MICROBIOLOGY INDONESIA
ISSN 1978-3477, eISSN 2087-8587
Vol.9, No.1, March 2015, p 35-43

Available online at
http://jurnal.permi.or.id/index.php/mionline
DOI: 10.5454/mi.9.1.5

Antibacterial Activity and Mode of Action of (+)-2,2'-Epicytoskyrin A

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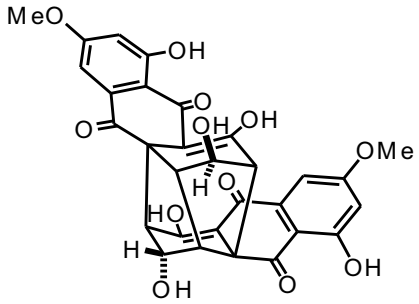
Antibacterial activity of (+)-2,2'-epicytoskyrin A, a main metabolite from culture of fungal endophyte *Diaporthe* sp. GNBP-10, was investigated against several strains of Gram-positive, Gram-negative bacteria, and one strain of *Mycobacterium smegmatis*. (+)-2,2'-Epicytoskyrin A exhibited prominent activity against *Staphylococcus aureus* BCC 1452 with minimum inhibitory concentration (MIC) value of 0.06 µg mL^{-1}. The effect of (+)-2,2'-epicytoskyrin A treatment to *S. aureus*, resulted in alteration of bacterial cell membrane with an increase of cation efflux, while the cytoplasmic content was not leaked out and finally resulted in cell damage.

Key words: (+)-2,2'-epicytoskyrin A, antibacterial activity, *Diaporthe* sp. GNBP-10, *Uncaria gambier*

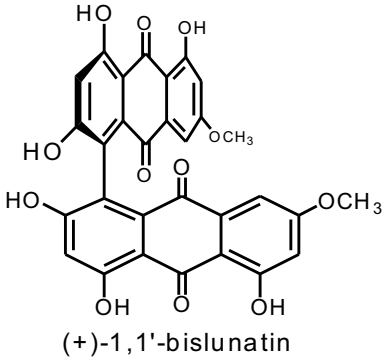
Aktivitas antibakteri (+)-2,2'-episitoskirin A, yaitu suatu metabolit utama pada kultur jamur endofit *Diaporthe* sp. GNBP-10 telah diinvestigasi terhadap bakteri gram positif dan gram negatif beserta satu strain *Mycobacterium smegmatis*. (+)-2,2'-Episitoskirin A memperlihatkan aktivitas paling menonjol melawan *Staphylococcus aureus* BCC 1452 dengan nilai konsentrasi hambat minimum (KHM) sebesar 0.06 µg mL^{-1}. Pemaparan (+)-2,2'-episitoskirin A terhadap *S. aureus* mengakibatkan perubahan pada membran sel bakteri dengan meningkatkan pelepasan kation tanpa meningkatkan sekresi cairan sitoplasma yang berujung pada kerusakan sel.

Kata kunci : (+)-2,2'-episitoskirin A, aktivitas antibakteri, *Diaporthe* sp. GNBP-10, *Uncaria gambier*

Prior to this present study, the endophytic fungus *Diaporthe* sp. GNBP-10 associated with a gambier plant *Uncaria gambier* Roxb. was reported have ability to produce (+)-1,1'-bislunatin (Fig 1) when cultivated onto potato dextrose agar (PDA) medium. This metabolite possessing a weak antibacterial activities against some pathogenic bacterial including *Bacillus subtilis*, *Staphylococcus aureus*, *Eschericia coli*, *Micrococcus luteus*, *Shigella flexneri*, *Proteus vulgaris* and *P. mirabilis* (Praptiwi *et al*. 2013). Together with (+)-1,1'-bislunatin, another main metabolite was also isolated from the culture of *Diaporthe* sp. GNBP-10, and identified as (+)-2,2'-epicytoskirin A (Fig 1). The isolation and chemical structure determination of a natural (+)-2,2'-epicytoskyirin A was reported in 2006 for the first time. This metabolites was found as a major metabolite in the culture of an endophytic fungus *Diaporthe* sp. F isolated from a tea plant of *Camellia sinensis* L. The cytotoxic effect on KB cell (inhibitory concentration, IC_{50} 0.5 µg/mL) of the metabolite were also report in the same article (Agusta *et al*. 2006). The total synthesis of (+)-2,2'-epicytoskyrin A was described by Nicolau and his co-workers (Nicolaou *et al*. 2005). To the best of our knowledge, there is no antibacterial activity of (+)-2,2'-epicytoskyrin A has been reported yet. In this paper we reported antibacterial properties of (+)-2,2'-epicytoskyrin A.



(+)-2,2'-epicytoskyrin A



(+)-1,1'-bislunatin

Fig 1 Chemical structure of (+)-2,2'-epicytoskyrin A and (+)-1,1'-bislunatin.

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MATERIALS AND METHODS

Fungal Strain and Cultivation. Endophytic fungus *Diaporthe* sp. GNBP-10 isolated from young stem of gambier plant (*Uncaria gambier* Roxb.) and deposit at Indonesian Culture Collection (InaCC). The isolation process and identification of fungal strain were dercribed on Ilyas *et al*. (2007). The endophytic fungus *Diaporthe* sp. GNBP-10 was cultivated on Potato Dextrose Agar (PDA) (25 x 250 mL) medium and then extracted with ethyl acetate as described on the previous paper (Praptiwi *et al*. 2013).

Bacterial Strains. Tested pathogenic bacterial used in this study were originated from three sources, Balitvet Culture Collection (BCC), Indonesian Culture Collection (InaCC) LIPI, and several clinical isolates from Faculty of Medicine, University of Indonesia (FKUI). The strains were comprised of Bacillus *subtilis* BCC 2558, *B. subtilis* InaCC B1, *Staphylococcus aureus* BCC 1452, *S. aureus* InaCCB4, *Escherichia coli* BCC 2606, *E. coli* InaCC B5, *Micrococcus luteus* LIPIMC 0076, *Klebsiella pneumoniae* BCC 1758, *Salmonella typhimurium* BCC 2366, *Salmonella enteritidis* BCC 2586, *Salmonella paratyphi* B, and *Mycobacterium smegmatis* LIPIMC 0358. The clinical isolates were *S. aureus*, *B. subtilis*, *Streptococcus pneumoniae*, *K. pneumoniae*, *E. coli*, *S. typhii*, *S. paratyphii*, *S. entiritidis*, and *Pseudomonas aeruginosa*.

Isolation and Indentification of (+) -2,2 '-Epicytoskyrin A. The crude extract (1.21 g) was then separated through Sephadex LH-20 (300 mL, Amersham Biosciences) column chromatography and eluted with methanol as reported in previous paper (Praptiwi et al., 2013). (+)-2,2'-Epicytoskyrin A was appeared as fraction 1 (F1, 0.219 g), and further purified by precipitation in 60 % methanol in water at room temperature. Its purity was determined by a reversed-phase HPLC analysis equipped with a Capcell-Pak C18 column (Shiseido) and run with isocratic solvent system of 55 % of acetonitrile at 40 ^{o}C for 30 min at flow rate of 1 mL min^{-1} and wavelength of 254 nm. Furthermore, determination of the chemical structure of (+)-2,2'-epicytoskyrin A was deduced base on their ^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) data (JEOL ECA-500, DMSO-d_6) and compared with published data of (+) -2,2 '-epicytoskyrin A previously isolated from Diaporthe sp. E (Agusta et al., 2006).

Antibacterial Activity Tests and Determination of Minimum Inhibitory Concentration (MIC) of (+) -2,2 '-Epicytoskyrin A. Preliminary antibacterial activity test were perform by using a paper dick method described by Praptiwi *et al.* (2013). Determination of MIC values were conducted by Broth Microdilution Method that has been previously validated (Scwable *et al*. 2007) and describe in our previous paper (Praptiwi *et al.* 2013). The bacterial used were covered Gram-negative bacteria, and Gram-positive bacteria including *Escherichia coli* InaCC B5, *Bacillus subtilis* InaCC B1, *Staphylococcus aureus* InaCC B4, *Micrococcus luteus* LIPIMC 0076, and wild-strain bacteria from Balitvet Culture Collection (BCC) : *Bacillus subtilis* BCC2558 (isolated from healthy chicken caecal mucosa, Bogor), S*taphylococcus aureus* BCC1452 (isolated from cow's milk, West Java), *Salmonella typhimurium* BCC2366 (isolated from duck, South Kalimantan), *Escherichia coli* BCC2606 (isolated from piglet diarrhea stool, Jakarta). Concentrations of (+) -2,2'-Epicytoskyrin A were in the range of 128 - 0.015 µg mL^{-1}.

Analysis of (+)-2,2-Epicytoskyrin A Influence on Bacterial Cells. The possible mechanism of bacterial groth inhibition was elucidated based on the protein and nucleic acid contents in the culture media. *Staphylococcus aureus* BCC 1452 was cultivated in Muller Hinton Broth medium and incubated at room temperature for 48 h. Preparation of tested bacterial culture medium for analysis of nucleic acid, protein, ion Ca^{2+} and ion K^{+} and together with preparation of microbial cell pellet for scanning electron microscope observation were perform according to Castillo *et al.* (2006) that described in our previous paper (Praptiwi *et al.* 2013). Data on the ammount of nucleic acids and proteins leaked from the cells were further analyzed using SPSS Ver. 22.0 program at significance level of 5 %.

RESULTS

Cultivation of the Fungal, Isolation and Characterization of (+)-2,2'-Epicytoskyrin A. The results of fungal cultivation and Sephadex LH-20 column chromatography process were described on the previous paper (Praptiwi *et al.* 2013). The recent target metabolite, (+)-2,2'-Epicytoskyrin A was appeared as a single spot on fraction 1 (F1: 0.219 g). The purity of metabolite in F1 is 99.85 % determined by HPLC analysis and calculated based on the peak areas of the chromatogram (Fig 2). Finally, the chemical structure of (+)-2,2'-Epicytoskyrin A was confirmed by comparing its chemical shift values in the ^{1}H- and ^{13}C-

NMR spectra as shown on Fig 3 and 4 and Table 1. Base on the above data it was confirmed that the metabolite in F1 was (+)-2,2'-epicytoskyrin A.

Antibacterial Activity and Minimum Inhibitory Concentration (MIC) of (+) -2,2 '-Epicytoskyrin A. In the preliminary observation of antibacterial activities through disc diffussion assay, the isolated metabolite (+)-2,2'-epicytoskyrin A showed activities against *E. coli* and *S. aureus*. Further investigation applying the microdillution broth method, (+)-2,2'-epicytoskyrin A showed a strong antibacterial growth inhibition activities against some pathogenic bacteria. The MIC values of (+)-2,2'-epicytoskyrin A against tested bacteria were listed in Table 2. The MICs against several pathogenic Gram-positive bacteria were in the range of 0.06 to 32 µg mL^{-1}, while against Gram-negative bacteria were in the range of 1 to 32 µg mL^{-1}. (+)-2,2'-Epicytoskyirin A showed prominent activity against *S. aureus* BCC1452 and B. subtilis BCC2558 with MIC value of 0.06 and 2 µg mL^{-1} respectively. It also showed good activity against several strains of *Salmonella* compared to chloramphenicol and erythromycin. The similar results was observed against clinical isolates bacteria. Most of the MICs were lower than positive control which indicated a good activity of (+)-2,2'-epicytoskyrin A. It inhibited the growth of pneumonia causing bacteria, *S. pneumoniae* and *K. pneumoniae*, with MIC value of 1 and 2 µg mL^{-1} respectively. In addition, it showed moderate activity against *Mycobacterium smegmatis* with MIC value of

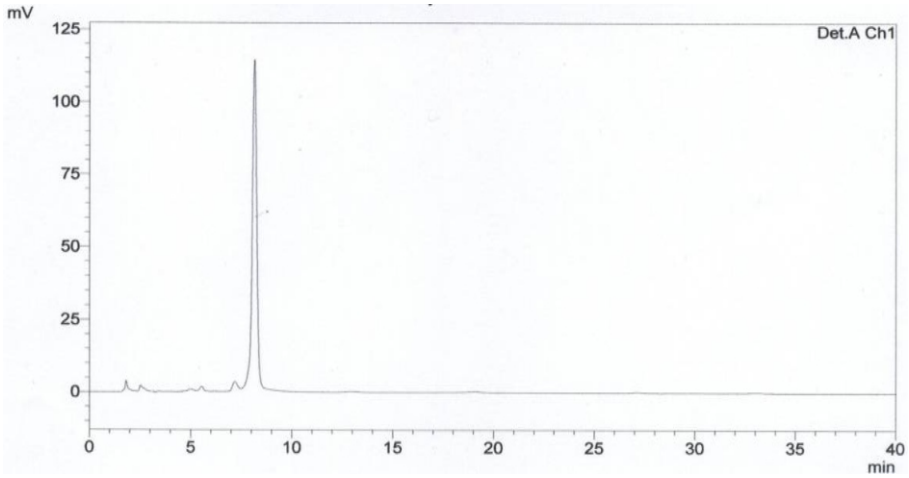

Fig 2 HPLC chromatogram for purified (+)-2,2'-epicytoskyrin A. The purity level of (+)-2,2'-epicytoskyrin A was 99.85 % calculated from the peak area on the HPLC chromatogram.

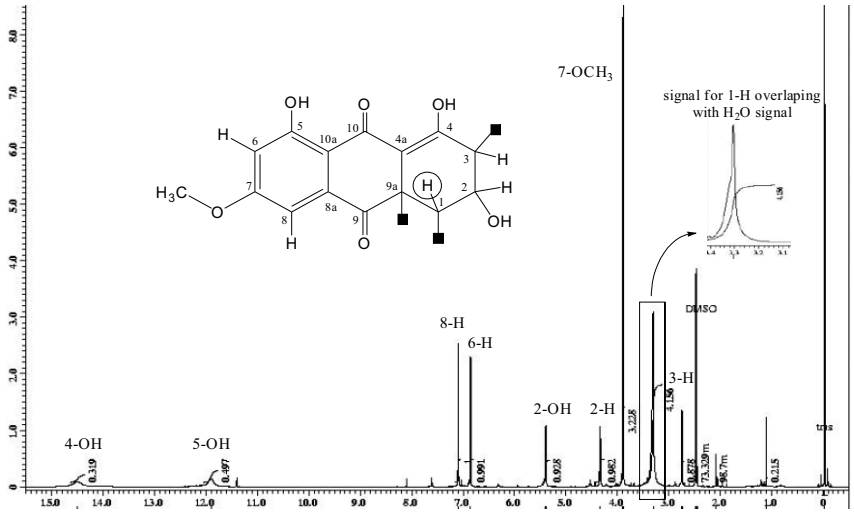

Fig 3 ^{1}H NMR spectrum of purified (+)-2,2'-epicytoskyrin A. Only nine proton signals were appeared on the ^{1}H NMR spectrum due to the symetrical dimeric of the monomer.

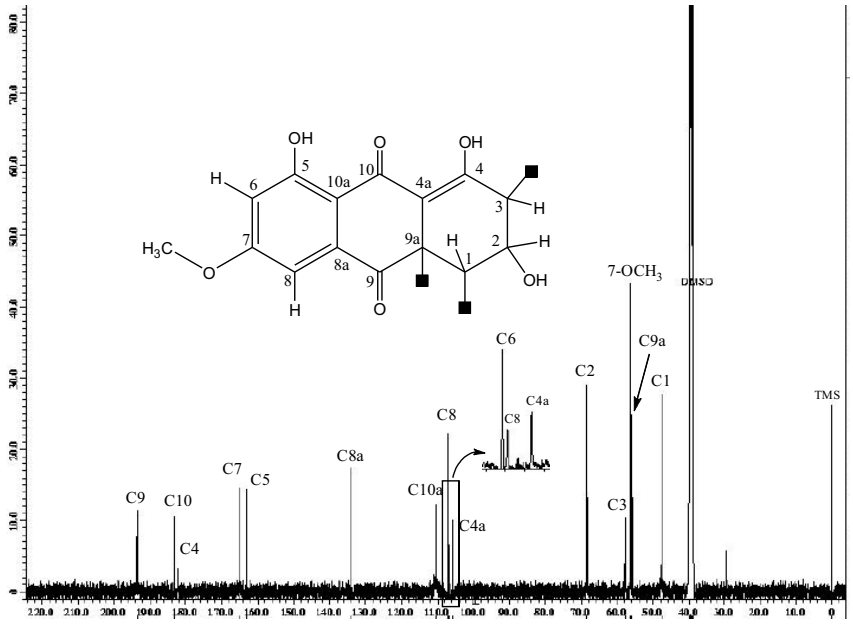

Fig 4 ^{13}C NMR spectrum of purified (+)-2,2'-epicytoskyrin A. Only fourteen carbon signals were appeared on the ^{13}C NMR spectrum due to the symetrical dimeric of the monomer.

Table 1 ^{13}C- and ^{1}H-NMR data in DMSO-d_6 for (+)-2,-epicytoskyrin A isolated from culture of endophytic fungus Diaporthe sp. GNBP-10

No. atom	(+)-2,2'-Epicytoskirin A from *Diaporthe* sp GNBP-10		(+)-2,2'-Epicytoskirin A from *Diaporthe* sp E*	
	^{13}C-NMR	^{1}H-NMR	^{13}C-NMR	^{1}H-NMR
1,1'	47.8	3.30 (2H, br s)	47.7	3.34 (2H, br s)
2,2'	68.4	4.33 (2H, br d, *J*=5.0 Hz)	68.3	4.37 (2H, br d, *J*=5.0 Hz)
2,2'-OH		5.40 (2H, br s)		5.44 (2H, br s)
3,3'	57.9	2.75 (2H, d, *J*=5.0 Hz)	57.8	2.79 (2H, d, *J*=5.0 Hz)
4,4'	182.3		182.2	
4,4'-OH		14.50 (2H, br s)		14.52 (2H, br s)
4a,4a'	105.7		105.6	
5,5'	163.1		163.0	
5,5'-OH		11.90 (2H, br s)		11.92 (2H, br s)
6,6'	107.1	6.86 (2H, d, *J*=2.1 Hz)	107.0	6.89 (2H, d, *J*=2.3 Hz)
7,7'	165.3		165.2	
8,8'	106.9	7.10 (2H, d, *J*=2.1 Hz)	106.8	7.14 (2H, d, *J*=2.3 Hz)
8a,8a'	133.9		133.8	
9,9'	193.6		193.5	
10,10'	55.9		55.8	
10a,10'	183.2		183.0	
7,7'-OCH$_3$	110.5		110.3	
7,7'-CH$_3$	56.3	3.90 (6H, s)	56.2	3.92 (6H, s)

* Agustus *et al*. 2006

Table 2 MIC values of (+)-2,2'-epicytoskyrin A toward some of bacteria isolates

No.	Tested Organism	MIC (μg mL^{-1})		
		(+)-2,2'-Epicytoskyrin A	Chloramphenicol	Erythromycin
1	*Bacillus substilis* BCC 2558	2	4	32
2	*Bacillus subtilis* InaCC B1	8	8	0,03
3	*Escherichia coli* BCC2606	16	8	64
4	*Escherichia coli* InaCC B5	8	8	32
5	*Klebsiella pneumoniae* BCC 1758	4	8	64
6	*Micrococcus luteus* LIPIMC 0076	8	8	16
7	*Mycobacterium smegmatis* LIPIMC 0358	16	(*)	(*)
8	*Salmonella enteritidis* BCC 2586	4	64	64
9	*Salmonella paratyphi* B	8	64	64
10	*Salmonella typimurium* BCC 2366	8	8	64
11	*Staphylococcus aureus* BCC 1452	0.06	2	1
12	*Staphylococcus aureus* InaCC B4	16	16	0.06
	clinical isolates from human:		Amoxicillin	Vancomycin
1	*Bacillus subtilis*	8	2	> 64
2	*Escherichia coli*	1	> 64	> 64
3	*Klebsiella pneumoniae*	2	> 64	> 64
4	*Pseudomonas aeruginosa*	16	> 64	> 64
5	*Salmonella entiritidis*	2	> 64	> 64
6	*Salmonella typhii*	32	4	> 64
7	*Salmonella paratyphii*	8	8	32
8	*Staphylococcus aureus*	32	> 64	4
9	*Streprococcus pneumoniae*	1	> 64	> 64

16 μg mL^{-1}.

Inhibition of Bacterial Growth. In revealing the alteration of (+)-2,2'-epicytoskyrin A to bacterial membrane integrity, *S. aureus* BCC 1452 was used as a model for Gram-positive bacteria. Several parameters including concentration of Ca^{2+}, K^{+}, nucleic acid, protein, and the morphology of the (+)-2,2'-epicytoskyrin A-treated and -untreated cell were evaluated. These parameters are important in indicating the membran integrity of the cell. The leakage of Ca^{2+} and K^{+} ions were observed after (+)-2,2'-epicytoskyrin A treatment. The ion leakage increased as the concentration of (+)-2,2'-epicytoskyrin A increased (Fig 5). The Ca^{2+} concentration released by the cells increased up to 3 and 6 times compared to control after (+)-2,2'-epicytoskyrin A treatment at 1- and 2-MIC respectively. In addition, the release of K^{+} ion reached 334.53 ppm after (+)-2,2'-epicytoskyrin A treatment at 2-MIC, four times than that of control which only 75.85 ppm.

The presence of nucleic acid and protein in the supernatant released by cells were (revealed by UV absorbance at 260 and 280 nm) exhibited small difference between treated and untreated bacteria (Fig 6). In order to determine the significance difference of the value among groups, analysis of variance was performed followed by Tukey test. It was revealed that the nucleic acid and protein of bacteria treated with either (+)-2,2'-epicytoskyrin A or erythromycin were not significantly differ from those of control ($p < 0.05$). On the other hand, the nucleic acid and protein concentration released by bacteria treated with chloramphenicol were significantly higher than those of control ($p < 0.05$), which indicate the loss of cytoplasmic materials from the cell due to membrane damage.

The morphology of normal or untreated *S. aureus* BCC1452 cell under SEM photograph showed a round with smooth surface, while bacteria cell treated with (+)-2,2'-epicytoskyrin A at 2 MIC exhibited damage indicated by some small holes on the cell membrane (Fig 7). The similar effect was observed in the cell treated with commercial antibiotic chloramphenicol and erythromycin.

DISCUSSION

In the term of bilogical activity as an antibacterial, it was clearly show (+)-2,2'-epicytoskirin is more

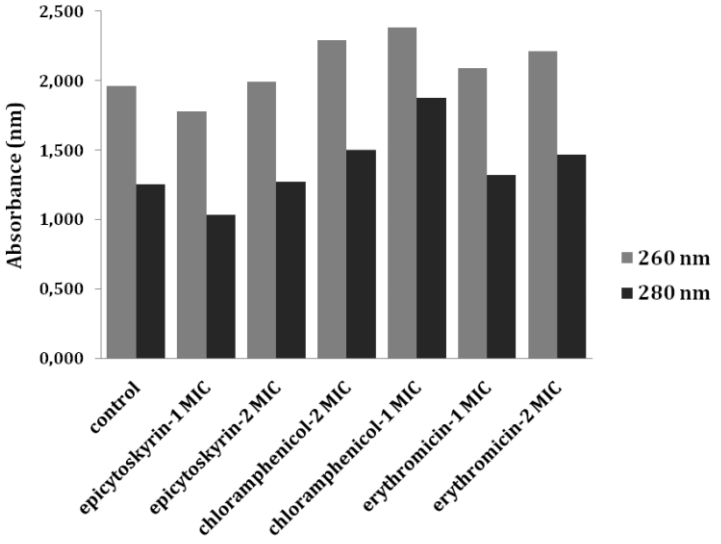

Fig 5 Nucleic acids and proteins content in solution post treatment of *S.aureus* BCC1452. The nucleic acids and proteins content in the medium of treated and untreated cell (control) were not significantly different indicated (+)-2,2'-epicytoskyrin A did not seem to cause the membrane damage of bacteria.

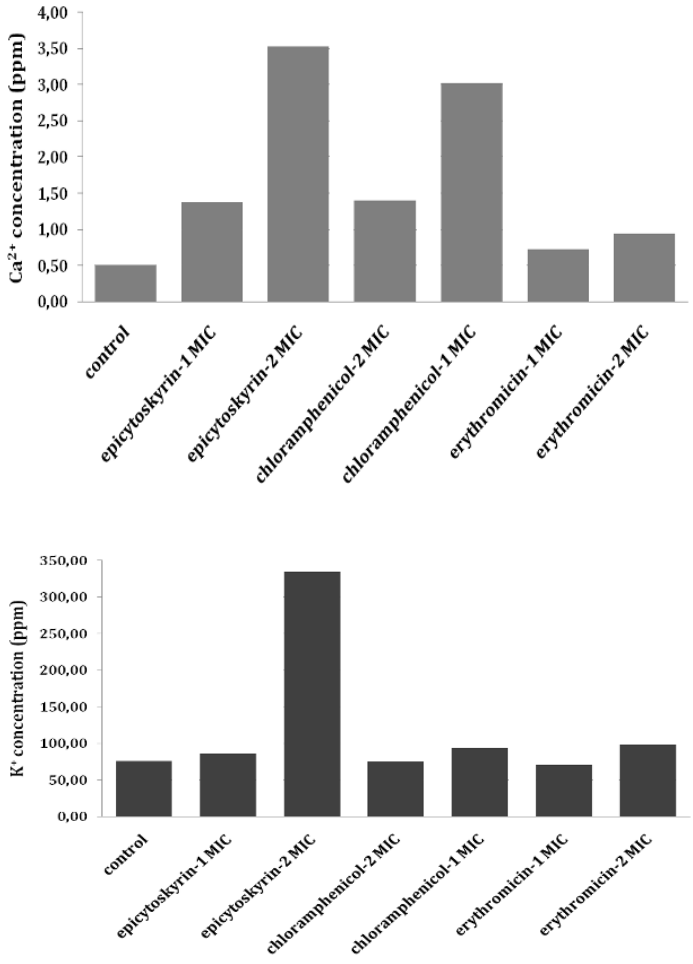

Fig 6 Ca^{2+} and K^{+} content in solution post treatment of *S.aureus* BCC1452. The highest Ca2+ and K+ content were found in the medium of *S. aureus* BCC1452 culture treated by 2MIC of (+)-2,2'-epicytoskyrin A that revealed disrupting of the cell mebrane permeability.

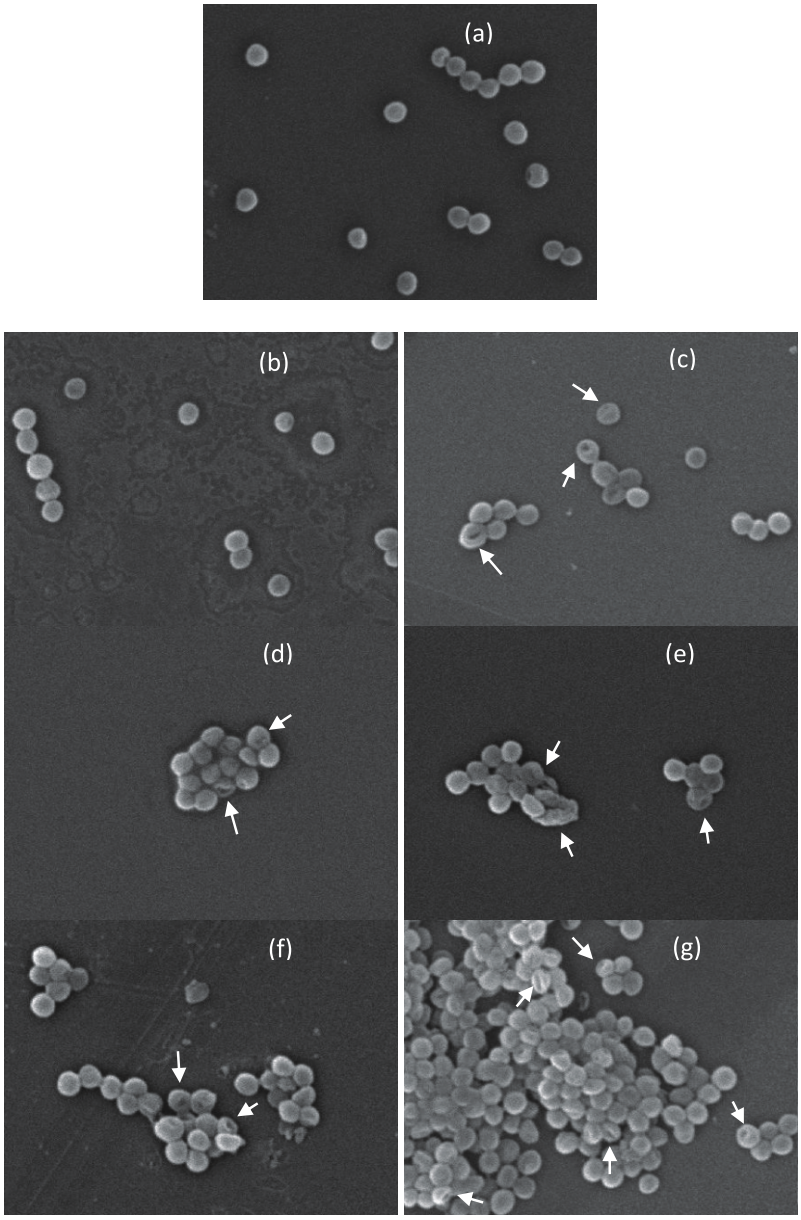

Fig 7 Cell morphology of *S. aureus* B1452, 1000 times of magnificent. (a) control (b) (+)-2,2'-epicytoskyrin A 1 MIC (c) (+)-2,2'-episitoskyrin A 2 MIC (d) chloramphenicol 1 MIC (e) chloramphenicol 2 MIC (f) erythromycin 1 MIC (g) erythromycin 2 MIC.

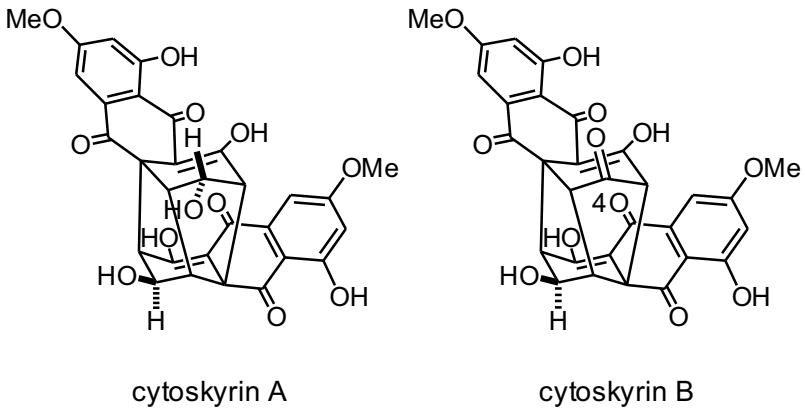

Fig 8 Chemical structure of cytoskyrin A and cytoskyrin B. Both chemical compounds just differ only on hydroxyl moeity on C-4 posistion on cytoskyrin A and keton moeity on cytoskyrin B.

potent compare than another bisanthraquinone (+)-1,1'-bislunatin (Praptiwi *et al*. 2013) that produce by the same endophytic fungus. (+)-2,2'-Epicytoskyrin A likewise its epimer cytoskyrin A showed potent antibacterial activity against Gram-positive bacteria, S. aureus. The MIC values against *S. aureus* was 0.06 µg ml^{-1}, the same value as MIC of cytoskyrin A (Singh *et al.* 2007). Although the *S. aureus* strains used in both studies were different, this could indicate that their bioactivity was relatively similar.

Based on their chemical structure, (+)-2,2'-epicytoskyrin A differs from cytoskyrin A only in hydroxyl groups at position C-2 and 2'. The result of this study showed that the difference in those positions did not affect the activity against *S. aureus* significantly. On the contrary, hydroxyl moiety at C-4 position have significant role to the activity as demonstrated earlier by Singh *et al.* 2007. Cytoskyrin B which have a carbonyl moiety instead of hydroxyl at C-4 position was inactive against both of Gram-positive and Gram-negative bacteria.

The activity of (+)-2,2'-epicytoskyrin A, however, differ from cytoskyrin A in inhibiting *K. pneumoniae*, a Gram-negative bacteria that caused some nocosomial infections including pneumonia. The MIC of (+)-2,2'-epicytoskyrin A against this bacteria (2 µg mL^{-1}) was much less than cytoskyrin A (>64 µg mL^{-1}) (Singh *et al.* 2007). In addition, the low MIC of (+)-2,2'-epicytoskyrin A (1 µg mL^{-1}) against pneumococcal bacteria, *S. pneumoniae*, suggested a new opportunity for pneumococcal infections therapy.

The mechanism of action of (+)-2,2'-epicytoskyrin A was investigated by observing morphology of *S. aureus* BCC1452 cell under SEM as well as monitoring the cytoplasmic materials and ion released from the cell. The absorbance at 260 and 280 nm of the cell treated with (+)-2,2'-epicytoskyrin A was insignificantly differ from that of untreated cell. However, high amount cation was released from the treated cell at 2 MIC up to 6 times of the untreated cell. The absorbance at 260 and 280 nm represent the macromolecule nucleic acid and protein respectively (Woo *et al*. 2000; Carson *et al*. 2002). The presence of both substances in the media of bacterial suspension, indicated by the high absorbance at 260 and 280 nm, suggested the cell damage at the membrane level (Woo *et al*. 2000). Thus based on the results, the addition of (+)-2,2'-epicytoskyrin A in this study did not seem to cause the membrane damage of bacteria.

The extensive of Ca^{2+} and K^{+} leakage at 2 MIC indicated that (+)-2,2'-epicytoskyrin A could have disrupted the membrane permeability. The bacterial membrane is selective permeable to some ions and organic molecules, thus provide an important role in protecting cell. The slight alteration on this permeability can affect the cell metabolism and eventually result in cell death (Cox *et al.* 2001). Disruption of the normal functioning mechanism of the selective membrane permeability caused the cations to be transported across their concentration gradient (Henie *et al*. 2009).

(+)-2,2'-Epicytoskyrin A as antibacterial could have act in a similar way as its epimer, cytoskyrin A. Cytoskyrin A was demonstrated to cause DNA damage in bacteria cell by a low concentration as 12 ng/spot in biochemical induction assay (BIA) (Singh *et al*. 2007). The effect of (+)-2,2'-epicytoskyrin A to the membrane integrity of the bacteria cell could have been the result of its damaging action to DNA bacteria. However, other possibilities are present because some anthraquinones have known to exhibit different mechanism of action as antibacterial. They can disrupt the redox processes of bacteria and at high concentration can disrupt the cell membrane as demonstrated by cationic anthraquinone such as naphthotriazole-4,9-dione (Chan *et al*. 2011), Some anthraquinones including rubiadin, damnacanthal, and 5,5'-bisoranjidiol are direclty linked to the increase of reactive oxygen species (ROS) level, thus lead to oxidation process of biomolecules and eventually to cell damage (Comini *et al*. 2011). The precise way in which (+)-2,2'-epicytoskyrin A alter the integrity of bacteria cell membrane still need to be studied further.

To conclude, (+)-2,2'-Epicytoskyrin A exhibited excellent activity against some Gram-positive bacteria and Gram-negative bacteria. The effect of its treatment to Gram-positive bacteria model, *S. aureus*, resulted in alteration of bacteria cell membrane with an increase of cation efflux, while the cytoplasmic content was not leaked out. (+)-2,2'-epicytoskyrin A was also resulted in cell damage, indicated as small hole over the bacteria cell, yet the cause of this process still needs to be studied further.

ACKNOWLEDGMENTS

The authors thank to Balitvet Culture Collection and Microbiology Department, University of Indonesia for supply the clinical microbial isolates used in this work. Many thanks also deliver to Andi Saptaji Kamal, and Zurrahmi Ulya for technical assistant. This work financialy supported by a

Kompetitif LIPI research project.

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