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Antibacterial Activity of Bisanthraquinone (+)-1,1'-Bislunatin

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Endophytic fungi has been known as a source of biologically active compunds with a broad sprectrum of activities. One of the endophytic fungi isolated from young stem of gambier plant (*Uncaria gambier* Roxb.:Rubiaceae), *Diaporthe* sp. GNBP-10, produced (+)-1,1'-bislunatin when cultivated on potato dextrose agar (PDA). (+)-1,1'-Bislunatin showed moderate antibacterial activity against 7 bacteria (*Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Micrococcus luteus*, *Shigella flexneri*, *Proteus vulgaris*, and *P. mirabilis*) with MIC value in the range of 32-64 μg mL^{-1}. The wall disruption and morphological changes in the cells affected by exposure of (+)-1,1'-bislunatin are also discussed in this article.

Key words: (+)- 1,1'-bislunatin, antibacterial, *Diaporthe* sp. GNBP-10, effect on bacterial cells, endophytic fungus, *Uncaria gambier* Roxb.

Fungi endofitik telah diketahui sebagai pengahasil senyawa biologi aktif yang memiliki spektrum aktivitas yang luas. Salah satu fungi endofitik diisolasi dari akar tanaman gambir yang masih muda (*Uncaria gambier* Roxb.:Rubiaceae), *Diaporthe* sp. GNBP-10 menghasilkan (+)-1,1'-bislunatin ketika ditumbuhkan pada media *Potato Dextrose Agar* (PDA). (+)-1,1'-Bislunatin menunjukkan aktivitas aktivitas antibakterial yang moderat terhadap 7 spesies bakteri patogen (*Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Micrococcus luteus*, *Shigella flexneri*, *Proteus vulgaris*, dan *P. mirabilis*) dengan nilai penghambatan minimum antara 32-64 μg mL^{-1}. Pemberian (+)-1,1'-bislunatin terhadap sel bakteri menimbulkan kerusakan dinding sel dan perubahan morfologi sel bakteri.

Kata kunci: (+)- 1,1'-bislunatin, antibakterial, *Diaporthe* sp. GNBP-10, efek terhadap sel bakteri, fungi endofitik, *Uncaria gambier* Roxb.

Endophytic microbes are microorganisms that live inside the plant tissues without causing negative effects to residential plant (Petrini 1991). Endophytic microbes, particularly endophytic fungi in healthy plant tissues are known to produce broad spectrum and highly diversed bioactive secondary metabolites (Zhang *et al*. 2006). Various bioactive compounds have been reported to be isolated from endophytic microbes associated with various plant species, for examples anticancer compound, taxol, which is produced by fungus *Taxomyces andreanae* (Stierli *et al.* 1993), leusinostatin A and leusinostatin A α-*O*-glucoside produced by endophytic fungus *Acremonium* sp. Tbp-5 (Strobel *et al*. 1997; Strobel *et al*. 1997) and antibiotic sitosporon D and E produced by endophytic fungi *Cytospora* sp. CR 200 (Brady *et al*. 2000) and many other examples.

An important metabolite from endophytic fungus *Diaporthe* sp. GNBP-10 isolated from gambier plant *Uncaria gambier* Roxb.: Rubiaceae (Ilyas *et al.* 2009) that possesses strong activity against pathogenic bacteria *Escherichia coli* and *Micrococcus luteus* was isolated and characterized in this current study. The metabolite was identified as (+)-1,1'-bislunatin (Fig 1), which is a dimeric form of lunatin, an anthraquinone produced from *Curvularia lunata* (Jadulco *et al*. 2002). Prior to this present study the compound was isolated from endophytic fungus *Diaporthe* sp. F associated with a tea plant *Camellia sinensis* (L.) Kuntze and reported to produce cytotoxicity against KB cells with IC_{50} 3.5 μg mL^{-1} (Agusta *et al*. 2006). To the best of our knowledge, there is no antibacterial activity of (+)-1,1'-bislunatin reported yet. In this paper we deal with the antibacterial activity of (+)-1,1'-bislunatin against several bacteria and its effect on the cell morphology of the tested bacteria.

MATERIALS AND METHODS

Fungus Cultivation. The endophytic fungus *Diaporthe* sp. GNBP-10 isolated from young stem of gambier plant (*Uncaria gambier* Roxb. : Rubiaceae) was cultivated on potato dextrose agar (PDA) (25 x 200 mL) (Becton, Dickinson and Company), and

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incubated at room temperature for one month. After the incubation fungal biomass including the medium, were cut into small pieces and soaked overnight with ethyl acetate before being filtered. The filtrates were evaporated using rotary evaporator to afford 1.21 g of dark red crude extract.

Isolation and Identification of (+)-1,1'-Bislunatin. The crude extract (1.21 g) was then separated through Sephadex LH-20 (300 mL, Amersham Biosciences) column chromatography and eluted with methanol. Eluate from column chromatography was analyzed TLC silica gel plate (Merck, type 60, F_{254}) using mixture of tetrahydrofuran-chloroform-water (6:4:0.5) as solvent. The eluate that has similar spot was combined into 1 fraction. Analysis of purity level and identification of (+)-1,1'-bislunatin were performed through High Performance Liquid Chromatography (HPLC) (Shimadzu, CBM-20A). The HPLC analysis was performed using CapcellPak C18 column (5 µm, 25 cm x 4,6 mm, Shiseido) with solvent gradient 1 - 70 % acetonitrile (v/v in water) at 40 °C. The flow rate was set at 1 ml min^{-1} and UV detector at 254 nm. Compound identity was analyzed by ^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) (JOEL ECA-50, Japan) using DMSO-$_{d6}$ solvent and compared with the published data of (+)-1,1'-bislunatin previously isolated from *Diaporthe* sp. F (Agusta *et al*. 2006).

Antibacterial Activity Assay and Determination of Minimum Inhibitory Concentration (MIC). *Bacillus subtilis* LIPIMC 0073, *Staphylococcus aureus* LIPIMC 0114, *Eschericia coli* LIPIMC 0186, *Micrococcus luteus* LIPIMC 0076, *Shigella flexneri* (clinical isolate), *Proteus vulgaris* (clinical isolate), and *P. mirabilis* (clinical isolate) had been used in this study. The antibacterial activity of (+)-1,1'-bislunatin were assayed by paper disc (diam. 6 mm, Whatman) method. Ten microliters of (+)-1,1'-bislunatin (1 mg mL^{-1} in acetone) was put onto paper disc and dried for 10 min, before the discs were placed on Muller Hinton Medium that had previously been inoculated with the bacteria to be tested. Then incubation was performed at 37 °C for 24 h. Ten microliters acetone was put on paper disc as negative control. The antibacterial activity of the substance was characterized by the formation of clear zone around the paper disc.

Minimum Inhibitory Concentration (MIC) was determined by Broth Microdilution Method that has been previously validated (Pfaller *et al.* 2002). Microbial isolate was grown at 35 °C in Muller Hinton Broth (Becton, Dickinson and Company). The population density used for antimicrobial testing was 1×10^5 CFU mL^{-1}. (+)-1,1'-Bislunatin was disolved in dimethyl sulfoxide (DMSO, Phyto Technology Laboratory). The stock solution was prepared through double concentration in YMA medium (Becton, Dickinson and Company). The concentrations of (+)-1,1'-bislunatin used for MIC test ranged between 128.0 to 0.25 µg mL^{-1}. The MIC value of (+)-1,1'-bislunatin that inhibited the bacterial growth was determine by adding 15 µl (0.5 mg mL^{-1}) of *p*-iodonitrotetrazolium violet indicators (INT, Sigma-Aldrich) into each well. Each measurement was done in triplicate. Chloramphenicol and erythromycin were used as positive controls.

Analysis of (+)-1,1'-Bislunatin Influence on Bacterial Cells. The mechanism of bacterial growth inhibition was determined based on the protein and nucleic acid contents in the culture media. *Escherichia coli* and *B. subtilis* were cultured in Muller Hinton Broth medium at room temperature. After 48 h, (+)-1,1'-bislunatin (in DMSO) was added into culture medium then incubated again for 24 h. Ten ml bacteria suspension was centrifuged at 4 °C, 3 500 rpm for 15 min, and the filtrate was discarded. Pellet was washed with phosphate buffer pH 7.4 twice, then resuspended with 9 mL phosphate buffer and 1 mL of (+)-1,1'-bislunatin at 1 MIC concentration followed by incubation in shaker incubator at 100 rpm, and 37 °C for 18 - 24 h. The suspension was centrifuged again for 15 min, at 3 500 rpm. Supernatant was filtered, and its absorbance was measured using Spectrophotometer UV-Vis Shimadzu at 260 nm and 280 nm (Castillo *et al*. 2006).

Ca^{2+} and K^{+} ions that leaked out from cell membrane due to (+)-1,1'-bislunatin exposure was detected by Atomic Absorption Spectrophotometer (AAS, Perkin Elmer). Supernatant was prepared as for the analysis of protein and nucleic acid (Castillo *et al*. 2006).

E. coli suspension which has been previously treated with (+)-1,1'-bislunatin was centrifuged for 5 min at 5 000 rpm. Supernatant was discarded and pellet then was fixed with 2% glutaraldehyde for 2 h and centrifuged again. Cocodylate buffer 0.2 M pH 7.2 was added to the pellet and left for 20 min, then 1% osmium tetraoxide was added into the cocodylate buffer and left in refrigerator for an hour before being centrifuged again. This pellet was then suspended in 70, 80, and 96% alcohol, for 10 min each respectively. Then, the pellet was finally suspended in butanol. One loopful of suspension was placed on a cover glass glued to an

aluminium stub and frozen. Then it was freeze-dried for 1 h. The dried cell suspension on the cover glass was then vacuum coated (6 to 7 Pa) with gold for 20 min and analysed with Scanning Electrone Microscope (SEM) JEOL 6300.

RESULTS

Cultivation of the Fungi, Isolation and Characterization of (+)-1,1'-bislunatin. After one month incubation culture media (including the fruiting bodies) were isolated with ethylacetate and dried at low pressure to obtain the dark red extract. Separation of 1.21 g crude extract using Sephadex-LH20 column chromatography produced six fractions *ie.* fraction 1 (F1: 0,219 g), fraction 2 (F2: 0,0358 g), fractions 3 (F3: 0,235 g), fraction 4 (F4: 0,1168 g), fractions 5 (F5: 0,0019 g), and fraction 6 (F6: 0,3611 g) respectively. The target metabolite (+)-1,1'- bislunatin appeared as a single spot on F6. Further analysis by co-chromatography technique showed 99.94 % purity of the obtained (+)-1,1'-bislunatin based on HPLC chromatogram peak area (Fig 4). Finally, the chemical structure of (+)-1,1'-bislunatin was confirmed by comparing its chemical shift values in the ^{1}H- and ^{13}C-NMR spectra as shown on Fig 5 and 6 and Tabel 1.

Antibacterial Activity of (+)-1,1'-Bislunatin. In the preliminary disc difussion assay, the isolated metabolite (+)-1,1'-bislunatin showed antibacterial activities against *E. coli* and *S. aureus*. Further investigation implementing dilution method also showed a moderate antibacterial activities against some pathogenic bacteria with MIC values ranging from 32 to 64 µg mL^{-1}. Interestingly, (+)-1,1'-bislunatin (MIC 64 µg mL^{-1}) showed conspicuously stronger activity against clinical isolate *Proteus mirabilis*. It was even more obvious than the widely known commercial antibiotics chloramphenicol (MIC 113.78 µg mL^{-1}) and erythromicin (MIC 113.78 µg mL^{-1}). On the other hand, antibacterial activity of (+)-1,1'-bislunatin (MIC 64 µg mL^{-1}) was conspicuously stronger than erythromycin (MIC 113.78 µg mL^{-1}) but equally strong with chloramphenicol (MIC 64 µg mL^{-1}) against clinical isolate of *P. vulgaris*.

Inhibition Mechanism of Bacterial Growth. The leakages of protein, nucleic acid, Ca^{2+}, dan K^{+} were the parameters observed in the bacterial cells membrane breakdown when exposed to (+)-1,1'-bislunatin. The result showed that, when exposed to (+)-1,1'-bislunatin, the concentration of protein, and nucleic acid in *E.coli* and *B.subtilis* culture media significantly increased compared to the control treatment (Fig 2).

Table 1 13C- and 1H-NMR data in DMSO-d6 for (+)-1,1'-bislunatin isolated from the culture of *Diaporthe* sp. GNBP-10 and *Diaporthe* sp. F

Atom	(+)-1,1'-bislunatin from *Diaporthe* sp. GNBP-10		(+)-1,1'-bislunatin from *Diaporthe* sp. F*	
	13C-NMR	1H-NMR	13C-NMR	1H-NMR
1,1'	124.0		123.7	
2,2'	164.2	6.70, 2H,s	164.0	6.74, 2H,s
3,3'	107.4		107.3	
4,4'	163.7		163.6	
4a,4a'	108.6		108.6	
5,5'	164.2	6.78, 2H,d,J=2.1 Hz	164.1	6.73,2H,d,J=2.1 Hz
6,6'	106.7	6.96,2H,d,J=2.1 Hz	106.5	6.96,2H,d,J=2.1 Hz
7,7'	165.6		165.5	
8,8'	106.9		106.9	
8a,8a'	135.0		134.9	
9,9'	181.6		181.4	
9a,9a'	130.9		130.9	
10,10'	188.5		188.5	
10a'10a	109.4	3.82,6H,s	109.2	3.81,6H,s
7,7'-OCH_3	56.2		56.0	
OH		12.28, 12.82, 2x2H,s		12.24, 12.82, 2x2H,s

*Agusta *et al.* 2006

Table 2 MIC values of (+)-1,1'-bislunatin against tested bacteria

Microbial isolates	(+)-1,1'-bislunatin	MIC values (ug mL^{-1}) chloramphenicol	erythromycin
B. subtilis LIPIMC0073*	64	8	0.03
S. aureus LIPIMC0114	64	16	0.06
E. coli LIPIMC0186	64	16	32
M. luteus LIPIMC0076	64	16	16
S. flexneri (clinical isolate)	32	1	4
P. vulgaris (clinical isolate)	64	64	113.78
P. mirabilis (clinical isolate)	64	113.78	113.78

*LIPIMC: Lembaga Ilmu Pengetahuan Indonesia Collection.

Observation of *E. coli* and *B. subtilis* cell morphology when exposed to (+)-1,1'-bislunatin indicated abnormalities. Treatment *E.coli* (Fig 3B) and *B.subtilis* (Fig 3D) with of 64 µg/ml (+)-1,1'-bislunatin caused uneven cell surface.

DISCUSSION

The isolation and characterization of (+)- 1,1'-bislunatin was done by Sephadex LH-20 column chromatography. In order to validate the purity of (+)-1,1'-bislunatin, the F6 was analysed using HPLC by co-chromatography technique using (+)-1,1'-bislunatin as the internal standard. The HPLC chromatogram indicated that the only single peak in F6 was identical with the (+)-1,1'-bislunatin standard, and had 99.94% purity. The chemical structure of F6 was elucidated by 1D-NMR analisys. Both the ^{1}H-NMR and ^{13}C-NMR spectra of the purified F6 showed identical chemical shift value with (+)-1,1'-bislunatin isolated from the culture of *Diaporthe* sp. F (Agusta *et al*. 2006). Based on the above data, it was clear that the metabolite in F6 was (+)-1,1'-bislunatin.

The isolated (+)-1,1'-bislunatin was tested for antibacterial activity against several bacterial isolates including clinical isolate *P. mirabilis*. *P. mirabilis* is a species of gram-negative bacteria that causes cystitis, acute pyelonephritis,urinary stones production (Mobley Research Laboratory 2013). Usually, Gram negative bacteria are less sensitive to antibiotic substances due to their cell membrane structure that is obviously different from Gram positive bacteria. Nevertheless, the MIC value of (+)-1,1'-bislunatin against *P. mirabilis* was still lower than that of erythromycin. The result of the present study showed that generally, the MIC value of bacteria exposed to (+)-1,1'-bislunatin was greater than any of those standard antibiotics. This indicates that (+)-1,1' bislunatin possesses weaker antibacterial activity compared to any of commercial erythromycin-based antibiotics.

In order to determine the mechanism of (+)- 1,1'-bislunatin inhibition of *E.coli* and *B.subtilis* growth, protein, nucleic acid, Ca^{2+} and K^{+} leakage from cells were observed. The protein leakage was observed at the wavelength of 280 nm, while the nucleic acid leakage was observed at 260 nm (Castillo *et al*. 2006). There were increasing concentration of protein and nucleic acid in *E.coli* and *B.subtilis* culture media of when exposed to (+)- 1,1'- bislunatin. This indicates the presence of protein and nucleic acid transfer from cytosol in the bacteria cells into culture medium. This is in conformity with the increase of the contents of metal ions Ca^{2+} and K^{+} in same medium (Fig 2). The result of this study is in accordance with (Castillo *et al.* 2006) that the potassium leakage as the first sign of membrane damage in microorganisms, a process that leads to the increase presence of protein, nucleic acid, and ions Ca^{2+} and K^{+} in the culture medium transferred there from the cytosol cell due to the breaking down of bacteria cell walls. Lavlinesia (2007) also noted that

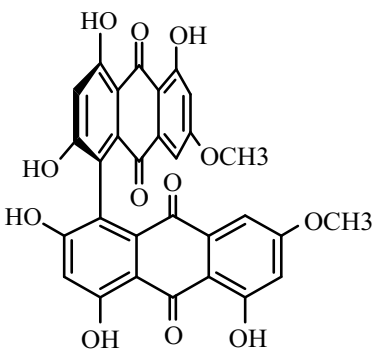

Fig 1 Molecular structure of (+)-1,1'-bislunatin.

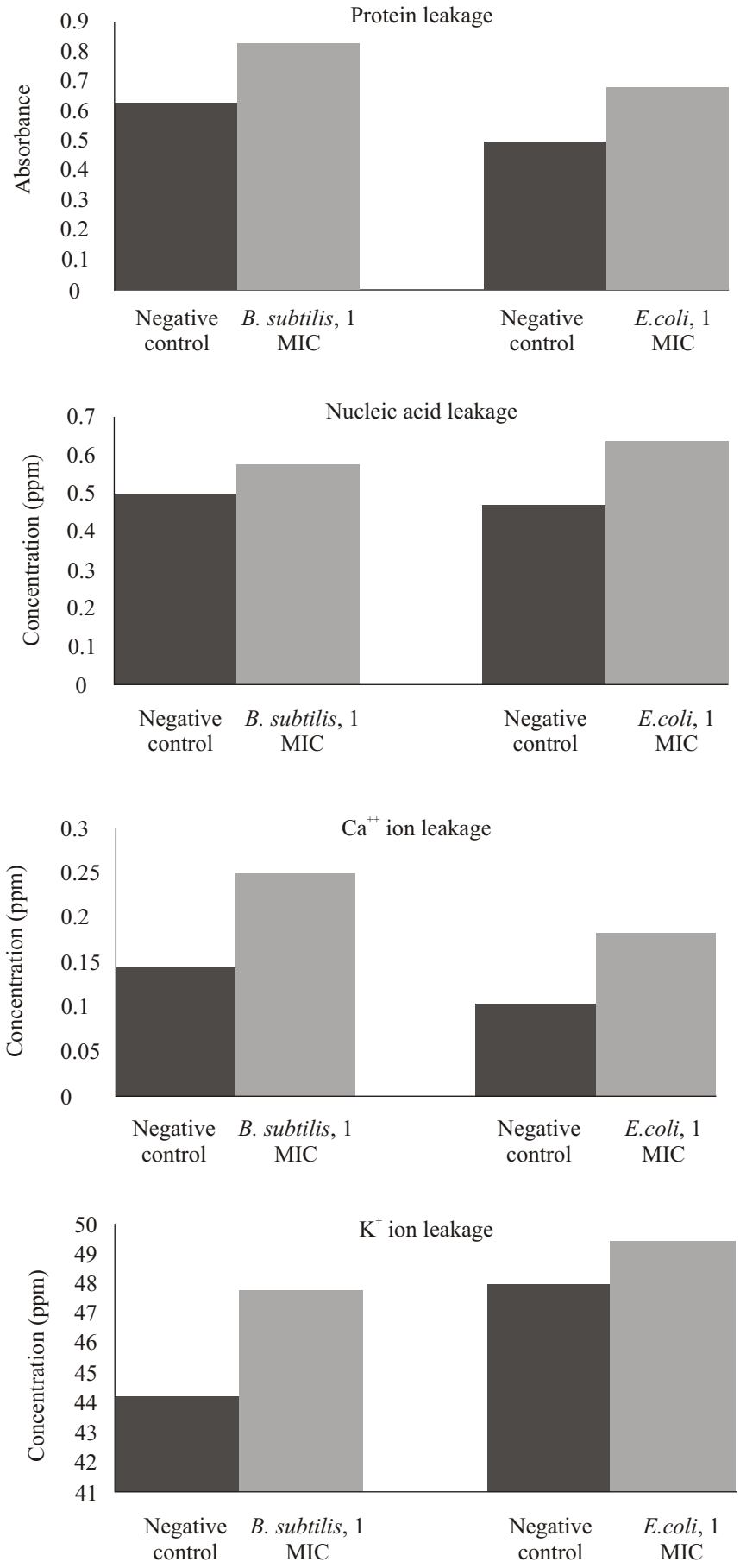

Fig 2 The quantities of (Ca^{2+}, K^{+}), protein and nucleic acid leakages from bacterial cells after being exposed by (+)-1,1'-bislunatin on 1 MIC values.

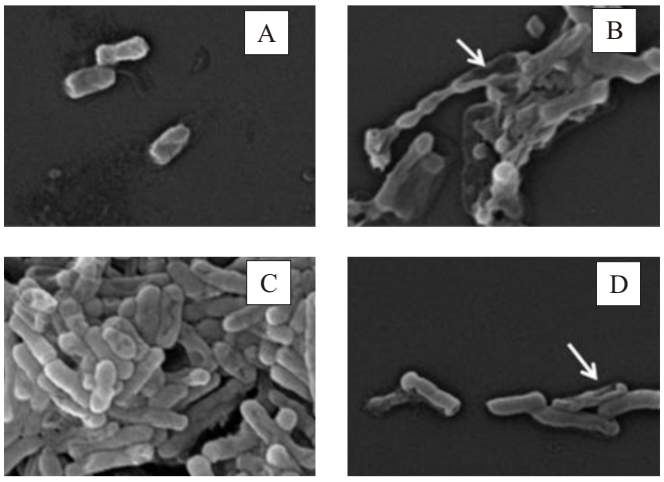

Fig 3 SEM micrograph of the cell morphology of *E. coli* without (A) and after being treated (+)-1,1'-bislunatin (B). The same treatment in *Bacillus subtilis* cells without (C) and after being treated (+)-1,1'-bislunatin (D). The arrows indicate the deformed shrinking cells.

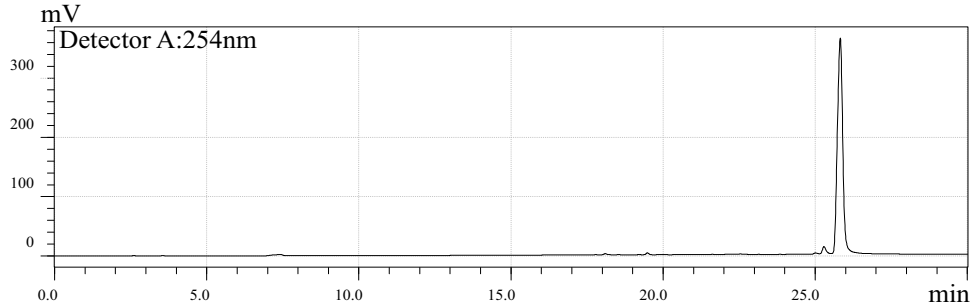

Fig 4 HPLC Chromatogram of purified (+)-1,1'-bislunatin.

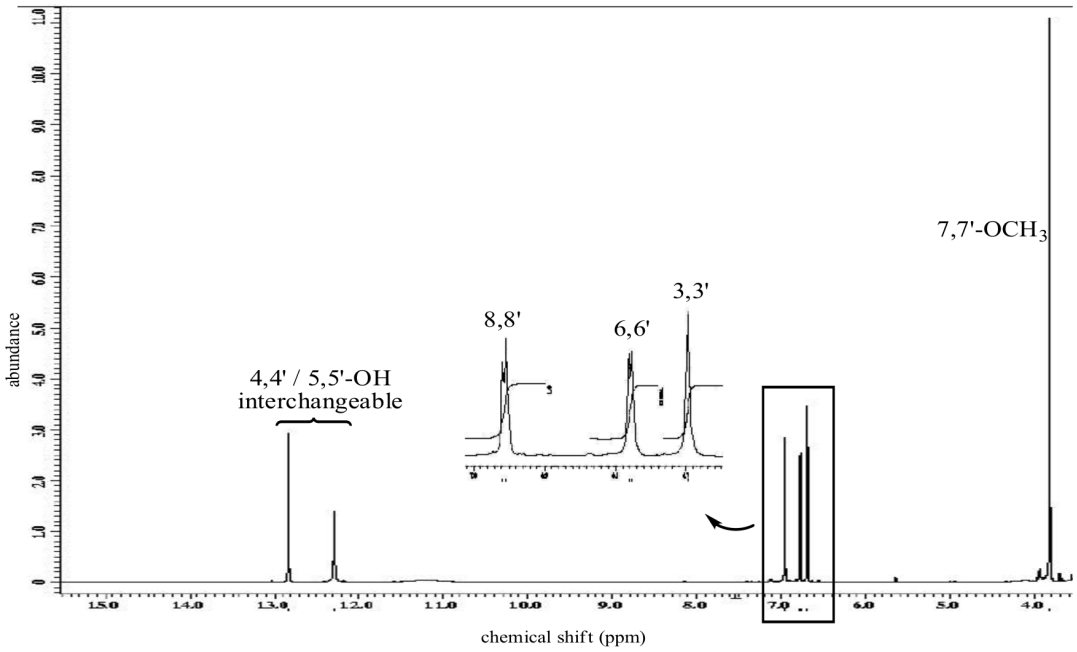

Fig 5 ^{1}H NMR spectrum of purified (+)-1,1'-bislunatin.

the excessive cytoplasm loss was due to membrane leakage. It also supports the previous results of Henie *et al.* (2009) which stated that the release of bacterial protein, nucleic acids, and K^{+} were indicators of the disruption in the bacterial membranes.

This indicated the damage in the bacterial cells.

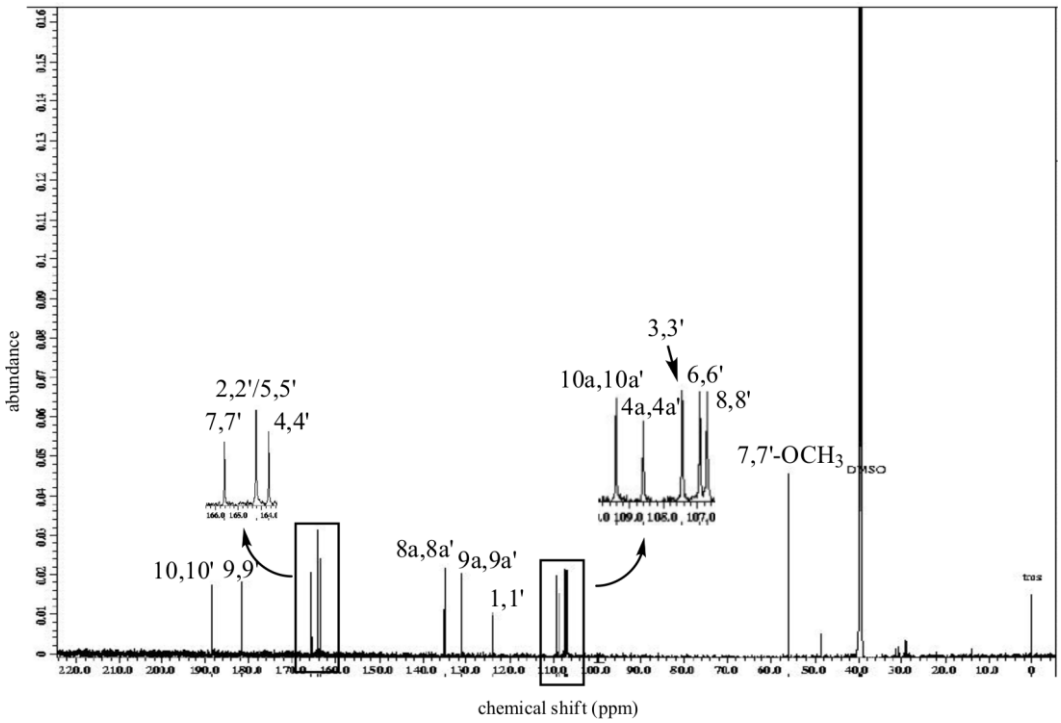

Fig 6 ^{13}C NMR spectrum of purified (+)-1,1'-bislunatin.

Evidently exposure to (+)-1,1'-bislunatin caused an obvious change in the permeability of bacterial cell membrane and degeneration of cell membrane. According to Kvam *et al*. (2012) the cell poration and lysis are believed to be indicators of gradual cellular protein and nucleic acid leakage from the cell.

The observation on cellular morphology showed that the bacteria cells deflated after treatment with (+)-1,1'-bislunatin, causing losses of cell wall function as protector or barrier between organs and cellular fluid. As a consequence the cell loss most of its contents due to the destruction of the bacterial cell wall or necrosis and the cellular fluid migrate irresistibly into the culture medium. This is in accordance with our results in which the concentration of proteins and nucleic acids, Ca^{2+} and K^{+} increased in the culture medium of the cells after being treated with (+)-1,1'-bislunatin.

To conlclude (+)-1,1'-bislunatin isolated from endophytic fungus *Diaporthe* sp. GNBP-10, obtained from young stem of gambier plant (*Uncaria gambier* Roxb.: Rubiaceae) showed moderate antibiotic activity against several bacterial isolates. Exposure of (+)- 1,1'-bislunatin caused disruption of the cell wall and changes in the cell morphologies of *B. subtilis* and *E. coli* cells.

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