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Cyclo(D-Tyr-D-Phe): a new antibacterial, anticancer, and antioxidant cyclic dipeptide from *Bacillus* sp. N strain associated with a rhabditid entomopathogenic nematode

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A new microbial cyclic dipeptide (diketopiperazine), cyclo(D-Tyr-D-Phe) was isolated for the first time from the ethyl acetate extract of fermented modified nutrient broth of *Bacillus* sp. N strain associated with rhabditid Entomopathogenic nematode. Antibacterial activity of the compound was determined by minimum inhibitory concentration and agar disc diffusion method against medically important bacteria and the compound recorded significant antibacterial against test bacteria. Highest activity was recorded against *Staphylococcus epidermis* (1 μg/ml) followed by *Proteus mirabilis* (2 μg/ml). The activity of cyclo(D-Tyr-D-Phe) against *S. epidermis* is better than chloramphenicol, the standard antibiotics. Cyclo(D-Tyr-D-Phe) recorded significant antitumor activity against A549 cells (IC_{50} value: 10 μM) and this compound recorded no cytotoxicity against factor signaling normal fibroblast cells up to 100 μM. Cyclo(D-Tyr-D-Phe) induced significant morphological changes and DNA fragmentation associated with apoptosis in A549 cells. Acridine orange/ethidium bromide stained cells indicated apoptosis induction by cyclo(D-Tyr-D-Phe). Flow cytometry analysis showed that the cyclo(D-Tyr-D-Phe) did not induce cell cycle arrest. Effector molecule of apoptosis such as caspase-3 was found activated in treated cells, suggesting apoptosis as the main mode of cell death. Antioxidant activity was evaluated by free radical scavenging and reducing power activity, and the compound recorded significant antioxidant activity. The free radical scavenging activity of cyclo(D-Tyr-D-Phe) is almost equal to that of butylated hydroxyanisole, the standard antioxidant agent. We also compared the biological activity of natural cyclo(D-Tyr-D-Phe) with synthetic cyclo(D-Tyr-D-Phe) and cyclo(L-Tyr-L-Phe). Natural and synthetic cyclo(D-Tyr-D-Phe) recorded similar pattern of activity. Although synthetic cyclo(L-Tyr-L-Phe) recorded lower activity. But in the case of reducing power activity, synthetic cyclo(L-Tyr-L-Phe) recorded significant activity than natural and synthetic cyclo(D-Tyr-D-Phe). The results of the present study reveals that cyclo(D-Tyr-D-Phe) is more bioactive than cyclo(L-Tyr-L-Phe). To the best of our knowledge, this is the first time that cyclo(D-Tyr-D-Phe) has been isolated from microbial natural source and also the antibacterial, anticancer, and antioxidant activity of cyclo(D-Tyr-D-Phe) is also reported for the first time. Copyright © 2013 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: apoptosis; antibacterial; antitumor; antioxidant; *Bacillus* sp; cyclo(D-Tyr-D-Phe); diketopiperazine

Introduction

An increase in the number of people in the world with health problems caused by various drug-resistant bacteria, cancers, parasitic protozoans, and fungi is a cause for alarm [1]. Throughout the ages, natural products have been the most consistently successful source of lead compounds that have found many applications in the fields of medicine, pharmacy, and agriculture [2]. It has been well established that microorganisms are an unlimited source of natural products, many of which have potential therapeutic applications. The increase in the frequency of multidrug resistant pathogenic bacteria has created an urgent demand in the pharmaceutical industry for more rational approaches and strategies in the screening of new antibiotics [3].

Despite recent advances in our knowledge of the molecular pathogenesis and targeted therapy of cancer, it remains one of the most malignant diseases threatening human health and quality of life. The World Health Organization has defined cancer as one of the top ten leading causes of mortality worldwide [4]. Moreover, increasing recurrence of mammalian tumors and severe side effects of chemotherapeutic agents reduce the clinical efficacy of a large variety of anticancer agents that are currently being used. Thus, there is always a constant need to develop alternative anticancer drugs with minimal side effects [5]. Recently, natural products have gained increasing attention from a therapeutic point of view and have become the most consistently successful source of new potential drugs [6]. Although many anticancer drugs have been developed, a limited number of these are currently utilized for treatment because of the toxic effects of such drugs on normal cells.

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On the other hand, it is known that free radicals play a fundamental role in several diseases. The biochemical damages caused by free radicals to cells and tissues lead to the development of many diseases such as arteriosclerosis, high blood pressure, cancer, inflammation, renal failure, and liver disease [7]. Antioxidants also play an important role in the later stages of cancer development. There is increasing evidence that oxidative processes promote carcinogenesis, although the mechanisms for this are not well understood. The antioxidants may be able to cause the regression of premalignant lesions and inhibit their development into cancer. Preliminary studies have indicated that some antioxidants may be of benefit in the treatment of precancerous conditions such as oral leukoplakia, possibly a precursor of oral cancer [8].

In the course of studies on entomopathogenic nematode (EPN), a new EPN 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. A specific bacterium was found associated with the nematodes. The cell-free culture filtrate of the bacteria was found to inhibit several pathogenic bacteria, fungi, and a plant parasitic nematode (*Meloidogyne incognita*) [9], suggesting that it could be a rich source of biologically active compounds. Bacteria of the genus *Bacillus* are known for their prolific production of diverse metabolites with a variety of biological activities [10,11].

In our continuous quest for new bioactive secondary metabolites from bacteria associated with EPN, cyclo(D-Tyr-D-Phe) diketopiperazine (DKP) was isolated from a *Bacillus* sp. N strain, using a bioassay-guided chromatography. The present manuscript also describes the antibacterial, anticancer, and antioxidant activity of cyclo(D-Tyr-D-Phe), and the activity was also compared with the synthetic cyclo(D-Tyr-D-Phe) and cyclo(L-Tyr-L-Phe). This article represents the first report that documents the production of cyclo(D-Tyr-D-Phe) by the *Bacillus* sp. and their biological properties.

Materials and Method

Chemicals

1,1-Diphenyl-2-picryl-hydrazyl (DPPH), nicotinamide adenine dinucleotide (NADH), butylated hydroxyanisole (BHA), potassium ferricyanide, and trichloroacetic acid (TCA) were purchased from Sigma (Sigma–Aldrich, GmbH, Sternheim, Germany). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (Steinheim, Germany). Dulbecco's modified Eagle's medium was obtained from Life Technologies (Grand Island, NY, USA). For column chromatography, silica gel (230–400 mesh) was used, and TLC was monitored on a precoated silica gel 60 GF_{254} plates. Microbiological media was purchased from Hi-Media Laboratories Limited, Mumbai, India. The standard antibacterial agent chloramphenicol was purchased from Sigma-Aldrich. All other reagents were of analytical grade and other chemicals used in this study were of highest purity.

General Experiment Procedures

^{1}H and ^{13}C nuclear magnetic resonance spectroscopy (NMR) were recorded on a Bruker DRX 500 NMR instrument, Bruker, Rheinstetten, Germany at 500 MHz and 125 MHz, respectively. All spectra were recorded at room temperature in DMSO-*d6* solvent. Chemical shifts are given in parts per million (ppm) and coupling constants in hertz. High-resolution electrospray ionization mass spectroscopy data were measured using an electrospray ionisation mode of a Thermo Scientific Exactive Mass Spectrometer with ions given in *m/z*. The melting point of the pure compound was measured between 30 °C and 300 °C using a differential scanning calorimeter (DSC) with a Mettler Toledo DSC 822e instrument (Mettler-Toledo, Schcoerfenbach, Switzerland). UV-visible spectrum of the compounds was recorded on a Systronics double beam spectrophotometer 2201, India at room temperature (scanning range 190–800 nm). Optical rotation of the compounds was measured using a Rudolph Research Autopol III polarimeter (Hackettstown, NJ, USA) at 25 °C in acetone. The software used for the chemical structure drawing was Chemsketch Ultra, Toranto, Canada.

Bioactive Compound Producing Bacteria

Bacillus sp. N strain was isolated from the hemolymph of nematode infested *Galleria mellonella* larvae. The strain was identified as *Bacillus* sp. N strain (Accession No. CP001407) on the basis of 16S rDNA and BLAST analysis. The strain was currently deposited in Institute of Microbial Technology, Chandigarh, India, and the accession number is MTCC 5234.

Fermentation and Extraction

For isolation and identification of bioactive metabolites, a loopful culture of *Bacillus* sp. N strain was cultivated in nutrient broth (seed broth) (peptone 5.0 g/l, meat extract 1.0 g/l, yeast extract 1.0 g/l, NaCl 5.0 g/l, water 1000 ml) supplemented with 1% beef extract and incubated on a rotary shaker (250 rpm) at 30 °C. 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 dark for 96 h. The culture media were then centrifuged (10 000 *g*, 20 min, 4 °C) followed by filtration through a 0.45 μm filter, to obtain cell-free culture filtrate. The culture filtrates (25 l) obtained after cultivation of the strain in 1 l conical flasks for 96 h were extracted twice with ethyl acetate and concentrated to dryness under a vacuum at 35 °C.

Purification of Bioactive Compound

The oily yellow residue (2.1 g from 25 l) obtained after drying was then 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). Each fraction collected were separately analysized for antimicrobial activity.

Thin-layer Chromatography

Thin-layer chromatography was carried out on silica gel plates with benzene: acetone (60 : 40 v/v) as mobile phase. The spot was located by exposing the plate to iodine fumes.

Analytical High Performance Liquid Chromatography

High-performance liquid chromatography (HPLC) analysis of pure compound was carried out on 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.

Structural Elucidation

Apart from ^{1}H, ^{13}C, and DEPT-135 NMR experiments, 2D NMR sequences used in structural elucidation involve correlation spectroscopy, heteronuclear single quantum coherence, and heteronuclear multiple-bond correlation. Heteronuclear single quantum coherence information was utilized in transferring assigned numbers from carbon to proton. The integrations in ^{1}H NMR were verified by DEPT-135. Partial structures were formed by correlation spectroscopy sequence, which was utilized in creating substructures using heteronuclear multiple-bond correlation sequence.

Synthesis of Cyclo(D-Tyr-D-Phe) and Cyclo(L-Tyr-L-Phe)

Cyclo(L-Tyr-L-Phe) was synthesized from L-tyrosine methyl ester and L-phenyl alanine methyl ester (Sigma-Aldrich) and cyclo (D-Tyr-D-Phe) was synthesized from D-tyrosine methyl ester, and D-phenylalanine methyl ester (Sigma-Aldrich) by using the procedure reported by Tani *et al*. [12]. Purity of the compounds was determined by HPLC and TLC.

Acid Hydrolysis of the Compound

The absolute configuration of the amino acids in the compounds was determined using Marfey's FDAA (1-fluoro-2, 4-dinitrophenyl-5-L-alanine amide) derivatisation method [13]. A sample (1 mg) of compound was heated with 0.1 ml 6N HCl at 120 °C for 20 h. The hydrolysate was evaporated to dryness and dissolved in H_2O. Analytical HPLC analysis of the hydrolysate was carried out under isocratic conditions on Shimadzu LC-20AD, C-18 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.

Antibacterial Activity

Test microorganisms

Gram-positive bacteria includes the following: *Staphylococcus aureus* MTCC 902, *Staphylococcus epidermidis* MTCC 435; Gram-negative bacteria includes the following: *Escherichia coli* MTCC 2622, *Klebsiella pneumoniae* MTCC 109, *Proteus mirabilis* MTCC 425, *Vibrio cholerae* MTCC 3905, *Pseudomonas aeruginosa* MTCC 2642. All the test microorganisms were purchased from Microbial Type Culture collection Centre, Institute of Microbial Technology, Chandigarh, India. The test bacteria were maintained on nutrient agar slants.

Minimum inhibitory concentrations

The minimum inhibitory concentration of natural and synthetic compounds was determined by a broth dilution method [14,15]. The test bacterial strains were grown in nutrient broth to exponential phase at a concentration of 10^8 CFU/ml. Bacterial culture was diluted with NA broth to give a final concentration of 10^6 CFU/ml. Test compounds was dissolved in DMSO at a concentration of 1 mg/ml, which were diluted to 500, 250, 125, 64, 32, 16, 8, 4, 2, and 1 μg/ml. One hundred microlitre of each concentration was added into wells of 96 well plates containing 100 μl diluted bacterial suspension. MICs were determined from five independent experiments performed in duplicate. The wells containing 100 μl of bacterial inoculates were used as bacterial control. The plates were incubated at 30 °C for 24 h; inhibition of bacterial growth was determined by measuring the absorbance at 600 nm using an ELISA reader.

Agar disc diffusion assay

Agar disc diffusion assay of compounds were tested against seven strains of bacteria using standard disc diffusion assay [16]. All bacterial cultures were first grown in Mueller Hinton broth (18 h, 35 °C); Sterile 6 mm diameter circular discs of filter paper (Whatman No. 1) were loaded with MIC concentration of test compounds dissolved in methanol, evaporated to dryness, and then placed onto the bacterial lawn. Chloramphenicol was used as the positive controls. The agar plates were incubated for 24 h at 35 °C until bacteria had developed in a confluent film. The diameter of zone of inhibition (millimeter) between the discs was measured. The experiment was run in three replicates.

Antitumor and Cytotoxicity Activity

Cancer cell lines tested, source, and maintenance

The following cancer and normal cell lines were used in the study. (i) Breast cancer cell line (MDAM B-231); (ii) Cervical cancer cell line (HeLa); (iii) Lung cancer cell line (A549); (iv) Colon cancer cell line (HTL 116); and (v) factor signaling (FS) normal fibroblast. All cell lines were purchased from National Centre for Cell Science, Pune, India and maintained in DMEM supplemented with 10% FBS with antibiotics and antimycotics at 37 °C in a CO_2 incubator.

Determination of Cell Survival MTT Test

The MTT (3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) assay was used to determine the anticancer and cytotoxicity property of natural and synthetic DKP. Briefly, cells (5×10^3/well) 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) 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 (Bio-Rad ELISA reader 680, California, USA). The relative cell viability in percentage was calculated (A_{570} of treated sample / A_{570} of untreated sample× 100) [17].

Phase-contrast Microscopy

A549 and FS normal fibroblast cells were plated at a density of 2×10^4 cells/cm^2 into a 24 well plate. A549 was treated with 10 μM test compound, where as FS normal fibroblast was treated with 100 μM test compound for 48 h. Cells were viewed by phase-contrast light microscopy (Nikon, TMS, Japan) and photographs were taken using a Nikon camera (Japan).

Acridine Orange/Ethidium Bromide Staining

Morphological changes characteristic of apoptosis were assessed by fluorescent microscopy using acridine orange/ethidium bromide staining method. Briefly, A549 cells were seeded in 96 well plates and treated with test compound as in MTT assay, but for 24 h. After washing once with PBS, the cells were stained with 100 µl of a 1:1 mixture of acridine orange/ethidium bromide (4 µg/ml) solutions, immediately washed with PBS, and photomicrographed under a Nikon inverted fluorescent microscope (TE-Eclipse 300).

Cell Cycle Analysis

Flow cytometry

Distribution of a population of cells to the different stages of the cell cycle can be determined by the quantitative measurement of nuclear DNA content. Propidium iodide is a fluorescent dye that intercalates into the major groove of double-stranded DNA and excitation at 488 nm results in a broad emission centered around 600 nm. When added to a suspension of permeabilized single cells, propidium iodide incorporation will be determined by measuring total fluorescence emission from each cell using a flow cytometer.

The A549 cell lines (70% confluent) were treated with varying concentration of cyclo(D-Tyr-D-Phe) in DMEM medium for 48 h. The cells were trypsinized thereafter, washed twice with cold phosphate buffered saline (PBS), and centrifuged. The cell pellet was resuspended in 300 µl cold PBS to which ice cold ethanol (700 µl) was added and the cells were incubated for 1 h at 4 °C. The cells were centrifuged at 5000 rpm for 10 min; pellet washed twice with cold PBS, suspended in 250 µl PBS, and incubated with 100 mg/ml RNAase A for 30 min. The cells were chilled over ice for 10 min and incubated with propidium iodide (50 mg/ml) and analyzed by flow cytometry (BD Biosciences).

Caspase-3 activity assay

Caspase-3 activity in the A549 cell lysates was measured using a colorimetric assay kit by following the instructions from the manufacturer (Sigma-Aldrich). Cells were incubated with and without cyclo(D-Tyr-D-Phe) for 24 h. After the incubation period, the cells were collected by trypsinization and lysed using the lysis buffer provided in the kit (250 mM HEPES, pH 7.4 containing 25 mM CHAPS and 25 mM DTT). The assay was based on the hydrolysis of the peptide substrate acetyl-Asp-Glu-Val-Asp p-nitroaniline (Ac-DEVD-pNA) by caspase-3 resulting in the release of p-nitroaniline (pNA) moiety. The cell lysates were mixed with the substrate and read at 405 nm in a microplate reader (Bio-rad). The results were expressed as micromoles of pNA released per minute per milliliter.

Antioxidant Activity

DPPH (2′-2′ diphenyl–2′ picrylhydrazyl) radical scavenging assay

The free radical scavenging capacity of the natural and synthetic compounds was measured by the DPPH radical scavenging method of Yen and Chen [18] with slight modifications. The method involves the reaction of the test compound with the stable DPPH in 0.1 mM methanol solution. Briefly, the reaction mixture contained 300 µl of test compound of varying concentrations (10–100 µg/ml) and 2 ml of DPPH solution. After 10 min, the change in absorbance was recorded at 517 nm in a spectrophotometer against a blank, which did not contain the test compound. BHA was used as a positive control. The DPPH radical scavenging capacities were expressed as BHA antioxidant capacity in µg/ml of test compound.

The percent DPPH scavenging activity was calculated by the following equation:

$$\text{DPPH Scavenging Effect } (\%) = [(\text{A0}–\text{A1})/\text{A0} \times 100],$$

Where A0 is the absorbance of the control reaction and A1 is the absorbance in the presence of the extracts or standards. In order to calculate the IC_{50} value, which is the amount of sample necessary to decrease the absorbance of DPPH radical by 50%, the decolourization was plotted against the concentration of the test compound.

Reducing power assay

Reducing power of the natural and synthetic compounds was determined following the method of Oyaizu [19]. Briefly, to different concentrations of DKPs (1 ml) were added 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of potassium ferricyanide (1%). The reaction mixture was allowed to incubate at 50 °C for 20 min. Then 2.5 ml of trichloroacetic acid (10%) was added to the reaction mixture, which was then centrifuged at 9500 rpm for 10 min. The upper layer of solution (2.5 ml) was recovered and mixed with 2.5 ml distilled water and 2.5 ml $FeCl_3$ (0.1%). BHA was used as a positive control. The absorbance was recorded at 700 nm in a spectrophotometer. An increase in the absorbance of reaction mixture indicated the increased reducing power.

Results

Isolation of Antibacterial Compound

The ethyl acetate extract of the cell-free culture filtrate of the bacteria grown in modified nutrient broth medium supplied with 1% beef extract recorded significant antibacterial activity against *B. subtilis*, the initial test organism used to know whether the crude have antibacterial property or not. Isolation of the bioactive compounds from ethyl acetate extract by silica gel column chromatography yielded a white colored crystal compound which eluted at 70% ethyl acetate in CH_2Cl_2. After recrystallisation of compound over hexane/benzene (1:1), the bioactivity was shown to be retained against *B. subtilis*. In HPLC, compound was eluted with a retention time (R_t) of 5.121 [Figure 1(A)]. In TLC the compound possessed an R_f value of 0.44.

Structure Elucidation of the Active Compound

Based on the spectroscopic characteristics of ^1H and ^{13}C NMR spectra, the compound belong to cyclic dipeptide (DKP) and was identified as cyclo(D-Tyr-D-Phe) [Figure 1 (B)].

Cyclo(D-Tyr-D-Phe): (3S,6S)-3-benzyl-6-(4-hydroxybenzyl)piperazine-2,5-dione was obtained as a UV-absorbing white solid. Melting point of the compound is 175.03 °C, the optical rotation is $[\alpha]^{30}D$-85.3° (*c* 0.10, EtOH), and the UV max: 210 nm (MeOH). ^1H NMR (500 MHz, DMSO-δ6): 9.06 (1H, s), 7.67 (2H, br.s), 7.29 (2H, t, J = 7.5), 7.25 (1H, t, J = 7.5), 7.08 (2H, d, J = 7.0), 6.89 (2H, d, J = 8.5), 6.69 (2H, d, J = 8.5), 3.95 (1H, m), 3.99 (1H, m), 2.63 (1H, dd, J = 14.0, 7.0), 2.34 (1H, dd, J = 14.0, 7.0). ^{13}C NMR (125 MHz, DMSO-*d6*, ppm): 166.1(C), 165.9 (C), 154.7 (C), 136.8 (C), 131.6

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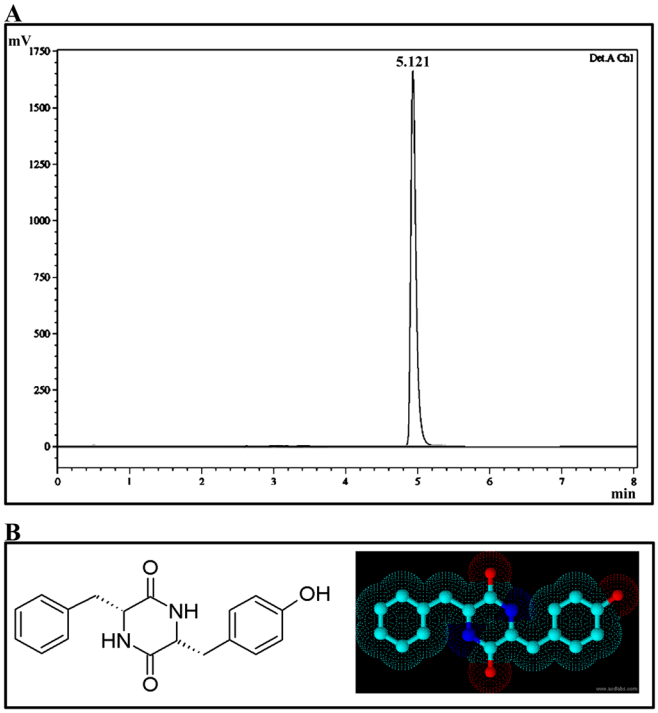

Figure 1. (A) High-performance liquid chromatography profile of cyclo (D-Tyr-D-Phe). (B) Structure of cyclo(D-Tyr-D-Phe.

(2CH), 129.1 (2 CH), 128.9 (2 CH), 126.7 (C), 126.4 (CH), 115.1 (2 CH), 55.9 (CH), 55.5 (CH), 39.3 (CH2), 37.86 (CH2) [Figure 2(A)]. The 2D ^{1}H–^{1}H and ^{1}H–^{13}C experiments allowed the assignment of two fragments to tyrosine and phenylalanine. Cyclo(D-Tyr-D-Phe) have a molecular formula of $C_{11}H_{19}O_5N_2$ as determined by high-resolution electrospray ionization mass spectroscopy ([M + H] at *m/z* 311.34719; calcd. 310.34712) [Figure 2(B)]. Thus, the structure of the compound is established as cyclo(D-Tyr-D-Phe).

Synthetic of Cyclo(L-Tyr-L-Phe) and Cyclo(D-Tyr-D-Phe)

The synthesis of cyclo(L-Tyr-L-Phe) and cyclo(D-Tyr-D-Phe) showed relatively good yields of 79 and 84%, respectively. Purity of the synthetic compounds was confirmed by HPLC and TLC. HPLC analysis yielded a single peak with retention times of 5.745 and 5.245 min for cyclo(L-Tyr-L-Phe) and cyclo(D-Tyr-D-Phe), respectively (Data not shown). TLC analysis showed single spot with R_f values of 0.51 and 0.44 for cyclo(L-Tyr-L-Phe) and cyclo(D-Tyr-D-Phe), respectively. NMR analysis confirmed the structures of the synthesized compounds corresponding to those proposed in Figure 3(A) and 3(B).

Determination of Absolute Configuration of Compounds

The modified Marfey's method was successfully applied to determine the absolute configuration of compounds. All the derivatized obtained from the hydrolysis of the test compounds were compared with the retention times of the derivatized standard D-amino and L-amino acids. The retention time (R_t, min) of HPLC analysis of FDAA derivatives of Tyr and Phe in the hydrolysate of natural cyclo(Tyr-Phe) were 20.514 and 30.562 min, respectively, and the retention time matched with that of authentic D-tyrosine and D-phenylalanine [Figure 4(A)]. Thus, the compound isolated from natural source was identified as cyclo (D-Tyr-D-Phe). In the similar way, the HPLC analysis of synthetic cyclo(L-Tyr-L-Phe) was also carried out and HPLC analysis of FDAA derivatives of Tyr and Phe in the hydrolysate of synthetic cyclo(L-Tyr-L-Phe) were 26.367 and 33.465 min, respectively, and the retention time matched with that of authentic L-tyrosine and L-phenylalanine [Figure 4(B)].

Antibacterial Activity

Antibacterial activity of natural cyclo(D-Tyr-D-Phe) and synthetic cyclo(L-Tyr-L-Phe) and cyclo(D-Tyr-D-Phe) is shown in Table 1 against the test organisms. The compounds were less effective as compared with commercially available antibacterial agent chloramphenicol. But against *S. epidermidis*, natural and synthetic cyclo(D-Tyr-D-Phe) recorded potent antibacterial activity than the chloramphenicol. An MIC of 1 μg/ml was exhibited by this natural and synthetic compound against *S. epidermidis*, a human pathogen responsible for causing infection in immunocompromised patients and also forms biofilms in plastic devices placed, catheters, or other surgical implants within the body. The zone of diameter of inhibition was determined by the MIC concentration of test compounds by paper disc diffusion method and the results are also shown in Table 1. In disc diffusion method, greater activity was recorded against *S. epidermidis* (33 mm) [Figure (5)]. But synthetic cyclo(L-Tyr-L-Phe) recorded lower activity. Among the reported cyclo(D-Tyr-D-Phe) dipeptide stereoisomers, to date, the antibacterial property was not pursued. Therefore, the present study exemplifies the first report on antibacterial evaluation of cyclo(D-Tyr-D-Phe) dipeptide class.

Antitumor and Cytotoxicity Activity

MTT assay

Cell viability assay of natural cyclo(D-Tyr-D-Phe) and synthetic cyclo(L-Tyr-L-Phe) and cyclo(D-Tyr-D-Phe) was determined by MTT assay after 72 h of treatment and are shown in Figure 6(A). We observed a dose-dependent growth inhibition in cell lines, when exposed to the natural and synthetic cyclo(D-Tyr-D-Phe) in the range, 1–100 μM. Natural and synthetic cyclo(D-Tyr-D-Phe) recorded similar pattern of activity and the best activity was recorded against A549 cell (IC_{50}–10 μM) [Figure 6(A) and 6 (B)]. Similar to antibacterial activity, synthetic cyclo(L-Tyr-L-Phe) recorded lower activity in cancer cell lines. More of these compounds recorded no cytotoxicity against human normal cell (FS normal fibroblast) up to 100 μM [Figure 6(C)].

As A549 cells turned out to be the most sensitive cell line (IC_{50}–10 μM) by both natural and synthetic cyclo(D-Tyr-D-Phe), it was selected for further studies by using its natural cyclo(D-Tyr-D-Phe) [Figure 6(A)].

Morphological Changes by Phase-contrast Microscopy

We compared the morphological effects of cyclo(D-Tyr-D-Phe) in A549 cells and FS normal fibroblasts. Cyclo(D-Tyr-D-Phe) induced nuclear membrane damage characteristic of apoptosis in A549 cells at 10 μM, no significant change was observed in normal fibroblast at 100 μM [Figure 7(A)].

Acridine Orange/Ethidium Bromide Staining

The morphological study showed chromatin condensation and blebbing in A549 [Figure 7(B)]. When A549 cells were stained

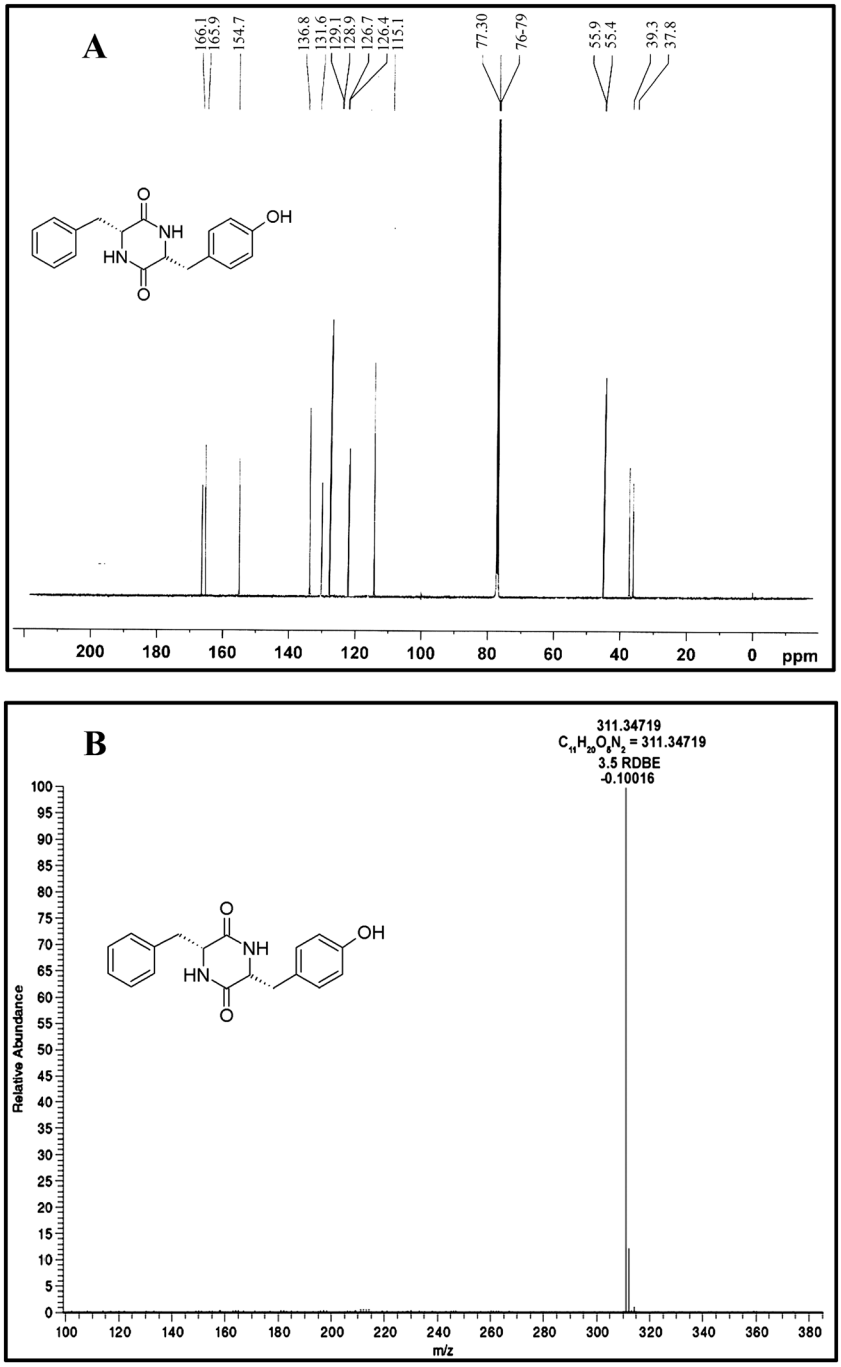

Figure 2. Spectral data of cyclo(D-Tyr-D-Phe). (A) ^{13}CNMR; and (B) HRMS.

with acridine orange/ethidium bromide, an increase of orange-stained cells with nuclear condensation was clearly observed in both A549 cell line. When A549 cell was treated with the different doses of the cyclo(D-Tyr-D-Phe), morphological changes of the cell was detected by acridine orange staining. Apoptotic cells showing DNA segmentation of nucleus were counted and inhibition of apoptosis was then calculated for the average of three independent determinations.

Cell Cycle Analysis

To explore whether the growth-inhibitory effect of cyclo(D-Tyr-D-Phe) on A549 cells is mediated through cell cycle arrest, we analyzed the distribution of cells in different phases of the cell cycle, by measuring intracellular DNA content in each phase. It was very interesting to note that cyclo(D-Tyr-D-Phe) failed to influence any stage of the cell cycle even at very high concentrations [Figure 7(C)]. However, there was an increase in the number of cells in sub G0 phase, when treated with cyclo(D-Tyr-D-Phe), again indicative of enhancement in apoptosis [Figure 7(C)]. The percentage of apoptotic cells (Sub G0) increased from 2.4% in control plates to 6.8% in cyclo(D-Tyr-D-Phe) treated with 25 μM, suggesting that cyclo(D-Tyr-D-Phe) causes apoptosis in A549 cells

Hence, our results clearly indicate that cyclo(D-Tyr-D-Phe) induces cytotoxicity in A549 cells through apoptosis and is independent of cell cycle arrest.

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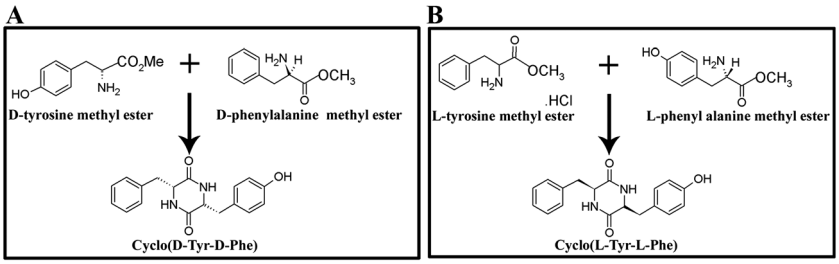

Figure 3. Synthesis of cyclo(Tyr-Phe). (A) Cyclo(D-Tyr-D-Phe); and (B) Cyclo(L-Tyr-L-Phe).

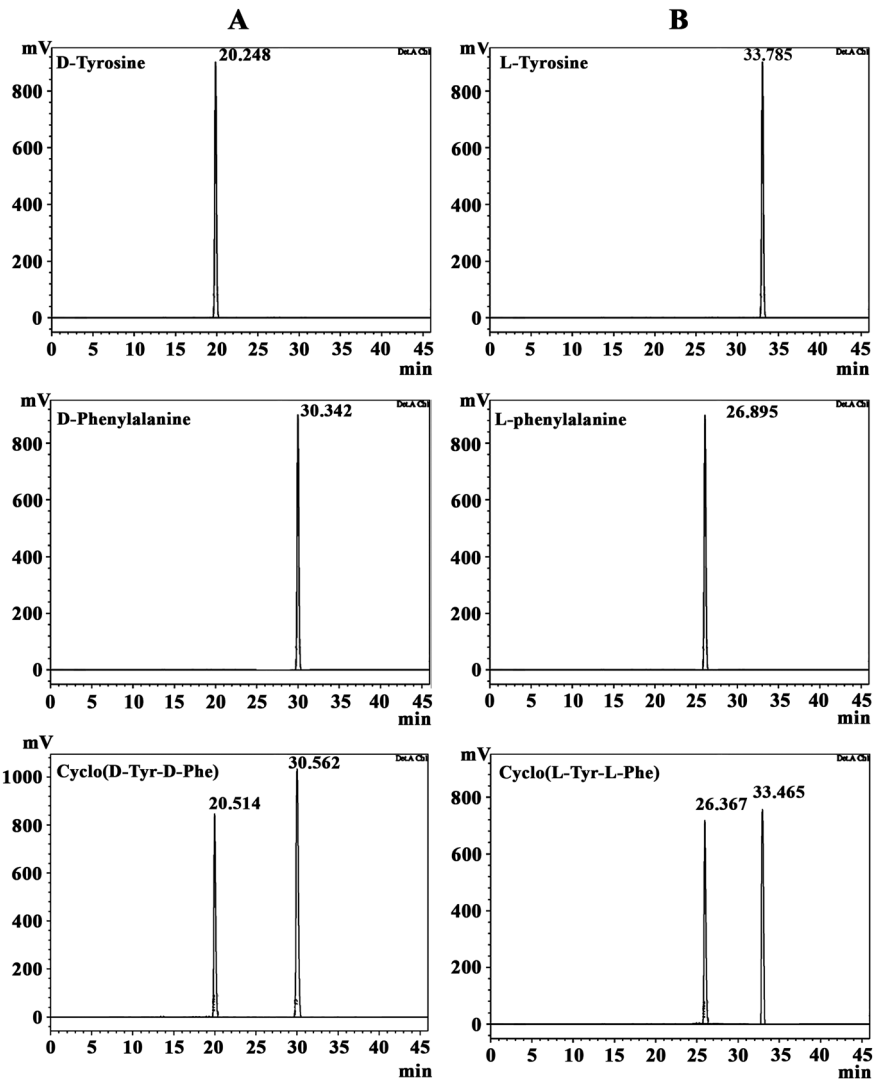

Figure 4. High-performance liquid chromatography profile of FDAA derivatives of the acid hydrolysates of cyclo(Tyr-Phe) and corresponding authentic amino acids. (A) Cyclo(D-Tyr-D-Phe); and (B) Cyclo(L-Tyr-L-Phe).

Caspase- 3

Caspase-3 is a key mediator in the apoptotic pathways because of its ability to cleave a vast array of proteins. Here, we have checked the activity of caspase-3 by using a colorimetric assay. A549 cell lysates of control and cyclo(D-Tyr-D-Phe) treated cells were prepared and incubated with a caspase-3 specific substrate (Ac-DEVD-pNA) with the reaction buffer and the release of pNA was measured at 405 nm in a spectrophotometer. Significant increase in the caspase-3 activity was observed in cyclo(D-Tyr-D-Phe)-treated conditions compared with control, indicating the involvement of caspase-3 in cyclo(D-Tyr-D-Phe)-induced cell death. The results are given in Figure 8.

Antioxidant Activity

DPPH radical scavenging activity

In the present study, cyclo(D-Tyr-D-Phe) was effective in reducing the stable DPPH radical to yellow-colored diphenylpicrylhydrazine,

Table 1. Antibacterial activity of natural and synthetic cyclo(Tyr-Phe) against test bacteria

Test bacteria	MIC (μg/ml)				Zone of inhibition (Diameter in mm)			
	1	2	3	Chloramphenicol	1	2	3	Chloramphenicol
B. subtilis	64	64	250	2	20 ± 1.12	20 ± 1.12	12 ± 0	28 ± 0
S. aureus	16	16	125	2	15 ± 0	15 ± 0	9 ± 1	29 ± 1
E. coli	64	64	—	1	13 ± 1	13 ± 1	—	29 ± 0
P. aeruginosa	32	32	—	2	17 ± 0	17 ± 0	—	30 ± 0
S. epidermis	1	1	64	4	33 ± 1.5	33 ± 1.5	22 ± 2.1	31 ± 1.15
K. pneumonia	4	4	125	1	30 ± 2.1	30 ± 2.1	21 ± 1.15	32 ± 0
P. mirabilis	2	4	64	2	21 ± 1	21 ± 1	13 ± 1	30 ± 0
V. cholerae	16	16	64	2	16 ± 1.15	16 ± 1.15	11 ± 0	30 ± 1

MIC, minimum inhibitory concentrations.
Values represent mean of three replications, (—) no MIC up to 1000 μg/ml.
1-Natural cyclo(D-Tyr-D-Phe), 2- Synthetic cyclo(D-Tyr-D-Phe), and 3-Synthetic cyclo(L-Tyr-L-Phe).

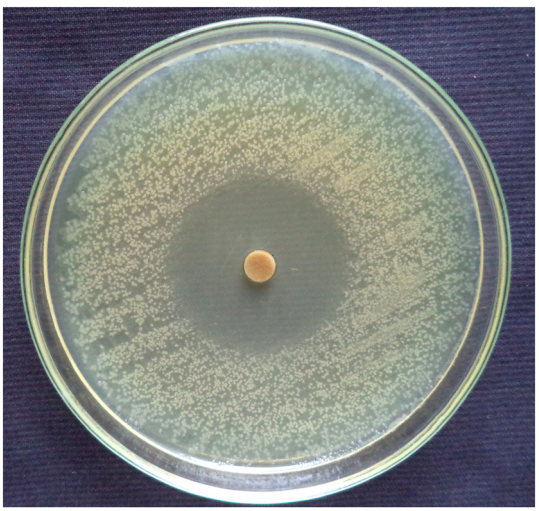

Figure 5. Antibacterial activity of cyclo(D-Tyr-D-Phe) against *S. epidermis* by disc diffusion assay.

indicating that this compound is active in DPPH radical scavenging [Figure 9(A)]. BHA and cyclo(D-Tyr-D-Phe) have almost similar scavenging activity. In this study, we expressed DPPH antioxidant capacity results by considering kinetic parameters by testing different initial concentrations (20–100 μg/ml) of the test samples. Even at 20 μg/ml, more than 50% of the activity was observed for the cyclo(D-Tyr-D-Phe) [Figure 9(A)].

Reducing power assay

Reducing power is based on the reduction of Fe^{3+} to Fe^{2+} in the presence of reductants present in the test DKPs. Figure 9(B) shows the relative reducing power of test DKPs. It is clear from the results that the BHA exhibited maximum reducing potential, followed by synthetic cyclo(L-Tyr-L-Phe). Whereas natural and synthetic cyclo(D-Tyr-D-Phe) recorded similar pattern of activity, which is lower than synthetic cyclo(L-Tyr-L-Phe). Reducing power assay is an important parameter used in evaluating antioxidant activities of natural compounds. It inhibits lipid peroxidation by donating a hydrogen atom, resulting in termination of free radical chain reaction.

Discussion

Diketopiperazines are forming a class of cyclic organic compounds that result from peptide bonds between two amino acids to form a bis-lactam. DKPs are the smallest cyclic peptides and commonly biosynthesized from amino acids in different macroorganisms and microorganisms. Although cyclic dipeptides are extensively obtained by extraction from natural sources, they may be easily prepared by conventional synthetic procedures, because of the relative structural simplicity of their essential nucleus [20].

Diketopiperazines are not only a class of naturally occurring privileged structures that have the ability to bind to a wide range of receptors, but they also have several characteristics that make them attractive scaffolds for drug discovery [21]. They are small, conformationally constrained heterocyclic scaffolds in which diversity can be introduced at up to six positions and stereochemistry controlled at up to four positions, and they are stable to proteolysis. They are important in drug discovery because they have a rigid backbone, which can mimic a preferential peptide conformation and contain constrained amino acids embedded within their structures without the unwanted physical and metabolite properties of peptides [22]. These three-dimensional molecular skeletons having diverse substituent groups overcome one of the limitations of conventional medicinal chemical agents, namely, the typical planarity found in most known pharmaceutical substances discovered through organic synthesis [21].

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 [23,24]. Cyclic dipeptides provide excellent models for theoretical studies as well as the development of new pharmaceutical compounds because of 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 [25]. DKPs have long been disregarded, however, more recently, they have received an increasing amount of attention in drug discovery [26]. They also were claimed to have many biological activities such as antitumour [27], antiviral [28], antifungal [29], antibacterial [30], and antihyperglycaemic [31] agents.

Most Gram-negative bacteria, but also Gram-positive bacteria, fungi, and higher organisms are able to produce DKPs [32,33]. *B. subtilis* [34] and several unidentified *Bacillus* species [35,36]

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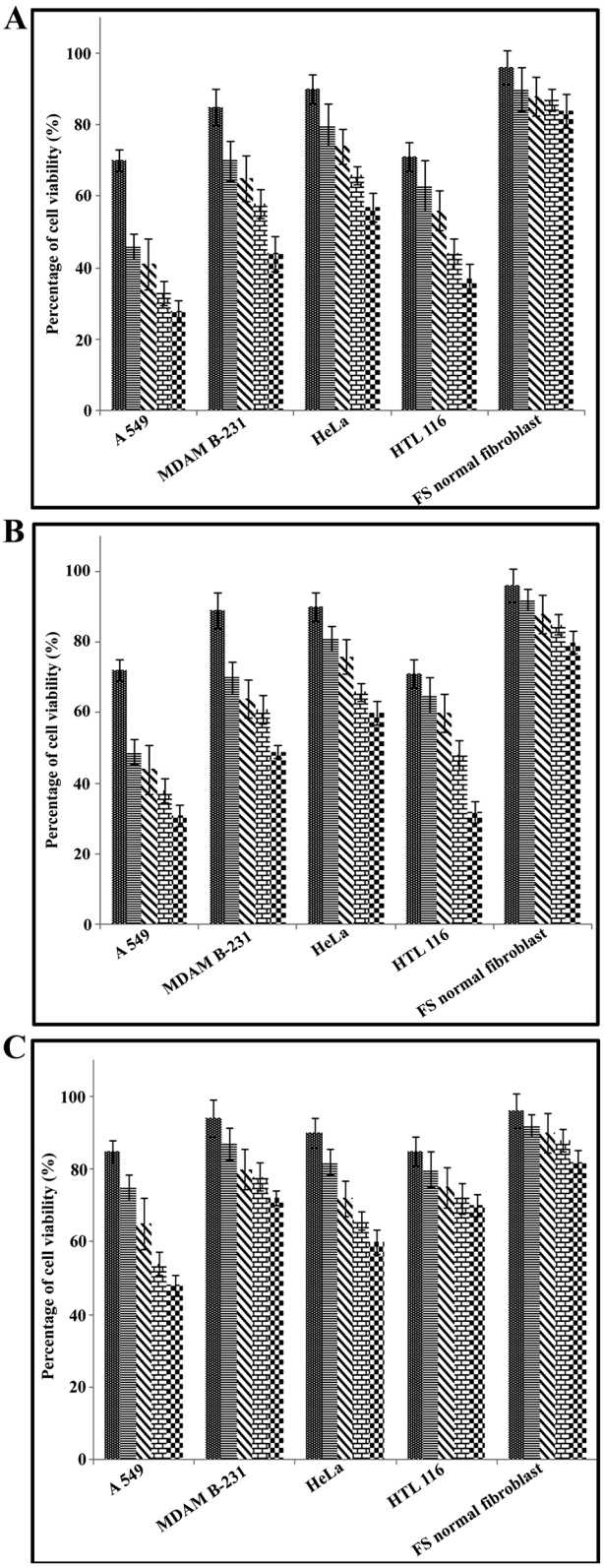

Figure 6. MTT assay of cyclo(Tyr-Phe) against human cancer and normal cell lines. (A) Natural cyclo(D-Tyr-D-Phe); (B) Synthetic cyclo(D-Tyr-D-Phe); and (C) Synthetic cyclo(L-Tyr-L-Phe). All the measurements were done in three replicates and results are expressed as arithmetic mean ± standard error on the mean.

were found to produce several DKPs. Recently, we have reported six antimicrobial proline based DKP from LB and TSB ethyl acetate extract of *Bacillus* sp. [36,37]. The production of cyclo (D-Tyr-D-Phe) was previously reported from marine *Bacillus subtilis* [34]. The antimicrobial activity of various cyclic dipeptides has been reported earlier [37–40]. The antimicrobial activity of cyclo (D-Tyr-D-Phe) is also reported for the first time.

The antibiotic bicyclomycin is a DKP, and structure activity studies revealed that the unique nature of this compound that was finally developed for clinical applications and bicyclomycin is the only known selective inhibitor of Rho. Rho is a member of the RecA-type ATPase class of enzymes that couple oligonucleotide translocation to ATP hydrolysis Bicyclomycin inhibition the secondary RNA binding site of transcription termination factor Rho, a hexameric RNA/DNA helicase/transcolase that terminates the translocation of selected gene in bacteria [41]. Inhibition of Rho is a novel mechanism of action an antimicrobial drugs and our compound is structurally different from bicylomycin. Thus, the action of our compound may be through a novel route and this should be elucidated in future. Another cyclic peptide bactenecin exhibited stronger binding to LPS and induced perturbation of the inner membrane of *Burkholderia pseudomallei*. Interaction of bactenecin with model membranes resulted in changes in correlates with efficient membrane permeabilization membrane fluidity and permeability, leading to leakage of dye across the membrane at levels [42].

The roles of many proteins and peptides have been identified as novel targets in cancer therapy, allowing for the design of more selective agents [43]. Milne *et al*. [44] observed the effects of cyclo(Phe-Pro), cyclo(Tyr-Pro), and cyclo(Trp-Trp) on undifferentiated human colon carcinoma cells (HT-29). These compounds significantly enhanced the expression of the biochemical differentiation markers and retarded the growth of these cells. Graz *et al*. [45] observed the ability of the DKPs to induce differentiation of neoplastic cells to non-neoplastic cells as well as stimulating faster recovery of these cells through histone acetylation and phosphorylation while allowing the recovery of normal cells. Our results showed that, cyclo(D-Tyr-D-Phe), exhibited the highest cytotoxicity to A549 cells. The growth inhibition exhibited by cyclo(D-Tyr-D-Phe) was shown to be dose dependent. Our results also show, for the first time, that cyclo (D-Tyr-D-Phe) induces apoptotic cell death in A549 cells. The apoptotic programme is characterized by particular morphological features such as chromatin condensation and nuclear fragmentation [46]. Leist and Jäättelä [47] go further to associate these morphological features with that of apoptosis observed when caspases, particularly caspase-3, are activated, that is, caspase-dependent programmed cell death or apoptosis in its most classic form. In our study, also caspase-3 has activated and this clearly indicated that cyclo(D-Tyr-D-Phe) is caspase-dependent cell death. Ideal anticancer drugs would eradicate cancer cells without harming normal tissues while evading the mechanisms of drug resistance. Similar in our study, also cyclo(D-Tyr-D-Phe) kill cancer cells without killing the normal cell (FS normal fibroblast cells). The anticancer activity of the cyclo(D-Tyr-D-Phe) is never reported earlier and to our best knowledge this is first report of the anticancer activity of this compound.

The antioxidant activity of DKP were reported for cyclo(His-Pro) [48] and cyclo(Cys-Leu) [49]. In the present study, cyclo(D-Tyr-D-Phe) exhibited significant radical scavenging activity and the activity is almost equal to the activity of BHA. The ability of the cyclo(D-Tyr-D-Phe) compounds to act as antioxidants depends

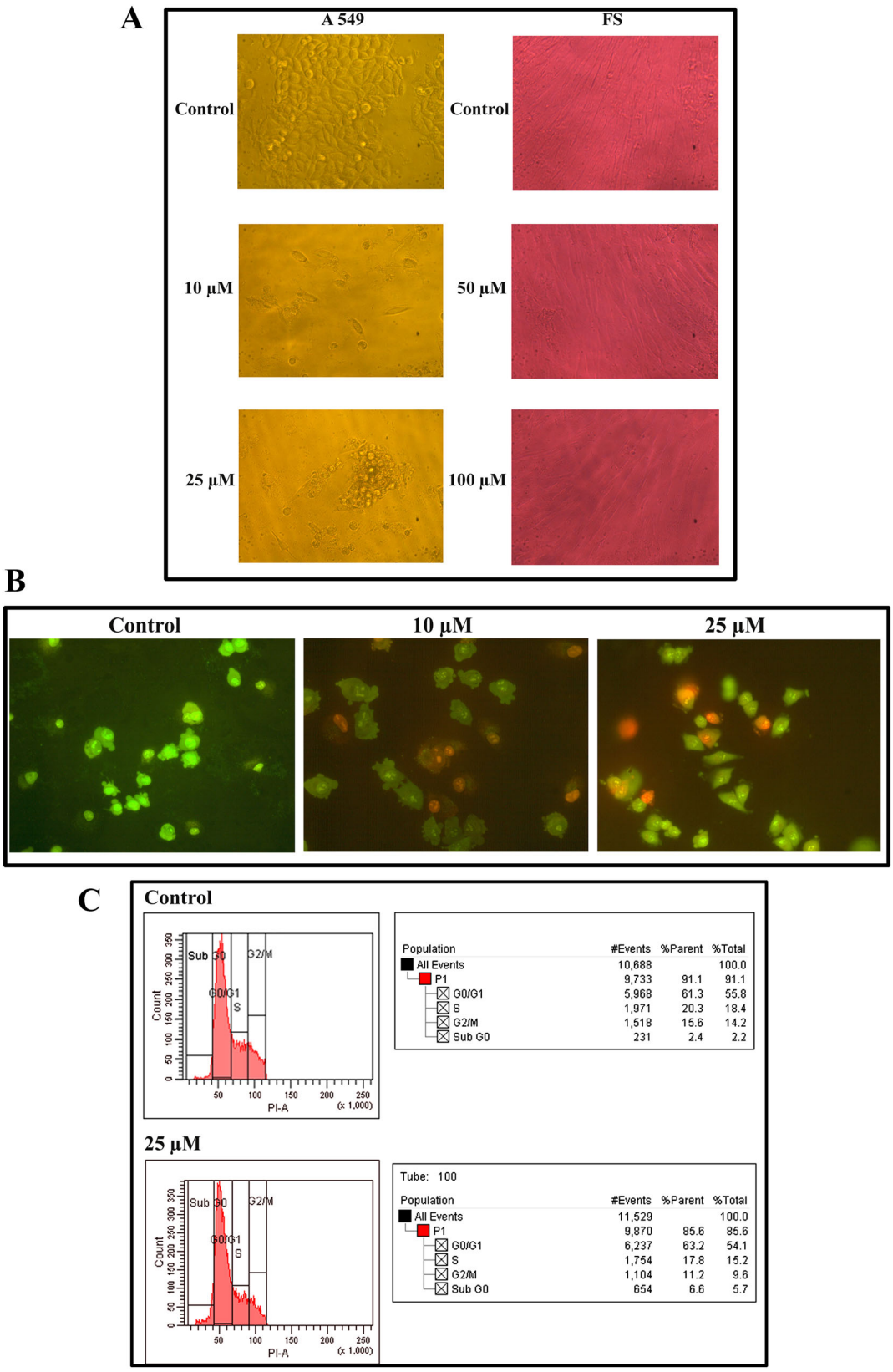

Figure 7. Effect of cyclo(D-Tyr-D-Phe) on A549 cells. (A) Phase-contrast light microscopy image of A549 and factor signaling cells. (B) Acridine orange/ethidium bromide staining of A549 cells to detect apoptosis: Live cells were observed as green, whereas the apoptotic cells as orange-red because of co-staining with ethidium bromide because of loss of membrane integrity. Magnification 40×. (C) Effect of cyclo(D-Tyr-D-Phe) against A549 on cell cycle. Representative histograms on the left-hand panel indicate the percentages of cells in G1, S, G2/M, and sub-G0 phases of the cell cycle. The total cell populations and the populations in different phases are given the right-hand panel (in table). The percentage of cells with sub-G0 DNA content was taken as a measure of the apoptotic cell population. The data provided are representatives of three independent experiments.

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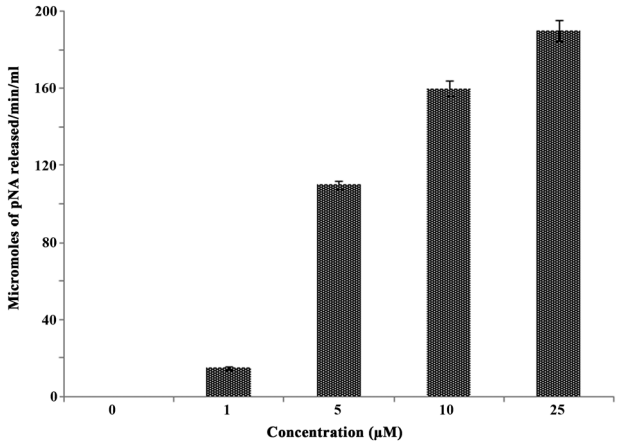

Figure 8. Effect of cyclo(D-Tyr-D-Phe) on caspase 3 release in A549 cells. Activity was determined in the lysates of control and treated cells using colorimetric assay. Values presented were the mean ± SD of three independent experiments.

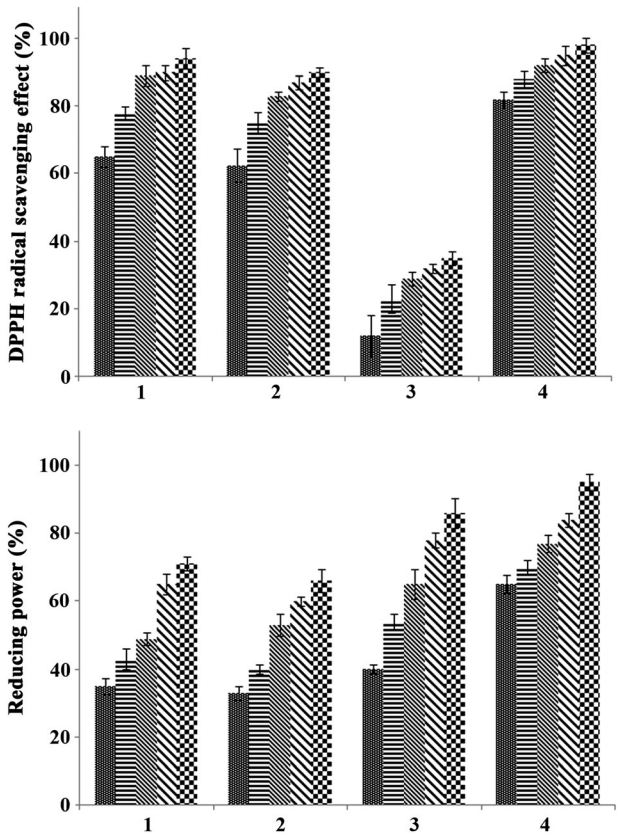

Figure 9. Antioxidant activity of cyclo(D-Tyr-D-Phe). (A) Free radical scavenging; and (B) reducing power assay. All the measurements were carried out in three replicates, and results are expressed as arithmetic mean ± standard error on the mean.

on the redox properties of their hydroxyl groups present in the structure and the potential for electron delocalization across the chemical structure. To the best of the knowledge and from the cited literature, this is the first time that the antioxidant activity of cyclo(D-Tyr-D-Phe) has been reported.

Various phenylalanine containing cyclic dipeptide like cyclo (L-Phe-L-Pro), cyclo(D-Phe-D-Pro), cyclo(D-Phe-L-Pro), cyclo(L-Phe-2-OH-Pro), cyclo(L-Phe-L-trans-4-OH-Pro), and cyclo(Gly-L-Phe) are reported to have various bioactivity [50–53]. Similarly, tyrosine containing cyclic peptides like cyclo(L-Pro-L-Tyr), cyclo (D-Pro-D-Tyr), cyclo(L-Pro-D-Tyr), cyclo(L-Pro-L-Tyr), cyclo(Gly-L-Tyr), cyclo(D-4-OH-Pro-L-Tyr), cyclo(glycyl-L-tyrosyl)-4,4-dimethylallyl ether are reported to have bioactivity [54–56]. In the aforementioned phenylalanine and tyrosine containing cyclic dipeptide, D-amino acids containing cyclic dipeptides recorded more bioactivity than the L-amino acids. Similarly our compound contains both D-tyrosine and D-phenylalanine, which may be one of the reasons of enhanced bioactive when compared with the corresponding enantiomer.

The DKPs are heterocyclic compounds that exist in several stereoisomeric forms and also in DD, DL, and LL forms. The stereochemistry appears to be important for biological activity. Antibiotic activities of DD-DKPs and DL-DKPs against *Vibrio anguillarum* were found where cyclo(D-Pro-D-Val) 17 had a MIC of 0.05 μg/ml and cyclo (D-Pro-L-Val) of 0.11 μg/ml [57]. The LL-enantiomer was also described in the literature and tested against other bacteria than *Vibrio anguillarum* showing weak activity against *S. aureus* and *Micrococcus luteus* [58]. The authors concluded that because of the three DKP enantiomers (LL, DL, and DD) the DD-enantiomer showed the highest antibiotic activity D-amino acids are more active than their L-enantiomers. In the present study, cyclo (D-Tyr-D-Phe) is more bioactive than cyclo(L-Tyr-L-Phe) and this may be because of the chirality changes of the tyrosine and phenylalanine that make the change of binding orientation of cyclo (Tyr-Phe) against the target. Compared with cyclo(L-Tyr-L-Phe), cyclo(D-Tyr-D-Phe) make a different spatial orientation that may make tyrosine side chain containing hydroxyl group pointing toward the active site, giving an enhanced bioactivity. However, the different binding orientation of cyclo(L-Tyr-L-Phe) means that the tyrosine is no longer able to make a hydrogen bond to a water molecule. This may be one of the reasons of enhanced activity of D-isomer. The higher the activity of cyclo(D-Tyr-D-Phe) suggests that the different orientation of the compounds could play some role. Stereochemistry play an important role in bioactive has been previously reported on the inhibition of *Bacillus* sp. chitinase by cyclo(L-Arg-L-Pro) and cyclo(L-Arg-D-Pro), where cyclo(L-Arg-D-Pro) is more active due to the presence of D-proline in active site [59]. But comparing other D-DKPs and L-DKPs reported in the literature, no clear connection between the enantiomers and the biological activities can be deduced. A systematic search for all possible combinations of amino acids and their enantiomers could shed more light on the antimicrobial potential of these simple dipeptides.

Conclusions

The DKP cyclo(D-Tyr-D-Phe) is a new biologically active natural product, isolated from the culture filtrate of *Bacillus* sp. N strain. To the best of our knowledge, cyclo(D-Tyr-D-Phe) has not been isolated from any other natural source. In our hands, cyclo (D-Tyr-D-Phe) recorded prominent antibacterial activity against test bacteria. The antibacterial activity of cyclo(D-Tyr-D-Phe) is also reported for the first time. Our results also showed that cyclo (D-Tyr-D-Phe) significantly inhibits the growth of A549. Furthermore cyclo(D-Tyr-D-Phe) induced apoptotic cell death in A549 cells, suggesting the potential to inhibit the growth of tumors *in vivo*. The detailed anticancer activity of cyclo(D-Tyr-D-Phe) is also reported for the first time. Finally, we have compared the

biological activity of cyclo(D-Tyr-D-Phe) with cyclo(L-Tyr-L-Phe). The data clearly show that cyclo(D-Tyr-D-Phe) is more active than its cyclo(L-Tyr-L-Phe). The reason behind this may be because of the spatial orientation of the compound against target site and the exact reason behind this is still unknown. The comparative study of the antimicrobial, anticancer, and antioxidant activity of stereoisomer cyclo(Tyr-Phe) is also reported for the first time. Our findings warrant further investigation into the effects of cyclo(D-Tyr-D-Phe) and related DKPs in the context of cancer chemoprevention or chemotherapy in humans. Further assessment is required to determine precise intracellular or extracellular targets and the mechanism of action by which cyclo(D-Tyr-D-Phe) induces apoptosis.

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