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มหาวิทยาลัยรังสิต
เมืองเอก ก.พหลโยธิน
จ.ปทุมธานี 12000

Rangsit University
Muang-Ake, Paholyothin Rd.
Pathumthani 12000, Thailand

T. (66) 2997 2200-30
F. (66) 2533 9470
E. info@rsu.ac.th

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Dear Panita Khlaychan,

I am pleased to inform that paper entitled

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of the authors named
Panita Khlaychan, Acharawan Thongmee, Patamaporn Sukplang, Nitirat Chimnoi and Supanna Techasakul

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Thank you for submitting your work to our journal.

Yours sincerely,

Wipaporn Kajornwongwattana

Wipaporn Kajornwongwattana
Managing Editor
Interprofessional Journal of Health Sciences (Interprof. J. Health Sci.)
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Rangsit University, Thailand

Antimicrobial Activity of Coronarin D against *Staphylococcus aureus* and its Synergistic Potential with Antibiotics

Panita Khlaychan[1], Acharawan Thongmee*[2], Patamaporn Sukplang[2]
Nitirat Chimnoi[3], and Supanna Techasakul[3]

[1]Biomedical Sciences Graduate Program, Department of Medical Science, Faculty of Science, Rangsit University, Pathum Thani 12000, Thailand
[2] Department of Medical Science, Faculty of Science, Rangsit University, Pathum Thani 12000 , Thailand
[3] Chulabhorn Research Institute, Kamphaeng Phet 6 Road, Talat Bang Khen, Lak Si, Bangkok 10210, Thailand

*Corresponding author: acharawan@rsu.ac.th

ABSTRACT

Staphylococcus aureus causes various infections in humans such as food poisoning, skin and soft tissue infection, and sepsis. Effective new drugs are needed to control *S. aureus* associated infections due to the emergence and rapid antibiotic resistance. In the present study, the antibacterial activity of Coronarin D, the main compound in *Hedichium coronarium*, was investigated. Antibacterial activity of coronarin D was tested against methicillin-susceptible *Staphylococcus aureus* (MSSA) and methicillin-resistant *Staphylococcus aureus* (MRSA) strains by broth dilution method. Synergistic effects were also determined with a combination of coronarin D and 5 antibacterial agents.

It was found that the MIC and MBC of coronarin D against both MSSA and MRSA were 15.6-50 μg/mL and 50-100 μg/mL, respectively. In addition, the combination of coronarin D and some antibiotics showed more potent antibacterial activity against MSSA and MRSA than antibiotics or coronarin D alone. The best antibacterial activity was seen in the combination of coronarin D-penicillin G at FICI value of 0.25 against MSSA and coronarin D-polymyxin B at FICI value of 0.25 against MRSA. These results showed that coronarin D had antibacterial activity against *S. aureus* and showed the synergistic effect with certain antibiotics.

Keywords: coronarin D, MRSA, antimicrobial agent, synergism

INTRODUCTION

Staphylococcus aureus can cause a variety of potentially serious infections both in community-acquired infections and hospital-acquired infections. *S. aureus* is the causative agent of multiple human infections including bacteremia, infective endocarditis, osteomyelitis, septic arthritis, pneumonia, gastroenteritis, meningitis, toxic shock syndrome, and urinary tract infections, skin, and soft tissue infections, e.g., impetigo, folliculitis, furuncles, carbuncles, cellulitis, scalded skin syndrome **(Tong *et al.* 2015)**. *S. aureus* infections can be treated with many antimicrobial agents. Penicillin is one of the active antibacterial agents, but many *S. aureus* strains now resist penicillins. Penicillin-resistant *S. aureus* is associated with the production of penicillinase (β-lactamase) which hydrolyzes the β-lactam ring of penicillin forming no antimicrobial activity product named penicillonic acid **(Lowy, 2003)**. The semi-synthetic β-lactamase-resistant penicillins such as methicillin and oxacillin first introduced in 1959 created a general decline in the penicillin-resistant *S. aureus* during early 1960. However, in 1961

methicillin-resistant *Staphylococcus aureus* (MRSA) were first found in a hospital in the United Kingdom and the first reported case of MRSA in the United States was in 1968. MRSA is a mutated form of *S. aureus* resistant to antibiotics, known as β-lactams such as methicillin, oxacillin, and penicillin. In addition, MRSA also resists sulfonamides, erythromycin, amino-glycosides, tetracyclines, and clindamycin. Vancomycin and teicoplanin are used for the treatment of MRSA infections. However, the vancomycin-intermediate *S. aureus* (VISA) was first found in Japan in 1996, and the vancomycin-resistant *S. aureus* (VRSA) was found in India in 2011**(Chambers H.F.&DeLeo F.R., 2009)**.

The emergence of MRSA has led to the development of novel antimicrobial agents. Among the potential sources of new antimicrobial agents, natural products could be an interesting alternative. One of the important sources of natural products is plants or herbs which are rich in a wide variety of active compounds such as tannin, terpenoids, alkaloids, and flavonoids. These compounds have been found in vitro to have antimicrobial properties with lesser side effects and reduced toxicity when compared to synthetic agents. Therefore, many plant extracts have been interested as sources of antimicrobial agents. One of the interesting plant extracts is Coronarin D which is extracted from *Hedychium coronarium* or Zingiberaceae family.

Coronarin D is a labdane type diterpene mainly isolated from the rhizomes of *H. coronarium* which is known in Thai as "Mahahong. ". Coronary D shows many biological activities such as anti-inflammatory properties **(Kiem *et al.* 2011)** and anticancer activity **(Kunnummakkara *et al.* 2002)**. In addition, Coronarin D can induce apoptotic cell death of hepatocellular carcinoma cells **(Lin *et al.* 2018)**. Furthermore, Coronarin D has antifungal activity against *Candida albicans* **(Kaomongkolgit *et al.* 2012)**.

However, there are few reports about the antibacterial activity of coronarin D, especially against drug-resistant strains. Thus, the antibacterial activity of coronarin D against drug- resistant bacterial strains such as MRSA should be evaluated. In addition, an alternative approach to treat infectious diseases is the use of plant extracts in the combination with antibiotics in order to decrease the amount of antibiotics and reduce the adverse effects while the antimicrobial activity is still the same. Therefore, the efficiency of coronarin D and the synergism between coronarin D and some antibiotics to inhibit MRSA will be determined in this study.

MATERIALS AND MET.0HODS

Bacterial Strains

Methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-susceptible *S. aureus* (MSSA) strains were kindly provided by the culture collection of the Microbiology Unit, Department of Medical Sciences, Faculty of Science, Rangsit University.

These isolates were identified as *S. aureus* by standard microbiological methods including gram stain, catalase, coagulase, and growth on Mannitol salt agar (MSA). *S. aureus* ATCC25923 and *S. aureus* ATCC43300 were also included in this study as reference strains for MSSA and MRSA, respectively.

All *S. aureus* isolates were screened for MRSA and MSSA by using a 1 µg oxacillin disk diffusion test. In addition, Polymerase chain reaction (PCR) was performed to detect the *mecA* gene.

Coronarin D

Coronarin D was kindly provided by Nitirat Chimnoi, Chulabhorn Research Institute. It was extracted from the rhizome of *H. coronarium*. The extraction method and the chemical structure of this compound were previously described (**Chimnoi *et al.*, 2008, Chimnoi *et al.*, 2009)**.

Antimicrobial susceptibility testing

Coronarin D was screened for its inhibitory activity against MRSA and MSSA according to the Clinical and Laboratory Standards Institute **(CLSI, M100-S23, 2015)**. Briefly, a lawn 6*culture of approximately 10^8 cells/mL of the test bacteria was made on Mueller Hinton agar (MHA) plates. Then sterile 6 mm in diameter filter paper disks loaded with 10 µL of coronarin D dissolved in undiluted dimethyl sulfoxide (DMSO) at the concentration of 10 mg/mL was placed on the MHA plates. The plates were then incubated at 37 °C for 24 hours and the diameter of the inhibition zone was measured. The zone of inhibition of coronarin D was compared with DMSO as controls. Each susceptibility experiment was performed in triplicates. The diameters of the zone of growth inhibition around the disks measured in millimeters were averaged mean values.

Determination of MIC and MBC

MIC was determined by the microdilution method. MRSA and MSSA were cultured in 5 mL Mueller Hinton broth (MHB) and incubated in a shaker incubator at 37˚C for 18-24 hours. The bacteria were adjusted to the 0.5 McFarland standards with MHB solution to achieve a concentration of approximately 10^8 cells/mL. Then this bacterial culture was further diluted in MHB to obtain 10^6 cells/mL suspensions. Twenty microliters of coronarin D were added into the first well of the 96-well plate containing 180 µl of 10^6 cells/mL bacterial suspension in MHB. Then the mixture from the first well was two-fold serial diluted with MHB containing 10^6 cells/mL bacterial suspension. Then the plate was incubated at 37˚C for 18-24 hours. Each dilution was done in duplicate. The MIC was taken as the lowest concentration that inhibited the growth of bacteria. Inhibition of microbial growth in the 96-well plate containing coronarin D was judged by comparison with the growth control i.e., bacteria suspended in 10% DMSO without the coronarin D. The MIC results were expressed in µg/mL.

The minimal bactericidal concentration (MBC) is defined as the lowest concentration of coronarin D that kills the bacteria. All wells that showed no growth in the MIC studies were determined for MBC. Ten microliters of the clear suspension were transferred and spotted onto MHA that contain no test agent and incubated at 37˚C for 18-24 hours. The lowest concentration that showed no growth on MHA was taken as MBC **(Wiegand, Hilpert & Hancock, 2008)**.

Synergistic effects

The screening test of synergistic activity was modified from **Kaur *et al.* (2013)**. A lawn culture of approximately10^8 cells/mL of the test bacteria was placed on MHA plates. The filter paper disk (10 µL/disk) containing 10 µL coronarin D dissolved in DMSO and the different concentrations of each antibiotic agent was placed on the MHA plates, and the distances were 10 mm for each disk. The plates were incubated at 37˚C for 18-24 hours. An increase in the inhibition

zone between the disks of coronarin D and antibiotic was considered positive for a synergistic effect. Each experiment was performed in triplicates

Checkerboard broth microdilution method

Synergistic activity was determined by the checkerboard broth microdilution method in order to detect minimal inhibitory concentration (MIC) of the combination of antibiotic and coronarin D as modified from **Reuk-ngam *et al.* (2014)**. The test bacteria were adjusted to the 0.5 McFarland standards with MHB to achieve a concentration of approximately 10^8 cells/mL. Then this bacterial culture was further diluted in MHB to obtain 10^6 cells/mL. The chosen antibiotic and coronarin D were two-fold serial diluted with MHB containing 10^6 cells/mL of test bacteria in 96 well plates. The antibiotic was diluted from right to left, and coronarin D was diluted from bottom to top. Then the plates were incubated at 37°C for 18-24 hours. Each combination was repeated in triplicates. The result was taken as the minimum concentration of the test reagent that inhibited the growth of bacteria.

The interaction between coronarin D and antimicrobial agents was estimated by calculating the fractional inhibitory concentration (FIC index) of the combination. The concentration of the individual compound in the combination of coronarin D and antibiotic in which the growth of bacteria is completely inhibited is taken as the MIC of the individual compound in the combination. The fractional inhibitory concentration was calculated as follows:

$$\text{FIC of compound } \mathbf{a}\ (\text{FIC}_a) = \frac{\text{MIC of compound } \mathbf{a} \text{ in combination}}{\text{MIC of compound } \mathbf{a} \text{ alone}}$$

$$\text{FIC of compound } \mathbf{b}\ (\text{FIC}_b) = \frac{\text{MIC of compound } \mathbf{b} \text{ in combination}}{\text{MIC of compound } \mathbf{b} \text{ alone}}$$

The sum of fractional inhibitory concentration (FIC_I) indices of two compounds in the combination was calculated as follows:

$$FIC_a + FIC_b = FIC_I$$

FIC index values were interpreted according to European committee for antimicrobial susceptibility testing **(EUCAST 2000)**. Synergism interaction was defined as an FIC index of 0.5 or less; additive interaction was defined as an FIC index of more than 0.5 and less than 1; an Indifferent effect was defined as an FIC index of more than 1 or less than 2 and antagonism interaction was defined as FIC index of more than 2.

RESULTS AND DISCUSSION

Antibacterial activity of antibiotics and coronarin D against MRSA and MSSA

Coronarin D and antibiotics were determined for antimicrobial activities against MRSA and MSSA by the agar diffusion method. The results revealed that coronarin D and antibiotics possessed antibacterial activity against MRSA and MSSA as shown in Table 1 and Table 2.

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of coronarin D and antibiotics

Coronarin D inhibited MSSA with the MIC values range from 15.6 μg/mL to 50 μg/mL, and killed MSSA with the MBC values range from 50 μg/mL to 100 μg/mL as shown in Table 3. The MIC and MBC values of coronarin D against MSSA were higher than penicillin G and vancomycin but lower than polymyxin B and colistin. In addition, coronarin D inhibited MRSA with the MIC values range from 15.6 μg/mL to 50 μg/mL, and killed MSSA with the MBC values range from 31.25 μg/mL to 50 μg/mL as shown in Table 4. Since the MBC values of coronarin D when tested with MSSA and MRSA are not more than 4xMIC, coronarin D was considered as bactericidal effect **(Pankey G.A. & Sabath L.D, 2004)**.

The antibacterial activity of coronarin D could be explained by the ability of this compound to cross the bacterial cell membrane and cause bacterial cell damage. Coronarin D, a labdane-type diterpene consisting of decalin ring and unsaturated lactone ring with one hydroxyl group was considered as a hydrophobic molecule; therefore, it could penetrate more easily into and interrupt the cell membrane of Gram-positive bacteria than that of Gram-negative bacteria. Gram-positive bacteria allowed the hydrophobic compounds to penetrate and/or damage the cell membrane more easily. **(Urzúa *et al.* 2008, Kaomongkolgit *et al.*, 2012, and Reuk-ngam *et al.*, 2014)**. Besides antibacterial activity, coronarin D also has antifungal activity. Coronarin D showed antifungal activity against *C. albicans* with the MIC values of 2 mg/mL **(Kaomongkolgit *et al.*, 2012 and Reuk-ngam *et al.*, 2014).**

Synergistic effect of coronarin D with antibiotics against MRSA and MSSA

The synergistic effects of coronarin D with antibiotics such as penicillin G, vancomycin, polymyxin B, and colistin against MRSA and MSSA were determined by disk diffusion method. The combination of coronarin D-Polymyxin B showed synergistic effects against all test MRSA and MSSA strains as shown in Table 5 and Table 6 and Figure 1. In addition, good synergistic effects against MSSA were found in the combination of coronarin D-colistin.

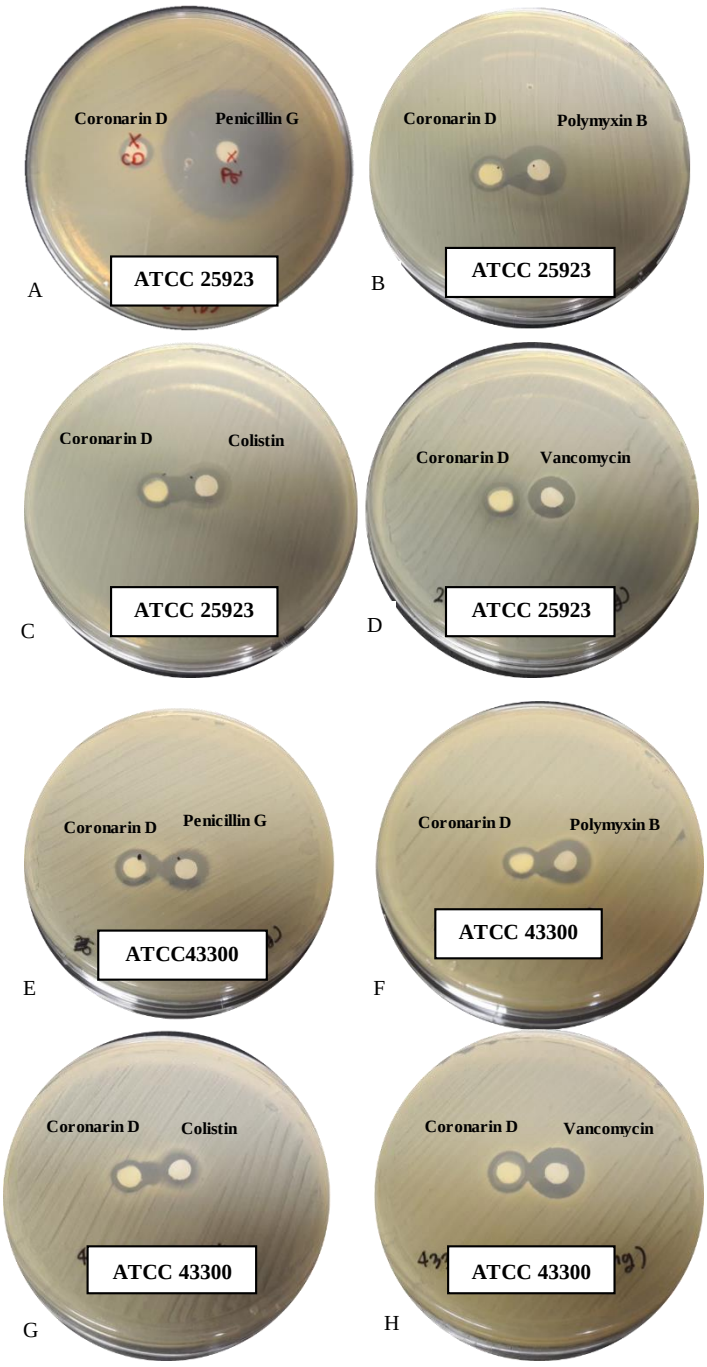

Figure 1 Synergistic effect of Coronarin D and antibiotics against MRSA and MSSA (A, B, C, D) Combination of coronarin D with penicillin G, polymyxin B, colistin and vancomycin, respectively, against *S. aureus* ATCC 25923 (E, F, G, H) Combination of coronarin D with penicillin G, polymyxin B, colistin and vancomycin, respectively, against *S. aureus* ATCC 43300.

Table 1 Antibacterial activity of coronarin D and antibiotics against methicillin-susceptible *Staphylococcus aureus*

	Inhibition zone of coronarin D and antibiotics against methicillin-susceptible S. *aureus* (mm)				
Strains	Coronarin D	Penicillin G	Vancomycin	Polymyxin B	Colistin
ATCC 25923	9.3±1.0	29.5±1.0	15.3±0.6	6.7±1.2	7.3±0.6
MSSA1	11.7± 2.1	19.0±1.0	15.3±0.6	10.3±2.5	8.3±0.6
MSSA2	10.0 ± 1.0	14.0±1.0	14.3±1.2	10.3±2.1	7.0±1.0
MSSA3	11.7±1.2	14.7±0.6	150.±0.0	10.0±0.0	6.3±0.6
MSSA4	10.0±1.0	35.3±4.2	15.3±0.6	14.0±1.0	7.0±0.0
MSSA5	9.3±0.6	13.0±1.7	14.3±0.6	9.7±0.6	6.7±0.6
MSSA6	10.0±1.7	13.3±0.6	15.0±0.0	9.7±0.6	6.7±0.6
MSSA7	9.7±1.2	27.3±6.4	14.7±0.6	12.0±0.0	6.7±0.6
MSSA8	9.7±0.6	15.0±1.0	15.0±0.0	9.3±1.5	7.0±1.7
MSSA9	11.7±1.2	35.3±1.2	15.0±0.0	12.7±0.6	7.0±1.7
MSSA10	9.0±1.0	9.3±0.6	15.0±0.0	12.0±1.0	7.3±1.2

Table 2 Antibacterial activity of coronarin D and antibiotics against Methicillin-Resistant *Staphylococcus aureus*

	Inhibition zone of coronarin D and antibiotics against methicillin-resistant *S. aureus* (mm)				
Strains	Coronarin D	Penicillin G	Vancomycin	Polymyxin B	Colistin
ATCC 43300	9.3±1.0	9.0±1.0	15.3.0±0.6	6.7±1.2	7.3±0.6
MRSA1	9.0±1.0	10.3±0.6	14.0±1.0	12.0±2.0	8.3±1.5
MRSA2	9.0±1.0	8.67±0.6	14.3±2.1	11.0±1.0	7.3±0.6
MRSA3	9.3±0.6	10.0±0.0	13.7±0.6	12.0±1.0	8.3±1.2
MRSA4	10.7±2.1	10.3±1.5	14.0±1.7	11.0±2.0	7.3±0.6
MRSA5	8.67±2.1	11.3±1.5	13.7±0.6	14.7±3.8	8.0±1.0
MRSA6	9.7±1.5	9.7±1.2	13.0±1.0	10.67±0.6	7.3±0.6
MRSA7	10.0±1.0	8.3±1.2	13.0±1.7	12.3±0.6	8.0±1.0
MRSA8	9.3±1.2	8.0±1.0	13.0±1.0	9.7±0.6	7.3±0.6
MRSA9	13.0±5.3	8.0±1.0	15.7±1.2	11.0±0.0	6.0±0.0
MRSA10	9.67±1.2	11.0±1.7	15.3±0.6	10.3±1.5	6.0±0.0

Table 3 MIC and MBC values (µg/mL) of antibiotics and coronarin D against methicillin-susceptible *Staphylococcus aureus* (MSSA)

	MIC and MBC of Coronarin D and Antibiotics (µg/mL)									
	Coronarin D		**Penicillin G**		**Vancomycin**		**Polymyxin B**		**Colistin**	
Strains	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
ATCC 25923	15.6	62.5	0.015	0.046	1.95	1.95	62.5	125	250	416.67
MSSA1	25	50	12.5	50	1.56	100	25	100	100	500
MSSA2	50	100	3.125	6.25	1.56	6.25	25	100	100	250
MSSA3	50	50	12.5	25	1.56	12.5	25	100	100	250
MSSA4	50	50	0.049	3.125	1.56	100	25	100	100	250
MSSA5	25	50	3.125	25	0.78	100	12.5	100	50	250
MSSA6	25	50	1.56	12.5	0.78	100	12.5	199	50	250
MSSA7	25	50	0.195	3.125	1.56	100	12.5	100	50	250
MSSA8	25	50	12.5	50	1.56	100	12.5	100	25	250
MSSA9	25	50	0.049	3.125	0.78	50	12.5	100	50	250
MSSA10	25	50	6.25	25	1.56	6.25	25	50	25	250

Table 4 MIC and MBC values (μg/mL) of antibiotics and coronarin D against methicillin-resistant *Staphylococcus aureus* (MRSA)

	MIC and MBC of Coronarin D and Antibiotics (μg/mL)									
	Coronarin D		Penicillin G		Vancomycin		Polymyxin B		Colistin	
Strains	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
ATCC 43300	15.6	31.25	125	250	1.95	3.9	62.5	125	250	500
MRSA1	50	50	12.5	208.3	1.56	250	25	125	50	250
MRSA2	50	50	12.5	250	3.125	250	50	250	100	500
MRSA3	50	50	12.5	250	3.125	250	50	250	100	500
MRSA4	50	50	12.5	250	6.25	250	25	125	100	500
MRSA5	50	50	12.5	250	3.125	250	25	250	100	250
MRSA6	50	50	12.5	250	3.125	250	25	250	100	250
MRSA7	50	50	12.5	250	3.125	250	50	250	100	250
MRSA8	50	50	12.5	250	3.125	250	50	250	100	500
MRSA9	50	50	12.5	250	6.25	250	50	250	100	500
MRSA10	50	50	6.25	250	1.56	125	12.5	250	100	500

Table 5 Synergistic effects of coronarin D and antibiotics against methicillin-susceptible *Staphylococcus aureus* (MSSA)

	Numbers of MSSA strain		
Combination	Synergy	Indifference/additive	Antagonism
Coronarin D-Penicillin G	6	5	0
Coronarin D-Vancomycin	4	7	0
Coronarin D-Polymyxin B	11	0	0
Coronarin D-Colistin	11	0	0

Synergy effects (bringing of the zone of inhibition and appearance of the zone of inhibition in between coronarin D and an antibiotic); Indifference/additive (no effect on a zone of inhibition); Antagonism (flattening of a zone of inhibition)

Table 6 Synergistic effects of coronarin D and antibiotics against methicillin-resistant *Staphylococcus aureus* (MRSA)

	Numbers of MRSA strain		
Combination	Synergy	Indifference/additive	Antagonism
Coronarin D-Penicillin G	4	7	0
Coronarin D-Vancomycin	1	10	0
Coronarin D-Polymyxin B	11	0	0
Coronarin D-Colistin	10	1	0

Synergy effects (bringing of the zone of inhibition and appearance of the zone of inhibition in between coronarin D and an antibiotic); Indifference/additive (no effect on a zone of inhibition); Antagonism (flattening of a zone of inhibition)

Synergistic effect of coronarin D and antibiotics determined by the checkerboard method

The synergistic effect of coronarin D and antibiotics such as penicillin, vancomycin, polymyxin B, and colistin were also determined by the fractional inhibitory concentration index (FIC_I). The FIC_I values were calculated according to the research of **Didry *et al.* (1993)**. Synergism interaction was defined as an FIC index of 0.5 or less; additive interaction was defined as an FIC index of more than 0.5 and less than 1; an Indifferent effect was defined as an FIC index of more than 1 or less than 2 and antagonism interaction was defined as FIC index of more than 2. The results summarized in Table 7 were shown potential synergism of coronarin D and four antibiotics against MSSA by the FIC index; Out of 4 strains in 11 strains had synergistic effects and 7 strains had indifferent effect interaction for the combination between coronarin D and penicillin G, 6 strains in 11 strains had a synergistic effect and 5 strains had indifferent effect interaction for the combination between coronarin D and vancomycin, 7 strains had a synergistic effect and 4 strains had indifferent effect interaction for the combination between coronarin D and polymyxins B. Out of 9 strains had a synergistic effect and 2 strains had indifferent effect interaction for the combination between coronarin D and colistin.

The FIC index of synergism coronarin D with four antibiotics against MRSA was displayed in Table8. Out of 5 strains in 11 strains had synergistic effects and 6 strains had indifferent effect interaction for the combination between coronarin D and penicillin G, 9 strains in 11 strains had a synergistic effect and 2 strains had indifferent effect interaction for the combination between coronarin D and vancomycin. All of 11 strains had a synergistic effect for the combination between coronarin D and polymyxins B. In addition, the combination of coronarin D-polymyxin B and coronarin D-penicillin G showed the best synergistic effect against MRSA and MSSA, respectively.

The investigation of synergistic effects between natural products and antibiotics is an alternative approach to fight pathogens. Due to the combination, the MIC value of antibiotics is decreased which was very beneficial because toxicity and/or side effects from antibiotics usage were reduced, and the emerging of resistance strains was prevented or prolonged. In addition, the process of developing a new drug is very expensive and time-consuming; therefore, using well-known drugs in combination with herbal substances is a good option to combat infectious diseases. **(Reuk-ngam *et al.*, 2014).** In this study, to screen for the synergistic effects of coronarin D and antibiotics, the agar diffusion method was employed. The results revealed that the combination of coronarin D-Polymyxin B showed synergistic effects against all test MRSA and MSSA strains. In addition, good synergistic effects against MSSA were found in the combination of coronarin D-Colistin.

Furthermore, the synergistic effects of coronarin D with antibiotics were determined by the checkerboard method. The fractional inhibitory concentration index (FIC_I) value was utilized to assess the synergism (total synergism, $FIC_I \leq 0.5$; additive interaction, $0.5 < FIC_I \leq 4$; and antagonism $FIC_I > 4$). The results revealed that all of the combinations were showed synergistic effects against MRSA and MSSA. The best synergism was obtained from the combinations of coronarin D with penicillin G with FIC_I values at 0.25 against MSSA. In addition, when tested with MRSA the best synergism was obtained from the combinations of coronarin D with polymyxin B with FIC_I values at 0.25. **Reuk-ngam *et al.* (2014)** studied synergistic effects between coronarin D and 9 antibiotics against *S. aureus*. Their report was in agreement with our results that coronarin D showed a synergistic effect with polymyxin B against *S. aureus* ATCC25923 strain.

Table 7 Fractional inhibitory concentration index (FIC_I) of antibiotics in combination with coronarin D against methicillin-susceptible *Staphylococcus aureus* (MSSA).

Bacteria strain	ATCC 25923	MSSA 1	MSSA 2	MSSA 3	MSSA 4	MSSA 5	MSSA 6	MSSA 7	MSSA 8	MSSA 9	MSSA 10
MIC of coronarin D	15.6	25	50	50	50	25	25	25	25	25	25
MIC of penicillin G	0.02	12.5	3.13	12.5	0.05	3.13	1.56	0.2	12.5	0.05	6.25
Concentration of coronarin D in combination (gain)	1.95 (8)	6.25 (4)	5.21 (9.6)	25 (2)	12.5 (2)	25 (1)	12.5 (2)	12.5 (2)	12.5 (2)	16.67 (1.5)	16.67 (1.5)
Concentration of penicillin G in combination (gain)	0.001875 (8)	1.3 (9.6)	0.52 (6)	6.25 (2)	0.010 (4.9)	0.0122 (256)	0.39 (0.4)	0.024 (8.125)	2.6 (4.8)	0.0164 (3)	2.09 (3)
FIC_I	0.25	0.35	0.27	1	0.46	1	0.75	0.63	0.71	1	1
MIC of vancomycin	1.95	1.56	1.56	1.56	1.56	0.78	0.78	1.56	1.56	0.78	1.56
Concentration of coronarin D in combination (gain)	15.6 (1)	16.67 (1.5)	25 (2)	9.375 (5.3)	10.42 (4.8)	3.65 (6.9)	25 (1)	2.1 (11.9)	3.125 (8)	12.5 (2)	25 (1)
Concentration of vancomycin in combination (gain)	0.0076 (256)	0.522 (3)	0.134 (12)	0.26 (6)	0.26 (6)	0.1625 (4.8)	3.125 (0.25)	0.585 (2.7)	0.39 (0.4)	0.13 (6)	0.0061 (256)
FIC_I	1	1	0.59	0.35	0.38	0.35	0.38	0.46	0.38	0.67	1
MIC of polymyxin B	62.5	25	25	25	25	12.5	12.5	12.5	12.5	12.5	25
Concentration of coronarin D in combination (gain)	3.9 (4)	6.25 (4)	10.417 (4.8)	10.42 (4.8)	6.25 (8)	20.83 (1.2)	3.125 (8)	6.25 (4)	1.5625 (16)	3.125 (8)	25 (1)
Concentration of polymyxin B in combination (gain)	7.8125 (8)	1.56 (16)	5.21 (4.8)	5.21 (4.8)	2.62 (9.5)	2.12 (5.9)	3.125 (4)	3.125 (4)	6.25 (2)	6.25 (8)	0.0976 (256)
FIC_I	0.38	0.31	0.42	0.42	0.23	1	0.38	0.5	0.56	0.63	1
MIC of colistin	250	100	100	100	100	50	50	50	25	50	25
Concentration of coronarin D in combination (gain)	3.9 (4)	1.5625 (16)	5.21 (9.6)	3.125 (16)	3.125 (16)	5.21 (4.8)	3.125 (8)	3.125 (8)	3.125 (8)	3.125 (8)	10.42 (2.4)
Concentration of colistin in combination (gain)	31.25 (8)	50 (2)	16.67 (6)	14.58 (6.8)	12.5 (8)	8.33 (6)	3.125 (16)	6.25 (8)	4.17 (6)	5.21 (9.6)	6.25 (4)
FIC_I	0.38	0.56	0.25	0.21	0.19	0.38	0.19	0.25	0.29	0.23	0.58

Table 8 Fractional inhibitory concentration index (FIC_I) of antibiotics in combination with coronarin D against Methicillin-Resistant *Staphylococcus aureus* (MRSA)

Bacteria strain	ATCC 43300	MRSA 1	MRSA 2	MRSA 3	MRSA 4	MRSA 5	MRSA 6	MRSA 7	MRSA 8	MRSA 9	MRSA 10
MIC of coronarin D	15.6	50	50	50	50	50	50	50	50	50	50
MIC of penicillin G	125	12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5	6.25
Concentration of coronarin D in combination (gain)	15.6 (1)	33.33 (1.5)	12.5 (4)	12.5 (4)	33.33 (1.5)	16.67 (3)	10.42 (4.8)	12.5 (4)	33.33 (1.5)	16.67 (3)	33.33 (1.5)
Concentration of penicillin G in combination (gain)	0.488 (256)	0.08 (156)	1.5625 (8)	1.5625 (8)	0.08 (156)	2.21 (5.65)	1.5625 (8)	1.5625 (8)	0.08 (156)	1.172 (10.67)	0.0366 (170)
FIC_I	1	0.67	0.38	0.38	0.67	0.51	0.25	0.25	0.67	0.43	0.67
MIC of vancomycin	1.95	1.56	3.13	3.13	6.25	3.13	3.13	3.13	3.13	6.25	1.56
Concentration of coronarin D in combination (gain)	15.6 (1)	25 (2)	12.5 (4)	12.5 (4)	12.5 (4)	12.5 (4)	12.5 (4)	12.5 (4)	12.5 (4)	12.5 (0.5)	12.5 (4)
Concentration of vancomycin in combination (gain)	0.0076 (256)	0.442 (3.5)	0.52 (6)	0.7813 (4)	1.3 (4.8)	0.39 (8)	0.39 (8)	0.52 (6)	0.28 (11.2)	0.58 (10.77)	0.195 (8)
FIC_I	1	0.67	0.42	0.5	0.46	0.38	0.38	0.42	0.34	0.34	0.38
MIC of polymyxin B	62.5	25	50	50	25	25	25	50	50	50	12.5
Concentration of coronarin D in combination (gain)	1.95 (8)	6.25 (8)	8.33 (6)	6.25 (8)	8.33 (6)	6.25 (8)	8.3 (6)	8.33 (6)	8.33 (6)	6.25 (8)	6.25 (8)
Concentration of polymyxin B in combination (gain)	7.8125 (8)	2.60 (9.6)	2.60 (19)	6.25 (8)	2.34 (10.68)	3.125 (8)	4.17 (6)	5.20 (9.6)	8.33 (6)	6.25 (8)	1.5625 (8)
FIC_I	0.25	0.23	0.22	0.25	0.26	0.25	0.33	0.27	0.33	0.25	0.25
MIC of colistin	250	50	100	100	100	100	100	100	100	100	100
Concentration of coronarin D in combination (gain)	3.9 (4)	12.5 (4)	12.5 (4)	6.25 (8)	6.25 (8)	8.33 (6)	5.21 (9.6)	9.375 (5.33)	6.25 (8)	6.25 (8)	4.17 (12)
Concentration of colistin in combination (gain)	31.25 (8)	2.08 (24)	3.125 (32)	12.5 (8)	6.25 (16)	12.5 (8)	9.375 (10.67)	8.5938 (11.6)	6.25 (16)	8.33 (12)	5.21 (19.19)
FIC_I	0.38	0.29	0.31	0.25	0.19	0.29	0.2	0.35	0.19	0.21	0.14

It has been shown that polymyxin B and colistin (polymyxin E) are narrow antibacterial spectrum and most effective against Gram-negative bacteria. Since polymyxin B and colistin have a positive charge, they bind with a negative charge of the lipopolysaccharide (LPS) of the gram-negative bacteria and cause the instability of the cell membrane and the leakage of fluid within the cell, inducing cells death **(Ezadi F., Ardebili A., & Mirnejadc R., 2019).** This study showed that in combination with coronary D, polymyxin B or colistin, showed inhibition efficacy against gram-positive bacteria better than a single compound. Coronarin D can inhibit gram-positive bacteria and has a mechanism of action on the cell membrane of gram-positive bacteria. Coronarin D can penetrate and interrupt the cell membrane by the structure of coronarin D, as a hydrophobic molecule. For this reason, Polymyxin B and colistin combined with Coronarin D were easier led to Gram-positive bacteria and causing damage in Gram-positive bacteria **(Reuk-ngam *et al.*, 2014)**.

CONCLUSION

In conclusion, coronarin D could be used as an antibacterial agent against MRSA and MSSA. The synergistic effect was observed in the combination of coronarin D to certain antibiotics, such as polymyxin B, penicillin G, and colistin. The mode of action of this molecule may involve cell membrane disruption.

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