage in a mutualistic relationship in which the host provides food and shelter, while endophytes protect chemicals. Endophytes, according to the research that has been done so far on their role in host plants, can improve the plant's ability to withstand environmental stress, increase disease resistance, and stimulate plant growth [1, 2]. Due to their potential as a source of secondary metabolites, endophytic fungi are intriguing and have proven useful for drug discovery. Endophytic microorganisms found in plants have been linked to

antimicrobial activity in a number of studies. The intercellular and intracellular spaces of plants can be colonized by fungi; however,systematic and extensive colonization is more likely to take place in the roots than in the aerial leaves or stems [3]. The host's apoplastic fluid serves as the primary source of nutrients for colonization in aerial organs, supporting normal EF reproduction. Fungi interact with their hosts in three distinct ways during an infection: Depending on the physiological state or specific circumstances of the host plant, mutualistic endophytes (beneficial endophytes), commensalistic endophytes (nonbeneficial/virulent endophytes), and pathogenic pathogens (virulent pathogens) areall possible [3, 4]. Fungal strains can increase, have no discernible effect on, or decrease hostfitness according to these three modes of action.

Throughout their life cycles, different microorganisms occupying the same habitat mustcompete for its resources for nutrition, living space, reproduction, and other needs. When resources are insufficient to meet the needs of the community, adaptable microorganisms are more likely to obtain adequate resources and increase their abundance than microorganisms with poor adaptability. The first might not be able to survive in bad conditions because of this. Organisms have developed two effective waysto compete in order to survive. One is to make allelochemicals that stop their rivals fromgrowing and eliminate the toxic effects their rivals have nearby. The other option is to make allelochemicals that help their producers form alliances with symbionts or hosts throughsymbiotic relationships. Even in extremely hostile environments, these symbiotic relationships enable both parties to survive and reproduce safely [1-4].

***HELIOTROPIUM INDICUM***

The genus *Heliotropium indicum* Linn belongs to the Boraginaceae family. Some of the heliotropes are common garden plants, while others are weeds. There are approximately 250 species in the genus *Heliotropium*, which is found on all continents in tropical, subtropical, and warm temperate zones. *H. indicum*, also known as "Indian heliotrope," is found in Bangladesh, India, and some parts of Africa [5-7]. The flowering period lasts for a very long time, andnew flowers grow apically within the cyme while mature nutlets are already present at the inflorescence's base. The flowers are sessile, regular, white or violet in color.

*H. indicum* has been used to treat a wide range of illnesses in various folk and traditional medical traditions. According to the findings of an ethnopharmacological survey, traditional healers in the Kancheepuram district of Tamil Nadu, India. *H. indicum* is used to treat stomachaches, nervous disorders, skin diseases, poison bites, and other ailments [8].According to the findings of another ethnopharmacological survey conducted in a few African nations, *H. indicum* is thought to be effective for the treatment of malaria, abdominal pain, and dermatitis. The treatment of malaria received the most usages (22 %) [9-12]. In Rayalseema, an Indian village in Andhra Pradesh, the leaf paste is used externally to treat rheumatism.

**BACTERIAL BLIGHT**

Green, snap, field, lima, scarlet runner, mung, and tepary beans are all susceptible to bacterial blight, which thrives in hot, humid environments. Small, angular, pale green, water-soaked spots on the leaves are the first signs. Large brown blotches with dry centers (typically surrounded by a narrow yellow zone) are formed as these gradually expand and merge into one. The leaves may, in extreme cases, become scorched, wither,and fall off. Cracks or water-soaked cankers that produce a yellowish liquid and are highly contagious can also appear on the stems. Spots additionally show up on the units,beginning little, however expanding to shape dry, brown indented patches. Both the common blight (*Xanthomonas campestris pv phaseoli*) and the halo blight (*Pseudomonas syringae pathovar phaseolicola*) are widespread bacterial blights that affect the majority of bean varieties [13. 14].

**LABLAB BEAN - PLANT DESCRIPTION**

Beans from Lablab (*Lablab purpureus* L.; Family: Fabaceae) is a type of bean that comes from Africa and is grown for food all over the tropics. It is the only species in the genus Lablab, which is monotypic. The inflorescence is comprised of racemes of numerous blossoms. White flowers are found in some cultivars, while others may be blue or purple [15]. Depending on the cultivar, the seeds may be black, brown, red, or white,with or without a white hilum at times. Wild plants have mottled seeds. The seed has alength of about a centimeter. Lablab is prone to bacterial blight (Fig 1). *Xanthomonas axonopodis pv. Phaseoli* is responsible for bacterial blight, also known as common bean blight. On the opposite sides of the primary leaves, seedlings exhibit

angular, water-soaked lesions. Necrose forms in water-soaked spots on leaves. The pathogen can be eradicated through seed treatment with the right antibiotics. Spraying plants with copper-based fungicide before symptoms appearis also effective in controlling bacterial blight [16, 17].

**Fig1** Bacterial blight infection in the leaves of *Lablab purpureus*

## MATERIALS & METHODS

### PLANT MATERIAL

In January 2023, a healthy *H. indicum* plant was collected. Before being surface-sterilized, the roots, stems, and leaves were washed in tap water for 10 to 15 min and cut into small pieces of about 2 cm × 2 cm. Four steps were taken to sterilize the surface. Tap water was used to rinse the samples first. After that, the samples were submerged in 70% ethanol for one min, then in 5.3% NaOCl for five min,then in 70% ethanol for thirty sec, and finally washed with sterile water [18].

### ISOLATION OF ENDOPHYTIC FUNGI

Potato dextrose agar was used to isolate endophytic fungi from *H. indicum* (Himedia, Mumbai, India). In a Petri dish containing the selective medium, thesurface-sterilized sample was incubated for a week at 27 °C. All fungi isolates were transferred into freshly prepared potato dextrose agar (Himedia, Mumbai, India) following a week of incubation [18].

### MORPHOLOGICAL EXAMINATION

The lactophenol cotton blue (LPCB) staining method allowed for the morphological identification of the isolated endophytic fungi. The fungi were grown for a week at 27 °C in potato dextrose agar. Both the macroscopic (the appearance of the colony) and the microscopic (mycelia) characteristics were examined after one week of incubation [18].

### SECONDARY METABOLITES PRODUCTION

The 5 isolates were inoculated in 50 mL of PDA broth and incubated at 28 °C for 7 days at room temperature. After incubation, the broth filtrate were separated and extracted with equal volume of ethyl acetate for secondary metabolites extraction. The upper organic phase was separated and allowed to evaporate the solvent to yield dry crude extracts. The DMSO dissolved EF9 crude secondary metabolites were separated by thin layer chromatography to identify the number of compounds presence. 5 µL of crude extract was potted in TLC plate (Merck TLC plate, Silica gel 60 F254). After compounds wereseparated, the TLC plates were visualized under UV Illuminator (Deep Vision - UV inspection cabinet) at 365 nm. The solvent system Chloroform: Methanol (4: 1) was mobile phase for isolate (EF9) crude extract. The dried crude extracts were dissolved in DMSO and all the crude extracts of isolates were tested against two *Xanthomonas sp* that isolated from *L. purpureus* (from infected leaves). The Agar well diffusion method was followed to study antibacterial activity of crude extracts. Based on anti-bacterial activity, the fungal isolateEF9 have exhibited high activity against tested pathogens with significant zone of inhibition (± 38 mm).

### EXTRACTION OF SECONDARY METABOLITES

The liquid–liquid extraction method was used to extract the fermentation broth twice with equal volumes of ethyl acetate. After that, the collected ethyl acetate phase was pressure-evaporated until a crude extract was

obtained. Before being analyzed by Gas Chromatography Mass Spectrometry (GCMS), the crude extract that was obtained fromthe extraction of the fermentation broth was dissolved in ethyl acetate and filtered through a membrane filter of 0.22 µm [18].

**ANTI-BACTERIAL ACTIVITY**

The leaves of lablab plants displaying anthracnose symptoms were collected washed with tap water and surface sterilized with 75% ethanol for 10 sec and 0.1% sodium hypochlorite for 3 min. The leaf sections were then dried on sterile filter paper, rinsed three times with sterile distilled water, and incubated for one week at 25°C on culture medium supplemented with streptomycin sulfate (300 mg L–1) (w/v) [19]**.** The agar well diffusion method was used to test the crude extract of secondary metabolite' s antagonistic activity against the bacterial pathogens that were isolated from *L. purpureus* (L.) leaves. Each concentration ofcrude extract—50 mg/mL and 100 mg/mL—was inoculated into each wells (6 mm) that were placed on the Nutrient agar test plates. Test plates were made by combining nutrient agar medium with a cell suspension of a plant bacterial pathogen. After two days of incubation at room temperature, the widths of the inhibition zones were measured to determine the antagonistic activity [20].

**GCMS PROFILING OF SECONDARY METABOLITES**

For the GC-MS analysis, crude extract was used. The GC-MS analysis was carried out in Shimadzu's QP 2010 Plus system, Kyoto, Japan. An auto sampler (AOC-20i) and a gas chromatograph are connected to a mass spectrometer at 250°C forthe ion source and 280°C for the interface. For GC-MS detection, a threshold desorption system with ionization energy of 1000 eV and a mass range of 50-650 m/z was utilized. The injection temperature was 280°C, and the column oven temperature was 50°C, at a pressure of 53.5 kPa. The carrier gas was helium (99.99 percent) in a constantflow rate-total flow: Column flow: 1.00 ml/min, or 54.0 ml/min. The GC-MS program was finished in 28 minutes. The mass spectra and chromatograms were analyzed with the Turbo mass software. To determine the proportion of each component, the averagepeak area of each component was compared to the total area. Information translation onmass reach GC-MS was driven using the informational collection of NIST library. Thespectra of the known compounds in the NIST library were compared with the mass range of the unknown compounds.

**RESULTS AND DISCUSSION**

**SAMPLE COLLECTION**

Phytocompounds have notable biological properties like being antimicrobial, analgesic, antipyretic, antitumor, wound healing, etc., which makes them useful against a variety of human diseases [7, 21-23]. *Heliotropium indicum* plant was collected and used to extract the endophyte (Fig 2).

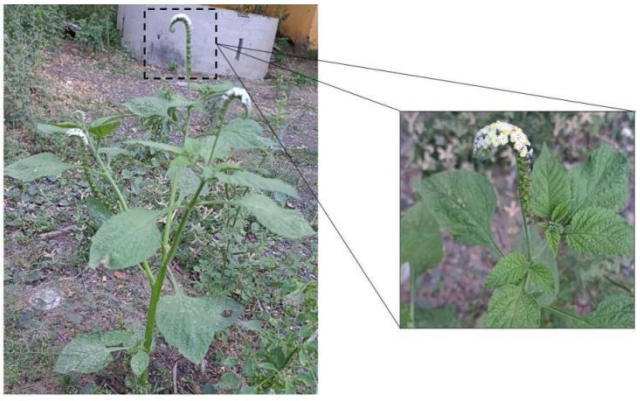


**Fig 2** *Heliotropium indicum*

This plant has been used for centuries to treat a wide range of diseases, such as wound healing, bone fracture, febrifuge, eye infection, menstrual disorders, nerve disorders, kidney problems, and antiseptic purposes. *H. indicum* contains numerous significant phytochemicals like tannins, saponins, steroids, oils, and

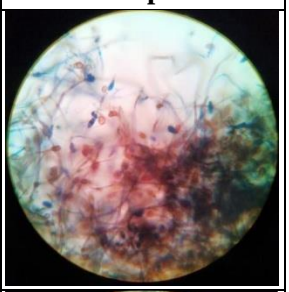
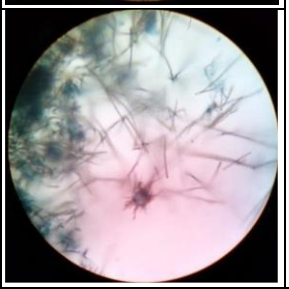
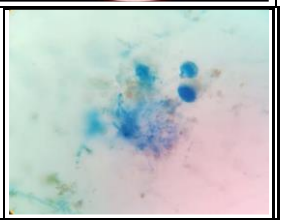
glycosides [6, 12]. Indicine N-oxide, heliotrine, and other pyrrolizidine alkaloids were isolated by Schoental [24], from this plant. According to research, *H. indicum* has anticataract, anticancer, antiinflammatory, and wound-healing properties, among other important pharmacological properties [25-30].

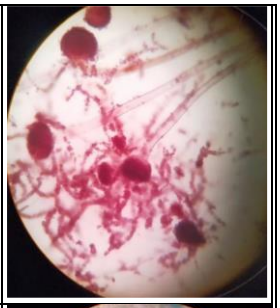
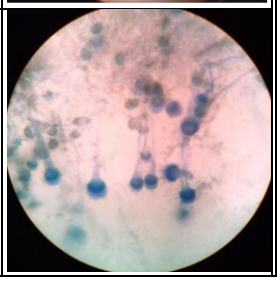
**MORPHOLOGICAL EXAMINATION OF ENDOPHYTIC FUNGI**

Five fungal endophytic isolates were isolated from *H. indicum* leaves, stem,flowers, and roots (Fig 3). On the basis of morphological and microscopic examination, the endophytes have been identified as *Curvalaria sp* (EF4) (from leaves), *Chaetomium sp* (EF6) (from Stem), *Aspergillus sp* (EF9) (from roots), *Aspergillus sp* (EF13) and *Aspergillus sp* (EF5) (both from root). *Aspergillus species* (EF9) (isolate from the root) was identified as the fungal isolate (isolate from the root) with the highest anti-bacterial activity against the bacterial pathogen that causes Lablab disease in this study. Shape, color, and size were all distinguishing characteristics of the isolate's colonies. The conidiophore morphology (including their length and width) and conidia size differences between endophytic fungi were confirmed by microscopic examination of the stained mycelium slides. Table 1 provides a summary of the microscopic images of all of the isolated endophytic fungi.

**Fig 3** Isolation of Endophytic fungi from Heliotropium indicum (Leaf, Stem,Flower, and Root)

**Table 1 Microscopic View of all Endophytic Fungi Isolates from *Heliotropiumindicum***

| S.No. | Endophytic Fungi(Isolates) | Microscopic View |
|---|---|---|
| 1. | *Curvalaria species* (EF4) |  |
| 2. | *Chaetomium species* (EF6) |  |
| 3 | *Aspergillus species* (EF9) |  |

| | | |
|---|---|---|
| 4. | *Aspergillus species* (EF13) |  |
| 5. | *Aspergillus species* (EF5) |  |

**SECONDARY METABOLITES PRODUCTION**

Endophytic microorganisms that are associated with these plants have received little attention despite the fact that the genus *Aspergillus sp* have been cultivated for a considerable amount of time and are known to be resistant to a variety of climatic conditions and pathogens. We investigated the anti-bacterial activity of fungal isolates from the different parts of *H. indicum* and pulp as well as the composition of the isolate's secondary metabolites in order to test the hypothesis regarding the potential contribution of endophytes to the *Aspergillus sp*. There are currently few studies of the endophytic microflora from *H. indicum*. Endophytic fungi from *H. indicum* plant parts have been reported to be capable of producing antibacterial secondary metabolites, according to known data.

Endophytic fungi from the genus *Aspergillus* have been reported as rich sources of bioactive secondary metabolites with applications in various fields. Such secondary metabolites include Dianhydro-aurasperone C, fonsecinone A, asperazine, rubrofusarin B, and (R)-3- hydroxybutanonitrile, Neosartorin, Ergosterol, cerevisterol,5-hydroxymethylfuran-3-carboxylic acid, allantoin, trypacidin, and monomethylsulochrin [31].

**EXTRACTION OF SECONDARY METABOLITES**

In our work, endophytic fungi were found in roots and leaves of *H. indicum* and represented by three different genera (*Curvalaria sp, Chaetomium sp,* and *Aspergillus sp*). From the identified fungal isolate EF9 attributed were active against morethan the other fungal isolates; however, potential of *Aspergillus terreus* (EF9) from root part was shown highest anti-bacterial activity against *Xanthomonas sp* isolated from infected leaves of *L. purpureus*. The ethyl acetate solvent system was used toextract the secondary metabolites from the broth culture of all fungal isolates. The crude secondary metabolite (Fig 4 A) produced by EF9 isolate inhibited growth of the phytopathogenic bacteria in *L. purpureus*. The thin layer chromatography analysis has confirmed that crude secondary metabolites have diverse compounds (Fig 4 B). InTLC, there are two *Xanthomonas sp* strains that were used as the test pathogens, since they parasitize on various plants. Since fungal species are able to enter into a variety of symbiotic relationships with various host plants, expressing either a mutualistic or pathogenic mechanism, there is no contradiction here.

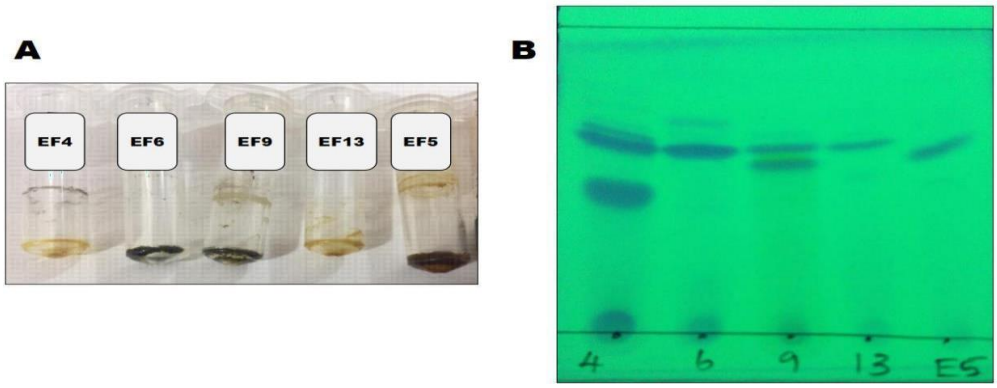



**Fig 4 A:** Extraction of secondary metabolites from fungal isolates (EF4, EF6, EF9,EF13 and EF5) isolated from *Heliotropium indicum*. **Fig 4 B:** Thin Layer Chromatography of all endophytic fungi crude secondary metabolites. A) Ethyl acetate crude secondary metabolites extract of respective fungal isolates and B) Thin layer chromatogram of Ethyl acetate crude secondary metabolites extract separated using a mobile phase of Chloroform and Methanol (4:1) ratio.

**ANTI-BACTERIAL ACTIVITY**

The metabolic capacity of endophytic microorganisms, particularly fungi, to produce a wide range of bioactive molecules that can protect the plant from pathogens is one of their most important characteristics. Secondary metabolites produced by endophytic fungi have been accounted for as inhibitors of a various plant pathogens [32-34]. The anti-bacterial activity of crude secondary metabolites from *Aspergillus sp* (EF9) was studied using *Xanthomonas sp* (Isolate I and II) from infected leaves of *L. purpureus*. Both isolates growth was inhibited by crude extract. The maximum inhibition was observed at 70 mg/mL concentration (Fig 5; Table 2).


**Fig 5:** Anti-bacterial activity using EF9 (*Aspergillus sp*) isolate from root of *H. indicuma* against *Xanthomonas sp* isolates.

**Table 2:** Anti-bacterial Activity of EF9 secondary metabolites against *Xanthomonas sp*

| Test Pathogens | Zone of Inhibition in mm | | | | |
|---|---|---|---|---|---|
| | **30 mg** | **50 mg** | **70 mg** | **+ Ve** | **- Ve** |
| *Xanthomonas species* - Isolate I | - | 30 | 38 | 14 | - |
| *Xanthomonas species* - Isolate II | 22 | 26 | 32 | 16 | - |

**GCMS PROFILING OF SECONDARY METABOLITES**

From the anti-bacterial activity, it was found that fungal isolate (EF9) *Aspergillus sp* showed highest activity and hence that isolate was subjected to secondary metabolites production in a 50 mL of Potato Dextrose Broth and was analyzed for GCMS profiling to know the compounds present in crude secondary metabolites (Fig 6). The pool of diverse chemical compounds, including fatty acids, alkaloids, esters and ethers, alcohols, and aldehydes, was produced by *Aspergillus sp* (EF9), according to the GC–MS assay. The findings of the study

confirm that the genus *Aspergillus sp* is known tobe capable of biosynthesis of various secondary metabolites. It is possible that the 2,4- Ditert-Butylphenol, which is a significant component of ethyl acetate extract's crude secondary metabolites, has antibacterial properties. It has been reported that the natural compound 2,4-Ditert-butylphenol (DTBP) has various biological activities, including antibacterial, antifungal, and anticancer properties. Even though these results are good,we still don't know much about how DTBP works to fight cancer.

**Fig 6:** Secondary metabolites production using EF9 (*Aspergillus sp*) isolatefrom root part of *Heliotropium indicum*.

The major secondary metabolites presented in crude secondary metabolites were α-Pinene, Trans-hexahydro-2(3H)-benzofuranone, 2-Chloro-5-methylbenzene-1,3diol, 2,4-Ditert- Butylphenol, 9-Octadecene, E-14-Hexadecenal, 5-(Morpholino) Pent-2en4ynal, o-Tolylamino-acetic acid [1-(2,5-dihydroxy-phenyl)-ethylidene]-hydrazide, Diisobutyl phthalate, Eicosane,1H-2-Benzopyran-1-one,(3-methoxymethoxy1,4Dimethyl- Pent-1-enyl)-benzene, 9, 10-Anthracenedione. The GCMS profiling of the crude extractof EF9 isolate was depicted in (Fig 7).

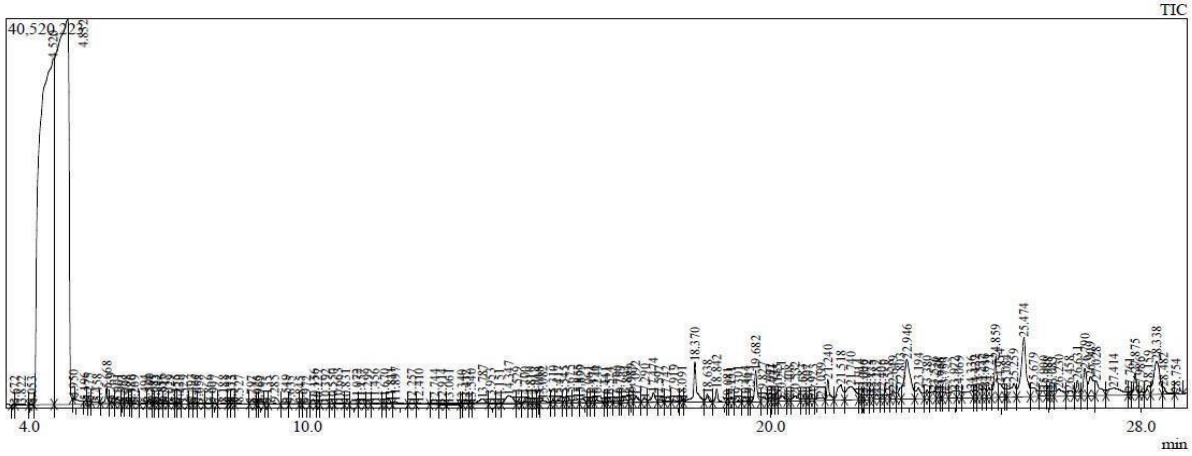



**Fig 8** GCMS Profiling of Crude Secondary Metabolites extracted from EF9isolate (*Aspergillus terreus*)

**Table 3:** The major secondary metabolites identified in Crude SecondaryMetabolites extracted from EF9 isolate (*Aspergillus terreus*)

| S. No | Interpretation | Volatile compounds in secondary metabolites | Area % |
|---|---|---|---|
| 1. | Formula:$C_{10}H_{16}$, CAS:80-56-8, Mol.Weight:136,R.Time:6.465, MassPeaks:466 | α-Pinene | 0.05 |
| 2. | Formula: $C_8H_{12}O_2$CAS:24871-12-3 | Trans-Hexahydro-2(3H)benzofuranone | 0.11 |

| | | | |
|---|---|---|---|
| | MolWeight:140<br>R.Time:14.585<br>MassPeaks:483 | | |
| 3 | Formula:$C_7H_7ClO_2$CAS:56021-31-9<br>Mol.Weight:158<br>R.Time:17.995<br>MassPeaks:447 | 2-Chloro-5-methylbenzene-1,3diol | 1.60 |
| 4 | Formula:$C_{14}H_{22}O$ CAS:0-<br>00-0 Mol.Weight:206R.Time:18.355<br>MassPeaks:502 | 2,4-Ditert-Butylphenol | 3.19 |
| 5 | Formula:$C_{18}H_{36}$ CAS:7206-<br>25-9 Mol.Weight:252R.Time:19.345<br>MassPeaks:455 | 9-Octadecene | 2.62 |
| 6 | Formula:$C_{16}H_{30}O$ CAS:330207-53-9<br>Mol.Weight:238R.Time:22.540<br>MassPeaks:517 | E-14-Hexadecenal | 2.90 |
| 7 | Formula:$C_9H_{11}NO_2$ CAS:0-<br>00-0 Mol.Weight:165R.Time:23.060<br>MassPeaks:492 | 5-(Morpholino)Pent-2-en-4-ynal | 2.87 |
| 8 | Formula:$C_{17}H_{19}N_3O_3$CAS:0-00-0<br>Mol.Weight:313R.Time:23.375<br>MassPeaks:531 | o-Tolylamino-acetic acid [1-(2,5-<br>dihydroxy-phenyl)-ethylidene]-<br>hydrazide | 0.21 |
| 9 | Formula:$C_{14}H_{22}O$ CAS:0-<br>00-0 Mol.Weight:206R.Time:18.355<br>MassPeaks:502 | 2,4-Ditert-Butylphenol | 3.19 |
| 10 | Formula:$C_{18}H_{36}$ CAS:7206-<br>25-9 Mol.Weight:252R.Time:19.345<br>MassPeaks:455 | 9-Octadecene | 2.62 |
| 11 | Formula:$C_{16}H_{30}O$ CAS:330207-53-9<br>Mol.Weight:238R.Time:22.540<br>MassPeaks:517 | E-14-Hexadecenal | 2.90 |
| 12 | Formula:$C_9H_{11}NO_2$ CAS:0-<br>00-0 Mol.Weight:165R.Time:23.060<br>MassPeaks:492 | 5-(Morpholino)Pent-2-en-4-ynal | 2.87 |
| 13 | Formula:$C_{17}H_{19}N_3O_3$CAS:0-00-0<br>Mol.Weight:313R.Time:23.375<br>MassPeaks:531 | o-Tolylamino-acetic acid [1-(2,5-<br>dihydroxy-phenyl)-ethylidene]-<br>hydrazide | 0.21 |

**CONCLUSION**

Five fungal endophytes were isolated from *H. indicum* in our research. All of the *Aspergillus sp* fungal isolates tested were moderately effective againstphytopathogens. However, the anti-bacterial activity of the root isolate *Aspergillus sp.* was more anti-bacterial activity against *Xanthomonas sp* isolates from *L. purpureus* (infected leaves).. The presence of several compounds with known antibacterial activity in the secondary metabolites of the cultured isolate of EF9 suggests that the root-associated endophytes' biosynthetic potential merits additional investigation. Additionally, the findings suggest that the endophytic community of plants that are immune to pathogens and adverse conditions may be an abundant source of novel bioactive compounds. In addition, our findings suggest that *H. indicum* medicinal properties may be due to the plant's endophytic microorganisms' capacity to produce biologically active secondary metabolites, and that endophytic fungi from the roots of *H. indicum* have the potential to be used in agricultural industry because they produce antimicrobial compounds. In order to discover new antibiotics, additional research is

required to identify the produced activecompounds.

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