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ORIGINAL ARTICLE

Bioactive natural products from *Pseudonocardia endophytica* VUK-10

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KEYWORDS

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Abstract Two proline containing cyclic dipeptides (CDPs), cyclo (L-Pro-L-Tyr) (**1**) and cyclo (L-Pro-L-Phe) (**2**) were isolated from the fermentation broth of *Pseudonocardia endophytica* VUK-10 originating from the Nizampatnam mangrove ecosystem on the south coast of Andhra Pradesh, India. The structures of the compounds were established by 1H NMR and 13C NMR spectroscopy, FTIR and EIMS. The antimicrobial and cytotoxic activities of the compounds were tested against a variety of medicinally and agriculturally important bacteria and fungi as well as on the MDA-MB-231, OAW-42, HeLa and MCF-7 human cell lines. *Xanthomonas malvacearum* was most sensitive toward **1** (MIC 4 μg/ml), whereas compound **2** had good antibacterial activity against *Xanthomonas campestris* (MIC 8 μg/ml). *Fusarium solani* was highly sensitive toward **1** (MIC 16 μg/ml). The compounds were cytotoxic against the human cell lines at micro molar concentrations; the highest activity (IC50 $<$ 10 μM) of **1** was recorded against the MDA-MB-231 cancer cell line.

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1. Introduction

Effective novel drugs are in great demand to restrain the spread of antibiotic-resistant pathogens. Natural products and their derivatives are invaluable sources of therapeutic agents. Their multitude, chemical diversity and complexity confer potent biological activities that allow the discovery of new drugs for the control and treatment of human illnesses [1–4]. Actinobacteria are a valuable source of bioactive natural products, and great efforts are made to search undisturbed natural habitats for such microorganisms to enhance the discovery of novel bioactive metabolites [5,6]. Soils of such sources have been widely exploited, but there are only few and inconclusive investigations of actinobacteria from mangrove sediment available, even though they are reliable sources for the discovery of new bioactive compounds [7,8]. In recent years, there has been a growing awareness of the value of mangrove sediments as sources of actinobacteria.

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Among prokaryotes, actinobacteria are considered to have the highest economical and biotechnological potential, as they produce a wide range of promising bioactive secondary compounds including antibiotics, antitumor agents, immune suppressive drugs and enzymes. The genus *Pseudonocardia* belongs to the order Actinomycetales, members of which are well known for their proven ability to produce bioactive metabolites and other molecules of pharmaceutical interest, but relatively few natural products have been described from species of this particular genus [9]. Antibiotics discovered from *Pseudonocardia* spp. include deoxyniboquinone, three diazaanthraquinone derivatives, the pseudonocardians A-C [10], dentigerumycin [11], phenazostatin D [12], and the polyketide NPP [13]. Of these, deoxyniboquinone, the pseudonocardians A-C and phenazostatin D are produced by marine *Pseudonocardia* spp. isolated from deep sea sediment of the South China Sea and the littoral of Mauritius (Indian Ocean), respectively. They have potent antimicrobial, cytotoxic and neuroprotective properties. Dentigerumycin is a cyclic depsipeptide that was isolated from a *Pseudonocardia* sp. associated with a fungus growing in the ant *Apterostigma dentigerum* and NPP is an antifungal polyketide produced by the soil actinomycete *Pseudonocardia autotrophica* KCTC 9441. Based on the above description, the genus *Pseudonocardia* have been proved as the best dependable resource for active metabolites, though the genus was less explored for natural products.

In the course for the search of bioactive metabolites, the morphologically distinct actinobacterial strain VUK-10, having good antimicrobial potential, was selected from 55 actinobacterial strains isolated from sediment samples of the Nizampatnam mangrove ecosystem (Andhra Pradesh, India). The strain VUK-10 was classified as *Pseudonocardia endophytica* (JN087501) by polyphasic taxonomy [14]. Two bioactive compounds, 4-(2-acetamidoethyl) phenyl acetate and 4-((1,4-dioxooctahydropyrrolo [1,2-a] pyrazin-3-yl) methyl) phenyl acetate with potent anti-microbial and cytotoxic activities were reported from this strain [15]. In the ongoing research, we report here the isolation, structural elucidation and biological evaluation of two further bioactive compounds from *Pseudonocardia endophytica* VUK-10.

2. Materials and methods

2.1. Fermentation, isolation and identification of metabolites

A seed culture of *Pseudonocardia endophytica* VUK-10 was grown in YMD broth (seed broth) and incubated on a rotary shaker (250 rpm) at 35 °C for 48 h. Seed culture at concentration of 10% (100 ml of the seed culture in 1000 ml of the production medium) was transferred into the optimized fermentation medium (glucose [1%, w/v], tryptone [0.5%], K_2HPO_4 [0.05%], NaCl [3%] and $FeSO_4$ [0.001%]; pH adjusted to 7.0) [16]. After cultivation of the strain for 96 h, the culture filtrates (50 L) were extracted twice with ethyl acetate, the extract concentrated by rotary evaporation and then freeze-dried to yield 7.6 g of a dark brown residue of the crude extract.

The ethyl acetate crude extract was subjected to Sephadex LH-20 gel filtration chromatography using dichloromethane/methanol (1:1, v/v) as eluent, resulting in yielding nine fractions. Based on the ^{1}H NMR spectral data and bioassay, fraction VII (3.34 g) was selected for further studies and subjected to silica gel column chromatography (25 × 5 cm, Silica gel 60, Merck) which afforded fractions 1–5. Based on TLC monitoring, NMR spectral data and antibacterial activity against *B. subtilis*, sub fractions 1 (294 mg) and 3 (340 mg) were selected for further purification by silica gel column chromatography (22 × 5 cm, Silica gel 100, Merck) using *n*-hexane/isopropanol (95:5) and dichloromethane/ethyl acetate/acetone (2:1:1), which yielded compound **1** (32 mg) and **2** (22 mg), respectively. The structures of **1** and **2** were elucidated by nuclear magnetic resonance (Avance 300 MHz) (^{1}H and ^{13}C NMR; model: Varian Gemini 200 and samples were made in $CCL_4/CDCL_3$ + DMSO using tetra methyl silane as internal standard; NMR Spectroscopy Division IICT-Hyderabad) spectroscopy, EIMS (Electron ionization mass; model: micromass VG-7070H, 70 eV spectrophotometer; Mass Spectroscopy Division IICT-Hyderabad) and FTIR (Fourier transform infra-red; Thermo Nicolet Nexus 670 spectrophotometer with NaCl optics).

2.2. Test micro organisms

Gram positive bacteria: *Bacillus cereus* (MTCC 430), *Streptococcus mutans* (MTCC 497), *Staphylococcus aureus* (MTCC 3160), *Staphylococcus epidermis* (MTCC 120), *Bacillus subtilis* (ATCC 6633), *Bacillus megaterium* (NCIM 2187); Gram negative bacteria: *Escherichia coli* (ATCC 35218), *Pseudomonas aeruginosa* (ATCC 9027), *Proteus vulgaris* (MTCC 7299), *Serratia marcescens* (MTCC 118) *Xanthomonas campestris* (MTCC 2286), *Xanthomonas malvacearum* (NCIM 2954) and *Salmonella typhi* (ATCC 14028); medically important dermatophytes: *Candida albicans* (ATCC 10231) and *Epidermophyton floccosum* (MTCC 145); medically and agriculturally important filamentous fungi: *Aspergillus niger* (ATCC 1015), *Aspergillus flavus* (ATCC 9643), *Fusarium oxysporum* (MTCC 3075), *Fusarium solani* (MTCC 4634), *Penicillium citrinum* (MTCC 6489), *Verticillium alboatrum* and *Alternaria alternata* (MTCC 6572). The test microorganisms were procured from ATCC, Manassas, VA, USA, and MTCC, Chandigarh, NCIM, Pune, India, and preserved at 4 °C.

2.3. MIC assay

Minimum inhibitory concentrations (MIC) of the test compounds against bacteria and fungi were determined in an agar plate diffusion assay [17] in triplicates for each concentration. Mueller–Hinton agar and Czapek–Dox agar media were prepared to grow the bacteria and fungi, respectively. The purified compounds were dissolved in dimethyl sulfoxide at concentrations ranging from 0 to 1000 μg/ml. The inoculated plates were examined after 24–48 h of incubation at 37 °C for bacteria and 48–72 h at 28 °C for fungi. The lowest concentration of the compounds exhibiting significant antimicrobial activity against the test micro organisms was taken as the MIC of the compound. The standard antibiotics used in this study were Tetracycline against bacteria, Griseofulvin against dermatophytes and Amphotericin-B against fungi.

2.4. MTT assay

The cytotoxicity of the compounds against human cancer cell lines was assessed in 96 well plates by measuring the reduction

of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) to the water insoluble formazan crystals [18]. The cell lines were human breast adenocarcinoma (MDA-MB-231) (cell line reported to be resistant to cancer drugs) [19], human cervical cancer (HeLa), human ovarian cyst adenocarcinoma (OAW-42) and human breast adenocarcinoma (MCF-7) cell lines that had been obtained from the National Centre for Cell Science, Pune, India. The cell lines MDA-MB-231, HeLa and OAW-42 were cultivated in Dulbecco's modified Eagle's medium supplemented with fetal bovine serum (10%; v/v), L-glutamine (2 mM), penicillin (10 units/ml) and streptomycin (10 µg/ml), while the MCF-7 cell line was cultivated in Roswell Park Memorial Institute medium 1640 with the same supplements, all in a humidified atmosphere (95%) with 5% of CO_2 at 37 °C. Cells were seeded in 96-well microtiter plates at a density of 5×10^3 per well containing 100 µl medium. After overnight incubation, the test compounds were added at 10, 100, 1000 and 5000 nM concentrations, each in triplicate. After 24 h of further incubation, cell viability was assessed by adding 20 µl of MTT (5 mg/ml in PBS) per well, and the plates were incubated at 37 °C for 4 h. The formazan crystals formed in the cells were dissolved by addition of 100 µl of 0.1% acidified isopropanol, and the optical density was measured at 570 nm using a micro plate reader. The IC_{50} values (50% inhibitory concentration) of the compounds were calculated using Sigma Plot software with reference to that of taxol as standard. All the experiments were carried out in triplicate.

3. Results

3.1. Fermentation, isolation and identification of metabolites

Culture filtrates obtained after 96 h fermentation of *Pseudonocardia endophytica* VUK-10 were extracted with ethyl acetate and concentrated to yield a dark brown residue, which in turn was subjected to gel filtration chromatography using the solvent system of dichloromethane/methanol. Among the nine fractions collected, fraction VII exhibiting good antimicrobial activity was rechromatographed on a silica gel column and yielded 5 fractions. Further purification of active sub fractions 1 and 3 by sequential chromatographic purifications on silica gel yielded the two antimicrobial compounds **1** and **2**.

Compound (**1**) was obtained as white amorphous powder, wholly soluble in dimethyl sulfoxide, methanol, ethanol and chloroform, $[\alpha]_D^{25}$-87.1 (c0.8, MeOH). The ^{1}H NMR spectrum of compound showed signals at δ 7.04 (d, 2H, J = 8.49 Hz); 6.78 (d, 2H, J = 8.49 Hz); 6.57 (bs, 1H); 5.8 (s, 1H); 4.22 (dd, 1H, J = 2.83, 10.19 Hz); 4.08 (t, 1H, J = 7.17 Hz); 3.69–3.44 (m,3H); 2.74 (dd, 1H, J = 10.19, 14.54 Hz); 2.37–2.27 (m,1H); 2.05–1.84 (m,3H) (see Supplementary Material, Fig. 1); while ^{13}C exhibited 12 signals at δ 169.691; 165.243; 155.81; 130.34; 126.59; 116.008; 59.06; 56.27; 45.34; 35.93; 28.26 and 22.32 (see Supplementary Material, Fig. 2). EIMS analysis of the compound gave a molecular ion m/z at 283 (M + Na) (see Supplementary Material, Fig. 3). The IR spectrum exhibited absorption bands at V_{max} 3243, 2961, 1662, 1514, 1445, 1261, 800 cm^{-1} (see Supplementary Material, Fig. 4). Based on the above spectral data, the compound **1** was identified as (3S, 8aS)-3-(4-hydroxybenzyl) hexahydropyrrolo [1,2-a] pyrazine-1,4-dione [Cyclo(L-Pro-L-Tyr)], with the molecular formula of $C_{14}H_{16}N_2O_3$ (Fig. 1A).

Compound (**2**) was obtained as a white solid, freely soluble in dimethylsulfoxide, methanol, ethanol and chloroform, $[\alpha]_D^{25}$-93.4 (c 1.1, MeOH). The ^{1}H NMR spectrum of the compound showed signals at δ 7.30 (m,5H); 5.70 (s, 1H); 4.28 (dd, 1H, J = 2.83, 10.38 Hz); 4.07 (t, 1H, J = 6.79 Hz); 3.69–3.52 (m,3H); 2.77 (dd, 1H, J = 10.38, 14.35 Hz); 2.39–2.29 (m,1H); 2.08–1.80 (m,3H) (see Supplementary Material, Fig. 5); while ^{13}C exhibited 12 signals at δ 166.3; 165.1; 135.8; 129.1 (2C); 129.0 (2C); 127.3; 59.0; 56.2; 45.3; 36.6; 28.2 and 22.3 (see Supplementary Material, Fig. 6). EIMS analysis of the compound gave a molecular ion m/z at 245 (M + H) and 267 (M + Na) (see Supplementary Material, Fig. 7). The IR spectrum displayed absorption bands at V_{max} 3257, 2927, 1665, 1436, 1219 and 755 cm^{-1} (see Supplementary Material, Fig. 8). Based on the supra mentioned spectral data, the compound **2** was identified as 3-benzyl hexahydropyrrolo [1,2-a] pyrazine-1,4-dione [Cyclo(L-Pro-L-Phe)], with the molecular formula of $C_{14}H_{16}N_2O_2$ (Fig. 1B).

3.2. Biological assays

3.2.1. MIC assay of antibacterial and antifungal activities

MIC assay. Antibacterial activities of the bioactive compounds (**1** and **2**) in terms of MIC are shown in Table 1. The bioactive compounds exhibited antibacterial activity against a variety of gram-positive and gram-negative bacteria, for which the MIC values ranged from 4 to 128 µg/ml. Among the opportunistic and pathogenic bacteria, compound 1 was active against all the bacteria tested, and the best activity of this compound was recorded against *Xanthomonas malvacearum* (4 µg/ml), followed by *Staphylococcus aureus* and *Escherichia coli* (8 µg/ml). Compound **2** presented the highest sensitivity to *Xanthomonas campestris* (8 µg/ml) followed by *Streptococcus mutans* and *Pseudomonas aeruginosa* (16 µg/ml). Tetracycline served as the positive control for bacteria. Compared with the standard drug tetracycline, compound **1** displayed high sensitivity against *Xanthomonas malvacearum* and *Staphylococcus aureus* and **2** against *Xanthomonas campestris, Streptococcus mutans* while in some cases compound **1** (*Escherichia coli*) & **2** (*Staphylococcus aureus*) recorded similar sensitivity like positive control (Table 1). Tetracycline, in other cases showed good antibacterial activity over the metabolites of the strain.

Antifungal activities of the bioactive compounds against dermatophytes and filamentous fungi and the corresponding MIC values are recorded in Table 2. Compound **1** exhibited significant MIC value against *Candida albicans* (16 µg/ml), whereas compound **2** recorded activity at 32 µg/ml against *Epidermophyton floccosum*. Among the filamentous fungi tested, *Fusarium solani* recorded sensitivity of 16 µg/ml toward

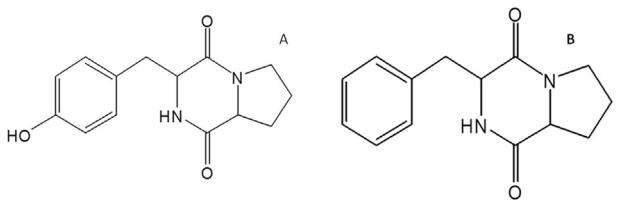

Figure 1 Molecular structures of 3-(4-hydroxy benzyl) hexahydropyrrolo [1,2-a] pyrazine-1,4-dione and 3-benzyl hexahydropyrrolo [1,2-a] pyrazine-1,4-dione.

Table 1 Minimum inhibitory concentrations (MIC, μg/ml) of compounds **1** and **2** produced by *Pseudonocardia endophytica* VUK-10 against gram positive and gram negative bacteria.

Test microorganisms		**1**[a]	**2**[b]	Tet[c]
Staphylococcus aureus	(MTCC 3160)	8	32	32
Streptococcus mutans	(MTCC 497)	32	16	32
Staphylococcus epidermis	(MTCC 120)	32	32	16
Xanthomonas campestris	(MTCC 2286)	32	8	16
Xanthomonas malvacearum	(NCIM 2954)	4	32	8
Bacillus subtilis	(ATCC 6633)	64	64	32
Bacillus megaterium	(NCIM 2187)	32	32	16
Bacillus cereus	(MTCC 430)	64	64	8
Escherichia coli	(ATCC 35218)	8	32	8
Pseudomonas aeruginosa	(ATCC 9027)	64	16	8
Serratia marcescens	(MTCC 118)	64	128	32
Proteus vulgaris	(MTCC 7299)	128	128	16
Salmonella typhi	(ATCC 14028)	64	64	8

[a] 1: Cyclo(L-Pro-L-Tyr).
[b] 2: Cyclo(L-Pro-L-Phe).
[c] Tet: tetracycline.

Table 2 Minimum inhibitory concentration (MIC, μg/ml) of compounds **1** and **2** produced by *Pseudonocardia endophytica* VUK-10 against dermatophytes and filamentous fungi.

Dermatophytes		**1**[a]	**2**[b]	Antibiotic[c]
Candida albicans	(ATCC 10231)	8	64	16
Epidermophyton floccosum	(MTCC 145)	64	32	16
Filamentous fungi				
Aspergillus niger	(ATCC 1015)	128	64	16
Aspergillus flavus	(ATCC 9643)	64	128	8
Fusarium oxysporum	(MTCC 3075)	32	32	16
Fusarium solani	(MTCC 4634)	16	32	32
Penicillium citrinum	(MTCC 6489)	64	64	8
Verticillium alboatrum	(ATCC 13641)	512	ND[d]	64
Alternaria alternata	(MTCC 6572)	>512	256	32

[a] 1: Cyclo(L-Pro-L-Tyr).
[b] 2: Cyclo(L-Pro-L-Phe).
[c] Antibiotic: Griseofulvin against Yeast and Amphotericin-B against fungi.
[d] ND: not detected.

compound **1** while *Alternaria alternata* that recorded no activity up to 512 μg/ml. Compound **2** was active against *Fusarium solani* and *Fusarium oxysporum* at 32 μg/ml, and for this compound *Verticillium alboatrum* recorded no activity up to 1000 μg/ml. Compound **1** displayed high activity against *Fusarium solani* compared to standard drug while in some cases **1** (*Candida albicans*) and **2** (*Fusarium solani*) recorded similar sensitivity like positive control (Table 2). In other cases both compounds recorded lower antifungal activity than the standard fungicides, Griseofulvin against dermatophytes and Amphotericin-B against fungi.

3.2.2. MTT assay of cytotoxic activities

Compounds **1** and **2** were active against all four human cancer cell lines tested (Figs. 2 and 3). Cell lines displaying IC_{50} values of <10 μM (71.5%) (MDA-MB-231), <100 μM (71.3%, 55%) (HeLa and MCF-7), <1000 μM (61.4%) (OAW-42) for compound 1 (Fig. 2A–D).

The activity of compound **2** against MDA-MB-231, HeLa, MCF-7 and OAW-42 cell lines is presented in Fig. 3A–D. Compound **2** exhibited significant cytotoxicity with MDA-MB-231, HeLa, MCF-7 and OAW-42 cell lines, exhibiting IC_{50} values of <100 μM (59.6%, 69.1%) (MDA-MB-231, HeLa) and <1000 μM (54%, 56.7%) (MCF-7, OAW-42). Taxol, an anti-cancer drug used as the standard, recorded an IC_{50} value of 10 μM (59%, 60%, 57% and 63%) against the MDA-MB-231, HeLa, OAW-42 and MCF-7 cell lines.

4. Discussion

Diketopiperazines (DKPs), the smallest cyclic dipeptides resulting from peptide bonds between two amino acids,

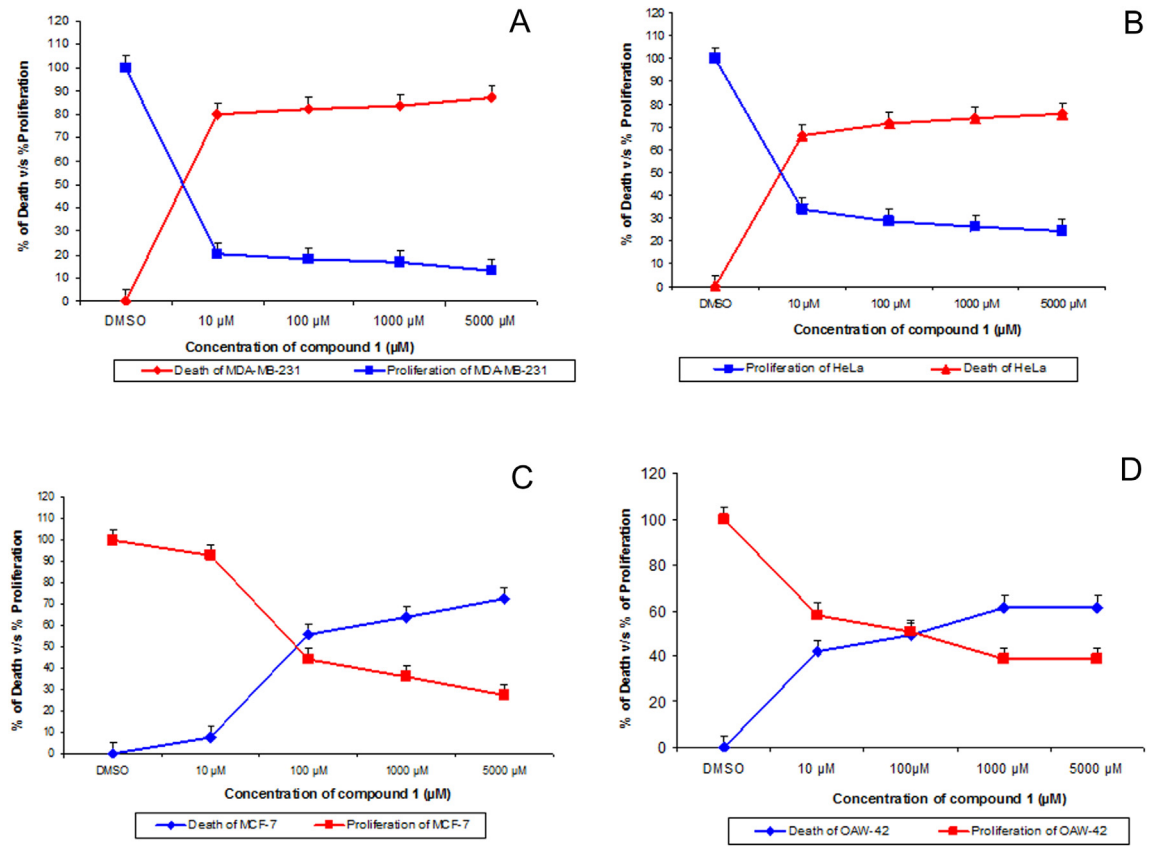

Figure 2 Growth of (A) MDB-MB-231, (B) HeLa, (C) MCF-7, (D) OAW-42 cancer cell lines as a function of the concentration of compound 1.

provide excellent models for the development of pharmaceutical compounds. Due to their relatively simple and rigid structure, stability, chiral nature and varied side chains, DKPs have been of research interest for their diverse bioactivities [20–22]. Some DKPs have diverse effects of pharmaceutical interest including antibacterial [23], antifungal [24], antitumor [25], antiviral [26], and anti-hyperglycemic [27] activities, as well as other bioactivities including quorum-sensing signaling, plant-growth promotion and inhibition of aflatoxin production [28]. DKPs containing proline residues form the core of several interesting natural product classes and exhibit promising bio-activities [29,30]. Therefore, diketopiperazines are privileged structures for the discovery of new lead compounds and are also considered ideal for the rational development of new therapeutic agents. In the present study, two compounds *viz*., 3-(4-hydroxy benzyl) hexahydropyrrolo [1,2-a] pyrazine-1,4-dione [cyclo (L-Pro-L-Tyr)] and 3-benzyl hexahydropyrrolo [1,2-a] pyrazine-1,4-dione [cyclo (L-Pro-L-Phe)] active against facultative and pathogenic bacteria, fungi and cancer cell lines, were isolated from the culture broth of *Pseudonocardia endophytica*.

Cyclo (L-Pro-L-Tyr) has been reported as a bioactive compound from terrestrial and marine organisms including sea urchin-derived *Bacillus* spp. [31], *Streptomyces* spp. TN256 strain [32], and sponge associated *Pseudomonas aeruginosa* [33]. It has been reported to inhibit the growth of *Saccharomyces cerevisiae* by inhibiting family 18 chitinases [34]. A MIC of 32 µg/ml was reported against *Staphylococcus aureus* [35], but we observed a somewhat greater potency against similar bacteria with MIC values of 16 µg/ml. The isolation of compound 2 was previously reported from *Cladosporium phlei*, a pathogenic fungus of *Phleum pratense* [36] and from *Bacillus pumilus*, a marine bacterium [37]. It exhibited antifungal activity against *Aspergillus fumigatus* and *Penicillium roqueforti* [38] and antibacterial activity against *B. subtilis*, *S. aureus*, *E. coli* and *P. aeruginosa* [35]. So far, the antimicrobial activity of compounds **1** and **2** was reported only on few microorganisms. We are providing here the first report of their antimicrobial activity against medicinally and agriculturally important bacteria. Compound **1** is a good potential inhibitor against *Xanthomonas malvacearum* (causes angular leaf spot of cotton) (4 µg/ml), *Candida albicans* (causes oral thrush and vaginal infection) (16 µg/ml) and *Fusarium solani* (responsible for root rot in beans), *Fusarium* (crown and foot rot of squash and pumpkin, black rot of potato) (16 µg/ml). In our hands, **2** effectively inhibited growth of *Xanthomonas campestris* (causes bacterial leaf spot on peppers and tomatoes) (8 µg/ml), *Epidermophyton floccosum* (causes tinea dermatophytoses), *Fusarium solani* and *Fusarium oxysporum* (responsible for fusarium wilt, fungal keratitis and onychomycosis) with an MIC of 32 µg/ml.

Cyclo (L-Pro-L-Tyr) and Cyclo (L-Pro-L-Phe) were known compounds [39] and the spectral data of the both compounds is quite agreement with the described literature. These two compounds are previously reported for anti-cancer activity against HeLa and MCF-7 cell lines [40]. In our study we observed dependable potency against similar cell lines.

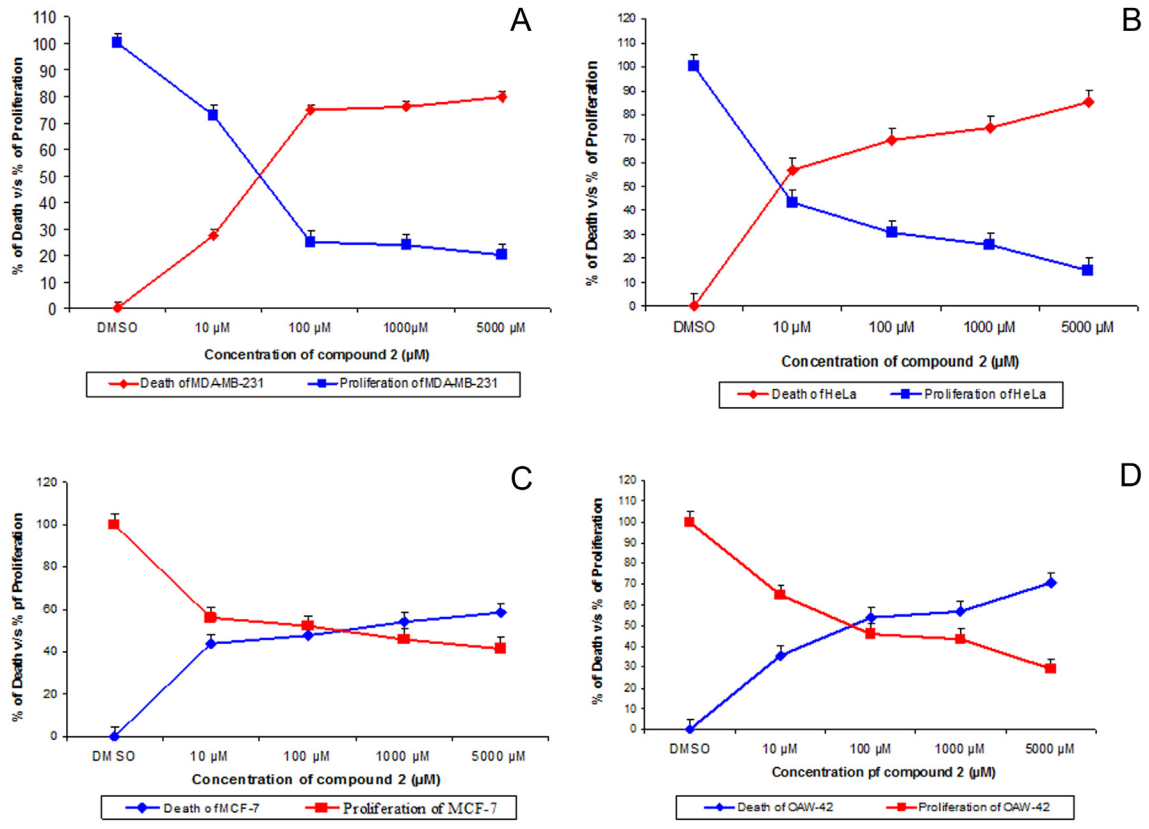

Figure 3 Growth of (A) MDB-MB-231, (B) HeLa, (C) MCF-7, (D) OAW-42 cancer cell lines as a function of the concentration of compound 2.

Compounds **1** and **2** displayed IC_{50} values of < 100 µM (71.3%; 69.1%) against HeLa, whereas **1** exhibited IC50 value of < 100 µM (55%) and **2** recorded IC_{50} value of < 1000 µM (54%) against MCF-7 cell lines. In addition, cytotoxic activity of **1** and **2** against MDA-MB-231 and OAW-42 cell lines is reported here for the first time. Both compounds showed good inhibitory potentiality against all tested cell lines, with remarkable display of activity against MDA-MB-231 (< 10 µM) by compound 1. The results of the present study show that the compounds exhibit potent cytotoxic activities at impressively low concentrations.

This is the first report of the isolation and characterization of Cyclo (L-Pro-L-Tyr) (**1**) and Cyclo (L-Pro-L-Phe) (**2**) from the genus *Pseudonocardia*.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jgeb.2016.10.002.

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