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Three bioactive cyclic dipeptides from the *Bacillus* sp. N strain associated with entomopathogenic nematode

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ABSTRACT

In continuation of our search for new bioactive secondary metabolites from *Bacillus cereus* associated with entomopathogenic nematode (EPN), three cyclic dipeptides (CDPs), cyclo(L-Leu-D-Arg) (1), cyclo(2-hydroxy-Pro-L-Leu) (2), and cyclo(L-Val-L-Pro) (3) were purified from the ethyl acetate extract of *B. cereus*. The chemical structure of the compounds was identified by 1D, 2D NMR and HR-ESI-MS. Cyclo(L-Leu-D-Arg) recorded best antifungal activity and the highest activity was recorded against *Cryptococcus neoformans* (1 μg/mL), which is better than the standard antifungal agent amphotericin B. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used for finding cell proliferation inhibition and cyclo(L-Leu-D-Arg) recorded significant activity against breast cancer cell line (MDAM-B231) (IC_{50} value: 25 μM) and the three cyclic dipeptides recorded no toxicity against normal human cell (fore skin (FS) normal fibroblast) up to 50 μM except cyclo(L-Val-L-Pro). Cyclo(L-Leu-D-Arg) induced significant morphological changes and DNA fragmentation associated with apoptosis in MDAM-B231 cells by acridine orange/ethidium bromide staining and flow cytometry analysis. Out of three cyclic dipeptides tested only cyclo(2-hydroxy-Pro-L-Leu) recorded significant antioxidant activity. The hydroxyl radical scavenging activity of cyclo(2-hydroxy-Pro-L-Leu) is greater than BHA, the standard antioxidant agent. Cyclo(L-Leu-D-Arg) was isolated for the first time from a natural source with a D-arginine residue. To the best of our knowledge, this is the first time that the bioactivity of the isolated cyclic dipeptides is reported against medically important fungi and cancer cells. This study is a significant contribution to the knowledge of cyclo(L-Leu-D-Arg) from *B. cereus* as potential sources of new drugs in the pharmacological industry, especially as potent antifungal and anticancer agent.

1. Introduction

Q3 At the beginning of the 20th century, bacterial epidemics were a global and important cause of mortality. In contrast, fungal infections were almost not taken into account. Since the late 1960s when antibiotic therapies were developed, a drastic rise in fungal infections was observed, and they currently represent a global health threat to human being [38]. In the present decade fungal infections range from superficial conditions of the skin (e.g. ringworm and athlete's foot) and nails (onychomycoses) to disseminate life threatening diseases. Serious invasive fungal infections caused by *Candida* spp., *Cryptococcus neoformans*, *Aspergillus* spp., *Pneumocystis carinii* and *Histoplasma capsulatum*, represent an increasing threat to human health. Despite modern antifungal therapy, the mortality rates for invasive infections with the three most common species of human fungal pathogens are *Candida albicans*, 20–40% [19]; *Aspergillus fumigatus*, 50–90% [19]; and *C. neoformans*, 20–70% [29]. This increasing incidence of infection is influenced by the growing number of immunodeficient cases related to AIDS, cancer, old age, diabetes, cystic fibrosis, organ transplants and other invasive surgical procedures [38]. The prevalence of these systemic fungal infections has increased significantly during the past decade. Unfortunately, our repertoire of antifungal agents is limited, particularly in comparison to the number of agents available for fungal infections. Thus the need of new antifungal agents is needed to compete against the fungal infections.

Cancer continues to be one of the major causes of death worldwide and only modest progress has been made in reducing the morbidity and mortality of this disease [13]. Current estimates from the American Cancer Society and from the International Union against cancer indicate that 12 million cases of cancer were diagnosed very year, with 7 million deaths worldwide; these numbers are expected to double by 2030 (27 million cases with 17 million deaths) [1]. Nature is an attractive source of new therapeutic

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candidate compounds as a tremendous chemical diversity is found in millions of species of plants, animals, marine organisms and microorganisms as potential anti-cancer agents [6,28].

During our studies on entomopathogenic nematodes (EPN), we isolated a new EPN *Rhabditis* sp. from sweet potato weevil grubs collected from Central Tuber Crops Research Institute (CTCRI) farm, Thiruvananthapuram [9]. A specific bacterium was also isolated from the haemolymph of nematode infested *Galleria mellonella* larvae and was found to be pathogenic to a number of insect pests [9]. Molecular analyses revealed that the bacterium resembles *Bacillus* sp. Bacteria of the genus *Bacillus* are known for their prolific production of diverse metabolites with a variety of biological activities [31]. In this article, we describe the isolation, structure elucidation and absolute configuration of a new antifungal cyclic dipeptide cyclo(L-Leu-D-Arg) from bacterial natural source along with two known cyclic dipeptides, using extensive spectroscopic analyses. The antifungal, anticancer and antioxidant activity of the cyclic dipeptides were also studied. The taxonomic study of the antifungal produced strain has been described in previous reports [31].

2. Materials and methods

2.1. Test fungal culture

Medically important fungi *Cryptococcus neoformans* (MTTC 1347), *Aspergillus fumigatus* (MTCC 3376), *Cryptococcus gastricus* (MTCC 1715), *Candida albicans* (MTCC 3017), *Trichophyton rubrum* (MTCC 296), *Candida tropicalis* (MTCC 184) and *Aspergillus flavus* (MTCC 873) were used in the present study. The fungus was grown on potato dextrose medium at 28 °C and maintained with periodic sub-culturing after every 15 days.

2.2. General experimental procedures

HR-ESI-MS data were recorded using electrospray ionization mode of a Thermo Scientific Extractive Spectrometer with ions given in m/z. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR spectra were measured on a Bruker DRX 500 spectrometer (Bruker, Rheinstetten, Germany) in DMSO-δ_6 and CD_3OD. The chemical shifts are expressed in δ values with TMS as an internal standard. ^{1}H–^{1}H COSY, HSQC, and HMBC were obtained by conventional methods. UV–vis spectrum of the compounds was recorded on a Systronics double beam spectrophotometer (model 2201) at room temperature (scanning range 190–800 nm). Optical rotation of the compounds was measured using a Rudolph Research Autopol III polarimeter at 25 °C in acetone. The software used for the chemical structure drawing was Chemsketch Ultra (Toronto, Canada).

2.3. Bioactive metabolite producing bacteria and growth conditions

The *B. cereus* was isolated from 3rd stage infective juveniles of the nematode sample collected from sweet potato weevil grubs or from the haemolymph of nematode infested *G. mellonella* larvae. The strain was identified as *B. cereus* (Accession No. CP001407) based on 16S rRNA and BLAST analysis and deposited in IMTECH (Institute of Microbial Technology, Chandigarh, India) and the accession number is MTCC 5234. The mature slant culture of *B. cereus* was inoculated into Erlenmeyer flasks (250 mL), containing 50 mL of seed medium consisting of 1% peptone, 0.5% yeast extract, 1.0% dextrose, 0.5% NaCl and 0.5% $CaCO_3$ (pH 7) after autoclaving. The flasks were cultivated on a rotary shaker at 150 rpm for 18 h at 30 °C. The seed culture was inoculated into Erlenmeyer flasks (1 L), containing 400 mL of the production medium. The fermentation medium contained the same ingredients as the seed medium. The cultivation was carried out for 96 h at 30 °C on a shaking incubator.

2.4. Isolation of cyclic dipeptides from the culture broth of B. cereus

After 96 h of fermentation at 30 °C, the culture broth was centrifuged. The supernatant fluid was extracted twice with ethyl acetate. The ethyl acetate layer was dried over Na_2SO_4 and evaporated to dryness under reduced pressure yielding 2.4 mg of extract, which was dissolved in chloroform and chromatographed on a silica gel 60 column (230–400 mesh) with 200 mL of linear gradient consisting of hexane and dichloromethane (v/v, 75:25 to 25:75), 200 mL of dichloromethane, 200 mL of linear gradient of dichloromethane and chloroform (v/v, 90:10 to 10:90), 200 mL of chloroform. About 32 fractions measuring 100 mL were collected and concentrated using rotary evaporator.

2.5. Thin-layer chromatography (TLC)

TLC of the pure cyclic dipeptides was carried out on silica gel plates (Merck 60, F_{254}) using a mixture of benzene and acetone (70:30, v/v). The spots were located by exposing the TLC plate to iodine fumes.

2.6. Reverse phase-high pressure liquid chromatography (RP-HPLC)

Pure cyclic dipeptides were analyzed on an LC-10AT liquid chromatography (LC; Shimadzu, Singapore) equipped with a C-18 column (5 μm, 4.6 mm × 250 mm) using the following gradient program: solvent A, water; solvent B, MeCN; linear gradient 0 min 50% B, 15 min 75% B, 25 min 100% B; UV detection at 210 nm with a flow rate of 1 mL/min.

2.7. Acid hydrolysis of compounds

Compounds (1 mg) each were dissolved in 0.1 mL 6 mol/L HCl and hydrolyzed at 110 °C for 20 h. The hydrolysate was evaporated to dryness and dissolved in H_2O 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 mol/L $NaHCO_3$ (40 μL). The reaction mixture was heated at 47 °C for 1 h, cooled to room temperature, and then acidified with 2.0 mol/L HCl (20 μL). In a similar fashion, standard D- and L-amino acids were derivatized separately. RP-HPLC of the hydrolysate used an Shimadzu LC-20AD, C-18 column (5 μm, 4.6 mm × 250 mm) and a flow rate of 1.0 mL/min at 30 °C using an eluent system consisting of 0.2% aqueous TFA (eluent A) and MeCN (eluent B). A linear gradient from 25% eluent B (0 min) to 60% eluent B (40 min) and to 100% eluent B (45 min) was applied. Absorbance was recorded at 340 nm [23].

2.8. Antifungal assay

2.8.1. Minimum inhibitory concentration (MIC)

MICs were determined by using Broth micro dilution method [8]. A stock solution of 2 g/L of the test compound was prepared, which was further diluted with DMSO to give the required concentrations from 1 mg/mL to 1 μg/mL and then was added to broth media of 96-wells of microtiter plates using twofold serial dilution. Thereafter 100 μL inoculum of standard size (1×10^5) was added to each well. Fungal suspension alone without compounds was used as negative control, while broth containing standard drug was used as positive control. The microtiter plates were incubated at 27 ± 2 °C. The MIC values were taken as the lowest concentration

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of the extracts in the well of the microtiter plate that showed no turbidity after incubation. The turbidity of the wells in the microtiter plate was interpreted as visible growth of microorganisms. Each experiment was repeated thrice.

2.8.2. Minimum fungicidal concentration (MFC)

Minimum fungicidal concentration (MFC) was determined by the microdilution method in culture broth as recommended by NCCLS with low modifications. Minimum fungicidal concentration (MFC) was determined by adding 50 μl from the tubes that did not show growth in MIC to 150 μl of freshly prepared potato dextrose broth and incubating at 35 °C for 48 h. The MFC was regarded as the lowest concentration of test sample which fungus failed to grow in potato dextrose broth inoculated with 50 μl of suspension. All tests were performed in triplicates [3,27].

2.8.3. Agar disk diffusion assay

In vitro antifungal activity of the compounds was measured using agar disk diffusion assay for fungi [12]. The sterile disks were impregnated with MIC concentration of test compounds. The amphotericin B was used as positive reference standards. The antimicrobial activity was evaluated by measuring the zone of growth inhibition surrounding the disks. All the assays were carried out in triplicate.

2.9. Anticancer activity

2.9.1. Cell lines

The breast cancer cell line (MDAM-B231), cervical cancer cell line (HeLa), lung cancer cell line (A549), skin cancer cell lines (A431), colon cancer cell line (HTL 116) and normal human cell (FS normal fibroblast) were obtained from the National Centre for Cell Science, Pune, India. Both cancer cell lines were maintained in the recommended RPMI-1640 medium supplemented with 10% heat-inactivated (56 °C) fetal bovine serum (FBS), L-glutamine (3 mM), streptomycin (100 mg/mL), penicillin (100 IU/mL), and 25 mM HEPES and adjusted to pH 7.2 by bicarbonate solution. Cells were grown in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C.

2.9.2. Determination of cytotoxicity of cyclic dipeptides in cancer and normal cell line

2.9.2.1. MTT assay. The MTT assay was used to determine the cytotoxicity (IC_{50}) in FS normal fibroblast cells at concentrations of 5–100 μg/mL. After 72 h of exposure, viability was assessed on the basis of cellular conversion of MTT into a formazan product [2]. Absorbance (*A*) at 570 nm was measured 24 h later. To get cell survival (%), *A* of a sample with cells grown in the presence of various concentrations of the investigated extracts was divided with control optical density (the *A* of control cells grown only in nutrient medium), and multiplied by 100. It was implied that *A* of the blank was always subtracted from *A* of the corresponding sample with target cells. IC_{50} concentration was defined as the concentration of an agent inhibiting cell survival by 50%, compared with a vehicle-treated control. All experiments were done in triplicate.

2.9.3. Morphological studies using phase-contrast microscopy

MDAM-B231 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 was treated with 100 fibroblast 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).

2.9.4. Apoptotic studies using acridine orange/ethidium bromide staining

Morphological changes characteristic of apoptosis were assessed by fluorescent microscopy using acridine orange/ethidium bromide staining method. Briefly, cells were seeded in 96-well plates and treated with test samples 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).

2.9.5. Cell cycle analysis using flow cytometry

Cell cycle analysis helps in distinguishing the distribution of a population of cells in the various stages of cell cycle. Briefly, cells were treated with cyclic dipeptide for 24 h or 48 h followed by trypsinization. The cell pellets were fixed in 70% ice-cold ethanol, treated with 100 mg/mL RNAase A and 50 mg/mL propidium iodide, followed by flow cytometry analysis (BD Biosciences).

2.10. Antioxidant activity

2.10.1. DPPH (2′-2′-diphenyl-2′-picrylhydrazyl) radical scavenging assay

The free radical scavenging capacity of three cyclic dipeptides was measured by the DPPH radical scavenging method of Yen and Chen [39] with slight modifications. The method involves the reaction of cyclic dipeptides with the stable DPPH in 0.1 mM methanol solution. Briefly, the reaction mixture contained 300 μL of test compound of varying concentrations (20–100 μM) 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. Butylated hydroxyanisole (BHA) was used as a positive control. The % DPPH scavenging activity was calculated by the equation:

$$\text{DPPH Scavenging Effect}\ \ (\%) = \left[\frac{A0 - A1}{A0} \times 100\right],$$

where *A*0 is the absorbance of the control reaction and *A*1 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 stilbene.

2.10.2. Hydroxyl radical-scavenging activity

The hydroxyl radical scavenging activity of the peptide was measured by the deoxyribose method [32]. The reaction mixture contained 0.45 mL of 0.2 M sodium phosphate (pH 7.4), 0.15 mL of 10 mM 2-deoxyribose, 0.15 mL of 10 mM $FeSO_4$–EDTA, 0.15 mL of 10 mM hydrogen peroxide, 0.525 mL of distilled water and 0.075 mL (20–100 μM) of cyclic dipeptides in a tube. The reaction was started by the addition of hydrogen peroxide. After incubation at 37 °C for 4 h, the reaction was stopped by adding 0.75 mL of 2.8% (w/v) trichloroacetic acid (TCA) and 0.75 mL of 1.0% (w/v) of thiobarbituric acid (TBA). The mixture was boiled for 10 min, cooled in ice and then measured at 520 nm. The reaction mixture not containing test sample was used as control. BHA was used as standard for comparison purposes.

$$\text{Hydroxyl radical scavenging activity}\ \ (\%) = 1 - \left(\frac{\text{absorbance of sample}}{\text{absorbance of blank}}\right) \times 100$$

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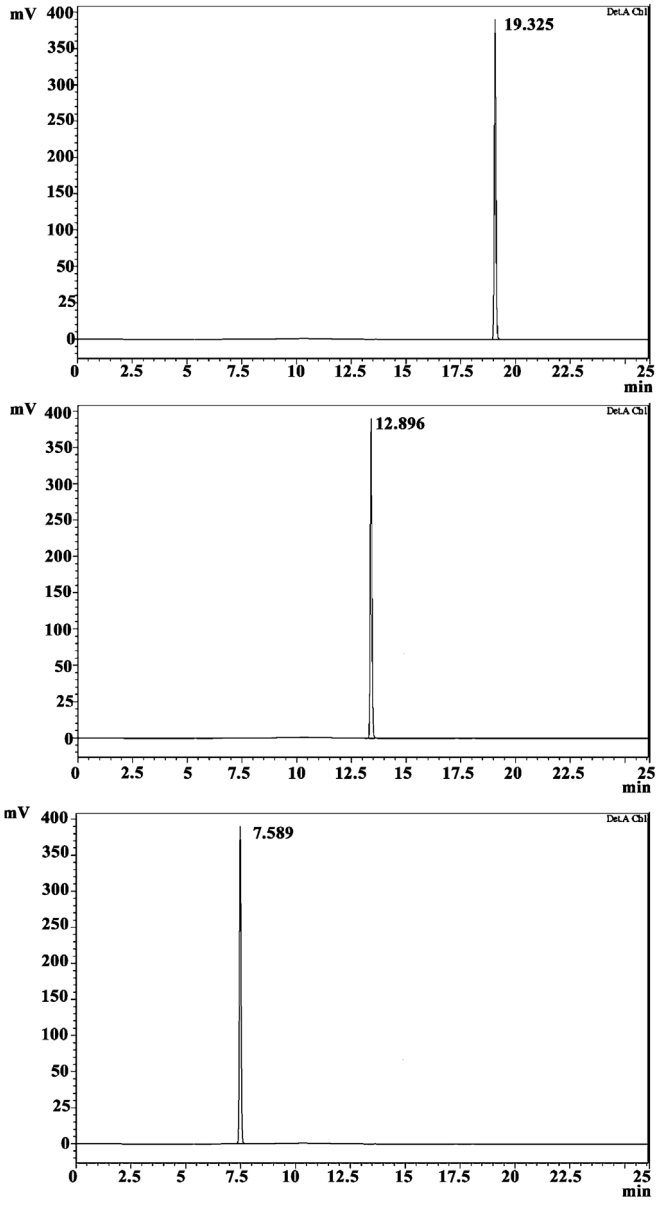

Fig. 1. HPLC profile of cyclic dipeptides. (A) Cyclo(L-Leu-D-Arg), (B) cyclo(2-hydroxy-Pro-L-Leu) and (C) cyclo-(L-Pro-L-Val).

3. Results

3.1. Purification and structure elucidation of bioactive compound

Bioassay-guided fractionation of the crude ethyl acetate extract yielded three compounds (1–3). Purity of the compounds was established on the basis of TLC profile. In TLC profile the compounds 1–3 possessed refractive indices (R_f) of 0.38, 0.26 and 0.35, respectively. HPLC analysis of the three compounds was performed by reverse phase and compounds were eluted as single peaks (Fig. 1). The purity of the compounds reached greater than 95% according to the peak area and the compounds have a retention time (R_t) of 19.325, 12.896 and 7.589 min, respectively (Fig. 1).

Based on 1D and 2D NMR and mass spectrometry the compounds were identified as cyclic dipeptides (diketopiperazines) (CDP 1–3). The first two compounds were identified as cyclo(L-Leu-D-Arg) (1) and cyclo(2-hydroxy-Pro-L-Leu) (2) (Fig. 2) which had not been isolated from natural products earlier. Furthermore, one known cyclic dipeptide cyclo(L-Pro-L-Val) (3) (Fig. 2) were also isolated and purified.

CDP 1: Cyclo(L-Leu-D-Arg): 1-{3-[(2*R*,5*S*)-5-(2-methylpropyl)-3,6-dioxopiperazin-2-yl]propyl}guanidine was obtained as a yellowish solid with UV-absorbing. Optical rotation of the compound was $[\alpha]_D^{30}$ +89.3° (*c* 0.10, EtOH). Yield of the compound is 29 mg. In DMSO, the ^{13}C and HSQC spectra showed 11 carbon signals. From the ^{13}C data, it was possible to elucidate two carbonyl groups (δ_c 170.8 and 167.0), three sp^3-hybridised carbons having an electronegative heteroatom (δ_c 59.0, 53.1 and 45.3), four sp^3-hybridised carbon (δ_c 38.3, 27.9, 24.6 and 22.9) and two methyl groups (δ_c 23.3 and 22.4). The 2D ^1H–^1H and ^1H–^{13}C experiments permitted the assignment of two fragments to leucine and arginine. The presence of a NH (δ_H 7.96) in ^1H NMR and observed HMBC correlations helps to identify compound 1 as cyclo(L-Leu-D-Arg). The molecular formula of this compound was determined to be $C_{12}H_{23}O_2N_5$ by HR-ESI-MS at *m*/*z* 270.2235 [M+H].

Cyclo(L-Leu-D-Arg): ^1H NMR (DMSO-d_6, 500 MHz) δ 7.96 (1H, br s, Arg-NH), δ 4.19 (1H, dd, *J* = 8.1, 8.0 Hz, Arg-H2), δ 4.01 (1H, dd, *J* = 6.9, 5.9 Hz, Leu-H2), δ 3.41 (1H, m, Arg-H5), δ 3.28 (1H, m, Arg-H5), δ 2.13 (1H, m, Arg-H3), δ 1.92 (1H, m, Arg-H3), δ 1.90 (2H, m, Leu-H4), δ 1.79 (2H, m, Arg-H4), δ 1.77 (1H, m, Leu-H3), δ 1.37 (1H, m, Leu-H3), δ 0.88 (3H, d, *J* = 7.0 Hz, Leu-H5), δ 0.87 (3H, d, *J* = 7.0 Hz, Leu-H50); ^{13}C NMR (DMSO-d_6, 125 MHz) 170.8 (C, Arg-C1), 167.0 (C, Leu-C1), 59.0 (CH, Arg-C2), 53.1 (CH, Leu-C2), 45.3 (CH_2, Arg-C5), 38.3 (CH_2, Leu-C3), 27.9 (CH_2, Arg-C3), 24.6 (CH, Leu-C4), 23.3 (CH_3, Leu-C5), 22.9 (CH_2, Arg-C4) and 22.4 (CH_3, Leu-C5).

CDP (2): Cyclo(2-hydroxy-Pro-L-Leu): (3*S*)-8a-hydroxy-3-(2-methylpropyl)hexahydropyrrolo[1,2-*a*]pyrazine-1,4-dione was obtained as a white solid. Optical rotation of the compound was $[\alpha]_D^{30}$ −119.3° (*c* 0.10, EtOH). Yield of the compound is 23 mg. The ^{13}C NMR spectrum displayed the characteristic structure of a diketopiperazines ring system, which included two amide carbonyl signals at d 168.3 and 167.2 and a methine signal at d 54.9. The ^1H NMR spectrum clearly indicated the presence of leucine residue, which included two methyl signals at d 0.87 (3H, d, *J* = 6.3 Hz, H-12) and 0.90 (3H, d, *J* = 6.3 Hz, H-13); a methylene signal at d 1.77 (1H, m, H-10a) and 1.55 (1H, m, H- 10b); and two methine signals at d 3.66 (1H, m, H-3) and 1.77 (1H, m, H-11). And it also displayed the characteristic structure of a proline residue, which included three methylene signals at d 3.42 (2H, m, H-9), 2.01 (1H, m, H-8a), 1.77 (1H, m, H-8b), and 2.01 (2H, m, H-7). The HMBC correlations of hydroxyl proton with C-5 (d 167.8) and the chemical shift value of C-6 (d 86.2) suggested the presence of a hydroxyl attached to C-6. The molecular formula was determined to be $C_{11}H_{18}O_3N_2$ by HR-ESI-MS at *m*/*z* 227.1210 [M+H]. On the basis of the above data, compound 2 was determined as cyclo(2-hydroxy-Pro-L-Leu).

Cyclo(2-hydroxy-Pro-L-Leu): ^1H NMR (DMSO-d_6, 500 MHz) δ 3.66 (1H, m), δ 8.33 (1H, d, *J* = 4.2), δ 2.01 (2H, m)a, δ 1.77 (1H, m), δ 2.01 (1H, m), δ 3.42 (2H, m), δ 1.55 (1H, m), δ 1.77 (1H, m)a, δ 1.77 (1H, m), δ 0.87 (3H, d, *J* = 6.3), δ 0.90 (3H, d, *J* = 6.3), δ 6.56 (1H, br s); ^{13}C NMR (DMSO-d_6, 125 MHz) 167.7 (C2), 55.4 (C3), 167.8 (C5), 86.2 (C6), 36.5 (C7), 19.2 (C8), 44.9 (C9), 44.6 (C10), 23.9 (C11), 21.6 (C12), 23.1 (C13).

CDP (3): Cyclo(L-Val-L-Pro): (3*S*,8a*S*)-3-(propan-2-yl)hexahydropyrrolo[1,2-*a*]pyrazine-1,4-dione was obtained as a white amorphous solid. Optical rotation of the compound was $[\alpha]_D^{30}$ −79.3° (*c* 0.10, EtOH). Yield of the compound is 20 mg. HR-ESI-MS at *m*/*z* 197.9 [M+H].

Cyclo(L-Val-L-Pro): ^1H NMR (500 MHz, $CDCl_3$) d 0.92 (3H, d *J* = 7.6 Hz, CH_3), 1.07 (3H, d *J* = 7.3, CH_3), 1.87–1.95 (1H, m, H-4a), 2.00–2.11 (1H, m, H-4b), 2.35–2.41 (2H, m, H-5), 2.59–2.67 (1H, m, H-10), 3.51–3.68 (2H, m, H-3), 3.94 (1H, s, H-9), 4.08 (1H, t *J* = 7.6 Hz, H-6), 5.99 (1H, br s, N-H). ^{13}C NMR (100 MHz, $CDCl_3$) d 169.71 (s, C-1), 45.07 (t, C-3), 22.33 (t, C-4), 28.48 (t, C-5), 60.27 (d, C-6), 164.60

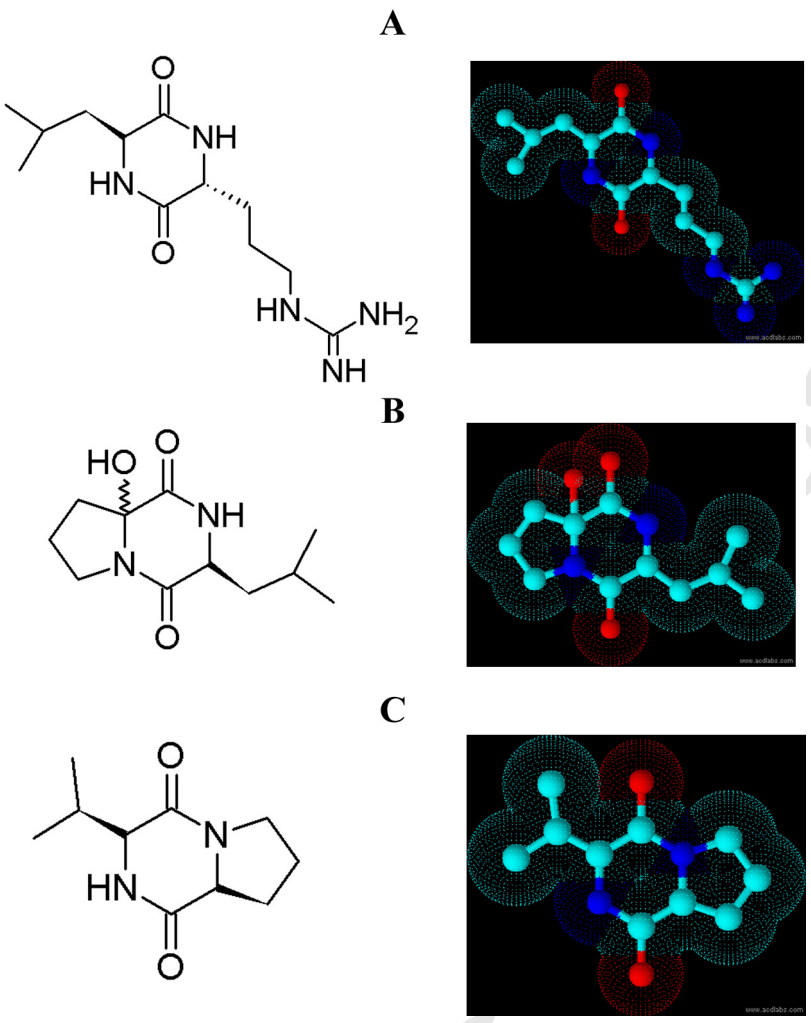

Fig. 2. Structure of cyclic dipeptides. (A) Cyclo(L-Leu-D-Arg), (B) cyclo(2-hydroxy-Pro-L-Leu) and (C) cyclo-(L-Pro-L-Val).

(s, C-7), 58.72 (d, C-9), 28.32 (d, C-10), 19.22 (q, C-11) and 16.03 (q, C-110).

HPLC analysis of the CDP 1-hydrolysate indicated the presence of leucine and arginine and retention time were 40.344 and 18.453 min, respectively. This retention time matches with the retention time of standard L-leucine and D-arginine (Fig. 3). HPLC analysis of the CDP 3 hydrolysate contains both L-proline and L-valine. The retention times (R_t, min) of the corresponding amino acids matched those of authentic L-proline (17.45) and L-valine (16.12), and were clearly different from the respective enantiomers. The stereochemistry of leucine in the CDP 2 was determined as L-configuration. However, the stereochemistry at 2-hydroxyproline was not able to determined by Marfey's method because the hydroxyproline residue decomposed under acidic conditions.

3.2. Antifungal activity

CDPs recorded significantly good activity against test fungi (Table 1). CDPs showed significantly higher activity against medically important fungi, *viz. T. rubrum, C. albicans, C. tropicalis* and *C. gastricus*. Highest activity of 1 μg/mL was recorded by CDP 1 against *C. neoformans* followed by *C. tropicalis* (Table 1). CDP 1 was more active than the standard antifungal agent amphotericin B, especially against *C. neoformans*. Infection with *C. neoformans* is termed

Table 1
MIC of diketopiperazines against test fungi.

Test fungi	μg/mL							
	CDP 1		CDP 2		CDP 3		Amphotericin B	
	MIC	MFC	MIC	MFCC	MIC	MFC	MIC	MFC
Cryptococcus neoformans	1	1	32	64	125	129	2	2
Aspergillus fumigatus	16	16	64	64	–	–	4	8
Trichophyton rubrum	4	8	32	32	125	250	8	8
Candida albicans	8	16	32	64	250	500	2	4
Candida tropicalis	2	4	16	16	–	1–	4	4
Cryptococcus gastricus	16	16	32	64	–	–	16	32
Aspergillus flavus	32	32	64	64	–	–	125	125

–, no activity up to 1000 μg/mL.

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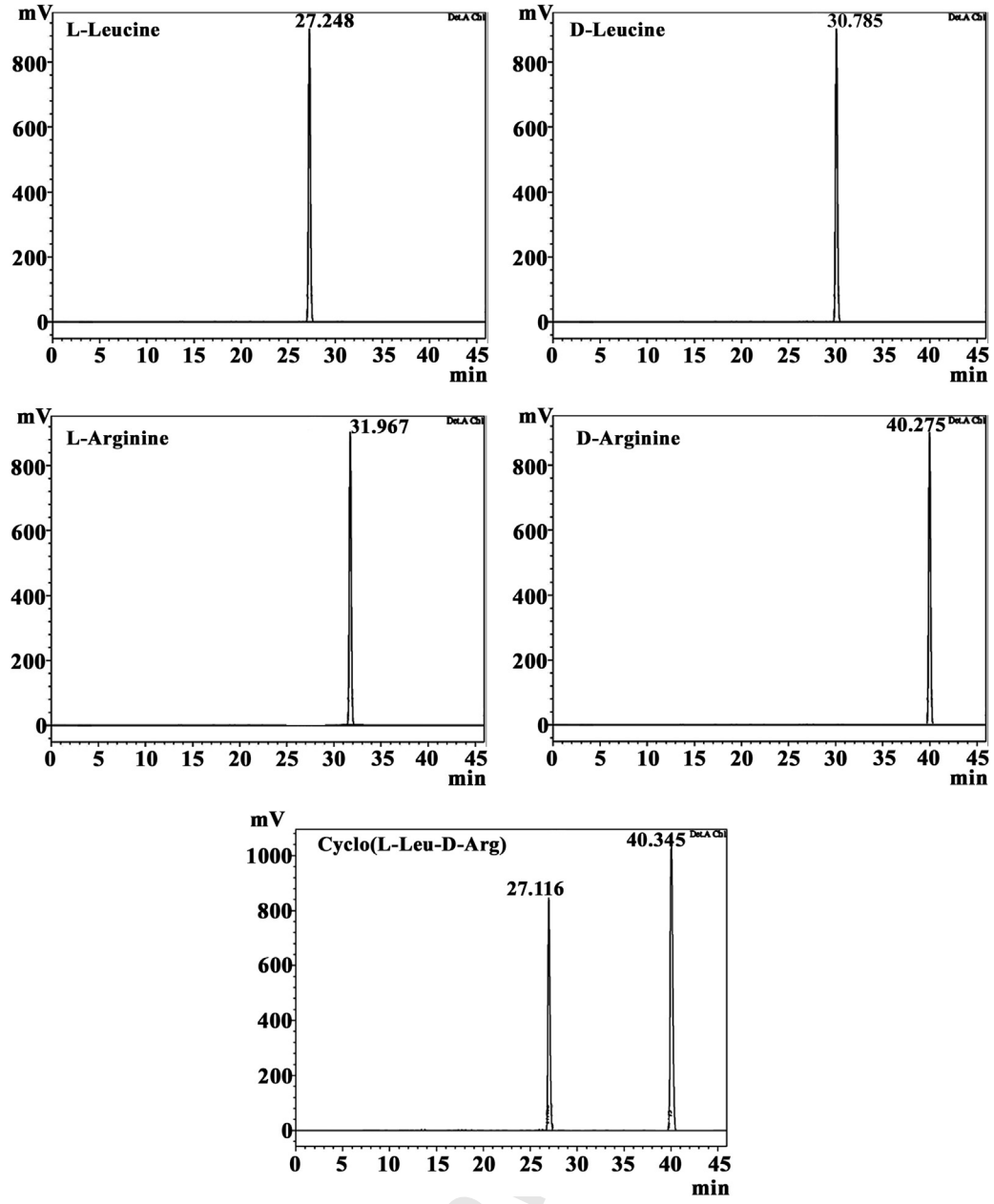

Fig. 3. HPLC profile of FDAA derivatives of the acid hydrolysates of cyclo(L-Leu-D-Arg) and standard L and D amino acids.

cryptococcosis. Most infections with *C. neoformans* consist of a lung infection. However, fungal meningitis and encephalitis, especially as a secondary infection for AIDS patients, are often caused by *C. neoformans* making it a particularly dangerous fungus. In the present study CDP 1 recorded activity against *C. neoformans* in impressive low concentration, which will help to develop a new antifungal agent based on cyclic dipeptides in near future. Disk diffusion assay of the CDPs using the MIC concentration of the

Table 2
Antifungal activity of diketopiperazines.

Test fungi	Zone of inhibition (dia. in mm)			
	Compound 1	Compound 2	Compound 3	Amphotericin B
Cryptococcus neoformans	29 ± 0	19 ± 0	12 ± 0	25 ± 0
Aspergillus fumigatus	27 ± 1	20 ± 0	–	20 ± 0.52
Trichophyton rubrum	26 ± 0.52	18 ± 0.52	16 ± 1	24 ± 1.15
Candida albicans	22 ± 1.15	16 ± 1.15	14 ± 0.52	25 ± 0.52
Candida tropicalis	21 ± 1.5	21 ± 0.52	–	28 ± 1.15
Cryptococcus gastricus	25 ± 0.52	18 ± 1.15	–	20 ± 0
Aspergillus flavus	21 ± 1.15	16 ± 1.5	–	18 ± 0.52

–, not tested.

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compounds showed that CDP 1 recorded best activity (Table 2). For this compound *C. neoformans* recorded best activity (29 mm) followed by *A. fumigatus* (27 mm).

3.3. Anticancer activity

3.3.1. Cytotoxicity test

Cell viability assay of cyclic dipeptides was determined by MTT assay after 72 h of treatment. We observed a dose-dependent growth inhibition in cell lines, when exposed to the cyclic dipeptides in the range, 1–100 μM. Cyclo(L-Leu-D-Arg) recorded best cytotoxicity followed by cyclo(2-hydroxy-Pro-L-Leu). Cyclo(L-Leu-D-Arg) recorded best activity against MDAM-B231 (IC_{50} – 25 μM) followed by A549 (IC_{50} – 50 μM) (Fig. 4A). Cyclo(2-hydroxy-Pro-L-Leu) also recorded activity against MDAM-B231 (IC_{50} – 100 μM) (Fig. 4B). The three diketopiperazines are nontoxic to FS normal fibroblast cell up to 50 μM (Fig. 4C). But mild toxicity was observed at 100 μM for CDP 3. As MDAM-B231 cells turned out to be the most sensitive cell line (IC_{50} – 10 μM), it was selected for further studies (Fig. 4A).

3.3.2. Morphological changes by phase-contrast microscopy

We compared the morphological effects of cyclo(L-Leu-D-Arg) in MDAM-B231 cells and FS normal fibroblasts. Cyclo(L-Leu-D-Arg) induced nuclear condensation and membrane damage characteristic of apoptosis in MDAM-B231 cells at 25 μM, but no significant change in the morphology was observed in normal fibroblast up to 100 μM (Fig. 5).

3.3.3. Acridine orange/ethidium bromide (AO/EB) staining

The AO/EB staining recorded chromatin condensation and blebbing in MDAM-B231 (Fig. 6A). When MDAM-B231 cells were stained with AO/EB, an increase of orange-stained cells with nuclear condensation was clearly observed in MDAM-B231 cells. When MDAM-B231 cell was treated with the different doses of the cyclo(L-Leu-D-Arg), 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.

3.3.4. Cell cycle analysis

Cell cycle analysis showed that apoptosis induced by cyclo(L-Leu-D-Arg) is not associated with a cell cycle arrest since there was no accumulation of cells in any phase of cell cycle, following cyclo(L-Leu-D-Arg) treatment (Fig. 6B). An increase in the percentage of cells in sub G0 phase from 1.2% to 40.2% at 24 h upon cyclo(L-Leu-D-Arg) treatment provides clear evidence about the induction of apoptosis in MDAM-B231 cells.

Hence our results clearly indicate that cyclo(L-Leu-D-Arg) induces cytotoxicity in MDAM-B231 cells through apoptosis and is independent of cell cycle arrest.

3.4. Antioxidant activity

3.4.1. DPPH radical scavenging assay

Out of three CDPS tested only cyclo(2-hydroxy-Pro-L-Leu) showed the highest antioxidant activity in the DPPH radical scavenging model (Fig. 7A). Cyclo(2-hydroxy-Pro-L-Leu) had excellent DPPH radical scavenging activity (84% at 100 μM) which was nearer to the BHA value of 94.19% at 100 μM.

3.4.2. Hydroxyl radical-scavenging activity

Similar to DPPH assay cyclo(2-hydroxy-Pro-L-Leu) recorded significant hydroxyl radical scavenging activity. The cyclo(2-hydroxy-Pro-L-Leu) had strongest hydroxyl scavenging activity of

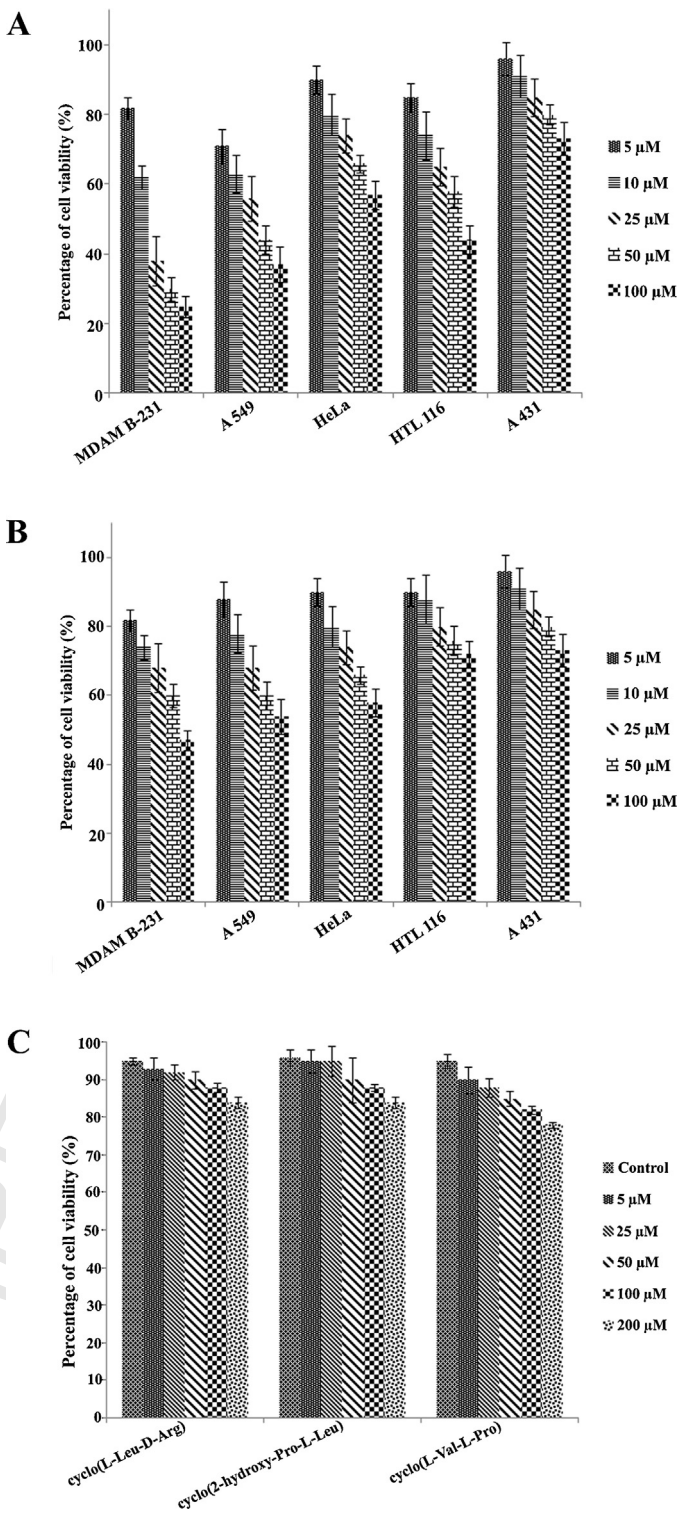

Fig. 4. MTT assay of cyclic dipeptides against human cancer and normal cell lines. All the measurements were done in three replicates and results are expressed as arithmetic mean ± standard error on the mean. (A) Cyclo(L-Leu-D-Arg), (B) cyclo(2-hydroxy-Pro-L-Leu), (C) MTT activity of the cyclic dipeptides against FS normal fibroblast cells.

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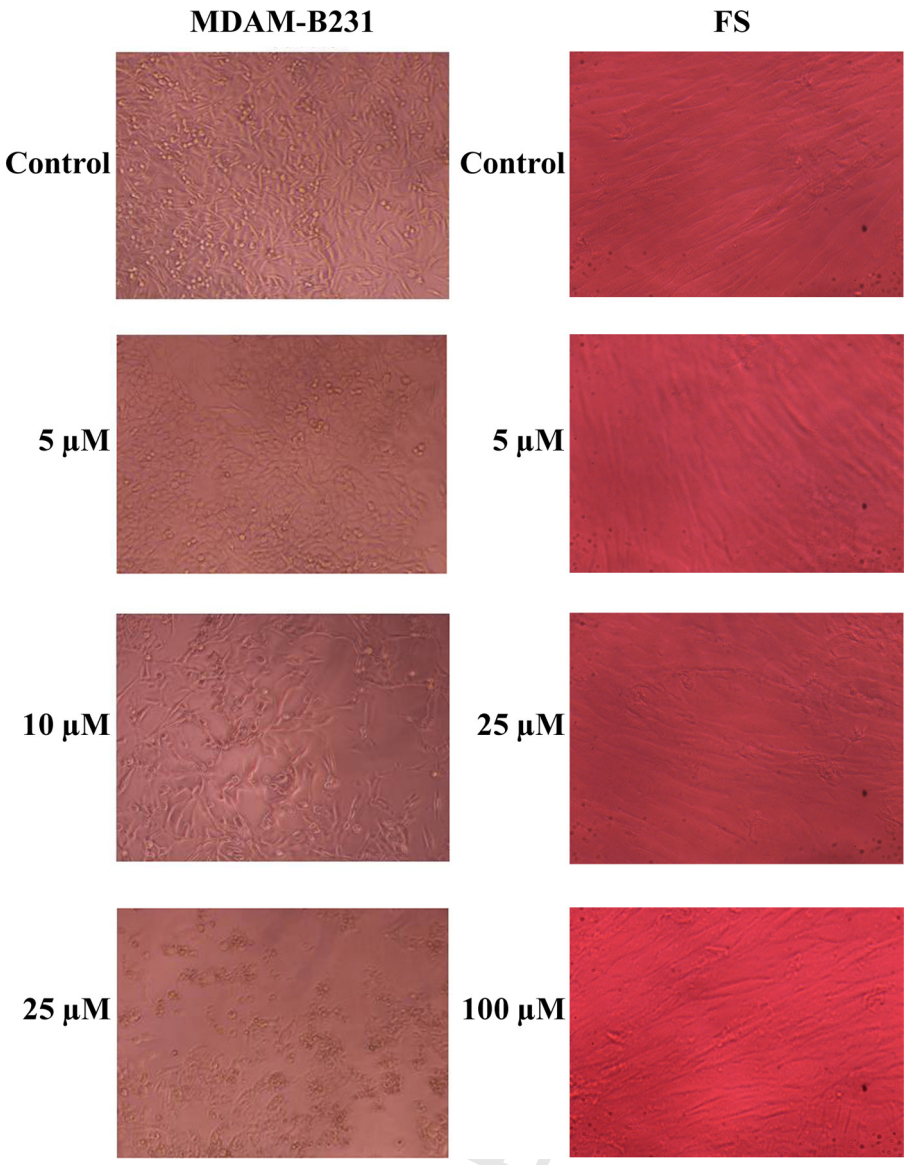


Fig. 5. Phase-contrast light microscopy image of MDAM-B231 and FS normal fibroblast cell after treatment with cyclo(L-Leu-D-Arg).

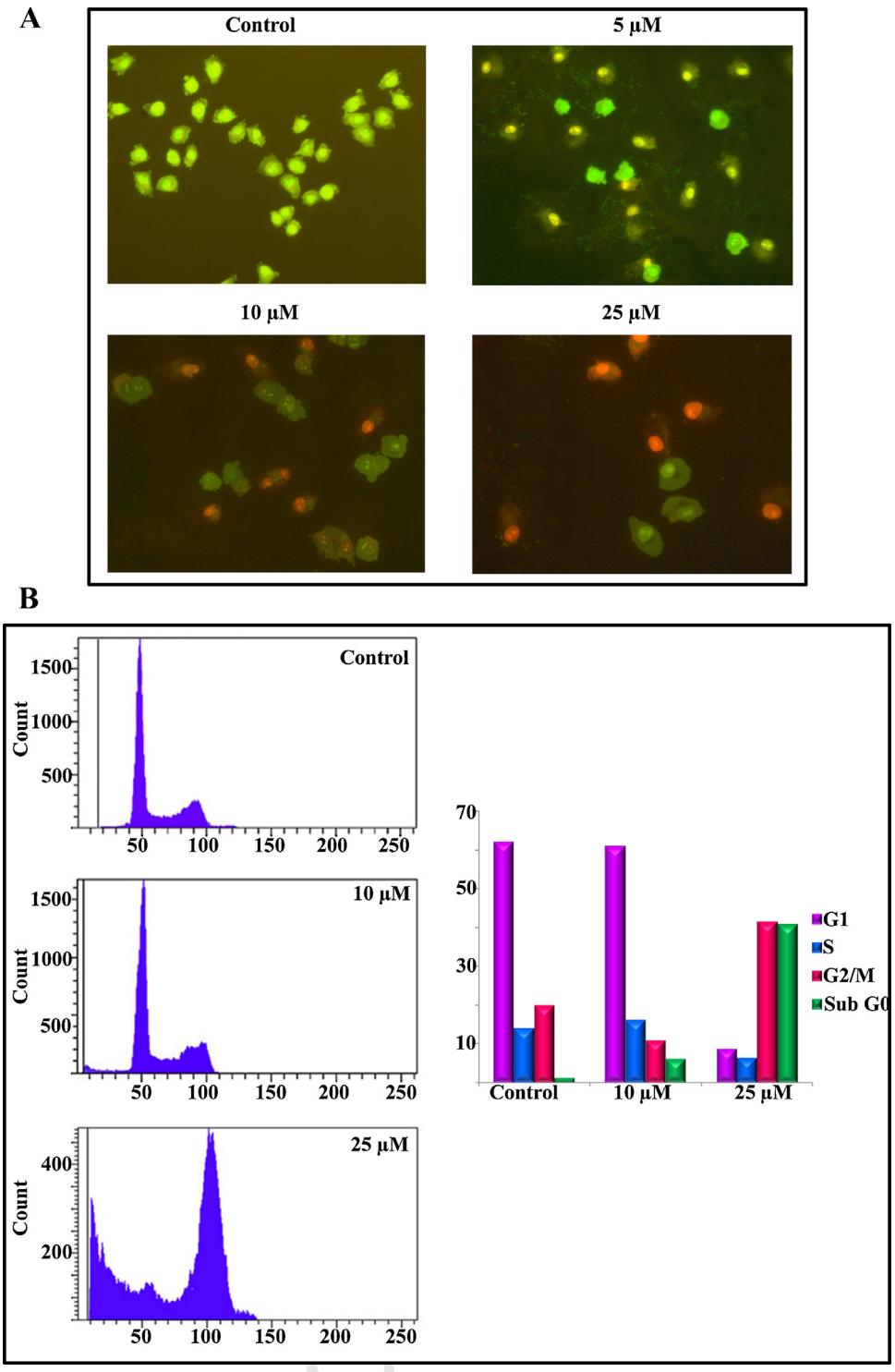
93.87% ± 0.17 at 100 μM, which is higher than the BHA, the standard antioxidant (47% ± 0.13) (Fig. 7B).

4. Discussion

Peptides and proteins constitute a diverse family of endogenous compounds that regulate a wide range of important biological functions [5]. Peptides have played a significant role in pharmaceutical research as biomedically useful agents or as lead compounds for drug development [4]. Many natural cyclopeptides have novel structures and exhibit significant bioactivity [26,34].

Diketopiperazines are forming a class of cyclic organic compounds that result from peptide bonds between two amino acids to form a bis-lactam. They are the smallest possible cyclic peptides. Diketopiperazines are commonly biosynthesized from amino acids by different organisms, including mammals, and are considered to be secondary metabolites [24]. Some proteases, such as dipeptidyl peptidases, cleave the terminal ends of proteins to generate dipeptides, which naturally cyclize to form diketopiperazines [10]. Due to their rigid structure, chiral nature and varied side chains, diketopiperazines are an attractive scaffold for drug design. For both natural and synthetic diketopiperazines, a wide variety of biological activities was reported, including antitumor. Some of their most important biological activities are related to the inhibition of plasminogen activator inhibitor-1 [10] and the alteration of cardiovascular and blood-clotting functions [25]. They also were claimed to have activities as antitumour [14], antiviral [33], antifungal [7], antibacterial [11] and antihyperglycaemic agents [18].

Diketopiperazine (DKP) derivative, produced naturally by many organisms and microorganisms, display a very wide diversity of structures and biological functions, making them useful chemical entities for the discovery and development of new drugs. Useful biological properties have already been demonstrated for some of them, such as antibacterial, fungicidal, herbicidal, antiviral, immunosuppressor, antitumour activities, etc. [22]. Cyclic peptides have inherent physiological advantages, including stability (resistance to enzymatic degradation) compared to their linear counterparts, conformational rigidity and improved receptor site selectivity and pharmacological specificity [36]. It is known that cyclic dipeptides have many potential biological functions. The



Q6 Fig. 6. (A) Acridine orange/ethidium bromide staining of MDAM-B231 cells to detect apoptosis: live cells were observed as green, whereas the apoptotic cells as orange-red due to co-staining with ethidium bromide due to loss of membrane integrity. Magnification 40×. (B) Effect of cyclo(L-Leu-D-Arg) against MDAM-B231 on cell cycle. Representative histograms on the right-hand panel indicate the percentages of cells in G1, S, G2/M and sub-G0 phases of the cell cycle. 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. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

investigation of the preferred conformations of cyclic dipeptides is very important to explore the functionary mechanism and discover the biological characteristics of cyclic dipeptides.

Cyclo(L-Leu-L-Arg) has been previously described as a natural product from *Streptomyces* species [35,37] or obtained by chemical synthesis [30]. Cyclo(L-Leu-D-Arg) is not reported from natural source and is reported here for the first time from *Bacillus* sp. Antibacterial activities against Gram-positive and Gram-negative bacteria, as well as antifungal activities of cyclo(L-Leu-L-Arg) have also reported [20]. Smaoui et al. [35] reported the antifungal activity of cyclo(L-Leu-L-Arg) only against *Fusarium oxysporum* by simple disk diffusion assay. But in the present study we reported the

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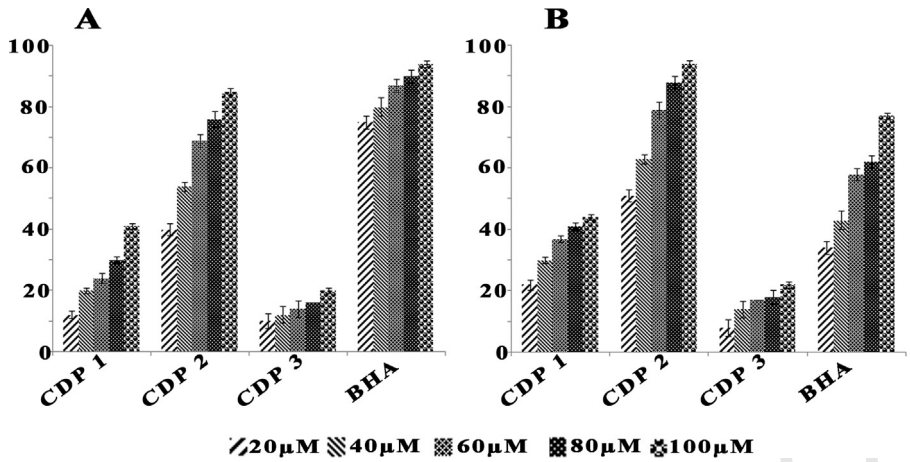

Fig. 7. Antioxidant activity of cyclo(2-hydroxy-Pro-L-Leu). (A) DPPH free radical scavenging. (B) Hydroxyl activity. All the measurements were done in three replicates and results are expressed as arithmetic mean ± standard error on the mean.

antifungal activity of cyclo(L-Leu-D-Arg) against seven medically and agriculturally important fungi. Antifungal activity of cyclo(L-Leu-D-Arg) is not reported earlier. Cyclo(2-hydroxy-Pro-L-Leu) has previously reported from marine-derived *Streptomyces* [20]. Antifungal and antioxidant activity of this compound was not reported earlier. But in our study cyclo(2-hydroxy-Pro-L-Leu) recorded significant antioxidant activity and the activity of this compound may be due to the presence of hydroxyl group. Cyclo(Pro-Val) has been previously reported from *Pseudomonas fluorescens* GcM5-1A carried by the pine wood nematode and spectral data of this compound was similar with our cyclo(Pro-Val) [12]. Previously we have reported proline containing cyclic dipeptides produced by the same *Bacillus* sp. associated with EPN having antimicrobial activity [16,17].

The present study also investigated whether the three CDPs could inhibit the growth of cancer cell lines and whether any of these CDPs could induce apoptosis in MDAM-B231 cancer cells. Our results showed that, out of three CDPs, cyclo(L-Leu-D-Arg) exhibited the highest growth inhibitory effect on cancer cell lines (Fig. 4A). The growth inhibition exhibited by cyclo(L-Leu-D-Arg) was shown to be dose dependent (Fig. 4A). Our results also show, for the first time, that cyclo(L-Leu-D-Arg) induces apoptotic cell death in MDAM-B231 cancer cells. The apoptotic program is characterized by particular morphological features such as chromatin condensation and nuclear fragmentation [15]. Cyclo(2-hydroxy-Pro-L-Leu) also recorded antitumor activity. Similar antitumor activity of cyclo(2-hydroxy-Pro-L-Leu) has been previously reported against HL-60 cell lines [20]. Previously cyclo(His-Phe) and cyclo(His-Ala) has been reported for significant biological activity in the treatment of cancer, infectious and cardiovascular-related diseases [23,25]. It possesses significant anti-tumor activity, causing greatest reduction of cell viability in cervical carcinoma cells [25].

5. Conclusions

The cyclo(L-Leu-D-Arg) is a new natural cyclic dipeptides, isolated from the culture filtrate of *Bacillus cereus* along with two known cyclic dipeptides. To the best of our knowledge, cyclo(L-Leu-D-Arg) has not been isolated from any bacteria. In our hands, cyclo(L-Leu-D-Arg) recorded prominent antifungal activity against medically important fungi. The antifungal activity of cyclo(L-Leu-D-Arg) is also reported for the first time. Our results also recorded that cyclo(L-Leu-D-Arg) significantly inhibits the growth of MDAM-B231 suggesting the potential to inhibit the growth of cancer cells *in vivo* by apoptosis. Our findings warrant further investigation into the effects of cyclo(L-Leu-D-Arg) and related CDPs 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(L-Leu-D-Arg) induces apoptosis. More over cyclo(2-hydroxy-Pro-L-Leu) recorded significant antioxidant activity and hydroxyl scavenging activity of this compound is better than the standard antioxidant compound BHA.

Uncited reference

[21].

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