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Isolation, Purification, and Characterization of Five Active Diketopiperazine Derivatives from Endophytic *Streptomyces* SUK 25 with Antimicrobial and Cytotoxic Activities

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In our search for new sources of bioactive secondary metabolites from *Streptomyces* sp., the ethyl acetate extracts from endophytic *Streptomyces* SUK 25 afforded five active diketopiperazine (DKP) compounds. The aim of this study was to characterize the bioactive compounds isolated from endophytic *Streptomyces* SUK 25 and evaluate their bioactivity against multiple drug resistance (MDR) bacteria such as *Enterococcus raffinosus, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumanii, Pseudomonas aeruginosa,* and *Enterobacter* spp., and their cytotoxic activities against the human hepatoma (HepaRG) cell line. The production of secondary metabolites by this strain was optimized through Thornton's medium. Isolation, purification, and identification of the bioactive compounds were carried out using high-performance liquid chromatography, high-resolution mass liquid chromatography-mass spectrometry, Fourier transform infrared spectroscopy, and nuclear magnetic resonance, and cryopreserved HepaRG cells were selected to test the cytotoxicity. The results showed that endophytic *Streptomyces* SUK 25 produces four active DKP compounds and an acetamide derivative, which were elucidated as *cyclo*-(ʟ-Val-ʟ-Pro), *cyclo*-(ʟ-Leu-ʟ-Pro), *cyclo*-(ʟ-Phe-ʟ-Pro), *cyclo*-(ʟ-Val-ʟ-Phe), and *N*-(7-hydroxy-6-methyl-octyl)-acetamide. These active compounds exhibited activity against methicillin-resistant *S. aureus* ATCC 43300 and *Enterococcus raffinosus,* with low toxicity against human hepatoma HepaRG cells. Endophytic *Streptomyces* SUK 25 has the ability to produce DKP derivatives biologically active against some MDR bacteria with relatively low toxicity against HepaRG cells line.

Keywords: Cytotoxicity, diketopiperazines, *Enterococcus raffinosus,* HepaRG, MRSA, *Streptomyces* SUK 25

Introduction

The development of antibiotic resistance among pathogenic bacteria has become a serious problem worldwide. The overuse of antibiotics in a number of situations is contributing to the increase in antibiotic resistance in microorganisms; therefore, the need for the detection and development of new and effective natural antibiotics is

important. *Streptomyces* represent a large group of micro-organisms that are ubiquitous in nature. Strain Universiti Kebangsaan 25 (SUK 25) is one strain that belongs to the genus *Streptomyces* sp., which was isolated from the root of *Zingiber spectabile* [1, 2]. SUK 25 exhibited potential inhibitory activity against some bacteria, especially against the methicillin-resistant *Staphylococcus aureus* (MRSA) strain during primary and secondary screenings. From SUK 25, several diketopiperazines (DKPs) were isolated, which were described from various natural resources [3]. DKPs create a large class of secondary metabolites synthesized mostly by human, plants, and microorganisms. DKPs are a class of cyclic dipeptides and characterized by a heterocyclic piperazine-2,5-dione synthesized from two amino acid units. Recently, the interest in these compounds has significantly increased because of their diverse and remarkable activity, and 90% of Gram-negative bacteria can produce DKPs [4]. Furthermore, DKPs have been isolated from Gram-positive bacteria [5], fungi [6], and higher marine organisms [7] as well.

The current study aimed to isolate and elucidate bioactive secondary metabolites from the endophyte *Streptomyces* SUK 25. The antimicrobial activity and cytotoxicity of some of the isolated compounds were evaluated. Cytotoxicity assay was carried out using the human hepatoma cell line named HepaRG, which is a unique cell line that differentiates canaliculae-like and hepatocyte-like cells retaining a drug metabolism capacity comparable to that of primary human hepatocytes and hepatoma carcinoma (HepG2) cells without inter-donor variability and functional instability with time in culture, which has been observed in primary cells [8–10]. In addition, these cells were obtained from a liver tumor cell of a female patient suffering from hepatocarcinoma and hepatitis C infection [11].

Materials and Methods

The fermentation, production, and extraction of secondary metabolites as well as chromatographic fractionation and purification of isolated compounds were carried out using reverse-phase open column chromatography and thin-layer chromatography (TLC) as described earlier [3].

Biological Activity
The antibacterial activity of the obtained compounds was tested against the multidrug resistance (MDR) pathogens *Enterococcus raffinosus, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumanii, Pseudomonas aeruginosa,* and *Enterobacter* sp., collectively referred to by the acronym ESKAPE. All strains were obtained from the culture collection of the Novel Antibiotic Laboratory,

Faculty of Health Sciences, Universiti Kebangsaan Malaysia. Antimicrobial assays were carried out using the disc diffusion agar method. Antibacterial bioassays were conducted according to Dobretsov and Qian [12]. Compounds were dissolved in 10% MeOH at a concentration of 1 mg/ml. Aliquots of 20 µl were soaked on filter paper discs (6 mm, No. 2668; Schleicher & Schüll, Germany). A standard antibiotic disc of gentamicin (20 µg/disc) was used as the positive control and 10% MeOH as the negative control for comparison. The paper discs were air dried at room temperature under sterilized conditions. Antibacterial effects were determined after 24 h of incubation at 37°C by measuring the diameter of the zone of inhibition.

MTT Cell Viability Assay for Cytotoxicity Testing
Cell viability was measured by the MTT colorimetric method [13]. This assay is based on the reduction of the soluble yellow 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) tetrazolium salt to a blue MTT formazan product by mitochondrial dehydrogenases. This assay was performed to determine the cytotoxicity of the two compounds namely, *cyclo*-(L-Val-L-Pro) and *cyclo*-(L-Phe-L-Pro). In this study, we followed the protocol from the manufacturer of cryopreserved HepaRG cells and media supplements (Millipore, USA). HepaRG cells were seeded in 96-well plates at a density of 10^4 cells per well and incubated in 5% CO_2 at 37°C for 24 h. The cells were then treated with varying concentrations from 5 to 120 µg/ml and incubated in 5% CO_2 at 37°C for 24 h. To determine the cell viability, 20 µl of 5 mg MTT/ml in PBS was added to each well and incubated in 5% CO_2 for 4 h at 37°C. The yellow MTT dye was reduced by succinic dehydrogenase in the mitochondria of viable cells to purple formazan crystals. Finally, the medium was carefully replaced with 200 µl of DMSO and mixed thoroughly to dissolve the formazan crystal product. Absorbance measurements were conducted utilizing the microplate spectrophotometer Nano-Quant infinite M200PRO at A_{570}. A positive control composed of 10% DMSO was used as a known cytotoxic natural agent that gives 100% lethality under the same conditions. The samples also included a blank (medium alone). All experiments were carried out with hepatocytes from three independent cell preparations for each compounds' concentration.

Cell viability was calculated using the following formula: Cell viability (%) = $\frac{\text{Mean OD of treated cells}}{\text{Mean OD of control cells}} \times 100$, where OD is the optical density. The half inhibitory concentration (IC_{50}) was defined as 50% reduction of absorbance compared with the control assay.

Statistical Analysis
The results were expressed as the mean ± SD for all experiments. Triplicate experiments of three independent HepaRG isolations were run, and three wells were used for each set of conditions. IC_{50} values were calculated using GraphPad Prism 5.

Spectroscopy and Structure Determination of Compounds
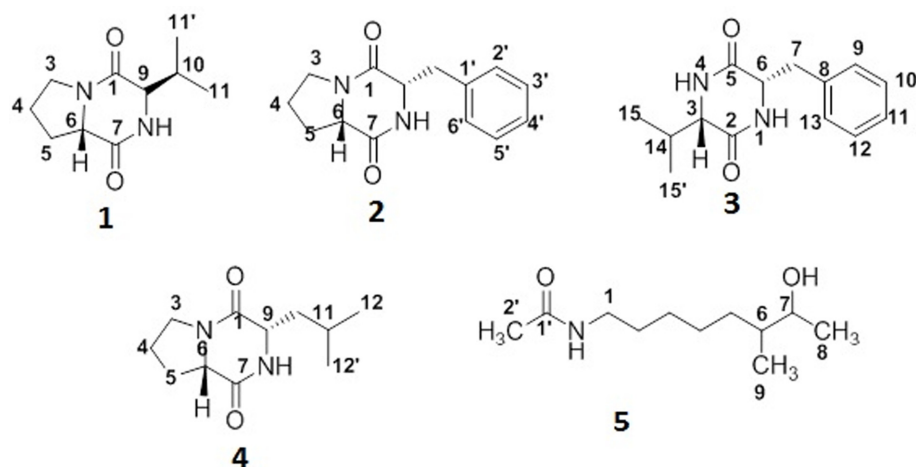Separation and final purification of the crude extract of each

Fig. 1. *Cyclo*-(L-Val-L-Pro) (**1**), *cyclo*-(L-Phe-L-Pro) (**2**), *cyclo*-(L-Leu-L-Pro) (**3**), *cyclo*-(L-Val-L-Phe) (**4**), and *N*-(7-hydroxy-6-methyl-octyl)-acetamide (**5**).

fraction were performed using high-performance liquid chromatography (HPLC) on an Agilent 1200 HPLC system equipped with a C-18 column (size 4.6 × 250 mm, 5 μm). In addition, the structure determination of compounds was determined using nuclear magnetic resonance (NMR) spectroscopy and FT–NMR spectroscopy at 600 MHz with Cryoprobe AVANCE III (Bruker, USA). For dereplication and exact mass measurements, HR-LCMS was measured on an Accela 600 HPLC pump with Accela autosampler coupled to a UV/Vis detector and an Orbitrap Exactive mass spectrometer (Thermo Fisher Scientific, Inc., USA). Analysis of samples was done using similar protocols described earlier by MacIntyre *et al*. [14]. Initially, the raw HR-LC/ESI/MS data were sliced into two data sets according to their ionization mode using the Mass Convert tool from ProteoWizard (http://proteowizard.sourceforge.net/). The sliced data were imported to MZMine 2.10 (http://sourceforge.net/projects/mzmine/), a software developed for the differential analysis of mass spectrometry data. The data processing step was performed in the same manner as explained previously by MacIntyre *et al*. [14] and Abdelmohsen *et al*. [15], albeit with slightly modified parameters. In this analysis, the data set was crop filtered from 0.1 to 35 min, and the retention time normalizer was not applied because only one batch of data was used. The processed data from MZMine was incorporated into the customized library through the built-in Excel MACRO for peak identification and dereplication. An algorithm was employed to use the molecular formula data set from Antibase 2013. The monoisotopic exact masses for each metabolite were then calculated to be used for the customized library. "Hits" and identified peaks were checked against the MS raw data in Xcalibur 2.2.

The FT-IR spectra were recorded on an FT-IR spectrophotometer (Perkin Elmer, USA) using the potassium bromide (KBr) disc method. Optical rotation was measured on an Autopol VI automatic polarimeter (Rudolph Research Analysis, USA). The melting point was measured on a Stuart apparatus (Bibby Scientific, UK).

Results

The ethyl acetate extracts from endophytic *Streptomyces* SUK 25 produced four DKP compounds and an acetamide derivative (Fig. 1). They were identified as *cyclo*-(L-Val-L-Pro (**1**), *cyclo*-(L-Phe-L-Pro) (**2**), *cyclo*-(L-Leu-L-Pro) (**3**), *cyclo*-(L-Val-L-Phe) (**4**), and *N*-(7-hydroxy-6-methyl-octyl)-acetamide (**5**), respectively, and were all isolated from fraction number II (FII; Table 1) after a series of chromatographic separation and purification using preparative HPLC. Structure elucidation was done by 1D and 2D NMR, as well as HR-LC/ESI/MS, as shown in Tables 2−5. The isolated DKPs **1**, **2**, **3**, and **4** were obtained as colorless solids from FII with melting points of 216−221°C. DKPs showed a UV absorbing band at 280 nm and turned violet upon spraying with anisaldehyde/sulfuric acid on a TLC silica plate. The NMR spectral data were comparable to those found in the literature [16, 17]. The NMR data (Tables 3 and 4) of the isolated DKP compounds gave characteristics ^{13}C NMR shifts at approx. 165 and 170 ppm for the amide unit in the ring system. The ^{1}H NMR resonance at the 4 ppm region is typical for a methine residue of an α-proton of an amino acid unit, and the DKPs are synonymous to a cyclic dipeptide. The obtained NMR data for the proline-bearing

Table 1. Pure weight, diameter of inhibition zone, and MIC and MBC values of active fractions of fraction II against MRSA ATCC 43300.

Fraction No.	Weight (mg)	I.Z (mm)	MIC (μg/ml)	MBC (μg/ml)
FII	51	15	8	64

Table 2. Name, formula, *m/z*, R$_T$, and O.R° of five active diketopiperazine compounds isolated from fraction II.

Compound name	Formula	*m/z*	R$_T$	O.R°-[α]D (*c* = 0.1, MeOH)
Cyclo-(L-Val-L-Pro) (**1**)	$C_{10}H_{16}N_2O_2$	197.1285	3.45	−324.6
Cyclo-(L-Phe-L-Pro) (**2**)	$C_{14}H_{16}N_2O_2$	245.1284	5.33	−138.2
Cyclo-(L-Leu-L-Pro) (**3**)	$C_{11}H_{18}N_2O_2$	211.1441	6.09	−310.4
Cyclo-(L-Val-L-Phe) (**4**)	$C_{14}H_{18}N_2O_2$	247.1440	7.64	−95.84
N-(7-Hydroxy-6-methyl-octyl)-acetamide (**5**)	$C_{11}H_{23}NO_2$	202.1802	1.25	−119.9[a]

[a](*c* 0.25 in CHCl$_3$).

Table 3. ^{1}H NMR and ^{13}C NMR spectral data of compounds **1–3** in MeOH-d_4.

Position	δ ^{13}C (ppm) **1**	δ ^{13}C (ppm)[a] **1**	δ ^{1}H (ppm), multiplicity (*J* in Hz) **1**	δ ^{13}C (ppm) **2**	δ ^{13}C (ppm)[a] **2**	δ ^{1}H (ppm), multiplicity (*J* in Hz) **2**	δ ^{13}C (ppm) **3**	δ ^{13}C (ppm)[a] **3**	δ ^{1}H (ppm), multiplicity (*J* in Hz) **3**
1	172.6 (s)	169.7		169.2	169.4		170.3	170.2	
3A	46.6 (t)	45.7	3.49 (m)	44.6	45.4	3.13–3.58	45.5	45.8	3.64–3.54
3B			3.54 (m)			3.13–3.58			3.64–3.54
4	23.6 (t)	22.3	1.92 (m)	21.6	22.5	1.24–1.84	22.7	23.6	2.22–1.98
5A	29.9 (t)	28.5	2.31 (ddd, 11.9, 6.9, 1.3)	27.9	28.3	2.31–2.37	28.1	28.4	4.04
5B			2.00 (m)			2.31–2.37			4.04
6	61.2 (d)	60.3	4.19 (dd, 6.9, 1.3)	58.7	59.0	4.46 (dd 10.6, 2.7)	59.0	59.2	4.15
7	167.6 (s)	164.6		165.7	164.7		166.1	166.2	
9	59.7 (d)	58.7	4.02 (d, 2.5)	56.3	56.1	4.10 (t, 7.6)	53.4	53.6	2.37
10	29.6 (d)	28.3	2.47 (q, 6.9, 6.9, 2.5)	36.3	36.7	3.19	38.6	38.9	2.22-1.98
11	16.4 (q)	16.0	0.93 (d, 6.9)				24.7	25.0	1.55
11′	18.6 (q)	19.2	1.08 (d, 6.9)						
12							23.3	23.1	1.03
12′							21.2	21.5	0.96
1′				136.4	135.6	7.22-7.38			
2′/6′				129.7	129.0	7.22-7.38			
3′/5′				128.0	128.8	7.22-7.38			
4′				127.0	127.3	7.22-7.38			

[a]NMR data were compared with those acquired by Li *et al.* [16] in CDCl$_3$.

DKPs were comparable to the L-DKPs described in the literature [17]. All optical rotation values for proline-containing DKPs (Table 2) gave a negative sign, indicating that the proline unit had the *S* configuration [18]. The IR spectrum (KBr film) V_{max} of the DKPs exhibited absorption at 3,348, 2,479, 2,070, 1,394, 1,092, 1,121, and 972 cm^{-1}, which indicated the presence of a C-N stretch and H-C-H-asymmetric stretch.

Compound **5**, *N*-(7-hydroxy-6-methyl-octyl)-acetamide, was obtained as a colorless oil from FII as well, showing an UV absorbing band at 280 nm. It gave a green coloration with anisaldehyde/sulfuric acid when sprayed on TLC silica plate. Data of the IR spectrum (KBr film) yielded V_{max} absorbance at 3,356, 3,256, 2,966, 2,931, 1,683, 1,650, 1,530, and 1,447 cm^{-1}. The optical rotation exhibited an [α]D^{25} value of −119.9 (*c* 0.25 in CHCl$_3$). ^{1}H and ^{13}C NMR spectroscopies were used for the characterization of compound **5**, which was elucidated as an aliphatic branched-amide. The NMR data (Table 5) suggested that this subfraction is similar to that of *N*-(7-hydroxy-6-methyl-octyl)-acetamide isolated from terrestrial *Streptomyces* sp. GT 2005/079 [19]. The *m/z* value of this compound was 202.1802 [M + H]$^+$ eluting at 1.25 min. The molecular formula $C_{11}H_{23}NO_2$ was supported by the integration and multiplicity obtained from the ^{1}H and ^{13}C NMR data, respectively. *N*-(7-Hydroxy-6-methyl-octyl)-acetamide was earlier described to exhibit moderate

Table 4. ^{1}H NMR and ^{13}C NMR data of compound **4** in MeOD[b].

Position	δ ^{1}H (ppm), multiplicity (J in Hz)	δ ^{13}C (ppm)	δ 13C (ppm)[a]
1-N*H*	8.0 brs		
2		168.4 (s)	167.41
3	3.66 (dd, 4.4. 1.6)	59.2 (d)	60.1
4-N*H*	8.0 brs		
5		169.1 (s)	67.3
6	3.34 (dt, 4.9, 1.4)	55.0 (d)	55.9
7A	3.26 (dd, 1.1, 3.8)	37.8 (t)	38.7
7B	3.01 (dd, 13.8. 4.7)		
8		136.3 (s)	137.2
9/13	7.22 (d, 6.9)	130.3 (d)	131.2
10/12	7.28 (t, 6.8)	127.9 (d)	128.8
11	7.23 (t, 7.2)	126.4 (d)	127.3
14	1.6 m	31.0 (d)	31.9
15	0.79, (t, 6.9)	18.2 (q)	19.1
15′	0.42, (t, 6.9)	16.2 (q)	17.1

[a]NMR data were compared with those acquired by Stark and Hofmann [17] in DMSO-d$_6$.

[b]MeOD, Deuterated methanol.

Table 5. ^{1}H NMR and ^{13}C NMR data of compound **5** in MeOD[a].

Position	δ ^{1}H (ppm) multiplicity (J in Hz)	δ ^{13}C (ppm)
1	3.66	39.6
2	1.25–1.38	32.3
3	1.25–1.38	26.7
4	1.25–1.38	27.1
5	1.25–1.38	29.5
6		39.9
7	3.22	71.6
8	1.18	19.4
9	1.18	14.5
1′		170.1
2′	1.97	23.3

[a]MeOD, Deuterated methanol.

activity against *Mucor miehei* [19]. However, in this current study, compound **5** displayed activity against *Enterococcus raffinosus*.

Biological Activity

Fraction FII exhibited activity against MRSA and *Enterococcus raffinosus*. The isolated pure compounds from

Table 6. Antimicrobial activity of the subfraction FII against ESKAPE pathogens by the disk diffusion method.

Name of bacteria (ATCC)	1	2	3	4	5	Gentamicin
ER (49464)	n.a.	n.a.	13	14	13	20
MRSA (43300)	15	n.a.	n.a.	n.a.	n.a.	22
KP	n.a.	n.a.	n.a.	n.a.	n.a.	18
AB	n.a.	n.a.	n.a.	n.a.	n.a.	17
PA (87853)	n.a.	n.a.	n.a.	n.a.	n.a.	17
EA (51697)	n.a.	n.a.	n.a.	n.a.	n.a.	20

The determined inhibition zone was in milliliter unit.

ER, *Enterococcus raffinosus*; SA, *Staphylococcus aureus*; KP, *Klebsiella pneumoniae*; AB, *Acinetobacter, baumanii*; PA, *Pseudomonas aeruginosa*; and EA, *Enterobacter aerogenes*; and n.a., no activity.

Table 7. Data analysis from GraphPad Prism 5 to determine the IC$_{50}$ of compounds **1** and **2** against the HepaRG cell line.

Log (inhibitor) vs. normalized response - Variable slope	1	2
Log IC$_{50}$	2.042	2.014
Hill slope	1.242	1.137
IC$_{50}$	110.1	103.2
Log IC$_{50}$	0.02299	0.01765
Hill slope	0.1191	0.08189
Confidence intervals	95%	95%
Log IC$_{50}$	1.983 to 2.101	1.968 to 2.059
Hill slope	0.9354 to 1.548	0.9261 to 1.347
IC$_{50}$	96.09 to 126.2	92.96 to 114.6
Degrees of freedom	5	5
R²	0.9843	0.9913
Absolute sum of squares	34.8	19.82
Sy.x	2.638	1.991

FII were tested against ESKAPE pathogens. Compound **1** showed bioactivity only against MRSA 43300 with a zone of inhibition at 15 mm. Compounds **3, 4,** and **5** were active only against *Enterococcus raffinosus* with a zone of inhibition at 13, 14, and 13 mm, respectively. The pure compounds were examined against the different test microorganisms, as shown in Table 6.

Cytotoxic Effects of C1 and C2 on HepaRG

Tested compounds **1** and **2** showed no toxicity towards HepaRG cells, with IC$_{50}$ values of 110.1± 1.2 and 103.2 ± 1.24 µg/ml for **1** and **2**, respectively, as shown in Table 7 and Fig. 2.
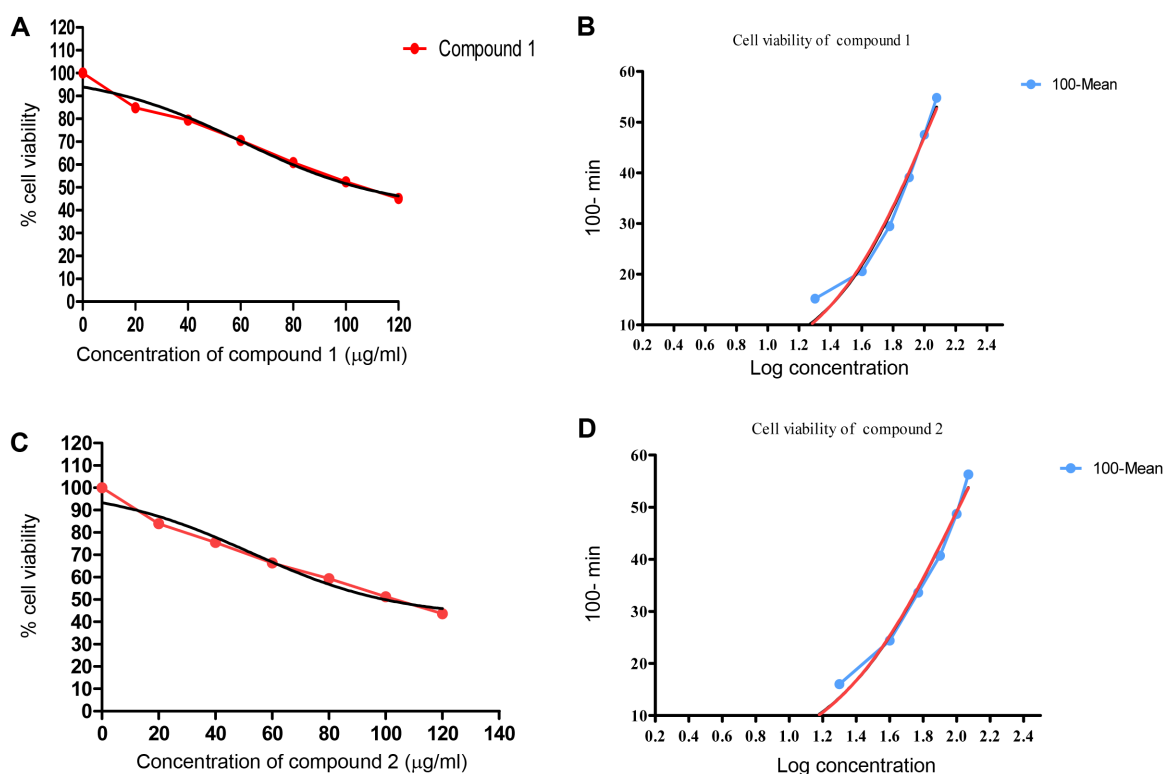
Fig. 2. Cytotoxic effects of compounds **1** and **2** on a cancer cell line (HepaRG).

Data are represent by the mean±SD of three independent experiments with each experiment conducted in triplicate. (**A**) and (**C**) present IC_{50} values of compounds **1** and **2**; 110.1 ± 1.2 and 103.2 ± 1.2 respectively. (**B**) and (**D**) IC_{50} values of compounds **1** and **2** using GraphPad Prism in Log concentration Log (inhibitor) vs. normalized response - Variable slope.

Discussion

The antimicrobial bioactivity and cytotoxicity of the isolated compounds were evaluated using several types of bacteria and HepaRG cells, respectively. The ethyl acetate extract of *Streptomyces* SUK 25 produced four active DKP compounds and a *N*-(7-hydroxy-6-methyl-octyl)-acetamide. The DKPs were identified as *cyclo*-(L-Val-L-Pro) (**1**), *cyclo*-(L-Phe-L-Pro) (**2**), *cyclo*-(L-Leu-L-Pro) (**3**), and *cyclo*-(L-Val-L-Phe) (**4**). All pure compounds were isolated from fraction FII after a series of chromatographic separation and purification by HPLC. Structure elucidation was accomplished by HR-LC ESI MS and NMR. The findings from this study are simillar to an earlier work [20] on the isolation of four cyclic DKP antibiotics, identified as streptocidins A−D, from the mycelial extract of *Streptomyces* sp. Tü 6071. Moreover, *cyclo*-(valyl-prolyl) has been isolated from the marine actinomycete B 1758 [21], as well as *cyclo*-(L-Leu-L-Pro) from *Streptomyces* sp. KH-614, which is taxonomically related to *Streptomyces lydicus*. *Cyclo*-(L-Leu-L-Pro) demonstrated antibacterial activity against VRE strains such as *E. faecalis*

(K-99-34), *E. faecalis* (K-00-184), and *E. faecalis* (K-00-221) with MIC values of 12.5 µg/ml. Moreover, *cyclo*-(L-Leu-L-Pro) was also described to be effective against three leukemic cell lines at concentrations below 100 µg/ml [22]. *Cyclo*-(L-Gly-L-Pro) and *cyclo*-(L-Phe-4-OH-L-Pro) were isolated from cultures of the marine bacterium *Pseudoalteromonas luteoviolacea* [23]. A combination of *cyclo*-(L-Leu-L-Pro) and *cyclo*-(L-Phe-L-Pro) exhibited increased bioactivity against *Escherichia coli*, *Staphylococcus aureus*, *Micrococcus luteus*, *Candida albicans*, and *Cryptococcus neoformans* with MIC values of 0.25−0.5 µg/ml [24]. Furthermore, seven DKPs known as *cyclo*-(L-Leu-L-Pro), *cyclo*-(L-Phe-L-Pro), *cyclo*-(Gly-L-Pro), *cyclo*-(L-Pro-L-Pro), *cyclo*-(L-Pro-L-Val), *cyclo*-(L-Leu-L-trans-4-OH-Pro), and *cyclo*-(L-Phe-L-*trans*-4-OH-Pro) were isolated from a fermentation broth of *Aspergillus fumigatus*. These fungal DKPs exhibited weak antibacterial activity, inhibiting the growth of *Staphylococcus aureus* and *Micrococcus luteus* at the concentration of 2.9 mmol/l [25]. In addition, *cyclo*-(L-Leu-L-Pro), also known as gancidin W, from two *Streptomyces* species inhibited the growth of VRE-resistant *Enterococcus faecalis* strains with MIC values of 12.5 µg/ml

[26]. The marine sponge *Axinella* sp. also yielded six DKPs identified as *cyclo*-(threonyl-prolyl), *cyclo*-(seryl-prolyl), *cyclo*-(alanyl-prolyl), *cyclo*-(leucyl-prolyl), *cyclo*-(valyl-prolyl), and *cyclo*-(glycyl-prolyl) [27]. Along with the cyclic tetrapeptide staphylopeptide A, five DKPs, *cyclo*-(L-Val-L-Pro), *cyclo*-(L-Tyr-L-Pro), *cyclo*-(L-Ala-L-Pro), and (L-Phe-L-Trp) were isolated from the culture broth of the marine bacterium *Staphylococcus* sp. no. P-100826-4-6 [28].

HepaRG cells were obtained from a liver tumor of a female patient suffering from hepatocarcinoma and hepatitis C infection [11]. The HepaRG cell line has been described as a tool in the prediction of hepatotoxicity in preclinical drug development [11, 29]. The ability of these cells to express cytochrome P450 as well as nuclear receptors, the major hepatic membrane transporters, phase II enzymes, and antioxidant enzymes, particularly in their differentiated state, makes them a valuable in vitro model to perform drug metabolism and toxicity studies [9]. In the present work, compounds **1** and **2** showed no toxicity towards HepaRG cells, with IC_{50} values of 110.1 ± 1.2 μg/ml, and 103.2 ± 1.24 μg/ml, respectively, as shown in Table 7 and Fig. 2. The isolated compounds were categorized as non-toxic as recommended by the National Cancer Institute. IC_{50} values of crude extracts over 30 μg/ml were defined as negative cytotoxicity. The results from this study is comparable to an earlier work where a cyclodipeptide mixture obtained from *Pseudomonas aeruginosa* strain PAO1 gave IC_{50} values of 530 and 660 μg/ml, against HeLa and Caco-2 cells, respectively [30].

The most important biological activities of DKPs, such as tryprostatins A and B, were demonstrated to treat tsFT210 cells at a IC_{50} concentration of 6.25, 50, and 12.5 μg/ml, respectively. The DKPs arrested tsFT210 cells in the G2/M phase [31, 32]. DKPs have displayed the ability to act as a plasminogen activator inhibitor-1 (PAI-1) [33, 34]. The mechanism of action of DKPs may lead to cause alteration of cardiovascular and blood-clotting functions [35]. The effects of acetylapoaranotin, acetylaranotin, and deoxyapoaranotin DKPs on cell proliferation were tested in vitro using five cancer cell lines (HCT116, AGS, A549, MCF-7, and HepG2) and showed potent inhibition of cell proliferation in four cancer cell lines with the exception of HepG2 cells [36]. A mixture of DKPs that consisted of *cyclo*-(L-Pro-L-Tyr), *cyclo*-(L-Pro-L-Val), and *cyclo*-(L-Pro-L-Phe) isolated from the *P. aeruginosa* PAO1 strain was applied to human cells grown in serum containing CM medium. The results obtained showed that these DKPs caused a decrease in the viability of HeLa and Caco-2 cell cultures, exhibiting 75% dead cells following treatment with the DKP mix at 100 mg/ml. The IC_{50} for that mix was 0.53 and 0.66 mg/ml for HeLa and Caco-2 cells, respectively. In addition, DKPs have the capability to inhibit the activity of regulatory LuxR-type proteins that are involved in *N*-acylhomoserine lactone dependent quorum sensing signaling [30].

In conclusion, this study demonstrates that endophytic *Streptomyces* SUK 25 has the ability to produce various DKP derivatives with potential bioactivity against some pathogenic bacteria such as MRSA and *Enterococcus raffinosus,* with low toxicity against HepaRG cells. This finding presents a good potential natural source of new drugs for the treatment of some infectious diseases.

Acknowledgments

References

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