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Purification, structural elucidation and bioactivity of tryptophan containing diketopiperazines, from *Comamonas testosteroni* associated with a rhabditid entomopathogenic nematode against major human-pathogenic bacteria

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ABSTRACT

The cell free culture filtrate of a *Comamonas testosteroni* associated with an Entomopathogenic nematode (EPN), *Rhabditis (Oscheius)* sp. exhibited promising antimicrobial activity. The ethyl acetate extract of the bacterial culture filtrate was purified by silica gel column chromatography to obtain five diketopiperazines or cyclic dipeptides (DKP 1–5). The structure and absolute stereochemistry of the compounds were determined based on extensive spectroscopic analyses (HR-MS, ^{1}HNMR, ^{13}CNMR, ^{1}H–^{1}H COSY, ^{1}H–^{13}C HMBC) and Marfey's method. Based on the spectral data the compounds were identified as Cyclo-(L-Trp-L-Pro) (1), Cyclo-(L-Trp-L-Tyr) (2), Cyclo-(L-Trp-L-Ile) (3), Cyclo-(L-Trp-L-Leu) (4) and Cyclo-(L-Trp-L-Phe) (5), respectively. Three diketopiperazines (DKP 2, 3 and 5) were active against all the ten bacteria tested. The highest activity of 0.5 μg/ml by Cyclo-(L-Trp-L-Phe) was recorded against *Vibrio cholerae* followed by *Salmonella typhi* (1 μg/ml) a human pathogen responsible for life threatening diseases like profuse watery diarrhea and typhoid or enteric fever. The activity of this compound against *V. cholerae* and *S. typhi* is more effective than ciprofloxacin and ampicillin, the standard antibiotics. Cyclo-(L-Trp-L-Phe) recorded significant antibacterial activity against all the test bacteria when compared to other compounds. Five diketopiperazines were active against all the test fungi and are more effective than bavistin the standard fungicide. Diketopiperazines recorded no cytotoxicity to FS normal fibroblast and VERO cells (African green monkey kidney) except DKP 3 and 4. To our best knowledge this is the first report of antimicrobial activity of the tryptophan containing diketopiperazines against the human pathogenic microbes. The production of cyclic dipeptides by *C. testosteroni* is also reported here for the first time. We conclude that the *C. testosteroni* is promising sources of natural bioactive secondary metabolites against human pathogenic bacteria which may receive great benefit in the field of human medicine in near future.

1. Introduction

Antibiotics are one of the pillars of modern medicine. Throughout the ages, natural products have been the most consistently successful sources of lead compounds that have found many applications in the fields of human medicine, pharmacy and agriculture [52,54]. Microbial natural products have been the source of most of the antibiotics in current use for the treatment of various infectious diseases. Many terrestrial born bacteria are reported to produce secondary metabolites having antimicrobial property [5]. However, bacterial resistance emerges when an antimicrobial agent is introduced into the market or just after its introduction due to misuse [4]. So there is a continuous need for new chemotherapeutants, especially novel antibiotics, to combat new diseases and drug-resistant pathogens that are becoming a significant threat to public health [56]. The discovery and development of new drugs from natural products (NPs) has played a significant role over the last few decades. Over 28% of the new chemical entities and 42% of the anticancer drugs introduced into the market can be traced back to NPs [42]. The majority of these have been isolated from terrestrial-borne microbes. Meanwhile, the emergence of severe resistance to antibiotics in microbial pathogens, such as methicillin and vancomycin-resistant *Staphylococcus aureus* (MRSA & VRSA) and vancomycin-resistant *Enterococci* (VRE), and the current

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increase in the number of new diseases/pathogens, e.g., Gram-negative New Delhi metallo-beta-lactmase (NMD-1) bacteria have caused a revival of interest in the discovery of Microbial based Natural Products (MNPs) with unique scaffolds to meet the urgent demand for new drugs [57]. Recent trends in drug discovery underline that terrestrial microorganisms are a potentially productive source of novel secondary metabolites and have great potential to increase the number of NPs in clinical trials.

Entomopathogenic nematodes carrying symbiotic bacteria represent one of the best non-chemical strategies supporting insect control [36,59]. In the soil, the infective juveniles of these nematodes (Heterorhabditis or Steinernema genera) actively seek the host, penetrate through insect's natural openings, travel to the hemocoel and release symbiotic bacterial cells (*Photorhabdus* and *Xenorhabdus* spp.). The bacteria multiply and release a number of virulence factors, including complexes of toxins, hydrolytic enzymes, hemolysins, and antimicrobial compounds that kill the insect host within 48 h [20], thus providing nutrients for the nematodes development and reproduction within the insect cadaver. 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. These metabolites have been used in biological pesticides and therapeutic agents such as antibiotic, antimycotic, insecticidal, nematicidal, antiulcer, antineoplastical and antiviral for many decades [55]. The antimicrobial nature of metabolites produced by *Xenorhabdus* and *Photorhabdus* spp. is known, and several of such compounds with antibiotic activity have been isolated and identified [21].

During our studies on EPN, a new entomopathogenic nematode belonging to the genus *Rhabditis* and subgenus *Oscheius* was isolated from soil samples collected from Central Tuber Crops Research Institute (CTCRI) farm, Thiruvananthapuram [41]. The nematodes can be cultured on laboratory reared *Galleria mellonella* larvae and maintained alive for several years. The specific symbiotic bacteria were found to be associated with the nematode [41] and can be isolated from the haemolymph of nematode infested *G. mellonella* larvae. Based on molecular characteristics, *nematode* resembles *Rhabditis (Oscheius)* at D2 and D3 (nucleotide sequence region) expansion segments of 28S rDNA [18]. The cell free culture filtrate of the bacteria was found to inhibit several pathogenic bacteria and fungi [41], suggesting that it could be a rich source of biologically active compounds. In the present study we reported the taxonomic study of the symbiotic bacteria, purification and structure elucidation of five tryptophan containing diketopiperazines, its antimicrobial activity with special reference to medically important bacteria. The present study also reported the antimicrobial tryptophan containing diketopiperazines for the first time from *Comamonas testosteroni.*

2. Materials and methods

2.1. Chemicals and media

All the chemicals used for extraction, column chromatography and high performance liquid chromatography (HPLC) were from Merck Limited, 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 Limited, Germany. Microbiological media were from Hi-Media Laboratories Limited, Mumbai, India. All other reagents and chemicals used in this study were of the highest purity. The standard antibiotics ciprofloxacin, ampicillin and amphotericin B were purchased from Sigma Aldrich (USA). The software used for the chemical structure drawing was Chemsketch Ultra, Toronto, Canada.

2.2. Pathogen microbial targets

Gram positive bacteria: *Bacillus subtilis* MTCC 2756, *S. aureus* MTCC 902, *Staphylococcus epidermis* MTCC 435 and *Staphylococcus simulans* MTCC 3610; Gram negative bacteria: *Escherichia coli* MTCC 2622, *Klebsiella pneumoniae* MTCC 109, *Proteus mirabilis* MTCC 425, *Vibrio cholerae* MTCC 3905, *Pseudomonas aeruginosa* MTCC 2642, and *Salmonella typhi* MTCC 3216; medically important fungi: *Aspergillus flavus* MTCC 183, *Candida tropicalis* MTCC 184, *Candida albicans* MTCC 277, *Cryptococcus gastricus* MTCC 1715 and *Trichophyton rubrum* MTCC 296 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 test fungi were maintained on potato dextrose agar slants.

2.3. 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 chamber for 10 min. Then larvae were opened with sterile needles and scissors, care being taken not to damage the gut, and a drop of the oozing haemolymph was streaked 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.

2.4. 16S rRNA sequencing and phylogenetic analysis

Genomic DNA was extracted from bacterial cultures through enzymatic hydrolysis [43]. The complete 16S rRNA gene (1.4–1.5 kp) was amplified via PCR, using universal bacterial primers 27 F (5′-AGA GTT TGATCC TGG CTC AG-3′) and 1541R (5′-AAG GAG GTG ATC CAG CCG CA-3′). The amplification was carried out on a DNA thermal cycler (BIORAD, USA). The 50 μl PCR reactions contained 4 μl of 2.5 U/μl Taq DNA polymerase (Genei, Bangalore, India), 5 μl of 10× buffer (Genei), 1 μl of 20 mM dNTPs (Genei), 37 μl of SDW, 1 μl of 50 μM each primer, and 1 μl of template.

The PCR conditions were initial denaturation at 94 °C for 4 min, 30 amplification cycles of 94 °C for 1 min, 56 °C for 1 min, and 72 °C for 2 min, and a final elongation at 72 °C for 10 min. 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, transformed into consensus sequences with Geneious Pro software version 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 [51]. Sequences were aligned using the computer package ClustalW [53] and were analyzed to determine the relationships between isolates by the neighbor-joining method [44] using the Maximum Composite Likelihood model. Bootstrap values were generated using 2000 replicates.

2.5. 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 1000 ml) supplemented by 0.1% tryptophan. A single colony of *Comamonas testosteroni* from

the agar plate was inoculated into the flask containing 100 ml sterile media. The flasks were incubated in a gyrorotatory shaker (120 rpm) at 28 °C in dark for 24 h. When the optical density of the culture at 600 nm was approximately 1.7, the bacterial cultures were transferred aseptically into 400 ml sterile medium and incubated in the gyrorotatory shaker at 28 °C in dark for 72 h. After fermentation, the culture media was centrifuged (10,000 × *g*, 20 min, 4 °C) followed by filtration through a 0.22 μm filter, to obtain cell free culture filtrate. Thirty liters of cell free culture filtrate were neutralized with concentrated hydrochloric acid and extracted with an equal volume of ethyl acetate thrice. Then the ethyl acetate layers were combined, dried over anhydrous sodium sulphate, and concentrated at 35 °C using a rotary flash evaporator.

2.6. Fractionation of the crude ethyl acetate extract

Activated silica gel (230–400 mesh) was packed onto a glass column (600 × 30 mm) using n-hexane solvent 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. About 82 fractions measuring 100 ml each were collected and concentrated by using the rotary evaporator.

2.7. Thin-layer chromatography (TLC)

An aliquot of the pure compounds were loaded on the activated silica gel TLC plates (20 cm × 20 cm). The plates were developed using 40% benzene in acetone. Spots were located by exposing the plate to iodine fumes.

2.8. Analytical HPLC

HPLC analysis of pure compounds was carried out on a LC-10AT liquid chromatography (LC; Shimadzu, Singapore) equipped with a C-18 column (5 μm, 4.6 mm × 250 mm) (150 mm × 3.9 mm, 5 mm). Mobile phase: 100% methanol. Flow rate: 1.0 ml/min. Detection: 220 nm (diode array detector).

2.9. Structure determination of antimicrobial compound

2.9.1. UV spectrophotometer

UV–visible spectrum of the pure compounds was recorded on a Systronics double beam spectrophotometer 2201, India at room temperature (scanning range 190–800 nm).

2.9.2. Nuclear magnetic resonance (NMR)

The structure of the compounds was determined using nuclear magnetic resonance (NMR) spectroscopy (Bruker DRX 500 NMR instrument, Bruker, Rheinstetten, Germany) equipped with a 2.5-mm microprobe. NMR Spectrometer using $CDCl_3$ was deployed to measure ^1H and ^{13}C and 2D NMR. All spectra were recorded at 23 °C. One-dimensional ^1H NMR experiments as well as two-dimensional ^1H–^1H correlation spectroscopy, ^1H–^{13}C heteronuclear multiple bond correlation, and ^1H–^{13}C heteronuclear multiple quantum coherence (HMQC) experiments were performed according to Bruker standard pulse sequences. Chemical shifts are reported relative to the solvent peaks. ($CDCl_3$: ^1H δ 7.24 and ^{13}C δ 77.23).

2.9.3. High resolution mass spectrophotometer (HRMS)

HRMS was performed on a Thermo Scientific Exactive Orbitrap LC-Mass Spectrometer with an electrospray ionization mode.

2.9.4. Optical rotations

Optical rotation of the compounds was measured using a Rudolph Research Autopol III polarimeter (Hackettstown, NJ, USA) at 25 °C in acetone.

2.9.5. Differential scanning calorimetry

The melting point of the compounds was measured using a differential scanning calorimeter in a Mettler Toledo DSC 822e instrument (Mettler-Toledo, Schcoerfenbach, Switzerland). Temperature ranges from 25 °C to 300 °C were employed.

2.10. Absolute configuration determination of compounds

A solution of 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 was 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 analytical HPLC analysis (Shimadzu LC-20AD, C18 column; 5 μm, 4.6 mm × 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 [39].

2.11. Antibacterial activity

2.11.1. Minimum inhibitory concentration (MIC)

The minimum inhibitory concentration of the compounds was determined according to the method described by the Clinical and Laboratory Standards Institute [14], with some modifications. Two fold serial dilutions of the antibiotics and peptide compounds were made with Mueller Hinton broth (MHB) to give concentrations ranging from 0.12 to 1000 μg/ml. Hundred microliters of test bacterial suspension were inoculated in each tubes to give a final concentration of 1×10^5 CFU/ml. The tubes were incubated for 24 h at 37 °C. The control tube did not have any antibiotics or test compounds, but contained the test bacteria and the solvent used to dissolve the antibiotics and compounds. The growth was observed both visually and by measuring OD at 600 nm. The lowest concentration of the test compound showing no visible growth was recorded as the MIC. Triplicate sets of tubes were maintained for each concentration of the test sample. Ciprofloxacin and ampicillin were used as positive control.

2.11.2. Minimum bactericidal concentration (MBC)

Minimum bactericidal concentration was determined according to the method of Smith-Palmer et al. [48]. About 100 μl from the tubes not showing bacterial growth in the MIC test were serially diluted and plated on Nutrient agar. The plates were incubated at 37 °C for 24 h. Minimum bactericidal concentration is defined as the concentration at which bacteria failed to grow on nutrient agar inoculated with 100 μl test bacterial suspensions.

2.11.3. Antibacterial assay by disk diffusion technique

The antimicrobial activity of the pure compounds was determined by the disk diffusion method [15] against bacteria. The test cultures maintained in nutrient agar slant at 4 °C were sub-cultured in nutrient broth to obtain the working cultures approximately containing 1×10^6 CFU/ml. The MIC concentration of the peptide compounds was incorporated in a 6 mm sterile disk. Mueller Hinton (MH) agar plates were swabbed with each bacterial strain and the test disks were placed along with the control disks.

Ciprofloxacin disks (5 μg/disk) were used as positive control. Plates were incubated overnight at 37 °C for 24 h. Clear, distinct zone of inhibition was visualized surrounding the discs. The antimicrobial activity of the test agents (compounds and antibiotics) was determined by measuring the zone of inhibition expressed in mm.

2.12. Antifungal assay

2.12.1. Minimum inhibitory concentration (MIC)

The MIC was performed by broth microdilution methods as per the guidelines of Clinical and Laboratory Standard Institute [10,13], with RPMI 1640 medium containing L-glutamine, without sodium bicarbonate and buffered to pH 7.0 (both from Sigma). Twofold serial dilutions of the peptide compounds were prepared in media in amounts of 100 μl per well in 96-well microtiter plates (Tarson, Mumbai, India). The test fungal suspensions were further diluted in media, and a 100 μl volume of this diluted inoculums was added to each well of the plate, resulting in a final inoculum of 0.5×10^4 to 2.5×10^4 CFU/ml for *Candida* species and 0.4×10^4 to 5×10^4 CFU/ml for other fungi. The final concentration of peptide compounds ranged from 1 to 1000 μg/ml. The medium without the agents was used as a growth control and the blank control used contained only the medium. Amphotericin B served as the standard drug controls. The microtiter plates were incubated at 35 °C for 48 h for *Candida* species and 30 °C for 72 h. for other fungi. The plates were read using ELISA, and the MIC was defined as the lowest concentration of the antifungal agents that prevented visible growth with respect to the growth control.

2.12.2. Antifungal assay by disk diffusion technique

Compounds were screened for their antifungal activity against test fungi by disk diffusion method [11,12]. The fungal cultures were grown on potato dextrose broth. The mycelia mat of fungi of 7-day old culture was suspended in normal saline solution and test inoculum was adjusted to 5×10^5 CFU/ml. Inocula (0.1 ml) were applied on the surface of the potato dextrose agar plate and spread by using a cotton swab. Subsequently, filter paper discs (6 mm in diameter, Hi-media) containing MIC concentrations of test compounds were placed on the agar plates and incubated at 35 °C for 24–48 h. Afterwards, the diameter of the inhibition zone was measured.

2.13. Cytotoxicity test

The MTT (3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) assay was used to determine the cytotoxicity of diketopiperazines. Lung cancer cell line (A 549) and colon cancer cell line (HTL 116), FS normal fibroblast and African green monkey kidney cell line (VERO) were used for testing. Briefly, cells (5×10^3 well^{-1}) were seeded in 0.2 ml of the medium (DMEM with 10% PBS) in 96 well plates, treated with drugs for 72 h. and after incubation, cytotoxicity was measured. For this after removing the drug containing media, 25 μl of MTT solution (5 mg/ml in PBS) and 75 μl of complete medium were added to wells (untreated and treated) and incubated for 2 h. At the end of incubation MTT lysis buffer was added to the wells (0.1 ml well^{-1}) and incubated for another 4 h at 37 °C. At the end of incubation, the optical densities at 570 nm were measured using a plate reader (Biorad ELISA reader 680, California, USA). The relative cell viability in percentage was calculated (A_{570} of treated sample/A_{570} of untreated sample × 100) [2].

2.14. Statistical analysis

All statistical analyses were performed with SPSS (Version 17.0; SPSS, Inc., Chicago, IL, USA). Data for disk diffusion assay was presented as means ± standard deviations.

2.15. Sequence submitted to GenBank

Partial sequence data for the 16S rRNA gene of *Comamonas testosteroni* have been deposited in the GenBank (NCBI) nucleotide database under the ac. no. (HQ200410).

3. Results

3.1. Identification of bacterium

The bacteria isolate (*Comamonas testosteroni* strain) was identified based on 16S rRNA gene sequencing. PCR amplification yielded ~1500 bp amplicon. Blast analysis showed 99% similarity to *C. testosteroni* sequence available in the Genbank database and thus the bacterium was identified as *C. testosteroni.* Partial sequence data for the 16S rRNA gene have been deposited in the GenBank (NCBI) nucleotide database under the ac. no. (HQ200410). The phylogram clearly portrayed the relationships of the isolates used for the analysis. The present bacteria isolate (*C. testosteroni* strain) was successfully grouped along with other *C. testosteroni* 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).

3.2. Isolation and purification of bioactive compounds

The ethyl acetate extract of the cell free culture filtrate of the bacterium showed significant antibacterial activity against *B. subtilis*. Silica gel column chromatography of this extract yielded five crystal compounds. The column solvent and yield were shown in Table 1. These crystal compounds were further purified by crystallization using hexane and benzene. Bioactivity of these compounds was confirmed by testing against the indicator test microorganism *B. subtilis.* TLC of these purified crystal compounds recorded single spots and R_F value is presented in Table 1. HPLC analysis of the compounds was performed by reverse phase and compounds were eluted as single peaks (Table 1 and Fig. 2). The purity of the compounds reached greater than 90% according to the peak area.

3.3. Identification of bioactive compound

The five pure compounds were subjected to various spectroscopic analyses, i.e. UV, HR-MS and NMR. The structure of these five compounds corresponded to different diketopiperazines (DKPs) or cyclic dipeptides, The diketopiperazines identified are Cyclo-(L-Trp-L-Pro) (1), Cyclo-(L-Trp-L-Tyr) (2), Cyclo-(L- Trp-L-Ile) (3), Cyclo-(L-Trp-L-Leu) (4) and Cyclo-(L-Trp-L-Phe) (5), respectively (Fig. 3).

Table 1
Isolation and purification details of pure compounds.

Compound	Column solvent	Yield (mg)	R_F value	Retention time (min)
1	25% DCM in hexane	16	0.33	2.579
2	100% DCM	18	0.39	4.167
3	10% ethyl acetate in DCM	36	0.27	2.927
4	30% ethyl acetate in DCM	30	0.44	3.234
5	65% ethyl acetate in DCM	23	0.37	3.719

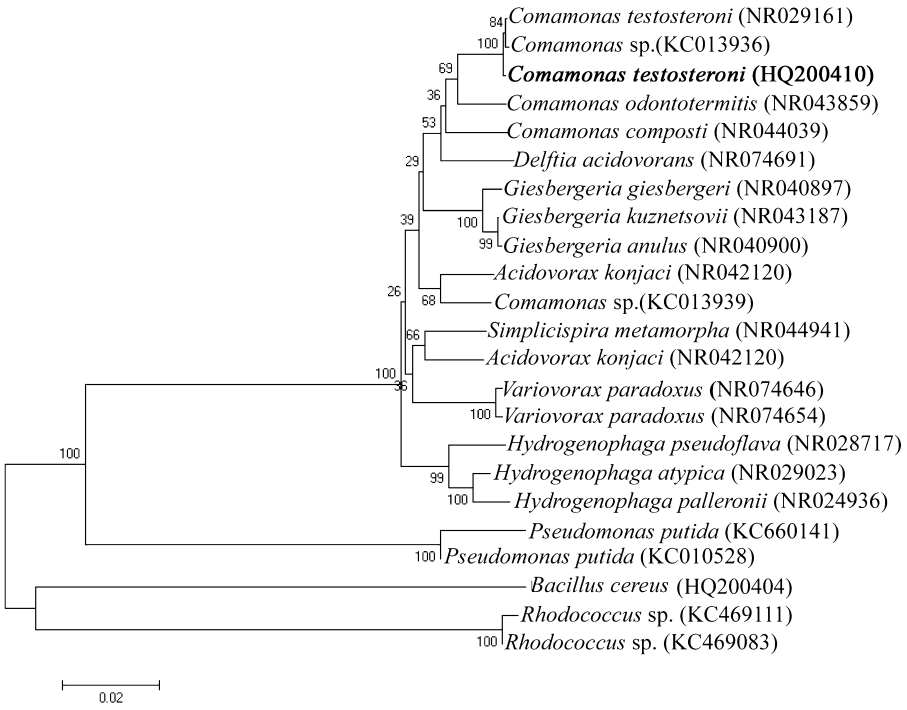

Fig. 1. Phylogenic relationships of *Comamonas testosteroni* strain isolated from *Rhabditis (Oscheius)* sp. and known bacterial relatives based on 16S rRNA gene sequences (neighbor-joining method).

DKP 1: Cyclo(L-Trp-L-Pro), (3S,8aS)-3-(1H-indol-3-ylmethyl)hexahydropyrrolo[1,2- a]pyrazine-1,4-dione: was obtained as white crystalline powder; Melting point: 285.58 °C; $[a]_D$ −128 (c, 0.02, MeOH); UV max: 220 nm (MeOH); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 1.79–2.15 (2H, m, H-4), 2.42–2.52 (2H, m, H-5), 2.90 (1H, dd *J* = 10.7 Hz/15.1 Hz, Ha-10), 3.54(2H, m, H-3), 3.71(1H, dd *J* = 3.9 Hz/15.0 Hz, Hb-10), 4.28 (1H, t *J* = 8.0 Hz, H-6), 4.28 (1H, dd *J* = 2.7 Hz/10.6 Hz, H-9); 5.84 (1H, brs, N—H), 7.12–7.70 (5H, m, Ar—H), 8.32 (1H, brs, N—H (Indole). ^{13}C NMR ($CDCl_3$, 125 MHz), 169.99, 46.30, 23.52, 29.23, 60.12, 166.22, 55.34, 27.79, 124.15, 110.76, 119.37, 120.17, 123.16, 112.12, 127.34, 136.18. Based on the spectral data the molecular formula was determined to be $C_{16}H_{18}N_3O_2$ by HRMS (*m/z* 284.34167 [M + H]), calcd. for $C_{16}H_{17}N_3O_2$, 283.32345.

DKP 2: Cyclo-(L-Trp-L-Tyr) (3S,6S)-3-(4-hydroxybenzyl)-6-(1H-indol-3-ylmethyl)piperazine-2,5-dione: was obtained as colorless amorphous solid. Melting point: 265.58 °C; [a]$_D$ −118 (c, 0.02, MeOH); UV max: 222 nm (MeOH); ^{1}H NMR ($CDCl_3$, 500 MHz): 2.64 (dd, *J* = 11.9, 8.4 Hz, 1H, H-15a), 1.54 (dd, *J* = 11.2, 8.3 Hz, 1H, H-15b), 3.24 (dd, *J* = 14.6, 7.9 Hz, 1H, H-8a), 2.83 (dd, *J* = 14.8, 7.9 Hz, 1H, H-8b), 3.93 (m, 1H, H-12), 4.23 (m, 1H, H-9), 6.72 (d, *J* = 7.0 Hz, 2H, H-18 and H-20), 6.54 (d, *J* = 7.0 Hz, 2H, H-17 and H-21), 7.21 (s, 1H, H-2), 7.13 (dd, *J* = 7.4, 7.3 Hz, 1H, H-5), 7.23 (dd, *J* = 7.3, 7.3 Hz, 1H, H-6), 7.34 (d, *J* = 7.4 Hz, 1H, H-7), 7.64 (d, *J* = 7.3 Hz, 1H, H-4). ^{13}CNMR (125 MHz in $CDCl_3$): 169.9, 169.6, 156.7, 138.8, 132.3, 129.4, 128.9, 126.7, 123.8, 121.3, 120.8, 116.4, 112.7, 109.9, 58.0, 57.4, 40.8, 30.9. Based on the spectral data the molecular formula was determined to be $C_{20}H_{20}N_3O_3$ by HRMS (*m/z* 350.14584 [M + H]), calcd. for $C_{20}H_{19}N_3O_3$, 349.14573).

DKP 3: Cyclo-(L-Trp-L-Ile) (6S)-3-(butan-2-yl)-6-(1H-indol-3-ylmethyl) piperazine-2, 5-dione: was obtained as colorless crystals. Melting point: 205.58 °C; $[a]_D$ −102 (c, 0.02, MeOH); UV max: 214 nm (MeOH); ^{1}H NMR ($CDCl_3$, 500 MHz): 7.59 (1H, dd, *J* = 8.7, 1.0 Hz, H-7), 7.29 (1H, dd, *J* = 8.6, 1.0 Hz, H-4), 6.99 (1H, ddd, *J* = 8.3, 7.0, 1.0 Hz, H-6), 7.01 (1H, s, H-2), 6.92 (1H, ddd, *J* = 8.1, 7.0, 1.0 Hz, H-5), 4.26 (1H, m, H-9), 3.70 (1H, dd, *J* = 14.4, 4.2 Hz, H-8), 3.16 (1H, dd, *J* = 14.4, 4.1 Hz, H-8), 2.84 (1H, dd, *J* = 2.7, 0.8 Hz, H-12), 1.80 (1H, m, H-15), 1.13 (2H, m, H-16), 0.69 (3H, d, *J* = 6.5 Hz, H-18), 0.58 (3H, t, *J* = 7.3 Hz, H-17); ^{13}C NMR ($CDCl_3$, 125 MHz): 172.3, 171.3, 138.2, 128.8, 126.8, 122.9, 120.7, 120.7, 113.5, 110.3, 59.2, 58.3, 39.9, 32.3, 27.2, 14.9, 12.0. Based on the spectral data the molecular formula was determined to be $C_{17}H_{22}N_3O_2$ by HRMS (*m/z* 299.18143 [M + H]), calcd. for $C_{17}H_{21}N_3O_2$, 299.16344).

DKP 4: Cyclo-(L-Trp-L-Leu) (3S,6S)-3-(1H-indol-3-ylmethyl)-6-(2-methylpropyl)piperazine-2,5-dione: was obtained as colorless crystals. Melting point: 217.28 °C; $[a]_D$ −63 (c, 0.02, MeOH); UV max: 208 nm (MeOH); ^{1}H NMR ($CDCl_3$, 500 MHz) ä 7.59 (1H, dd, *J* = 8.1, 1.0 Hz, H-7), 7.32 (1H, dd, *J* = 8.1, 1.0 Hz, H-4), 7.01 (1H, ddd, *J* = 8.1, 7.0, 1.0 Hz, H-6), 6.99 (1H, s, H-2), 6.96 (1H, ddd, *J* = 8.1, 7.0, 1.0 Hz, H-5), 4.22 (1H, dd, *J* = 4.1, 3.8 Hz, H-9), 3.41 (1H, dd, *J* = 14.6, 3.8 Hz, H-8), 3.23 (1H, dd, *J* =14.6, 4.1 Hz, H-8), 1.57 (1H, m, H-12), 1.44 (2H, dd, *J* = 7.8, 4.6 Hz, H-15), 1.43 (1H, m, H-16), 0.74 (3H, d, *J* = 6.2 Hz, H-18), 0.58 (3H, d, *J* = 6.2 Hz, H-17); ^{13}C NMR ($CDCl_3$, 125 MHz): 172.3, 172.1, 138.8, 129.1 126.0, 123.7, 120.7, 119.9, 112.9, 110.1, 58.1, 54.3, 42.4, 31.7, 25.8, 23.5, 22.4. Based on the spectral data the molecular formula was determined to be $C_{17}H_{22}N_3O_2$ by HRMS (*m/z* 299.18142 [M + H]), calcd. for $C_{17}H_{21}N_3O_2$, 299.16347).

DKP 5: Cyclo-(L-Trp-L-Phe). (3S,6S)-3-benzyl-6-(1H-indol-3-ylmethyl)piperazine-2,5-dione: was obtained as colorless crystals. Melting point: 270.28 °C; $[a]_D$ −153 (c, 0.02, MeOH); UV max: 220 nm (MeOH); ^{1}HNMR (500 MHz, DMBO-d6): 61.89 (1H, dd, *J* = 13.49, 7.02 Hz, H-17), 2.48 (1H, dd, *J* = 13.49, 4.70 Hz, H-17), 2.63(1H, dd, *J* = 14.47, 5.68 Hz, H-10), 2.89 (1H, dd, *J* = 14.47, 4.46 Hz, H-10), 3.94 (1H, m, H-14), 3.98 (1H, m, H-11), 6.80 (2H, m, H-20, 22), 7.01 (1H, d, *J* = 2.20 Hz, H-2), 6.98 (1H, ddd, *J* = 7.17, 7.45, 1.45 Hz, H-5), 7.18 (1H, ddd, *J* = 7.73, 7.45, 1.00 Hz, H-6), 7.26 (2H, m, H-19, 23), 7.29 (1H, m, H-21), 7.32 (1H, dd, *J* = 7.73, 7.45 Hz, H-7), 7.54 (1H, dd, *J* = 7.17, 1.00 Hz, H-4), 7.83 (1H, d, *J* = 2.00 Hz, H-15), 7.97 (1H, d, *J* = 2.00 Hz, H-12), 11.29 (1H, s, H-1)), ^{13}CNMR (125 MHz, DMSO-d6): 30.69, 39.96, 56.21, 55.64, 109.15, 111.33, 119.47, 119.91, 121.83, 124.41, 126.36,

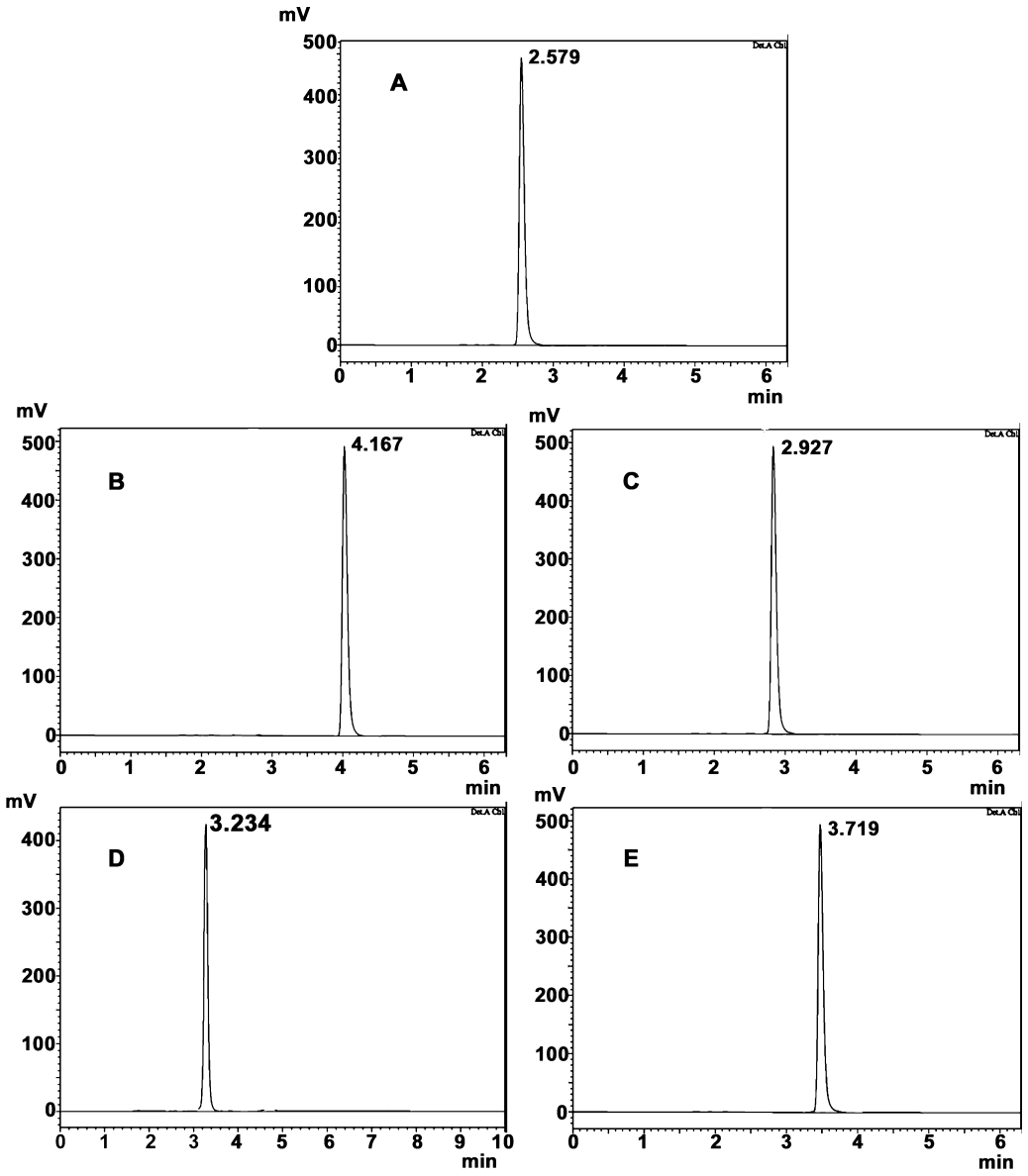

Fig. 2. Analytical HPLC profile of diketopiperazines. (A) Cyclo-(L-Trp-L-Pro), (B) Cyclo-(L-Trp-L-Tyr), (C) Cyclo-(L- Trp-L-Ile), (D) Cyclo-(L-Trp-L-Leu) and (E) Cyclo-(L-Trp-L-Phe).

127.54, 128.03, 129.70, 136.07, 136.56, 166.22, 167.18. Based on the spectral data the molecular formula was determined to be $C_{20}H_{20}N_3O_2$ by HRMS (*m/z* 334.38765 [M + H calcd, for $C_{20}H_{19}N_3O_2$, 333.14752).

3.4. 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, all the compounds contain L amino acids except DKP 1, which contain L-proline and D-tryptophan (Supplementary Figs. S1–S5). All the derivatized obtained from the hydrolysis of the diketopiperazines were compared with the retention times of the derivatized standard D and L-amino acids. The retention times for the FDAA derivatives of standard amino acids are presented in Table 2. The FDAA derivatives of Trp and Pro in the hydrolysate of DKP 1 were 40.344 and 18.453 min, respectively (Fig. S1). The FDAA derivatives of Trp and Tyr in the hydrolysate of DKP 2 were 37.188 and 25.123 min (Fig. S2), respectively. The FDAA derivatives of Trp and Ile in the hydrolysate of DKP 3 were 37.188 and 25.123 min (Fig. S3), respectively. The FDAA derivatives of Trp and Leu in the hydrolysate of DKP 4 were 37.456 and 27.567 min (Fig. S4), respectively. The FDAA derivatives of Trp and Phe in the hydrolysate of DKP 5 were 37.346 and 26.448 min (Fig. S5), respectively.

3.5. Bioactivity

3.5.1. Antibacterial activity

The isolated compounds were tested for antibacterial activity against ten bacterial strains using standard methods. MIC and MBC values were also determined and are shown in Table 3. The microorganism that presented highest sensitivity toward DKP 1 was *B. subtilis* and *S. aureus*. DKP 2 was active against all the test bacteria and best activity of this compound was recorded against *S. epidermis* (8 μg/ml), followed by *P. aeruginosa* and *S. typhi* (16 μg/ml). DKP 3 presented highest activity against *E. coli* and *S. typhi* (4 μg/ml). But DKP 4 is active only against four test bacteria and highest activity was recorded against *S. aureus* (125 μg/ml). Interestingly

Table 2
HPLC reversed-phase retention times of the L and D-amino acid standard derivatives.

Amino acid derivative	Retention time (min)	Amino acid derivative	Retention time (min)
L-Trp	37.342	D-Trp	40.232
L-Pro	17.451	D-Pro	18.344
L-Tyr	20.514	D-Tyr	33.084
L-Ile	25.234	D-Ile	23.567
L-Leu	27.248	D-Leu	24.045
L-Phe	26.897	D-Phe	31.456

Table 3
MIC and MBC of diketopiperazines against bacteria.

Test bacteria	MIC (μg/ml)													
	DKP 1		DKP 2		DKP 3		DKP 4		DKP 5		Ampicillin		Ciprofloxacin	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
B. subtilis	16	32	32	64	16	32	250	500	4	8	100	100	2	2
S. aureus	16	32	64	64	8	8	125	250	8	8	250	250	2	2
E. coli	500	500	32	32	4	4	500	1000	8	16	100	250	1	2
P. aeruginosa	125	250	16	16	32	32	–	–	16	16	500	1000	2	4
S. epidermis	–	–	8	16	32	64	–	–	2	2	125	125	2	4
P. mirabilis	–	–	32	64	64	128	–	–	2	2	64	125	4	8
V. cholerae	–	–	125	250	32	32	–	–	0.5	1	250	500	2	4
K. pneumonia	250	500	64	64	16	16	–	–	16	16	–	–	2	4
S. simulans	64	125	32	64	32	64	125	125	16	32	–	–	4	8
S. typhi	–	–	16	32	4	8	–	–	1	1	–	–	4	4

Values represent mean of three replications. –, no MIC upto 1000 μg/ml.

DKP 5 recorded significantly good activity against test pathogens in impressive low concentration and best activity was recorded against *V. cholerae* (0.5 μg/ml) followed by *S. typhi* (1 μg/ml). It appeared that effective MIC also represents the effective bactericidal concentration of the bacteria tested. The activity of the test compounds was higher than the standard antibiotic ampicillin but lower than that of ciprofloxacin. But the activity of DKP 5 against *V. cholerae* and *S. typhi* was significantly better than ciprofloxacin (Table 3).

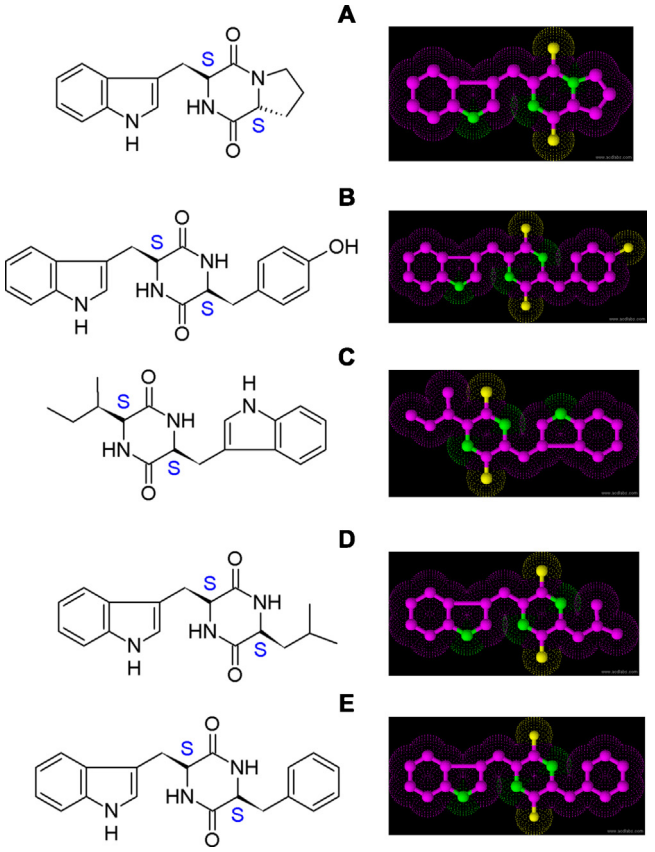

Fig. 3. Structure of five tryptophan diketopiperazines. (**A**) Cyclo-(L-Trp-L-Pro), (B) Cyclo-(L-Trp-L-Tyr), (C) Cyclo-(L- Trp-L-Ile), (D) Cyclo-(L-Trp-L-Leu) and (E) Cyclo-(L-Trp-L-Phe).

3.5.2. Antifungal activity

Antifungal activity against eight fungi and corresponding MIC values are indicated in Table 4. Five compounds recorded good antifungal activity against all the tested fungi. DKP 1 and 2 exhibited best MIC value against *P. expansum* (8 μg/ml and 32 μg/ml). DKP 3 showed significantly higher activity against *R. solani* and *P. expansum* (4 μg/ml), followed by *C. albicans*, *C. gastricus* and *C. tropicalis* (8 μg/ml). DKP 4 recorded significant activity against all fungi except *C. gastricus*. Whereas DKP 5 was highly active against all the eight test fungi and highest activity was recorded against *F. oxysporum* and *R. solani* (2 μg/ml). Amphotericin B showed antifungal activity at 16 μg/ml for *T. rubrum*, whereas DKP 5 recorded activities at 4 μg/ml. This activity is significantly better than the activity of Amphotericin B. DKPs recorded higher antifungal activity than the standard fungicide Bavistin against certain fungi (Table 4).

3.5.3. Agar disk diffusion assay

The result of disk diffusion assay of DKPs against test bacteria and fungi is presented in Table 5. Best activity was recorded by DKP 5 against *S. typhi* (35 mm), followed by *K. pneumonia* (33 mm) (Table 5 and Fig. 4). DKP 1 recorded best against *T. rubrum* (31 mm), followed by *P. expansum* (26 mm). For DKP 2 and 3 best activity was recorded against *S. epidermis* (34 and 32 mm). Where as DKP 4 recorded best activity against *R. solani* (27 mm).

3.6. Cytotoxicity activity

Cytotoxicity activity of DKPs against cancer and normal cell lines was determined by MTT assay after 72 h of treatment and was shown in Fig. 5. DKP 2 and 5 recorded activity against A549 cells and the IC_{50} was recorded at 100 and 50 μg/ml (Fig. 5IA). Where as DKP 5 alone recorded activity against HTL 116 (Fig. 5IB).

Table 4
MIC of diketopiperazines against fungi.

Test fungi	MIC (μg/ml)						
	DKP 1	DKP 2	DKP 3	DKP 4	DKP 5	Bavistin	Amphotericin B
A. flavus	32	250	32	16	32	100	–
C. albicans	64	250	8	16	16	–	32
F. oxysporum	16	64	32	8	2	16	–
R. solani	16	64	4	32	2	32	–
P. expansum	8	32	4	32	4	64	–
C. gastricus	32	–	8	64	8	–	16
C. tropicalis	128	500	8	64	8	–	8
T. rubrum	64	500	16	–	4	–	16

Values represents mean of three replications. –, not tested.

Table 5
Antimicrobial activity of diketopiperazines against test bacteria and fungi.

Test organisms	Zone of inhibition (dia. in mm)							
	DKP 1	DKP 2	DKP 3	DKP 4	DKP 5	Ciprofloxacin	Bavastin	Amphotericin B
B. subtilis	17 ± 1	23 ± 0	18 ± 0	17 ± 1	25 ± 0	26 ± 0	[a]	[a]
S. aureus	18 ± 1	17 ± 0.57	16 ± 0.57	20 ± 0.57	27 ± 1	27 ± 1	[a]	[a]
E. coli	20 ± 1.52	29 ± 0	19 ± 1.15	21 ± 1.52	26 ± 1.15	32 ± 1.15	[a]	[a]
P. aeruginosa	21 ± 1	32 ± 0.57	30 ± 1.15	–	30 ± 1.15	30 ± 0	[a]	[a]
S. epidermis	–	34 ± 0.57	32 ± 1.15	–	20 ± 0.57	29 ± 1	[a]	[a]
P. mirabilis	–	21 ± 0	15 ± 1	–	24 ± 1	31 ± 0.57	[a]	[a]
V. cholerae	–	22 ± 0.57	22 ± 1.73	–	23 ± 0	31 ± 0	[a]	[a]
K. pneumonia	22 ± 0	29 ± 0.57	21 ± 0	–	33 ± 1.52	28 ± 1.52	[a]	[a]
S. simulans	16 ± 1.2	24 ± 0	–	–	30 ± 1.73	25 ± 0.57	[a]	[a]
S. typhi	–	19 ± 1.2	22 ± 1	–	35 ± 1.73	30 ± 1.52	[a]	[a]
A. flavus	15 ± 0	21 ± 1.2	27 ± 1.73	22 ± 1	25 ± 1.52	[a]	24 ± 1.52	[a]
C. albicans	23 ± 1	25 ± 1	15 ± 1	26 ± 0	23 ± 0	[a]	[a]	24 ± 1.73
F. oxysporum	25 ± 0.57	22 ± 1	19 ± 0	21 ± 1.52	29 ± 1	[a]	16 ± 0	[a]
R. solani	24 ± 1.73	21 ± 0	25 ± 1.15	27 ± 1.15	27 ± 0	[a]	19 ± 1.52	[a]
P. expansum	26 ± 1	27 ± 0	23 ± 1.15	15 ± 1.52	30 ± 0.57	[a]	24 ± 1.15	[a]
C. gastricus	20 ± 0	–	22 ± 1	22 ± 1	25 ± 1.15	[a]	[a]	23 ± 1.15
C. tropicalis	18 ± 0	17 ± 0	20 ± 0	21 ± 0	23 ± 0	[a]	[a]	36 ± 1.73
T. rubrum	31 ± 0.57	29 ± 0.57	21 ± 0	17 ± 1	32 ± 1	[a]	[a]	27 ± 1

–, not tested as the MIC value is above 1000 μg/ml.
[a] Not tested.

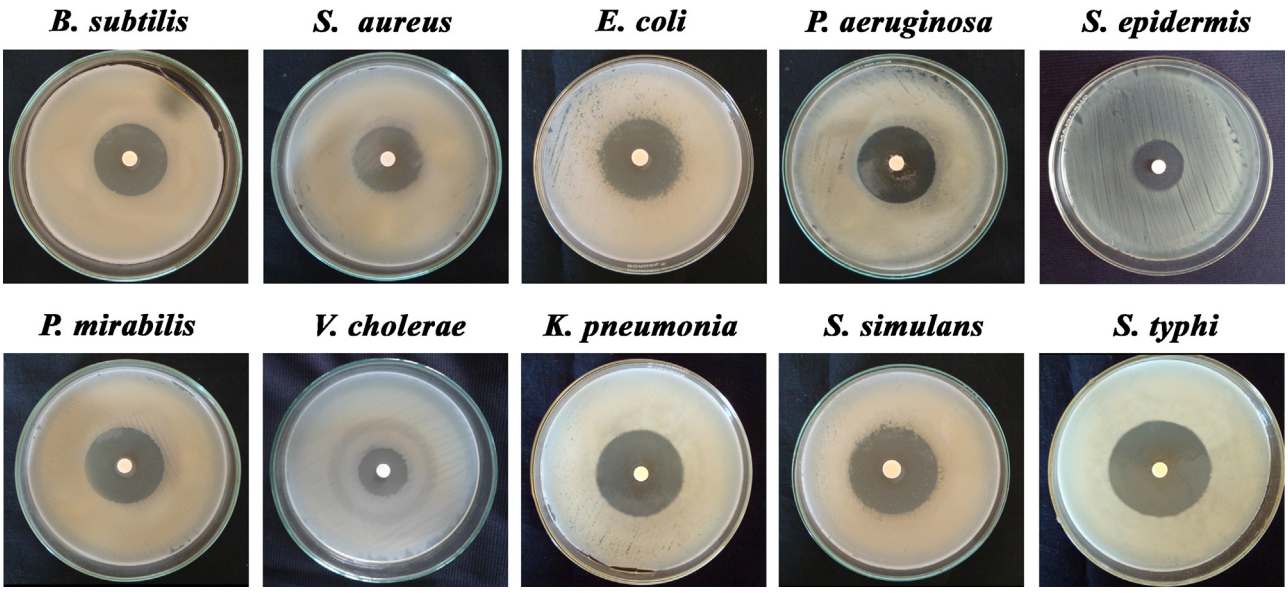

Fig. 4. Antibacterial activity of DKP 5 by disk diffusion method.

When exposed to DKPs in the range, 5–100 μg/ml, DKPs recorded no cytotoxicity against VERO cells except DKP 4 (Fig. 51IA). DKP 4 recorded significant cytotoxicity in VERO cells at 100 μg/ml. Where as DKP 3 and 4 recorded significant cytotoxicity against FS cells (Fig. 5 1IB).

4. Discussion

Diketopiperazines are the smallest cyclic peptides known, commonly biosynthesised from amino acids by a large variety of organisms, including mammals [45]. The ability of microorganisms

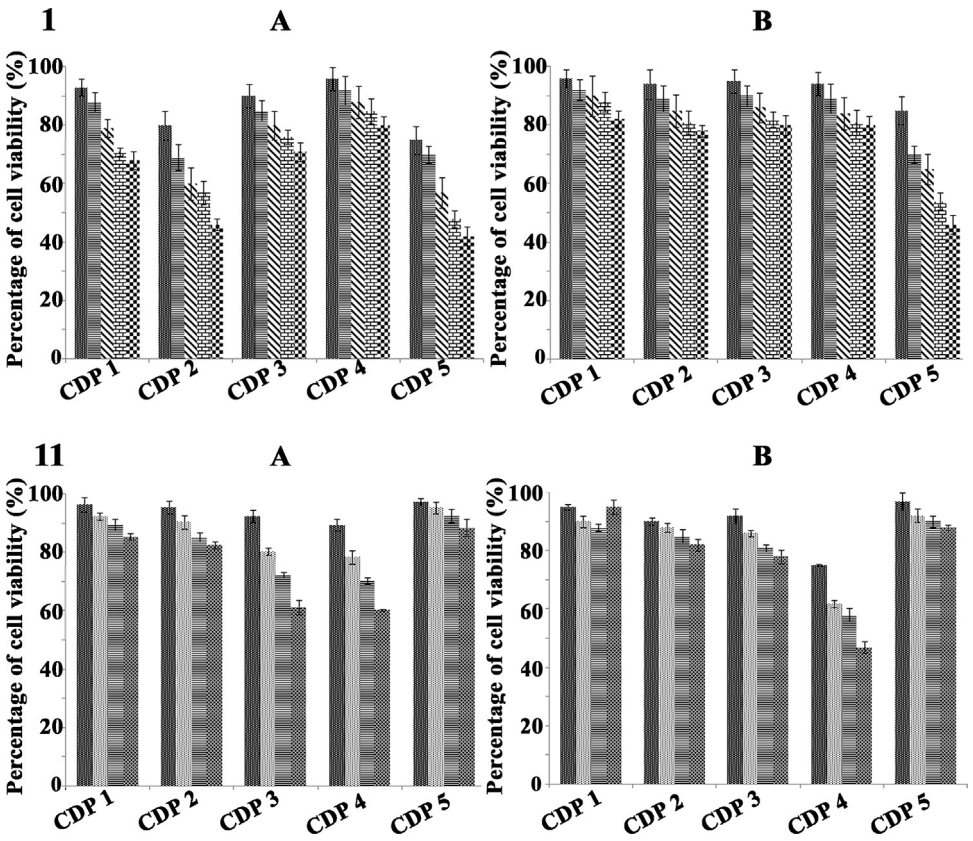

Fig. 5. Cytotoxicity of five diketopiperazines against cancer and normal cell lines. (1) Cancer cell lines, (A) A549 and (B) HTL 116. 5, 10, 25, 50 and 100 μM. (11) Normal cell lines (A) FS normal fibroblast and (B) VERO. 10, 25, 50 and 100 μM.

to produce diketopiperazines is widespread and published data have shown that about 90% of Gram-negative bacteria produce cyclic peptides [23]. Diketopiperazines have also been isolated from Gram-positive bacteria [17,50], fungi [7] and higher marine organisms [28]. Although cyclic dipeptides are extensively obtained by extracting from natural sources, they may be easily prepared by conventional synthetic procedures, due to the relative structural simplicity of their essential nucleus [23]. The conformationally constrained DKP scaffold is constituted of a six-membered ring that orientates its substituents in a spatially defined manner and represents a significant pharmacophore in medicinal chemistry because of its stable structural characteristics [17,50]. In addition, owing to their restricted conformational freedom and structural simplicity, DKPs have been extensively used as valuable models for conformational studies either in solution or in solid state, particularly with regard to the consequences (i.e. cis, trans isomerism) deriving from the relative configuration of amino acid residues [7,28].

Diketopiperazines (DKPs) are a relatively unexplored class of bioactive peptides that may hold great promise for the future. In recent years considerable interest has been focused on the cyclic dipeptides, because of their remarkable bioactivity. Important biological activities of diketopiperazines are activities as antitumour [32], antiviral [47], antifungal, antibacterial [54], antiprion [6], antihyperglycemic [49] and glycosidase inhibitor [3] agents. A number of studies are directed to important biological activities of diketopiperazines related to the inhibition of plasminogen activator inhibitor-1 [25] and alteration of cardiovascular and blood-clotting functions [9]. Recently, it was shown that diketopiperazines are able to activate or antagonize LuxR-mediated quorum-sensing systems of bacteria, and they are considered to influence cell-cell signaling [1]. Furthermore, they have been reported as part of antibacterial nucleosides [29]. Therefore, the DKPs scaffold is considered a useful tool for the discovery of new lead compounds and the suitable properties of DKPs make them attractive and promising agents for the rational development of new therapeutic agents [24,27].

DKPs are found to be ideal lead compounds for the rational design of an agent capable of preventing many diseases and disorders. Cyclic dipeptides provide excellent models for theoretical studies as well as the development of new pharmaceutical compounds due to their simplicity and limited conformational freedom. In recent years there has been a growing awareness to understand the specific function of each peptide and their structure. It is known that the biological functions of the peptides in living systems are related to their three-dimensional structures. Cyclic peptides are more bioavailable and more stable against degradative peptidases than linear peptides, the relevant research is fundamental to many aspects of peptide chemistry. The Diketopiperazines are heterocyclic compounds which exist in several stereoisomeric forms, and also in DD, DL, LD, and LL forms. The stereochemistry appears to be important for the biological activity of the cyclic dipeptides [22].

Cyclo(Pro-Trp) was previously reported from octocoral associated bacterium *Pseudoalteromonas* sp. [40], marine *B. subtilis* [38], *Sulfitobacter* Strain [37]. The antimicrobial activity of this compound was reported against some bacterial strains [26]. Other biological activity reported by this was hepatotoxicity [31]. Antifungal activity of this compound against filamentous fungi is not reported earlier. But in our study cyclo(Pro-Trp) recorded significant antifungal activity. The production cyclo(Trp-Tyr) was previously reported from marine algous endophytic fungus *Aspergillus niger* [58], *Microbispora aerata* strain [30], *Streptomyces* sp. H7372 [8]. Cyclo(D-Trp-L-Tyr) isolated from endophytic fungus *A. niger* did not show antimicrobial activity against *B. subtilis*, *S. aureus*, *Streptomyces viridochromogenes*, *E. coli*, *C. albicans* and

Mucor miehei [58], but low antiproliferative and cytotoxic effect was recorded. But in the present study cyclo(L-Trp-L-Tyr) recorded significant antimicrobial activity against the test microorganisms.

Cyclo-(D-Ile-L-Trp) and Cyclo-(D-Leu-L-Trp) having plant growth regulators activity was reported from *Penicillium brevi-compactum* [33]. To the best of our knowledge this is the first report on the antimicrobial activity of Cyclo-(D-Ile-L-Trp), and Cyclo-(D-Leu-L-Trp) and this is the second report that these compounds were reported from a natural source. In the case of cyclo (L-Trp-L-Phe), it was first isolated from an unidentified *Penicillium* sp. and proved to be with the biological function of regulating the growth of plants [34]. In 2008, cyclo (L-Trp-L-Phe) was isolated from fungal EF8 which was isolated from the conchocelis of *Porphyra yezoensisit* by Ding et al. [19] and proved to exhibit a moderate cytotoxicity against 37 human tumor cell lines with the average IC_{50} value of 3.3 μg/ml. In 2011 cyclo (L-Trp-L-Phe) was reported from South China Sea sponge *Holoxea* sp. associated fungus *Aspergillus versicolor* strain TS08 [16]. But till now, few reports on the production of DKPs by microbial cultivation have been found [46]. This study was the first to find the cyclo (L-Trp-L-Phe) from the bacterium-associated entomopathogenic nematode and the antimicrobial activity of this compound is also reported for the first time. Previously we have reported three DKPS [cyclo(L-Pro-L-Leu), cyclo(D-Pro-L-Leu) and cyclo-(D-Pro-L-Tyr)] produced by a bacterium associated with EPN having antifungal activity against plant pathogenic fungi [35].

The human pathogen *V. cholera*, which causes profuse watery diarrhea, *S. typhi*, causing typhoid or enteric fever fusarium wilt, *S. epidermis*, associated various diseases in patients with compromised immune systems were strongly inhibited by diketopiperazines and the activity is better than the standard antifungal agent ampicillin.

5. Conclusion

Already a large diversity of natural diketopiperazines has been reported but because only still a small fraction of organisms has been explored and much more can be expected. The chemical diversity of natural diketopiperazines is high and there are also several recent reports on novel diketopiperazines of unknown functions and activities which widens the diversity of this class of compounds. The microbial activities of the cyclic dipeptides especially against human pathogenic bacteria *V. cholera*, *S. typhi*, *S. epidermis*, and *P. mirabilis* have never been known. Here, we identified these compounds as a new antifungal agent against human pathogenic bacteria. Antibacterial activity of compounds is an encouraging bioprobe to develop new antimicrobial therapeutics from such type of small molecules in the near future.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.peptides.2013.09.019.

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