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FUNGAL MICROBIOLOGY

Antimicrobial Potential of Thiodiketopiperazine Derivatives Produced by *Phoma* sp., an Endophyte of *Glycyrrhiza glabra* Linn.

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Abstract During the screening of endophytes obtained from *Glycyrrhiza glabra* Linn., the extract from a fungal culture designated as GG1F1 showed significant antimicrobial activity. The fungus was identified as a species of the genus *Phoma* and was most closely related to *Phoma cucurbitacearum*. The chemical investigation of the GG1F1 extract led to the isolation and characterization of two thiodiketopiperazine derivatives. Both the compounds inhibited the growth of several bacterial pathogens especially that of *Staphylococcus aureus* and *Streptococcus pyogenes*, with IC_{50} values of less than 10 μM. The compounds strongly inhibited biofilm formation in both the pathogens. In vitro time kill kinetics showed efficient bactericidal activity of these compounds. The compounds were found to act synergistically with streptomycin while producing varying effects in combination with ciprofloxacin and ampicillin. The compounds inhibited bacterial transcription/translation in vitro, and also inhibited staphyloxanthin production in *S. aureus*. Although similar in structure, they differed significantly in some of their properties, particularly the effect on the expression of pathogenecity related genes in *S. aureus* at sub-lethal concentrations. Keeping in view the antimicrobial potential of these compounds, it would be needful to scale up the production of these compounds through fermentation technology and further explore their potential as antibiotics using in vivo models.

Keywords Licorice · *Glycyrrhiza* · *Phoma* · Antimicrobial activity · Biofilm disruption · Endophyte · Fungal secondary metabolite

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Introduction

Endophytic fungi, microorganisms residing asymptomatically within the plant tissues, play diverse ecological functions thus affecting plant nutrition, growth rate, resistance to stress conditions, as well as plant survival and distribution [1–3]. Due to the constant process of strain development by passing through various stages of plant growth and development, and their ecological functions, endophytes are proficient producers of bioactive secondary metabolites [4–6]. Endophytes especially assist the host plants in evading the pathogens by producing antimicrobial secondary metabolites. The ability of these organisms to produce compounds with antimicrobial activities, may be attributed to their evolution over millions of years in diverse ecological niches and natural habitats in which extreme competition for survival is needful [4, 7, 8]. There is an unending demand for novel antimicrobial agents due to rampant increase in drug resistant microbes, life threatening infections and recurring infectious diseases [9, 10]. Thus, endophytic microorganisms are being widely explored for bioactive natural products with antimicrobial potential.

Glycyrrhiza glabra Linn. is a well-known medicinal plant used in traditional medicine across the globe for its ethanopharmacological value to cure varieties of ailments [11].

Modern scientific investigations have provided strong evidence for the medicinal value of this plant and its chemical constituents [12–14]. For example, a long-term intermittent glycyrrhizin therapy successfully reduced hepatocellular carcinogenesis in patients with HCV-related chronic liver disease [15]. The medicinal value of this plant coupled with its natural resistance to plant pathogens inspired us to explore its endophytes for bioactive natural products, especially antimicrobial compounds.

In this study, two thiodiketopiperazine derivatives were isolated from an endophytic fungus, *Phoma* sp., associated with *G. glabra*. These compounds were found to possess potential antimicrobial activities against several human pathogens, particularly *Staphylococcus aureus* and *Streptococcus pyogenes*. In this paper, we present a detailed study on the antimicrobial and antibiofilm potential of these compounds.

Materials and Methods

Isolation and Cultivation of the Endophyte

The endophytic fungus, designated as GG1F1, was isolated according to the previously described methods [16, 17]. Briefly, leaves of *G. glabra* (licorice plant), obtained from Jammu (J&K), Jammu (32° 43′ 48″; 74° 50′ 58″), were thoroughly washed with running tap water and surface sterilized with 1 % sodium hypochlorite (Sigma-Aldrich, St. Louis, MO, USA) for 3 min followed by 70 % ethanol for 2 min. Before the treatment with alcohol, traces of sodium hypochlorite were removed by washing in sterile distilled water. The tissues were cut into small pieces of 0.5 to 1 cm and plated on water agar. The plates were incubated at 25 °C for 3 weeks. Hyphal tips of the fungus, emerging out of the plant tissue, were picked and grown on potato dextrose agar in pure culture. The culture was also submitted to the National Fungal Culture Collection of India under the voucher no. NFCCI 3729. For the extraction of secondary metabolites, the culture was grown in shake flasks on PD broth at 25 °C and 200 rpm for 15 days, in an incubator shaker (Daihan Labtech Co. Ltd., Namyangju-si, Gyeonggi-do, Korea).

For morphological analysis, microscopic slides of the endophyte were prepared by staining with lactophenol-cotton-blue and examined under light microscope (Olympus BX51). All media, antibiotics and stains used were from HiMedia, Mumbai, Maharashtra, India, unless stated otherwise.

Phylogenetic Analysis of GG1F1 by ITS1-5.8S-ITS2 Ribosomal Gene Sequencing

Phylogenetic analysis of GG1F1 was carried out by the acquisition of the ITS1-5.8S-ITS2 ribosomal gene sequencing. The ITS region of the fungus was amplified with the universal ITS primers, ITS4 (5′TCCTCCGCTTATTGATATGC3′) and ITS5 (5′GGAAGTAAAAGTCGTAACAA3′) (Sigma-Aldrich), using the polymerase chain reaction (PCR) [17, 18]. All the PCR chemicals were from Invitrogen, Waltham, MA, USA. The amplified product was sequenced and aligned with the sequences in the GenBank by BLASTN program to find out the sequence homology with closely related organisms [19]. Sequences from the closely related organisms were used to construct the phylogenetic tree [20, 21]. The ITS1-5.8S-ITS2 ribosomal gene sequence of GG1F1 has been deposited in the GenBank under the accession no. KU168143.

Extraction and Isolation of the Compounds

The 10-L volume of the culture broth of GG1F1, extracted with ethyl acetate (2 L × 3) and concentrated under reduced pressure, yielded a total of 0.8 g of the extract. This was subjected to column chromatography over sephadex LH-20 (Amersham Biosciences AB, Uppsala, Sweden) using MeOH to give five (F1–F5) fractions (pooled based on TLC profiles). All reactions were monitored by thin layer chromatograph (TLC) on silica gel 60 F_{254} (0.25 mm thick, Merck, Kenilworth, NJ, USA) and Silica gel GF $_{254}$ (for preparative TLC, Merck) with spot visualized by UV 254 and 365 nm, and ceric ammonium reagent was used as development agent.

Further, the active fraction F-5 (50 mg) was separated on preparative TLC using $CHCl_3$: MeOH (9:1) to yield the pure compounds, **1** (5 mg, R_f 0.7) and **2** (30 mg, R_f 0.4).

Evaluation of the Extract and the Compounds from Antimicrobial Activity

The extract prepared from the endophyte and the isolated compounds were evaluated for antimicrobial activity against a panel of nineteen pathogens including a fungal pathogen, *Candida albicans* (Table 1), using the microtiter plate assay [22]. The extract or the compound was added to 100 μl of Mueller-Hinton broth at different concentration *viz*., 100, 50, 25, 12.5, and 6.25 μg/ml or μM. Each well was inoculated with 10^4 cells of the relevant test organism and incubated at 37 °C for 24 h. Each plate had a set of controls as well: a column with a broad-spectrum antibiotic (ciprofloxacin/nystatin) as positive controls, a column without the test extract, and one without the relevant test organism. Absorbance was measured spectrophotometrically at 620 nm and IC_{50} of the extracts was calculated from the average percent inhibition of three replicates of each concentration as described previously [17]. Nystatin was obtained from Sigma-Aldrich.

Microtiter Biofilm Formation Assay

Biofilm formation assay was performed in presence and in absence of the test compounds, so as to assess their potential to

Table 1 A summary of the antimicrobial activities of compounds **1** and **2**, and the GG1F1 ethyl acetate extract against a panel of microbial pathogens

Serial number	Pathogens	IC_{50} (μM)		IC_{50} (μg/ml)	IC_{50} (μM)
		Compound 1	Compound 2	Extract	(Ciprofloxacin/nystatin[a])
1	*Staphylococcus aureus* ATCC 29213	5.8 ± 0.45	3.8 ± 0.40	4.1 ± 0.41	0.362 ± 0.09
2	MRSA	5.6 ± 0.99	8.4 ± 1.01	5.2 ± 0.38	9.33 ± 2.6
3	*Bacillus cereus* IIIM 25	9.9 ± 0.81	9.2 ± 0.77	38.2 ± 1.8	0.12 ± 0.009
4	*Klebsiella pneumoniae* ATCC 75388	4.5 ± 0.77	19.1 ± 1.1	4.7 ± 0.67	0.015 ± 0.0006
5	*Bacillus thuringiensis* MTCC 809	19 ± 0.84	14.8 ± 0.28	2.1 ± 1.13	0.003 ± 0.001
6	*Yersinia enterocolitica* MTCC840	65.3 ± 1.6	38 ± 1.7	7.5 ± 0.65	3.5 ± 0.202
7	*Erwinia herbicola* MTCC3609	14.2 ± 1.4	15.4 ± 2.7	46.2 ± 0.7	0.006 ± 0.0009
8.	*Shigella dysenteriae* NCTC 11311	–	82.3 ± 1.3	89.3 ± 1.3	0.006 ± 0.0003
9	*Lactococcus lactis* MTCC 440	39.4 ± 1.1	28.7 ± 1.7	14.2 ± 1.4	0.006 ± 0.001
10	*Staphylococcus epidermidis* MTCC 35	23.4 ± 1.5	22.6 ± 2.2	44.7 ± 2.3	0.06 ± 0.006
11	*Alcaligenes faecalis* MTCC126	–	–	54.5 ± 0.4	1.2 ± 0.06
12	*Staphylococcus warneri* MTCC 4436	7.5 ± 0.4	5.05 ± 0.4	6.1 ± 0.3	2.4 ± 0.105
13	*Pseudomonas fluorescens* MTCC 103	26.1 ± 2.7	18.4 ± 0.3	1.7 ± 0.3	0.151 ± 0.051
14	*Xanthobacter flavus* MTCC 132	–	98.3 ± 1.1	–	2.3 ± 0.021
15	*Streptococcus pyogenes* MTCC 442	3.1 ± 0.15	1.8 ± 0.2	1.6 ± 0.3	0.015 ± 0.0006
16	*Shigella boydii* NCTC 9357	26.7 ± 0.9	31.5 ± 1.2	75 ± 1.2	1.12 ± 0.063
17	*Clostridium pasteurianum* MTCC116	54.0 ± 0.5	92.3 ± 0.4	96.6 ± 1.8	0.015 ± 0.003
18	*Salmonella typhimurium* MTCC 98	86.2 ± 1.9	–	–	0.015 ± 0.003
19	*Candida albicans* MTCC 4748	35.8 ± 1.4	–	17.5 ± 1.17	1.5 ± 0.022

[a] Ciprofloxacin was used as the standard antibiotic against the bacterial cultures whereas nystatin was used against *C. albicans*

disrupt the biofilm formation. The film formation was observed by using a modified protocol [23]. Briefly, the culture was grown overnight and it was diluted to obtain a suspension of 10^8 CFU/ml. Two hundred mircoliters of Mueller-Hinton broth was added to each well in the first column of a 24-well plate and 100 μl of media was added to the rest of the wells. Compounds were added to the first row at a concentration of minimum inhibitory concentration (MIC) and serially diluted to make 2-fold dilutions. Each well was inoculated with 10^4 cells and incubated at 37 °C for 24 h. For staining, the culture broth was discarded by simply turning the plate over and shaking out the fluid. Plate was submerged in the small tub of water and then dried. A volume of 125 μL of 0.4 % crystal violet (CV) was added to each well and allowed to stand at room temperature for 10–15 min. The plate was then rinsed three to four times with water by submerging. After drying the plate by blotting, 125 μl of 30 % acetic acid was added to each well and incubated for 10–15 min at room temperature so as to solubilize the dye. The solubilized CV was transferred to a new 96-well plate; absorbance was measured at 570 nm to calculate percent biofilm inhibition.

The biofilm was visualized by light microscopy using sterilized glass pieces of 1/1 cm placed in 24-well plate. Assays were performed as described above. Stained glass pieces were observed under light microscope and visible biofilms were photographed for documentation.

Time Kill Kinetics

To evaluate the efficiency of cidal activity of the drug, the time-kill kinetics assay was employed as per the National Committee for Clinical Laboratory Standards guidelines [24]. Tubes containing MHB with compounds at different concentrations (1× MIC and 2× MIC) were inoculated with test organism to a density of 10^6 cells/ml in a final volume of 10 ml and incubated at 37 °C for 24 h. Aliquots were collected at time 0 and after intervals of 2 h each till 24 h. Samples were serially diluted in normal saline solution (NSS) and plated on MHA plates for determination of viable counts. The percentage reduction and $\log_{10}$ reduction from initial microbial population for each time interval was calculated to assess the change (reduction or increase) of the microbial population relative to a starting inoculum. The change was determined as follows: % Reduction = [(Initial count − Count at *x* time interval)/Initial count] × 100. The Log_{10} reduction was calculated as follows: Log_{10} reduction = Log_{10} (initial count) − Log_{10} (count at *x* time interval). The values represent the mean of three similar experiments.

Post Antibiotic Effect

The post antibiotic effect (PAE) of both the compounds was evaluated by the plate dilution method [25]. Briefly, tubes with 2 ml of the MH broth containing the compounds at 0, 0.5 MIC and MIC concentrations were inoculated with 10^6 cells of *S. aureus* and incubated for 2 h. Samples were centrifuged at 14,000×*g* for 5 min, the supernatant was discarded and fresh media was added. This was done twice to ensure the complete removal of the compound. The pellet was re-suspended in the fresh culture medium, diluted 1:10 with a final volume of 5 ml in each tube (control, 0.5MIC, and MIC) and incubated at 35 °C. Aliquots from each tube were collected at time 0 and every hour

until visual cloudiness and plated on MH agar plates to obtain CFU/ml. The PAE was calculated using the following equation: PAE = T − C, where T is the time required for the initial bacterial culture to increase by 1 $\log_{10}$ CFU/ml after the removal of the antimicrobial, and C represents the time required for bacterial cultures not treated with an antimicrobial to increase 1 $\log_{10}$ CFU/ml.

Checkerboard Microdilution Assay

To analyze the possible interaction between the isolated compounds and known antibiotics, a checkerboard microdilution assay was carried out. The range of concentrations of the compounds and the antibiotic used was such that it encompasses the MIC of each drug. Two hundred microliters of Mueller-Hinton Broth was added to each well in the first column of a 96-well microtiter plate and 100 μl to the rest of the wells. The antibiotic was serially diluted in vertical direction whereas the compound was horizontally diluted, resulting in a checkerboard with a combination of both drugs at different concentrations in each well. An inoculum equal to a 0.5 McFarland turbidity standard was prepared in normal saline solution (NSS) and each well was inoculated with 10^4 cells of the test organism which was incubated at 37 °C for 24 h. The fraction inhibitory concentration index (FICI) was calculated for each combination using the following formula: $FICI = FICI_A + FICI_B$, where $FICI_A$ = (MIC of drug A in combination/MIC of drug A alone), and $FICI_B$ = (MIC of drug B in combination/MIC of drug B alone). FICI values < 0.5 represent synergism in the interaction between drugs. FICI values between 0.5 and 4.0 are classified as indifferent, and FICI values > 4.0 are classified as antagonism [26].

In Vitro Transcription/Translation Assay

In vitro transcription and/or translation reactions were performed using S30 *Escherichia coli* extract as per the manufacturer's instructions (Promega, Fitchburg, WI, USA). The reaction mixture included S30 premix, S30 extract, amino acids, template DNA, each compound (at IC_{50} and MIC concentrations) and sterile deionized water. An amount of 0.5 μg/μl of plasmid DNA template was used for each reaction. The reactions were incubated at 37 °C for 60 min followed by 5 min incubation on ice. In a 96-well white solid plate, 75 μl of distilled water was added to each well, one well for each reaction, whereas 100 μl was added in one well for estimation of background luminescence. The 25 μl product of the above reaction was added to the corresponding well and then 100 μl of Steady-Glo Reagent was added to each well. The net luminescence was obtained by subtracting the background luminescence from the experimental luminescence in the test reactions. Luminescence was measured with a Multimode BioTek, Synergy, Mx100 equipment (BioTek, Winooski, VT, USA). After measuring luminescence, the percent inhibition for each of the compounds was calculated. The values represent the mean of three individual experiments.

Staphyloxanthin Biosynthesis Inhibition Assay

Staphyloxanthin, an important virulence factor which is a brightly colored carotenoid pigment, was extracted and quantified [27]. Briefly, the culture of *S. aureus* was grown with the compounds at 0.5 MIC concentration. Equal number of cells (values were normalized by measuring OD at 600 nm) were then washed with normal saline solution (NSS). The final pellet was extracted with methanol and allowed to stand at 40 °C for 25 min. Samples were centrifuged and the supernatant was read at 450 nm. Further, percent inhibition in biosynthesis of staphyloxanthin was calculated using the following equation: $[(X–Y)/X] \times 100$, where X is the OD of the control and Y is the OD of treated. The data represent mean of three independent experiments.

Quantitative Real-Time Polymerase Chain Reaction

To determine the effect of both the compounds on gene expression of virulent factors of *S. aureus*, a quantitative real-time PCR assay was performed. *S. aureus* ATCC 29213 was incubated with or without the sub-minimal inhibitory concentration (0.5 MIC) of both the compounds for 24 h. Total RNA was isolated from the samples by using TRIzol® Reagent (Ambion, Austin, TX, USA) according to the manufacturer's instructions. Prior to cDNA synthesis, RNA was treated with DNase I (Qiagen, Hilden, Germany), to remove trace amounts of DNA. The cDNA was synthesized using the First strand cDNA synthesis kit (Fermentas, Waltham, MA, USA). The primer pairs and reaction conditions used in this study were described by previous reports and are listed in Table 2 [28]. 16S rRNA was used as an internal control gene. real-time PCR reactions were run on a Stratagene Mx3000p instrument with Jump Start SYBR Green Ready Mix (Sigma-Aldrich).

Results

Isolation and Characterization of the Endophyte

The endophyte was obtained from a healthy leaf of *G. glabra* and designated as GG1F1. The fungus produced conidiomata and the cultural and morphological analysis revealed that the isolate was a species *Phoma* Sacc. emend. Boerema & G.J. Bollen. The ITS1-5.8S-ITS2 ribosomal gene showed maximum sequence similarity of 99 % with different *Phoma* species, among which the highest score was displayed for *Phoma* sp. strain UASWS0884 followed by *Stagonosporopsis cucurbitacearum* strain LHG-8 (Syn. *Phoma cucurbitacearum*) (Supplementary Table S1). The phylogenetic position of GG1F1 is presented in the Fig. 1. This indicates that GG1F1 is most

Table 2 Oligonucleotide primers used for gene expression studies by quantitative real-time PCR

Serial number	Gene description	Oligonucleotide primer sequence 5′-3′
1	Normalizing internal standard (16S rRNA gene)	Forward: ACTGGGATAACTTCGGGAAA
		Reverse: CGTTGCCTTGGTAAGCC
2	Staphylococcal enterotoxin A (*sea*)	Forward: ATGGTGCTTATTATGGTTATC
		Reverse: CGTTTCCAAAGGTACTGTATT
3	Accessory gene regulator A (*agrA*)	Forward: TGATAATCCTTATGAGGTGCTT
		Reverse: CACTGTGACTCGTAACGAAAA

closely related to *P. cucurbitacearum* among different species of *Phoma*.

Characterization of Secondary Metabolites

We obtained two pure compounds from the extract of GG1F1. The compounds were characterized by the analyses of HRESIMS, NMR (1D and 2D spectroscopy), and their comparison with the known compounds [29].The compounds **1** and **2** exhibited the molecular ion peak (+)-HRESIMS *m/z* 481.0989 $[M + H]^+$ (calcd for $C_{23}H_{21}N_4O_4S_2$, *m/z* 481.1004) and **2** (+)-HRESIMS *m/z* 513.0712 $[M + H]^+$ (calcd for *m/z* $C_{23}H_{21}N_4O_4S_3$, 513.0725), respectively (see Electronic Supplementary Material, ESM) and both were found to be thiodiketopiperazine derivatives (Fig. 2). Total quantities of 5 and 30 mg of the compounds were obtained from 10-L volume of the culture broth, respectively.

Antimicrobial Activity of the Extract and the Compounds

The extract of GG1F1 inhibited eight, among the 18 bacterial pathogens tested, with IC_{50} values of less than 10 μg/ml (Table 1), covering both Gram-positive and Gram-negative bacteria. The fungal pathogen *C. albicans* was also inhibited significantly with an IC_{50} value of 17.5 μg/ml. Compounds **1** was effective against several pathogens especially, *S. aureus*, Methicillin-resistant *S. aureus*, and *S. pyogenes* with IC_{50} values of 5.8, 5.6, and 3.1 μM, respectively, and the Compounds **2** also inhibited these pathogens with IC_{50} values of 3.8, 8.4, and 1.8 μM in the same order.

Biofilm Inhibition

The compounds **1** and **2** also strongly inhibited biofilm formation in *S. aureus*. At the MIC of compound **1** (12.5 μM)

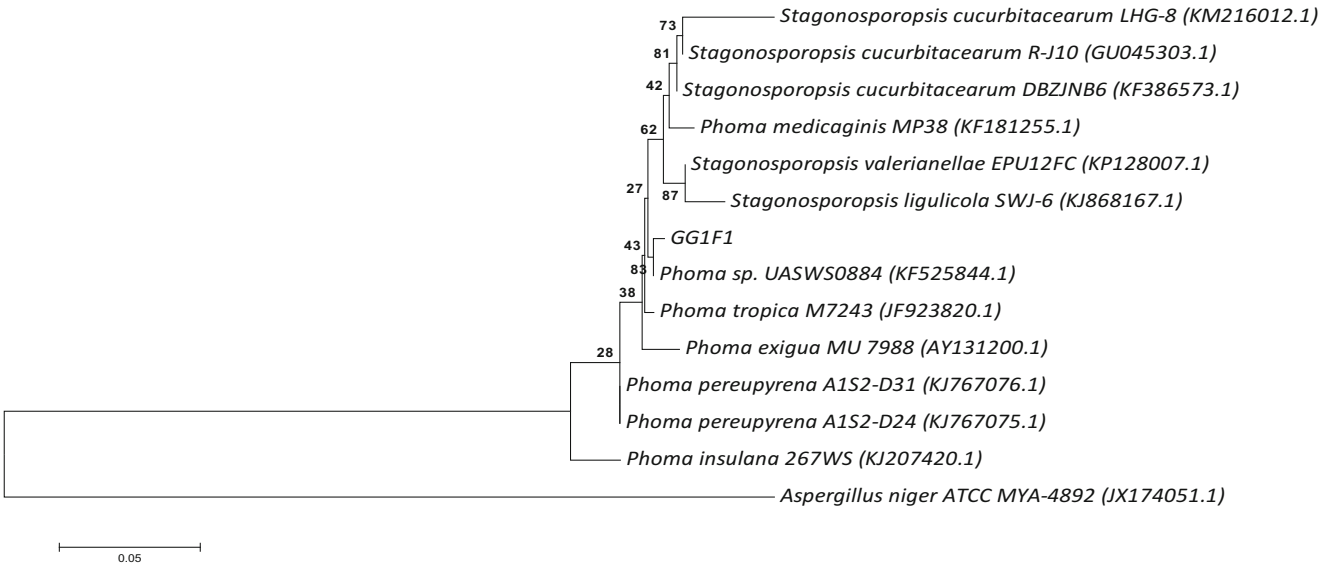

Fig. 1 Molecular phylogenetic analysis of GG1F1. The evolutionary history was inferred by using the maximum likelihood method based on the Tamura-Nei model (1993). There were a total of 584 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 [21]. *Aspergillus niger* was used as an outgroup. The GenBank Accession number of each sequence is given in parenthesis

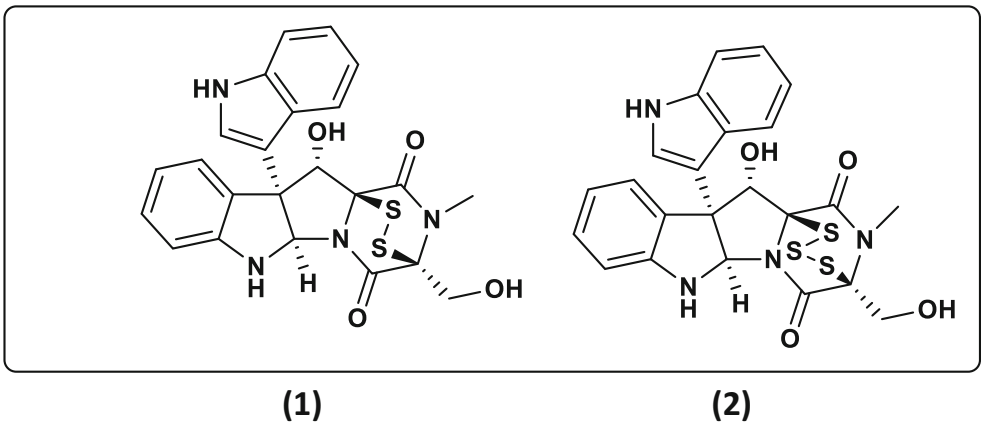

Fig. 2 Thiodiketopiperazine derivative compounds, **1** and **2**, isolated from GG1F1

and compound **2** (15 μM), the biofilm formation was inhibited by 68.8 and 70.3 %, respectively. The biofilm formation was inhibited by more around 54 % at the IC_{50} of both the compounds (Table 3). At 0.78- and 0.94-μM concentrations (one sixteenth MIC) of compound **1** and compound **2**, respectively, the biofilm was inhibited by around 30 %. The microscopic analysis of the biofilm also indicates efficient biofilm disruption properties of these compounds (Fig. 3). The compound **1** caused around 85 % decrease in the biofilm formation at all the concentrations tested against *S. pyogenes* whereas compound **2** inhibited it to a similar extent at one fourth MIC up to the MIC level. Compound **2** again showed stronger biofilm inhibition in *S. pyogenes* inhibiting it by around 60 % at 0.94 μM concentrations (one-sixteenth MIC).

Time Kill Kinetics

When *S. aureus* was grown in presence of the compound **1** at MIC and 2 × MIC, $\log_{10}$ reductions of 0.6 and 1.0 were observed at 24 h, corresponding to 74.6 and 91.6 % growth reduction, respectively (Fig. 4a). Similarly, at the corresponding concentrations of compounds **2**, $\log_{10}$ reductions of 1.35 and 1.5 were observed at 24 h, accounting for 95.6 and 97.1 % growth reduction, respectively. At 2 × MIC $\log_{10}$ reduction of 1 was observed at 14 h (Fig. 4b). Further, the compound **1** inhibited *S. pyogenes* more effectively. Log_{10} reductions of 1.4 and 1.7 were observed at 24 h, corresponding to 96.5 and 97.8 % growth reduction, respectively. Log_{10} reduction of 1.0 was observed at 14 and 10 h, respectively, at MIC and 2 × MIC (Fig. 5a). Compound **2** was similarly active against *S. pyogenes*. Log_{10} reductions of 1.5 and 1.7 were observed at 24 h, corresponding to 96.8 and 97.9 % growth reduction, respectively, at MIC and 2 × MIC (Fig 5b). Log_{10} reduction of 1.0 was observed at 14 and 10 h at the respective concentrations.

Antimicrobial Potential of Compounds 1 and 2 in Combination with Standard Antibiotics

Combinations of each compound and each antibiotic (ciprofloxacin, streptomycin, and ampicillin) were tested by the checkerboard microdilution assay to evaluate if there was any interaction between the two antimicrobials. In the checkerboard test with ciprofloxacin, FICI value for compound **1** was 0.28 indicating that there is a synergistic

Table 3 Biofilm inhibition caused by compound **1**and Compound **2** in *S. aureus* and *S. pyogenes*

S. aureus				*S. pyogenes*			
Compound 1		Compound 2		Compound 1		Compound 2	
Concentration (μM)	Biofilm inhibition (%)	Concentration (μM)	Biofilm inhibition (%)	Concentration (μM)	Biofilm inhibition (%)	Concentration (μM)	Biofilm inhibition (%)
12.5[a]	68.8 ± 1.7	15.0[a]	70.3 ± 1.3	15.0[a]	86.6 ± 1.01	15.0[a]	86.84 ± 0.2
6.25	54.4 ± 3.3	7.50	57.2 ± 2.1	7.50	85.9 ± 0.00	7.50	85.65 ± 0.6
3.13	41.1 ± 0.3	3.75	54.4 ± 2.0	3.75	85.7 ± 0.45	3.75	85.73 ± 0.7
1.56	36.4 ± 0.4	1.88	42.0 ± 1.0	1.88	84.9 ± 1.91	1.88	78.91 ± 0.2
0.78	30.1 ± 3.7	0.94	28.5 ± 3.2	0.94	86.4 ± 1.46	0.94	60.75 ± 3.9

[a] These are the MICs of the respective compounds against *S. aureus* and *S. pyogenes*

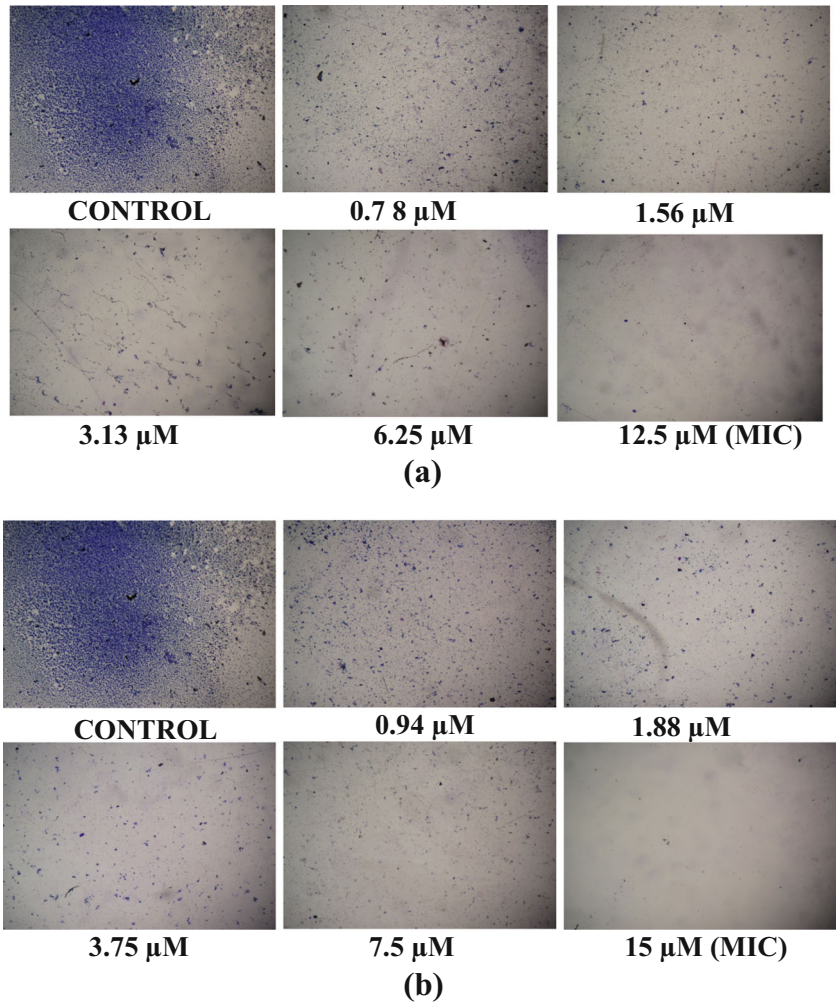

Fig. 3 Microscopic documentation of biofilm inhibition at different concentrations of **a** compound **1** and **b** compound **2**. Both the compounds exhibited potential biofilm inhibition activity against *S. aureus*

interaction between the compound and ciprofloxacin against *S. aureus* whereas FICI value for compound **2** was 0.89 indicating no synergistic or antagonistic interactions between the two antimicrobials. With streptomycin, FICI values of 0.37 and 0.33 were obtained respectively for compounds **1** and **2**, thus displaying a synergistic relationship. FICI values of 4.01and 0.95 were observed from compounds **1** and **2** in combination with ampicillin, indicating indifferent and synergistic activities, respectively.

Inhibition of Transcription/Translation

In order to understand the mode of inhibition of bacterial growth by the compounds of GG1F1, in vitro transcription/translation reactions were performed at two different concentrations. Compound **1** inhibited transcription/translation reactions by 70.0 % (±0.12) and 24.6 % (±0.7), whereas compound 2 by 90.2 % (±0.4) and 10.9 % (±0.38) at MIC and IC_{50}, respectively (Fig. 6).

Post Antibiotic Effect and Staphyloxanthin Production

After 2-h treatment of *S. aureus* with 0.5MIC and MIC of the compounds **1** and **2**, the culture took 3 and 5 h, and 2 and 5 h, respectively to attain $\log_{10}$ growth of 1, whereas, the control attained it in only 1 h.

Staphyloxanthin production by *S. aureus* decreased by 32.4 % (±0.6) and 42.1 % (±0.8) when the culture was grown with compound **1** and **2** at 0.5 MIC, respectively.

Expression of Pathogenecity-Related Genes in *S. aureus* Under the Influence of the Compounds

The comparative expression of Staphylococcal enterotoxin A (*sea*) gene and the accessory gene regulator A (*agrA*) was studied by the quantitative RT-PCR assay. The expression of *sea* gene was increased by 22.3-fold with compound **1**, while its expression (0.93-fold) was not affected significantly by compound **2**. The expression of *agrA* gene was also not affected significantly in *S. aureus* with the treatment of these compounds (Fig. 7).

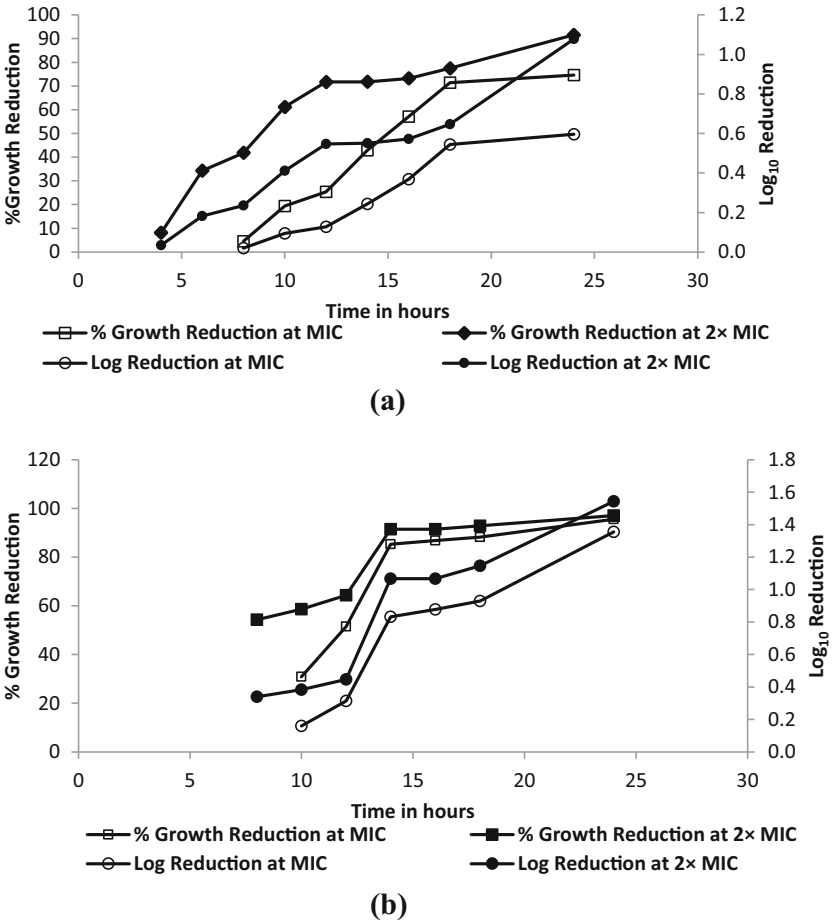

Fig. 4 **a** Time kill curves of *S. aureus* at MIC (12.5 μM) and 2 × MIC (25 μM) of compound **1**. Log_{10} reductions of 0.59 and 1.0 were observed at 24 h, corresponding to 74.6 and 91.6 % growth reduction, respectively. **b** Time kill curves of *S. aureus* at MIC (15 μM) and 2 × MIC (30 μM) of compound **2**. Log_{10} reductions of 1.35 and 1.5 were observed at 24 h, corresponding to 95.6 and 97.1 % growth reduction, respectively. At 2 × MIC Log_{10} reduction of 1 was observed at 14 h

Discussion

The genus *Phoma* is a complex assemblage of more than 3000 infrageneric taxa [30]. The species of *Phoma* may parasitize other fungi, lichens, insects, vertebrates as well as plants. Further, in plants, *Phoma* species may exist as both pathogenic as well as endophytic in nature [17, 31, 32]. Although *S. cucurbitacearum* is known to produce gummy stem blight of cucurbits, there are no reports of any disease caused by it in *G. glabra* [33].

Several bioactive compounds have been characterized from the species of *Phoma*, including those with potential antimicrobial activities [31, 34, 35]. However, this is the first report of isolation of compounds **1** and **2** from *Phoma*/*Stagonosporiopsis* sp. and their antimicrobial potential. These compounds have been earlier reported from *Tilachlidium* sp. and were shown to possess potential cytotoxic activity against P388 leukemia cells [29]. Subsequently, compound **1** was prepared synthetically and was found having broad range of cytotoxic activity against several cancer cell lines [36]. Compound 2 was also reported from two other fungal sources, *Plectosphaerella cucumerina* and *Acrostalagmus luteoalbus* (syn. *Verticillium luteoalbum*) and was found to have strong cytotoxic activity [37, 38]. Thus, it seems that several fungi have the ability to produce thiodiketopiperazine derivatives. However, no data is available on the antimicrobial activity of these compounds.

Potential antimicrobial activity of the GG1F1 extract prompted us to characterize its secondary metabolites. Both the compounds isolated belonged to the same class and possessed similar chemical structures. Thus, the antimicrobial profiles of these compounds were similar but with differences in active concentrations. For example, compound **1** was active against *Klebsiella pneumoniae* at a much lower concentration than Compound 2 (IC_{50} 4.5 vs. 19.1 μM). Further, compound **1** was active against *C. albicans* (IC_{50} 35.8 μM) whereas compound **2** did not inhibit this fungal pathogen.

Many forms of bacterial infections, especially recurrent and chronic are associated with the formation of bacterial biofilms. This study shows that both the compounds produced by GG1F1 were efficient in reducing biofilm formation at sub-MIC concentrations. This makes them efficient antimicrobial agents against biofilm forming pathogens. The Agr quorum sensing system also controls biofilm formation in *S. aureus* [39]. However, no significant change in the expression of *agr* gene in the treated cells indicates that the biofilm inhibition in

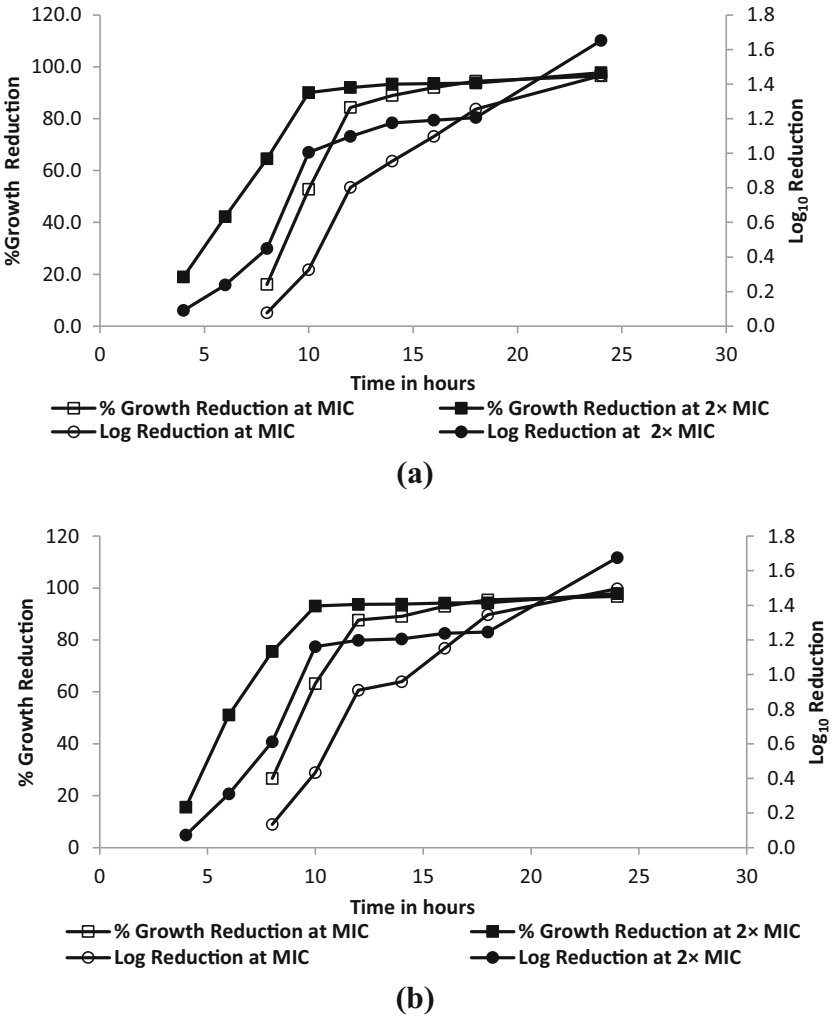

Fig. 5 **a** Time kill curves of *S. pyogenes* at MIC (15 μM) and 2 × MIC (30 μM) of compound **1**. Log_{10} reductions of 1.4 and 1.7 were observed at 24 h, corresponding to 96.5 and 97.8 % growth reduction, respectively. Log_{10} reduction of 1.0 was observed at 14 and 10 h, respectively, at MIC and 2 × MIC. **b** Time kill curves of *S. pyogenes* at MIC (15 μM) and 2 × MIC (30 μM) of compound **2**. Log_{10} reductions of 1.5 and 1.7 were observed at 24 h, corresponding to 96.8 and 97.9 % growth reduction, respectively. Log_{10} reduction of 1.0 was observed at 14 and 10 h, respectively, at MIC and 2 × MIC

this case is independent of this system. Thus, there are certainly other mechanisms by which these compounds are inhibiting the biofilm formation.

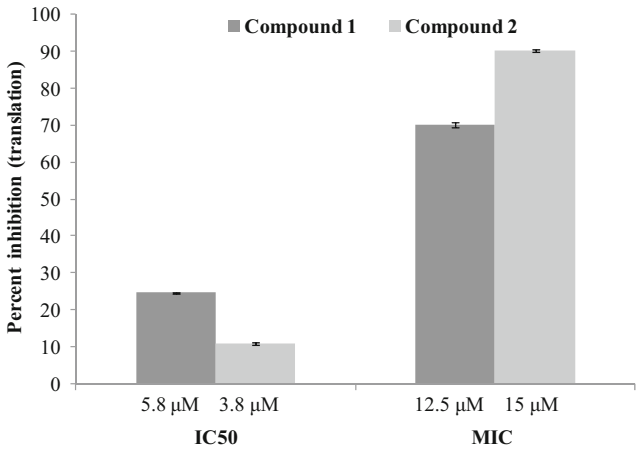

Fig. 6 Percent translation/transcription inhibition. Compound **1** inhibited transcription/translation by 70.0 % (±0.12) and 24.6 % (±0.7), whereas compound **2** by 90.2 % (±0.4) and 10.9 % (±0.38) at MIC and IC_{50}, respectively

The time kill kinetics shows that both the compounds are efficiently bactericidal against both the pathogens. In each case, the bactericidal activities were concentration dependent.

The interaction of compound **1** and **2** with ciprofloxacin and ampicillin showed that these compounds act differently in combination with these antibiotics. Ciprofloxacin and ampicillin act on different targets, DNA gyrase and cell wall synthesis, respectively. However, with the protein synthesis inhibitor, streptomycin, both the compounds acted synergistically. This indicates that these compounds may be acting in a similar fashion to that of streptomycin. Thus, these compounds were evaluated for transcription/translation inhibition in bacteria.

Our findings show clearly that the compounds inhibit the transcription/translation in bacteria potentially. This may be the main reason for the bactericidal activity of the compounds produced by GG1F1.

Staphyloxanthin, a brightly colored carotenoid pigment is an important virulence factor of *S. aureus*. The loss of this pigment renders the organism susceptible to reactive oxygen species. The inhibition of this pigment is a potential target

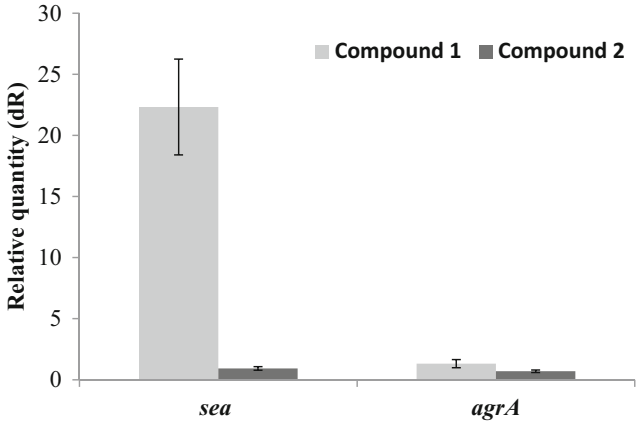

Fig. 7 Relative gene expression levels of Staphylococcal enterotoxin A (*sea*) and the accessory gene regulator A (*agrA*) in *S. aureus* after treatment with compound **1** and compound **2** at 0.5 MIC concentrations. The *sea* gene was expressed several fold on treatment with compound **1** while its expression was not affected significantly with compound **2**. The expression of *agrA* gene was also not affected significantly in *S. aureus* with the treatment of either of the compounds

against *S. aureus* [40]. Both these compounds inhibit the production of staphyloxanthin production, thus targeting the virulence factors of the pathogen.

As in case of the *agrA* gene, the expression of the *sea* gene also showed different response under the influence of the two compounds at sub-lethal concentrations. It is beneficial in certain conditions if the antibiotic decreases the production of *S. aureus* enterotoxins. However, compound **1** induces the expression of the *sea* gene at lower concentrations. β-Lactam antibiotics have also been found to induce the production of several enterotoxins in *S. aureus* [41]. Considering these results, the compound **2** has an advantage over compound **1** in inhibiting the virulence and growth of *S. aureus*.

In conclusion, two known thiodiketopiperazine derivatives were found to be produced by *Phoma* sp. GG1F1. These compounds possess potential antimicrobial and antibiofilm inhibition activities against several human pathogens, particularly *S. aureus* and *S. pyogenes*. Both the compounds acted synergistically with streptomycin and inhibited transcription/translation. The compounds differed significantly in some of their properties, particularly the effect on the expression of pathogenecity related genes in *S. aureus* at sub-lethal concentrations. Efforts should be made to scale up the production of these compounds through fermentation technology and explore their potential as antibiotics using in vivo models. The observations that were made during this study highlight the role of plant–microbe interactions in strain development of endophytes which enable them to produce potential bioactive natural products. Further, previously characterized natural products may possess hidden biological potential that may be unraveled through their evaluation for new biological activities. In nature, potential antimicrobial activity of the endophyte GG1F1 may be providing the host plant with resistance to bacterial pathogens.

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