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Bioactive Metabolites Produced by *Pseudonocardia endophytica* VUK-10 from Mangrove Sediments: Isolation, Chemical Structure Determination and Bioactivity S

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Chemical investigation of the actinobacterial isolate *Pseudonocardia endophytica* VUK-10 has led to the segregation of two known bioactive compounds, namely 4-(2-acetamidoethyl) phenyl acetate and 4-((1, 4-dioxooctahydropyrrolo [1, 2-a] pyrazin-3-yl) methyl) phenyl acetate. The strain was isolated from a sediment sample of the Nizampatnam mangrove ecosystem, south coastal Andhra Pradesh, India. The chemical structure of the active compounds was established on the basis of spectroscopic analysis, including ^{1}H NMR and ^{13}C NMR spectroscopies, FTIR, and EIMS. The antimicrobial and cytotoxic activities of the bioactive compounds produced by the strain were tested against opportunistic and pathogenic bacteria and fungi and on MDA-MB-231, OAW, HeLa, and MCF-7 cell lines. The compounds exhibited antimicrobial activities against gram-positive and gram-negative bacteria and fungi and also showed potent cytotoxic activity against MDA-MB-231, OAW, HeLa, and MCF-7 cell lines. This is the first example for this class of bioactive compounds isolated from *Pseudonocardia* of mangrove origin.

Keywords: Mangrove ecosystem, *Pseudonocardia endophytica*, bioactive compounds, 4-(2-acetamidoethyl) phenyl acetate, 4-((1, 4-dioxooctahydropyrrolo [1, 2-a] pyrazin-3-yl) methyl) phenyl acetate

Introduction

Bioactive molecules are secondary metabolites that are unimportant for growth and reproduction but probably form a defense mechanism to the producer microorganism to compete in nature [18]. Secondary metabolites are synthesized by pathways, which are often connected and controlled by primary metabolism. In fact, the intermediate metabolites from primary metabolism, serve as predecessors for the biosynthesis of secondary metabolites, and the formulation of the culture medium, closely connected with the metabolic capacities of the producing organism, greatly influences the biosynthesis of the bioactive molecules [9]. These active molecules are generally extracellular and their isolation in highest purity from the complex fermentation broth needs the application of a combination of various separation steps such as solvent extraction, chemical precipitation, ion exchange chromatography, and HPLC purification [28]. A critical element in a drug discovery based on microbial extracts is the isolation of unexploited group of microorganisms from unexplored habitats that are at the same time good producers of secondary metabolites [7].

The actinomycetes are an important group of bacteria with considerable importance as prolific producers of antibiotics

and other therapeutic compounds. Actinomycetes produce more than half of the bioactive compounds in the antibiotic database [19]. As the frequency of new bioactive compounds discovered from terrestrial actinomycetes decrease with time, while industrial programmes are increasingly screening actinomycetes from diverse environments for their ability to generate new metabolites [8, 15]. Recent studies revealed that huge investments are pumped into the development of marine biotechnology to uncover compounds for drug development over the next decade. It is evident that unexplored habitats are a rich source of varied new actinomycetes which can produce interesting till unfound bioactive compounds, including antibiotics. The mangrove ecosystems of tropical regions are the best pristine habitats that need to be explored as new promising sources for a wide range of biologically active and structurally complex natural products [1, 31]. The mangrove environments differ greatly from terrestrial habitats, the biochemical properties of mangrove actinobacteria are different from those of terrestrial ones. Studies on the biodiversity of mangrove actinobacteria are important not only in terms of basic research, but also for the biotechnological exploitation of such organisms [29]. Characterization of indigenous actinomycetes reflects their adaptations, resulting into secondary metabolite synthesis that can develop a new source of pharmaceutical compounds [16]. The actinobacterial strains subjected to the periodical changes due to abiotic factors observed in mangrove forests, such as salinity and tidal gradients, are presumed to be effective promoters for metabolic pathway adaptations and can generate unique metabolites [21].

The requirements for discovery and isolation of new lead structures from rare actinomycetes are undisputed. Remarkably, a vast number of the bioactive compounds are derived from a single actinomycetes genus, *Streptomyces*, and similar prolific taxa await discovery [17]. This goal may be reached by investigating new microbial sources for production of bioactive compounds, such as non-*Streptomyces* actinomycetes. Conventional isolation methods are hardly helpful to segregate rare actinomycetes genera. To isolate rare genera, factors like sample collection habitats, chemical and physical pretreatments of the samples, use of specific selective media, fine tuning of culture conditions and genus specific methodologies for screening of isolates need to be employed [11, 13, 25]. The genus *Pseudonocardia* was first recognized by Henssen [12] and kept along with nocardioform actinomycetes with a type IV cell wall and lack of mycolic acids. These actinobacteria are relatively unexploited to date and are potential sources of unidentified natural products for extensive use in medicine, agriculture, and industry. The genus *Pseudonocardia* is attracting wide attention as a new and an important source of antifungal, antibacterial, and cytotoxic compounds [3, 20, 22, 23, 26]. In the present study, we report the isolation, structure elucidation, and biological evaluation of two known bioactive compounds (PE-V1 and PE-V2) from the cell-free culture filtrate of *Pseudonocardia endophytica* VUK-10 of Nizampatnam mangrove sediments.

Materials and Methods

Isolation

The actinomycetes strain, VUK-10, was isolated from the Nizampatnam mangrove ecosystem by employing soil dilution plate technique on asparagine glucose agar medium. The strain was identified as *Pseudonocardia endophytica* by cultural, morphological, physiological and biochemical studies, along with 16S rDNA analysis. The rDNA sequence was deposited in the NCBI GenBank with the accession number JN087501 [14]. The strain was maintained on yeast extract-malt extract-dextrose (YMD) agar medium at 4°C for further study [30].

Fermentation, Extraction, Purification, and Structural Elucidation of Bioactive Metabolites

The twin activities i.e. isolation and identification of bioactive metabolites, a loopful culture of *Pseudonocardia endophytica* VUK-10 was cultivated in YMD broth (seed broth) and incubated on a rotary shaker (250 rpm) at 35°C. After 48 h incubation, the seed culture at the rate of 10% was transferred to the optimized fermentation medium consisting of glucose (8%), soy-peptone (1%), yeast extract (0.2%), meat extract (0.1%), $CaCO_3$ (0.3%), K_2HPO_4 (0.03%), $MgSO_4$ (0.1%), $FeSO_4$ (0.005%), and NaCl (3%), with pH adjusted to 7.0. The culture filtrates (40 L) obtained after cultivation of the strain for 96 h were extracted twice with ethyl acetate and concentrated in a rotavap, and freeze dried to yield a dark brown residue. The weight of total crude extract was 5.5 g. The ethyl acetate crude extract was subjected to Sephadex LH-20 gel filtration chromatography by using dichloromethane/methanol (1:1) as the eluent, resulting in seven fractions. Based on the ^{1}H-NMR spectral data and bio-active screening, fraction V (2.2 g) was selected for further studies and subjected to silica gel column chromatography (100−200 mesh), which afforded fractions 1−6. Based on TLC monitoring and NMR spectral data, fraction 6 was selected for further purification. In TLC monitoring, it was noticed that fraction 6 (188 mg) was a two-compound mixture with slight Rf difference. Fraction 6 was subjected to further purification by silica gel column chromatography using dichloromethane/isopropanol (95:5) that afforded subfractions A and B. The subfractions A and B were further purified by silica gel column chromatography by using chloroform/isopropanol (4:1) and benzene/ethyl acetate (1:1) and yielded PE-V1 (34 mg) and PE-V2

(5 mg), respectively. The structural elucidation of PE-V1 and PE-V2 was carried out by detailed interpretation of NMR, mass, FTIR spectroscopic data, and literature survey [4, 5].

Antimicrobial Activity of Bioactive Compounds

The antimicrobial spectra of the bioactive compounds of the strain were determined in terms of minimum inhibitory concentration (MIC) against a wide variety of gram-positive and gram-negative bacteria and fungi by using the agar plate diffusion assay [2]. Triplicate sets of the plates were maintained for each concentration of the test sample. Muller-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 1,000 μg/ml and used to assay against the test bacteria such as *Bacillus cereus* (MTCC 430), *Streptococcus mutans* (MTCC 497), *Staphylococcus aureus* (MTCC 3160), *Staphylococcus epidermis* (MTCC 120), *Bacillus subtilis* (ATCC 6633), *Bacillus megaterium* (NCIM 2187), *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); yeasts such as *Candida albicans* (ATCC 10231) and *Epidermophyton floccosum* (MTCC 145); and molds including *Aspergillus niger* (ATCC 1015), *Aspergillus flavus* (ATCC 9643), *Fusarium oxysporum* (MTCC 3075), *Fusarium solani* (MTCC 4634), *Penicillum citrinum* (MTCC 6489), *Verticillium alboatrum*, and *Alternaria alternata* (MTCC 6572). 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 bioactive metabolites exhibiting significant antimicrobial activity against the test microbes was taken as the MIC of the compound.

MTT Assay

The cytotoxicity of the compounds was assessed on the basis of the measurement of the *in vitro* growth in 96-well plates by cell-mediated reduction of tetrazolium salt to water-insoluble formazan crystals, as per the microculture MTT assay [24]. Cell lines for testing *in vitro* cytotoxicity included human breast adenocarcinoma cell line (MDA-MB-231), human cervical cancer cell line (HeLa), human ovarian cyst adenocarcinoma cell line (OAW-42), and human breast adenocarcinoma cell line (MCF-7) (cell lines reported to be resistant to cancer drugs) obtained from National Centre for Cell Science, Pune, India. Cell lines MDA-MB-231, HeLa, and OAW-42 were cultured on 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 breast cancer cell line MCF-7 was cultured on Roswell Park Memorial Institute medium 1640 supplemented with fetal bovine serum (10% (v/v)), L-glutamine (2 mM), penicillin (10 units/ml), and streptomycin (10 μg/ml), 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 (100 μl) containing 0.1 ml of medium. After overnight incubation, the cells were treated with different test concentrations of bioactive compounds (10, 100, 1,000, and 5,000 nM) at identical conditions with three replicates of each concentration. After 24 h of incubation, the 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 with 100 μl of 0.1% acidified isopropanol, and the rate of color development was measured at 570 nm using a microplate 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 triplicates.

Results and Discussion

Fermentation, Extraction, Purification, and Structural Elucidation of Bioactive Metabolites

Culture filtrates obtained after 96 h fermentation 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 seven fractions collected, the fraction V exhibiting good antimicrobial activity was rechromatographed on a silica gel column and yielded six fractions. Further purification of active subfraction 6 by sequential chromatographic purifications on silica gel yielded two active fractions A and B, which were subsequently purified to produce PE-V1 and PE-V2.

Compound PE-V1 was obtained as a white solid, wholly soluble in dimethylsulfoxide, methanol, ethanol, and chloroform. The ^{1}H-NMR spectrum of compound PE-V1 showed signals at δ 7.16 (d, 2H, J = 8.30 Hz), 6.99 (d, 2H, J = 8.30 Hz), 5.84 (bs, 1H), 3.46 (q, 2H, J = 6.78 Hz), 2.79 (t, 2H, J = 6.78 Hz), 2.28 (s, 3H), 1.93 (s, 3H) (Fig. S1); while ^{13}C exhibited 10 signals at δ 170.2, 169.5; 149.1, 136.4, 129.5 (2C), 121.5 (2C), 40.5, 34.8, 29.5, and 20.9 (Fig. S2). EIMS analysis of the compound gave a molecular ion *m/z* at 244 (M+Na) (Fig. S3). The IR spectrum exhibited absorption bands at V_{max} 3,289, 2,927, 1,749, 1,643, 1,100, 1,021, 914, and 726 cm^{-1} (Fig. S4). Based on the above spectral data, bioactive compound PE-V1 was identified as 4-(2-acetamidoethyl) phenyl acetate with the molecular formula of $C_{12}H_{15}NO_3$ (Fig. 1).

Compound PE-V2 was obtained as a pale yellow solid, freely soluble in dimethylsulfoxide, methanol, ethanol, and chloroform; $[\alpha]_D^{25}$-103.29 (c, 0.82, MeOH). The ^{1}H-NMR spectrum of the compound PE-V2 showed signals at δ 7.26 (d, 2H, J = 8.49 Hz), 7.06 (d, 2H, J = 8.49 Hz), 5.65 (bs, 1H), 4.28 (dd, 1H, J = 2.07; 9.63 Hz), 4.08 (t, 1H, J = 6.98 Hz), 3.71-3.52 (m, 3H), 2.81 (dd, 1H, J = 10.19; 14.54 Hz), 2.34 (m, 1H), 2.30 (s, 3H), and 2.09-1.82 (m, 3H) (Fig. S5), while ^{13}C

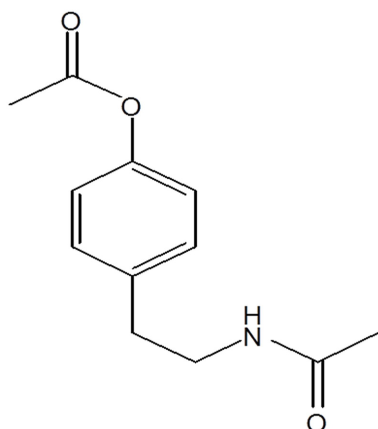

Fig. 1. Molecular structure of 4-(2-acetamidoethyl) phenyl acetate.

exhibited 13 signals at δ 169.4 (2C), 164.8, 149.9, 133.4, 130.2 (2C), 122.3 (2C), 59.0, 56.1, 45.4, 36.1, 28.3, 22.4, and 21.1 (Fig. S6). EIMS analysis of the compound gave a molecular ion *m/z* at 303 (M+H) and 325 (M+23) (Fig. S7). The IR spectrum displayed absorption bands at V_{max} 3,448, 2,926, 1,754, 1,664, 1,435, 1,370, 1,197, and 766 cm^{-1} (Fig. S8). Based on the supra mentioned spectral data, bioactive compound PE-V2 was identified as 4-((1, 4-dioxooctahydropyrrolo [1, 2-a] pyrazin-3-yl) methyl) phenyl acetate, with the molecular formula of $C_{16}H_{18}N_2O_4$ (Fig. 2). The spectral data of compound PE-V2 matched exactly with the spectral data of the same compound reported in the literature [4].

Minimum Inhibitory Concentration of Bioactive Compounds

Antibacterial activities of the bioactive compounds (PE-V1 and PE-V2) 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 8 to 256 µg/ml. Among the opportunistic and pathogenic gram-positive bacteria,

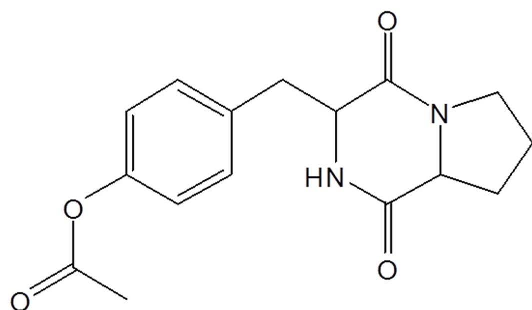

Fig. 2. Molecular structure of 4-((1, 4-dioxooctahydropyrrolo [1, 2-a] pyrazin-3-yl) methyl) phenyl acetate.

Table 1. Minimum inhibitory concentration (MIC) of bioactive compounds isolated from *Pseudonocardia endophytica* VUK-10 (MIC-(µg/ml)) against test bacteria.

Test microorganisms	PE-V1	PE-V2	Tet[a]
Staphylococcus aureus	8	64	32
Streptococcus mutans	16	32	32
Staph. epidermis	16	32	16
Xanthomonas campestris	32	32	16
X. malvacearum	64	32	8
Bacillus subtilis	32	64	32
B. megaterium	16	64	16
B. cereus	16	16	8
Escherichia coli	64	32	8
Pseudomonas aeruginosa	32	128	8
Serratia marcescens	128	64	32
Proteus vulgaris	128	128	16
Salmonella typhi	64	256	8

PE-V1: 4-(2-acetamidoethyl) phenyl acetate.
PE-V2: 4-((1, 4-dioxooctahydropyrrolo [1, 2-a] pyrazin-3-yl) methyl) phenyl acetate.
[a]Antibiotic: Tetracycline against bacteria.

compound PE-V1 was active against all the bacteria tested, and the best activity of this compound was recorded against *Staphylococcus aureus* (8 µg/ml), followed by *Streptococcus mutans*, *Staphylococcus epidermis*, *Bacillus megaterium*, and *Bacillus cereus* (16 µg/ml). Compound PE-V2 presented highest activity against *Bacillus cereus* (16 µg/ml), followed by *Streptococcus mutans* and *Staphylococcus epidermis* (32 µg/ml). Of the gram-negative bacteria, the microorganisms that presented highest sensitivity towards compound PE-V1 was *Pseudomonas aeruginosa* and *Xanthomonas campestris* (32 µg/ml), followed by *Salmonella typhi*, *Escherichia coli*, and *Xanthomonas malvacearum* (64 µg/ml). PE-V2 recorded highest activity against *Escherichia coli* and *Xanthomonas* sp. (*Xanthomonas campestris* and *Xanthomonas malvacearum*) (32 µg/ml). Tetracycline served as the positive control for the bacteria. Compared with the standard drug tetracycline, PE-V1 displayed high sensitivity against *Staphylococcus aureus* and *Streptococcus mutans*, whereas PE-V2 recorded sensitivity similar to the positive control (Table 1). Tetracycline, in other cases, showed good antibacterial activity over the metabolites of the strain.

Antifungal activity against dermatophytes and filamentous fungi and the corresponding MIC values are recorded in Table 2. The bioactive compounds showed low antifungal activity against the dermatophytes (*Candida albicans* and *Epidermophyton floccosum*) and the tested fungi. Among the

Table 2. Minimum inhibitory concentration (MIC) of bioactive compounds isolated from *Pseudonocardia endophytica* VUK-10 (MIC-(μg/ml)) against dermatophytes and fungi.

Dermatophytes	PE-V1	PE-V2	Antifungal[a]
Candida albicans	64	64	16
Epidermophyton floccosum	128	64	16
Fungi			
Aspergillus niger	128	128	16
Aspergillus flavus	64	64	8
Fusarium oxysporum	128	256	16
Fusarium solani	64	512	32
Penicillium citrinum	256	64	8
Verticillium alboatrum	ND	>512	64
Alternaria alternata	128	256	32

PE-V1: 4-(2-acetamidoethyl) phenyl acetate.

PE-V2: 4-((1, 4-dioxooctahydropyrrolo [1, 2-a] pyrazin-3-yl) methyl) phenyl acetate.

[a]Antifungal agent: Griseofulvin against yeast and Amphotericin-B against fungi.

ND: Not detected.

filamentous fungi tested, *Aspergillus flavus* and *Fusarium solani* recorded sensitivity of 64 μg/ml towards compound PE-V1, and *Verticillium alboatrum* recorded no activity up to 1,000 μg/ml. Compound PE-V2 was active against *Penicillium citrinum* and *Aspergillus flavus* at 64 μg/ml, and for this compound also *Verticillium alboatrum* recorded no activity up to 512 μg/ml. Both compounds recorded lower antifungal activity than the standard fungicides, Griseofulvin against dermatophytes and Amphotericin-B against fungi.

MTT Assay

The cytotoxicity of the purified compounds PE-V1 and PE-V2 was assayed against MDA-MB-231 (human breast adenocarcinoma cell line), HeLa (human cervical cancer cell line), MCF-7 (human breast adenocarcinoma cell line), and OAW-42 (human ovarian cyst adenocarcinoma cell line). The results exhibited that both compounds were active against the four cell lines.

The activity of PE-V1 against MDA-MB-231, HeLa, MCF-

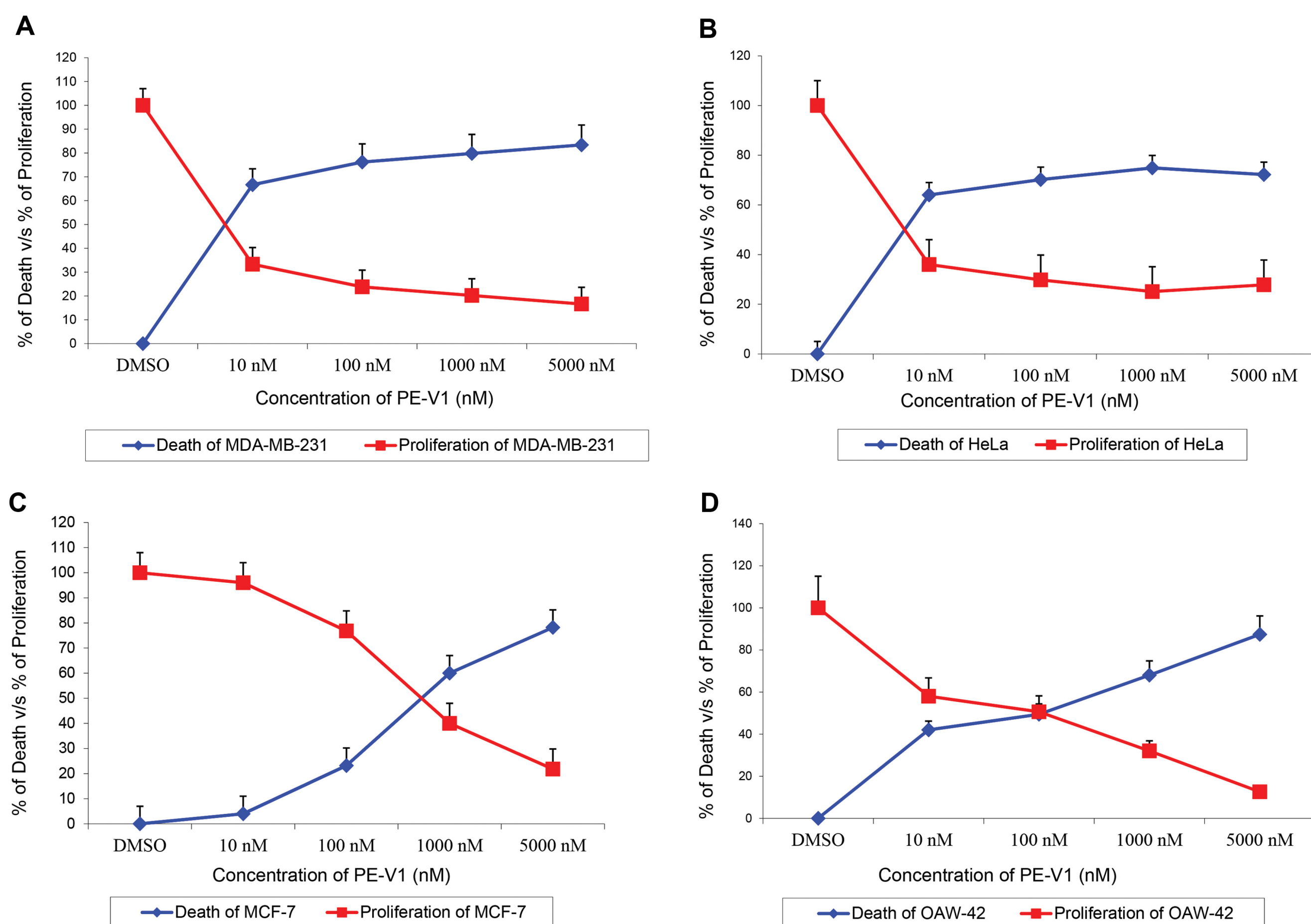

Fig. 3. Dose response curve of compound PE-V1 on the growth of (**A**) MDA-MB-231, (**B**) HeLa, (**C**) MCF-7, and (**D**) OAW-42 cancer cell lines.

7, and OAW-42 cell lines is presented in Figs. 3A, 3B, 3C, and 3D. Compound PE-V1 showed significant cytotoxicity when evaluated against MDA-MB-231, HeLa, MCF-7, and OAW-42 cell lines, displaying IC_{50} values of 10 nM (66.7%, 76.2%) (MDA-MB-231 and HeLa), 1,000 nM (60%) (MCF-7), and 100 nM (50%) (OAW-42).

The activity of PE-V2 against MDA-MB-231, HeLa, MCF-7, and OAW-42 cell lines is presented in Figs. 4A, 4B, 4C, and 4D. Compound PE-V2 exhibited potent cytotoxicity with MDA-MB-231, HeLa, MCF-7, and OAW-42 cell lines, exhibiting IC_{50} values of 10 nM (60.8%, 63.3%, 54%) (MDA-MB-231, HeLa, OAW-42) and 1,000 nM (50.2%) (MCF-7). Taxol, an anticancer drug used as the standard, recorded an IC_{50} value of 10 nM (59%, 60%, 57%, and 63%) against the MDA-MB-231, HeLa, OAW-42, and MCF-7 cell lines.

The genus *Pseudonocardia* is a prominent source of a new generation of potent bioactive compounds. Maskey *et al.* [23] isolated a new phenazine derivative, Phenazostatin D, from *Pseudonocardia* sp. B6273 that acts as a neuroprotective. Omura *et al.* [27] reported the antimicrobial activity of the antibiotics azureomycins A and B from *Pseudonocardia azurea* sp. Dekkar *et al.* [6] extracted new quinolone compounds from *Pseudonocardia* sp. with selective and potent anti *Helicobacter pylori* activity. New diazaanthraquinone derivatives such as Pseudonocardians A–C from a deep-sea actinomycete, *Pseudonocardia* sp. SCSIO 01299, exhibited potent antibacterial and cytotoxic activities [22]. Other bioactive compounds from *Pseudonocardia* include Pseudonocardones A, B, & C and 6-deoxy-8-*O*-methylrabilomycin [3], dentigerumycin [26], and NPP [20] that all exhibited potential antimicrobial and cytotoxic properties. In the present study, two bioactive metabolites, 4-(2-acetamidoethyl) phenyl acetate (PE-V1) and 4-((1, 4-dioxooctahydropyrrolo [1, 2-a] pyrazin-3-yl) methyl) phenyl acetate (PE-V2) of *Pseudonocardia endophytica* VUK-10, are reported. Comin and Keller [5] isolated 4-(2-acetamidoethyl) phenyl acetate (PE-V1) from the culture filtrates of *Streptomyces griseus*. The bioactive compound 4-((1, 4-dioxooctahydropyrrolo [1, 2-a] pyrazin-3-yl) methyl) phenyl

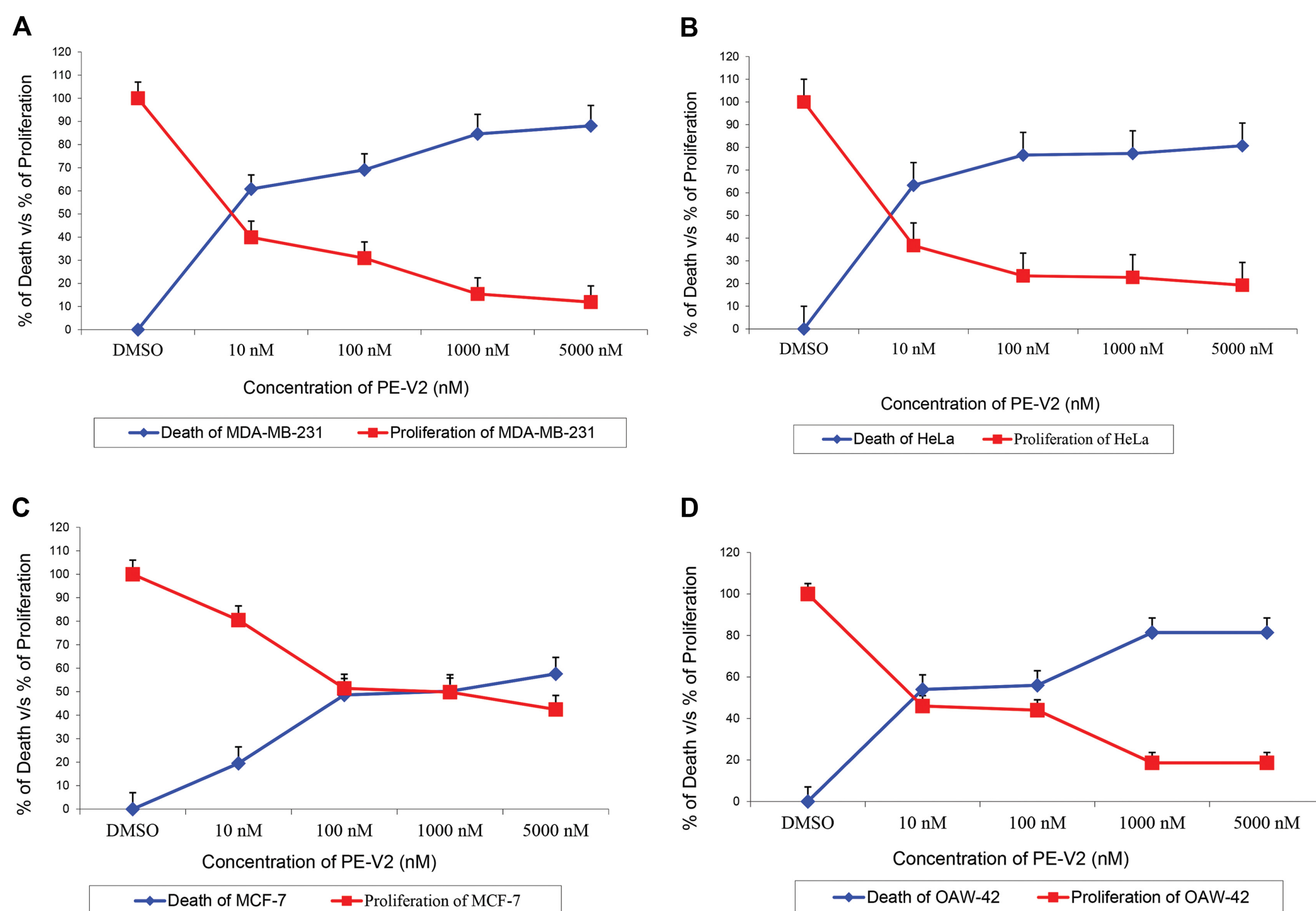

Fig. 4. Dose response curve of compound PE-V2 on the growth of (**A**) MDA-MB-231, (**B**) HeLa, (**C**) MCF-7, and (**D**) OAW-42 cancer cell lines.

acetate (PE-V2) isolated in this study is an identical derivative of a known compound, 3-(4-hydroxy benzyl) hexahydropyrrolo [1, 2-a] pyrazine-1, 4-dione [10, 32]. The two bioactive compounds of the present work, extracted from strain VUK-10, showed potent cytotoxic activity against MDA-MB-231, HeLa, MCF-7, and OAW-42 cell lines and antimicrobial activity against opportunistic and pathogenic bacteria and fungi. There is no information available on the antimicrobial and cytotoxic activities of the reported bioactive compounds, and this is the first report of the isolation and characterization of compounds PE-V1 and PE-V2 from genus *Pseudonocardia.*

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