Ann Microbiol (2014) 64:209–218
DOI 10.1007/s13213-013-0653-6

ORIGINAL ARTICLE

Isolation and identification of antimicrobial secondary metabolites from *Bacillus cereus* associated with a rhabditid entomopathogenic nematode

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Received: 21 September 2012 / Accepted: 23 April 2013 / Published online: 21 May 2013

Abstract The cell-free culture filtrate of *Bacillus cereus* associated with an entomopathogenic nematode, *Rhabditis* (*Oscheius*) sp., exhibited strong antimicrobial activity. The ethyl acetate extract of the bacterial culture filtrate was purified by silica gel column chromatography to obtain six bioactive compounds. The structure and absolute stereochemistry of these compounds were determined based on extensive spectroscopic analyses (LCMS, FABMS, ^{1}H NMR, ^{13}C NMR, ^{1}H $-^{1}$H COSY, ^{1}H $-^{13}$C HMBC) and Marfey's method. The compounds were identified as cyclo(D-Pro-D-Leu), cyclo(L-Pro-D-Met), cyclo (L-Pro-D-Phe), cyclo (L-Pro-L-Val), 3,5-dihydroxy-4-ethyl-trans-stilbene, and 3,5-dihydroxy-4-isopropylstilbene, respectively. Compounds recorded antibacterial activity against all four tested bacteria strains of *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa*. 3,5-dihydroxy-4-isopropylstilbene recorded activity only against Gram-positive bacteria while cyclo(L-Pro-L-Val) recorded no antibacterial activity. Best antibacterial activity was recorded by 3,5-dihydroxy-4-ethyl-trans-stilbene (4 μg/ml) against *Escherichia coli.* The six compounds recorded significant antifungal activities against five fungal strains tested (*Aspergillus flavus*, *Candida albicans*, *Fusarium oxysporum*, *Rhizoctonia solani* and *Penicillium expansum*) and they were more effective than bavistin, the standard fungicide. The activity of cyclo(D-Pro-D-Leu), cyclo(L-Pro-D-Met), 3,5-dihydroxy-4-ethyl-trans-stilbene, and 3,5-dihydroxy-4-isopropylstilbene against *Candida albicans* was better than amphotericin B. To the best of our knowledge, this is the first report of antifungal activity of the bioactive compounds against the plant pathogenic fungi *Fusarium oxysporum*, *Rhizoctonia solani*, and *Penicillium expansum*. We conclude that the *Bacillus cereus* strain associated with entomopathogenic nematode is a promising source of natural bioactive secondary metabolites which may receive great benefit as potential sources of new drugs in the agricultural and pharmacological industry.

Keywords *Bacillus cereus* · Secondary metabolite · Purification · Antimicrobial

Electronic supplementary material The online version of this article (doi:10.1007/s13213-013-0653-6) contains supplementary material, which is available to authorized users.

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Introduction

Entomopathogenic nematodes (EPN) belonging to the family Steinernematidae and Heterorhabditidae are one of the most important biocontrol agents against insect pests (Boszormeny et al. 2009). *Xenorhabdus* and *Photorhabdus* are Gram-negative bacteria belonging to the family Enterobacteriaceae that live in symbiosis with the nematodes *Heterorhabditis* and *Steinernema*, respectively (Tailliez et al. 2010). During the symbiotic stage, the bacteria are carried in the nematode gut, but after infection of an insect host, the nematodes inject the bacteria into the insect

hemocoel (Forst et al. 1997). The bacteria multiply rapidly and produce various metabolites which can overcome the insect immune system (Forst and Nealson 1996), kill the insect, and inhibit the growth of various fungal and bacterial competitors (Akhurst 1982; Chen et al. 1994, 1996). By doing so, the bacterial symbionts are believed to prevent putrefaction of the insect cadaver and establish conditions that favor the development of both the nematode and bacterial symbionts (Gaugler and Kaya 1990).

The secondary metabolites produced by *Xenorhabdus* spp. and *Photorhabdus* spp. are known, and several compounds with biological activity such as antibiotic, antimycotic, insecticidal, nematicidal, antiulceral, antineoplastical, and antiviral have been isolated and identified. These include indoles and stilbenes (Paul et al. 1981), xenorhabdins (McInerney et al. 1991a), xenocoumacin (McInerney et al. 1991b), nematophin (Li et al. 1997), benzylineacetone (Ji et al. 2004), xenortides and xenematide (Lang et al. 2008), and cyclolipopeptide (Gualtieri et al. 2009).

In the course of studies on EPN, a new entomopathogenic nematode belonging to the genus *Rhabditis* and subgenus *Oscheius* was isolated from sweet potato weevil grubs collected from Central Tuber Crops Research Institute (CTCRI) farm, Thiruvananthapuram (Mohandas et al. 2007). The nematodes could be cultured on laboratory-reared *Galleria mellonella* larvae and maintained alive for several years. The bacteria were found to be pathogenic to a number of insect pests (Mohandas et al. 2007) and could be isolated from 3rd stage infective juveniles of the nematode or from the hemolymph of nematode infested *G. mellonella* larvae. Based on molecular characteristics, *Rhabditis* (*Oscheius*) sp. resembles *Rhabditis* isolate Tumian 2007 at D2 and D3 (nucleotide sequence region) expansion segments of 28S rDNA (Deepa et al. 2010). The cell-free culture filtrate of the bacteria was found to inhibit several pathogenic bacteria, fungi, and a plant parasitic nematode (*Meloidogyne incognita*) (Mohandas et al. 2007), suggesting that it could be a rich source of biologically active compounds. The present study reveals the fermentation, isolation, characterization, and biological evaluation of the metabolites produced by bacterium along with taxonomic study.

Materials and methods

Chemicals and media

All the chemicals used for extraction and column chromatography were of analytical grade and high performance liquid chromatography (HPLC) grade methanol was from Merck, Mumbai, India. Silica gel (230–400 mesh) used for column chromatography and precoated silica gel 60 GF_{254} plates used for Thin Layer Chromatography (TLC) were from Merck, Germany. Microbiological media were from Hi-Media Laboratories, Mumbai, India. All other reagents were of analytical grade and the other chemicals used in this study were of the highest purity. The standard antibiotics ciprofloxacin and amphotericin B were purchased from Sigma Aldrich. The software used for the chemical structure drawing was Chemsketch Ultra, Toronto, Canada.

Test microorganisms

Gram-positive bacteria: *Bacillus subtilis* MTCC 2756, *Staphylococcus aureus* MTCC 902; Gram-negative bacteria: *Escherichia coli* MTCC 2622, and *Pseudomonas aeruginosa* MTCC 2642; medically important fungi: *Aspergillus flavus* MTCC 183, *Candida albicans* MTCC 277; and agriculturally important fungi: *Fusarium oxysporum* MTCC 284, *Rhizoctonia solani* MTCC 4634, and *Penicillium expansum* MTCC 2006. All the test microorganisms were purchased from Microbial Type Culture Collection Centre, IMTECH, Chandigarh, India. The test bacteria were maintained on nutrient agar slants and the test fungi were maintained on potato dextrose agar slants.

Bacterial isolation

The bacterium was isolated from the haemolymph of *G. mellonella* infected with IJs of *Rhabditis* (*Oscheius*). Dead *G. mellonella* larvae were surface-sterilized in 70 % alcohol for 10 min, flamed and allowed to dry in a laminar airflow cabinet for 2 min. Larvae were opened with sterile needles and scissors, care being taken not to damage the gut, and a drop of the oozing hemolymph was streaked with a needle onto nutrient agar plates. After 24–48 h incubation at 30 °C, single colonies on the nutrient agar plates were selected and aseptically transferred to fresh nutrient agar medium in slant tubes.

16S rDNA sequencing and phylogenetic analysis

Genomic DNA extraction and PCR amplification were performed according to the previously standardized protocol (Bavykin et al. 2004). The PCR product was purified using a QIAquick Gel extraction kit (QIAGEN, Tokyo, Japan) and sequenced in both directions using the same primers as for the PCR amplification. The nucleotide sequence obtained was processed to remove low quality reads, and transformed into consensus sequences with Geneious Pro software v.5.6. The resulted high-quality sequences were analyzed with BLASTn (NCBI) to confirm the authenticity of the bacterium. The sequences of related species and genus were downloaded from the Genbank database and a phylogenetic study was carried out with the program MEGA version 5

(Tamura et al. 2011). Sequences were aligned using the computer package ClustalW (Thompson et al. 1994) and were analyzed to determine the relationships between isolates by the neighbor-joining method (Saitou and Nei 1987) using the Maximum Composite Likelihood model. Bootstrap values were generated using 2,000 replicates.

Fermentation and extraction

The bacterial fermentation was carried out using modified tryptic soya broth (TSB) (tryptone 17 g/l, soytone 3.0 g/l, glucose 2.5 g/l, NaCl 5.0 g/l, meat peptone 10 g/l, water 1,000 ml). A single colony of *Bacillus cereus* N strain from the agar plate was inoculated into the flask containing 100 ml sterile media. The flasks were incubated in a gyrorotatory shaker (150 rpm) at 30 °C in the dark for 24 h. When the optical density of the culture at 600 nm was approx 1.7, the bacterial cultures were transferred aseptically into 400 ml sterile medium and incubated in the gyrorotatory shaker at 30 °C in the dark for 96 h. The culture media were then centrifugated (10,000 *g,* 20 min, 4 °C) followed by filtration through a 0.45-μm filter, to obtain cell-free culture filtrate. Thirty litres of cell-free culture filtrate were neutralized with concentrated hydrochloric acid and extracted three times with an equal volume of ethyl acetate. The ethyl acetate layers were combined, dried over anhydrous sodium sulphate, and concentrated at 30 °C using a rotary flash evaporator.

Purification of bioactive compounds

The crude extract (9.3 g) obtained after drying was loaded on a silica gel column (25×600 mm) previously equilibrated with hexane and eluted successively with 200 ml of 100 % hexane, 200 ml of linear gradient hexane: dichloromethane (v/v, 75:25 to 25:75), 200 ml of 100 % dichloromethane, 200 ml of linear gradient dichloromethane :ethyl acetate (v/v, 95:5 to 5:95), 200 ml of 100 % ethyl acetate and finally with 200 ml of 100 % methanol. Two fractions (100 ml each) were collected from each combination. The antimicrobial activity of each fraction was determined by agar well diffusion assay against *B. subtilis*, which was selected as initial test microorganism. An amount of 50 μl of crude extract was added to the wells (6 mm) and incubated for 24 h at 35 °C.

The final methanol fraction showed high antibacterial activity and was further purified using a second column. About 2.8 g of methanol fraction was loaded onto a silica gel column (10×300 mm) and eluted successively with 100 ml of 100 % chloroform, 100 ml of linear gradient chloroform: acetone (v/v, 75:25 to 25:75), 100 ml of 100 % acetone, 100 ml of linear gradient acetone: methanol (v/v, 75:25 to 25:75) and finally with 100 ml of 100 % methanol. The collected fractions were tested for antibacterial activity against *B. subtilis.*

The purity of the compounds were checked using TLC (silica gel) and HPLC, using LC-10AT liquid chromatography (LC; Shimadzu, Singapore) equipped with a C-18 column (5 μm, 4.6×250 mm) and 100 % methanol as a mobile phase with a flow rate of 1 ml/min. Ultraviolet (UV) detection was carried out with a diode array detector (Shimadzu).

Spectroscopic measurements

The structure of the compounds were determined using nuclear magnetic resonance (NMR) spectroscopy (Bruker DRX 500 NMR instrument; Bruker, Rheinstetten, Germany) equipped with a 2.5-mm microprobe. $CDCl_3$ was used as solvent to measure ^{1}H, ^{13}C and 2D NMR experiments and all spectra were recorded at 23 °C. ^{1}H NMR spectra were recorded in $CDCl_3$ using tetramethylsilane (TMS) as internal standard at 500 and 400 MHz, ^{13}C NMR spectra were recorded at 125 and 100 MHz, chemical shifts are given in parts per million and coupling constants in Hz. Chemical shifts are reported relative to the solvent peaks ($CDCl_3$: ^{1}H δ 7.24 and ^{13}C δ 77.23). High resolution mass spectrophotometer (HRMS) data were measured using an electrospray ionization mode of a Thermo Scientific Exactive Orbitrap LC-Mass Spectrometer with ions given in m/z. UV spectra and optical rotations were acquired on a Systronics double beam spectrophotometer 2201 UV–VIS spectrophotometer, India and a Rudolph Research Autopol III polarimeter, respectively. The melting point of the pure compounds were measured with a differential scanning calorimeter (DSC) with a Mettler Toledo DSC 822e instrument (Mettler-Toledo, Schcoerfenbach, Switzerland), and a temperature range of 30–300 °C were employed

Absolute configuration determination of compounds by Marfey's method

A solution of four compounds (1.5 mg) in 6 M HCl (1 ml) was heated to 120 °C for 24 h. The solution was then evaporated to dryness and the residue redissolved in H_2O (100 μl) and then placed in a 1-ml reaction vial and treated with a 2 % solution of FDAA (200 μl) in acetone followed by 1.0 M $NaHCO_3$ (40 μl). The reaction mixture was heated at 47 °C for 1 h, cooled to room temperature, and then acidified with 2.0 M HCl (20 μl). In a similar fashion, standard D- and L-amino acids were derivatized separately. The derivatives of the hydrolysates and standard amino acids were subjected to HPLC analysis (Shimadzu LC-20AD, C18 column; 5 μm, 4.6×250 mm; 1.0 ml/min) at 30 °C using the following gradient program: solvent A, water+0.2 % TFA; solvent B, MeCN; linear gradient 0 min 25 % B, 40 min 60 % B, 45 min 100 % B; UV detection at 340 nm (Marfey 1984).

Determination of antibacterial activity

Minimum inhibitory concentration (MIC)

MIC was determined by standard macro-dilution broth test as recommended by the National Committee for Clinical Laboratory Standards, USA (CLSI 2006) against all the four test bacteria. A stock solution of 2,000 μg/ml of the test compounds and standard antibiotics was prepared, which was further diluted with methanol to give the required concentrations 1,000 to 1 μg/ml. The tubes were incubated at 35 °C for 24 h. The MIC value was defined as the lowest concentration of the compound showing no visible growth. Triplicate sets of tubes were maintained for each concentration of the test sample.

Minimum bactericidal concentration (MBC)

MBC was determined according to the method of Smith-Palmer et al. (1998) against all the four test bacteria. About 100 μl from the MIC tubes not showing growth were serially diluted and plated on nutrient agar plates. The plates were incubated at 35 °C for 24 h. MBC is the lowest concentration at which bacteria failed to grow in nutrient agar inoculated with 100 μl of suspension. Triplicate sets of tubes were maintained for each concentration of the test sample.

Determination of antifungal activity

Minimum inhibitory concentration (MIC)

MIC was determined using potato dextrose agar media against the standard fungicide bavistin by the poisoned food technique (Rollas et al. 1993) against *Aspergillus flavus*, *Fusarium oxysporum*, *Rhizoctonia solani*; and *Penicillium expansum*. A stock solution of 1,000 μg/ml of the test compound was prepared, which was further diluted with methanol to give the required concentrations 1,000 to 1 μg/ml. One tube was used as solvent control. For *Candida albicans,* the broth dilution method was adopted using potato dextrose broth against the standard fungicide amphotericin B. The plates were incubated at 28 °C for 24–48 h. All experiments were in triplicate for each treatment against each fungus.

Agar disc diffusion method

In vitro antibacterial and antifungal activity of the compounds was measured using agar disc diffusion assay against the test bacteria and fungi (Murray et al. 1995; CLSI 2008). The sterile disks were impregnated with MIC concentration of test compounds. The ciprofloxacin (5 μg/disc) was used as positive reference standards for bacteria. Amphotericin B (Sigma) was used as reference standard for *Candida albicans.* Bavistin was used as reference standard against other fungi. The antimicrobial activity was evaluated by measuring the zone of growth inhibition surrounding the disks. All the assays were carried out in triplicate.

Statistical analysis

All statistical analyses were performed with SPSS (v.17.0; SPSS, Chicago, IL, USA). Data for disc diffusion assay was presented as means ± standard deviations. Statistical significance was defined as $p<0.05$.

Results

Identification of bacterium

The bacteria isolate (*Bacillus cereus* N strain) was identified based on 16S rDNA gene sequencing. PCR amplification yielded ~1,500 bp amplicon. Blast analysis showed 98 % similarity to *Bacillus cereus* sequence available in the Genbank database and thus the bacteria was identified as *Bacillus cereus.* Partial sequence data for the 16S rDNA gene have been deposited in the GenBank (NCBI) nucleotide database under the Ac. No. (HQ200404). The phylogram clearly portrayed the relationships of the isolates used for the analysis. The present bacterial isolate (*Bacillus cereus* N strain) was successfully grouped along with other *Bacillus cereus* isolates obtained from the Genbank database confirming the authenticity of the isolate (Fig. 1). The strain was currently deposited in IMTECH (Institute of Microbial Technology, Chandigarh; India) and the accession number is MTCC 5234.

Isolation and purification of bioactive compounds

The ethyl acetate extract of the cell-free culture filtrate of the bacteria showed antibacterial activity against *Bacillus subtilis*. Silica gel column chromatography of this extract yielded four fractions in the first column which were further purified by crystallization using hexane and benzene to get four white crystal compounds. The methanol fraction obtained from the first column recorded good antimicrobial activity and which was further purified using a second column chromatography to get two pure compounds. The column solvent and yield were shown in the Table 1. Initial bioactivity of these compounds was confirmed by testing against the indicator test microorganism *Bacillus subtilis.* Thin layer chromatography of the purified compounds revealed single spots and R_F value are presented in the Table 1. HPLC analysis of the six compounds was performed by reverse phase and compounds were eluted as single peaks (Table 1). The purity of the compounds reached greater than 90 % according to the peak area.

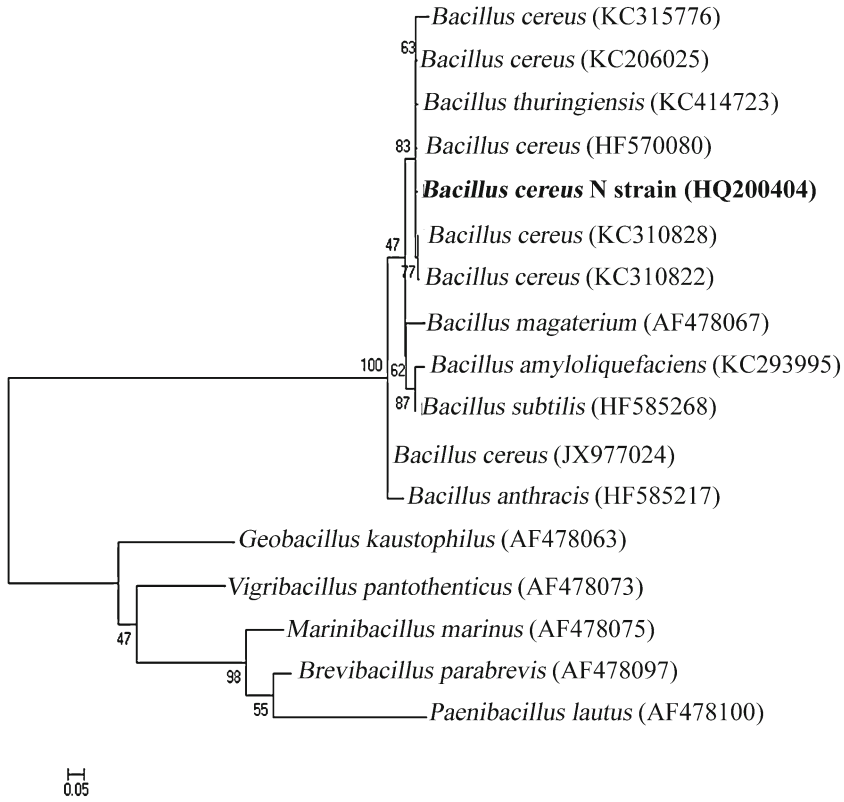

Fig. 1 Phylogenic relationships of *Bacillus cereus* N strain isolated from *Rhabditis* (*Oscheius*) sp. and known bacterial relatives based on 16S rRNA gene sequences (neighbor-joining method)

Identification of bioactive compound

The pure compounds were subjected to various spectroscopic analyses, i.e. UV, FABMS, and NMR. The structure of these six compounds corresponded to four different diketopiperazines (DKPs) and two stilbenes. The compounds identified are cyclo(D-Pro-D-Leu) (**1**), cyclo(L-Pro-D-Met) (**2**), cyclo (L-Pro-D-Phe) (**3**), cyclo (L-Pro-L-Val) (**4**), 3,5-dihydroxy-4-ethyl-trans-stilbene (**5**), and 3,5-dihydroxy-4-isopropylstilbene (**6**), respectively (Fig. 2 and Supplementary information).

Absolute configuration determination of compounds

The modified Marfey's method was successfully applied to the determination of the absolute configuration of compounds. Regarding the absolute stereochemistry, the compounds contain both D and L amino acids.

Bioactivity

Antibacterial activity

The isolated compounds were tested for antibacterial activity against four bacterial strains using standard methods. MIC and MBC values were also determined and are shown in Table 2. The microorganism that presented highest sensitivity towards compound 1 was *Bacillus subtilis* and *Escherichia coli* (8 μg/ml), followed by *Staphylococcus aureus* (16 μg/ml). Compound 2 was active against all the test bacteria and best activity of this compound was recorded against *Pseudomonas aeruginosa* (16 μg/ml), followed by *Escherichia coli* and *Bacillus subtilis* (32 μg/ml). Compound 3 presented highest activity against *Escherichia coli* (8 μg/ml). Compound 4 recorded highest activity against *Bacillus subtilis* (16 μg/ml). Compound 5 was active against test bacteria and highest

Table 1 Isolation and purification details of pure compounds

Compound	Column solvent	Yield (mg)	R_F value	Retention time (min)
1	20 % ethyl acetate in DCM	13	0.53	2.779
2	26 % ethyl acetate in DCM	16	0.44	2.753
3	38 % ethyl acetate in DCM	10	0.36	2.705
4	50 % ethyl acetate in DCM	11	0.50	2.679
5	15 % chloroform acetone[a]	18	0.33	2.522
6	45 % chloroform acetone[a]	14	0.26	2.954

[a]Compounds obtained in the second column chromatography

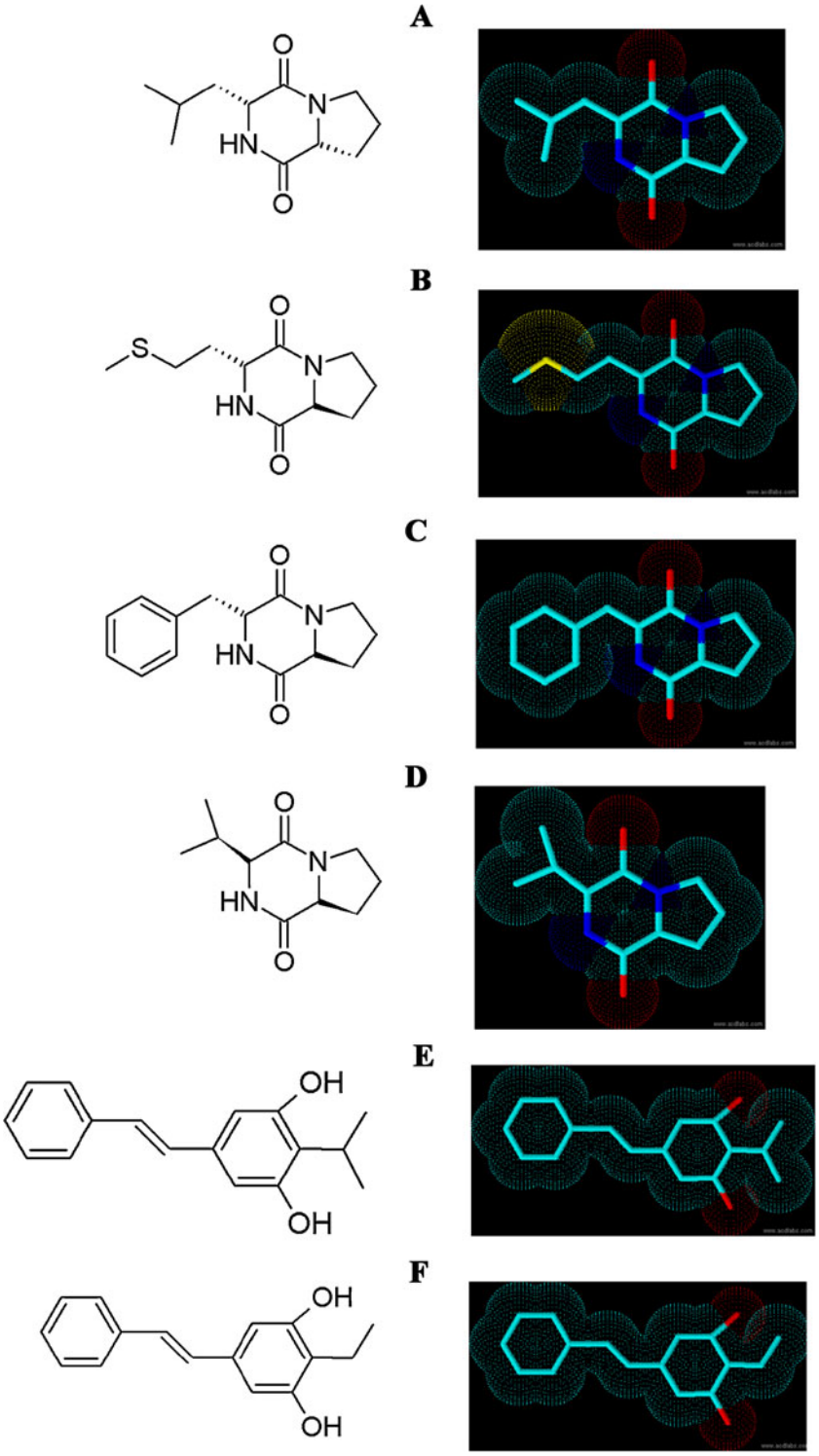

Fig. 2 Structure of diketopiperazines. **a** cyclo(D-Pro-D-Leu) (**1**), **b** cyclo(L-Pro-D-Met) (**2**), **c** cyclo (L-Pro-D-Phe) (**3**), **d** cyclo (L-Pro-L-Val) (**4**), **e** 3,5-dihydroxy-4-ethyl-trans-stilbene (**5**), and **f** 3,5-dihydroxy-4-isopropylstilbene (**6**)

activity was recorded by *Escherichia coli* (8 μg/ml). Compound 6 was active only against Gram-positive bacteria and presented the highest activity against *Staphylococcus aureus* (8 μg/ml). It appeared that effective MIC also represents the effective bactericidal concentration of the bacteria tested. The activity of the test compounds was lower than that of ciprofloxacin.

Antifungal activity

Antifungal activity against five fungi and corresponding MIC values are indicated in Table 3. The compounds exhibited good antifungal activity against all the tested fungi especially against *Penicillium expansum*, *Fusarium oxysporum* and *Rhizoctonia solani.* Compounds 1, 4 and 6 recorded highest

Table 2 MIC and MBC (μg/ml) of compounds against bacteria

Test compound	*B. subtilis*		*S. aureus*		*E. coli*		*P. aeruginosa*	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Compound 1	8	16	16	32	16	16	32	32
Compound 2	32	32	64	128	32	64	16	32
Compound 3	16	32	16	32	8	16	32	64
Compound 4	–	–	–	–	–	–	–	–
Compound 5	16	32	32	64	8	8	64	128
Compound 6	16	32	8	16	–	–	–	–
Ciprofloxacin	4	4	4	4	4	4	8	8

Values represent mean of three replications

. – no MIC up to 1,000 μg/ml

activity against *Penicillium expansum* (2 μg/ml) followed by compounds 3 and 5. Compounds recorded significant activity against plant pathogenic fungi. The antifungal activity of compounds against *Candida albicans* in comparison with amphotericin B, are in Table 3. Amphotericin B showed antifungal activity at 32 μg/ml, whereas compounds 1 and 5 recorded activity at 8 μg/ml. This activity is much better than the activity of amphotericin B. Compounds recorded higher antifungal activity than the standard fungicide bavistin against all the four fungi tested (Tables 3).

Agar disc diffusion assay

The result of disc diffusion assay is presented in the Table 4. Highest activity was recorded by compound 2 and 4 against P. expansum (34 mm).

Discussion

The 2,5-diketopiperazines (DKPs), head-to-tail dipeptide dimers, are a common naturally occurring skeleton (Prasad 1995). Diketopiperazines corresponding to cyclic dipeptides have been isolated from microorganisms, sponges, and a variety of tissues and body fluids (Rudi et al. 1994; Ström et al. 2002; De Rosa et al. 2003). Due to their relative simplicity (Anteunis 1978) and stability (Prasad 1995), diketopiperazines provide excellent models for theoretical studies as well as the development of pharmaceutical compounds. Diketopiperazines possess diverse biological activities such as antitumor (Nicholson et al. 2006; van der Merwe et al. 2008), antifungal (Houston et al. 2004), antibacterial (Fdhila et al. 2003), and antihyperglycemic (Song et al. 2003). Due to their chiral, rigid, and functionalized structures, they bind to a large variety of receptors with high affinity, giving a broad range of biological activities (Martins and Carvalho 2007). Therefore, diketopiperazines are attractive structures for the discovery of new lead compounds for the rational development of new therapeutic agents.

The isolation of cyclo(Pro-Leu) has been previously reported from *Streptomyces* sp. *Pseudomonas putida, Lactobacillus plantarum*, and *Achromobacter xylosoxidans* (Degrassi et al. 2002; Rhee 2002; Yan et al. 2004; Dal Bello et al. 2007). The antibacterial activity of cyclo(L-Pro-L-Leu) has been reported against *Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* (Rhee 2004). Our study reveals that cyclo(D-Pro-D-Leu) recorded significantly higher antimicrobial activity than that

Table 3 MIC of compounds against fungi

Testcompound	MIC (μg/ml)				
	A. flavus	*C. albicans*	*F. oxysporum*	*R. solani*	*P. expansum*
Compound 1	16	8	8	8	2
Compound 2	64	16	8	16	8
Compound 3	64	32	4	4	4
Compound 4	32	32	8	8	2
Compound 5	16	8	16	16	4
Compound 6	32	16	4	8	2
Bavistin	64	–	32	32	64
Amphotericin B	–	32	–	–	–

Values represents mean of three replications. –not tested

Table 4 Antimicrobial activity of compounds

Test organism	Zone of inhibition (diam. in mm)								
	Compound 1	Compound 2	Compound 3	Compound 4	Compound 5	Compound 6	Ciprofloxacin	Bavastin	Amphotericin B
A. flavus	19±1	23±0	18±0	23±0	21±1.52	21±1.52	X	24±1.52	X
C. albicans	14±1	17±0.57	16±0.57	18±0.57	18±1.15	20±0	X	X	24±1.73
F. oxysporum	20±1.52	29±0	21±1.15	29±0	22±0	24±1	X	16±0	X
R. solani	31±1	32±0.57	27±1.15	32±0.57	25±1.52	29±0.57	X	19±1.52	X
P. expansum	20±0.57	34±0.57	26±1.15	34±0.57	26±1	30±0.57	X	24±1.15	X
B. subtilis	24±1	21±0	15±1	–	25±1	28±1.15	31±0.57	X	X
S. aureus	23±0	22±0.57	18±1.73	–	24±1.73	24±2	31±0	X	X
E. coli	29±0.57	29±0.57	17±0	–	20±2	–	28±1.52	X	X
P. aeruginosa	24±0	24±0	18±1.15	–	18±1	–	25±0.57	X	X

– not tested as the MIC value is above 100 (μg/mL), X not tested

of the reported cyclo(L-Pro-L-Leu), which is due to the presence of D-amino acids in the compound. Cyclo(L-Pro-D-Leu) inhibiting the production of aflatoxin by *Aspergillus parasiticus* has also been reported (Yan et al. 2004). Cyclo(Leu-Pro) peptides having antifungal property have been isolated from *Lactobacillus plantarum* (Strom et al. 2002). Rhee (2003) also reported the antifungal activity of cyclo(L-pro-L-Leu) especially against rice blast fungus, *Pyricularia oryzae*.

The cyclo(Pro-Phe) was first to be isolated from *Alternaria alternata* (Stierle et al. 1988; Rosa et al. 2003). Various biological activities of cyclo(Pro-Phe) including antifungal (Wang et al. 1999; Strom et al. 2002), antimicrobial (Graz et al. 1999; Rhee 2006) and quorum sensing (Holden et al. 1999) properties have been reported previously. Cyclo(L-Pro-L-Phe) was previously reported from the Antarctic sponge-associated bacterium, *Pseudomonas aeruginosa* (Jayatilake et al. 1996), *Streptomyces fungicidicus* (Li et al. 2006), and marine bacteria *Bacillus subtilis* sp. 132 (Wang et al. 2010). Cyclo(L-Phe-L-Pro) is also involved in quorum sensing (Holden et al. 1999; Degrassi et al. 2002). Rhee (2004) also reported the antibacterial activity of cyclo(Pro-Phe) against various Gram-positive and -negative bacteria and the data are almost in agreement with our results. Cyclo-(L-Pro-L-Met) was first reported from the Antarctic sponge-associated bacterium, *Pseudomonas aeruginosa* (Jayatilake et al. 1996) and marine-derived actinomycete, *Nocardiopsis* sp. 03N67 (Shin et al. 2010). Isolation of cyclo(Pro-Val) has been previously reported from *Halobacillus litoralis* YS3106 (Yang et al. 2002), *Pseudomonas fluorescens* GcM5-1A carried by the pine wood nematode (Guo et al. 2007), and endophytes isolated from Taiwanese herbal plants (Hsieh et al. 2009). Yang et al (2002) reported that cyclo(Pro-Val) showed weak antifungal activity against human and plant pathogenic fungi. Toxicity of cyclo(Pro-Val) against Japanese black pine suspension cells was also reported (Guo et al. 2007).

3,5-dihydroxy-4-isopropylstilbene and 3,5-dihydroxy-4-ethyl-trans-stilbene have already been reported from the EPN bacteria *Photorhabdus* and *Xenorhabdus* (Paul et al. 1981; Richardson et al. 1988; Hu et al. 1998). Stilbenes are very simple compounds that nevertheless serve complex ecological roles and are the result of unusual biosyntheses. *Photorhabdus* is the only stilbene producer outside the plant kingdom. Moreover, in *Photorhabdus*, stilbenes are derived from a head-to-head condensation of two b-ketoacyl intermediates instead of the consecutive elongation of cinnamoyl-CoA by malonyl-CoA in the plant pathway (Joyce et al. 2008). Strong antifungal activity for 3,5-dihydroxy-4-isopropylstilbene against *Aspergillus flavus*, *Candida*

tropicalis, etc. has been reported (Li et al. 1995). Similar antifungal activity was also observed in the present studies. But the antimicrobial activity of 3,5-dihydroxy-4-ethyl-trans-stilbene is not reported in the literature and this is first report of the antimicrobial activity of this compound.

Conclusion

EPN bacteria produce a diverse group of secondary metabolites, of which a few have been isolated and identified. Further, there are also a number of EPN bacteria which have not been exploited for their bioactive metabolites. The results of the present study show that DKPs and stilbenes exhibited significant antifungal activity against test fungal strains. Therefore, these microbial secondary metabolites can be ideal candidates for use as potential antimicrobial agents, and entomopathogenic bacteria can be regarded as a novel source of potential pharmaceuticals. Thus, compounds isolated from bacteria are an encouraging bioprobe to develop new antimicrobial therapeutics from such types of small molecules in the near future.

Acknowledgments The authors are grateful to the Indian Council Medical Research (ICMR), Government of India for funding. We thank the Director, CTCRI, for providing facilities for the work.

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