ANTIBACTERIAL ACTIVITY OF CORONARIN D AND ITS SYNERGISTIC EFFECT WITH ANTIBIOTICS ON *STAPHYLOCOCCUS AUREUS*

BY

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โดย

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วิทยานิพนธ์ฉบับนี้จะไม่ประสบความสำเร็จลุล่วงไปได้ถ้าปราศจากช่วยเหลือและการให้คำปรึกษาอย่างดีจากหลายท่าน ขอขอบพระคุณอาจารย์ที่ปรึกษาวิทยานิพนธ์ ผศ. รตท. หญิง ดร. อัจฉราวรรณ ทองมี และอาจารย์ที่ปรึกษาร่วม ผศ. ดร. ปถมาพร สุกปลั่ง ได้ประสิทธิประสาทความรู้และคำแนะนำในการทำวิจัย ตลอดจนให้ความช่วยเหลือในการเขียนและตรวจสอบวิทยานิพนธ์จนประสบผลสำเร็จเป็นอย่างดี ผู้วิจัยขอกราบขอบพระคุณเป็นอย่างสูง

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งานวิจัยนี้สำเร็จได้เป็นอย่างดีเนื่องมาจากได้รับการสนับสนุนจากสถาบันวิจัยจุฬาภรณ์ รองศาสตราจารย์ ดร.สุพรรณนา เตชะสกุล และคุณนิติรัตน์ ฉิมน้อย ที่อนุเคราะห์สารทดสอบที่ใช้ทดลองรวมทั้งการสนับสนุนทางห้องปฏิบัติการและอุปกรณ์ต่างๆ ที่ใช้ในการทำวิจัยนี้ ผู้วิจัยขอกราบขอบพระคุณเป็นอย่างสูง

สุดท้ายนี้ขอขอบพระคุณครอบครัวและเพื่อนๆทุกคนที่ให้การสนับสนุนตลอดระยะเวลาในการทำงานวิจัยนี้

ปณิตา คล้ายจันทร์

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Abstract

S. aureus causes community-acquired and hospital-acquired infections. Many antibiotics are used to treat infected patients while the development of resistant bacteria increases. Therefore, many researchers are interested in searching new antimicrobial agents. Coronarin D is a natural compound as an antimicrobial agent and the main compound in *Hedichium coronarium* with anti-inflammatory, anticancer, and antimicrobial properties. The study, hence, aimed to investigate the antibacterial activity of coronarin D against methicillin-susceptible *Staphylococcus aureus* (MSSA), methicillin-resistant *Staphylococcus aureus* (MRSA) and to conduct a clinical analysis of bacterial strain profiles isolated from infected patients by broth dilution method. Synergistic effects were determined by the checkerboard method with a combination of 5 antibacterial agents with coronarin D. The antibacterial activities were tested by the time-kill assay.

It was found that the MIC and the MBC of coronarin D against MSSA and MRSA were 15.6-50 µg/mL and 50-100 µg/mL, respectively. The best antibacterial activity was seen in the combination of coronarin D-penicillin G at FIC value of 0.25 against MSSA and coronarin D-polymyxin B at FIC_I value of 0.25 against MRSA. Moreover, the best activity of the combination was presented by the killing rate. The combination of coronarin D-polymyxin B killed MRSA within 4 hours; however, coronarin D or polymyxin B alone could not kill MRSA. For MSSA, the killing rate of the combination of coronarin D and polymyxin B was faster than that of coronarin D or polymyxin B alone. Those results showed that coronarin D presented synergistic effects with antibiotics resulting in a more potent killing rate than antibiotics or coronarin D alone.

(Total 42 pages)

Keywords: MRSA, Antimicrobial Agent, Natural Product, Synergism

Student's Signature …..... Thesis Advisor's Signature .....

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ชื่อวิทยานิพนธ์ : ฤทธิ์ต้านการเจริญของแบคทีเรียและการเสริมฤทธิ์กับยาปฏิชีวนะของ Coronarin D ในการยับยั้ง *Staphylococcus aureus*

หลักสูตร : วิทยาศาสตรมหาบัณฑิต สาขาวิชาวิทยาศาสตร์ชีวการแพทย์

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บทคัดย่อ

S. aureus เป็นสาเหตุสำคัญของโรคติดเชื้อทั้งโรคติดเชื้อที่ได้รับจากชุมชนและที่ได้รับจากโรงพยาบาล ยาปฏิชีวนะหลายชนิดถูกพัฒนาเพื่อใช้ในการรักษาโรคติดเชื้อนี้ ขณะที่เชื้อก่อโรคมีการพัฒนาการดื้อยาเพิ่มขึ้น นักวิจัยจึงพยายามค้นหาสารที่มีฤทธิ์ต้านเชื้อเพิ่มขึ้น งานวิจัยนี้สนใจศึกษาสารที่ได้จากธรรมชาติคือ Coronarin D ซึ่งเป็นสารประกอบหลักที่พบในพืช *Hedichium coronarium* มีรายงานว่าสารนี้มีคุณสมบัติต้านการอักเสบ มีฤทธิ์ต้านเซลล์มะเร็งและมีฤทธิ์ต้านจุลชีพ ในงานวิจัยนี้ศึกษาฤทธิ์ต้านจุลชีพของ Coronarin D ต่อ Methicillin susceptible *Staphylococcus aureus* (MSSA) Methicillin resistant *Staphylococcus aureus* (MRSA) ที่แยกได้จากผู้ป่วย ด้วยวิธี Broth dilution ทดสอบประสิทธิภาพการเสริมฤทธิ์ของ Coronarin D กับยาปฏิชีวนะ 4 ชนิด โดยวิธี Checkerboard และทดสอบประสิทธิภาพการยับยั้งการเจริญของแบคทีเรียด้วย Time kill assay

ผลการวิจัยพบว่า Coronarin D มีฤทธิ์ยับยั้ง MRSA และ MSSA โดยค่าความเข้มข้นที่ต่ำสุดของ Coronarin D ที่มีฤทธิ์ยับยั้งเชื้อและฆ่าเชื้อทดสอบมีค่า 15.6 -50 ไมโครกรัมต่อมิลลิลิตร และ 50 -100 ไมโครกรัมต่อมิลลิลิตร ตามลำดับ และพบการเสริมฤทธิ์ของ Coronarin D กับยาปฏิชีวนะ โดย Coronarin D สามารถเสริมฤทธิ์กับยา Penicillin G ได้ดีที่สุดในการต้าน MSSA ซึ่งมีค่าดัชนีชี้วัดรวมอยู่ที่ 0.25 และออกฤทธิ์ร่วมกับยา Polymyxin B ได้ดีที่สุด ในการต้านเชื้อ MRSA ซึ่งมีค่าดัชนีชี้วัดรวมอยู่ที่ 0.25 และพบว่าการใช้ Coronarin D ร่วมกับ Polymyxin B สามารถฆ่าเชื้อ MRSA ได้ภายใน 4 ชั่วโมง ขณะที่หากใช้ สาร Coronarin D หรือยาปฏิชีวนะเพียงอย่างเดียวไม่สามารถฆ่าเชื้อนี้ได้ และพบว่าการใช้ Coronarin D ร่วมกับ Polymyxin B สามารถฆ่าเชื้อ MSSA ได้รวดเร็วกว่าเมื่อใช้ สาร Coronarin D หรือยาปฏิชีวนะเพียงอย่างเดียว สรุปได้ว่า Coronarin D มีฤทธิ์ในการฆ่าแบคทีเรียและมีการเสริมฤทธิ์กับยาปฏิชีวนะ ซึ่งฤทธิ์ในการฆ่าแบคทีเรียของ Coronarin D ร่วมกับยาปฏิชีวนะดีกว่าการใช้ Coronarin D หรือยาปฏิชีวนะเพียงอย่างเดียว

(วิทยานิพนธ์มีจำนวนทั้งสิ้น 42 หน้า)

คำสำคัญ: MRSA, สารต้านจุลชีพ, ผลิตภัณฑ์ธรรมชาติ, การเสริมฤทธิ์

ลายมือชื่อนักศึกษา ..... ลายมือชื่ออาจารย์ที่ปรึกษา .....

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CHAPTER 1

INTRODUCTION

1.1 Background of the Research Problem

Staphylococcus aureus is Gram positive cocci that 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 to penicillins. Penicillin resistant *S.aureus* is associated with production of penicillinase (β-lactamase) which hydrolyzes 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, a mutated form of *Staphylococcus aureus*, resist to antibiotics known as β-lactams such as methicillin, oxacillin and penicillin. In addition, MRSA also resist to other antibiotics, such as sulfonamides, erythromycin, aminoglycosides, 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.

The emergence of MRSA has led to the development of novel antibiotics. Among the potential sources of new antimicrobial agents, natural product could be an interesting alternative. An important source 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 interested 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 *Hedychium coronarium* which is known in Thai as "Mahahong. ". Coronarin D shows many biological activities such as anti-inflammatory properties (Kiem et al., 2011) and anticancer activity (Kunnumakkara et al., 2008). 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, Jamdee, Wongnai, Chimnoi, & Techasakol, 2012).

However, there are few reports about antibacterial activity of coronarin D, especially against drug resistant strains. Thus, the antibacterial activity of coronarin D against drug resistant bacterial strain 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 antibiotic to inhibit MRSA will be determined in this study.

1.2 Objectives of research

1.2.1 To study the antimicrobial activity of coronarin D against methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-susceptible *Staphylococcus aureus* (MSSA)

1.2.2 To study the synergistic effect of coronarin D with antibiotics against methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-susceptible *Staphylococcus aureus* (MSSA)

1.3 Research hypothesis

1.3.1 Coronarin D alone can inhibit clinical isolates of methicillin-susceptible *Staphylococcus aureus* (MSSA) and methicillin-resistant *Staphylococcus aureus* (MRSA).

1.3.2 Coronarin D combined with antibiotics could show synergistic effects on antibacterial activity against clinical isolates of methicillin susceptible *Staphylococcus aureus* (MSSA) and methicillin resistant *Staphylococcus aureus* (MRSA).

1.4 Scope of the research

Coronarin D extracted from the rhizome of *Hedychium coronarium* was evaluated for the antibacterial activity against methicillin susceptible *Staphylococcus aureus* (MSSA) and methicillin resistant *Staphylococcus aureus* (MRSA) by agar diffusion method. The minimal inhibitory concentration (MIC), minimal bactericidal concentration (MBC) and killing rate of coronarin D were also determined. In addition, the synergistic effects of coronarin D in combinations with antibiotics were tested against methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-susceptible *Staphylococcus aureus* (MSSA).

CHAPTER 2

LITERATURE REVIEWS

2.1 General characteristics of *Staphylococcus aureus*

Staphylococcus aureus is a Gram-positive round-shaped bacterium appear as grapelike. *S.aureus* is a usual member of the human microbiota, frequently found in the upper respiratory tract and on the skin. The bacterial colonies on nutrient agar are smooth, round, and have pale-yellow to orange pigment. *S.aureus* is non-motile, non-spore forming, and facultative anaerobic bacterium. This bacterium possesses catalase and nitrate reductase. *S. aureus* has several virulence factors, e.g., cell wall proteins such as protein A and fibronectin-binding protein, exotoxins such as staphylococcal enteroxin (type A, B, C_1, C_2, C_3, D, E,), toxic shock syndrome toxin-1, staphylococcal enteroxin F, pyrogenic exotoxin C, exfoliative toxin, epidermolysin, and cytolytic toxin such as hemolysin, staphylolysin α, β, δ, and staphylococcal leucocidin, Pental-Valentine leucocidin, and enzymes such as coagulase, hyaluronidase, lipase, DNase, RNase, phosphatase, and fibrinolysin (Foster, 1996). Therefore, *S.aureus* can cause infections in many organs due to these virulence factors.

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2.2 Clinical significance of *Staphylococcus aureus*

Staphylococcus aureus is a normal inhabitant in the anterior nares of 25–30% of people and can also reside transiently on the skin. Since *S. aureus* produces many extracellular proteins, enzymes and toxins, this bacterium can cause infectious diseases in many organs, such as staphylococcal pneumonia, osteomyelitis, food poisoning, scalded skin syndrome, bacteremia, sepsis, toxic shock syndrome, and skin and wound infection, such as cellulitis, erysipelas, impetigo, folliculitis, furuncle, carbuncle, and abscess. *S. aureus* is associated with community and hospital acquired diseases and has been considered as a major problem of public health. In addition, *S.*

aureus is a one of the important pathogens due to the rise in antibiotic resistance. The spread of methicillin-resistant *S. aureus* (MRSA) strains which are often multi-drug resistant bacteria in hospitals and subsequently in community resulted in significant mortality and morbidity. (Gnanamani, Hariharan, & Paul-Satyaseela, 2017).

2.3 Treatment of *Staphylococcus aureus* infections

The first line antibiotic used for the treatment of *S. aureus* infections was β-lactams such as penicillin. After the introduction of penicillin in 1940s, penicillin resistant *S. aureus* (PRSA) was first reported. Penicillin resistant *S. aureus* produced a penicillinase enzyme called β-lactamase that hydrolyze the β-lactam ring of penicillin. Since penicillin resistant *S. aureus* strains are emerging, β-lactamase stable or penicillinase resistant semi-synthetic penicillins such as methicillin, oxacillin, flucloxacillin, and dicloxacillin have been developed for the treatment of this bacterial infections.

The other used antibiotics for penicillin resistant *S. aureus* strains are first generation cephalosporins (cefazolin, cephalothin, and cephalexin), macrolides (erythromycin), lincosamides (clindamycin and lincomycin), TMX/SMX (cotrimoxazole), tetracyclines (doxycycline), fluoroquinolones (ciprofloxacin), and aminoglycosides (gentamicin). The first-generation cephalosporins are stable to β-lactamase but are not as bactericidal as penicillins, macrolides (erythromycin, clarithromycin, and roxithromycin). Azithromycin is a semi-synthetic macrolide antibiotic of the azalide class. Like other macrolide antibiotics, azithromycin inhibits bacterial protein synthesis by binding to the 50S ribosomal subunit of the bacterial 70S ribosome. Lincosamides (lincomycin and clindamycin) inhibit peptide chain initiation and protein synthesis by binding to the 50s ribosomal unit.

After methicillin was introduced for treatment of penicillin resistant strains of *S.aureus,* the methicillin resistant strains of *S. aureus* (MRSA) were noted. Therefore, penicillin resistant *S. aureus* infections remain clinical problem while the methicillin resistant strains of *S. aureus* (MRSA) are emerging (Gnanamani et al., 2017).

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The alternative protocol for treatment of antibiotic resistant *S. aureus* infections is the use of antibiotic combinations. The combination of antibiotics has been interested for the treatment of metastatic infections since it might reduce antimicrobial resistance. For example, the combination of gentamycin and nafcillin was used for the treatment of endocarditis caused by *S. aureus.* (Miller, Wexler, & Steigbigel, 1978). It was showed that the combination of gentamycin and nafcillin reduced infection better than gentamycin or nafcillin treatment alone. In addition, the combination of rifampicin and fluoroquinolone was used for the treatment of staphylococcal infections (Drancourt et al., 1993). Furthermore, the combination of penicillin and aminoglycoside can be used in uncomplicated *S. aureus* endocarditis therapy (Ribera et al.,1996).

2.4 Methicillin-resistant *Staphylococcus aureus* (MRSA)

Methicillin-resistant *Staphylococcus aureus* (MRSA) emerged after the treatment of penicillin-resistant with methicillin. The resistant bacterial strains were isolated from patients in the hospital. Shortly after in the 1990s, it spread throughout worldwide (Harkins et al., 2017). MRSA causes a diversity problem for global healthy and also resist to many antibiotics. MRSA can be classified two types, Hospital acquired-MRSA (HA-MRSA) and Community acquired-MRSA (CA-MRSA) (Chambers & DeLeo, 2009). HA-MRSA usually resist to many antibiotics because they are evoked in hospital. (Sheen, 2010). CA-MRSA is unlike HA-MRSA that it has not been infected from hospital or medicine devices. Major genomic elements in CA-MRSA are genes that demonstrate drug resistance which finds more than one resistant gene as inserted in the sequence, transposon or plasmid (Turner et al., 2019). CA-MRSA is usually resistant to methicillin and β-lactam and it is sensitive to other antibiotics such as trimethoprim, sulfamethoxazole, clindamycin and tetracycline. (Deresinsk, 2005).

MRSA infections are a major threat. Moreover, sometimes antibiotics used for the treatment these infectious diseases are associated with side effects on the host including nausea, vomiting, headache, hypersensitivity, immune-suppression or

allergic reactions and may cause severe damage to the liver and cause bone marrow depression. Developing new medicines is extremely important. Many efforts have been made to synthesize and develop new compounds which can be used as potential antimicrobial agents to combat MRSA infections. Therefore, the search for new effective antimicrobial agents is interesting.

2.5 Coronarin D

Coronarin D is extracted from the rhizomes of *Hedychium coronarium* or Zingiberaceae family. This plant is widely cultivated in Thailand and it is known as "Mahahong". Major component of coronarin D is a labdene diterpene. The structure of coronarin D was shown in figure 2.1. The analysis of coronarin D by mass spectroscopic method was shown in figure 2.2 (Chimnoi et al., 2009).

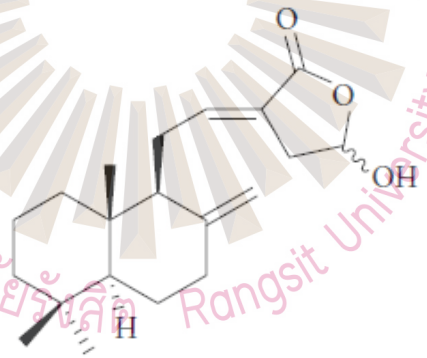
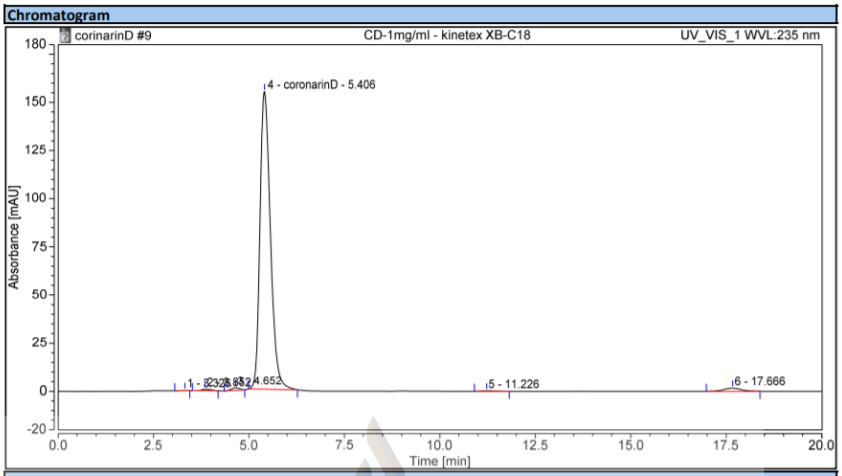


Figure 2.1 The structure of coronarin D

Source: Reuk-ngam, Chimnoi, Khunnawutmanotham, & Techasakul, 2014



Integration Results

No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Relative Area %	Relative Height %	Amount
1		3.326	0.028	0.150	0.06	0.09	n.a.
2		3.852	0.228	0.790	0.45	0.50	n.a.
3		4.652	0.314	1.232	0.62	0.78	n.a.
4	coronarinD	5.406	48.932	154.549	96.95	97.44	n.a.
5		11.226	0.087	0.227	0.17	0.14	n.a.
6		17.666	0.884	1.655	1.75	1.04	n.a.
Total:			50.473	158.603	100.00	100.00	

Figure 2.2 The purity of Coronarin D analyzed by mass spectroscopic method

Source: Researcher

2.6 Biological activity of coronarin D

Coronarin D demonstrates diverse biological activities such as anti-inflammatory activity, antifungal activity and antibacterial activity. Coronarin D inhibits the release of β- hexosamine vascular from RBL-2H3 cells. In addition, coronarin D inhibits both constitutive and inducible nuclear factor-KB pathway activation, leading to potentiation of apoptosis, inhibition of invasion, and suppression of osteoclastogenesis (Kunnumakkara et al., 2008). Coronarin D has ability to suppress cell proliferation and induce cell death of hepatocellular carcinoma cell by the JNK pathway (Lin et al., 2018).

Coronarin D also has antifungal activity against *Candida albicans*. It causes fungal membrane damage. The minimum inhibitory concentration (MIC) was 2 mg/mL, and the minimum fungicidal concentration (MFC) was 4 mg/mL. The time killing rate of coronarin D was more than clotrimazole and nystatin. (Kaomongkolgit et al., 2012). Antibacterial activity of coronarin D was also reported. Coronarin D

demonstrated antibacterial activity against Gram-positive bacteria such as *Staphylococcus aureus, Staphylococcus epidermidis, Enterococcus faecalis* and *Bacillus cereus* (Reuk-ngam et al., 2014).

2.7 Synergistic effects of antibiotics with plant extraction

Another approach to treat infectious diseases is the use of plant extracts in the combination with antibiotics. Plant extracts in combination with antibiotics are new strategy bacterial infection treatment since the synergistic effects of antibiotics and plant extracts lead to not only decreased amount of antibiotics but also more effective treatment with less side effects.

Adwan, Abu-shanab, and Adwan (2007) evaluated the interaction between ethanolic extracts of *Rhus coriaria* (leaf), *Psidium guajava* (Leaf), *Lawsonia inermis* (Leaf), and *Sacropoterium spinosum* (seed) and antimicrobial drugs including oxytetracyclin HCl, enrofloxacin, gentamicin sulphate, and sulphadimethoxin against 4 clinical isolates of methicillin-resistant *Staphylococcus aureus* (MRSA) by well-diffusion method. The results showed that crude extracts from these plants increased the inhibition zones of oxytetracyclin HCl, gentamicin sulphate, and sulphadi-methoxin, while the combinations between the plants extracts and enrofloxacin decreased inhibition zones (Adwan et al., 2007). In addition, antibacterial activity of the combinations of these plant extracts and antibiotics were tested with clinical isolates of MSSA and MRSA by well-diffusion method and broth dilution method. The results showed that these plant extracts can be synergist with antimicrobial agents against both MSSA and MRSA (Adwan, G. & Adwan, K., 2008).

In 2012, Gupta, et al. studied the antimicrobial activity of a clerodane diterpene 16α-hydroxycleroda-3,13 (14) -Z-dien-15,16-olide (CD) extracted from the leaves of *Polyalthia longifolia* (Sonn.) Thwaites (Annonaceae). The results revealed that this agent caused bacterial membrane damage (Gupta et al., 2012). Moreover, this agent combined with antibiotic groups, such as norfloxacin, ciprofloxacin, and ofloxacin showed the synergistic effects when tested with *S.aureus.*

Reuk-ngam et al. (2014) reported the synergistic effects of coronarin D with 9 antibiotics against Gram-positive bacteria by checkerboard assay. The study showed that by combining Coronarin D at the concentration of 0.25 minimal inhibitory concentration (MIC) with antibiotics, the activities of antibiotics were boosted to 4- to 128-fold.

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CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Bacterial Strains

Methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-susceptible *S. aureus* (MSSA) strains are 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.

3.1.2 Antibiotics

Penicillin G, vancomycin and colistin used in this study were purchased from Sigma Chemicals Co., St. Louis, while Polymyxin B was purchased from Calbiochem San dieco. CA USA.

3.1.3 Coronarin D

Coronarin D was extracted from the rhizome of *H. coronarium*. The extraction method was previously described by Chimnoi et al. (2009). The chemical structure of coronarin D was analyzed using mass-spectroscopic technique.

3.2 Methods

3.2.1 Antimicrobial susceptibility testing

Coronarin D was screened for its inhibitory activity against MRSA and MSSA. The disk diffusion assay was performed by making a lawn culture of approximately 10^8 cells/mL of the test bacteria on Mueller Hinton agar (MHA) plates. Then sterile 6 mm in diameter filter paper disks loaded with 10 µL of coronarin D dissolved in dimethyl sulfoxide (DMSO) at the concentration of 10 mg/mL. The disks were 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. Zone of inhibition of coronarin D was compared with corresponding solvent as controls.

Each susceptibility experiment was performed in triplicates. The diameters of the zone of growth inhibition around the disks measured in millimeter were averaged mean values.

3.2.2 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 suspension.

Twenty microliters of coronarin D was 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.

3.2.3 Synergistic effects

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/disc) containing 10 µL coronarin D dissolved in 10% DMSO and the different concentrations of each antibiotic agent were placed on the MHA plates, and the distances were 10 mm of each disc. The plates were incubated at 37˚C for 18-24 hours. An increase in the inhibition zone between the discs of coronarin D and antibiotic was considered as positive for synergistic effect. Each experiment was performed in triplicates

3.2.4 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 recommended by CLSI (CLSI, M7-A7, 2006). 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:

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

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

The sum of fractional inhibitory concentration (FIC_s) indices of two compounds in the combination was calculated as follows: $FIC_a + FIC_b = FIC_I$

Synergism interaction was defined as a FIC index of 0.5 or less while additive interaction was defined as a FIC index of more than 0.5 and less than 4, and antagonism interaction was defined as FIC index of more than 4.

3.2.5 Time kill assay

The 1.8 mL of 10^6 cells/mL bacterial suspension was mixed with the 0.2 mL coronarin D at the concentration of FIC combination and compound alone and then

incubated at 37 °C. After the incubation period of 0, 2, 4, 6, 8, 10, 12, and 24 hours, ten microliters of the mixture were pipetted for bacterial count. The experiments were carried out in triplicate. Time–kill curves were constructed by plotting $\log_{10}$ CFU/mL of survivals against time. The effectiveness of the coronarin D against MRSA and MSSA is the time that viable cells reduced $3\log_{10}$ meaning that the bactericidal activity reached 99.9%.

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CHAPTER 4

RESULTS

4.1 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 agar diffusion method. The results revealed that coronarin D and antibiotics possessed antibacterial activity against MRSA and MSSA as shown in Table 4.1 and Table 4.2 and Figure 4.1. In addition, the inhibition zone of coronarin D was larger than colistin but smaller than penicillin G, vancomycin and polymyxin B.

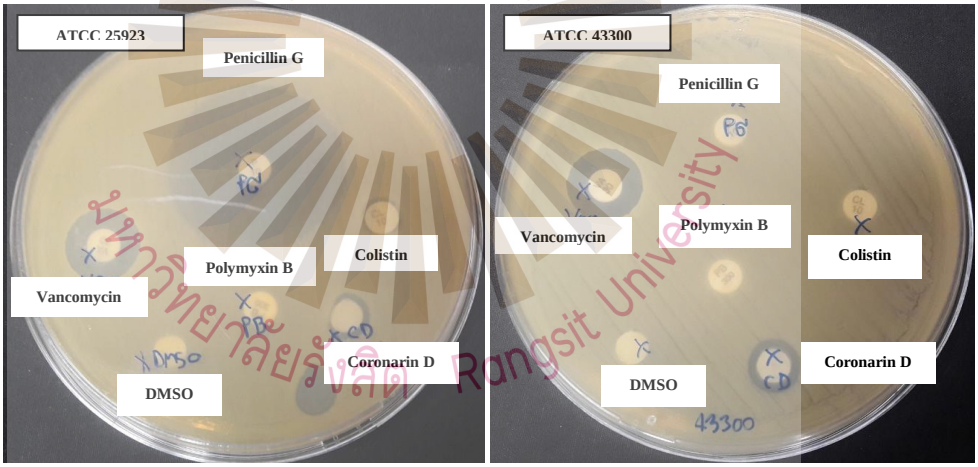

Figure 4.1 Antibacterial activity of antibiotics and coronarin D against *S.aureus*, ATCC25923 (MSSA) and ATCC43300 (MRSA)

4.2 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 4.3. 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 4.4. 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.

4.3 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 disc diffusion method. The combination of coronarin D-Polymyxin B showed synergistic effects against all test MRSA and MSSA strains as shown in Table 4.5 and Table 4.6 and Figure 4.2. In addition, the good synergistic effects against MSSA was found in the combination of coronarin D-colistin.

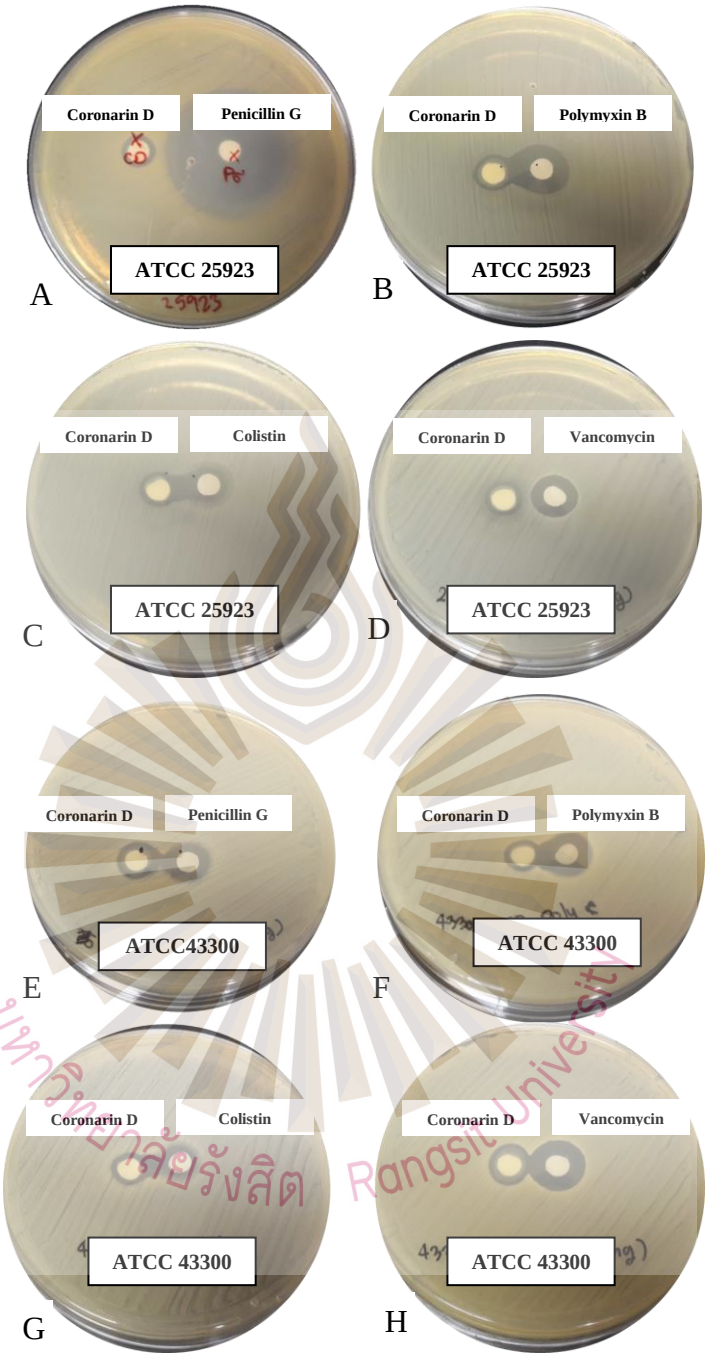

Figure 4.2 Synergistic effect of Coronarin D and antibiotics against MRSA and MSSA

(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 4.1 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(S)±1.0	15.3(S)±0.6	6.7±1.2	7.3±0.6
MSSA1	11.7± 2.1	19.0(R)±1.0	15.3(S)±0.6	10.3±2.5	8.3±0.6
MSSA2	10.0 ± 1.0	14.0(R)±1.0	14.3(S)±1.2	10.3±2.1	7.0±1.0
MSSA3	11.7±1.2	14.7(R)±0.6	15.0(S)±0.0	10.0±0.0	6.3±0.6
MSSA4	10.0±1.0	35.3(S)±4.2	15.3(S)±0.6	14.0±1.0	7.0±0.0
MSSA5	9.3±0.6	13.0(R)±1.7	14.3(S)±0.6	9.7±0.6	6.7±0.6
MSSA6	10.0±1.7	13.3(R)±0.6	15.0(S)±0.0	9.7±0.6	6.7±0.6
MSSA7	9.7±1.2	27.3(R)±6.4	14.7(S)±0.6	12.0±0.0	6.7±0.6
MSSA8	9.7±0.6	15.0(R)±1.0	15.0(S)±0.0	9.3±1.5	7.0±1.7
MSSA9	11.7±1.2	35.3(S)±1.2	15.0(S)±0.0	12.7±0.6	7.0±1.7
MSSA10	9.0±1.0	9.3(R)±0.6	15.0(S)±0.0	12.0±1.0	7.3±1.2

Interpretive criteria for Penicillin G; R = Resistant (Diameter ≤28),
I = Intermediate, and S = Sensitive (Diameter ≥29)
Interpretive criteria for Vancomycin; R = Resistant (Diameter ≤11),
I = Intermediate, and S = Sensitive (Diameter ≥12)

Table 4.2 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(R)±1.0	15.3(S)±0.6	6.7±1.2	7.3±0.6
MRSA1	9.0±1.0	10.3(R)±0.6	14.0(S)±1.0	12.0±2.0	8.3±1.5
MRSA2	9.0±1.0	8.67(R)±0.6	14.3(S)±2.1	11.0±1.0	7.3±0.6
MRSA3	9.3±0.6	10.0(R)±0.0	13.7(S)±0.6	12.0±1.0	8.3±1.2
MRSA4	10.7±2.1	10.3(R)±1.5	14.0(S)±1.7	11.0±2.0	7.3±0.6
MRSA5	8.67±2.1	11.3(R)±1.5	13.7(S)±0.6	14.7±3.8	8.0±1.0
MRSA6	9.7±1.5	9.7(R)±1.2	13.0(S)±1.0	10.67±0.6	7.3±0.6
MRSA7	10.0±1.0	8.3(R)±1.2	13.0(S)±1.7	12.3±0.6	8.0±1.0
MRSA8	9.3±1.2	8.0(R)±1.0	13.0(S)±1.0	9.7±0.6	7.3±0.6
MRSA9	13.0±5.3	8.0(R)±1.0	15.7(S)±1.2	11.0±0.0	6.0±0.0
MRSA10	9.67±1.2	11.0(R)±1.7	15.3(S)±0.6	10.3±1.5	6.0±0.0

Interpretive criteria for Penicillin G; R = Resistant (Diameter ≤28),
I = Intermediate, and S = Sensitive (Diameter ≥29)
Interpretive criteria for Vancomycin; R = Resistant (Diameter ≤11),
I = Intermediate, and S = Sensitive (Diameter ≥12)

Table 4.3 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

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Table 4.4 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

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Table 4.5 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 4.6 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)

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4.4 Checkerboard method

The synergistic effect of coronarin D and antibiotics such as penicillin G, vancomycin, polymyxin B and colistin were determined by Fractional inhibitory concentration index (FIC_I). The FIC_I values were calculated according research of Didry, Dubreuil, and Pinkas (1993). Synergism interaction was defined as an FIC index of 0.5 or less while additive interaction was defined as a FIC index of more than 0.5 and less than 4, and antagonism interaction was defined as FIC index of more than 4. The results of FIC_I were summarized in Table 4.7 and Table 4.8. When MSSA were tested, out of 44 combinations, 26 combinations had synergistic effect and 18 combinations had no effect, while 36 combinations had synergistic effect and 8 combinations had no effect against MRSA. In addition, the combination of coronarin D-polymyxin B and coronarin D-penicillin G showed the best synergistic effect against MRSA and MSSA, respectively. Therefore, these combinations were further selected for Time-kill assay.

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Table 4.7 Fractional inhibitory concentration index (FIC_I) of antibiotics in combination with coronarin D against methicillin-susceptible *Staphylococcus aureus* (MSSA)

Strains	MIC of coronarin D	MIC of penicillin G	Concentration of coronarin D in combination (gain)	Concentration of penicillin G in combination (gain)	FIC_I	MIC of vancomycin	Concentration of coronarin D in combination (gain)	Concentration of vancomycin in combination (gain)	FIC_I
ATCC 25923	15.60	0.02	1.95(8)	0.001875(8)	0.25	1.95	15.6 (1)	0.0076 (256)	1.00
MSSA1	25.00	12.50	6.25(4)	1.3(9.6)	0.35	1.56	16.67(1.5)	0.522(3)	1.00
MSSA2	50.00	3.13	5.21(9.6)	0.52(6)	0.27	1.56	25(2)	0.134(12)	0.59
MSSA3	50.00	12.50	25(2)	6.25(2)	1.00	1.56	9.375(5.3)	0.26(6)	0.35
MSSA4	50.00	0.05	12.5(2)	0.010(4.9)	0.46	1.56	10.42(4.8)	0.26(6)	0.38
MSSA5	25.00	3.13	25(1)	0.0122(256)	1.00	0.78	3.65(6.9)	0.1625(4.8)	0.35
MSSA6	25.00	1.56	12.5(2)	0.39(0.4)	0.75	0.78	25(1)	3.125(0.25)	0.38
MSSA7	25.00	0.20	12.5(2)	0.024(8.125)	0.63	1.56	2.1(11.9)	0.585(2.7)	0.46
MSSA8	25.00	12.50	12.5(2)	2.6(4.8)	0.71	1.56	3.125(8)	0.39(0.4)	0.38
MSSA9	25.00	0.05	16.67(1.5)	0.0164(3)	1.00	0.78	12.5(2)	0.13(6)	0.67
MSSA10	25.00	6.25	16.67(1.5)	2.09(3)	1.00	1.56	25(1)	0.0061(256)	1.00

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Table 4.7 Fractional inhibitory concentration index (FIC_i) of antibiotics in combination with coronarin D against methicillin-susceptible *Staphylococcus aureus* (MSSA).(cont.)

Strains	MIC of coronarin D	MIC of polymyxin B	Concentration of coronarin D in combination (gain)	Concentration of polymyxin B in combination (gain)	FIC_I	MIC of colistin	Concentration of coronarin D in combination (gain)	Concentration of colistin in combination (gain)	FIC_I
ATCC 25923	15.60	62.50	3.9(4)	7.8125(8)	0.38	250.00	3.9(4)	31.25(8)	0.38
MSSA1	25.00	25.00	6.25(4)	1.56(16)	0.31	100.00	1.5625(16)	50(2)	0.56
MSSA2	50.00	25.00	10.417(4.8)	5.21(4.8)	0.42	100.00	5.21(9.6)	16.67(6)	0.25
MSSA3	50.00	25.00	10.42(4.8)	5.21(4.8)	0.42	100.00	3.125(16)	14.58(6.8)	0.21
MSSA4	50.00	25.00	6.25(8)	2.62(9.5)	0.23	100.00	3.125(16)	12.5(8)	0.19
MSSA5	25.00	12.50	20.83(1.2)	2.12(5.9)	1.00	50.00	5.21(4.8)	8.33(6)	0.38
MSSA6	25.00	12.50	3.125(8)	3.125(4)	0.38	50.00	3.125(8)	3.125(16)	0.19
MSSA7	25.00	12.50	6.25(4)	3.125(4)	0.50	50.00	3.125(8)	6.25(8)	0.25
MSSA8	25.00	12.50	1.5625(16)	6.25(2)	0.56	25.00	3.125(8)	4.17(6)	0.29
MSSA9	25.00	12.50	3.125(8)	6.25(8)	0.63	50.00	3.125(8)	5.21(9.6)	0.23
MSSA10	25.00	25.00	25(1)	0.09765(256)	1.00	25.00	10.42(2.4)	6.25(4)	0.58

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Table 4.8 Fractional inhibitory concentration index (FIC_I) of antibiotics in combination with coronarin D against Methicillin-Resistant *Staphylococcus aureus* (MRSA)

Strains	MIC of coronarin D	MIC of penicillin G	Concentration of coronarin D in combination (gain)	Concentration of penicillin G in combination (gain)	FIC_I	MIC of vancomycin	Concentration of coronarin D in combination (gain)	Concentration of vancomycin in combination (gain)	FIC_I
ATCC 43300	15.60	125.00	15.6(1)	0.488(256)	1.00	1.95	15.6(1)	0.0076(256)	1.00
MRSA1	50.00	12.50	33.33(1.5)	0.08(156)	0.67	1.56	25(2)	0.442(3.5)	0.67
MRSA2	50.00	12.50	12.5(4)	1.5625(8)	0.38	3.13	12.5(4)	0.52(6)	0.42
MRSA3	50.00	12.50	12.5(4)	1.5625(8)	0.38	3.13	12.5(4)	0.78125(4)	0.50
MRSA4	50.00	12.50	33.33(1.5)	0.08(156)	0.67	6.25	12.5(4)	1.3(4.8)	0.46
MRSA5	50.00	12.50	16.67(3)	2.21(5.65)	0.51	3.13	12.5(4)	0.39(8)	0.38
MRSA6	50.00	12.50	10.42(4.8)	1.5625(8)	0.25	3.13	12.5(4)	0.39(8)	0.38
MRSA7	50.00	12.50	12.5(4)	1.5625(8)	0.25	3.13	12.5(4)	0.52(6)	0.42
MRSA8	50.00	12.50	33.33(1.5)	0.08(156)	0.67	3.13	12.5(4)	0.28(11.2)	0.34
MRSA9	50.00	12.50	16.67(3)	1.172(10.67)	0.43	6.25	12.5(0.5)	0.58(10.77)	0.34
MRSA10	50.00	6.25	33.33(1.5)	0.0366(170)	0.67	1.56	12.5(4)	0.195(8)	0.38

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Table 4.8 Fractional inhibitory concentration index (FIC_I) of antibiotics in combination with coronarin D against Methicillin-Resistant *Staphylococcus aureus* (MRSA) (cont.)

Strains	MIC of coronarin D	MIC of polymyxin B	Concentration of coronarin D in combination (gain)	Concentration of polymyxin B in combination (gain)	FIC_I	MIC of colistin	Concentration of coronarin D in combination (gain)	Concentration of colistin in combination (gain)	FIC_I
ATCC 43300	15.60	62.50	1.95(8)	7.8125(8)	0.25	250.00	3.9(4)	31.25(8)	0.38
MRSA1	50.00	25.00	6.25(8)	2.60(9.6)	0.23	50.00	12.5(4)	2.08(24)	0.29
MRSA2	50.00	50.00	8.33(6)	2.60(19)	0.22	100.00	12.5(4)	3.125(32)	0.31
MRSA3	50.00	50.00	6.25(8)	6.25(8)	0.25	100.00	6.25(8)	12.5(8)	0.25
MRSA4	50.00	25.00	8.33(6)	2.34(10.68)	0.26	100.00	6.25(8)	6.25(16)	0.19
MRSA5	50.00	25.00	6.25(8)	3.125(8)	0.25	100.00	8.33(6)	12.5(8)	0.29
MRSA6	50.00	25.00	8.3(6)	4.17(6)	0.33	100.00	5.21(9.6)	9.375(10.67)	0.20
MRSA7	50.00	50.00	8.33(6)	5.20(9.6)	0.27	100.00	9.375(5.33)	8.59375(11.6)	0.35
MRSA8	50.00	50.00	8.33(6)	8.33(6)	0.33	100.00	6.25(8)	6.25(16)	0.19
MRSA9	50.00	50.00	6.25(8)	6.25(8)	0.25	100.00	6.25(8)	8.33(12)	0.21
MRSA10	50.00	12.50	6.25(8)	1.5625(8)	0.25	100.00	4.17(12)	5.21(19.19)	0.14

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4.5 Time Kill assay

The time-kill curves of coronarin D, penicillin G, polymyxin B, colistin and coronarin D combined with antibiotics against MRSA and MSSA were showed in Figure 4.3- 4.7.

The combination of coronarin D-polymyxin B, coronarin D- penicillin G and coronarin D-colistin killed MSSA within 2 hours but coronarin D alone, polymyxin B, penicillin G and colistin alone cannot kill MSSA. For MRSA, killing rate of the combination of coronarin D- polymyxin B and coronarin D- colistin was faster than coronarin D, polymyxin B or colistin alone.

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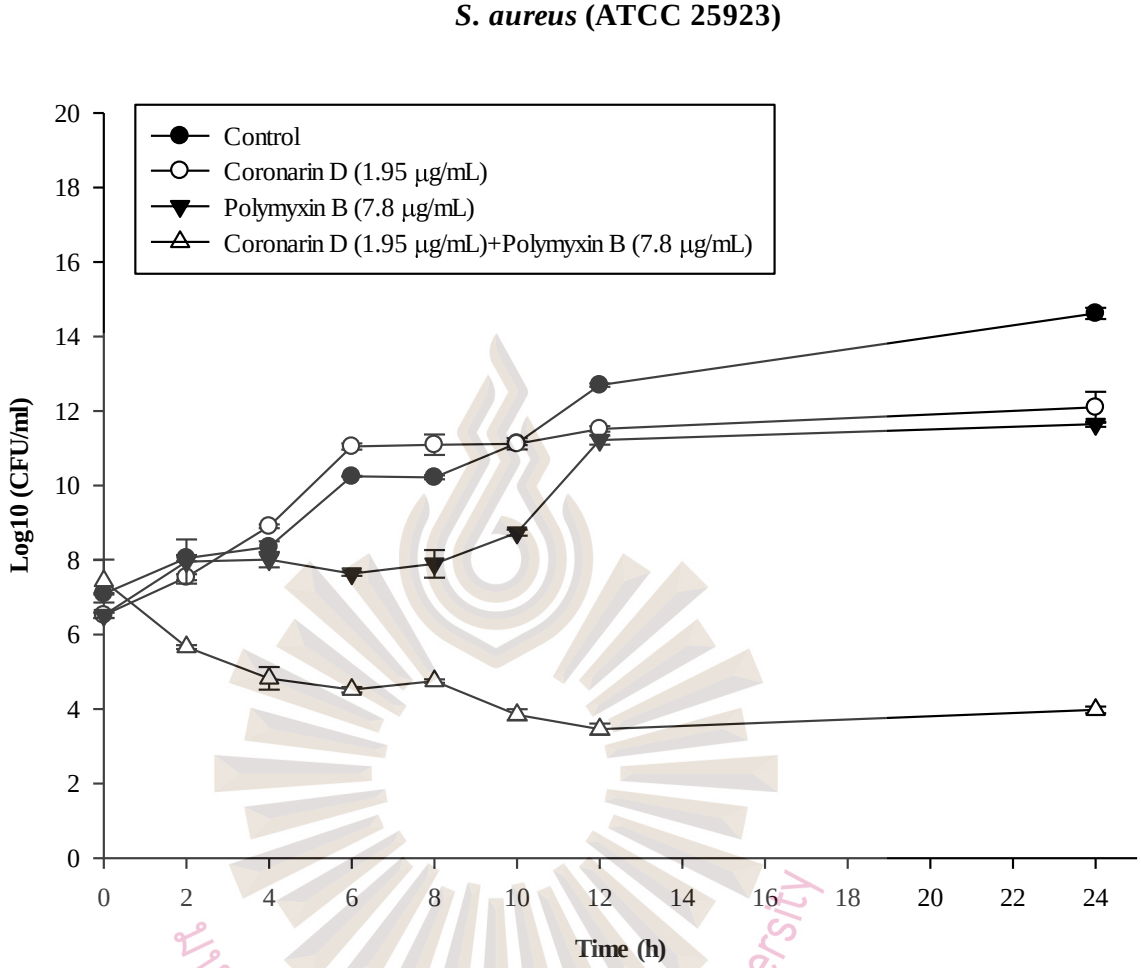

Figure 4.3 Time kill curve of coronarin D at concentration of 1.95 µg/mL, polymyxin B at concentration of 7.8 µg/mL and combination of coronarin D- polymyxin B at FIC value against MSSA strain (ATCC 25923)

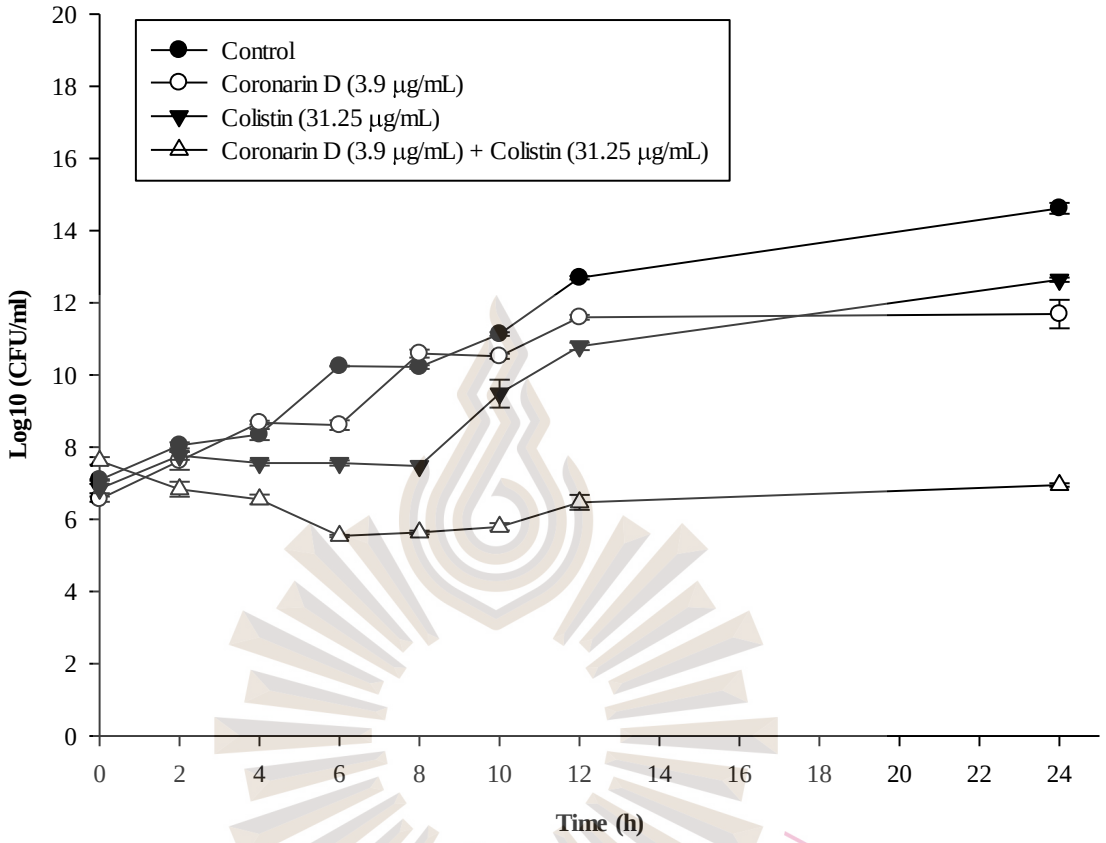

Figure 4.4 Time kill curve of coronarin D at concentration of 3.9 µg/mL, colistin at concentration of 31.25 µg/mL and combination of coronarin D- colistin at FIC value against MSSA strain (ATCC 25923)

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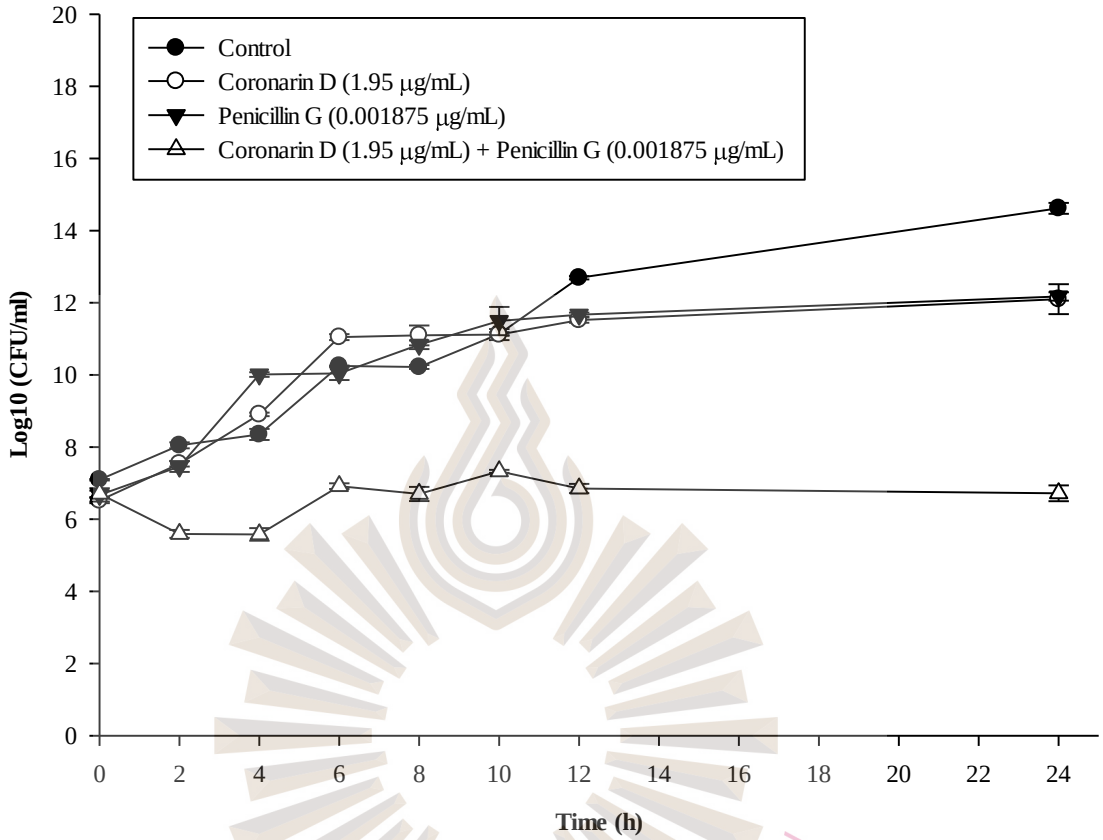

Figure 4.5 Time kill curve of coronarin D at concentration of 1.95 µg/mL, penicillin G at concentration of 0.001875 µg/mL and combination of coronarin D- penicillin G at FIC value against MSSA strain (ATCC 25923)

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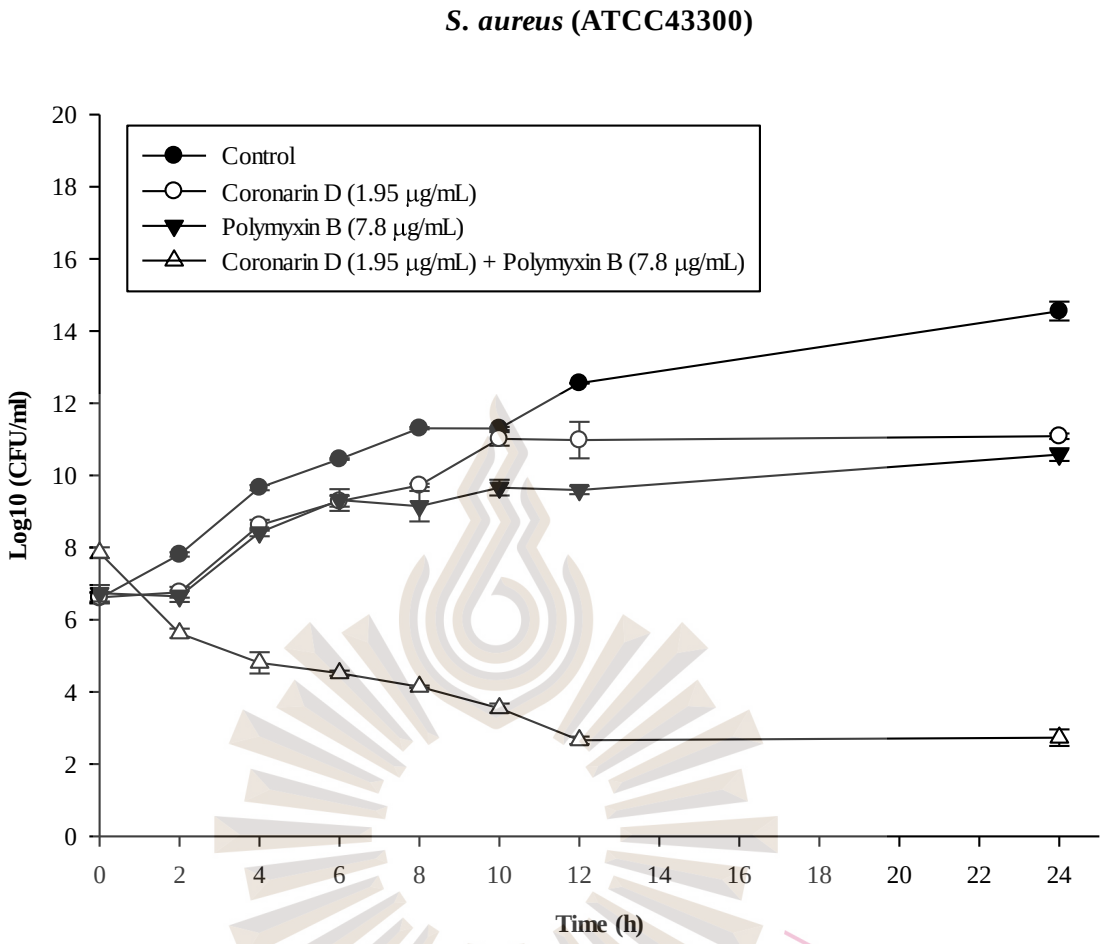

Figure 4.6 Time kill curve of coronarin D at concentration of 1.95 µg/mL, polymyxin B at concentration of 7.8 µg/mL and combination of coronarin D- polymyxin B at FIC value against MRSA strain (ATCC 43300)

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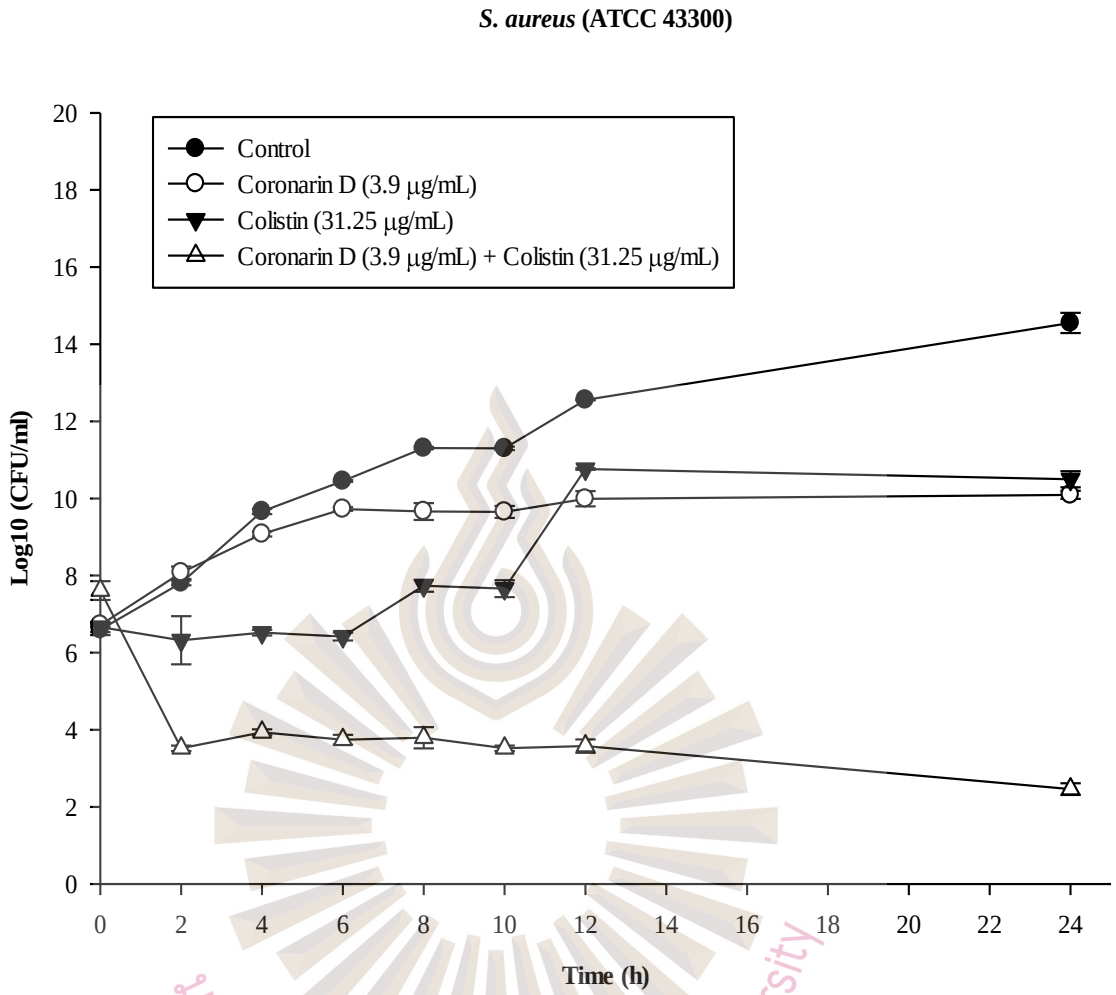

Figure 4.7 Time kill curve of coronarin D at concentration of 3.9 µg/mL, colistin at concentration of 31.25 µg/mL and combination of coronarin D- colistin at FIC value against MRSA strain (ATCC 43300)

CHAPTER 5

DISCUSSION AND CONCLUSIONS

Coronarin D, a labdane diterpene compound, was extracted from rhizomes of plant named *Hedychium coronarium* that has been cultivated in Chiangmai Province of Thailand. Coronarin D was extracted, purified and structural analyzed by Chimnoi et al. (2009). In addition, the structure of coronarin D was examined using NMR and was reported in 1988 by Itokawa (Itokawa et al., 1988). Coronarin D showed many biological activities such as anti-inflammatory (Lin et al., 2018), antifungal (Kaomongkolgit et al., 2012), anticancer (Kunnumakkara et al., 2002), and antimicrobial activity (Reuk-ngam et al., 2014).

The antibacterial activity of coronarin D against methicillin resistant *Staphylococcus aureus* (MRSA) and methicillin susceptible *Staphylococcus aureus* (MSSA) was examined in this study. Coronarin D showed antibacterial activities against MRSA and MSSA when determined by agar diffusion method. The MIC values of coronarin D against both strains ranged from 15.6 - 50 µg/mL. In addition, the MBC values of coronarin D against MRSA and MSSA ranged from 31.25 - 50 µg/mL and 50-100 µg/mL, respectively. Therefore, coronarin D was considered as bactericidal agent against MSSA and MRSA strains since the MBC values are not more than 4xMIC (Pankey & Sabath , 2004). These could be explained by the ability of this compound to cross 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 cell membrane more easily. (Kaomongkolgit et al., 2012; Reuk-ngam et al., 2014; Urzúa, Rezende, Mascayano, & Vasquez, 2008). Beside 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).

The investigation of synergistic effect between natural products and antibiotics is an alternative approach to fight pathogens. Due to the combination, 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 for 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, 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, the good synergistic effects against MSSA was found in the combination of coronarin D-Colistin.

Furthermore, the synergistic effects of coronarin D with antibiotics was determined by checkerboard method. The fractional inhibitory concentration index (FICI) 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 combination 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 this study results that coronarin D showed synergistic effect with polymyxin B against S. *aureus*. However, there is no report of synergistic effect against resistant bacteria.

The determination of the best combination of coronarin D with an antibiotic for the potential of synergistic effects against MSSA and MRSA strains, time-killing rate was employed. The combination of coronarin D-polymyxin B, coronarin D- penicillin

G and coronarin D-colistin killed MSSA within 2 hours but coronarin D alone polymyxin B, penicillin G and colistin alone cannot kill MSSA. For MRSA, killing rate of the combination of coronarin D- polymyxin B and coronarin D- colistin was faster than coronarin D, polymyxin B or colistin alone.

In conclusion, coronarin D could be used as antibacterial agent against MRSA and MSSA. 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 the cell membrane disruption.

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