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PRODUCTION, ISOLATION AND STRUCTURAL ELUCIDATION OF A NOVEL ANTIMICROBIAL METABOLITE FROM THE ENDOPHYTIC FUNGUS, *PHOMOPSIS/DIAPORTHE THEAE*

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ABSTRACT

The present study was aimed to isolate endophytic fungi from five different medicinal plants and to structurally elucidate an antimicrobial compound from Phomopsis theae (asexual synonym of Phomopsis theae (syn. Diaporthe theae), an endophyte is the first and hitherto the only recorded Phomopsis/Diaporthe species isolated from Vitex negundo in Tamilnadu. A total of 77 coelomycetous endophytic fungi were isolated and identified based on spore morphology and the mechanism of spore production. Among 77 endophytic fungi, the isolate, Phomopsis/Diaporthe theae showed excellent antimicrobial activity against all the human pathogens tested. Further, its fermentation conditions were optimized to enhance the antimicrobial metabolites production. Bioassay guided fractionation of crude ethyl acetate extract of Phomopsis/Diaporthe theae led to the isolation of a novel antibacterial and anticandidal compound, which was later identified as pyrimidine imino methylfuran derivative (1). This compound showed pronounced antimicrobial activity against Staphylococcus aureus, Pseudomonas aeroginosa and Candida albicans even at very low concentration (5 µg/mL) and the MIC was found to be 1.25 µg/mL against all the pathogens. Thus, the obtained results convincingly suggest that Phomopsis/Diaporthe theae could be a promising candidate for the discovery of new antibiotics.

KEY WORDS

Endophytic fungi, Phomopsis/Diaporthe theae, Antimicrobial compound, Human pathogens.

INTRODUCTION

Fungi that reside in the tissues of living plants without causing any disease and symptoms are referred to as endophytes. These fungal endophytes produce novel secondary metabolites as a resistance mechanism to overcome pathogenic invasion (Tan and Zou 2001; Venieraki et al. 2017). Endophytic fungi have been considered as a rich repository of novel bioactive compounds having unusual biological activities (Devi et al. 2012; Maheshwari et al. 2017). Even though a large number of studies have focused on the antimicrobial activity of natural products, the demand for new antibiotics is still increasing owing to the rapid emergence of resistant strains (Supaphon et al. 2013; Karam et al. 2016). Endophytic fungi have already been reported as prolific producers of novel antimicrobials (Katoch et al. 2014; Sharma et al. 2016). Searching natural products synthesized by fungal endophytes could be a promising way to solve the problem of drug resistance (Yashavantha Rao et al. 2015a).

Diaporthe (syn. *Phomopsis*) species are well-known as endophytes and also it has induced considerable interest in this genus (Dissanayake et al. 2015; 2017a, b). *Phomopsis* is a genus containing over 1000 species and they are commonly found as endophytes in tropical plants (Uecker 1988). The species of *Diaporthe* have

attracted considerable research interest across the world mainly due to their ability to produce different types of antimicrobial metabolites (Tong et al. 2014). Some of the reported antimicrobials from *Phomopsis* spp. were 3,11,12-trihydroxycadalene (Silva et al. 2006), phomoenamide (Rukachaisirikul et al. 2008) and dicerandrol C (Erbert et al. 2012). The anti-candidal competence of a novel ketone derivative was isolated from an endophytic fungus *Diaporthe* sp. ED2 dwell in *Orthosiphon stamieus* (Yenn et al. 2017). To the best of our knowledge, Aliyar has not previously been explored for endophytic fungal diversity and antimicrobial compounds. Therefore, the present study was aimed to isolate endophytic fungi from five different medicinal plants in the forest areas of Aliyar, Pollachi and to structurally elucidate an antimicrobial compound from *P./D. theae*

MATERIALS AND METHODS

Chemicals

All chemicals/regents used in this study were of analytical grade and were purchased from HiMedia Laboratories Pvt. Ltd, Mumbai, India. The human pathogenic microorganisms *Bacillus subtilis* (MTCC 441), *Staphylococcus aureus* (MTCC 96), *Micrococcus luteus* (MTCC 1541), *Escherichia coli* (MTCC 443), *Pseudomonas aeruginosa* (MTCC 424), *Klebsiella pneumoniae* (MTCC 109) and *Candida albicans* (MTCC 227) were obtained from the Microbial Type Culture Collection (MTCC) centre, Chandigarh, India and were maintained on nutrient agar slants at refrigerated condition.

Study area

In this present study, both young and mature leaf and stem samples were collected from five different medicinal plants *Anisomeles malabarica* (L.) R.Br., *Mesua ferrea* L., *Piper nigrum* L., *Rauvolfia tetraphylla* L. and *Vitex negundo* L. in the forest areas of Azhiyar (10.4739 ºN and 76.9728 ºE), Pollachi, South India. The samples were collected in sterile polypropylene bags and were brought to the laboratory for isolation of fungi (Fisher and Petrini 1987).

Isolation of endophytic fungi

The collected samples were washed thoroughly using running tap water. The segments (approx. 0.5 cm^2) of each leaf and stem tissues of each plant were surface sterilized and placed on potato dextrose agar (PDA) medium poured Petri plates for isolation of endophytic fungi (Dobranic et al. 1995; Schulz et al. 1998). The medium was also amended with chloramphenicol (25 µg/ mL) to minimize bacterial contamination. The Petri plates were incubated at 23 ± 2°C in a light chamber with 12 h light followed by 12 h dark cycle. The Petri dishes were observed at regular intervals from the second day onwards for the fungal growth. Individual colonies of fungi were isolated and maintained on PDA slants (Bills 1996).

Identification of endophytic fungi

The morphological identification of fungal isolates was determined by bright field microscopy observations of lacto phenol cotton blue stained fungal specimen at 40× magnification. The isolated fungal strains were identified on the basis of spore morphology and the mechanism of spore production down to genus level by standard mycological monographs (Sutton 1980; Nag Raj 1993). In this study, altogether, 94 different fungal isolates were obtained and identified. Of these, 77 isolates were belonged to coelomycetes, 9 isolates were belonged to hyphomycetes and the remaining 8 were sterile forms.

Screening of endophytic coelomycetous fungi for antimicrobial activity

In this study, all the 77 coelomycetous endophytic fungi were screened for antimicrobial activity by agar well diffusion method (Rios et al. 1988). The mycelial discs of about 9 mm were cut from the mother culture of all the coelomycetous fungi and inoculated separately in 100 mL saline bottle containing 25 mL of potato dextrose broth (PDB). The inoculated bottles were incubated at 23 ± 2°C for 21 days under static condition. After incubation, the culture was harvested, and the growth of each organism was measured in terms of mycelial dry weight. The culture filtrate was collected by passing through double layer of cheese cloth in a glass funnel and was centrifuged at 8,000 rpm for 20 min at 4°C. The cell free supernatant was filter sterilized using 0.45 µm filter paper and bioassayed against human pathogens. The bacterial pathogens were grown in Muller-Hinton agar (MHA) medium and the yeast pathogen was grown in Sabouraud dextrose agar (SDA) medium. A total of 100 µL of cell free supernatant of each fungus was loaded into each well of the Petri plates. 10% dimethyl sulphoxide (DMSO) was served as control. After inoculation, the plates were incubated at 37°C for 24 h and the zone of inhibition (ZOI) was measured in terms of millimeter. These assays were carried out in triplicate.

18S rDNA sequencing and phylogenetic analysis

Among the 77 coelomycetous fungi tested, the isolate, *Phomopsis/Diaporthe sp. GJJM 16* showed excellent antimicrobial activity against all the human pathogens; hence, it was further chosen for molecular identification. The fungal genomic DNA was isolated by CTAB extraction method (Sterling 2003) and PCR amplification was done using ITS 1 and ITS 2 primers (White et al. 1990). The part of ribosomal RNA spanning the 3' end of the 18S rDNA, the internal transcriber spacer, the 5.8S rDNA and a part of the 5' end of 28S rDNA was amplified. The purified PCR products were sequenced using ABI 3730xl Genetic Analyzer by BigDye terminator method (Applied Biosystems, USA). The 18S rDNA sequence of this fungus was aligned with reference sequences showing sequence homology from the NCBI database using multiple sequence alignment programme.

Optimization of antimicrobial compound production

Effect of different liquid media

Five different liquid media namely, potato dextrose broth (PDB), potato carrot broth (PCB), potato dextrose yeast extract broth (PDYEB), modified medium-1 broth (M-1DB) and Czapex-Dox broth (CDB) were inoculated separately with *P./D. theae* in conical flasks and incubated at 23 ± 2°C for 28 days. After incubation, the mycelial dry weight was estimated. Ethyl acetate extract was prepared, and antimicrobial activity was evaluated using agar well diffusion method as described earlier.

Effect of different temperature

P./D. theae was inoculated in M-1D medium with the initial pH of 6.8 and incubated at different temperatures such as 15, 25 and 35°C for 21 days. After incubation, the mycelial dry weight was estimated, and ethyl acetate extract was tested for antimicrobial activity using agar well diffusion method.

Effect of different pH

The pH of M-1D medium was adjusted to 5, 5.5, 6, 6.5, 7, 7.5 and 8 with 0.1 N NaOH or 0.1 N HCl. Each flask was inoculated with *P./D. theae* and incubated at 25°C for 21 days. After incubation, the mycelial dry weight was estimated, and ethyl acetate extract was tested for antimicrobial activity using agar well diffusion method.

Effect of different incubation period

P./D. theae was inoculated in M-1D medium with the pH of 6.5 and incubated for 7, 14, 21 and 28 days at 25°C under static condition. After incubation, the mycelial dry weight was estimated, and ethyl acetate extract was tested for antimicrobial activity using agar well diffusion method.

Effect of different substrates in M-1D medium

About 3% (w/v) of different carbon sources (glucose, fructose, xylose, mannose, lactose, sucrose, maltose, soluble starch and glycerol), 0.5% (w/v) of different nitrogen sources (bactotryptone, yeast extract, casein, Hi-Veg peptone, beef extract, soytone, sodium nitrate, malt extract, ammonium nitrate and ammonium tartate), 0.005% of different amino acids (aspartic acid, cysteine, glutamic acid, arginine, leucine, methionine, proline and threonine) and 0.005% of different vitamin sources (ascorbic acid, biotin, cyanocobalamine, folic acid, inocitol, nicotinic acid, pantothenic acid, riboflavin and thiamine HCl) were amended separately in M-1D broth. Combination of M-1D medium mineral source ($Ca_2(NO)_3$-0.0028%, KNO_3-0.008%, KCl-0.006%, $MgSO_4$-0.036%, NaH_2PO_4-0.002%, H_3BO_3-0.0001%, $MnSO_4$-0.0005%, $ZnSO_4$-0.0002%, KI-0.0007%) was amended with altered carbon (3.5% sucrose) and nitrogen (0.6% casein and 0.6% yeast extract) sources separately. M-1D medium without respective substrate was run as control. Each flask was inoculated with *P./D. theae* and incubated at 23 ± 2°C under static condition. The culture was harvested after 21 days and filtered through Whatman No.1 filter paper. Ethyl acetate extract was prepared, and antimicrobial activity was evaluated. The mycelial biomass was collected, and the dry weight was estimated.

Extraction and isolation of active fraction

P./D. theae was grown in the optimized M-1D medium (pH 6.4). From the mother culture, about 30 discs (9 mm)/L were inoculated in hopkin flask. After inoculation, the flask was kept at 25°C under static condition for 28 days. Totally, 20 L of culture filtrate was prepared. The culture filtrate of *P./D. theae* was extracted with ethyl acetate and concentrated *in vacuo* to get crude extract (5.0 g). The crude ethyl acetate extract was subjected on TLC using hexane: ethyl acetate (6:4, v/v), which exhibited 8 visible spots under UV with 254 and 365 nm representing different Rf values. Among them, the bio-autography exposed R_f value of 0.55 showed significant inhibition zones around the spots against *B. subtilis, M. luteus, S. aureus, P. aeroginosa, K. pneumoniae and C. albicans.* Further, the bioassay guided crude extract was fractionated by silica gel (60–120 mesh) column chromatography using hexane–ethyl acetate–chloroform–methanol gradient

elution with the flow rate of 3 mL/min. A total of 132 fractions, each 20 mL, were collected and the fractions showing similar pattern were pooled together. A total of eight partially purified fractions (I-VIII) were obtained and were bioassayed using TLC bio-autography. Among these, the fraction V showed excellent antimicrobial activity against all the pathogens. This particular fraction was further separated using preparative TLC. The 'b' band of fraction V (14.57 mg) with the R_f value of 0.59 was separated using chloroform and ethyl acetate (70:30, v/v).

Chemical characterization of the active fraction

The physical appearance of the purified compound was visually observed. UV spectrum was acquired using JASCO V-650 Spectrophotometer at the wavelength of 200-900 nm and the FT-IR spectrum was obtained using JASCO FT-IR-4100 spectrophotometer in a diffuse reflectance mode at a resolution of 400-4000 cm^{-1} in KBr pellet. The number of protons and carbon atoms present in the purified compound was recorded through NMR spectral study (^{1}H and ^{13}C) using Brucker 400 MHz with deuterated chloroform ($CDCl_3$) as the solvent and the chemical shifts were measured in δ scale in ppm. The ESI-MS spectrum of this compound was recorded on a Finnigan LCQ with quaternary pump Rheos 4000 (Flux Instrument).

Antimicrobial activity of the purified compound

Well diffusion assay

The test pathogens *S. aureus*, *P. aeroginosa* and *C. albicans* were grown in Muller-Hinton agar (MHA) medium. Well diffusion assay was performed to determine the antimicrobial activity of the purified compound (Ojo et al. 2007). The purified compound was dissolved in 10% DMSO and different concentrations of this compound ranging from 5–25 µg/mL were loaded into each well of the Petri plates. 10% DMSO (negative control) was used to compare the antimicrobial activity of the purified compound; also, seventeen different standard antibiotics were used as the positive controls. After inoculation, the MHA plates were incubated at 37°C for 24 h and the ZOI was measured in millimeter. These assays were carried out in triplicate.

Minimum inhibitory concentration

Minimum inhibitory concentration (MIC) was performed for the purified compound against the human pathogens using MTT assay (Sheena et al. 2003). In a 96-well micro plate, 100 µL of sterile nutrient broth was taken in each well of the plate and the purified compound dissolved in 10% DMSO was serially diluted with the nutrient broth. Control wells were maintained with 10% DMSO as the negative control and gentamicin and nystatin were used as the positive controls. A total of 10 µL of test organisms (CFU $1.5x10^8$ cells/mL) were loaded into each well and incubated at 37°C for 24 h. After incubation, 5 µl of 0.5% of methyl thiazoyldiphenyl-tetrazolium bromide (MTT) was added to all the wells and observed for color intensity at 575 nm. The mean of live cells was recorded using ELISA reader (Power wave XS biotek, USA). This experiment was performed in triplicate to check for reproducibility.

Statistical analysis

The data were subjected to one-way analysis of variance (ANOVA) to determine the significance of individual differences at $p < 0.05$ level. Significant means were compared by the Duncan's multiple range test. All statistical analyses were carried out using SPSS statistical software package (SPSS, Version 10.0, Chicago, USA).

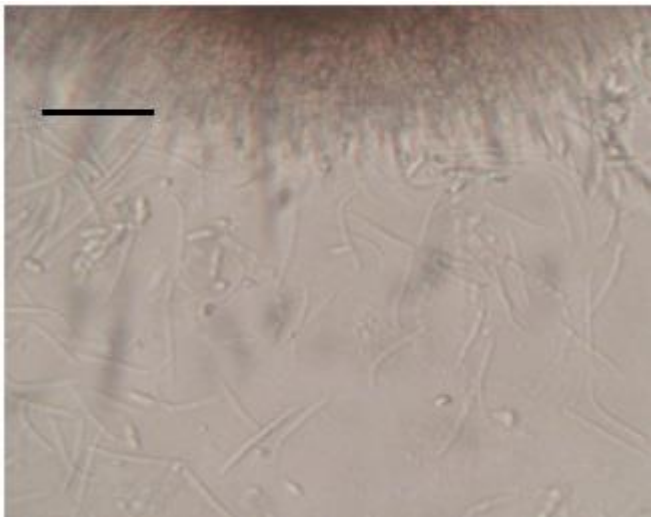
RESULTS

Screening of endophytic fungi for antimicrobial activity

Among 77 isolates tested, 67 isolates (87%) displayed antimicrobial activity against at least one human pathogen (Table S1). On the other hand, three isolates namely, *Phomopsis/Diaporthe sp. GJJM 06, Phomopsis/Diaporthe sp. GJJM 13 and Phomopsis/Diaporthe sp. GJJM 16* showed excellent antimicrobial activity against all of the seven pathogens, but 10 isolates did not show antimicrobial activity.

Among the 3 fungi, *Phomopsis/Diaporthe sp. GJJM 16* showed superior antimicrobial activity; hence, this fungus was further subjected to molecular characterization. Amplification and sequencing of the ITS region of fungal rDNA resulted in 588 base pair long nucleotide sequence and it was deposited in NCBI GenBank database (MD, USA) with the accession number JN638438.1. The molecular and classical taxonomy results strongly supported the identification of isolated strain as Phomopsis/*Diaporthe theae* (Fig. 1).

Fig. 1 Conidiogenous cells of *Phomopsis/ Diaporthe theae*, emitting prominent two types of conidia (α and β) under light microscope



Bar, 100 X

Fig. 2 ^{1}H-NMR spectrum of the purified compound MJK-2

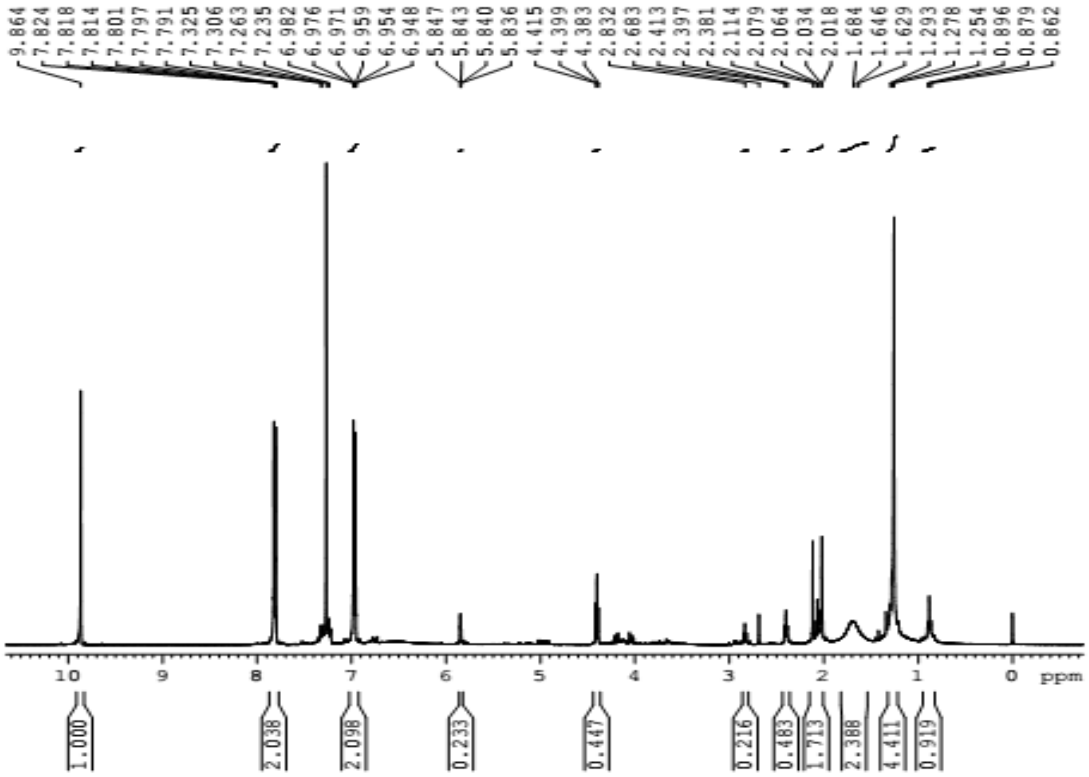


Fig. 3 ^{13}C-NMR spectrum of the purified compound MJK-2

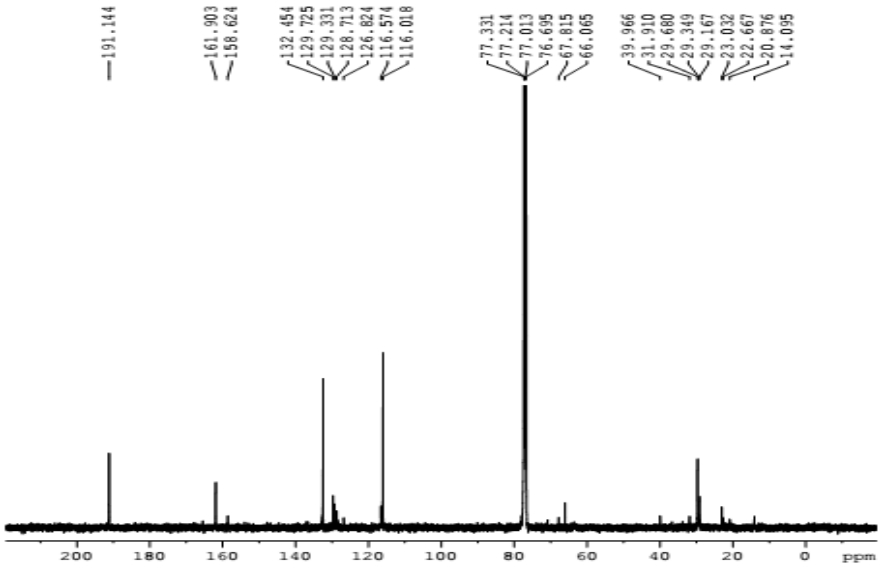


Fig. 4 Mass spectrum of the purified compound MJK-2

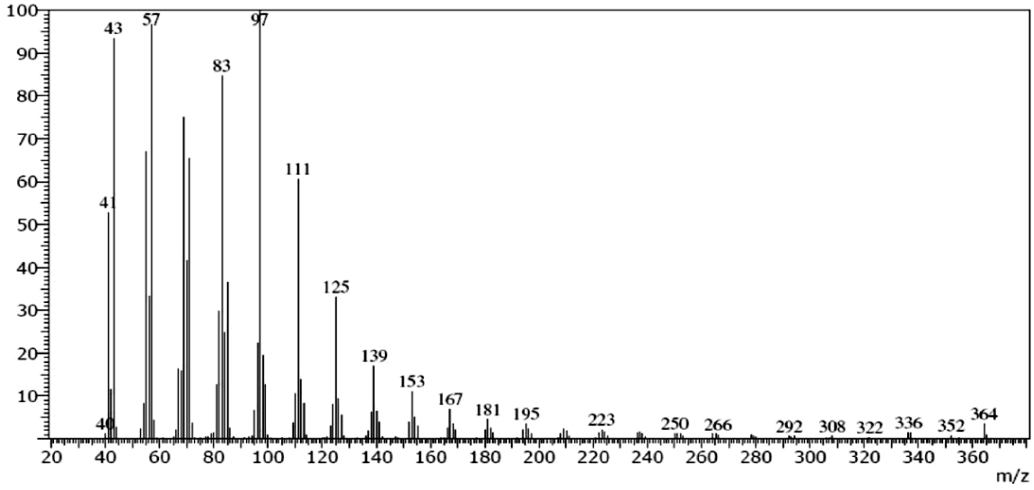
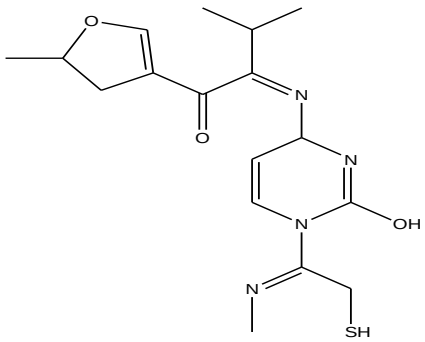


Fig. 5 Structure of the purified compound MJK-2

(2Z)-2-(1,4-dihydro-2-hydroxy-1-((E)-2-mercapto-1-(methylimino) pyrimidin-4-ylimino)-1-(4,5-dihydro-5-methyl furan-3-yl) methylbutane-1-one



Optimization of antimicrobial compound production

In this study, required conditions were optimized for the production of antimicrobial metabolites using *P./D. theae*. Among five different liquid media tested, M-1DB medium supported the maximum growth and antimicrobial activity followed by PDB and PDYEB (Table

1). However, rest of the two media (PCB and CDB) moderately supported the growth and antimicrobial activity of *P./D. theae*. Among different temperatures tested, 25°C was found to be optimum for the maximum growth (Fig. S1a) and antimicrobial activity (Fig. S1b). Among different pH tested, the pH 6.5 was found to be optimum for the maximum growth (Fig. S2a) and antimicrobial activity (Fig. S2b). Among different incubation periods tested, the maximum growth was achieved on 28th day with excellent antimicrobial activity (Figs. S3a and S3b).

Table 1 Growth and antimicrobial activity of *Phomopsis/ Diaporthe theae* on different liquid media against human pathogens

		***Phomopsis/ Diaporthe* sp. GJJM16**				
Media	**Biomass (g/100ml)**	***B.subtilis***	***S.aureus***	***M. luteus***	***P. aeruginosa***	***C. albicans***
PDYEB	1.50±0.28	14±0.50	12±0.50	13±0.28	11±0.12	13±0.26
M1DB	1.69±0.14	22±0.12	20±0.32	18±0.12	16±0.38	19±0.32
PDB	1.53±0.73	17±0.20	16±0.50	14±0.20	12±0.50	15±0.12
PCB	1.20±0.22	11±0.28	14±0.32	-	11±0.22	12±0.20
CDB	0.92±0.12	10±0.20	12±0.50	-	-	10±0.50

Values are the mean ± SD with three replicates

PDYEB, Potato Dextrose Yeast Extract Broth; M1DB, Modified medium-1 Broth; PCB, Potato carrot Broth; MEB, Malt Extract Broth; CDB, Czapex-Dox- Broth

Table 2 Bioactivity of standard antibiotics against human pathogens

Standard antibiotics	**Zone of Inhibition (mm)**		
	Gram positive bacteria	**Gram negative bacteria**	**Yeast**
	S. aureus	***P. aeruginosa***	***C.albicans***
Azithromycin (At) 15mcg	23±0.1	nt	32±0.2
Amikacin (Ak) 30 mcg	31±0.3	25±0.2	33±0.3
Lomefloxacin (Lo) 10 mcg	nt	30±0.2	nt
Cephadroxil (Cq) 30 mcg	17±0.1	28±0.2	22±0.2
Sparfloxacin (Sc) 5 mcg	31±0.2	35±0.3	43±0.4
Netillin /Netilmicin Sulphate (Nt) 30 mcg	nt	26±0.2	nt
Ceftazidime (Ca) 30 mcg	nt	29±0.2	nt
Ceftriaxone (Ci) 30 mcg	nt	17±0.1	nt
Ciprofloxacin (Cf) 5 mcg	40±0.3	38±0.4	38±0.4
Cephotaxime (Ce) 30 mcg	25±0.1	25±0.2	35±0.3
Gentamicin (G) 10 mcg	31±0.2	28±0.3	34±0.3
Cefaperazone (Cs) 75 mcg	34±0.3	24±0.2	36±0.2
Ampicillin/Sulbactam (As) 10 mcg	nt	29±0.1	nt
Ampicillin/Cloxacillin (Ax) 10mcg	34±0.2	nt	42±0.3
Cefuroxime (Cu) 30 mcg	46±0.4	nt	31±0.2
Roxithromycin (Ro) 30 mcg	42±0.3	nt	42±0.3
Clarithromycin (Cw) 15 mcg	40±0.4	nt	41±0.4

NT, Not Tested

Among nine different carbon sources tested, sucrose amended medium showed the maximum growth and antimicrobial activity against all the pathogens (Figs. S4a and S4b). Among the various concentrations of sucrose tested, 3.5% sucrose supported significant growth with excellent antimicrobial activity (Figs. S4c and S4d). Among ten different nitrogen sources tested, casein and yeast extract supplemented media showed the maximum yield of biomass and antimicrobial activity; however, inorganic nitrogen sources such as ammonium nitrate and ammonium tartate and soytone did not show significant effect on the growth and antimicrobial activity (Figs. S5a and S5b). Among the different concentrations of casein and yeast extract tested, 0.6% casein and 0.6% yeast extract amended media supported significant growth with excellent antimicrobial activity (Figs. S5c-f). In this study, all the supplemented amino acids showed lowest growth as

well as antimicrobial activity compared to control (without amino acid) (Figs. S6a and 6b). Similarly, all the supplemented vitamins gave lowest biomass as well as antimicrobial activity than the control (without vitamin) (Figs. S7a and 7b). Hence, both amino acid and vitamin were not subjected to further analysis.

Fig. S1a Effect of temperature on the growth of *Phomopsis/ Diaporthe theae*

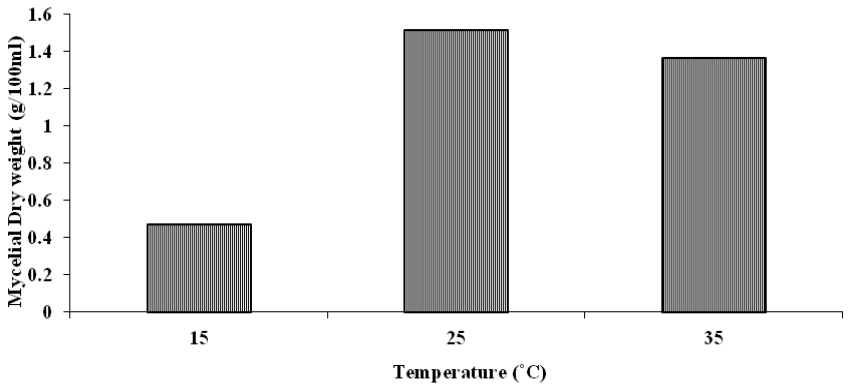


Fig. S1b Effect of temperature on the antimicrobial activity of *Phomopsis/ Diaporthe theae*

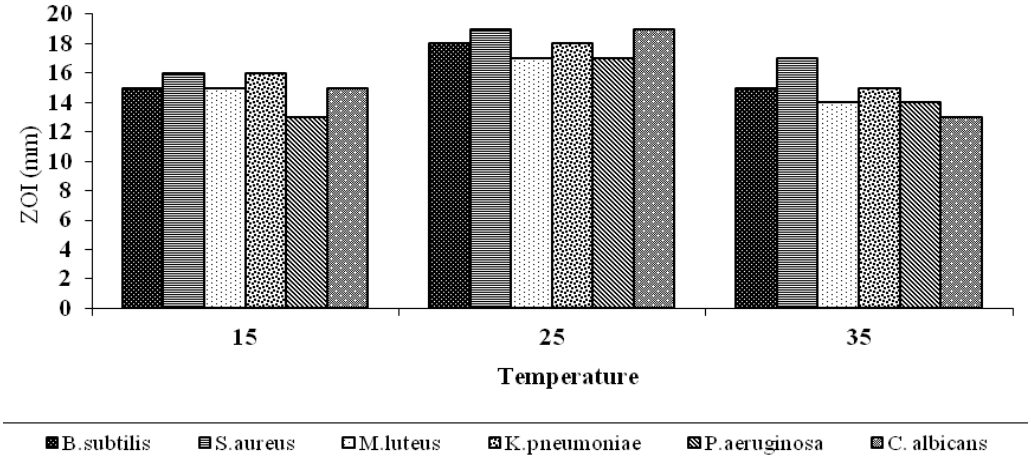


Fig. S2a Effect of pH on the growth of *Phomopsis/ Diaporthe theae*

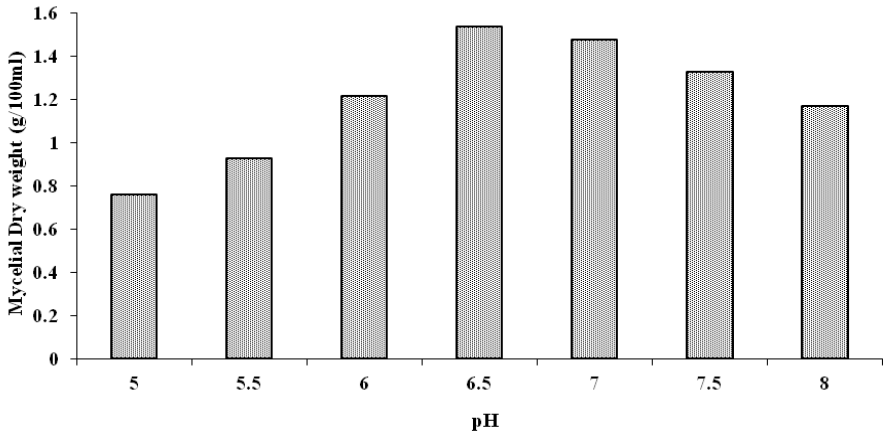


Fig. S2b Effect of pH on the antimicrobial activity of *Phomopsis/ Diaporthe theae*

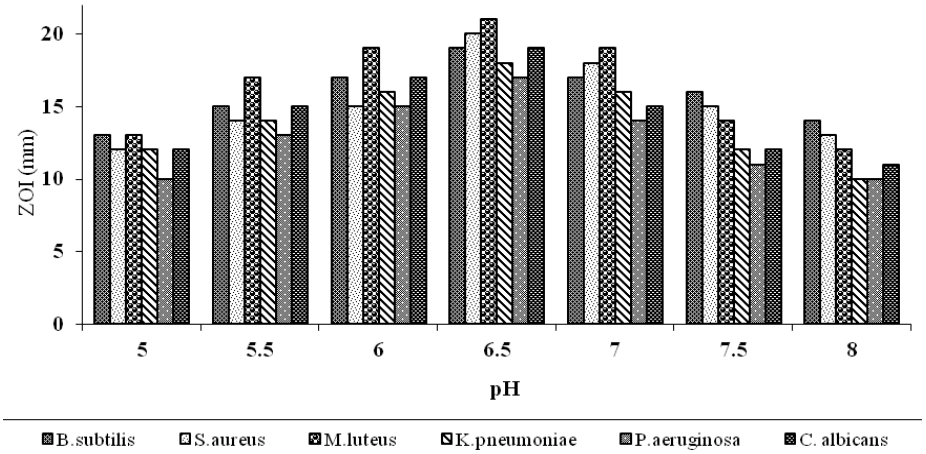


Fig. S3a Effect of incubation period on the growth of *Phomopsis/ Diaporthe theae*

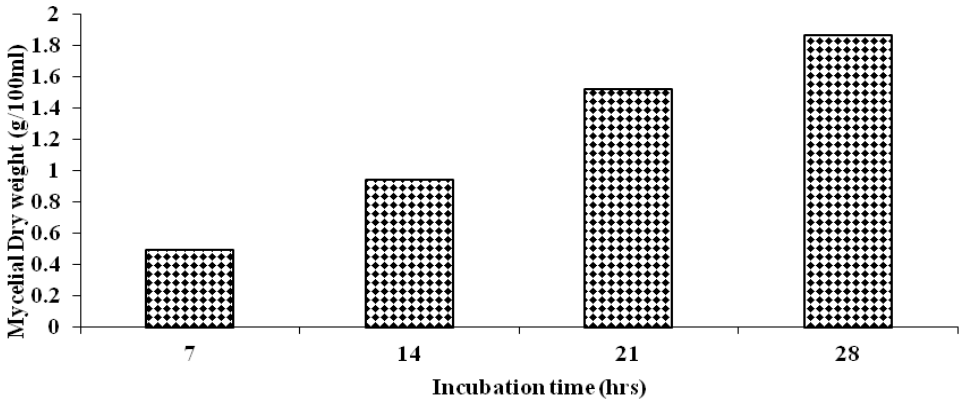


Fig. S3b Effect of incubation period on the antimicrobial activity of *Phomopsis/ Diaporthe theae*

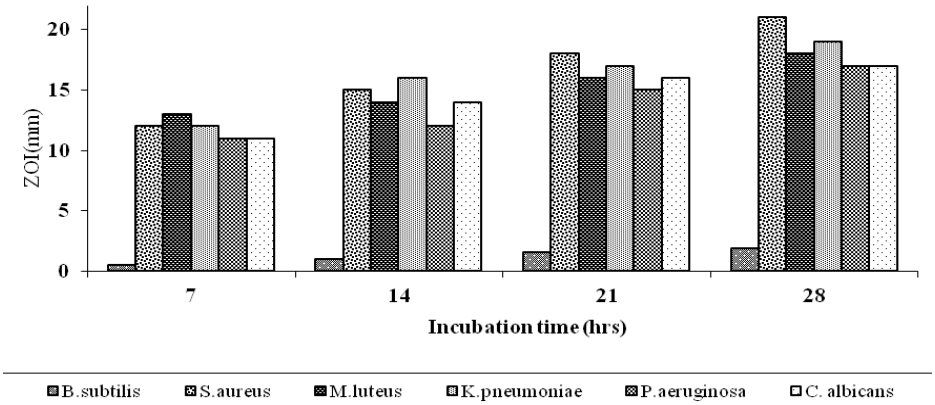


Fig. S4a Effect of various carbon sources on the growth of *Phomopsis/ Diaporthe theae*

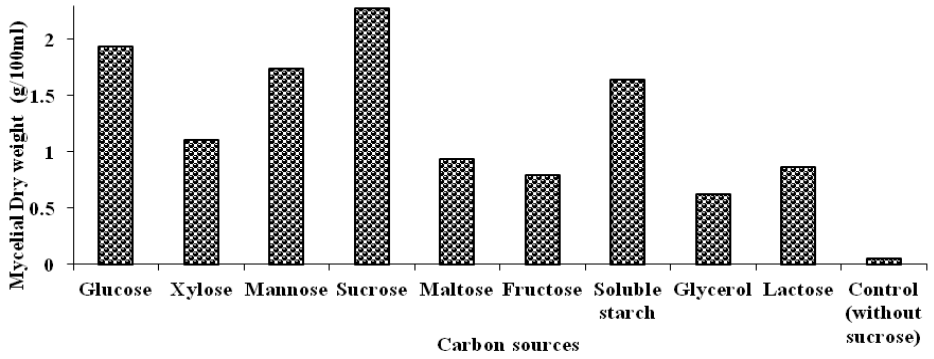


Fig. S4b Effect of various carbon sources on antimicrobial activity of *Phomopsis/ Diaporthe theae*

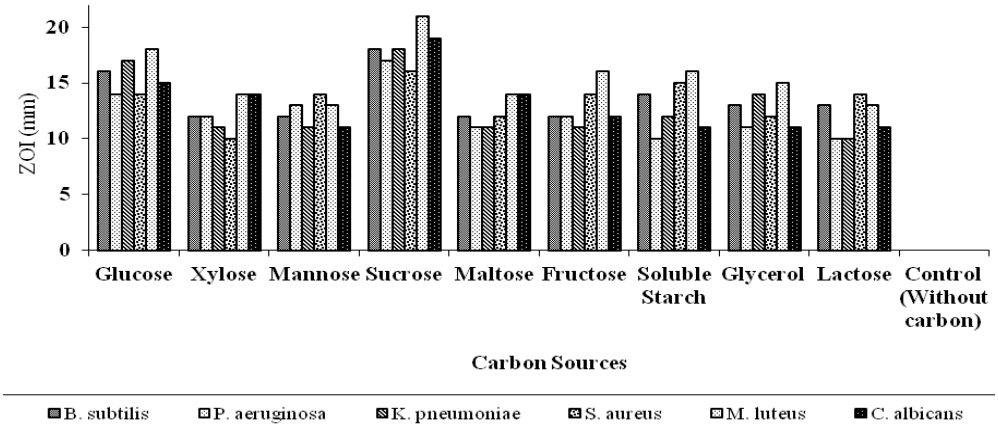


Fig. S4c Effect of different concentrations of sucrose on the growth of *Phomopsis/ Diaporthe theae*

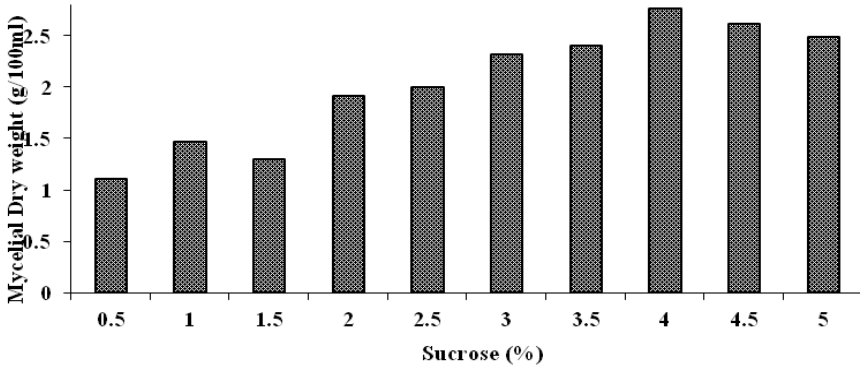


Fig. S4d Effect of different concentrations of sucrose on antimicrobial activity of *Phomopsis/ Diaporthe theae*

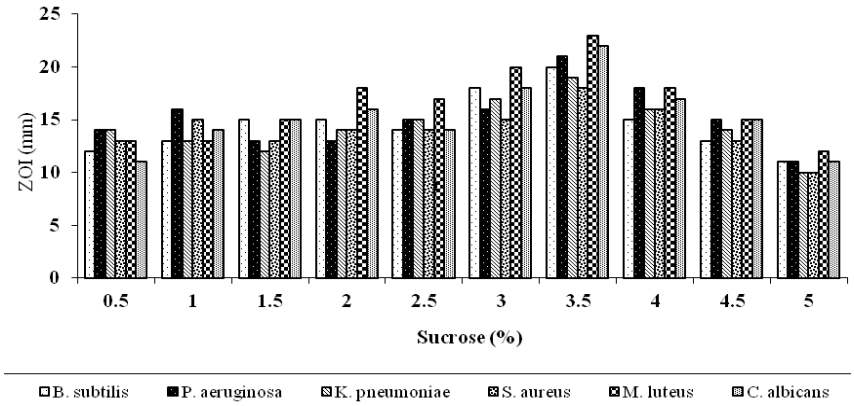


Fig. S5a Effect of various nitrogen sources on the growth of *Phomopsis/ Diaporthe theae*

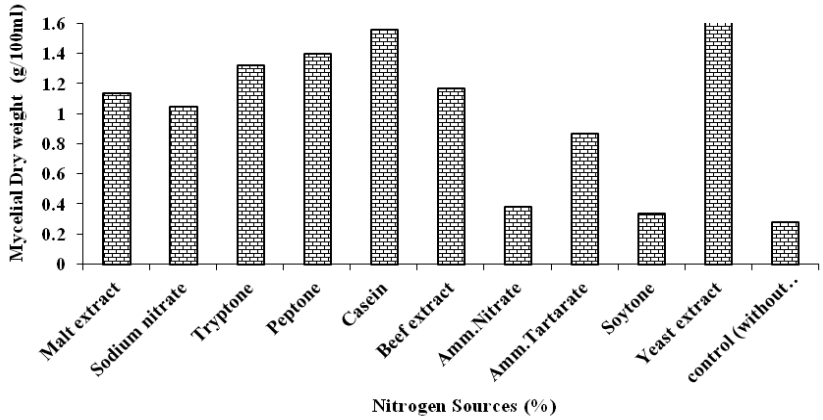


Fig. S5b Effect of various nitrogen sources on antimicrobial activity of *Phomopsis/ Diaporthe theae*

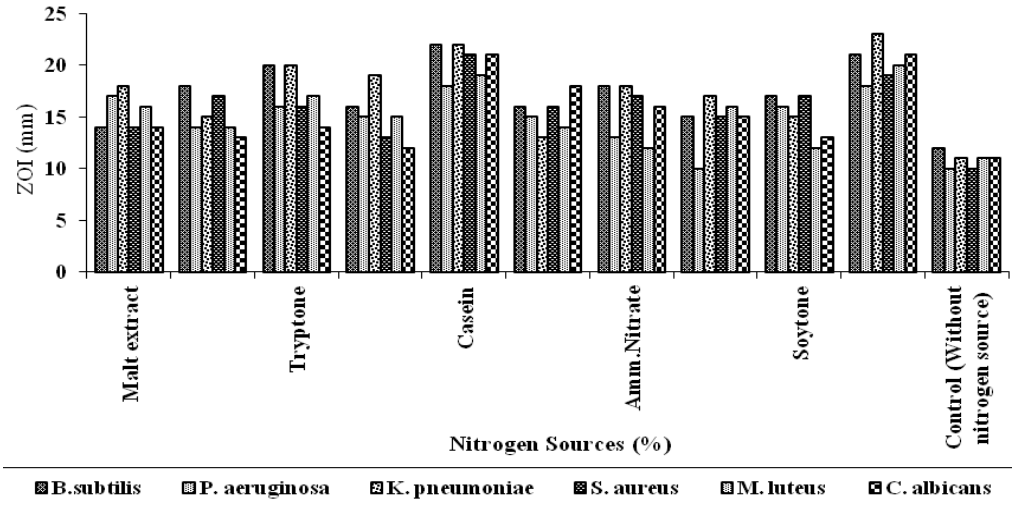


Fig. S5c Effect of different concentrations of casein on the growth of *Phomopsis/ Diaporthe theae*

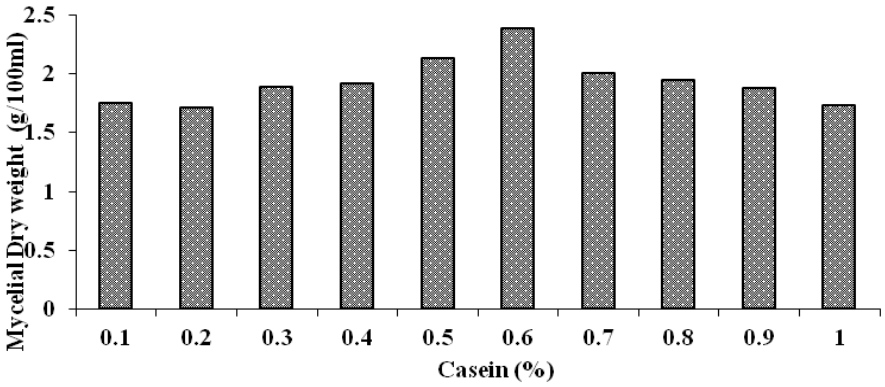


Fig. S5d Effect of different concentrations of casein on antimicrobial activity of *Phomopsis/ Diaporthe theae*

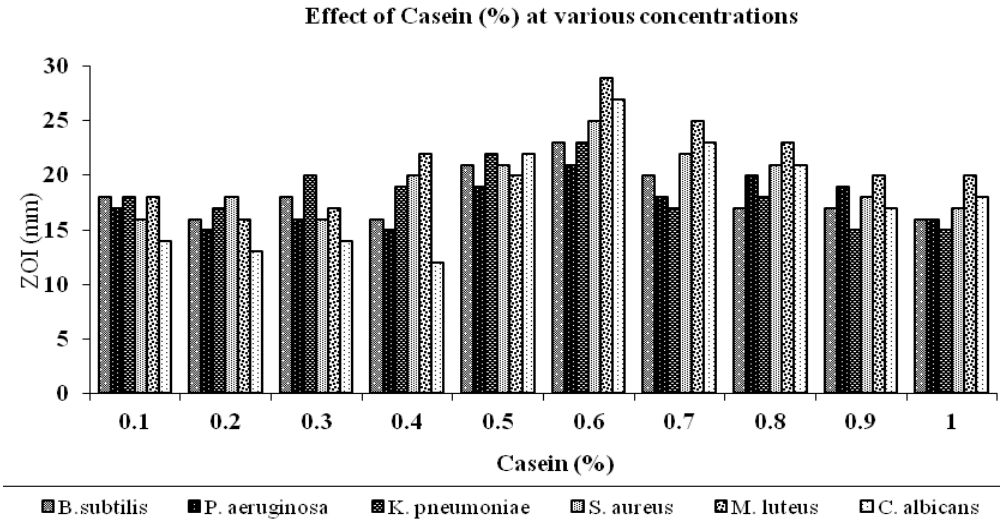


Fig. S5e Effect of different concentrations of yeast extract on the growth of *Phomopsis/ Diaporthe theae*

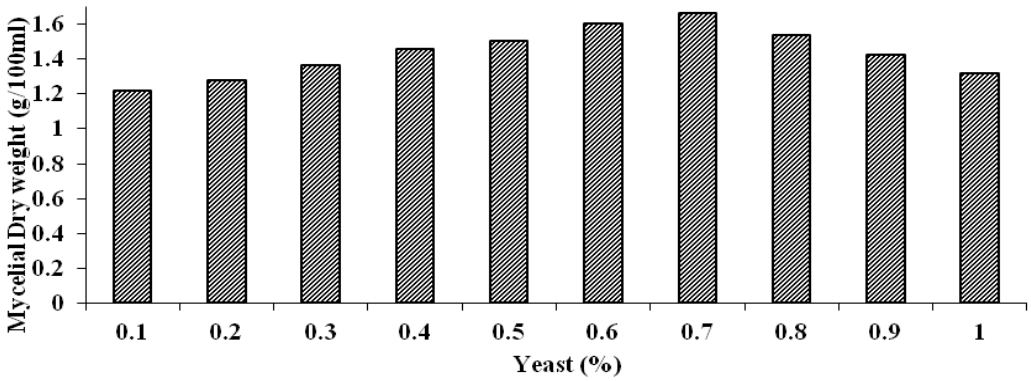


Fig. S5f Effect of different concentrations of yeast extract on antimicrobial activity of *Phomopsis/ Diaporthe theae*

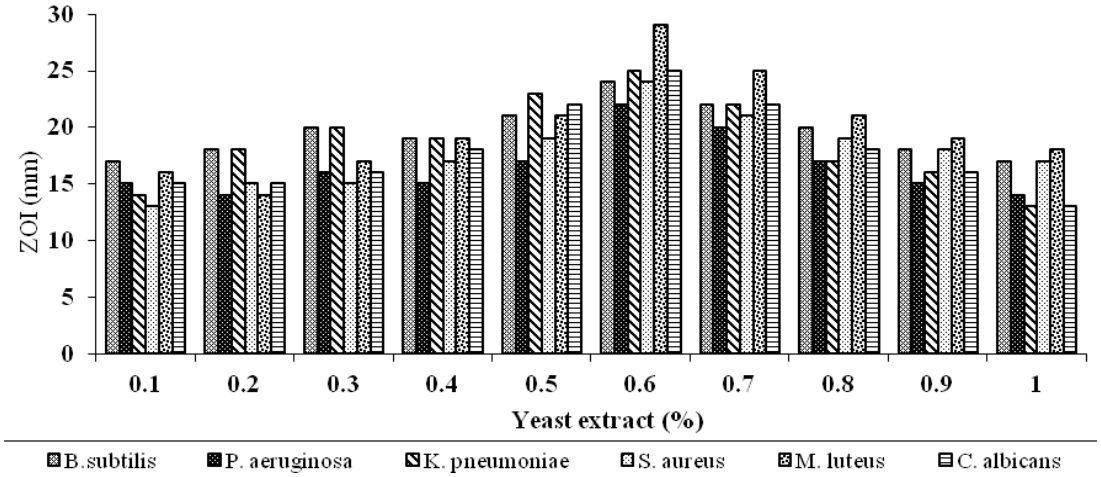


Fig. S6a Effect of various amino acid sources on the growth of *Phomopsis/ Diaporthe theae*

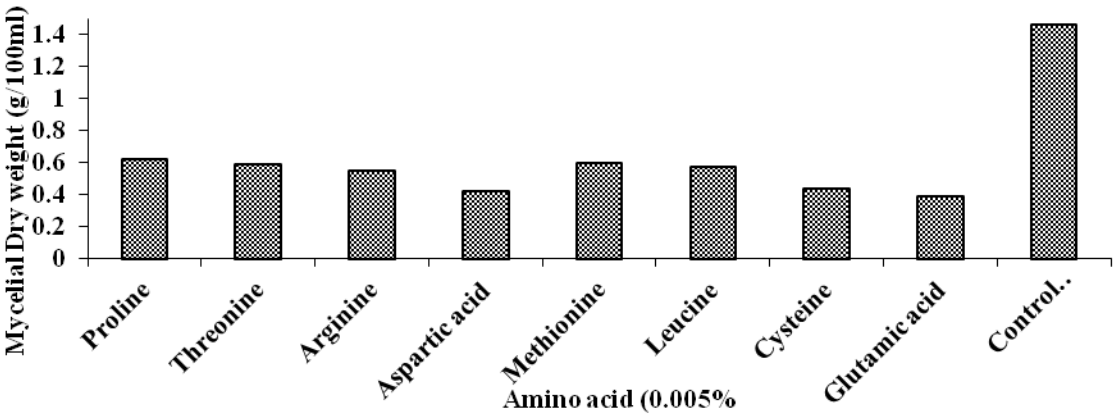


Fig. S6b Effect of various amino acid sources on antimicrobial activtiy of *Phomopsis/ Diaporthe theae*

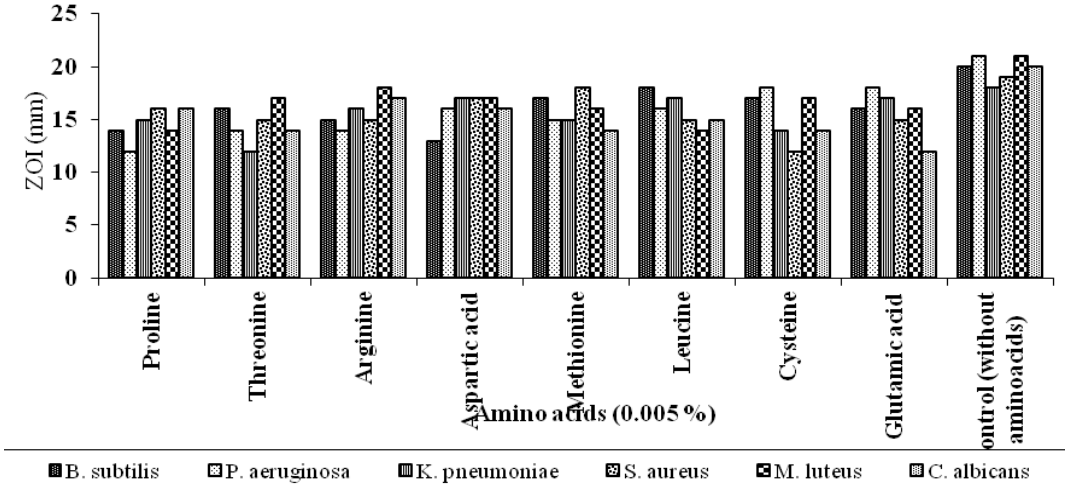


Fig. S7a Effect of various vitamin sources on the growth of *Phomopsis/ Diaporthe theae*

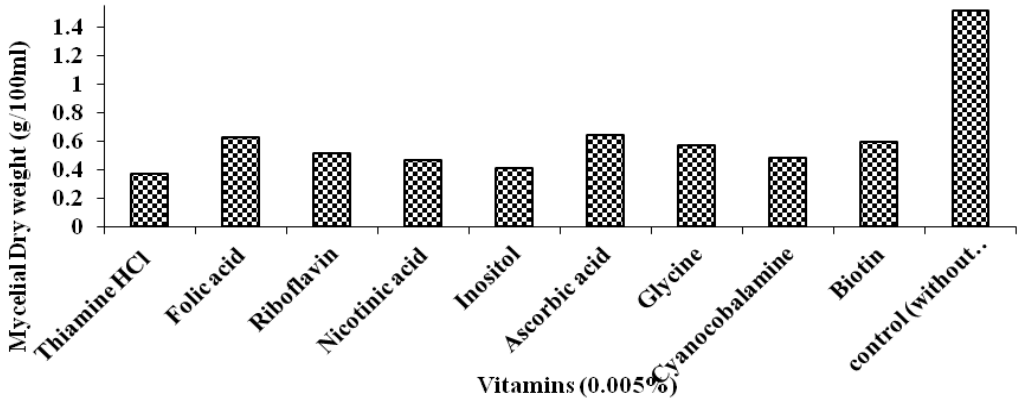


Fig. S7b Effect of various vitamin sources on antimicrobial activity of *Phomopsis/ Diaporthe theae*

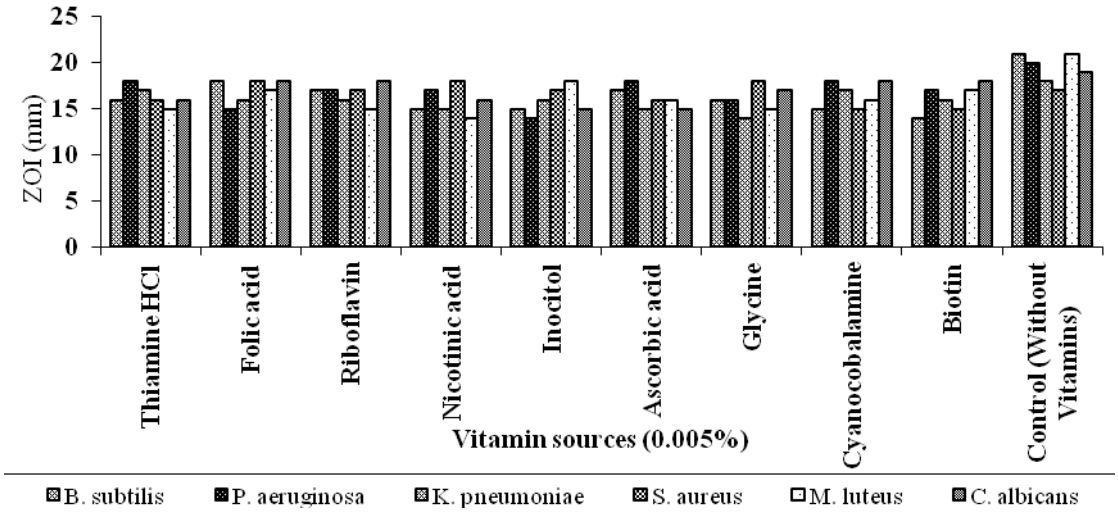


Fig. S8 UV spectrum of the purified compound MJK-2

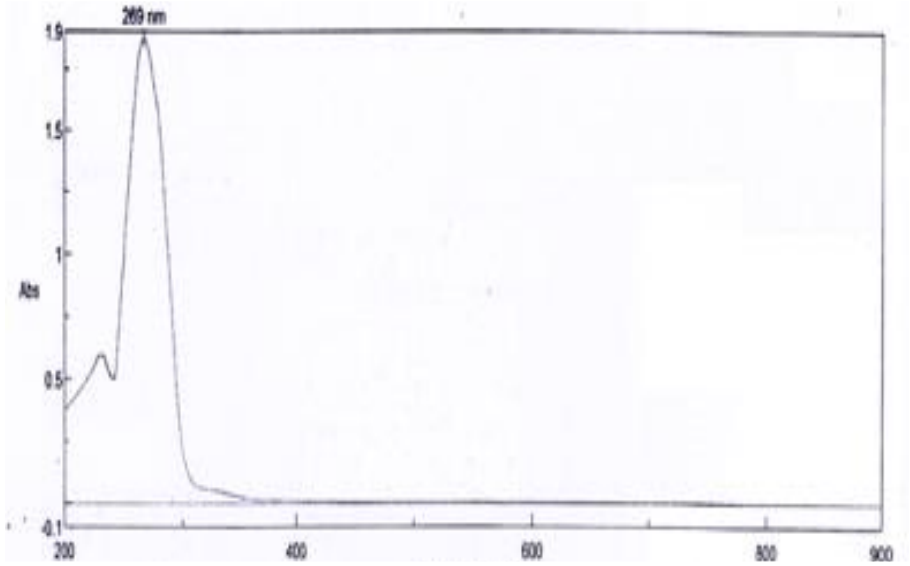


Fig. S9 IR spectrum of the purified compound MJK-2

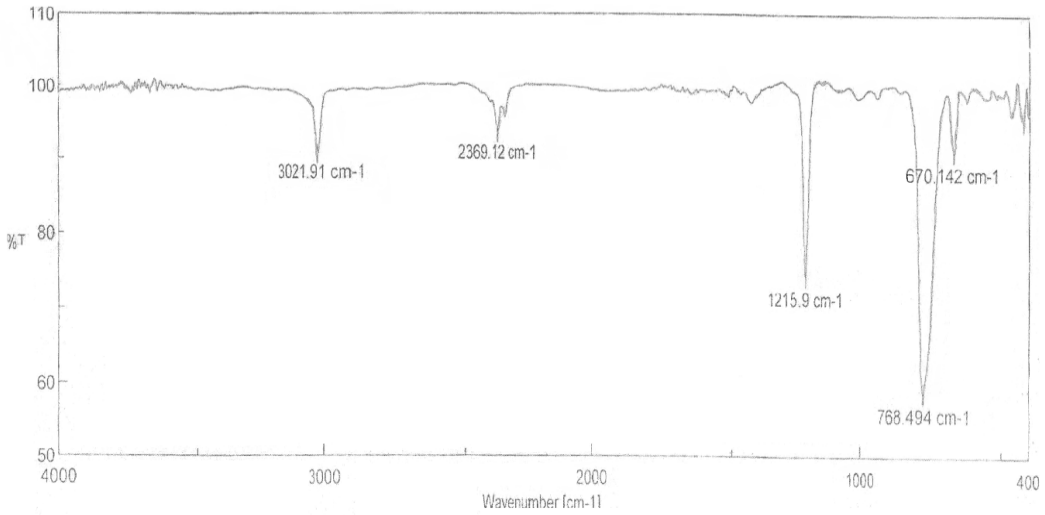
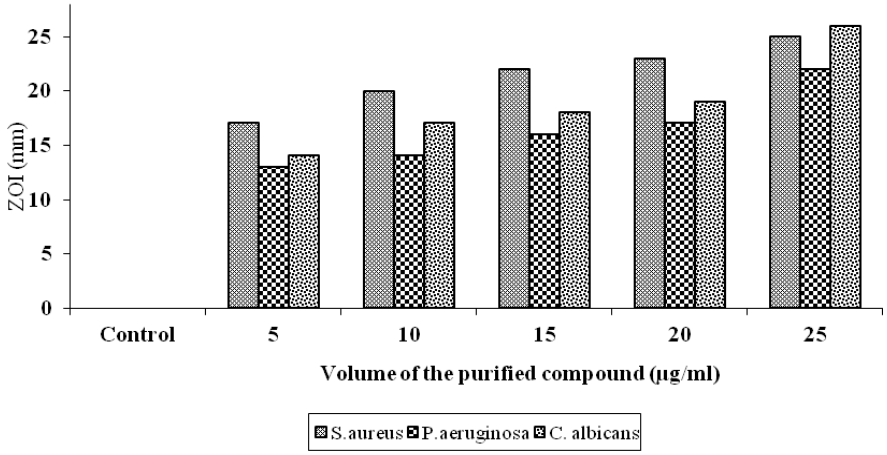


Fig. S10 Effect of purified compound MJK-2 on antimicrobial activity



Chemical characterization of the purified compound

Compound (1) was isolated as a pale yellow colored amorphous semi solid and was soluble in ethyl acetate and methanol. The UV spectrum of this compound showed maximum absorption at 269 nm (Fig. S8). The FT-IR spectra showed the characteristic peaks at 3021.91 cm^{-1} and 670.14 cm^{-1} due to the aromatic C-H stretch in addition to peak at 1215.9 cm^{-1} for C-N stretching (Fig. S9). The peak at 768.49 cm^{-1} showed the presence of C-H stretch of phenyl substitution bands. Aromatic amines exhibited moderate absorption bands (bending vibrations) at 2369.12 cm^{-1} (Fig. S9).

^{1}H-NMR data exhibited peaks at δ 0.86 to 1.20 ppm due to methylene protons (Fig. 2). The four equivalent methyl groups showed a singlet at δ 1.26 ppm. The peaks at δ 4.3 to 4.4 indicated the existence of Aryl CH proton and the H node proton shift at δ 5.8 ppm inferred the trans –N-C=R group. The proton appeared as a multiplet in the region of δ 5.8 ppm. A characteristic peak at δ 9.8 ppm revealed the proton of –OH group (Fig. 2). The off-resonance decoupled ^{13}C-NMR spectrum exhibited peaks at the region of δ 14.09-39.96 ppm due to the presence of methyl, methylene and methine carbons (Fig. 3). The ethylene carbon displayed a peak at δ 129.33 ppm and the ester carbonyl carbon resonated at δ 158.62 to 161.90 ppm confirmed the imino carbon. A single peak at δ 191.14 ppm inferred the presence of the carbonyl group (Fig. 3).

The ESI-MS spectroscopic analysis showed molecular ion peak at 364 m/z (Fig. 4) and supported our NMR data. The molecular formula of this compound was found to be $C_{17}H_{24}N_4O_3S$. On the basis of these data, the

structure of the compound (1) was confirmed as (2Z)-2-(1,4-dihydro-2-hydroxy-1-((E)-2-mercapto-1 (methylimino)ethyl) pyrimidine-4-ylimino)-1-(4,5-dihydro-5-methylfuran-3-yl)-3-methylbutane-1-one (Fig. 5). This compound was further identified as pyrimidine imino methylfuran derivative (1).

Antimicrobial activity of the purified compound

Well diffusion assay

In this study, compound (1) showed broad spectrum antimicrobial activity against all the tested human pathogens and the data was presented in Figure S10. Interestingly, this compound inhibited the growth of microbes even at very low concentration (5 µg/mL) and the antimicrobial activity was increased with increasing concentration of purified compound. The maximum ZOI was recorded against *C. albicans* (31 ± 0.5 mm) followed by *S. aureus* (27 ± 0.5 mm) and *P. aeroginosa* (25 ± 0.5 mm) at a test concentration of 25 µg/mL (Fig. S10). Table 2 clearly divulges the broad spectrum antimicrobial activity of the compound (1) against standard antibiotics (positive control).

Minimum inhibitory concentration

MIC was recorded as the lowest concentration at which no visible growth of test pathogens was observed. In this current study, the purified compound showed significant antimicrobial activity against *S. aureus*, *P. aeroginosa* and *C. albicans* exhibiting MICs of 1.25 µg/mL.

DISCUSSION

Endophytic fungi are often regarded as one of the versatile sources for novel antimicrobial metabolites. Forest environment is the largest reservoir of biological and chemical diversity. Hence, research focus on forest environment has been gaining increasing momentum in the recent past. Even so, forest areas have not yet fully explored for endophytic fungal diversity and antimicrobial metabolites. Therefore, the present study was aimed to evaluate the endophytic fungal diversity and antimicrobial compounds from the forest areas of Aliyar, Pollachi. In this study, a total of 77 coelomycetous endophytic fungi were isolated from five different medicinal plants and were screened for antimicrobial activity. The degree of antimicrobial activity varied greatly among the coelomycetous fungi. Among 77 isolates, 67 isolates (87%) displayed antimicrobial activity against at least one human pathogen. Several researchers have already reported similar antimicrobial activity of endophytic fungi against various human pathogens. Phongpaichit et al. (2006) have isolated 377 endophytic fungi from five different Garcinia plants, of which, as low as 18.56% showed antimicrobial activity against at least one pathogenic microorganism such as *S. aureus*, methicillin-resistant *S. aureus*, *C. albicans* and *Cryptococcus neoformans*. In another study, Desale and Bodhankar (2013) have obtained 17 different endophytic fungi, of which, 76% exhibited antibacterial activity against *E. coli*, *S. typhimurium*, *B. cereus*, *S. aureus*, *K. pneumoniae* and *B. subtilis*. Khiralla et al. (2016) isolated 15 endophytic fungi from five Sudanese medicinal plants. Therefore, it is clear from our study that the medicinal plants of unexplored Aliyar region are a potential source of antimicrobial-producing endophytic fungi. Among 77 isolates tested, *Phomopsis/Diaporthe sp. GJJM 06, Phomopsis/Diaporthe sp. GJJM 13 and Phomopsis/Diaporthe sp. GJJM 16* showed excellent antimicrobial activity against all the pathogens. Interestingly, the isolate, *Phomopsis/Diaporthe sp. GJJM 16* showed superior antimicrobial activity than the other two isolates. Using classical and molecular characterization, this particular isolate was further identified as *Diaporthe theae*. Much similar to our present results, many researchers have already reported the antimicrobial activity of *Phomopsis* spp. against various human pathogens (Jayanthi *et al*., 2011; Desale and Bodhankar 2013; Tong et al. 2014; Yashavantha Rao et al. 2015a). Based on the earlier reports and in the present study, it is here concluded that the species of *Phomopsis* are a rich source of antimicrobial-producing endophytic fungi.

Manipulation of nutritional and environmental factors which promote the secondary metabolites biosynthesis is critical in the microbial natural products discovery (Bills et al. 2008). Antibiotics produced by endophytic fungi can be enhanced by genetic modification, mixed culture fermentation, immobilization of the cells and optimization of fermentation conditions or enzymes induction (Ho et al. 2003; Yashavantha Rao and Satish 2015b). In this study, required conditions were optimized for the production of antimicrobial metabolites using *P./D. theae*. Among five different liquid media tested, M-1DB medium supported the maximum growth and antimicrobial activity followed by PDB and PDYEB. On the other hand, PCB and CDB media moderately supported the growth and antimicrobial

activity of *P./D. theae*. This is likely due to the need for certain nutritional supplements, which may serve as precursors, for the biosynthesis of intracellular metabolites (Tong et al. 2011). In addition to media composition, temperature, pH, culture vessel, aeration, cultivation time, light intensity can increase or reduce the production of bioactive compounds (Siqueria et al. 2011). Temperature has profound effect on the physiology, morphology, biochemistry and metabolites production of organisms (Vijayakumar et al. 2012). Among different temperatures tested, 25°C was optimum for the maximum growth and antimicrobial activity of *P./D. theae*. The change in pH of the culture medium induces the production of new products that adversely affect the antibiotic production (Vijayakumar et al. 2012). Among different pH tested, the pH 6.5 was optimum for the maximum growth and antimicrobial activity of *P./D. theae*. In case of incubation periods, the maximum growth was achieved on 28th day with excellent antimicrobial activity. In this study, nutritional array was also applied to understand the conditions in which *P./D. theae* could produce increased amount of antibiotics or secondary metabolites (Bills et al. 2008). Among nine different carbon sources tested, 3.5% sucrose supported significant growth with excellent antimicrobial activity. In case of nitrogen sources, 0.6% casein and 0.6% yeast extract amended media supported the maximum yield of biomass with excellent antimicrobial activity. Surprisingly, in this study, all the supplemented amino acids and vitamins gave lowest biomass as well as antimicrobial activity.

P./D. theae was grown in the optimized M1-D medium for isolation of antimicrobial metabolites. Among different solvent extracts tested, ethyl acetate extract showed excellent antimicrobial activity against all the pathogens (data not shown here). Hence, ethyl acetate was used for extraction and isolation of antimicrobial molecules from *P./D. theae*. Bioassay guided fractionation of crude ethyl acetate extract of *P./D. theae* led to the isolation of a novel bioactive compound, pyrimidine imino methylfuran derivative (1). To our knowledge, this compound was reported for the first time from *P./D. theae*. Interestingly, this compound showed superior antibacterial and anticandidal activity against all the human pathogens tested. On the contrary, Li et al. (2010) have isolated 8 novel compounds from *Phomopsis* sp. A123; surprisingly, all these compounds had no effect on the growth of tested bacteria and a yeast pathogen. In this present study, Gram positive bacterium was more susceptible than the Gram negative one. This result was found to be consistent with the earlier reports shown by Yenn et al. (2012), Tong et al. (2014) and Yashavantha Rao et al. (2015a). Conversely, in another study, the ethyl acetate extract of *Phomopsis* sp. showed highest antibacterial activity against *E. coli* followed by *S. typhimurium* and *S. aureus* (Desale and Bodhankar 2013). The difference in antimicrobial activity was likely due to the morphological differences between these two bacterial groups (Tong et al. 2014).

CONCLUSION

The present study clearly revealed the potential source of antimicrobial-producing endophytic fungi from Aliyar region. The compound, pyrimidine imino methylfuran derivative isolated using bioassay guided fractionation method, was reported for the first time from *P./D. theae* and it showed strong antibacterial and anticandidal activities against human pathogens including *P. aeroginosa, S. aureus* and *C. albicans*. Thus, the results of this study have increased the scope of finding novel antimicrobial natural products from endophytic fungi.

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