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Antibacterial activity of 1-methyl ester-nigericin from *Streptomyces hygroscopicus* BRM10; an endophyte in *Alpinia galanga*

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ABSTRACT

Strain BRM10 was isolated from the root tissues of *Alpinia galanga* Swartz. and identified as *Streptomyces hygroscopicus* BRM10 on the basis of morphology, chemotaxonomy and 16SrDNA sequencing. It was an antagonist of some bacteria, *Staphylococcus aureus* ATCC25932, *Bacillus cereus* ATCC6633, *Escherichia coli* ATCC10536 and *Pseudomonas aeruginosa* ATCC27853. The culture filtrate and the crude extract from *S. hygroscopicus* BRM10 were all inhibitory to tested bacteria. The major active ingredients from the crude extract were purified by silica gel column chromatography, thin-layer chromatography and identified to be 1-methyl ester-nigericin (compound **1**) and nigericin (compound **2**) by NMR and mass spectral data, respectively. Bioassay studies showed that compound **1** had antibacterial activity against Gram positive bacteria lower than compound **2**, and its minimum inhibitory concentrations against *Staphylococcus aureus* ATCC25932 and *Bacillus cereus* ATCC6633 were 0.5 µg/ml and 1.0 µg/ml, respectively and no inhibitory activity was observed against *Escherichia coli* ATCC10536 and *Pseudomonas aeruginosa* ATCC27853, at a concentration of 64 µg/ml.

INTRODUCTION

Endophytes are microorganisms that colonize inside plant tissue (Lodewyckx *et al*., 2002). They habitat in tissues below the plant's epidermal cell layers due to the fact that some host plant's tissues are transiently asymptomatic (Sardi *et al*., 1992; Tharek *et al*., 2011). Almost all vascular plants harbor several endophytic actinomycetes, which would help to improve the host plant's growth and promotional activity against diseases (Sachiko *et al*., 2006; Shimizu *et al*., 2000; Taechowisan *et al*., 2003). Bioactive compounds from endophytic actinomycetes have distinct chemical structures that may form the basis for synthesis of new drugs. Several reports refer to endophytic actinomycetes produced secondary metabolites and the novel antibiotics for example: Munumbicins from *Streptomyces* sp. NRRL 30562, an endophyte of *Kennedia nigriscans* (Castillo *et al*., 2002), Kakadumycins from *Streptomyces* sp. NRRL 30566, an endophyte of *Grevillea pteridifolia* (Castillo *et al*., 2003), Coronamycins from *Streptomyces* sp. MSU-2110, endophyte of *Monstera* sp. (Ezra *et al*., 2004), p-aminoacetophenonic acids from *Streptomyces griseus*, an endophyte of *Kandelia candel* (Guan *et al*., 2005), 6-alkylsalicylic acids from *Streptomyces laceyi* MS53, an endophyte of *Ricinus communis* L. (Kim *et al*., 2006) and p-aminoacetophenonic acids from *Streptomyces* sp. HK10552, an endophyte of *Aegiceras corniculatum* (Wang *et al*., 2010). We report here the isolation and identification of *Streptomyces hygroscopicus* BRM10, an endophyte of *Alpinia galanga* Swartz. Extraction of the culture medium of the strain BRM10 afforded 1-methyl ester-nigericin (a novel nigericin), which displayed strong antibacterial activity against Gram positive bacteria.

MATERIALS AND METHODS

Organisms and media

Streptomyces hygroscopicus BRM10 was isolated from the root tissues of *Alpinia galanga* Swartz. by the surface-sterilization technique (Taechowisan *et al*., 2003). Identification of the isolate to species level was based on morphology, chemotaxonomy, physiology and also 16S rDNA sequencing as described by Taechowisan and Lumyong (2003). Solid medium for sporulation used in this study was International Streptomyces Project Medium 4 (ISP-4) and the liquid medium used for fermentation was ISP-2 (Shirling and Gottlieb, 1966). The bacteria *Staphylococcus aureus* ATCC25932, *Bacillus cereus* ATCC6633, *Escherichia coli* ATCC10536 and *Pseudomonas aeruginosa* ATCC27853 were used for antibacterial assay. They were grown on nutrient agar (NA).

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Preparation of the crude extract

A spore suspension of the strain BRM10 was prepared in distilled water from cultures grown on ISP-4 medium at 30 ^{o}C for 10 days. The suspension, 10^8 spores per 100 ml of liquid medium, was added to ISP-2 broth in each 500-ml Erlenmeyer flask. Cultures were kept on a shaker at 120 rpm at 30 ^{o}C for 48 h and used as seed stocks. For large production of culture filtrates, the strain BRM10 was grown in a modified 3000 ml glass container containing 1500 ml of ISP-2 broth, and incubated in an orbital shaker for 5 days in the same condition. The 5-day-old cultures were filtrated by Whatman paper No. 1 under vacuum. The mycelial mats were washed with distilled water and separated by centrifugation at 5000 rpm for 20 min. The culture filtrate and mycelial mats of the strain BRM10 were extracted three times with 1/3 volumes of ethyl acetate. This organic solvent was pooled and then taken to dryness under flash evaporation at 40 ^{o}C. The yield of dry material per litre was about 1100 mg.

Purification of the compounds

The residue of 1000 mg was dissolved in 10 ml of hexane and fractionated on column chromatography (Merck silica gel 60, 35-70 mesh) with gradients hexane and ethyl acetate (2:1 and 1:1). The combined fractions eluted with 50% ethyl acetate in hexane, (274 mg) were further separated by TLC (Merck, Si gel 60, 0.5 mm; hexane : ethyl acetate = 60 : 40) to give 68 mg and 53 mg of pure compounds **1** and **2**, respectively.

Structure elucidation of the compounds

The structures of the active compounds have been identified using NMR and mass spectral data. The melting point of the compounds was determined on a Buchi-540 melting point apparatus. Optical rotations were measured on a Perkin-Elmer 241 polarimeter, IR spectra on a Perkin-Elmer 1 spectrometer, ^{1}H- and ^{13}C-NMR spectra on a Bruker DRX 500 spectrometer, and EI-MS and FAB-MS respectively on a Hewlett-Packard 5989 B and a Finnigan/Thermo Quest Mat 95 XL mass spectrometer.

Antagonism and antibacterial activity assay

An *in vitro* plate assay technique was used to test the antagonistic effect of the strain BRM10 on the bacteria as described in the previous report (Taechowisan *et al*., 2008). For antibacterial activity assay, the culture filtrate, crude extract, or purified compounds were tested for antibiosis against the bacteria using the paper disk method (Taechowisan *et al*., 2008). Two pieces of 8-mm sterile paper disks (Advantec, Toyo Roshi Kaisha, LTD., Japan) were respectively soaked in culture filtrate, crude extract and each of two purified compounds (50 µg/disc). The air-dried discs were places on NA plates. Each plate was then poured with melt soft agar containing 10^5 CFU/ml of the bacteria. The plates were incubated at 37^{o}C for 24 hr. The area of the zone of inhibition was measured. Each treatment consisted of three replicates.

Minimum inhibitory concentration

The minimum inhibitory concentrations of the compound was tested against *Staphylococcus aureus* ATCC25932, *Bacillus cereus* ATCC6633, *Escherichia coli* ATCC10536 and *Pseudomonas aeruginosa* ATCC27853 in a 96-well microtiter plate. The compound was twofold diluted from 0.5 µg/ml to 64 µg/ml in nutrient broth supplemented with 10% glucose containing 0.01% phenol red as colour indicator (NBGP). Bacteria was adjusted to 10^5 CFU/ml for each microtiter plate. The microtiter plates were incubated at 37^{o}C for 24 hours. Microbial growth was determined by observing the change of colour in the wells (red to yellow when there is microbial growth). The lowest concentration that showed no change of colour was considered as the minimum inhibitory concentrations (MIC). For the determination of the minimum bactericidal concentration (MBC), a portion of liquid (1 ml) from each well that showed no change of colour was plated on Mueller Hinton Agar (MHA) and incubated accordingly for 24 h. The lowest concentration that yielded no growth after this sub-culturing was taken as the MBC.

Identification of the strain BRM10

The morphology was observed under light microscope. The carbon utilization test was carried on 1% carbon source in basal medium (ISP-9). Diaminopimelic acid from the whole-cell extract was analyzed using the procedure of Becker *et al*. (1964). Genomic DNA was isolated using the procedure of Hopwood *et al*. (1985). 16S rDNA was amplified by PCR by the method of Taechowisan and Lumyong (2003). The purified PCR fragment was cloned into pTG19-T vector (Vivantis, Canada) and sequenced using the dideoxy chain termination method with a BigDye Terminator Cycle Sequencing kit (Applied Biosystems, USA). The sequence was compared with similar sequences from the reference organisms contained in the BLAST database (a nucleotide database of the National Center for Biotechnology Information. A phylogenetic tree was reconstructured by using MEGA 4.0 software (Tamura *et al*., 2007).

RESULTS AND DISCUSSION

Identification of endophytic Streptomyces strain BRM10

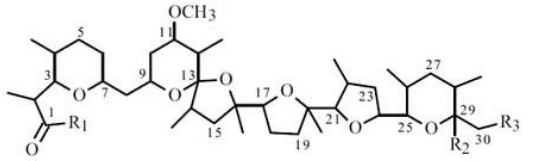
An endophytic *Streptomyces* strain BRM10 was isolated from a surface-sterilized root of *Alpinia galanga* Swartz. The morphological observation, spore chains are spirales, the spore surface is rough. The spore mass is gray, becoming black when it is mature. The reverse side of colony is pale yellow, becoming brown when it is mature. Melanoid pigments are not formed, and soluble pigments are not produced. The following carbohydrates are utilized for growth: D-fructose, D-glucose, D-mannitol, D-xylose, L-arabinose, *i*-inositol and rhamnose. Raffinose and sucrose are not utilized for growth. Based on results in morphological observation as well as on the presence of LL-type diaminopimelic acid in the whole-cell extracts, endophytic actinomycetes BRM10 was identified as belonging to the genus *Streptomyces*. Almost the complete 16S rDNA sequence was determined for the endophytic *Streptomyces* sp. BRM10 from position 8 to position 1523. BLAST search results for strain BRM10 came from GenBank; when reference sequences were

Table 1. Comparison of ^{1}H NMR and ^{13}C NMR Spectra of nigericin, 1,29-*O*-dimethyl-30-acetyl nigericin and 1-methyl ester-nigericin and in $CDCl_3$ (mult, *J*/Hz)

C atom position	C atom group	nigericin[a]		1,29-*O*-dimethyl-30-acetyl nigericin[a]		1-methyl ester-nigericin	
		δ_C (125 MHz)	δ_H (500 MHz)	δ_C (125 MHz)	δ_H (500 MHz)	δ_C (75 MHz)	δ_H (300 MHz)
1	C=O	177.5 s	-	176.4 s	–	176.5	-
MeO-1	OCH_3	-	-	51.7 q	3.74 (s)	51.7	3.71 (s)
2	CH	44.2 d	2.22 (m)	43.1 d	2.65 (m)	43.0	2.53 (m)
2a	CH_3	13.06 q	1.24 (m)	12.7 q	1.05 (m)	12.9	1.04 (m)
3	CH	73.0 d	3.63 (m)	73.6 d	3.77 (m)	73.7	3.76 (m)
4	CH	27.5 d	1.49 (m)	27.7 d	1.81 (m)	27.6	1.78 (m)
4a	CH_3	10.8 q	1.05 (m)	10.9 d	0.97 (d, J = 8.40)	10.5	0.93 (m)
5	CH_2	25.7 t	1.42 (m)	25.0 t	1.66 (m)	25.8	1.50 (m)
			1.49 (m)		1.96 (m)		1.90 (m)
6	CH_2	23.1 t	1.74 (m)	21.5 t	2.06 (m)	21.9	2.04 (m)
7	CH	69.0 d	3.84 (m)	69.6 d	4.10 (m)	69.5	4.12 (m)
8	CH_2	35.2 t	1.13 (m)	36.6 t	0.97 (m)	36.5	0.96 (m)
			2.50 (m)		2.50 (m)		2.53 (m)
9	CH	60.3 d	4.03 (m)	60.4 d	4.19 (m)	60.4	4.14 (m)
10	CH_2	31.7 t	1.90 (m)	36.4 t	2.31 (m)	33.0	2.24 (m)
11	CH	78.0 d	3.27 (m)	78.8 d	3.40 (m)	78.6	3.29 (m)
MeO-11	OCH_3	57.4 q	3.34 (s)	58.2 q	3.31 (s)	57.8	3.34 (s)
12	CH	37.1 d	1.49 (m)	36.5 d	1.81 (m)	36.9	1.73 (m)
12a	CH_3	13.13 q	1.12 (m)	12.93 q	1.03 (m)	13.10	1.02 (m)
13	C	108.2 s	-	107.3 s	–	107.9	-
14	CH	39.0 d	1.74 (m)	39.5 d	2.07 (m)	39.5	2.11 (m)
14a	CH_3	13.3 q	0.88 (m)	12.99 q	0.90 (d, J = 8.54)	13.13	0.95 (m)
15	CH_2	43.2 t	1.49 (m)	43.2 t	1.60 (m)	41.4	1.55 (m)
			1.42 (m)		1.78 (m)		1.85 (m)
16	C	81.5 s	-	83.6 s	–	82.6	-
16a	CH_3	28.0 q	2.18 (s)	23.9 q	1.25 (s)	26.2	1.23 (s)
17	CH	82.4 d	3.36 (m)	80.0 d	3.40 (m)	82.1	3.59 (m)
18	CH_2	26.1 t	1.49 (m)	27.6 t	1.49 (m)	26.6	1.51 (m)
			1.42 (m)		1.81 (m)		1.79 (m)
19	CH_2	31.0 t	1.35 (m)	33.0 t	1.37 (m)	32.1	1.37 (m)
			1.75 (m)		2.20 (m)		2.17 (m)
20	C	83.5 s	-	83.7 s	–	83.8	-
20a	CH_3	22.7 q	2.11 (s)	23.0 q	1.15 (s)	22.9	1.12 (s)
21	CH	85.8 q	3.76 (m)	86.1 d	3.87 (m)	86.2	3.90 (m)
22	CH	35.1 d	1.80 (m)	35.7 d	2.20 (m)	34.6	1.81 (m)
22a	CH_3	15.6 q	0.87 (m)	15.8 q	0.87 (d, J = 7.12)	15.6	0.91 (m)
23	CH_2	32.3 t	1.35 (m)	36.4 t	1.35 (m)	33.9	1.31 (m)
			1.80 (m)		2.20 (m)		1.82 (m)
24	CH	76.7 d	4.20 (m)	77.3 d	4.25 (m)	76.9	4.29 (m)
25	CH	74.4 d	3.74 (m)	76.8 d	4.07 (m)	75.8	3.80 (m)
26	CH	32.5 d	1.35 (m)	32.6 d	1.37 (m)	32.7	1.37 (m)
26a	CH_3	17.3 q	0.86 (m)	17.5 q	0.84 (d, J = 8.34)	17.3	0.88 (m)
27	CH_2	37.2 t	1.35 (m)	39.6 t	1.46 (m)	37.1	1.37 (m)
28	CH	35.7 d	1.42 (m)	35.8 d	1.40 (m)	35.4	1.70 (m)
28a	CH_3	16.3 q	0.92 (m)	16.0 q	1.66 (m)	16.1	0.91 (m)
29	C	97.0 s	-	98.1 s	-	97.2	-
MeO-29	OCH_3	-	-	48.3 q	3.27 (s)	-	-
30	CH_2	68.3 t	3.73 (m)	64.9 t	3.27 (s)	67.7	3.58 (m)
			4.25 (m)	21.0 q	4.25 (m)		4.30 (m)
AcO-30	$CH_3\underline{C}OO$			171.1 s	-		
	$\underline{CH_3}COO$			48.3 q	2.09 (s)		

[a] reported by Wu *et al*. (2009).

chosen. The BLAST search results and the phylogenetic tree generated from representative strains of the related genera showed that strain BRM10 had high levels of sequence similarity to species of *Streptomyces hygroscopicus* subsp. *hygroscopicus* (accession number: AB045864) (Figure 1). 16S rDNA analysis revealed that strain BRM10 is phylogenetically closely related to *Streptomyces hygroscopicus* (the sequence similarity levels were 99%). The nucleotide sequence data reported in this paper appeared in the GenBank, EMBL and DDBJ databases with accession number AB723804.



nigericin : $R_1 = R_2 = R_3 = OH$
1,29-*O*-dimethyl-30-acetyl nigericin : $R_1 = OCH_3$, $R_2 = OH$, $R_3 = CH_3COO$
1-methyl ester-nigericin : $R_1 = OCH_3$, $R_2 = R_3 = OH$

Fig 1. Chemical structures of nigericin and 1-methyl ester-nigericin.

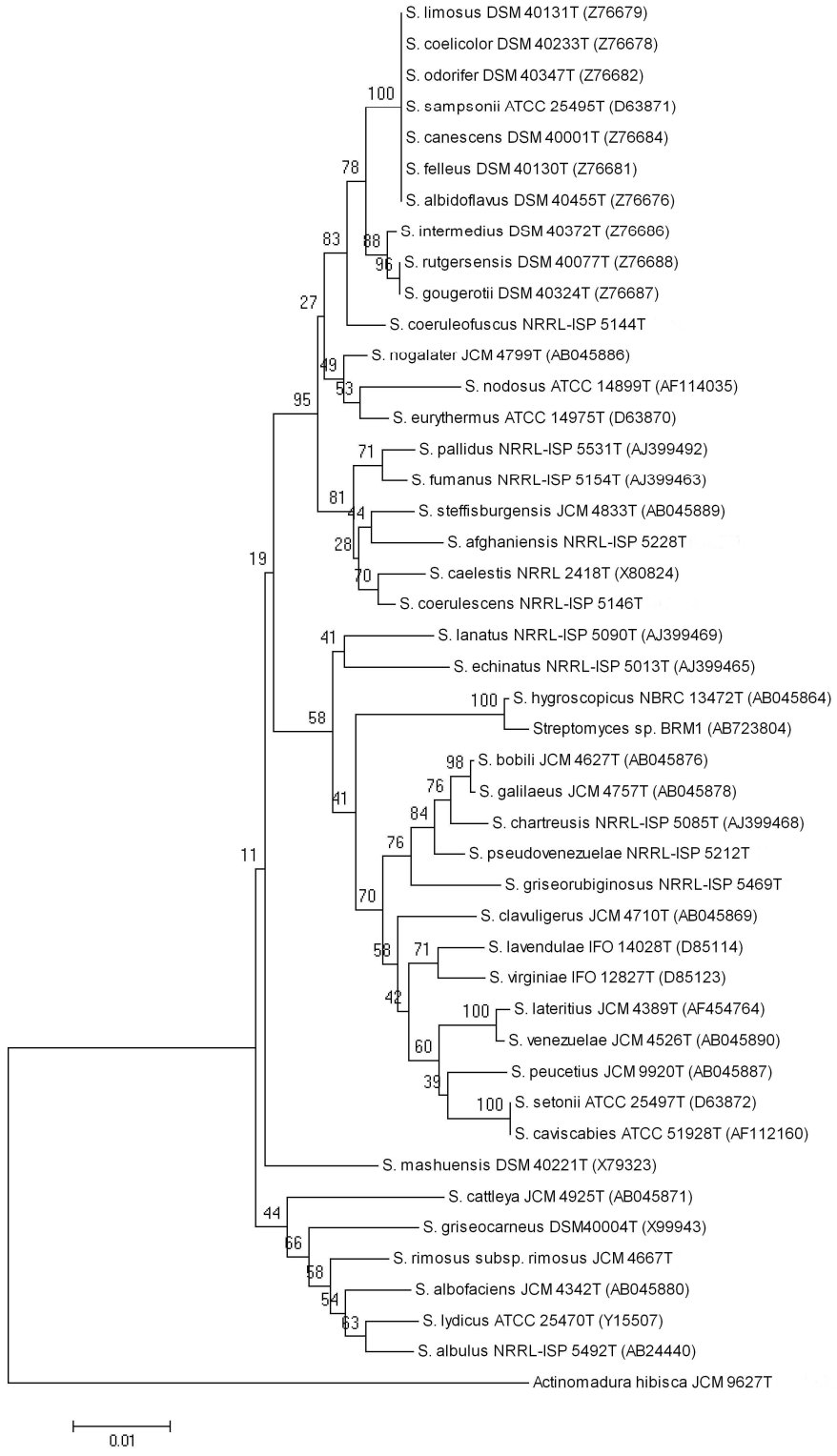


Fig 2. Neighbor-joining phylogenetic tree of *Streptomyces* sp. strain BRM10, including representatives of the most closely-related type strains which were retrieved from GenBank, and accession numbers appear in parentheses. Bootstrap (1,000 replicates) values are given in percentage. Bar, 0.01 substitutions per

Isolation and structure elucidation of the compounds

The purified compound 1 was isolated as a white powder. Its molecular formula was determined to be $C_{41}H_{70}O_{11}$ by HRESIMS based on a quasi-molecular ion peak at *m/z* 761.48 $[M+Na]^+$ and NMR spectra. According to some empirical characteristic signals (Wu *et al.*, 2009), compound 1 has a very similar structure to nigericin except for only one methyl (MeOOC-1). HMQC and HMBC showed the singlet proton of methyl group at δ 3.74 ppm has a direct correlation with carbon at δ 51.7 (MeOOC-1) and long-range correlation with the carbonyl group

(C-1) at δ 176.5 ppm. The multiplet proton of CH (C-2) at 2.49 ppm has a direct correlation with the carbon at δ 43.0 ppm, correlation with the carbonyl group (C-1) at δ 176.5 ppm, HC-O (C-3) at δ 73.8 ppm and methyl group (C-2a) at δ 12.9 ppm. In the data comparison with those of nigericin, the carboxyl at C-1 was esterified in compound 1. The compound 1 was determined to be 1-methyl ester-nigericin. The detailed comparison is shown in Figure 2 and Table 1.

The purified compound 2 was isolated as a white powder. Its molecular formula was determined to be $C_{40}H_{68}O_{11}$ by HRESIMS based on a quasi-molecular ion peak at *m/z* 749.26 $[M+Na]^+$ and NMR spectra. It was identified as nigericin by comparison with its authentic sample (Steinrauf *et al*., 1968; Seto and Otake, 1982; Wu *et al*., 2009).

Antibacterial activity of purified compounds

Results of the antibacterial screening indicated that, the crude extract showed a wide range of activity, being effective against Gram positive and Gram negative bacteria. Nigericin showed strong activity against Gram positive bacteria, while 1-methyl ester-nigericin showed moderate activity. Both compounds showed no activity against Gram negative bacteria. The antibacterial activity of the crude extract and purified compounds were evaluated and the results are shown in Table 2.

Table. 2: Antibacterial activity of crude extract and purified compounds from *Streptomyces hygroscopicus* BRM10.

Test substances	Potential of growth inhibition			
	S.a.[a]	B.c.	E.c.	P.a.
Crude extract	4+[b]	4+	2+	1+
Compound 1	3+	3+	0	0
Compound 2	4+	4+	0	0

Growth inhibition was carried out by using the paper disk method at 50 µg/disc. [a]: S.a.; *Staphylococcus aureus* ATCC25932, B.c.; *Bacillus cereus* ATCC6633, E.c.; *Escherichia coli* ATCC10536 and P.a.; *Pseudomonas aeruginosa* ATCC27853. [b]0: No activity; 1+ (weak activity), 5-10 mm halo diameter; 2+ (weak activity), 10.1-15 mm halo diameter; 3+ (moderate activity), 15.1-20 mm halo diameter; 4+ (strong activity), >20 mm halo diameter.

1-methyl ester-nigericin showed antibacterial activity, the MIC against *Staphylococcus aureus* ATCC25932 and *Bacillus cereus* ATCC6633 were 0.5 µg/ml and 1.0 µg/ml, respectively and no inhibitory activity was observed against *Escherichia coli* ATCC10536 and *Pseudomonas aeruginosa* ATCC27853, at a concentration of 64 µg/ml, while nigericin showed strong antibacterial activity greater than 1-methyl ester-nigericin (Table 3). As with 1-methyl ester-nigericin, the presence of methyl group at the C-1 resulted in decreasing of antibacterial activity. The results confirm that methylation of C-1 position of nigericin resulted in the loss of activity.

According to other studies, nigericin was used as an antibiotic active against Gram positive bacteria, it had MIC against *S. aureus* ATCC25923 and *B. cereus* 1126 were 0.125 µg/ml and 0.25 µg/ml, respectively and no inhibitory activity was observed against *E. coli* CMCC44103, at a concentration of 25 µg/ml. For the MBC against *S. aureus* ATCC25932 and *B. cereus* ATCC6633 were 32 µg/ml and 64 µg/ml, respectively (Wu *et al*., 2009).

Table. 3: Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the crude extract and purified compounds.

Test agents	MIC (µg/ml)				MBC (µg/ml)			
	S.a.[a]	B.c.	E.c.	P.a.	S.a.	B.c.	E.c.	P.a.
Crude extract	0.5	0.5	32	64	8	8	>64	>64
Compound 1	0.5	1	>64	>64	32	64	ND	ND
Compound 2	0.5	0.5	>64	>64	16	16	ND	ND

[a]S.a.; *Staphylococcus aureus* ATCC25932, B.c.; *Bacillus cereus* ATCC6633, E.c.; *Escherichia coli* ATCC10536 and P.a.; *Pseudomonas aeruginosa* ATCC27853.

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