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Original article

# Antifungal activity of *Streptomyces* sp. SLR03 against tea fungal plant pathogen *Pestalotiopsis theae*

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ABSTRACT

*Objectives:* The search for new biocontrol agents, especially from natural sources, to control plant pathogens is a key area in tea plant protection research.
*Methods:* Starch casein nitrate (SCN) agar was used to isolate the actinomycetes from the soil samples. These isolates were tested with modified version of the dual-culture method for antagonistic activity against *Pestalotiopsis theae*. The most bioactive isolate of actinomycets has been identified by biochemical, phisological, and morphological characterisation. Bioactivity of *Streptomyces* sp. SLR03 metabolites was measured *in vitro* and *in vivo*. The extract was eventually analyzed by GC–MS.
*Results:* For the first time, an attempt has been made to isolate actinomycete species with bio-control potential from the river soil samples. A total of one hundred and seven actinomycete strains isolated and were evaluated for antagonistic potential against *Pestalotiopsis theae* using a dual-culture assay. Among the strains isolated, one strain SLR03 that showed potential and it was characterized. Further, the strain SLR03 was evaluated for antagonistic activity against *P. theae* both *in vitro* and *in vivo*. In *in vitro* assay, 86.15% and 93.85% mycelial growth inhibition was observed with cell-free filtrate and ethyl acetate extract of *Streptomyces* sp. SLR03, respectively. The ethyl acetate extract was further evaluated for its biocontrol activity against *P. theae*, it exhibited 80.39% reduction of disease incidence compared to the control. Further, the ethyl acetate extract were analyzed using GC–MS. The GCMS chromatogram exhibited 24 intense peaks consistently with 19 different compounds, 10 of which contain antifungal activity.
*Conclusions:* This present study illustrates that *Streptomyces* sp. strain SLR03 is a prospective candidature for forthcoming biological control programme.
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## 1. Introduction

Grey blight in tea caused by *Pestalotiopsis theae* has become a challenging disease in tea-cultivating countries, and 17% crop loss has been reported during monsoon seasons (Sanjay et al., 2008; Sanjay, 2017). Chemical fungicides, such as mancozeb, Companion and Carbendazim, are used to control *P. theae* in tea fields. The continual use of chemical fungicides leads to residue issues, disease resistance and cost constraints to the planters. To overcome these problems and effectively control plant pathogens, there is a necessity to identify antifungal compounds using a biological approach. An investigation of novel antifungal compounds from actinomycetes is of significant research around the globe.

Microbes are economically sustainable sources for the synthesis of antifungal compounds (Jiménez-Reyes et al., 2019; Janette et al., 2019). Among microorganisms, the actinobacteria are one of the most fascinating candidates of bioactive compounds that inhibit various plant pathogenic fungi (Betancur et al., 2017; Djinni et al., 2019). Actinobacteria represent an extensive array of important and renowned resources for bioactive metabolites, among the members of *Streptomyces* and it contributed more than 60% of the antimicrobials compounds to date (Das et al., 2018; Sholkamy et al., 2020).

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Therefore, in the current era, the search for novel actinobacteria from diverse locations to explore the novel and prospective antifungal compounds have become importance. An attempt has been made to isolate actinomycetes from the fresh water river basin and evaluate *in vitro* and *in vivo* biocontrol activity of *Streptomyces* sp. SLR03 against *P. theae* it is an economically important tea pathogen.

## 2. Materials and methods

### 2.1. Collection of soil samples

Soil samples were collected on January 2012 from the Sholayar river, located at 76° 39′ 0″ E, 10° 27′ 30″ N longitude and 76° 44′ 35″ E, 10° 27′ 0″ N latitude. The Samples were procured from 4 to 5 cm of the soil profile. The soil samples were collected at different locations using clean, dry polyethylene bags and spatulas. The soil samples were stored at 4 °C temperature in refrigerator until further process it.

### 2.2. Actinomycete isolation

Actinomycetes were isolated from the collected soil samples using standard protocols (Kuster and Williams 1964) on starch casein nitrate (SCN) agar augmented with cycloheximide (25 μg/ml) and rifampicin (50 μg/ml) and incubated at 30 °C for 5 days. Actinomycete colonies showing different morphological characteristics were picked and sub-cultured into International *Streptomyces* Project (ISP) 2 agar slants. Stock cultures of isolates were maintained as spore and mycelial suspensions in 20% glycerol at −20° C.

### 2.3. Screening of antagonistic actinomycetes

The antagonistic effects of actinomycete isolates were assessed by modified version of the dual-culture method (Landa et al., 1997). The actinomycete isolates were streaked on a one corner of the SCN agar plate and incubated for 5 days at 30° C. A mycelial disk (5 mm in diameter) of

*P. theae* was then inoculated on the plate, which was incubated for an additional 72 h at 30° C. The mycelial growth of *P. theae* toward (a) and away (b) from the actinomycetes was then measured to determine the effective antagonistic activity of actinomycete isolates and select appropriate isolates for further studies.

### 2.4. Characterization of prospective isolate Streptomyces sp. SLR03

The actinomycete isolate showing the most potent fungal antagonism was characterized based on morphological, biochemical and physiological characteristic features (Miyadoh et al., 1997). The morphological characteristics included colony shape and color, sporulation and pigment diffusion on ISP and TS (tryptic soy) agar (Shimizu et al., 2000). The physiological and biochemical properties of isolates were investigated according to the methods of (Shirling and Gottlieb, 1966) and Holt et al., (2000).

### 2.5. Production and extraction of bioactive metabolites

The seed inoculums were prepared by culturing of *Streptomyces* sp. SLR03 in ISP 2 medium for 24 h. The seed culture (10%) was inoculated to TS medium containing (g/l) tryptone 17.0, soybean meal 3.0, dextrose 2.5, $NaCl_2$ 5.0 and $K_2HPO_4$ 2.5, pH 7.1 ± 0.2, and incubated on a rotary shaker for 5 days at 29° C. The 20 L of culture broth obtained after filtration was extracted twice with ethyl acetate and concentrated under reduced pressure to yield a crude extract that was dried in a rotary evaporator (Kavitha et al., 2009).

### 2.6. In vitro antagonistic activity

The agar well diffusion method was performed using the technique of Bauer et al., (1966), on double-layered PDA plates to test the non-culture filtrate (NCF) and ethyl acetate extract (EAE) of *Streptomyces* sp. SLR03 isolate. Three wells were made in the top layer of solidified PDA plates by punching the top agar layer 3 times with a cork borer (5 mm diameter) along a radius approximately 3 cm from the center of the plate. One agar disc (5 mm diameter) of *P. theae* culture was cut from the marginal colony of a 5-day-old culture cultivated in PDA plates and moved into the middle of the plate. Fifty microliters of NCF, EAE (50 μg/ml) or trypticase soy broth (TSB) was put in one of the 3 wells, and the plates were incubated at 28° C for 5 days. The standard fungicides Bavistin (0.05%), Dithane (0.3%) and Companion (1%) were used at appropriate concentrations. The results were observed and assessed the percent inhibition of colony growth (PICG), according to modified methods of Lokesha and Benagi (2007). Each test was repeated three times, and the average was calculated.

### 2.7. In vivo antagonistic activity

The *in vivo* antagonistic activity was evaluated using greenhouse conditions as reported by Kim et al., (2003). The EAE compound was dissolved in 1 ml of dimethyl sulfoxide (DMSO) and diluted with 49.5 ml of 1% Tween 80. Aliquots of 50 ml were sprayed onto one set of tea plants (consist of six plants). The treated tea saplings were maintained in a greenhouse for 1 day prior to inoculation with *P. theae*. The standard fungicides and 1% DMSO were used as a positive and negative control respectively. The tea plantlets were kept under greenhouse conditions suitable for establishment of disease. The disease intensity was determined according to the method described by (Wang et al., 2009). The tea saplings were spraying with spore suspension of *P. theae* ($1X10^6$ spores/ml) at the first stage (one plant/pot). The spore suspensions were prepared using 0.85% saline mixed with 15 days old culture spores. All the experiments were conducted in triplicate.

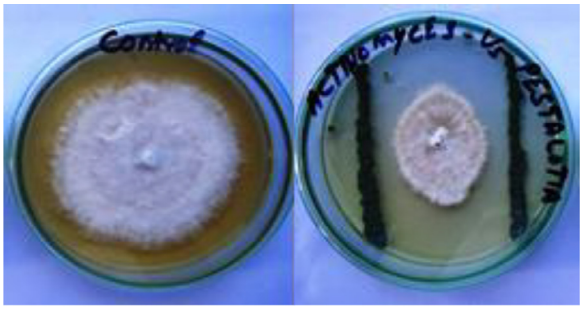
### 2.8. Gas chromatography-mass spectrometry (GC–MS) analysis

Partially purified *Streptomyces* extract was analyzed by using GC–MS with a fused silica capillary column (C18, 30 × 0.25 mm ID, film thickness 0.5 μm). The data were processed with GC–MS ChemStation (Agilent Technologies, 6890-N series GC with 5990 series II). The column conditions were as follows: column oven temperature 150° C (4 min) – 4° C/min, injection port temperature 250° C and detector port temperature 280° C (Roy et al., 2006). The sample peaks detected with gas chromatography were subjected to mass spectral analysis. The spectra were analyzed using available library data and with National Institute of Standards and Technology (NIST) MS search (version 2.0; included with NIST '02 mass spectral library and accessed with Agilent p/n G1033A).

## 3. Results

### 3.1. Isolation and screening of antagonistic actinomycetes

A total of 107 actinomycetes isolated from 15 different soil samples, of 107 isolates, the 6 most prominent isolates (isolates that were found abundantly, produced pigments and inhibited adjacent colonies on SCN agar plates) were further screened for their antagonistic potential against *P. theae* using an *in vitro* dual-culture




a. b.

**Fig. 1.** *P. theae* using cross plug method incubated for 7 days at 28° C on Potato Dextrose agar medium; (a) control plate of *P. theae*; (b) antagonism activity of *Streptomyces* sp. SLR03 against *P. theae*.

assay. Among, six actinomycete isolates, only one strain (SLR03) was found to have antagonistic potential against *P. theae* (Fig. 1).

The strain SLR03 grew on SCN agar media and showed the typical morphology of *Streptomyces* sp. The color of the aerial and substrate mycelium tended to be green and light green respectively. The surface of the mycelium dusty with rough in nature (Table 1). The isolate SLR03 not produced any diffusible pigments. Further, isolate SLR03 Gram positive with filamentous in nature aerial hyphae were differentiated into long looped chains of spores with spiral spore morphology (Fig. 2). The biochemical and physiological results as follows; indole negative, methyl red negative, Voges Proskauer negative, citrate positive, starch and casein positive. The sugar utilization results showed with positive for glucose and sucrose, negative for lactose and mannitol (Table 2). The strain *Streptomyces* sp. SLR03 was evaluated further for its antagonistic activity against *P. theae* under *in vitro* and *in vivo* conditions.

### 3.2. In vitro antagonistic activity

The antagonistic activity of *Streptomyces* sp. SLR03 was measured by testing SLR03-derived NCFs and EAEs against *P. theae*. After 5 days, EAEs showed the maximum inhibition of *P. theae* colony growth (93.08%), whereas NCFs showed minimum inhibition (93.85%). The commercial fungicides Bavistin (0.05%), Dithane (0.3%) and Companion (1.0%) inhibited colony growth by 97.08%, 96.11% and 95.15%, respectively, and the control showed no inhibition of fungal colony growth (0.0%) (Fig. 3 and Table 3).

### 3.3. In vivo antagonistic activity

The results of *in vivo* studies in a greenhouse with infected plants are displayed in Table 4 and Fig. 4. The partially purified compound and ethyl acetate extract from *Streptomyces* sp. SLR03 efficiently controlled gray blight disease and was exhibited maximum 89.39 and 81.09% of disease control efficiency. The maximum percentage of disease control efficiency was observed at 7 days compared to the *P. theae* infected tea plants. The maximal reduction of disease incidence was observed with partially purified compound (89.39%), followed by EAEs (81.09%), but the commercial fungicides such as Bavistin, Dithaneand Companion showed reduction in disease incidence by 62.28%, 75.73% and 76.72%, respectively (Table 4 and Fig. 4).

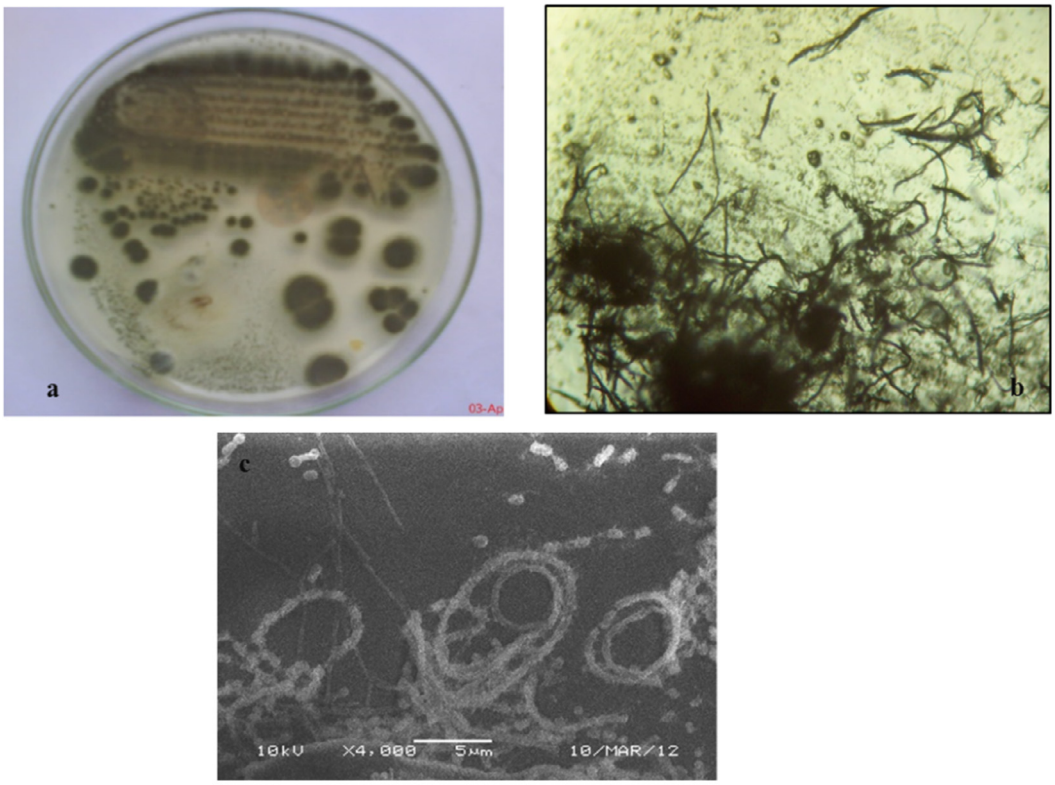



**Fig. 2.** *Streptomyces* SLR03 a) colony morphology on starch casein nitrate medium (SCN) in a 5 days old culture, b) micrograph of branched aerial mycelium 7 days old culture (x 1000), c) SEM image of isolate SLR03 spore morphology.

**Table 1**
Cultural, morphological and physiological characteristics of antagonistic *Streptomyces* sp. SLR03 on SCN agar.

| Characteristic features | Observation | | | |
|---|---|---|---|---|
| **Cultural** | **Mycelium** | **Colony nature** | **Colony colour** | **Pigmentation** |
| | Aerial | Dusty with spores | Green | No pigment |
| | Substrate | Rough surface | Light green | |
| **Microscopic** | **Gram staining** | | **Mycelial nature** | |
| | Gram positive filamentous | | Long looped chain of spore | |
| **Biochemical** | **Indole** | **MR** | **VP** | **Citrate** |
| | Negative | Negative | Negative | Positive |
| **Physiological** | **Oxidase** | **Catalase** | **Starch** | **Casein** |
| | Negative | Negative | Positive | Positive |
| **Sugar Utilization** | **Glucose** | **Sucrose** | **Lactose** | **Mannitol** |
| | Positive | Positive | Negative | Negative |

**Table 2**
Biochemical characteristics of *Streptomyces* sp. strain SLR03 after 5 days incubation at 28 °C.

| Biochemical tests | SLR03 | Carbohydrate utilization | SLR03 | Biopolymer hydrolysis | SLR03 |
|---|---|---|---|---|---|
| Indole | – | Dextrose | + | Gelatin | – |
| Methyl red | – | Fructose | + | Starch | + |
| VP | – | Sucrose | + | Casein | + |
| Citrate | + | Galactose | – | | |
| TSI | A/K | Lactose | – | | |
| Oxidase | – | Mannitol | – | | |
| Catalase | – | | | | |

'+' - positive; '–' – negative.

### 3.4. GC–MS analysis

GC–MS analysis of the partially purified *Streptomyces* extract revealed 19 different compounds. Only 10 of these compounds have antimicrobial activities such as tetradecane ($C_{14}H_{30}$), nonadecane ($C_{19}H_{40}$), 10-Henicosene ($C_{21}H_{42}$), 3-Eicosene ($C_{20}H_{40}$), 1-Hexadecanol ($C_{16}H_{34}O$), 1-iodo-2-methylundecane ($C_{12}H_{25}I$), Tetradecane, 2,6,10- trimethyl ($C_{17}$ $H_{36}$), Decane – 2,3,5,8 trimethyl ($C_{14}$, $H_{30}$), Pterin-6-carboxylic acid ($C_7H_5N_5O_3$) and 1-octadecanesulfonylchloride ($C_{18}H_{37}ClO_2S$) as in Table 5. The nature and activity of each chemical compound are also depicted in Table 5. The GC–MS spectrum confirmed the presence of 10 major components with retention times of 4.10, 5.09, 6.05, 7.00, 8.04, 9.06, 10.08, 11.00, 12.05, 14.02, 15.05 and 16.00 min (Fig. 5). Mass spectral data obtained from GC–MS were interpreted using the NIST database.

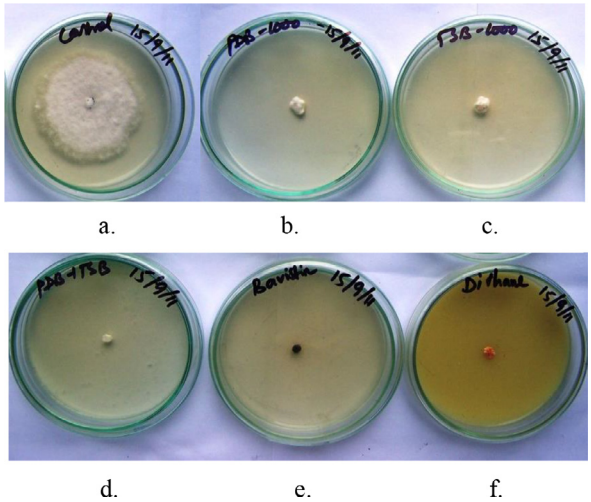



**Fig. 3.** Antagonistic activity of *Streptomyces* sp. SLR03 against *P. theae* grown on PDA plates; (a) control plate of *P. theae* on PDA plates, (b) PDA plate mixed with 1ml of non-culture filtrate, (c) PDA plate mixed with 1ml of ethyl acetate extract, (d) PDA plate mixed with 0.05 % of bavistin, (e) PDA plate mixed with 0.3 % of dithane, (f) PDA plate mixed with 1.0 % of companion.

**Table 3**
Antagonistic activity of *Streptomyces* sp. SLR03 filtrates against *P. theae*.

| S. No. | Treatment | % of inhibition fungal colony growth |
|---|---|---|
| 1 | Control | 0.00 |
| 2 | 1 ml of Non-culture filtrate | 93.85 |
| 3 | 1 ml of Ethyl acetate filtrate | 93.08 |
| 4 | Bavistin (0.05%) | 97.08 |
| 5 | Dithane (0.3%) | 96.11 |
| 6 | Companion (1.0%) | 95.15 |
| | SE | 3.84 |
| | CD | 7.88 |

## 4. Discussion

The overuse of chemical fungicides and fertilizers is common in agricultural farming systems of most parts of the world which affects, it threatening the environments and health of individuals. In general pesticides persists in the environment and also destroys the beneficial microorganisms which have positive effects on soil fertility and plant growth. Biological controls with use of microorganisms are an appropriate substitute for such negative impacts on environment and also disease management. Such biological control measures with use of microorganisms are well adapted to the soil ecology and also determinedly exert effective antagonistic activity against soil-dwelling plant pathogens (Kohl et al., 2019).

Over the years, the use of microbial secondary metabolites for crop protection received greater attention, and these metabolites are used as an alternative to chemical compounds (Singh et al., 2017; Cardoso et al., 2019). Since, these metabolites are biologically synthesized, it contains specific activity against the plant pathogens and also environment friendly (Heydari and Pessarakli, 2010; Masi et al., 2018). Thus, worldwide interest has been renewed, and approximately 40 different plant diseases and asso-


**Fig. 4.** *In vivo* Biocontrol activity of *Streptomyces* sp. SLR03 against *P. theae*; (a) control tea plants (un-inoculated, disease free), (b) inoculated with *P. theae* (diseased), (c) *P. theae* inoculated plants with ethyl acetate extract, (d) *P. theae* inoculated plants with partially purified compound, (e) *P. theae* infected tea plants with Bavistin (0.05 %), (f) *P. theae* infected tea plants with Dithane (0.3 %), (g) *P. theae* inoculated plants with companion (1.0 %).

**Table 4**
Control of gray blight disease incidence on tea plants by *Streptomyces* sp. SLR03 under greenhouse conditions.

| S.No | Treatments | Average disease increased in shoots (mm) | % disease control |
|---|---|---|---|
| 1. | Un-inoculated (disease free) | | – |
| 2. | Inoculated (control) | 16.57 | – |
| 3. | Ethyl acetate extract | 3.25 | 81.09 |
| 4. | Partially purified compound | 5.33 | 89.39 |
| 5. | Bavistin (0.05%) | 6.25 | 62.28 |
| 6. | Dithane (0.3%) | 7.83 | 52.73 |
| 7. | Companion (1.0%) | 3.86 | 76.72 |
| | SE | | 3.64 |
| | CD | | 7.47 |

ciated pathogenic organisms are currently managed with microbial metabolites (Pal and Gardener, 2006; Panth et al., 2020). Several microbial bioactive secondary metabolites have also been discovered as agrochemicals, and many of them are currently commercially available (Sanjai, 2014).

The current investigation on *in vitro* and *in vivo* using an existing natural resource, a *Streptomyces* strain that showed effective antagonistic characteristics against *P. theae*. Ara et al., (2012) reported a similar antifungal activity of actinomycetes against *P. mangifera*, a causal agent of mango brown rot. The results of Ponmurugan et al., (2011) revealed that most isolates of *Streptomyces* spp. from tea rhizosphere soil showed potential antagonists activity against tea pathogens. Several other researchers have reported satisfactory results using *Streptomyces* spp. against plant pathogens *in vitro* (Kim and Song, 2016; Lyu et al., 2017; Kim et al., 2019).

*Streptomyces* sp. strain SLR03 suppressed fungal disease in inoculated tea saplings under greenhouse condition. Greenhouse experiments showed that the biocontrol efficacy of *Streptomyces* sp. strain SLR03 correlated with anti-*P. theae* activity. The results confirm the importance of the fresh water *Streptomyces* isolates as biocontrol agents and emphasize the importance of indigenous *Streptomyces* sp. as biocontrol agents against tea fungal phytopathogens. The present results corroborate with the previous studies which determined the biocontrol potential of microorganisms under artificial conditions in greenhouse experiments (Lia et al., 2012; Zhou et al., 2014; Besset-Manzoni et al., 2019).

GC–MS analysis of *Streptomyces* sp. strain SLR03 EAE showed 24 retention peaks, most of which were reported to have antifungal metabolites. Due to a lack of authentic samples and library data for the corresponding compounds, some of the GC–MS peaks

**Table 5**
GC/MS analysis of *Streptomyces* sp. SLR03 partially purified extract.

| S.No. | Compounds | Bioactive propertiesAs per literatures |
|---|---|---|
| 1. | Hexadecane ($C_{16}$ $H_{34}$) | |
| 2. | Tetradecane ($C_{14}$ $H_{30}$) | Antifungal and antibacterial |
| 3. | Decane-2,4,6 trimethyl ($C_{13}$ $H_{28}$) | |
| 4. | Nonadecane ($C_{19}$ $H_{40}$) | Antifungal |
| 5. | 3-Trifluroacetoxytetradecane ($C_{16}H_{29}F_3O_2$) | |
| 6. | 10-Henicosene ($C_{21}$ $H_{42}$) | Anti-phytopathogenic fungal pathogen |
| 7. | Trichloroacetic acid ($C_{16}$ $H_{29}Cl_3O_2$) | |
| 8. | 3-Eicosene ($C_{20}$ $H_{40}$) | Antifungal |
| 9. | 1-Hexadecanol ($C_{16}$ $H_{34}$ O) | Antifungal/Antimicrobial |
| 10. | 1-iodo -2-methylundecane ($C_{12}$ $H_{25}I$) | Antifungal/Antimicrobial |
| 11. | Tetradecane, 2,6,10-trimethyl ($C_{17}$ $H_{36}$) | Anti-inflammatory and Antifungal |
| 12. | Decane-2,3,5,8 trimethyl ($C_{14}$ $H_{30}$) | Anti-phytopathogenic fungal pathogen |
| 13. | 4-Trifluroacetoxypentadecane($C_{17}H_{31}F_3O_2$) | |
| 14. | 3-Trifluroacetoxytetradecane ($C_{16}H_{29}F_3O_2$) | |
| 15. | 2-Trifluroacetoxytetradecane ($C_{16}H_{29}F_3O_2$) | |
| 16. | 5-Eicosene (E)($C_{20}$ $H_{40}$) | |
| 17. | Pterin 6 carboxylic acid ($C_7H_5N_5O_3$) | Antifungal |
| 18. | 1octadecanesulfonylchloride($C_{18}H_{37}$ $ClO_2S$) | Antifungal |
| 19. | Ethanol-2(hexadecyloxy) ($C_{18}$ $H_{38}$ $O_2$) | |

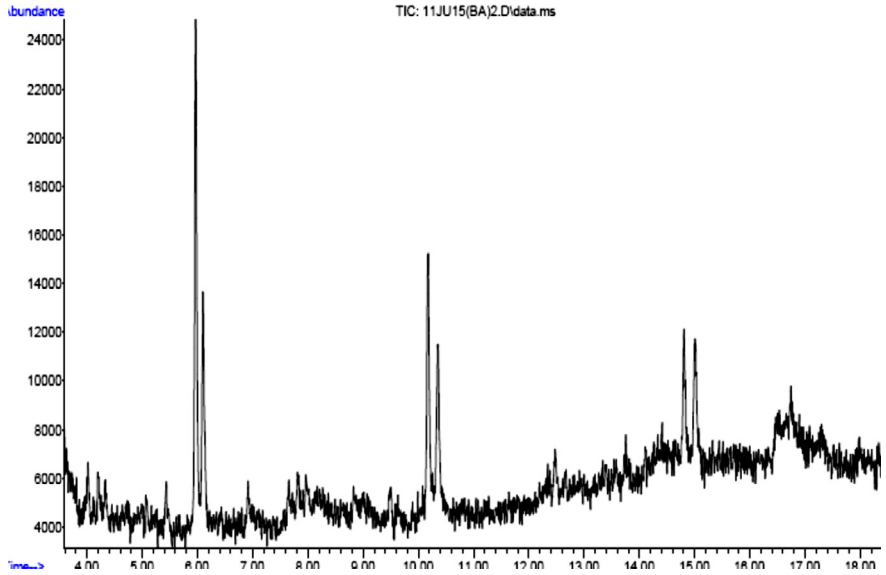



**Fig. 5.** GC MS analysis of *Streptomyces* sp. SLR03 partially purified extracts.

remained unidentified. The chemical constituents of extracts and the important functional groups they contribute are characteristic of bioactive compounds and validate the use of *Streptomyces* sp. strain SLR03 as an important candidate antifungal agent. Most of the compounds were aromatic, and has varied biological activity, including antifungal activity (Sholkamy, 2014). The investigation confirmed the potential richness of antifungal agents from *Streptomyces* sp. strain SLR03. Further investigation is needed to determine the structure of an active compound and scale up its production. From the results of primary screening, a phytopathogenic study and GC–MS analysis, it is evident that strain SLR03 has great potential for secondary metabolite production.

## 5. Conclusion

The present investigation reports the presence of bioactive actinomycetes in the Sholayar river basin (fresh water environment), which is surrounded by reserve forest and tea plantations and originates from the Western Ghats, South Asia. Locally available strains of bioactive actinomycetes can be explored and tapped as one of the potential source of novel antifungal antibiotics. The strain *Streptomyces* sp. SLR03 exhibited effective antifungal activities against *P. theae*. Further, the ethyl acetate extract showed presence of antifungal compound, when analyzed with GC–MS chromatography spectrum. This biological agent could be combined with fungicides for the effective control of foliar pathogens, such as *P. theae*, that cause grey blight leading dieback disease in tea. The future, the potential candidature of *Streptomyces* sp. SLR03 characterized further with NGS approach and bioactive principle compound was identified.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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