SYNERGISTIC EFFECT OF *BOESENBERGIA ROTUNDA* (L.) MANSF. ESSENTIAL OIL AND CLOXACILLIN ON METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* (MRSA) INHIBITION

CHITTADECH APINUNDECHA

A Thesis Submitted in Partial Fulfillment of the Requirements for the
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ผลการเสริมฤทธิ์กันระหว่างน้ำมันหอมระเหยจากกระชายและยาคลอกซาซิลลินต่อการยับยั้งเชื้อสแตปฟิโลคอคคัส ออเรียส ที่ดื้อต่อยาเมธิซิลลิน

นายชิษฎเดช อภินันท์เดชา

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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ปีการศึกษา 2566

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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

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ชิษฏเดช อภินันท์เดชา : ผลการเสริมฤทธิ์กันระหว่างน้ำมันหอมระเหยจากกระชายและยาคลอกซาซิลลินต่อการยับยั้งเชื้อสแตปฟิโลคอคคัส ออเรียส ที่ดื้อต่อยาเมธิซิลลิน SYNERGISTIC EFFECT OF *BOESENBERGIA ROTUNDA* (L.) MANSF. ESSENTIAL OIL AND CLOXACILLIN ON METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* (MRSA) INHIBITION. อาจารย์ที่ปรึกษา : ศาสตราจารย์ เภสัชกร ดร.เกรียงศักดิ์ เอื้อมเก็บ, 105 หน้า.

คำสำคัญ: ผลการเสริมฤทธิ์กัน/ กระชาย/ ความต้านทานต่อยาเมธิซิลลิน/ สแตปฟิโลคอคคัส ออเรียส/ ยาคลอกซาซิลลิน

ปัจจุบันการดื้อต่อยาปฏิชีวนะของแบคทีเรียได้แพร่กระจายออกเป็นวงกว้าง ส่งผลให้ยาปฏิชีวนะเสื่อมประสิทธิภาพลงและส่งผลให้เกิดปัญหาด้านสุขภาพต่าง ๆ ปัญหานี้ทำให้เกิดความตระหนักถึงความเร่งด่วนในการมองหาวิธีการรักษาทดแทน ในขณะที่การดื้อต่อยาปฏิชีวนะของแบคทีเรียยังคงเพิ่มขึ้น แต่ตัวเลือกในการรักษามีจำกัด ซึ่งเป็นเหตุให้เกิดการค้นหาทางเลือกใหม่ในการรักษา เพื่อจัดการกับปัญหาดังกล่าวงานวิจัยนี้มีวัตถุประสงค์เพื่อตรวจสอบประสิทธิภาพของน้ำมันหอมระเหยจากกระชายในการต้านเชื้อแบคทีเรียสแตปฟิโลคอคคัส ออเรียส ที่ดื้อต่อยาเมธิซิลลิน (MRSA) การวิเคราะห์ส่วนประกอบทางเคมีของน้ำมันหอมระเหยจากกระชายโดยใช้เทคนิคแก๊สโครมาโตกราฟี-แมสสเปกโทรเมทรีพบว่า มีสารประกอบทั้งหมด 24 ชนิด โดยสารประกอบหลักประกอบด้วย บีตาโอซิมีน ทรานส์เจอรานิออล แคมเฟอร์ และยูคาลิปตอล การทดสอบความสามารถในการต้านเชื้อแบคทีเรียโดยใช้น้ำมันหอมระเหยจากกระชาย และยาคลอกซาซิลลิน ต่อเชื้อ MRSA 3 สายพันธุ์ ได้แก่ MRSA สายพันธุ์ DMST 20649, 20651, และ 20652 ได้ใช้วิธีการหาความเข้มต่ำสุดในการยับยั้งการเจริญเติบโตของเชื้อแบคทีเรีย (MIC) ผลการทดลองแสดงให้เห็นว่าน้ำมันหอมระเหยจากกระชายมีค่า MIC เท่ากับ 4 มิลลิกรัมต่อมิลลิลิตร ในขณะที่ยาคลอกซาซิลลิน มีค่า MIC เท่ากับ 512 ไมโครกรัมต่อมิลลิลิตร สำหรับการตรวจสอบศักยภาพของน้ำมันหอมระเหยจากกระชายในการเสริมฤทธิ์กับยาคลอกซาซิลลินต้านเชื้อ MRSA ดังกล่าว การทดสอบอันตรกิริยาของยาถูกทดสอบด้วยด้วยวิธี chequerboard และ time-kill assay ผลการทดลองพบว่า มีการเสริมฤทธิ์กันระหว่างน้ำมันหอมระเหยจากกระชาย และยาคลอกซาซิลลิน โดย มีค่าดัชนีสัดส่วนการยับยั้ง (FICI) น้อยกว่า 0.5 และมีการลดลงของเชื้อแบคทีเรียในหน่วยซีเอฟยูต่อมิลลิลิตรมากกว่า $2\log_{10}$ ณ ชั่วโมงที่ 24 เมื่อเปรียบเทียบกับการทดสอบโดยใช้น้ำมันหอมระเหยจากกระชายซึ่งเป็นสารที่มีประสิทธิภาพมากที่สุดเพียงอย่างเดียว นอกจากนี้งานวิจัยนี้ได้ประเมินความสามารถในการยับยั้งการสร้างไบโอฟิล์มและพบว่าน้ำมันหอมระเหยจากกระชายทั้งแบบใช้เดี่ยวและใช้ร่วมกับยาคลอกซาซิลลินสามารถยับยั้งการ

สร้างไบโอฟิล์มได้ อีกประการหนึ่งนี้งานวิจัยนี้ได้ทดสอบการเปลี่ยนแปลงการซึมผ่านของเยื่อหุ้มเซลล์ของแบคทีเรีย รวมไปถึงการเปลี่ยนแปลงโครงสร้างในเซลล์แบคทีเรียโดยใช้กล้องจุลทรรศน์อิเล็กตรอนแบบส่องกราด (SEM) และแบบส่องผ่าน (TEM) การทดสอบการซึมผ่านของเยื่อหุ้มเซลล์ศึกษาจากการเปลี่ยนแปลงของค่าดูดกลืนแสงที่ความยาวคลื่น 260 นาโนเมตร ซึ่งในเซลล์ที่ถูกทดสอบด้วยน้ำมันหอมระเหยจากกระชาย และสารผสมระหว่างน้ำมันหอมระเหยจากกระชายและยาคลอกซาซิลลิน พบว่าเกิดการเพิ่มการซึมผ่านของเยื่อหุ้มเซลล์ เมื่อเปรียบเทียบกับเซลล์ที่ไม่ได้รับสารทดสอบ สำหรับภาพที่ได้รับจาก SEM และ TEM พบความเสียหายในผนังเซลล์และเยื่อหุ้มเซลล์จากเซลล์ที่ได้รับการทดสอบด้วยยาคลอกซาซิลลิน และเซลล์ที่ได้รับการทดสอบด้วยน้ำมันหอมระเหยจากกระชายตามลำดับ ภาพที่ได้จากการทดสอบด้วยสารผสมระหว่างน้ำมันหอมระเหยจากกระชายและยาคลอกซาซิลลินแสดงให้เห็นความเสียหายทั้งในผนังเซลล์และเยื่อหุ้มเซลล์ กล่าวโดยสรุป ผลการทดลองที่ได้กล่าวไว้ข้างต้นยืนยันว่าน้ำมันหอมระเหยจากกระชายอาจเป็นทางเลือกในการต้านเชื้อแบคทีเรียที่มีการดื้อต่อยาปฏิชีวนะ เช่น MRSA ในอนาคต และการเสริมฤทธิ์กันระหว่างน้ำมันหอมระเหยจากกระชายกับยาคลอกซาซิลลินสามารถสร้างองค์ความรู้ใหม่ที่มีคุณค่าเกี่ยวกับการประยุกต์ใช้ในการรักษาโดยใช้ยาสูตรผสมช่วยแก้ไขปัญหาการดื้อยาปฏิชีวนะในแบคทีเรียอีกด้วย

สาขาวิชาปรีคลินิก ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2566 ลายมือชื่ออาจารย์ที่ปรึกษา_____

CHITTADECH APINUNDECHA : SYNERGISTIC EFFECT OF *BOESENBERGIA ROTUNDA* (L.) MANSF. ESSENTIAL OIL AND CLOXACILLIN ON METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* (MRSA) INHIBITION. THESIS ADVISOR : PROF. GRIANGSAK EUMKEB, Ph.D. 105 PP.

Keyword: SYNERGISTIC EFFECT / *BOESENBERGIA ROTUNDA* / METHICILLIN RESISTANT / *STAPHYLOCOCCUS AUREUS* / CLOXACILLIN

Antibiotic resistance among bacteria has now become widespread, leading to reduced effectiveness of antibiotics and causing various health issues. This issue has resulted in a growing awareness of the urgent need for alternative treatments. As bacterial resistance increases, treatment options are limited, leading to the exploration of novel alternative treatments. To address this issue, this research focused on investigating the potential of essential oil extracted from *Boesenbergia rotunda* (BREO) in treating methicillin-resistant *Staphylococcus aureus* (MRSA). The analysis of the chemical composition of BREO using gas chromatography-mass spectrometry (GC-MS) identified 24 compounds, with main components including β-ocimene, trans-geraniol, camphor, and eucalyptol. Both BREO and cloxacillin (CLX) were investigated for their antibacterial activity through minimum inhibitory concentration (MIC) in the treatment of three strains of MRSA, including MRSA DMST 20649, MRSA DMST 20651, and MRSA DMST 20652. Results demonstrated that BREO had an MIC of 4 mg/mL, while CLX had an MIC of 512 µg/mL. To determine the potential of BREO in combination with CLX, drug interactions were conducted using a checkerboard and time-kill assays. The combination of BREO and CLX demonstrated a synergistic effect, with an FIC index < 0.5 and a bacterial CFU/mL decrease exceeding 2log10 CFU/mL when compared to the use of BREO (the most effective compound) individually at 24 h of incubation. In addition, this research evaluated the ability of BREO to inhibit biofilm formation and found that BREO, both alone and in combination with CLX, showed inhibition of biofilm formation. Furthermore, this research examined alterations in cytoplasmic membrane (CM) permeability and morphological alterations in bacterial cells using scanning electron microscopy (SEM) and transmission electron microscopy (TEM). The change in

OD_{260} in BREO and BREO+CLX-treated cells compared to the untreated cell indicates the alteration in CM. The images obtained from SEM and TEM revealed damage to the cell wall and cytoplasmic membrane in CLX-treated and BREO-treated cells, respectively. The image obtained from the combination of BREO and CLX exhibited damage on both the cell wall and CM. In conclusion, the findings suggest that BREO could be a promising alternative treatment option, particularly for solving the issue of antibiotic resistance in bacteria found in MRSA. The combination of BREO with traditional antibiotics might offer valuable knowledge into the application of combination therapy for solving antibiotic resistance in bacteria.

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Student's Signature C. Apinundecha
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Chittadech Apinundecha

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LIST OF ABBREVIATIONS

ABC	=	ATP-binding cassette
AME	=	Aminoglycoside modifying enzyme
ANOVA	=	Analysis of variance
ATCC	=	American Type Culture Collection
BRE	=	*Boesenbergia rotunda* extract
BREO	=	*Boesenbergia rotunda* essential oil
CA-MHB	=	Cation-adjusted Mueller Hinton broth
CA-MRSA	=	Community-acquired MRSA
CFU/mL	=	Colony forming unit per milliliter
CLSI	=	Clinical Laboratory Standard Institute
CLX	=	Cloxacillin
CRE	=	Carbapenem-resistant Enterobacteriaceae
DI water	=	Distilled water
DMSO	=	Dimethylsulfoxide
DMST	=	Department of Medical Sciences Thailand
DNA	=	Deoxyribonucleic acid
EPS	=	Extracellular polymeric substance
ESBL	=	Extended-spectrum β-lactamase
FIC	=	Fractional inhibitory concentration
GC-MS	=	Gas chromatography-mass spectrometry
GlcNAc	=	N-acetylglucosamine
HGT	=	Horizontal gene transfer
L	=	Liter
LPS	=	Lipopolysaccharide
MATE	=	Multidrug and Toxic Compound Extrusion
MBC	=	Minimum bactericidal concentration
MFS	=	Large Facilitator Superfamily

LIST OF ABBREVIATIONS (Continued)

MHA	=	Mueller Hinton agar
MIC	=	Minimum inhibitory concentration
MRCoNS	=	Methicillin resistant coagulase negative Staphylococcus
MRSA	=	Methicillin-resistant *Staphylococcus aureus*
MSSA	=	Methicillin-susceptible *Staphylococcus aureus*
MurNAc	=	N-acetylmuramic acid
NIS	=	Nisin
OD	=	Optical density
OECD	=	Organization for Economic Cooperation and Development
OEO	=	Oregano essential oil
OsO_4	=	Osmium tetroxide
PABA	=	Para-aminobenzoic acid
PBPs	=	Penicillin-binding proteins
PBP2a	=	Penicillin-binding protein 2a
Pa-A	=	Panduratin A
RNA	=	Ribonucleic acid
RND	=	Resistance-Nodulation-Cell Division
SCCmec	=	Staphylococcal cassette chromosome
SEM	=	Scanning electron microscopy
SEM	=	Standard error of the mean
SMR	=	Small Multidrug Resistance
TDR	=	Totally-drug resistant
TEM	=	Transmission electron microscopy
THF	=	Tetrahydrofolic acid
TSS	=	Toxic shock syndrome
TSST	=	Toxic shock syndrome toxin – 1
VPs	=	Volatile phenolics
VRSA	=	Vancomycin-resistant Staphylococcus aureus
WHO	=	World Health Organization

LIST OF ABBREVIATIONS (Continued)

g	=	Gram
h	=	Hour
mDAP	=	Meso-diaminopimelic
mL	=	Milliliter
mRNA	=	Messenger RNA
mg	=	Milligram
mg/mL	=	Milligram per milliliter
min	=	Minute
rpm	=	Revolution per minute
µL	=	Microliter
µg/mL	=	Microgram per milliliter

CHAPTER I

INTRODUCTION

1.1 Introduction

Antibiotic resistance in bacteria is a severe global health threat, reducing antibiotic effectiveness, limiting treatment options, and increasing mortality risks. Infections caused by antibiotic-resistant bacteria result in high medical costs, longer hospital stays, and higher mortality rates than those caused by susceptible strains (CDC, 2019).

Antibiotic resistance in these bacteria often arises from improper antibiotic use and inadequate sanitation and hygiene (Ramay et al., 2020; Ventola, 2015). Moreover, the release of antibiotics into the environment can contribute to the development of antibiotic resistance in bacteria and lead to the widespread dissemination of these resistant bacteria in the environment (Larsson and Flach, 2022).

The development of antibiotic resistance in bacteria involves several mechanisms. These include inactivating enzyme production, target modification, antibiotic uptake limitation, and the efflux pump systems (Reygaert, 2018).

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a strain of *Staphylococcus aureus* resistant to methicillin and other antibiotics, particularly β-lactams such as penicillins, cephalosporins, monobactams, and carbapenems. In addition, MRSA exhibits resistance to other categories of antibiotics, including ciprofloxacin, tetracycline, gentamicin, chloramphenicol, and linezolid. MRSA infection can potentially cause various diseases, including bacteremia, pneumonia, osteomyelitis, prosthetic joint infections, and skin infections (Vestergaard et al., 2019).

The resistance of MRSA to penicillin frequently occurs due to the modification of penicillin-binding proteins (PBPs), which leads to the synthesis of PBP2a, a process initiated by the expression of the *mec*A gene. PBP2a is responsible for decreasing the binding affinity of β-lactams to the bacterial cell wall, resulting in the synthesis of the

bacterial cell wall when exposed to β-lactams through the transpeptidation and transglycosylation reactions (Lim and Strynadka, 2002).

MRSA can produce biofilm, a protective mechanism for bacteria that allows them to accumulate on environmental surfaces, resulting in increased tolerance against the selective pressure from antibiotics. A biofilm is a protective layer produced by bacteria. This layer is synthesized from an extracellular polymeric substance (EPS) matrix composed of polysaccharides, proteins, lipids, and nucleic acids. This structure encases several layers of bacteria, which can hinder the penetration of antibiotics. Treating antibiotic-resistant bacteria enclosed within a biofilm requires higher antibiotic dosages compared to non-biofilm-forming bacteria (Cascioferro et al., 2021).

However, there is an alternative approach to treating biofilm formation. This approach involves the use of phytochemicals obtained from various medicinal plants. The use of phytochemicals represents an interesting approach to tackle the issue of antibiotic-resistant bacteria. These bioactive compounds play a crucial role in combating bacterial infections based on their distinct mechanisms of action. Plants naturally produce these chemicals as a defense mechanism against various stressors and threats, including herbivores, pathogens, and competitors (Lewis and Ausubel, 2006). Therefore, a novel approach to treating antibiotic-resistant bacteria requires an investigation into the antibacterial activity and mode of action of phytochemicals that are specifically related to the resistance mechanisms of these bacteria. These phytochemicals should interact synergistically with conventional antibiotics against antibiotic-resistant bacteria (Khare et al., 2021).

Essential oils are natural compounds extracted using the hydrodistillation method. These oils provide various therapeutic benefits due to their wide range of pharmacological properties, which contribute to combatting these pathogens. These oils attribute their antibacterial properties to diverse bioactive compounds, such as terpenes, phenols, aldehydes, and ketones (Tang et al., 2020; Yu et al., 2020).

Boesenbergia rotunda, commonly referred to as fingerroot, is a traditional medicinal plant that is extensively used in Southeast Asian countries such as Thailand, Indonesia, and Malaysia. This plant demonstrates various health benefits from diverse perspectives due to its range of pharmacological activities, including antibacterial, anti-

biofilm formation, antioxidant, antifungal, and anticancer properties (Eng-Chong et al., 2012).

Teethaisong et al. (2018) suggest that the crude extract of *B. rotunda* (BRE) displayed strong antibacterial effects against both susceptible strains of *Staphylococcus aureus* and MRSA, indicating its ability to combat MRSA. Furthermore, BRE was combined with cloxacillin against MRSA strains, resulting in a synergistic effect. This effect leads to stronger antibacterial activity compared to using the individual compounds. Bacterial cell damage to both the cell wall and cytoplasmic membrane was a result of the treatment of this combination (Teethaisong et al., 2018).

The synergy approach based on the distinct mode of action of plant compounds and conventional antibiotics has the potential to reverse antibiotic resistance in bacteria. This involves rendering bacteria susceptible to antibiotics again. Furthermore, combining conventional antibiotics with medicinal plant compounds has demonstrated greater effectiveness in antibacterial activity and could potentially decrease the adverse effects compared to using antibiotics or plant compounds individually in high doses during treatments (Khameneh et al., 2019).

Despite previous studies that have investigated the antibacterial activity and synergistic effects of *B. rotunda* crude extract against MRSA, there have been few available research focusing on the synergistic effect between *B. rotunda* essential oil (BREO) and conventional antibiotics against MRSA strains. Therefore, the objective of this research was to evaluate the antibacterial activity, synergistic effects, and mode of action demonstrated by BREO when used individually and when combined with cloxacillin (CLX).

1.2 Research objectives

1. To evaluate the antibacterial activity and synergistic effects of BREO and CLX against CLX-resistant MRSA**.**
2. To investigate modes of action of BREO and CLX, including effects on bacterial morphology.
3. To determine the potential of BREO to damage cell envelopes and inhibit biofilm formation.

1.3 Scope and limitation of the study

Isolates of MRSA, including DMST 20649, 20651, and 20652, were provided by the Department of Medical Sciences (DMST). The quality control strain was *S. aureus* ATCC 29213, which was purchased from the American Type Culture Collection (ATCC). Sigma-Aldrich, Germany, contributed all antibiotics used in this research, including CLX and nisin (NIS). Rhizomes of *B. rotunda* were acquired at the Suranakhon market in the Mueang district of Nakhon Ratchasima province, Thailand. The plant specimen was verified and confirmed by Dr. Santi Wattana of Suraneree University of Technology, Thailand. A voucher specimen (BKF NO. 192160) was deposited in the Forest Herbarium of Thailand.

CHAPTER II

LITERATURE REVIEW

2.1 The bacterial cell wall

Bacteria are single-celled organisms without a nucleus. Using the gram-staining method, these organisms are categorized into gram-positive or gram-negative based on differences in the structure of the cell wall. Gram-positive bacteria have a thick cell wall, while gram-negative bacteria have a thinner wall (Silhavy et al., 2010).

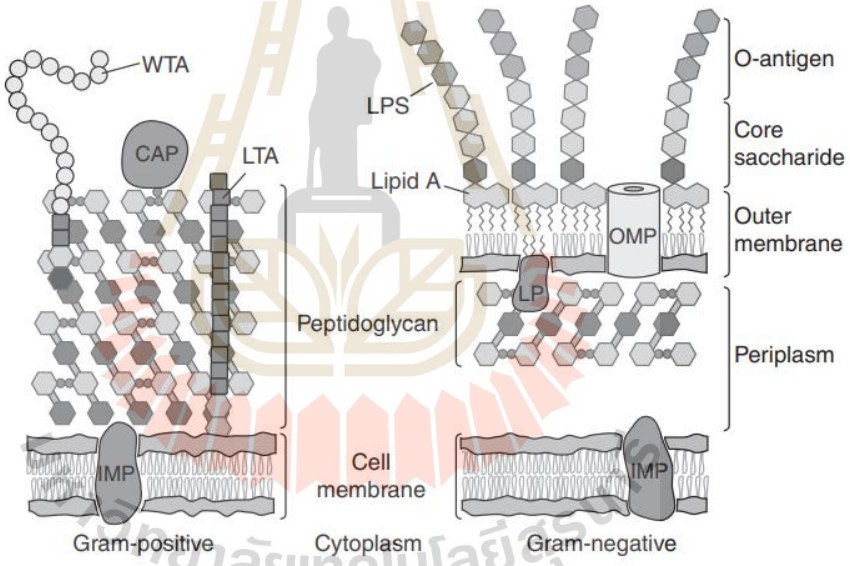

Figure 2.1 Cell envelopes of gram-positive and gram-negative bacteria (Silhavy et al., 2010).

2.1.1 The cell wall of gram-positive bacteria

The cell wall of gram-positive bacteria consists of approximately 90% peptidoglycan, which distinguishes them from gram-negative bacteria (Silhavy et al., 2010).

In the gram staining method, a blue to purple color appears after staining with crystal violet dye. The color intensity indicates the thickness of the peptidoglycan layer (Coico, 2006).

Furthermore, the cell wall of gram-positive bacteria contains polymers such as teichoic acid and teichuronic acid, which are composed of ribitol or glycerol (Brown et al., 2013). Teichoic acid attaches to peptidoglycan in some gram-positive bacteria, forming the teichoic wall. However, all gram-positive bacteria have lipoteichoic acid (Rohde, 2019). These structures mainly bind magnesium ions (D'Elia et al., 2009).

2.1.2 The cell wall of gram-negative bacteria

The cell wall of gram-negative bacteria is composed of approximately 5-10% peptidoglycan. This cell wall includes four major components: peptidoglycan, lipoprotein, outer membrane, and lipopolysaccharide (LPS) (Silhavy et al., 2010).

LPS maintains the stability and integrity of the outer membrane in gram-negative bacteria. It protects the cell from lysis and prevents phagocytosis by phagocytes. LPS is composed of lipid A, the core oligosaccharide, and the O-antigen. Additionally, LPS is an endotoxin released during cell lysis (Bertani and Ruiz, 2018).

2.1.3 Peptidoglycan synthesis

Peptidoglycan strengthens bacterial cells, protecting them from environmental stresses such as osmotic pressure and antibiotic effects. The synthesis of this component involves multiple steps, as described below.

Steps of peptidoglycan synthesis include glycan strand formation, formation of the pentapeptide chain, the addition of the peptide chain, and polymerization of glycan strands.

In glycan strand formation, N-acetylglucosamine (GlcNAc) attaches to N-acetylmuramic acid (MurNAc) via a β-1,4 glycosidic bond. For the formation of the pentapeptide chain, MurNAc attaches to a sequence comprising L-Ala, D-Glu, mDAP, and two D-Ala residues facilitated by transpeptidase. In gram-positive bacteria, L-Lys is

positioned in the third position. During the addition of the peptide chain, transglycosylase enzymes facilitate the attachment of MurNAc to peptide chains, forming the foundational structure of the cell wall. Lastly, peptidoglycan elongates at PBPs during polymerization, which is crucial for penicillin-binding and glycosidic bond formation (Garde et al., 2021).

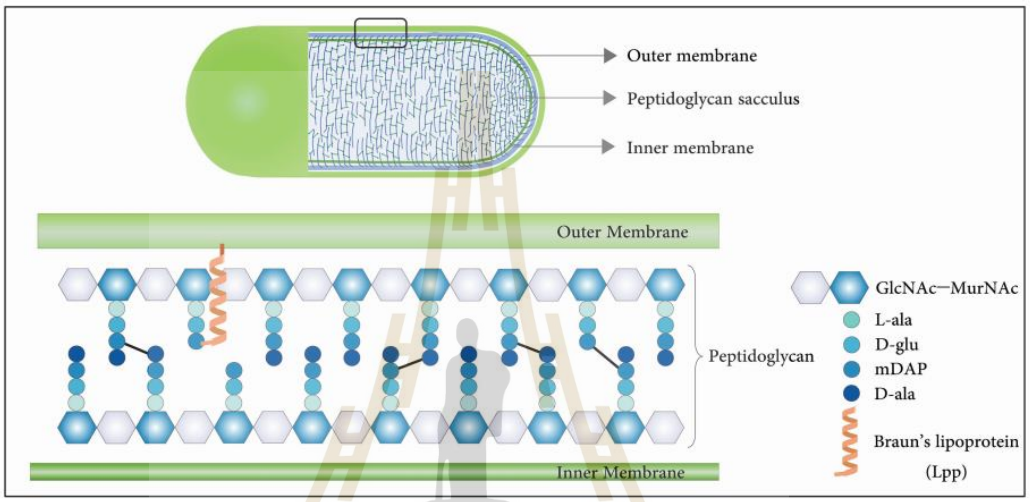

Figure 2.2 The structure of peptidoglycan (Garde et al., 2021).

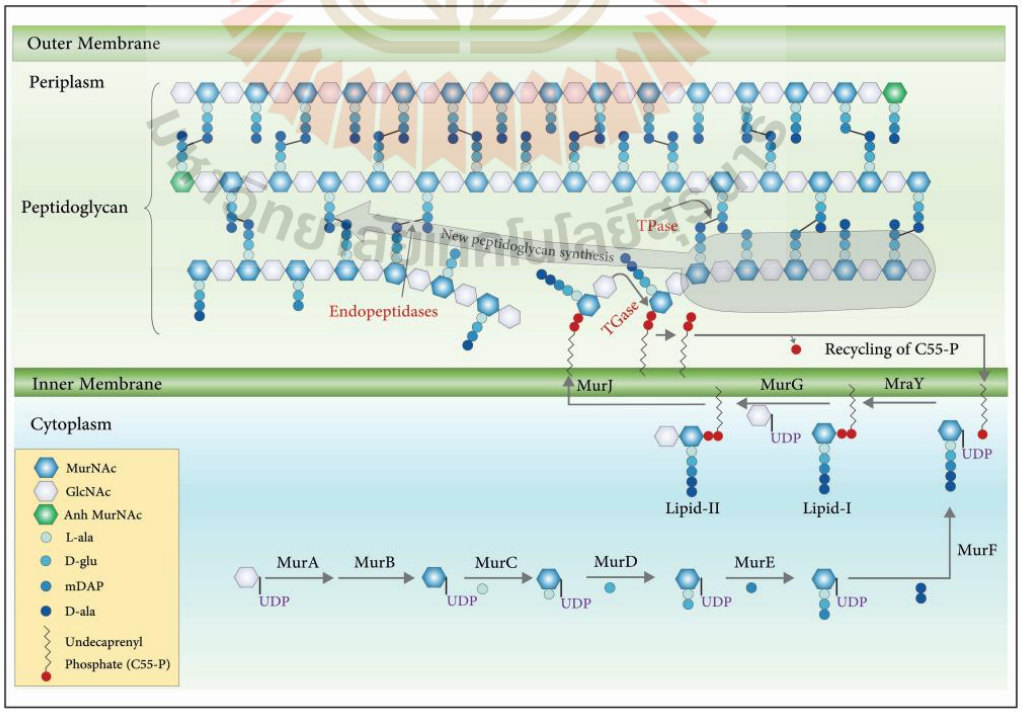

Figure 2.3 The scheme of peptidoglycan synthesis (Garde et al., 2021).

2.2 Antibiotics and their modes of action

Antibiotics are chemotherapeutic drugs that serve to treat bacterial infections by inhibiting or killing bacteria. Their effectiveness is based on targeting specific processes inside the bacteria (Julian Davies and Davies, 2010).

These drugs are categorized based on their mechanisms of action, targeting components such as the bacterial cell wall, membrane, protein production, DNA, and RNA (O'Rourke et al., 2020).

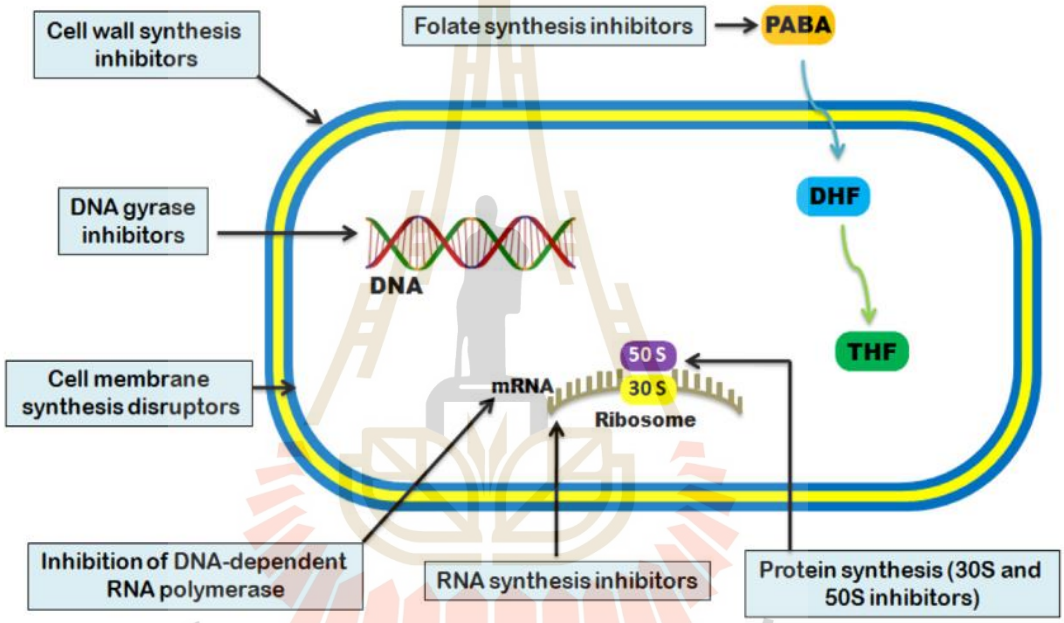

Figure 2.4 The mechanism of action of antibiotics (Uddin et al., 2021).

2.2.1 Cell wall synthesis inhibitors

Cell wall synthesis inhibitors are antibiotics that inhibit bacterial cell wall synthesis. These antibiotics include several classes, including β-lactams, glycopeptides, and peptides (Kapoor et al., 2017).

β-Lactams are antibiotics that contain at least one β-lactam ring in their structure. These antibiotics are commonly used to inhibit the cell wall synthesis of bacteria. Benzylpenicillin was identified as the first β-lactam. Subsequently, based on their structures, β-lactam derivatives were classified into several classes, including penicillins, cephalosporins, monobactams, and carbapenems. The mode of action of

this class of antibiotics involves the suppression of peptidoglycan biosynthesis through the interaction of β-lactams with PBPs. This interaction inhibits the transpeptidase enzyme, leading to the disruption of peptidoglycan polymerization (Bush and Bradford, 2016).

Due to the effectiveness of β-lactams in cell wall biosynthesis inhibition, bacteria develop resistance by producing enzymes, including β-lactamase, which includes penicillinases, AmpC, cephalosporinases, carbapenem-hydrolyzing enzymes, and extended-spectrum β-lactamase (ESBLs). These enzymes degrade β-lactams, allowing peptidoglycan synthesis (Bonomo, 2017).

Research and development efforts have resulted in the synthesis of β-lactamase inhibitors, such as clavulanic acid, sulbactam, and tazobactam. These compounds enhance the therapeutic potential of β-lactams against β-lactamase-producing bacterial strains (Tehrani and Martin, 2018).

Furthermore, bacterial strains, particularly MRSA, have developed resistant mechanisms against β-lactams by altering their PBPs. These modified PBPs, known as PBP2a, exhibit reduced binding affinity to β-lactams. The resistance is associated with the expression of the *mec*A gene on the staphylococcal cassette chromosome mec (SCCmec) (Shalaby et al., 2020).

2.2.2.1 Penicillins

Penicillins, a group of antibiotics, inhibit cell-wall synthesis. These antibiotics display differences in the R-group in their structure, which are associated with varying effectiveness of bacterial activity and spectrum (Mora-Ochomogo and Lohans, 2021).

The first clinically used penicillin is benzylpenicillin, which includes penicillin G and penicillin V. Both are for the treatment of susceptible streptococcal infections. (Bush and Bradford, 2016).

Subsequently, the emergence of penicillin resistance in bacteria, particularly penicillinase-producing staphylococci, led to the ineffectiveness of penicillin G. As a result, another category of penicillin, which is tolerant to penicillinase, including methicillin, oxacillin, cloxacillin, and nafcillin, was employed to treat these β-lactam-resistant bacteria (Kuriyama et al., 2014).

Because of their potential to inhibit cell wall synthesis, penicillin antibiotics are frequently used against gram-positive bacteria, characterized by their thick peptidoglycan layers (Cochrane and Lohans, 2020).

Monotherapy using penicillins, including ampicillin, amoxicillin, penicillin G, and penicillin V, is inadequate against β-lactamase-producing bacteria. Combination therapy involving these antibiotics has been developed with β-lactamase inhibitors, such as clavulanic acid, sulbactam, and tazobactam. These combinations are effective against such resistant strains. For instance, the combination of amoxicillin with clavulanic acid demonstrates synergistic inhibition of the growth of these bacteria (Bush and Bradford, 2016).

2.2.1.2 Cloxacillin

Cloxacillin is a semi-synthetic antibiotic belonging to the penicillin group. It has a modified R-group at position 6, resulting in the presence of a 2,6-dimethoxyphenyl group.

The modification in the R-group enhances the stability of the antibiotic against degradation or cleavage catalyzed by penicillinase and β-lactamase enzymes produced by bacteria, especially *S. aureus*. The mode of action of CLX involves inhibiting the final stage of cell wall synthesis after binding to PBPs. Despite its mechanism being similar to other penicillins, the modified R-group enables it to inhibit bacterial cell wall synthesis when exposed to the β-lactamase enzyme. Consequently, these bacteria become less able to tolerate osmotic pressure. Additionally, CLX can stimulate the activity of autolysins, leading to cell wall breakdown (NIH, 2023).

Based on its mechanism of action, CLX is utilized to treat various infectious diseases, including endocarditis, osteomyelitis, and skin and soft tissue infections. Nevertheless, CLX resistance in *S. aureus* has become a problem in Ghanaian hospitals (Labi et al., 2016). Hence, CLX cannot be employed as a monotherapy.

To reduce the chance of CLX resistance development, it is recommended to use CLX in combination therapy with other antibiotics such as ampicillin, gentamicin, and daptomycin for the treatment of endocarditis (Habib et al., 2015). Cloxacillin was co-administered with gentamicin or daptomycin in a rabbit model to treat endocarditis caused by methicillin-susceptible *Staphylococcus aureus* (MSSA) infection (García-de-

la-Mària et al., 2020). Moreover, a synergistic bactericidal effect was observed when cloxacillin was co-administered with vancomycin for the treatment of endocarditis caused by *S. aureus* infection (Castañeda et al., 2021). Similarly, ceftobiprole, a β-lactam that targets PBP2a, exhibits potent synergistic inhibition of MRSA when used in combination with CLX (Sharma and Gutheil, 2023).

2.2.2 Protein synthesis inhibitor

Protein synthesis is the fundamental process of translating messenger RNAs (mRNAs) into diverse functional proteins crucial for bacterial survival. This process is regulated by the activity of ribosomal protein subunits, specifically the 30S subunit (composed of 16S, 23S, and 5S ribosomal proteins) and the 50S subunit. These subunits assemble to form a functional 70S ribosome. Protein synthesis involves distinct steps, including initiation, elongation, and termination. These steps correspond to the structure of the functional ribosome, which includes three separate areas: the amino acid binding (A) site, the peptidyl-tRNA binding (P) site, and the exit (E) site (Noeske and Cate, 2012).

Protein synthesis inhibitors represent a class of antibiotics that specifically target bacterial protein synthesis through interaction with either the small (30S) or large (50S) ribosomal subunits. Those that bind to the 30S subunit, such as tetracyclines, streptomycin, and aminoglycosides, interfere with the A site of the ribosome, thereby disrupting the accurate base pairing between mRNA codons and anticodons at the entry site of the ribosome. In addition, other antibiotics, including kasugamycin, edeine, and amicoumacin A, also target the 30S subunit but demonstrate their inhibitory effects on protein synthesis at the P or E site, subsequently affecting mRNA translocation.

Conversely, antibiotics that target the 50S primarily affect the P and E sites. Specifically, chloramphenicol, linezolid, clindamycin, streptogramin A, and blasticidin S are known for affecting the P site. A separate class of inhibitors, the macrolides (e.g., erythromycin), are associated with interactions at the E site of the 50S subunit (Lin et al., 2018).

2.2.3 Nucleic acid synthesis inhibitor

Nucleic acid synthesis inhibitors are a specific group of antibiotics that target bacterial DNA and RNA synthesis, thereby inhibiting nucleic acid production. This category includes antibiotics such as rifamycin and fluoroquinolones.

Rifamycin works by forming a chemical bond with DNA-dependent RNA polymerase. This interaction inhibits the RNA elongation phase, effectively suppressing RNA synthesis.

On the other hand, fluoroquinolones interact with enzymes like DNA gyrase and topoisomerase IV. Inhibiting DNA gyrase activity disrupts bacterial cell division, while interference with topoisomerase IV inhibits DNA replication by blocking the proper separation of daughter DNA helices (Uddin et al., 2021).

2.2.4 Metabolic pathway inhibitor

Metabolic pathway inhibitors represent a distinct category of antibiotics functioning as competitive inhibitors. They specifically target bacterial metabolic enzymes integral to folic acid metabolism, particularly dihydrofolate synthase and dihydrofolate reductase.

The primary antibiotics that inhibit these enzymes are sulfonamides and trimethoprim. Sulfonamides demonstrate their antimicrobial activity by inhibiting dihydropteroate synthase. This inhibition results in the reduction of dihydropteroic acid synthesis, a compound produced when pteridine reacts with para-aminobenzoic acid (PABA).

Conversely, trimethoprim targets and inhibits dihydrofolate reductase, subsequently decreasing the synthesis of tetrahydrofolic acid (THF). This action interferes with the biosynthesis of essential proteins and nucleic acids.

To counteract resistance arising from mutations in dihydropteroate synthase and dihydrofolate reductase genes, a prevalent strategy is the co-administration of trimethoprim and sulfamethoxazole (Capasso and Supuran, 2014).

2.2.5 Cell membrane-targeted antibiotics

Antibiotics that specifically target bacterial membranes function by compromising both the structural integrity and permeability of these membranes.

Polymyxins, including polymyxin B and E, belong to the class of cationic

peptide antibiotics characterized by their hydrophobic chains. These antibiotics exhibit a strong affinity for the negatively charged constituents of bacterial membranes, particularly LPS found in gram-negative bacteria. This interaction induces an alteration in the lipid structure of the membrane.

Therefore, affected bacteria display a compromised membrane permeability. Moreover, the altered membrane exhibits an osmotic imbalance, as molecules and ions pass through the membrane without regulation. Increased osmotic pressure within the bacterial cell can Induce cell death via cell lysis (Mohapatra et al., 2021).

2.3 Antibiotic resistance in bacteria

Penicillin was the first antibiotic discovered by Alexander Fleming in 1928. However, by 1940, an enzyme known as penicillinase had been identified, which reduced the effectiveness of penicillin (Lobanovska and Pilla, 2017). Subsequently, penicillins were modified in their structure to enable them to maintain stability against degradation by penicillinase (Kong et al., 2010).

In Japan, the dissemination of antibiotic resistance among bacterial strains through conjugation mechanisms within the bacterial population was first observed and documented in the mid-1950s (J Davies, 1995). Furthermore, genes related to pathogenicity and various functional genes can also be disseminated among bacterial populations through horizontal gene transfer (HGT) mechanisms (von Wintersdorff et al., 2016). HGT is responsible for the transfer of genetic material among bacteria, and this includes the β-lactamase enzyme, which can also be transferred to other bacteria through HGT. Importantly, this mechanism has been associated with the emergence of multiple mutations related to these genes (Munita and Arias, 2016).

Superbugs exhibit resistance to several antibiotics and are found both in multi-drug resistant (MDR) and totally-drug resistant (TDR) strains (Khan and Khan, 2016). *Mycobacterium tuberculosis* is classified as a TDR-carrying superbug, which requires a multiple-drug regimen for effective treatment (Seung et al., 2015).

The World Health Organization (WHO) has brought attention to these bacteria due to problems related to limited treatment options and inadequate data for the effective management of these bacterial infections (Julian Davies and Davies, 2010).

Infections caused by MDR bacteria can result in various health problems and significant economic burdens. These infections are frequently associated with pathogens such as MRSA, carbapenem-resistant Enterobacteriaceae (CRE), and ESBL-producing *Escherichia coli* (van Duin and Paterson, 2020). Addressing the problem posed by infections from antibiotic-resistant bacteria is important. To effectively combat these infections, it is essential to thoroughly understand the mechanisms through which these bacteria develop resistance. This understanding is crucial in guiding the development of alternative treatment options. The prevalence of antibiotic-resistant bacteria and the ineffectiveness of last-resort antibiotics in treating some of these bacteria gives rise to a cause for concern related to this occurrence. Annually, hundreds of thousands of deaths are attributed to infections caused by these resistant bacteria (Ventola, 2015).

The growing antibiotic resistance in bacteria is a significant global health concern, with a critical impact expected to worsen by 2050. This includes a substantial increase in patient mortality due to infections from antibiotic-resistant bacteria that do not respond to treatment (O'neill, 2014).

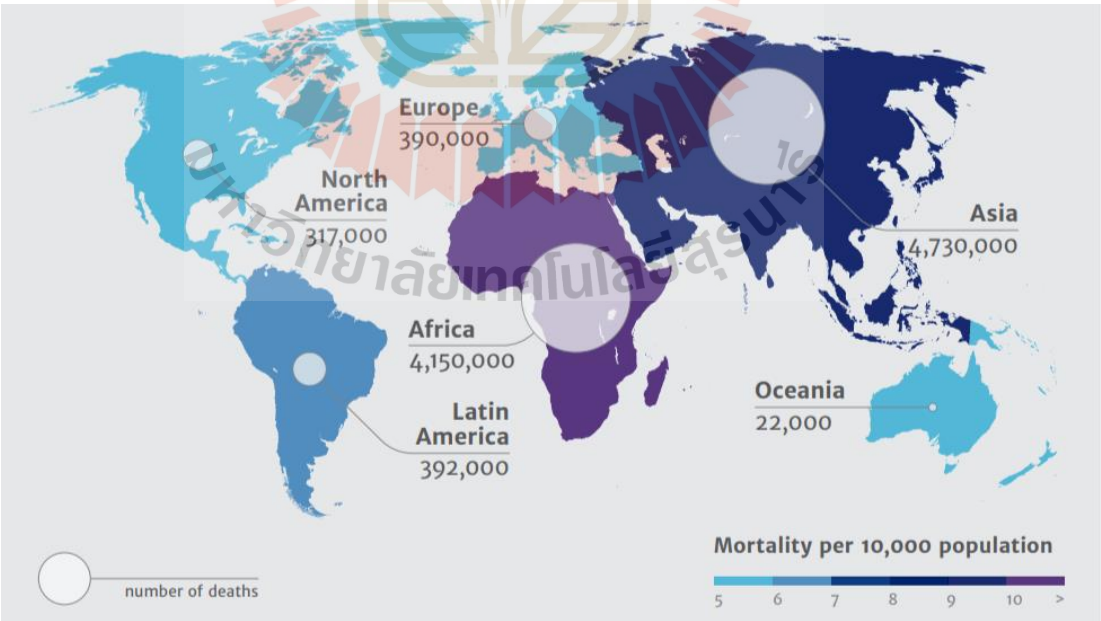

Figure 2.5 Annual deaths associated with antimicrobial resistance by the 2050s (O'neill, 2014).

Accordingly, it is expected to result in economic losses estimated at from 300 billion to 1 trillion USD, impacting healthcare, productivity, and national economies (Dadgostar, 2019). Even in Organization for Economic Cooperation and Development (OECD) countries, economic losses could reach 35 trillion USD (O'neill, 2014). Additionally, the United States only predicts significant combined losses, including 20 billion USD in healthcare costs and 35 billion USD in productivity losses. Developing countries might face a significant decline in GDP by approximately 5-7% (Ventola, 2015).

Antibiotic-resistant bacteria, illustrating the earlier mentioned problem, are mentioned in the 2021 WHO GLASS report (WHO, 2021). These bacteria, including *E. coli*, *S. aureus*, *Acinetobacter baumannii*, *Klebsiella pneumoniae*, and Salmonella spp., demonstrate resistance to many antibiotics and are globally prevalent, causing diseases related to gastrointestinal, bloodstream, and urinary tract infections (Pulingam et al., 2022).

Additionally, it is important to note that low- to middle-income countries encounter higher rates of antibiotic resistance in bacteria (Gerald et al., 2017).

Upon evaluating the economic burden, as outlined in the findings by Poudel et al., it was revealed that antibiotic-resistant infections have a high mortality rate. Additionally, patients affected by these infections show an increased likelihood of needing hospital readmissions, consistent with odds ratios indicating an average extension of hospital stays by approximately 7.4 days (Poudel et al., 2023).

The outcomes arising from resistant bacteria are as follows—A meta-analysis of several studies reveals a higher prevalence of antibiotic-resistant *Enterococcus faecalis* and *Enterococcus faecium* in the South-East Asia and Eastern Mediterranean regions compared to other regions (Jabbari Shiadeh et al., 2019).

According to the investigation conducted by Pormohammad et al. (2019), it was observed that *E. coli* exhibited an approximate amoxicillin resistance rate of 70.5% in humans (Pormohammad et al., 2019).

Salmonella enterica, a foodborne pathogen causing gastroenteritis, leads to an estimated 155,000 annual deaths, with identified resistance to nalidixic acid and ampicillin (Castro-Vargas et al., 2020).

In a study by Ezeh et al., resistance rates of *S. aureus* were observed in Nigeria,

spanning from 13% to 82%. Remarkably, high levels of resistance were evident in penicillin G, cloxacillin, amoxicillin, and cefuroxime, while other antibiotics such as erythromycin, chloramphenicol, methicillin, ofloxacin, rifampicin, and vancomycin exhibited moderate to low resistance (Ezeh et al., 2023).

Antibiotic resistance is a significant concern because bacteria can develop resistance to antibiotics through various mechanisms. These mechanisms include adaptations for survival in response to environmental factors and genetic changes within bacteria, such as altered gene expression. These mechanisms can be broadly categorized as extrinsic and intrinsic resistance (Peterson and Kaur, 2018).

Extrinsic resistance refers to the resistance that bacteria obtain from external factors, such as stressors, antibiotics, and other bacteria, often through mobile genetic elements, including plasmids, transposons, and integrons (van Hoek et al., 2011).

In contrast, intrinsic resistance is the resistance that bacteria naturally possess due to the expression of their genes. It can be classified into various mechanisms that vary among bacterial species, determining the types of resistance (Wright, 2007).

Both types of resistance can cause health problems by reducing the effectiveness of antibiotics and limiting treatment choices. To tackle these problems, understanding each resistance mechanism is important. These mechanisms include antibiotic entry limitation, production of inactivating enzymes, target modification, efflux pump systems, and biofilm formation (Peterson and Kaur, 2018).

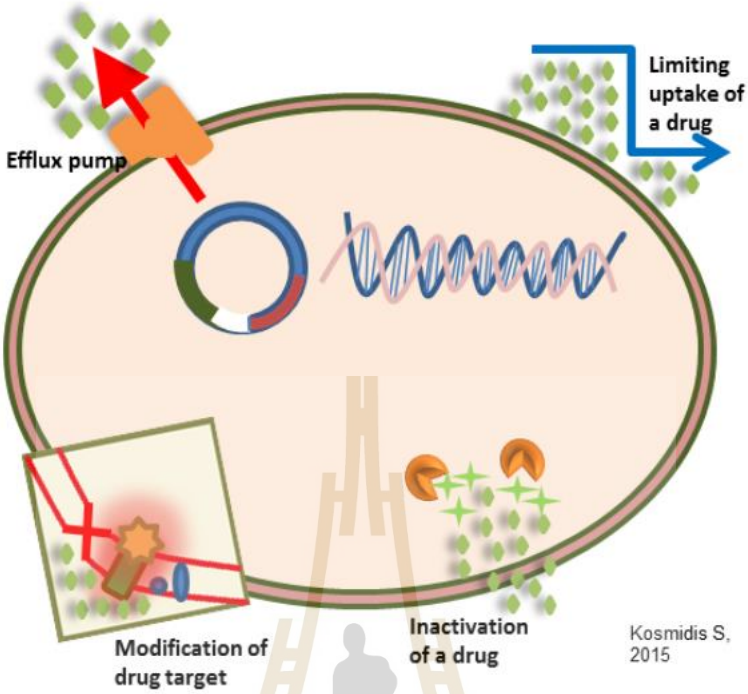

Figure 2.6 The antibiotic resistance mechanisms of bacteria (Reygaert, 2018).

2.3.1 Antibiotic entry limitation

Resistance mechanisms in bacteria related to modifying their cellular envelope result in reduced membrane permeability, thereby restricting the penetration of antibiotics. Specifically, alterations in LPS on the outer membrane of gram-negative bacteria are categorized as resistance mechanisms due to their ability to decrease antibiotic penetration, especially for hydrophilic antibiotics such as β-lactams, tetracyclines, rifampicin, and fluoroquinolones, due to the hydrophobic property of LPS (Breijyeh et al., 2020).

The antibiotic resistance of Mycoplasma is associated with the reduced effectiveness of antibiotics that target cell walls, such as β-lactams and glycopeptides, because this bacterium lacks cell walls (Gautier-Bouchardon, 2018).

Porin proteins are protein channels found in the outer membrane of gram-negative bacteria. Mutations that reduce the number of these proteins, including OmpF, lead to antibiotics penetrating less effectively (Choi and Lee, 2019).

Furthermore, biofilm formation serves as a protective barrier synthesized by

bacterial populations, comprising an EPS matrix. This matrix not only enhances the ability of bacteria to survive under severe conditions but also prevents invasion by the immune system, including phagocytes and the complement system. Consequently, eliminating a bacterial population within a biofilm necessitates a higher antibiotic concentration compared to treating non-biofilm-forming bacteria. This protective barrier contributes to a reduction in bacterial susceptibility due to the high abundance of bacteria within the biofilm, which can undergo a slow cell division process (Roy et al., 2018).

2.3.2 Efflux pump systems

Efflux pump systems are antibiotic resistance mechanisms that play a crucial role in the survival of bacteria, especially in the elimination of harmful substances such as antibiotics from the cells. These mechanisms function by actively pumping antibiotics out of cells using distinct families, including ATP-binding cassette (ABC), Small Multidrug Resistance (SMR), Multidrug and Toxic Compound Extrusion (MATE), Resistance-Nodulation-Cell Division (RND), and Large Facilitator Superfamily (MFS). The differences among these efflux pump families are described as follows (Nishino et al., 2021).

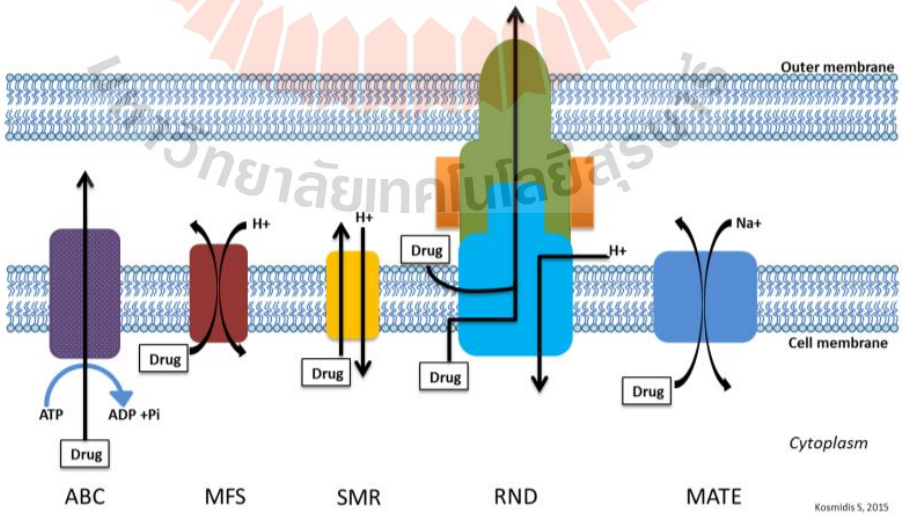

Figure 2.7 Five families of efflux pump system (Reygaert, 2018).

2.3.2.1 ABC family

The ABC family, particularly AcrB, which is often found in *E. coli*, pumps antibiotics across the lipid membrane using ATP hydrolysis.

2.3.2.2 SMR family

The SMR Family, which includes proteins with four transmembrane α-helical domains, such as EmrAB, transports antibiotics across cell membranes, a mechanism also found in *E. coli*.

2.3.2.3 MATE family

The MATE Family, represented by proteins such as NorM, expels cationic antibiotics from *Vibrio parahaemolyticus* cells by exchanging H+ or Na+ ions.

2.3.2.4 RND family

The RND Family, which includes AcrD and MdtABC, removes β-lactams through a coordinated efflux system.

2.3.2.5 MFS family

The MFS Family, which is the largest efflux pump family, comprises diverse secondary transporters, such as the AcrB protein found in *E. coli*.

2.3.3 Antibiotic inactivation

Antibiotic inactivation is a bacterial resistance mechanism that neutralizes the effects of antibiotics. This involves the production of enzymes that deactivate and chemically modify antibiotics (Reygaert, 2018).

The resistance mechanism that makes antibiotics ineffective is associated with reactions involving inactivating enzymes and the alteration of antibiotics through chemical bond addition. These reactions are a result of bacterial gene expression, which allows the production of enzymes or the transfer of this resistance to other bacteria (Munita and Arias, 2016).

β-Lactamases are enzymes that break down the structure of β-lactams, including the β-lactam ring found in antibiotics, such as penicillins and cephalosporins. Breaking the β-lactam ring by hydrolyzing the amide bond in its structure deactivates the function of these antibiotics (Tooke et al., 2019).

Tetracycline inactivation happens when the tet (34) gene is expressed, resulting in the production of an enzyme that deactivates tetracyclines. This gene shares

similarities with the xanthine-guanine phosphoribosyl transferase gene found in *Vibrio cholerae* (Roberts, 2005).

In addition to the degradation of antibiotics, another mechanism for deactivating antibiotics involves transferring chemical groups, such as phosphoryl, acetyl, and adenyl groups, to the antibiotics being targeted. Aminoglycoside modifying enzymes (AMEs) are a type of enzyme that utilizes this mechanism. These enzymes can transfer their chemical groups to modify the structure of aminoglycoside antibiotics, leading to their deactivation through activities such as acetyl and phosphoryl transfer (Peterson and Kaur, 2018).

2.3.4 Target modification

The modification of the target frequently results in a decrease in antibiotic binding affinity, leading to reduced antibiotic effectiveness. This resistance is related to changes in bacterial genetics, causing structural or functional alterations in the antibiotic target (Kapoor et al., 2017).

This resistance mechanism specifically affects β-Lactam antibiotics and typically involves the production of PBP2a, which modifies the structure of PBPs. The production of PBP2a is driven by the expression of the *mec*A gene, which encodes for this protein. PBP2a has a reduced affinity for β-Lactams, leading to a weaker binding between β-Lactams and PBP2a. Consequently, bacterial cell wall synthesis can continue even when these antibiotics are present (Peacock and Paterson, 2015).

Resistance to macrolides, streptogramins, and lincosamides occurs due to the expression of the erm gene family. This genetic expression leads to changes in the binding site of these antibiotics, causing a reduced specificity of the binding site for these antibiotics (Fyfe et al., 2016).

Resistance to fluoroquinolones is linked to mutations in DNA gyrase at the GyrA subunits, such as Ser83Trp and Ser83Leu, or in topoisomerase IV. These mutations cause structural alterations in these enzymes, reducing their ability to bind effectively to fluoroquinolones, which normally bind to gyrase-DNA complexes (Hooper and Jacoby, 2016).

2.4 *Staphylococcus aureus*

S. aureus is a Gram-positive bacterium with distinct characteristics, including a spherical shape, non-motile behavior, absence of spore formation, and the ability of some strains to produce capsules, resulting in a yellow pigment on agar plates. It typically clusters in grape-like formations based on its genus characteristics. *S. aureus* can grow in both aerobic and anaerobic environments under optimal conditions, with a temperature of 37°C and a pH of 7.4. Additionally, this bacterium yields positive results in tests for plasma coagulase, lactose fermentation, and deoxyribonuclease (Guo et al., 2020; Katzif et al., 2005; Masalha et al., 2001)

This pathogen is often found on human skin, in the throat, digestive systems, and mucous membranes, particularly in the nostrils. It is a major cause of skin and mucosal infections, such as otitis, pyoderma, and surgical site infections. *S. aureus* has developed resistance to various antibiotics, posing problems in treating diseases caused by this pathogen (Tong et al., 2015). These infections can occur in both hospital and community settings, including both hospital-acquired and community-acquired infections (Boucher and Corey, 2008).

S. aureus poses a significant public health threat due to its ability to adapt to various environmental conditions and its various antibiotic resistance mechanisms. These resistance mechanisms decrease the effectiveness of treatments against this bacterium (Howden et al., 2023). These resistance mechanisms can be categorized into two groups: intrinsic and extrinsic resistances.

2.4.1 Intrinsic resistance of *S. aureus*

In *S. aureus*, intrinsic resistance mechanisms often involve three mechanisms, including changes in outer membrane permeability, efflux systems, and the production of β-Lactamase (Guo et al., 2020).

Changes in outer membrane permeability: When the bacterial outer membrane becomes less permeable, it reduces the absorption of antibiotics, leading to decreased antibiotic effectiveness.

Efflux systems in *S. aureus* are driven by specific genes like QacA, NorA, and Smr, which encode transporter proteins. These proteins pump antibiotics out of the cell through an electrochemical gradient of H+ ions.

β-Lactamase enzyme, produced by antibiotic-resistant *S. aureus* strains such as MRSA, causes antibiotic resistance. This enzyme deactivates antibiotics through a hydrolysis process, binding to antibiotics at their target sites and making them ineffective.

2.4.2 Extrinsic resistance of *S. aureus*

Extrinsic resistance, also known as acquired antibiotic resistance, is a resistance mechanism that bacteria obtain from external sources. In *S. aureus*, examples of such resistance include acquiring resistance genes and forming biofilms (Reygaert, 2018).

Bacteria can acquire resistance mechanisms through plasmid-mediated processes, where resistance genes are inserted into the bacterial genome. This mechanism can also lead to excessive production of β-lactamase (Sultan et al., 2018). Furthermore, this resistance mechanism, associated with mutations in DNA gyrase, results in resistance, particularly against erythromycin (Vestergaard et al., 2019).

Biofilm is a complex structure composed of bacteria and protective layers, such as EPS substances. Bacteria inside this structure show greater resistance to antibiotics compared to bacteria that are free-floating (Mirghani et al., 2022). Moreover, the persistent cells within biofilms exhibit resistance to multiple antibiotics and play a role in the development of antibiotic resistance (D. Sharma et al., 2019).

Once, there was a suggestion to use penicillin to treat infections caused by *S. aureus*. However, the rise in penicillin-resistant bacteria resulted in the development and use of alternative treatments (Huemer et al., 2020). The emergence of vancomycin-resistant *S. aureus* (VRSA). Consequently, there is a need to develop alternative treatments against this bacterium because vancomycin has been reserved as the antibiotic suitable for the treatment of MRSA (Cong et al., 2020). Regarding the treatment of MRSA and VRSA, the recommended option is to use daptomycin due to its effectiveness against these bacteria (Lewis et al., 2018).

2.4.3 Staphylococcal infections

S. aureus can cause a wide range of infections, ranging from minor skin-related problems to severe systemic conditions that can be life-threatening (Tong et al., 2015). These infections can be contracted through contact with contaminated individuals or objects, and several cases result from asymptomatic colonization, including the

colonization of the nares, skin, and gastrointestinal tract (Sakr et al., 2018).

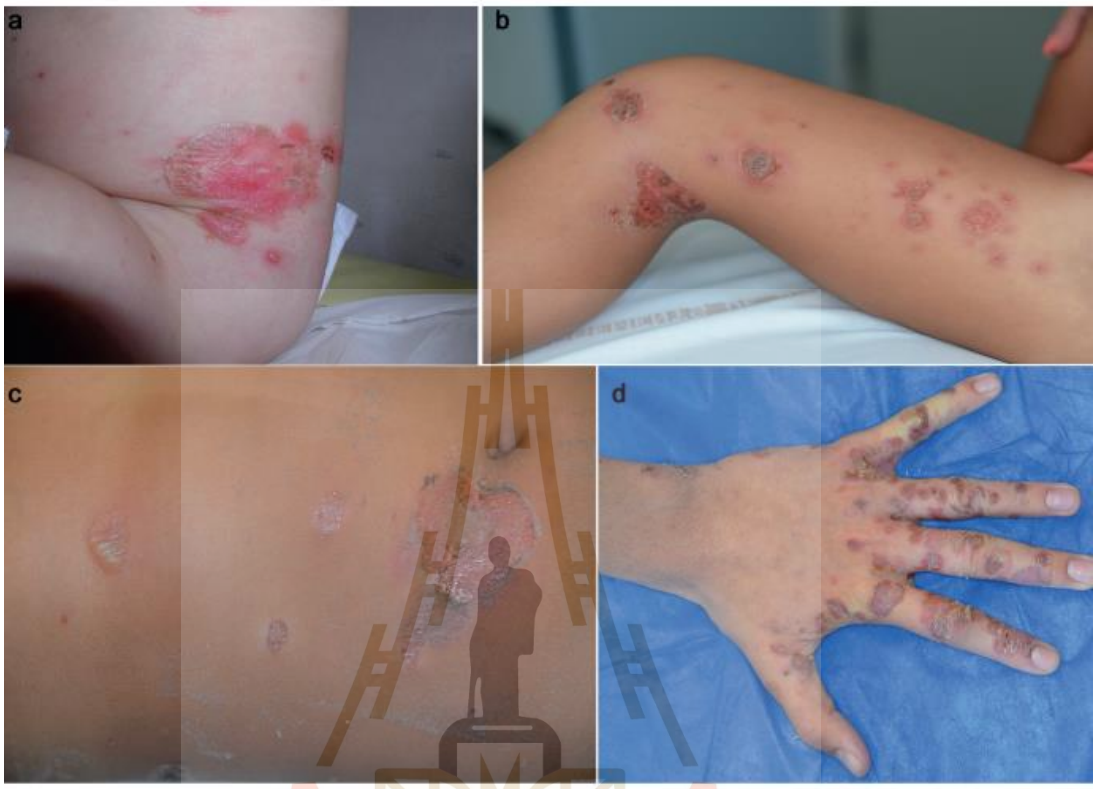

Figure 2.8 Skin infection (impetigo) caused by *S. aureus* (Del Giudice, 2020).

S. aureus contaminates the surfaces of medical equipment, leading to the formation of biofilm. Biofilm formation represents a virulence factor of this bacterium, leading to the occurrence of chronic infections. Anti-biofilm agents targeted at biofilms, which are composed of MDR *S. aureus*, have been developed (Archer et al., 2011).

Moreover, these biofilm-forming strains of S. aureus can release the toxic shock syndrome toxin-1 (TSST), the primary cause of staphylococcal toxic shock syndrome (TSS), a life-threatening condition resulting from *S. aureus* infection and leading to multiple organ dysfunctions (Cheung et al., 2021).

2.4.4 Biofilm formation of *S. aureus*

Biofilm formation is a factor that enhances the pathogenicity of bacteria. Biofilm is composed of complex structures that attach to environmental surfaces. This structure can inhibit the binding between antibiotics and bacteria, allowing them to

persist within the biofilm (Muhammad et al., 2020). Since bacteria are not exposed to the total effectiveness of antibiotics, they can increase their virulence and are susceptible to causing serious infectious diseases, especially in opportunistic infections in hospital settings where bacterial biofilms can attach to medical devices (Beceiro et al., 2013).

S. aureus demonstrates the ability to produce biofilms. These structures play a role as a defensive mechanism against stressors, especially antibiotics. Biofilms are formed under specific conditions, including the availability of nutrients, temperature, and water quantity (Archer et al., 2011). Bacteria within biofilms exhibit greater resistance to antibiotics compared to free-floating antibiotic-resistant bacteria. Generally, the formation of a biofilm involves several steps, which are described below (Mirghani et al., 2022).

2.4.4.1 Stages of biofilm formation

During this initial stage, biofilm-forming bacteria adhere to the surface, facilitated by adhesins, proteins that specifically bind to receptors on the surface. Once attached to the surface, bacteria initiate the production of EPS to form the protective barrier on this surface.

After attachment, bacteria increase EPS production and establish this protective barrier against bacterial stress, including antibiotics and immune responses. In addition, these bacteria can communicate with other bacteria through quorum sensing mechanisms, which play a role in bacterial communication and gene expression regulation.

As the biofilm progresses to the maturation phase, the EPS matrix thickens, forming organized structures with the production of channels and pores for nutrient and waste transport. Bacteria within the biofilm also differentiate, contributing to increased antibiotic resistance and motility.

In the final stage, bacteria within the biofilm differentiate into free-floating forms. Factors such as nutrient availability and temperature can accelerate this transformation, dispersing bacteria into the environment.

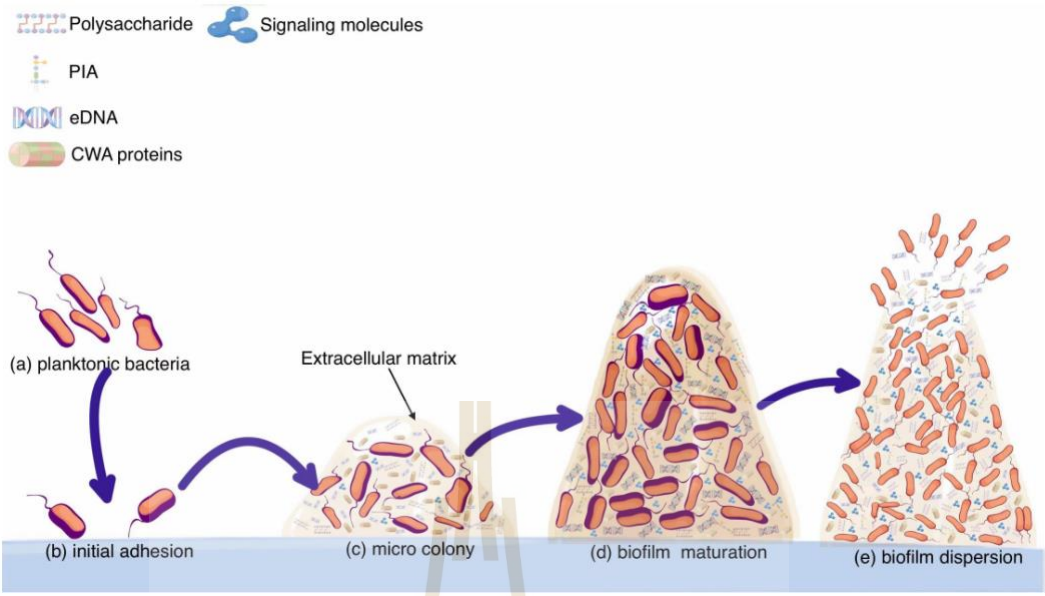

Figure 2.9 Stages of biofilm formation (Ma et al., 2022).

2.5 Methicillin-resistant *Staphylococcus aureus* (MRSA)

Antibiotic resistance in *S. aureus* emerged after Alexander Fleming discovered penicillin (Chambers and DeLeo, 2009). Methicillin was introduced in 1961 to treat penicillin-resistant strains of *S. aureus*. Approximately two years later, the prevalence of *S. aureus* strains resistant to this antibiotic was discovered (Lowy, 2003).

The resistance to methicillin is associated with the expression of the *mec*A gene, located on the SCCmec element of MSSA. This gene encodes the PBP2a protein, reducing the binding affinity of penicillin (Peacock and Paterson, 2015).

MRSA is a strain of *S. aureus* that is resistant to various antibiotics, particularly penicillins. This strain demonstrates multiple resistance mechanisms, including HGT, the production of inactivating enzymes, efflux pump systems, and the modification of bacterial proteins (Vestergaard et al., 2019).

Infections caused by MRSA can be found in both community and hospital settings; these infections can be categorized into hospital-acquired infections (HA-MRSA) and community-acquired infections (CA-MRSA) (Kateete et al., 2019). CA-MRSA is distinguished from HA-MRSA by its smaller SCCmec size and the frequent presence of Panton-Valentine leukocidin and cytotoxin. These substances contribute to the development of mild infections, such as skin and soft tissue infections (Watkins et al.,

2012). MRSA is a widely distributed pathogen found extensively in various regions, including Europe, the United States, North Africa, the Middle East, and East Asia, occurring in both CA and HA-MRSA forms (Lee et al., 2018). MRSA strains can be found in different parts of the body. CA-MRSA is more frequently found in areas outside the nostrils. For example, pediatric patients may have MRSA in their rectums, and MRSA colonization has also been observed in the throat (David and Daum, 2010).

The resistance mechanisms in MRSA, particularly those related to multidrug resistance (MDR), contribute to its ability to be resistant to antibiotics, including fluoroquinolones, tetracycline, macrolides, aminoglycosides, and lincosamides (Algammal et al., 2020). MRSA exhibits resistance to beta-lactam antibiotics through various mechanisms, including the production of beta-lactamase enzymes and PBP2a proteins. A major cause of antibiotic resistance in MRSA is often the production of PBP2a protein, which results from the expression of genes such as *mec*A, *mec*C, *mec*I, *mec*R1, *mec*R2, *blaZ*, *bla*I, and *bla*RI (Shalaby et al., 2020). Environmental stressors, such as oxidative stress, lead to the upregulation of genes like *mec*A and *mec*C in MRSA. In response to these stressors, bacteria activate resistance-related genes, including *mec*A and *mec*C (Peacock and Paterson, 2015). Additionally, the *mec*R1 and mecR2 genes play essential roles in regulating *mec*A and *mec*C genes by encoding regulatory proteins that facilitate their transcription. Conversely, repressor proteins encoded by the *mec*I genes help maintain a balance in the regulation of *mec*A and *mec*C expression by inhibiting their transcription (Miragaia, 2018). Furthermore, resistance to beta-lactam antibiotics is associated with the production of beta-lactamase enzymes, which are regulated by the expression of *blaZ*, *bla*I, and *bla*RI genes. Nevertheless, the regulation of these genes is under the control of the BlaI and BlaR1 proteins (Pence et al., 2015).

2.5.1 The Global Prevalence of MRSA

Based on a systematic review and meta-analysis conducted by Xu et al. (2023), patients with cystic fibrosis infected with MRSA exhibited significant resistance to erythromycin, requiring treatment with vancomycin and teicoplanin (Xu et al., 2023).

Findings from the Azzam et al. (2023) study indicated an overall prevalence rate of approximately 63% in Egypt. This prevalence was evaluated using PCR and

cefoxitin or oxacillin disc diffusion techniques. The PCR results detecting the mecA gene were consistent with the findings of disc diffusion experiments (Azzam et al., 2023).

The study by Wu et al. (2023) focused on clinical characteristics, antibiotic resistance patterns, and risk factors associated with invasive MRSA infections in newborn inpatients during the period of 2018-2019 showed that neonates who met the following criteria: 1. age at admission not exceeding 8 years, 2. congenital heart disease, and 3. birth weight less than 2500 g, were at an elevated risk of severe MRSA infection or invasive MRSA infections (Wu et al., 2023).

Hasanpour et al. (2023) conducted a comprehensive study involving the analysis of 119 articles sourced from 29 different countries covering the period from 1980 to 2022 and employed a random-effects model to estimate pooled prevalence rates, along with 95% confidence intervals (Hasanpour et al., 2023).

Additionally, Wu et al. (2019) presented findings regarding the prevalence of MRSA in the healthy population of China. An analysis of 37 articles revealed a high prevalence of MRSA among healthy individuals in China. This pathogen displayed resistance to penicillin, ampicillin, erythromycin, and clindamycin. The prevalence of this strain was dependent on several relevant factors, including younger age, attendance at daycare centers, receipt of flu vaccinations, recent antibiotic utilization, geographical location, visits to healthcare establishments, the presence of healthcare worker household members, and contact with livestock (Wu et al., 2019).

Shariati et al. (2020) provided valuable knowledge into the resistance rates of MRSA and methicillin-resistant coagulase-negative Staphylococcus (MRCoNS) to several antibiotics, including linezolid, tigecycline, daptomycin, and quinupristin/dalfopristin (Q/D). This research focuses on the observed increase in the prevalence of antibiotic resistance resulting from infections caused by nosocomial pathogens (Shariati et al., 2020).

Based on a comprehensive review of articles available in several databases, the findings indicate that resistance is less prevalent in North American and European countries when compared to countries in the African and Asian regions.

2.6 The use of medicinal plants

Recently, there has been a growing interest in the use of plants for therapeutic and medicinal purposes. Various parts of these plants have traditionally been used in many countries to treat health issues, especially bacterial infections. This growing interest is in response to global health challenges, such as the emergence of antibiotic-resistant bacteria. Such resistance can reduce the effectiveness of conventional antibiotics, leading to increased mortality and morbidity rates following infections by these resistant bacteria (Sofowora et al., 2013).

Based on the challenges posed by the emergence of antibiotic resistance in bacteria, there is an urgent need to develop alternative treatments. These treatments involve the use of medicinal plant compounds, known as phytochemicals and secondary metabolites. These are defensive substances synthesized by plants to fend off threats, such as insects and microbes. These compounds hold potential in the treatment of antibiotic-resistant bacteria (Keita et al., 2022).

Secondary metabolites include a variety of groups, such as alkaloids, phenols, polyphenols, flavonoids, quinones, tannins, coumarins, terpenes, lectins, polypeptides, saponins, and more. These secondary metabolites demonstrate a broad spectrum of therapeutic properties. Some of these properties might be essential in tackling the issue of antibiotic-resistant bacteria (Anand et al., 2019).

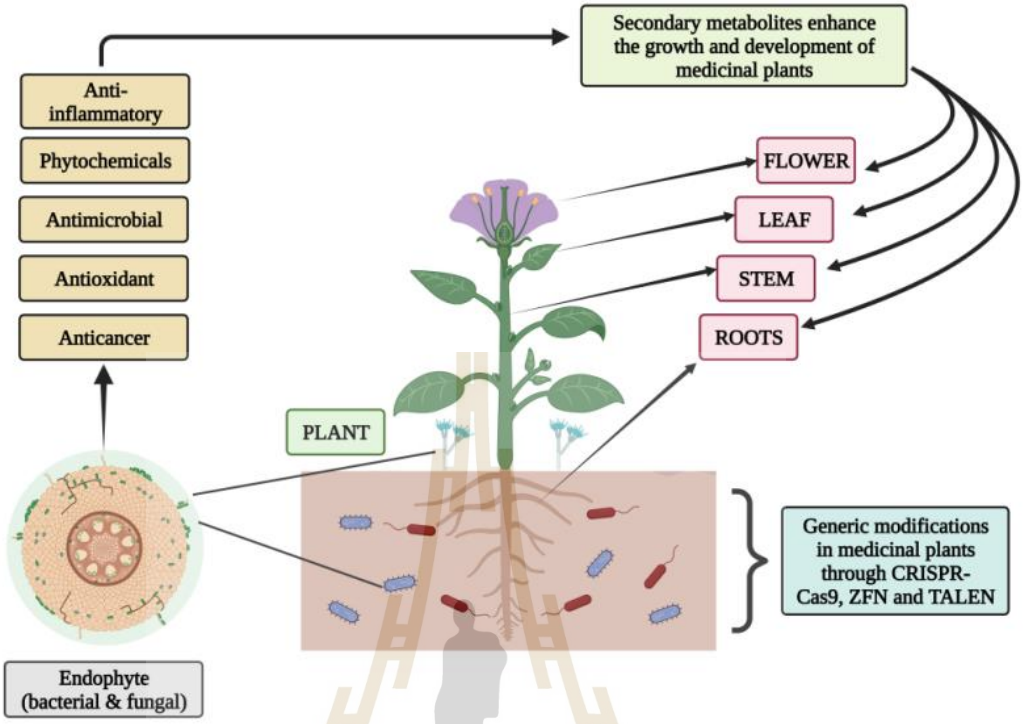

Figure 2.10 Secondary metabolites source and pharmacological properties (Kunwar et al., 2023).

In modern healthcare, medicinal plants play an essential role. These plants offer potential treatments against resistant strains of bacteria. Understanding bacterial resistance mechanisms and the properties of these beneficial plant compounds is essential to employing the potential of medicinal plants and developing novel therapies (Vaou et al., 2021).

Allium sativum, or garlic, is a common ingredient in many traditional dishes globally, particularly in soups and sauces. It is also used in traditional medicine for its health benefits, which include enhancing the immune response, regulating cholesterol levels, and reducing hypertension. The antibacterial activity of *A. sativum*, attributed to its bioactive constituents such as allicin and ajoene, results in a plant extract that exhibits a MIC of 150 mg/ml and a variable zone of inhibition ranging from 3 to 12.5 mm against *S. aureus* (Oyawoye et al., 2022).

Zingiber officinale Rosc., commonly known as ginger, has antimicrobial and anti-biofilm properties. The essential oil of this plant contains various constituents such as geranial, zingiberene, linalool, zingerone, and gingerol. Ginger exhibits strong antimicrobial effects, particularly against Gram-positive bacteria, such as *E. faecalis* and *S. aureus*, with a MIC of 3.125 μl/ml for these strains (Das et al., 2019).

Curcuma longa L., commonly known as turmeric, possesses strong antibacterial activity against MRSA. In a study conducted by Kang-Ju Kim et al., the antibacterial activity of its ethyl acetate extract was evaluated using MIC determination. The extract displayed MICs ranging from 0.125 to 2 mg/mL. A checkerboard assay revealed a synergistic effect when *C. longa* was combined with β-lactams, such as ampicillin and oxacillin (Kim et al., 2005).

Aloe barbadensis miller contains glycoproteins and polysaccharides. Glycoproteins reduce pain and inflammation, while polysaccharides support skin repair and potentially enhance immunity. Extracts from the inner leaves of the plant exhibit strong antibacterial properties. Bioactive compounds, including methoxyacetic acid and 3-tridecyl ester, have demonstrated effectiveness against bacteria such as *E. coli* and *Bacillus thuringiensis* (Das et al., 2020).

2.7 Benefits of the essential oil

Essential oils, often known as volatile oils, are mixtures of various volatile compounds derived from diverse plant parts such as leaves, flowers, stems, and roots. These compounds, including terpenes, sesquiterpenes, and esters, are typically extracted using the hydrodistillation technique (Aziz et al., 2018).

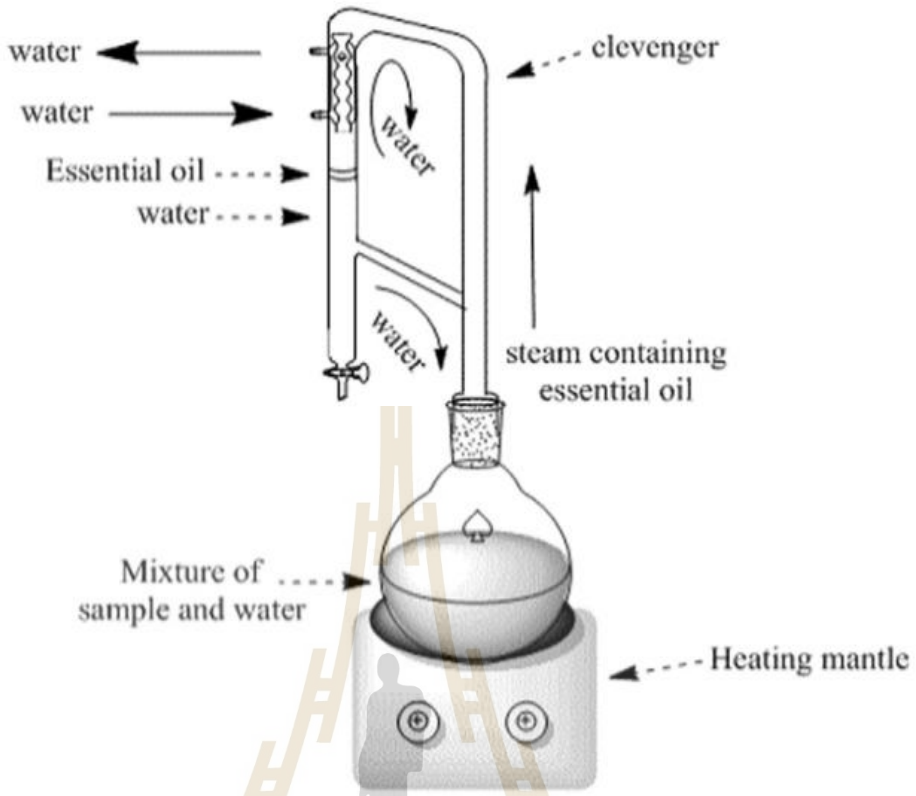

Figure 2.11 Essential oil extraction using a Clevenger-type apparatus system (Samadi et al., 2017).

Gas chromatography–mass spectrometry (GC-MS) is used to analyze the chemical constitution of these oils. The constituents of each essential oil are influenced by various environmental factors, such as the type of soil. These factors affect plant metabolism and lead to variations in the secretion levels of certain compounds (Chamorro et al., 2012).

Essential oils demonstrate antibacterial activities, providing a natural approach to treating health issues such as infectious diseases. According to their therapeutic properties, these oils are not only beneficial for health but are also utilized in food flavoring and the cosmetic industry due to their distinct biological activities (Swamy et al., 2016).

For example, artemisia, a member of the Asteraceae family, contains a wide range of biochemical constituents. Essential oils from this genus have been used for various therapeutic purposes. The essential oil extracted from *Artemisia scoparia* has

shown multiple therapeutic properties, including insecticidal, antibacterial, anticholesterolemic, antipyretic, antiseptic, cholagogue, diuretic, purgative, and vasodilatory effects. It is also effective in treating conditions including gall bladder inflammation, hepatitis, jaundice, malaria, and diabetes (Nigam et al., 2019).

2.7.1 Antibacterial properties of essential oil

Recently, there has been growing research into the antibacterial properties of essential oils for combating pathogenic bacteria, such as *Listeria monocytogenes*, *E. coli*, *S. aureus*, and *Salmonella typhimurium*. Myrtle, scientifically known as *Myrtus communis* L., has a history of traditional medicinal use against infectious diseases caused by bacteria such as *M. tuberculosis*, *E. coli*, and *S. aureus*. This antimicrobial effect is attributed to the bioactive components found in its essential oil, including oxygenated monoterpenes and polyphenols (Giampieri et al., 2020).

Among the essential oils studied, thyme and oregano have been found to be effective against *Salmonella choleraesuis*, *S. typhimurium*, and *E. coli* due to the presence of phenolic compounds like carvacrol and thymol (Swamy et al., 2016).

Origanum vulgare, commonly known as oregano, possesses a range of therapeutic properties. GC-MS analysis of oregano essential oil (OEO) identified 27 compounds, with carvacrol as the predominant constituent, comprising 84.38% of the total. Additionally, other major components, such as β-caryophyllene, γ-terpinene, p-cymene, and thymol, were found (Hao et al., 2021).

The essential oil derived from oregano has demonstrated antibacterial potential. It has shown activity against various pathogens, including *Clostridium perfringens*, *P. aeruginosa*, and *S. aureus* (Coccimiglio et al., 2016).

Ultree et al. (2002) reported that carvacrol has shown antibacterial activity against *Bacillus cereus* (Ultee et al., 2002). Furthermore, Septembre-Malaterre et al. (2020) mentioned that essential oils derived from *Artemisia annua*, which include 1,8-cineole, α-and-β-pinene, camphene, borneol, camphor, carvone, limonene, α-terpinene, and myrtenol exhibited inhibitory activity against *Enterococcus hirae* (Septembre-Malaterre et al., 2020).

The antibacterial activity of essential oils against gram-positive bacteria is more effective than against gram-negative bacteria. This activity is due to the presence of

phenols, alcohols, and terpenes, which are common constituents in most essential oils and exhibit a bactericidal effect, leading to bacterial cell death (Nazzaro et al., 2013).

The essential oil extracted from the leaves of *Melaleuca alternifolia*, commonly known as tea tree oil, exhibits antibacterial, antifungal, and anti-inflammatory properties. The antibacterial activity of this oil is primarily attributed to its major component, terpinene-4-ol. Additionally, this tea tree oil has shown beneficial effects, leading to its utilization in skincare products, particularly creams, for the treatment of skin wounds (Carson et al., 2006).

M. alternifolia, a native Australian plant belonging to the Myrtaceae family, serves as the primary source of Tea Tree Oil (TTO). TTO is known for its extensive antimicrobial properties, including antibacterial, antifungal, antiviral, and antiprotozoal activities. This plant has been used traditionally to treat various health conditions such as skin infections, the flu, and rheumatism (Qi et al., 2021).

M. alternifolia, commonly known as Tea Tree, is classified into six different chemotypes, with one of the most common types being the terpinen-4-ol chemotype. TTO is widely used in clinical applications, which are utilized for treating skin infections, in several regions, including Australia, Europe, and North America. This oil is employed in various products, such as hand cleansers and shampoos. Due to the strong antibacterial activity of TTO, it has been found effective against pathogenic bacteria, especially MRSA and *P. aeruginosa* (Brun et al., 2019).

TTO displayed MIC and MBC values of 0.25% and 0.5%, respectively, against *E. faecalis*. Its antibacterial mechanism involves damaging the membrane and inhibiting biofilm formation. This damage leads to the loss of membrane integrity in these bacteria, resulting in cell death (Qi et al., 2021).

Lavandula angustifolia, commonly known as lavender, belongs to the Lamiaceae family. Native to the Mediterranean regions, lavender holds significant value in Moroccan traditional medicine. The essential oil derived from this plant is primarily employed for its wound-healing properties in the treatment of skin wounds.

The essential oil derived from *L. angustifolia* primarily comprises three major constituents, including linalool, 1,8-cineole, and camphor. This oil has demonstrated

antibacterial activity in the treatment of skin wound infections caused by *E. coli*, *S. aureus*, and *P. aeruginosa* (Messaoudi Moussii et al., 2020).

Miguel (2010) described the various pharmacological properties of essential oils, including their anti-inflammatory, antioxidant, and antibacterial effects. These properties have been studied in vitro. One essential oil known for possessing these properties is *L. angustifolia* oil. Moreover, this oil has been found to stimulate the human immune system against bacteria that cause nosocomial infections (Miguel, 2010).

The antibacterial properties of essential oils lead to the suitable use of these oils as a preprocedural mouth rinse prior to dental procedures. These oils can inhibit the accumulation of plaque, a leading cause of dental problems. Listerine is one of the most preprocedural mouth rinses, containing essential oil, which has several active compounds such as thymol and menthol (Alshehri, 2018).

Moreover, essential oils derived from *Achillea clavennae* are employed as an alternative option for treating respiratory infections caused by bacteria, such *as K. pneumoniae* and *Streptococcus pneumoniae* (Bezić et al., 2003). This suggests that essential oils could potentially be used as natural sources for preventing and treating bacterial infections.

2.7.2 Synergistic approach of the essential oil

Several studies have investigated and found that phytochemicals, such as essential oils containing various secondary metabolites, possess effectiveness against MDR bacteria. Therefore, there is an opportunity to demonstrate the synergistic effect between essential oils and ineffective conventional antibiotics in combating multi-drug resistant bacteria. This is due to the interaction between ineffective antibiotics and secondary metabolites, which reveal multiple modes of action (Khare et al., 2021).

The use of the synergistic effect between natural compounds and conventional antibiotics has become an interesting trend in combating MDR bacteria. This approach has increased interest due to the limited treatment options and inadequate regulation in many countries (Cheesman et al., 2017).

The synergistic effect between phytochemicals and antibiotics, which exhibit distinct antibacterial mechanisms, has been shown to have greater antibacterial

potential compared to using a single drug or plant compound alone (Ayaz et al., 2019). Several groups of secondary metabolites, such as alkaloids and polyphenols, have been identified for their antibacterial properties (Othman et al., 2019). Additionally, essential oils, which contain hydrophobic volatile phenolics (VPs), have been found to enhance the antibacterial activity of hydrophilic antibiotics, such as vancomycin and β-lactams (Ahmad et al., 2021).

The synergistic effect can occur when antibiotics and essential oils target different components of bacterial cells, such as the cell wall, cytoplasmic membrane, protein, and nucleic acid. This effect leads to enhanced antibacterial effectiveness of antibiotics through the mode of action of essential oils (Yap et al., 2014).

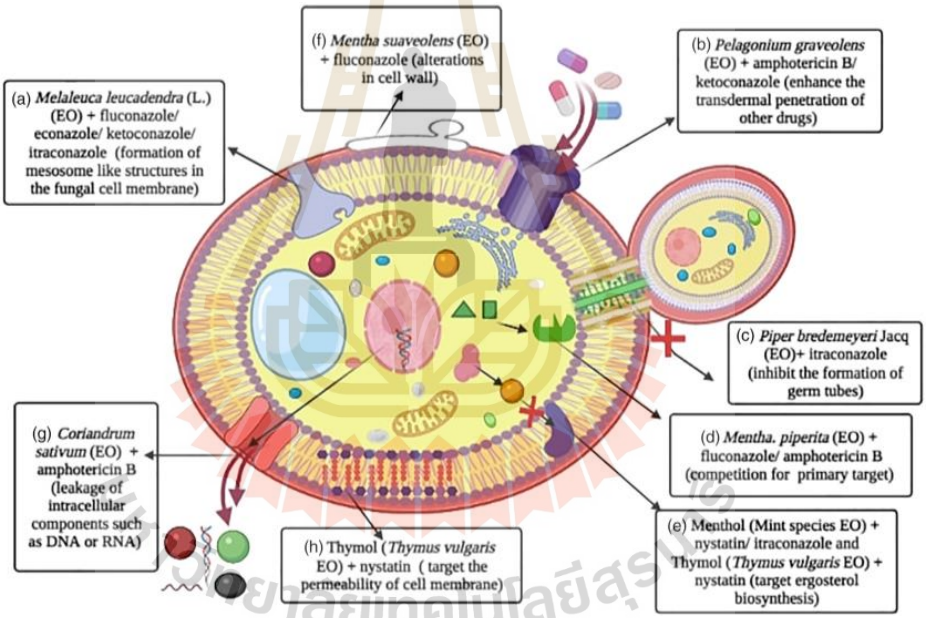

Figure 2.12 The synergistic effect of essential oils (Bhattacharya et al., 2021).

S. Purkait et al. (2020) conducted a study indicating that a combination of essential oils from clove (rich in eugenol) and cinnamon (abundant in cinnamaldehyde) exhibits synergistic antibacterial properties. This combination effectively combated *S. aureus*, *P. aeruginosa*, *S. typhimurium*, and *L. monocytogenes*. These compounds, constituents of this oil, are responsible for synergistic effects (Purkait et al., 2020).

OEO is rich in carvacrol, comprising 71.0% of its constituents. Other compounds such as γ-terpinene, β-caryophyllene, thymol, and p-cymene were also identified.

OEO demonstrated the inhibition of MDR A. baumannii with an MIC range of 1.75 to 3.50 mg/mL. This oil can synergistically enhance the effectiveness of Polymyxin B when used in combination to interfere with the membrane integrity of these strains. The FIC for the combination ranged between 0.18 and 0.37, reducing MIC values by up to approximately 16 times (Amaral et al., 2020).

The essential oil from Centaurea aerial parts contains over 86% oxygenated terpenes, with 11.45% fokienol and 8.8% thymol. This oil showed antibacterial activity against 8 out of 10 strains at 5 μg/mL but needed 15-1000 μg/mL for *K. pneumoniae* and *P. aeruginosa*. In combination with antibiotics, the extract enhanced its effectiveness against *E. coli* and *K. pneumoniae*. Combining cefixime with the extract increased efficacy against *K. pneumoniae* by 26% (Khleifat et al., 2019).

Analysis of *Thymus vulgaris* essential oil (TVEO) revealed the main constituents: thymol (34.5%), p-cymene (22.27%), and linalool (5.35%). TVEO exhibited potent activity against all multi-drug resistant strains, with inhibition zones from 24–40 mm/10μL and MIC values between 2.87–11.5 μg/mL. Combined with CTX, TVEO demonstrated synergistic effects against blaSHV-12-producing *E. coli* (FICI of 0.28) and additive effects against ESBL-producing *Enterobacter cloacae* (FICI of 0.987) (Benameur et al., 2019).

Analysis of several essential oils revealed their antibacterial properties against *Leuconostoc citreum*. Essential oils from cinnamon bark, oregano, and thyme thymol showed the highest effectiveness. A notable synergistic effect was seen when combining oregano and thyme thymol essential oils, which significantly reduced *L. citreum* concentration (Lee et al., 2020).

2.8 *Boesenbergia rotunda*

2.8.1 Overview of the *B. rotunda*

Boesenbergia rotunda, commonly referred to as 'Thai ginseng', is a medicinal herb native to Southeast Asia. As a member of the Zingiberaceae family, which consists of several medicinal plants, this herb can be identified by its unique rhizomes and leaves that are employed in both culinary and pharmacological traditions. The rhizomes, characterized by their distinct aroma, are frequently used in dishes such as

curries and soups. Besides adding flavor, these rhizomes are thought to enhance appetite and provide other health benefits (Ongwisespaiboon and Jiraungkoorskul, 2017).

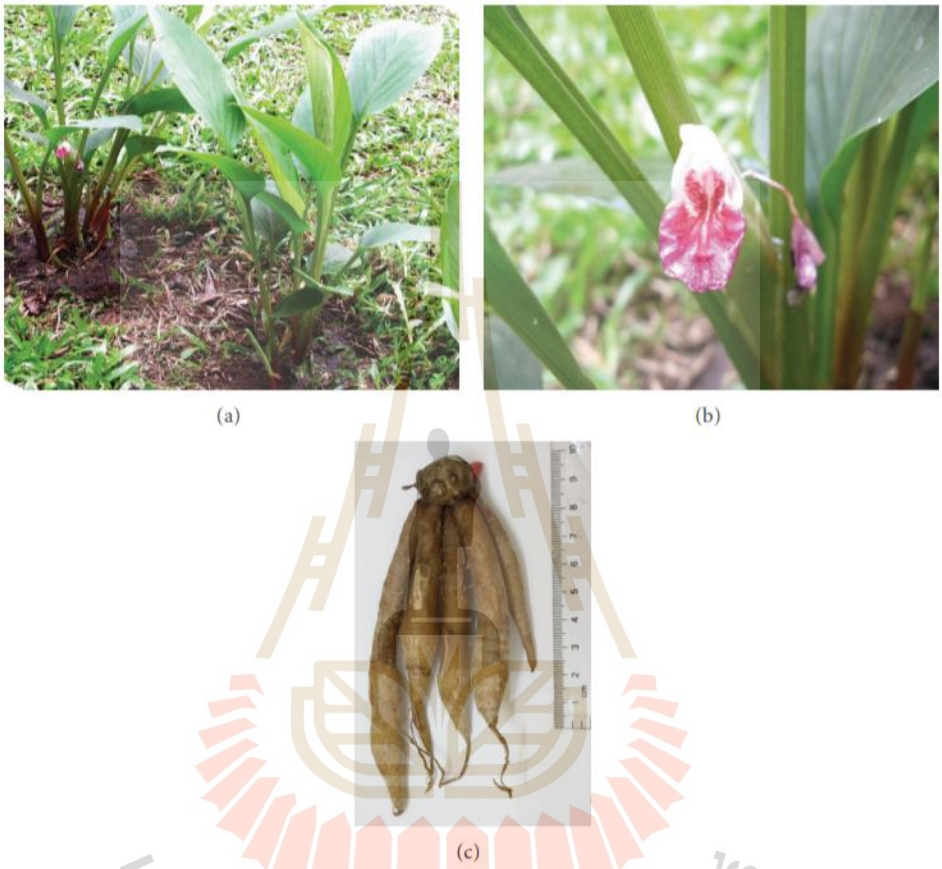

Figure 2.13 The appearance of different parts of *B. rotunda* (Eng-Chong et al., 2012).

This plant is known for treating various diseases and disorders, such as rheumatism, muscular pain, fever, gout, stomachache, dyspepsia, peptic ulcers, dental caries, dermatitis, tooth and gum disorders, inflammation, and diarrhea. These treatments are based on their pharmacological properties, which include antibacterial, antifungal, antiparasitic, wound healing, and anti-inflammatory activities (Eng-Chong et al., 2012; Pham et al., 2021).

Antibacterial properties of this plant have been effective against bacteria such as Salmonella, Listeria, and Staphylococcus. Moreover, its antifungal, wound healing, and anti-inflammatory effects demonstrate its diverse therapeutic applications. Several

studies suggest that *B. rotunda* can be as effective as current antibiotics, particularly against resistant strains. The extracts from this plant have demonstrated improved wound healing speed and quality in specific animal models. *B. rotunda* exhibits diverse therapeutic attributes, suggesting its potential for drug development. Its effectiveness against resistant bacteria is remarkable in modern medicine. Furthermore, its wound-healing properties may influence post-surgery care and ongoing wound treatment (Eng-Chong et al., 2012).

2.8.2 Antibacterial properties of *B. rotunda*

The antibacterial activity of *B. rotunda* has been extensively studied for its potential in treating infectious diseases caused by pathogenic bacteria. This plant has shown a MIC comparable to clarithromycin, a commonly used antibiotic for peptic ulcers caused by *Helicobacter pylori* (Wang, 2014). Furthermore, *B. rotunda* exhibited effective against other pathogenic bacteria, including *S. enterica*, *L. monocytogenes*, *B. cereus*, *S. aureus*, and *E. coli* (Pattaratanawadee et al., 2006).

The methanolic extract of *B. rotunda* was assessed for its antibacterial effects against *E. coli* ATCC 25922 and two *E. coli* strains from milk products. MIC values varied from 0.019 mg/mL to 2.5 mg/mL, while MBC values ranged from 0.039 mg/mL to 5.0 µg/mL. Remarkably, all *E. coli* strains were neutralized at 2x MIC within 2 h, emphasizing the extract's potent antibacterial activity against *E. coli* and its potential as a natural alternative to synthetic antibacterial agents (Zainin et al., 2013).

Furthermore, it promoted wound healing, accelerating wound contraction by day 12 and stimulating complete epidermal regeneration (Jitvaropas et al., 2012).

The ethyl acetate extract of *B. rotunda* exhibited notable antibacterial properties, particularly against gram-positive bacteria. This activity was evident in the inhibition zones that spanned from 8.5 to 11.8 mm when tested against bacteria such as *S. aureus* ATCC 25923, MRSA, *Staphylococcus epidermidis*, and *B. cereus* (Sopitthummakhun et al., 2021).

B. rotunda, known as fingerroot, contains potent anti-inflammatory compounds like panduratin A (Pa-A). Its extracts demonstrate antimicrobial effects against oral pathogens, reduce inflammatory mediators, and support bone health, suggesting its potential as an effective treatment for chronic periodontitis (Bailly, 2022).

The ethanol extract of *B. rotunda* rhizome yielded three flavanone compounds, exhibiting antioxidant activity with IC values: extract (92.64 µg/mL), compound-1 (46.66 µg/mL), compound-2 (62.84 µg/mL), and compound-3 (62.66 µg/mL). These compounds demonstrated antimicrobial potential, with a peak zone of inhibition of 13.20±0.76 mm against *E. coli* ATCC-11229 at 500 µg/mL and a MIC of 0.5 µg/mL. The rhizome contains compounds beneficial against several bacterial strains, including *E. coli* and *S. aureus* (Atun et al., 2018).

BRE effectively inhibited all tested Staphylococci with a MIC of 16 µg/mL. Despite some strains showing resistance to certain antibiotics, the combination of BRE and CLX yielded a fractional inhibitory concentration index (FICI) of 0.502 against resistant strains. TEM analysis revealed cell damage when treated with BRE, particularly in combination with CLX, resulting in significant leakage of cellular materials (Teethaisong et al., 2018).

As a result, the antibacterial properties of *B. rotunda* and its active components, as described in several studies, indicate their potential as alternative treatments for infectious diseases caused by pathogenic bacteria, particularly gram-positive bacteria.

2.8.3 Inhibition of biofilm formation property of *B. rotunda*

Rukayadi et al. (2010) observed that *B. rotunda* has anti-biofilm formation activity in the treatment of *E. faecalis* and *E. faecium*, which typically cause intestinal tract and urinary infections. Pa-A, the active component of this plant, showed a MIC of 2 µg/mL and a MBC of 8 µg/mL. This compound also demonstrated a bactericidal effect against bacteria after 30 minutes of incubation at the MIC (Rukayadi et al., 2010).

In addition, *B. rotunda*, with bioactive compounds pinostrobin (25% w/w) and pinocembrin (12% w/w), demonstrated significant inhibition against *Candida albicans* biofilm formation, with an IC_{50} of 17.7 µg/mL. Pinocembrin was especially effective in reducing the filamentous form, and both compounds impacted ALS3 mRNA expression, while only pinocembrin reduced ACT1 mRNA levels (Kanchanapiboon et al., 2020).

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals and equipment

The chemicals and equipment utilized in this study are listed in Tables 3.1 and 3.2.

Table 3.1 List of chemicals.

Name	Grade
Sodium chloride	AR
Cloxacillin	AR
95% Ethanol	Lab
Osmium tetroxide	Lab
Dimethyl sulfoxide (DMSO)	Lab
Uranyl acetate	Lab
Lead acetate	Lab
HEPE buffer	Lab
Phosphate buffer	Lab
Glutaraldehyde	Lab
Sodium sulfate	Lab

Table 3.2 List of equipment.

Name	Source
Filter paper	Whatman
Autoclave	Yamato
Hot plate	VELP scientifica
Microplate reader	Benchmark
CLASS II Biohazard safety cabinet	ESCO
96-well plate	Corning®
Micropipette (2-20 µL)	Corning®
Micropipette (20-200 µL)	Corning®
Micropipette (100-1000 µL)	Corning®
Refrigerated incubator	VELP scientifica

3.1.2 Antibiotics

CLX and nisin (NIS) were chosen for investigating sensitivity tests and drug interactions. The selection of antibiotics, especially CLX, was based on specific criteria, with a particular focus on β-lactams, specifically semi-synthetic β-lactams. Cloxacillin was chosen for the treatment of penicillin-resistant strains due to its continued usage in hospitalized patients. However, an important observation reveals a high resistance rate in MRSA strains (Adhikari et al., 2023).

3.1.3 Bacterial strains

MRSA isolates, including MRSA DMST 20649, 20651, and 20652, were chosen as treated strains due to their classification as serious threats in the Antibiotic Resistance (AR) Threats report by the CDC. The selection of MRSA strains DMST 20651 and 20652 was based on their resistance mechanisms, specifically related to the expression of *mec*A and *bla*Z, which encode the PBP2a and β-lactamase enzymes, respectively (Teethaisong et al., 2018). However, there is no available report on the resistance mechanism of MRSA DMST 20649. This strain was included in the study because a minimum of three bacterial strains was required for research publication.

Additionally, the quality control strain, *S. aureus* ATCC 29213, was obtained from the American Type Culture Collection (ATCC).

3.2 Methods

3.2.1 Plant specimen and the preparation of essential oil

The rhizomes of *B. rotunda* were selected to investigate antibacterial activity and synergistic effects in this study. These rhizomes were utilized in the form of dried samples to prevent potential issues related to bacterial and fungal contamination that may be present in these samples. DMSO was chosen as a solvent for dissolving several essential oils. This solvent is typically used at concentrations of 5-10%, as described in previous studies (Benali et al., 2020; Huang et al., 2021). DMSO demonstrates the potential to dissolve BREO in DI water or media. The preparation process involves mixing the substance with a vortex mixer for up to 30 seconds before transferring it into containers, such as 96-well plates or test tubes.

To obtain the essential oil of *B. rotunda*, hydrodistillation using a Clevenger-type apparatus was performed (Harborne, 1984). The process began by drying 200g of *B. rotunda* rhizomes in a hot air oven at 60°C for 48 h. Afterward, the dried samples of these plant rhizomes were extracted using hydrodistillation with 500 ml of distilled water for 4 h.

Upon obtaining a solution containing essential oil and distilled water, anhydrous sodium sulfate was used to dehydrate the distilled water. The essential oil of *B. rotunda* was then stored at 4 °C until it was utilized.

3.2.2 Analysis of chemical constituents of the essential oil

To identify the constituents of BREO, GC-MS was performed following the method described by (Adams, 2007) with slight modifications.

In this method, a Bruker 450 gas chromatograph, Bruker 320 mass-selective detector, and the Rtx-5MS fused silica capillary column (30 m length × 0.25 mm diameter x 0.25 µm film thickness) were used to analyze the essential oil in BREO.

The column was set at a temperature of 40°C for 2 minutes and then raised to 220°C at a rate of 30°C/min, followed by a 3-minute hold. A total of 1 L of carrier gas, helium, was injected into the column.

To identify the constituents of BREO, the chromatogram, which contains several peaks with specific retention times, was compared to the retention time of standard chemicals.

3.2.3 MIC determinations

MIC determination was employed to determine the antibacterial activity and sensitivity of bacteria to antibiotics. This technique was performed according to the protocol outlined by the Clinical Laboratory Standards Institute (CLSI) (CLSI, 2021).

Briefly, the MIC assay utilized serial dilutions (2-fold dilution) of cloxacillin (CLX) or BREO, along with 5% dimethyl sulfoxide (DMSO), in a 96-well plate containing Cation-Adjusted Mueller–Hinton broth (CA-MHB).

The quantity of MRSA strains was adjusted using spectrophotometry, where the bacterial inoculum quantity depended on the optical density (OD) value. The OD value of these strains was adjusted to 10^8 CFU/mL by comparing it to the OD value obtained from the standard growth curve of MRSA strains. Subsequently, the bacterial inoculum was diluted in normal saline to a concentration of 5×10^6 CFU/mL. The bacterial inoculum was then added to the wells of the 96-well plate, resulting in a final concentration of 5×10^5 CFU/mL.

The bacterial cells were incubated at 37°C for 18 h. Following the incubation period, the MIC value was determined as the lowest concentration of the antibacterial agents in a well where no turbidity or visible bacterial growth was observed, indicating the inhibition of bacterial growth.

Finally, the MIC of the antibiotic was compared to the MIC breakpoint defined by the CLSI guideline to determine the sensitivity of MRSA to antibiotics. The interpretation categories used are as follows: sensitive, intermediate, and resistant.

3.2.4 Chequerboard method

The chequerboard assay was used to determine drug interactions, which can be classified into synergism, additive, and antagonism, as described by Odds (2003) (Odds, 2003).

In this study, BREO and CLX were employed to assess drug interactions using the chequerboard assay. The assay was conducted in a 96-well plate, using a method quite similar to MIC determination.

However, instead of determining the minimum inhibitory concentration (MIC), this assay determined the combined concentration of BREO and CLX required to inhibit bacterial growth.

The drug interaction was evaluated after incubation at 37°C for 18 h. This evaluation was interpreted based on the fractional inhibitory concentration (FIC) value, which can be calculated using the equation mentioned below.

$$\text{FIC index} = FIC_A + FIC_B = \frac{\text{Conc. of A in MICs of A+B}}{\text{MIC of A alone}} + \frac{\text{Conc. of B in MICs of A+B}}{\text{MIC of B alone}}$$

The chequerboard assay was used to determine drug interactions, which can be classified into synergism, no interaction, and antagonism based on the fractional inhibitory concentration index (FICI) value. When the FICI value is ≤ 0.5, it indicates synergism between the drugs. A value ranging from > 0.5 to 4.0 indicates no interaction. However, when the FICI value is > 4.0, it indicates antagonism.

3.2.5 Time-kill assays

The time-kill assay was used in this study to evaluate and confirm the combination effect. Following the protocol described by Teethaisong et al. (2018) with slight modification, the bacterial inoculum was prepared with a concentration of 5 x 10^6 CFU/mL.

It was then treated with BREO, CLX, and BREO plus CLX for different incubation periods, including 0, 2, 4, 6, 8, and 24 h. After incubation, 10-fold dilutions were performed, creating aliquots with various dilutions such as 10^0, 10^{-1}, and 10^{-2} dilution factors. These aliquots were plated on Mueller Hinton agar (MHA) and incubated at 37°C for 18 h (Teethaisong et al., 2018).

Using the Miles and Misra method, plate counts were performed on agar plates with 3-50 colonies described by Hedges (2002) and Naghili et al. (2013) (Hedges, 2002; Naghili et al., 2013), which is a precise alternative to the traditional method. The number of colonies obtained from the plate counts was used to create time-kill curves, showing the relationship between the bacterial concentration (CFU/mL) and the incubation time. In addition, CFU/mL was calculated using the following formula.

$$\text{CFU/mL} = \text{Average number of colonies in each dilution} \times 100 \times \text{Dilution factor}$$

The time-kill assay is a method used to determine the antibacterial effect, which can be categorized into bacteriostatic and bactericidal effects based on $\log_{10}$

reduction values. The bacteriostatic effect is defined as a reduction of ≤ 3 $\log_{10}$ CFU/mL. On the other hand, the bactericidal effect is defined as a reduction of ≥ 3 $\log_{10}$ CFU/mL when comparing the CFU/mL after 24 h of incubation to the initial inoculum (Barry, 1999).

Moreover, the synergistic effect is described as a reduction of ≥ 2 log10 CFU/mL when comparing the CFU/mL of the combined drug to the most effective individual drug or compound after 24 h (Noel et al., 2021).

3.2.6 The cytoplasmic membrane (CM) permeability

The CM permeability experiment was performed to investigate the effects of BREO, both alone and in combination with CLX, on the cytoplasmic membranes of MRSA strains. This experiment was conducted in triplicate following the procedure described by Siriwong et al. (2015) (Siriwong et al., 2015) with some modifications.

CM alteration was evaluated based on the changes in the OD_{260} intensity of absorbing materials in bacterial cells. Evaluation of OD_{260} was performed using a UV-VIS spectrophotometer.

Briefly, after preparing the MRSA, it was collected and adjusted to a concentration of 5×10^6 CFU/mL in normal saline. Then, 5 mL of the adjusted inoculum was added to 45 mL of CAMHB supplemented with BREO, CLX alone at half MIC, or BREO plus CLX at FIC concentrations. A flask without any antibacterial agent served as the negative control, while a flask containing nisin was used as the positive control since it can enhance CM permeability, particularly in gram-positive bacteria.

These adjusted inoculums were incubated at 37°C in a shaking incubator. The OD_{260} values were then evaluated at different time intervals, including 0, 1, 2, 3, and 4 h. The change in OD_{260} values indicates the leakage of intracellular components, particularly DNA and RNA. These components consist of nitrogenous bases, including purine and pyrimidine bases, which exhibit maximum absorbance at a wavelength of 260 nm (Blanco and Blanco, 2022). This leakage suggests damage to the CM (Paul et al., 2011).

3.2.7 Biofilm formation inhibition

The biofilm formation inhibition assay was performed following the method described by He et al. (2022) (He et al., 2022) with minor adjustments.

Briefly, the bacterial inoculum was incubated at 37°C for 18 h and then adjusted to 5 x 10^6 CFU/mL in normal saline. 20 µL of adjusted inoculum was added to a 96-well plate containing 180 µL of CAMHB supplemented with 0.2% glucose, BREO, and CLX at a concentration of half the MIC, as well as BREO combined with CLX at the FIC concentration. These treatments were then incubated at 37°C for 48 h.

Afterward, CAMHB was removed from the 96-well plate, and distilled water was added to each well containing adherent cells. These cells were stained with 0.4% (w/v) crystal violet. After 30 minutes of being stained with crystal violet dye, the adherent cells were washed with distilled water and allowed to dry in the air at room temperature.

These dried cells were then washed with 100% ethanol. The solutions containing ethanol and bacterial cells, which were stained with crystal violet dye, were measured for OD at 595 nm (OD_{595}) using a microplate reader.

To evaluate the inhibition of biofilm formation, the percentage of inhibition was calculated using the formula mentioned in the study by Gómez-Sequeda et al. (2020) (Gómez-Sequeda et al., 2020).

3.2.8 Scanning electron microscopy (SEM)

The SEM was employed to evaluate the morphological changes of bacterial cells. The sample preparation in this experiment was slightly modified based on the procedure conducted by Hartmann et al. (2010).

MRSA DMST 20651 was incubated in CAMHB at 37°C for 18 h, and the final concentration was adjusted to 5 × 10^5 CFU/mL in normal saline. In this experiment, the treatment groups included exposing MRSA DMST 20651 to a concentration of half-MIC of CLX and BREO, as well as the FIC of CLX plus BREO groups at 37°C for 4 h. CAMHB containing only MRSA without antibiotics was chosen as the positive control.

After 4 h of incubation, all treatment groups and positive control groups were fixed with 2% glutaraldehyde supplemented with 0.15 M sodium phosphate buffer (pH 7.2). They were then resuspended in distilled water and stored at 4°C.

Next, these samples were fixed with 0.5% osmium tetroxide (OsO_4) for 2 h and dehydrated using an acetone solution with graded concentrations of 20%, 40%, 60%, 80%, and 100%. After the dehydration process, the samples were air-dried.

Afterward, the dried samples were mounted on a carbon stub and sputtered with gold. The images of these samples on the stub were investigated under SEM (Hartmann et al., 2010).

3.2.9 Transmission electron microscopy (TEM)

The TEM was performed to investigate the alteration of bacterial cells through cross-sectional images with higher magnification compared to SEM. The sample preparation procedure was conducted with some modifications based on the study of (Richards et al., 1993).

MRSA was incubated at 37°C for 18 h, and then the concentration was adjusted to 5×10^5 CFU/mL. These suspensions were exposed to BREO and CLX at a half-MIC concentration, as well as BREO plus CLX at the FIC concentration, and incubated at 37°C for 3 h in a shaking incubator set at a speed of 110 rpm.

After MRSA was exposed to BREO, CLX, and BREO plus CLX, the suspension in each treatment was centrifuged at 6000 × g at 4°C for 15 minutes. The supernatant was then removed, and the bacterial pellets were fixed for 12 h with 2.5% glutaraldehyde supplemented with 0.1 M phosphate buffer at pH 7.2. The MRSA cells in each treatment were washed twice in 0.1% phosphate buffer and fixed with 1% OsO_4 for 2 h at room temperature.

After OsO_4 fixation, the dehydration procedure was performed using an acetone solution with graded concentrations of 20%, 40%, 60%, 80%, and 100%, respectively. Each concentration was applied for 15 minutes to dehydrate the samples.

Then, the dried samples were embedded in epoxy resin and counterstained with 2% (w/v) uranyl acetate and 0.25% (w/v) lead citrate for 3 minutes and 2 minutes, respectively. The TEM images were captured using an 80kV TEM, and the cell area was calculated from these images using the equation: cell area = cell width x cell length (nm^2).

In this experiment, the positive control is MRSA DMST 20651, which was incubated in antibiotic-free CAMHB.

3.2.10 Statistical analysis

The IBM SPSS Statistics 22 was chosen to analyze the statistical data. The data was presented in the form of the mean ± standard error of the mean (S.E.M). One-way

analysis of variance (ANOVA) with a post hoc Tukey's HSD (Honestly Significant Difference) test, which was conducted at a P-value < 0.01, was used to determine significant differences in the data. These differences were observed in the data of CM permeability, biofilm formation, and cell area determination.

CHAPTER IV
RESULT AND DISCUSSION

4.1 GC-MS analysis

The GC-MS analysis was performed to identify the constituents of BREO, and it revealed that BREO contains 24 compounds that were present in the database of standard compounds. Among these compounds, β-ocimene (36.73%), trans-geraniol (25.29%), camphor (14.98%), and eucalyptol (8.99%) were considered the main constituents of BREO (Table 1).

Table 4.1 The result of the chemical composition of BREO.

Retention time (RT) (min)	Peak name	% Area
9.502	Tricyclene	0.16
9.872	alpha-Thujene	0.01
10.164	alpha-Pinene	0.65
10.969	Camphene	4.45
12.565	beta-Pinene	0.11
13.745	beta-Myrcene	1.00
14.380	alpha-Phellandrene	0.04
15.180	alpha-Terpinene	0.05
16.113	Eucalyptol	8.99
16.877	trans-beta-Ocimene	3.92
17.783	beta-Ocimene	36.73
18.108	gamma-Terpinene	0.06
19.987	Terpinolene	0.15
21.166	beta-Linalool	1.01

Table 4.1 The result of the chemical composition of BREO (Continued).

Retention time (RT) (min)	Peak name	% Area
23.807	Camphor	14.98
24.000	Camphene hydrate	0.36
24.665	Isoborneol	0.04
25.320	endo-Borneol	0.28
26.091	4-Terpineol	0.13
27.126	alpha-Terpineol	0.43
32.157	trans-Geraniol	25.29
32.716	alpha-Citral	0.15
39.831	Methyl cinnamate	1.01
51.294	Caryophyllene oxide	0.01
Total		100.00

4.2 MIC determinations

The minimum inhibitory concentration (MIC) determinations were conducted to assess the sensitivity of MRSA strains to CLX and BREO.

The results showed that all MRSA strains, including MRSA DMST 20649, 20651, and 20652, were resistant to CLX, with an MIC of 512 µg/mL.

However, *S. aureus* ATTC 29213 was susceptible to CLX. Regarding BREO, there is no available data from sensitivity tests conducted following CLSI guidelines for this essential oil. The MIC for BREO was found to be 4 mg/mL (Table 4.2).

Table 4.2 The result of MIC determinations of BREO and CLX in the treatment of *S. aureus* strains.

Bacterial strains	Minimum inhibitory concentrations (MICs)	
	CLX (μg/mL)	BREO (mg/mL)
MRSA DMST 20649	512^{R}	4^{ND}
MRSA DMST 20651	512^{R}	4^{ND}
MRSA DMST 20652	512^{R}	4^{ND}
S. aureus ATTC 29213*	0.125^{S}	2^{ND}

*A reference strain, ^{R} = resistant, ^{S} = susceptible, and ND = no data available.

Several studies suggest that *B. rotunda* exhibits potential in combating infections caused by antibiotic-resistant bacteria, such as MRSA. This plant demonstrates a synergistic effect when used in combination with conventional antibiotics, such as penicillin. This effect depends on the presence of a variety of secondary metabolites, including flavonoids, alkaloids, and essential oils (Eng-Chong et al., 2012; Teethaisong et al., 2018).

The GC-MS analysis revealed the presence of active compounds in abundant quantities within the essential oil of *B. rotunda*, including β-ocimene, trans-geraniol, camphor, and eucalyptol. These constituents have been reported for their efficacy in exhibiting antibacterial properties against various bacterial infections caused by both gram-positive and gram-negative bacteria.

The antibacterial activity of essential oils obtained from citrus leaves, such as *Citrus sinensis*, *C. grandis*, and *C. aurantifolia*, was observed against *S. aureus*, *B. cereus*, and *S. typhimurium*. This activity may be attributed to the presence of β-ocimene as a major constituent in these essential oils. The inhibition zones ranged from 20.1 ± 0.1 to 24.3 ± 0.1 mm, while the MIC values ranged from 5.25 to 21 mg/mL. In addition, *C. sinensis*, which consists of the highest content of β-ocimene compared to other citrus species, exhibited the highest anti-MRSA activity with a zone of inhibition of 23.2±0.2 mm and MIC of 5.25 mg/mL. These findings were reported by (Chi et al., 2020).

The study conducted by Jaradat et al. (2016) indicated that the essential oil

extracted from *T. bovei*, which contains a high content of trans-geraniol, revealed MIC values of 0.25 mg/mL and 0.5 mg/mL against *S. aureus* and *E. coli*, respectively (Jaradat et al., 2016).

Furthermore, the camphor oil obtained from *Cinnamomum camphora* showed antibacterial activity against Gram-positive bacteria, including *Streptococcus mutans* and *E. faecalis* (Rahman et al., 2016). Similarly, the essential oil extracted from the genus Artemisia, which consists of a rich content of camphor, exhibited antibacterial activity against *S. aureus* with a zone of inhibition ranging from 10 ± 0.0 to 25 ± 1.4 mm. In comparison, after treatment with methicillin and vancomycin, the zone of inhibition of these drugs against *S. aureus* strains was in the range of 8 ± 0.5 and 18 ± 1.0 mm, respectively (Lopes-Lutz et al., 2008).

Hamad Al-Mijalli et al. (2022) found that the essential oil extracted from *L. multifida* (LMEO) exhibited antibacterial activity with MIC values ranging between 0.78 to 1.56 mg/mL against *S. aureus*, *L. monocytogenes*, *B. subtilis*, and *E. coli*. This effect might be attributed to eucalyptol, which is one of the main components of LMEO (Hamad Al-Mijalli et al., 2022).

According to previous studies mentioned earlier, the antibacterial activity of BREO with the MIC of 4 mg/mL may be due to the antibacterial properties of its main constituents, including β-ocimene, trans-geraniol, camphor, and eucalyptol.

4.3 The chequerboard method

The drug interaction between BREO and CLX was evaluated using the chequerboard method, which demonstrated that when combined, BREO and CLX exhibited a synergistic effect against all MRSA strains, including MRSA DMST 20649, 20651, and 20652. The FIC index was < 5, with values of 0.28, 0.28, and 0.31, respectively. Furthermore, at the concentration represented by the FIC index, the combination of BREO and CLX showed lower values compared to the concentrations at MIC (Table 4.3).

Table 4.3 The results of the FIC index using the chequerboard assay of BREO and CLX in the treatment of *S. aureus* strains.

Bacterial Strains	MIC(a)		MIC(c)		FIC		FICI
	BREO (mg/mL)	CLX (µg/mL)	BREO (mg/mL)	CLX (µg/mL)	BREO	CLX	
MRSA DMST 20649	4^{ND}	512^{R}	1^{ND}	16^{R}	0.25	0.03	0.28^{*}
MRSA DMST 20651	4^{ND}	512^{R}	1^{ND}	16^{R}	0.25	0.03	0.28^{*}
MRSA DMST 20652	4^{ND}	512^{R}	1^{ND}	32^{R}	0.25	0.06	0.31^{*}

The symbol * refers to synergism, with the presence of FIC ≤ 0.5, where MIC(a) = MIC of an individual compound alone and MIC(c) = MIC of an individual compound in combination. The presence of the symbol related to the MIC criteria, including resistance (R), susceptibility (S), and no data available (ND).

According to the MIC results of BREO and CLX, both show low antibacterial activity due to MIC values greater than 1000 µg/mL. However, when these compounds are combined, they exhibit a synergistic effect with higher antibacterial activity and lower individual compound doses in the combination compared to using each compound alone. The synergistic effect has been observed in several previous studies, as described below.

Lira et al. (2020) reported a synergistic effect (FIC < 0.5) when combining geraniol and norfloxacin in the treatment of *B. cereus* and *S. aureus*. Similarly, this secondary metabolite exhibited a synergistic effect when combined with chloramphenicol against *E. coli*, *K. pneumoniae*, *Proteus mirabilis*, and *P. aeruginosa* (Lira et al., 2020).

Bekka-Hadji et al. (2022) suggested that the essential oil extracted from *A. herba* alba, which consists of abundant camphor, demonstrates a synergistic effect when combined with cephalosporins in the treatment of *S. aureus* (Bekka-Hadji et al., 2022).

Additionally, a synergistic effect was observed when combining 1 mg/mL of eucalyptol with 0.05 mg/mL of cinnamaldehyde, as well as 0.1 mg/mL of cinnamaldehyde against *L. innocua* (Requena et al., 2019).

Based on previous studies, the primary constituents of BREO such as geraniol, camphor, and eucalyptol, which are found in other sources, exhibit synergistic effects when combined with conventional antibiotics. This indicates that the synergistic effects of BREO, in combination with CLX, can be attributed to these constituents.

4.4 Time kill assay

The time-kill assay was performed to assess the antibacterial effects of compounds on bacteria, distinguishing between bacteriostatic (inhibiting bacterial growth) and bactericidal (killing bacteria) effects. In addition, this assay was used to confirm the result of drug interaction, which can be classified as synergistic, additive, or antagonistic based on Odd's criteria (Odds, 2003).

Figure 4.1 demonstrates the cell viability of MRSA DMST 20651 after exposure to BREO and CLX individually at a half-MIC concentration, and BREO plus CLX at an FIC concentration. The results indicate that the untreated control group displayed normal growth during the 0 to 24-h incubation, with no decrease in CFU/mL. The growth of MRSA DMST 20651 showed a slight decrease between 0 to 6 h after treatment with 2 mg/mL of BREO and 256 µg/mL of CLX individually. However, from 6 to 24 h, there was an increase in bacterial growth due to the increase of CFU/mL during this time interval.

However, when MRSA DMST 20651 was treated with a combination of 1 mg/mL of BREO and 16 µg/mL of CLX, the growth of this bacteria showed a slight decrease from 0 to 4 h and a dramatic decrease from 4 h until 24 h of incubation.

According to the drug interaction criteria, the combination of BREO and CLX group displayed a reduction > 2 $\log_{10}$ CFU/mL compared to the treatment of BREO at 24 h of incubation. This result indicates that BREO exhibits a synergistic effect when

combined with CLX against MRSA DMST 20651. Moreover, when considering the effect on inhibiting or killing bacteria, this combination also showed < 3 $\log_{10}$ reductions when compared to the initial CFU/mL at 0 h of incubation. This result suggests that BREO plus CLX exhibits a bacteriostatic effect against this MRSA strain.

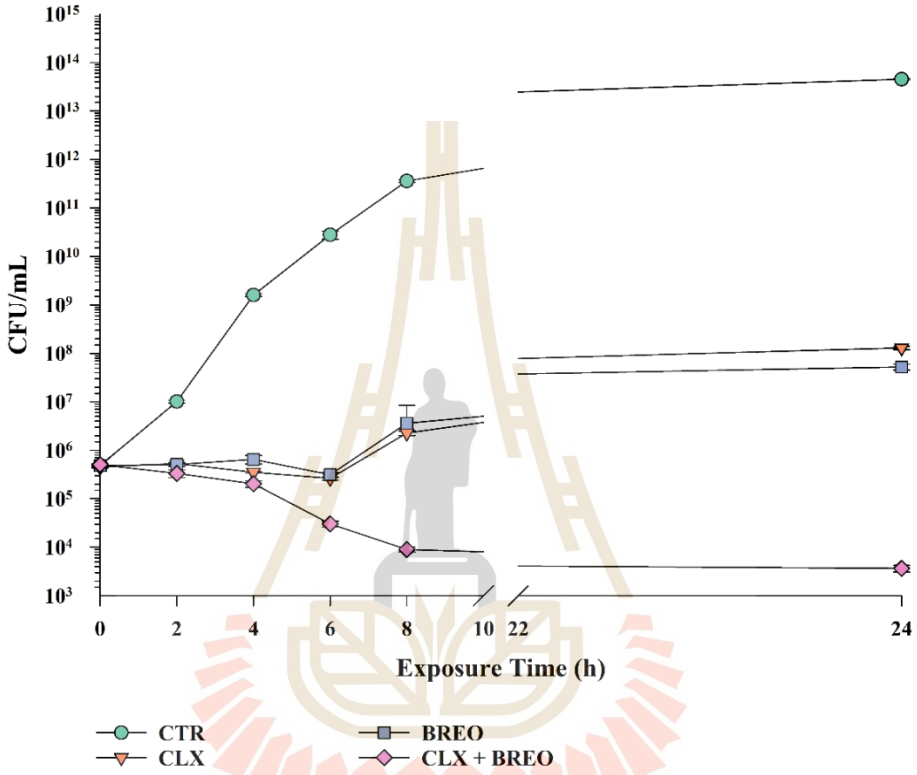

Figure 4.1 The results of the time-kill assay, the viability of MRSA DMST 20651 cells (CFU/mL) was plotted on the time-kill curve after exposure to BREO, CLX, and the combination of BREO and CLX. The treatment groups include CTR (control), CLX (256 µg/mL of CLX), BREO (2 mg/mL of BREO), and CLX + BREO (combination of 16 µg/mL of CLX and 1 mg/mL of BREO). The results were presented as the mean and standard error of the mean (SEM) from triplicate experiments.

The concentration used in the treatment groups was set at half of the minimum inhibitory concentration (MIC), which was found to be ineffective in inhibiting bacterial growth. This selection was due to the concentration at FIC of each compound in combination, which was frequently found below a half-MIC observed when the

compounds were used individually.

In this experiment, the combination of BREO and CLX demonstrated a synergistic effect, with a reduction in CFU/mL by >2 $\log_{10}$ CFU/mL after 24 h of incubation compared to BREO, which was the most effective compound. These results support a previous study where the individual compound concentrations in the combination were below half of the MIC of each compound when used individually (Siriwong et al., 2015). Furthermore, these results also support the findings of the chequerboard assay, which showed a synergistic effect when BREO was combined with CLX.

However, to acquire a more comprehensive understanding of the synergistic effect of this combination, investigations into their possible modes of action were conducted. These modes of action can verify that the synergistic effect is influenced by the distinct modes of action of BREO and CLX. The MRSA DMST 20651 strain was selected as the treated strain for investigating modes of action based on the lowest FIC value. This strain is known for its resistance mechanisms, which may be related to both the production of β-lactamase and the modification of the cell wall, as described in a previous study. (Teethaisong et al., 2018).

4.5 CM permeability

The CM permeability test was conducted by evaluating OD260 measurements for four treatment groups, including BREO, CLX, BREO plus CLX, and nisin (NIS), in comparison to the control (CTR) group. These OD260 values were plotted on a graph to demonstrate the relationship between OD_{260} and exposure time, as seen in Figure 4.2.

The results clearly demonstrate that the OD_{260} of CLX did not exhibit significant differences when compared to the OD_{260} of the CTR group. However, the OD_{260} values of the BREO, BREO plus CLX, and NIS groups were significantly higher than those of the CTR group after 1 h until 4 h of incubation ($p < 0.01$).

When comparing the BREO, BREO plus CLX, and NIS groups, no significant differences were found in OD260 values between the BREO plus CLX and NIS groups at all exposure times, except at the 3 h of exposure. However, significant differences

in OD_{260} values were observed at all exposure times when comparing the BREO and NIS groups.

The significant increase in OD_{260} compared to the untreated control group indicates an increase in CM permeability due to the leakage of cellular components of bacteria, especially DNA and RNA, which are UV-absorbing materials at the 260 nm wavelength.

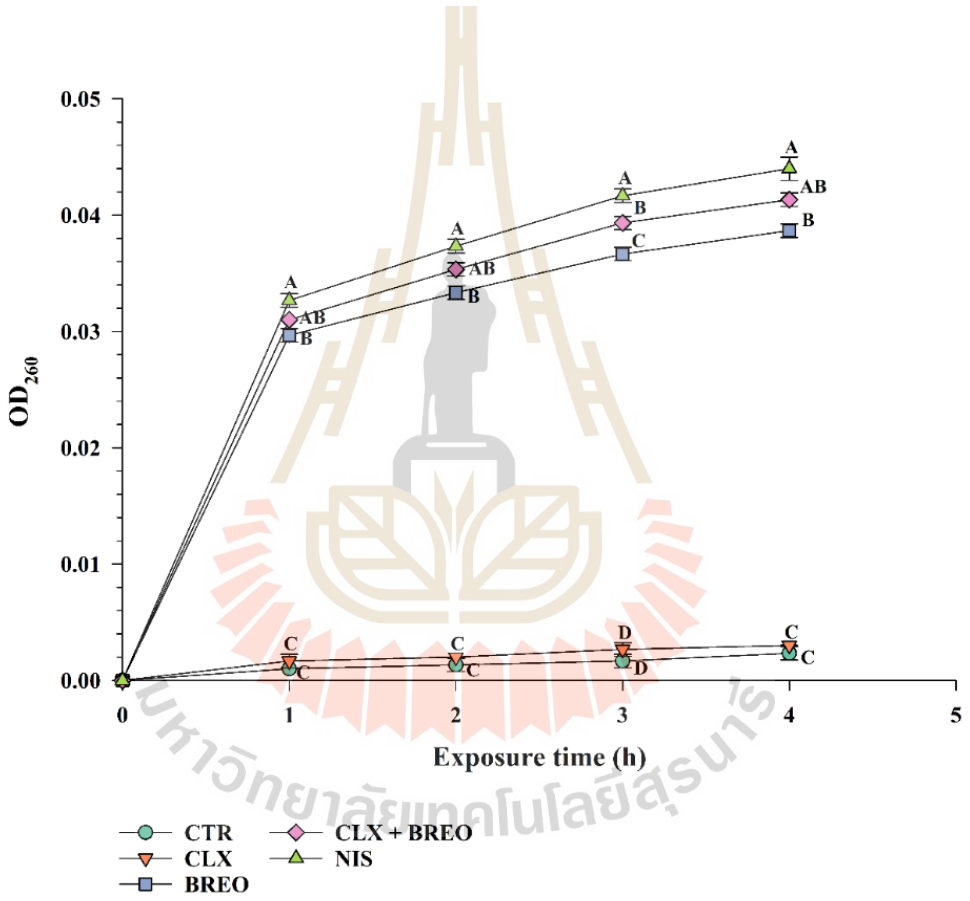

Figure 4.2 The results of the CM permeability assay, which presents the values of OD_{260} for the absorbing material after exposure to BREO, CLX, nisin (NIS), and the combination of BREO and CLX. The treatment groups include CTR (control), CLX (256 µg/mL of CLX), BREO (2 mg/mL of BREO), NIS (8 µg/mL of nisin), and CLX + BREO (16 µg/mL of CLX combined with 1 mg/mL of BREO). The results were presented as the mean and standard error of the mean (SEM) from triplicate experiments. The significant differences between groups were determined using one-way ANOVA and Tukey's HSD

Post-hoc tests, which describe the significant differences with different alphabetic symbols ($p < 0.01$).

According to the study conducted by Asker et al. (2020), it has been suggested that the alteration in CM permeability of *S. aureus* and *P. aeruginosa* is attributed to the mode of action of β-ocimene. This compound was found to enhance the inhibition of lipid synthesis in these bacteria. The degree of inhibition depends on β-ocimene concentration (Asker et al., 2020).

Tang et al. (2020) found that camphor can increase CM permeability, leading to the leakage of bacterial DNA and RNA. This compound is the main constituent of *Amomum villosum* Lour, comprising 20.94% of its quantity (Tang et al., 2020).

In addition, there was evidence reported by Lira et al. (2020) that geraniol plays a role in the increase of CM permeability, and the mode of action of this compound involves its adherence to the bacterial lipid membrane (Lira et al., 2020).

In our study, nisin was used as a positive control since it has been found in several previous studies (Cheypratub et al., 2018; Teethaisong et al., 2018), demonstrating its ability to affect the permeability of the CM and leading to CM damage. Furthermore, nisin has been considered as an alternative in combating MRSA infections (Shin et al., 2016).

According to the GC-MS result, β-ocimene, camphor, and geraniol were identified as the main constituents of BREO, and these compounds demonstrated the ability to alter CM permeability in several bacteria. Furthermore, when used alone or in combination with CLX, BREO increased CM permeability, indicating that this ability can be attributed to these compounds. Although BREO revealed a lower OD260 intensity compared to nisin when used alone, the combination of BREO and CLX showed no significant difference compared to nisin. This indicates that the main components of BREO, such as β-ocimene, camphor, and geraniol, can synergistically interact with CLX against MRSA DMST 20651.

4.6 Biofilm formation inhibition

The amount of biofilm formation was measured by comparing the percentage of inhibition (% inhibition) calculated from OD_{595} values, as shown in Figure 4.3. The results clearly demonstrated that the % inhibition values of the treatment groups, including BREO, CLX, and BREO plus CLX groups, were significantly different from the control group ($p < 0.01$). In addition, there were significant differences among all the treatment groups ($p < 0.01$).

Among these groups, the BREO plus CLX group exhibited the highest % inhibition with a value of 80.25 ± 0.60%, while the CLX group showed greater % inhibition compared to the BREO groups with values of 72.61 ± 0.61% and 67.58 ± 0.67%, respectively.

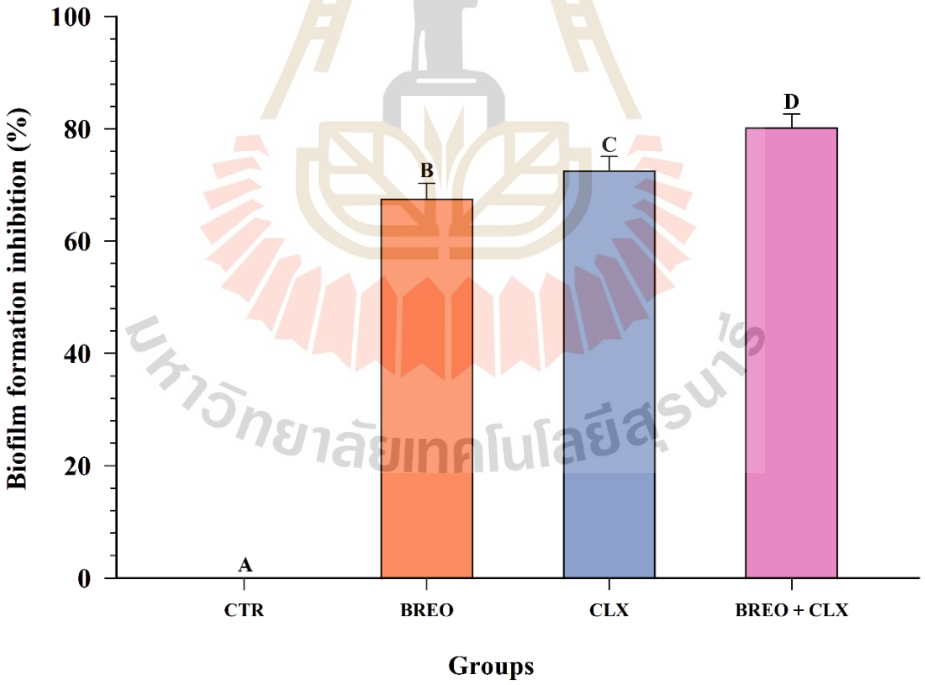

Figure 4.3 The results of the biofilm formation inhibition assay for BREO, CLX, and the combination of BREO and CLX in the treatment of MRSA DMST 20651. The treatment groups include CTR (control, n = 6), CLX (256 µg/mL of CLX, n = 6), BREO (2 mg/mL of

BREO, n = 6), and CLX + BREO (16 µg/mL of CLX combined with 1 mg/mL of BREO, n = 6). The results were presented as the mean and standard error of the mean (SEM). The significant differences between groups were determined using one-way ANOVA and Tukey's HSD Post-hoc tests, which describe the significant differences with different alphabetic symbols ($p < 0.01$).

Jain and Parihar (2018) suggested that geraniol can inhibit the biofilm formation of *S. aureus* with a percentage of inhibition of 86.13 ± 5.22. Meanwhile, camphor demonstrates 81.25 ± 1.63% inhibition against another strain of this bacteria (Jain and Parihar, 2018).

The study conducted by Vijayakumar et al. (2020) demonstrated that eucalyptol inhibited biofilm formation in *Streptococcus pyogenes* by 89% at 300 µg/mL. This compound also exhibited concentration-dependent inhibition against the biofilm-forming bacteria (Vijayakumar et al., 2020).

In our work, BREO demonstrated good biofilm formation inhibition with greater than 50% inhibition, following the criteria reported by Adeyemo et al. (2022). This essential oil can inhibit biofilm formation when used in a single and combined with CLX against MRSA DMST 20651. This activity can be attributed to the aforementioned active compounds, which are the main components of BREO (Adeyemo et al., 2022).

Furthermore, the combination of BREO and CLX showed a potential synergistic effect, as it demonstrated a significantly higher percentage of inhibition compared to CLX alone, which was the most effective in anti-biofilm formation activity ($p < 0.01$).

4.7 SEM and TEM

The morphological changes of MRSA DMST 20651 after treatment with BREO, CLX, and BREO plus CLX were observed using SEM images. The control group (CTR) consisted of MRSA DMST cells that were not exposed to any compound treatment. Cells in the CTR group revealed typical characteristics, including a round shape with a smooth and intact cell surface (Figure 4.4a). Figure 4.4b shows MRSA cells exposed to 256 µg/mL of CLX. The results demonstrated that several cell surfaces were dented (squared-tail arrow), while some cells exhibited roughness (dotted-tail arrow) and

shrinkage (arrow). The alterations observed in Figure 4.4c, when MRSA cells were treated with 2 mg/mL of BREO, included several furrows (dotted-tail arrow), bleb-like structures (squared-tail arrow), and a large amount of debris (arrow). In Figure 4.4d, the damages of MRSA cells were observed, including dents (arrows), shrinkage (squared-tail arrows), bleb-like structures (dotted-tail arrows), and a large amount of debris (rectangle-tail arrows). These damages were observed after the cells were exposed to 1 mg/mL of BREO and 16 µg/mL of CLX.

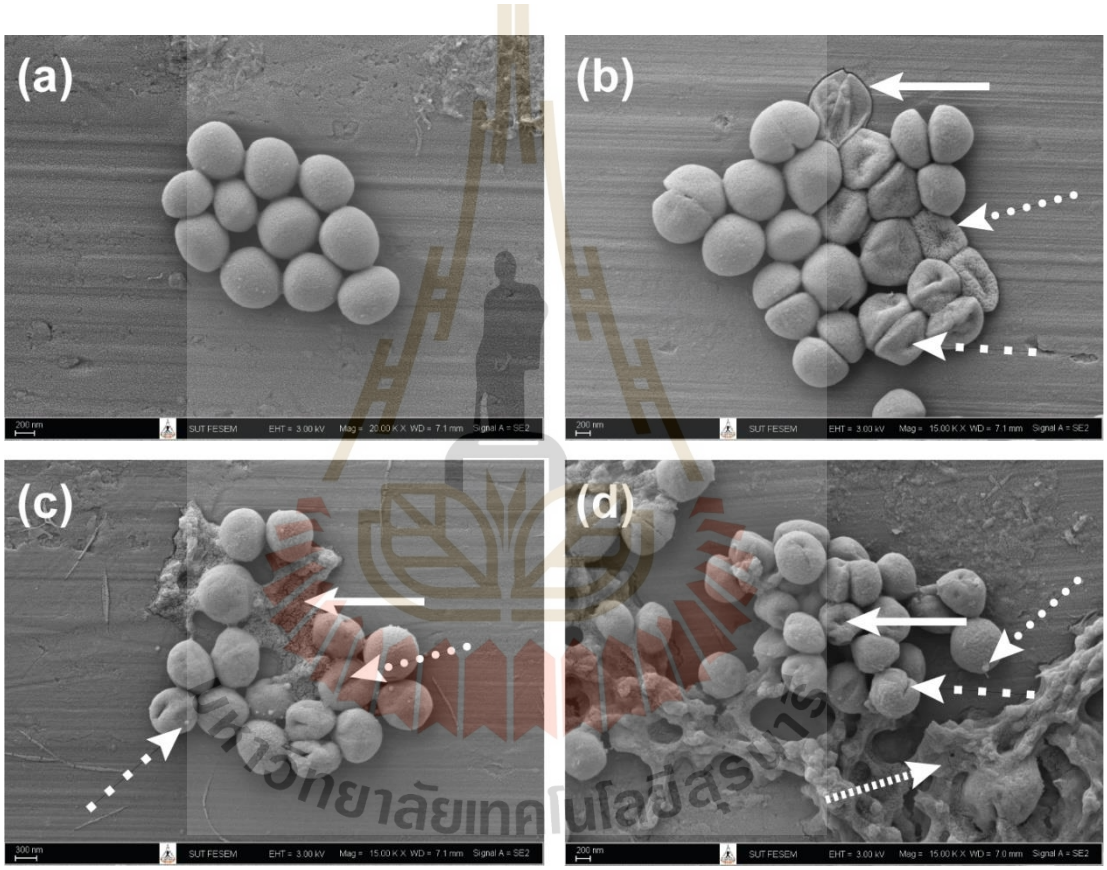

Figure 4.4 The results of SEM, presenting SEM images of MRSA DMST 20651 after treatment with BREO, CLX, and the combination of BREO and CLX. The treatment groups include CTR (untreated cells incubated in CAMHB, picture a), CLX (256 µg/mL of CLX, picture b), BREO (2 mg/mL of BREO, picture c), and BREO + CLX (1 mg/mL of BREO combined with 16 µg/mL of CLX, picture d). The scale bars and magnifications for pictures a, b, c, and d were 100 nm and 20,000x, 200 nm and 15,000x, 300 nm and 15,000x, and 200 nm and 15,000x, respectively.

TEM was employed as a method to observe damage in MRSA DMST cells. The samples were categorized into four groups, including CTR (untreated control), BREO (2 mg/mL), CLX (256 µg/mL), and a combination of BREO (1 mg/mL) with CLX (16 µg/mL). By using TEM, cross-sectional images of these samples were obtained, presenting the results of this study.

Figure 4.5a demonstrates the untreated cells used as the control group. These cells exhibited typical characteristics, such as a rounded shape with a distinct cell wall, cytoplasmic membrane, and cytoplasm, that were clearly distinguishable among these components.

After treating MRSA cells with 256 µg/mL of CLX, changes in both cell wall and cytoplasmic membrane were observed (arrow), resulting in deformed cell shapes compared to the untreated control group. These cellular changes have been presented in Figure 4.5b.

In Figure 4.5c, bacterial cells were exposed to 2 mg/mL of BREO, resulting in changes in the cytoplasmic membrane structure, resembling a hairpin (indicated by the arrow), which indicates the loss of cytoplasmic membrane integrity.

The alterations observed in Figure 4.5d indicate changes in the cell wall, cytoplasmic membrane, and cytoplasm of MRSA DMST 20651 cells. These changes were the result of exposure to a combination of BREO and CLX at concentrations of 1 mg/mL and 16 µg/mL, respectively. In this figure, some areas of the cell wall were detached from the cytoplasm (indicated by the arrow), resulting in a leakage of cytoplasm and subsequent loss of intracellular components (indicated by the dotted-tail arrow). Additionally, at the location of the squared-tail arrow, the cytoplasmic membrane was lost. These changes led to the deformation of MRSA DMST 20651 cells, resulting in an observed amorphous shape.

In investigating the impact of BREO alone and in combination with CLX on bacterial CM permeability, increased in CM permeability were observed that is consistent with the results of the CM permeability test. These increases are attributed to the leakage of OD_{260}-absorbing molecules or nucleic acids from the bacterial cells.

To validate the effect of BREO, CLX, and BREO plus CLX on MRSA DMST 20651, the measurement of cell area (Figure 6) was investigated. In this figure, the cell area of

the control group (CTR), BREO-treated group, CLX-treated group, and BREO plus CLX-treated group were $6.18 \times 10^5 \pm 3.51 \times 10^4$ nm^2, $5.20 \times 10^5 \pm 1.07 \times 10^4$ nm^2, $5.85 \times 10^5 \pm 1.31 \times 10^4$ nm^2, and $4.34 \times 10^5 \pm 1.76 \times 10^4$ nm^2, respectively.

Statistical analysis revealed no significant differences in cell areas among the CTR, BREO, and CLX groups ($p > 0.01$). However, when comparing the BREO plus CLX-treated group to these individual treatment groups, there was a significant decrease in cell area ($p < 0.01$). This suggests that the combination treatment of BREO and CLX affects the alteration of MRSA cell areas.

The morphological changes resulting from the effects of BREO, CLX, and the combination of BREO plus CLX may be used to determine the synergistic effect. There was a significant reduction in cell area in the combination of BREO and CLX compared to the individual compounds, including BREO alone and CLX alone.

In addition, the result of SEM and TEM may be used to determine the synergistic because the combination of each individual compound exhibits a distinct mode of action, including damage on CM and cell wall that is responsible for BREO and CLX, respectively.

These damages, attributed to distinct modes of action, can also be observed in the findings of Teethaisong et al. (2018), which reported that the combination of *B. rotunda* extract (BRE) and CLX demonstrated different cell damage on MRSA DMST 20651 cells (Teethaisong et al., 2018). Furthermore, the study conducted by Siriwong et al. (2015) suggested that the combination of compounds A and B exhibited synergistic effects through different damage occurring on the bacterial cell (Siriwong et al., 2015).

The findings in our study are consistent with the results from previous studies. Therefore, BREO can demonstrate a synergistic effect when combined with CLX against MRSA DMST 20651 based on the distinct mode of action. Additionally, our findings are consistent with the hypothesis of the study that BREO and CLX can demonstrate a synergistic effect.

Moreover, the necessary 4-hour incubation period before conducting SEM and TEM analysis is crucial to prevent misleading results caused by bacterial death occurring naturally after the log phase of the MRSA growth curve. Therefore, the

damage observed in these MRSA cells could not be associated with the natural mechanism of bacterial cell death.

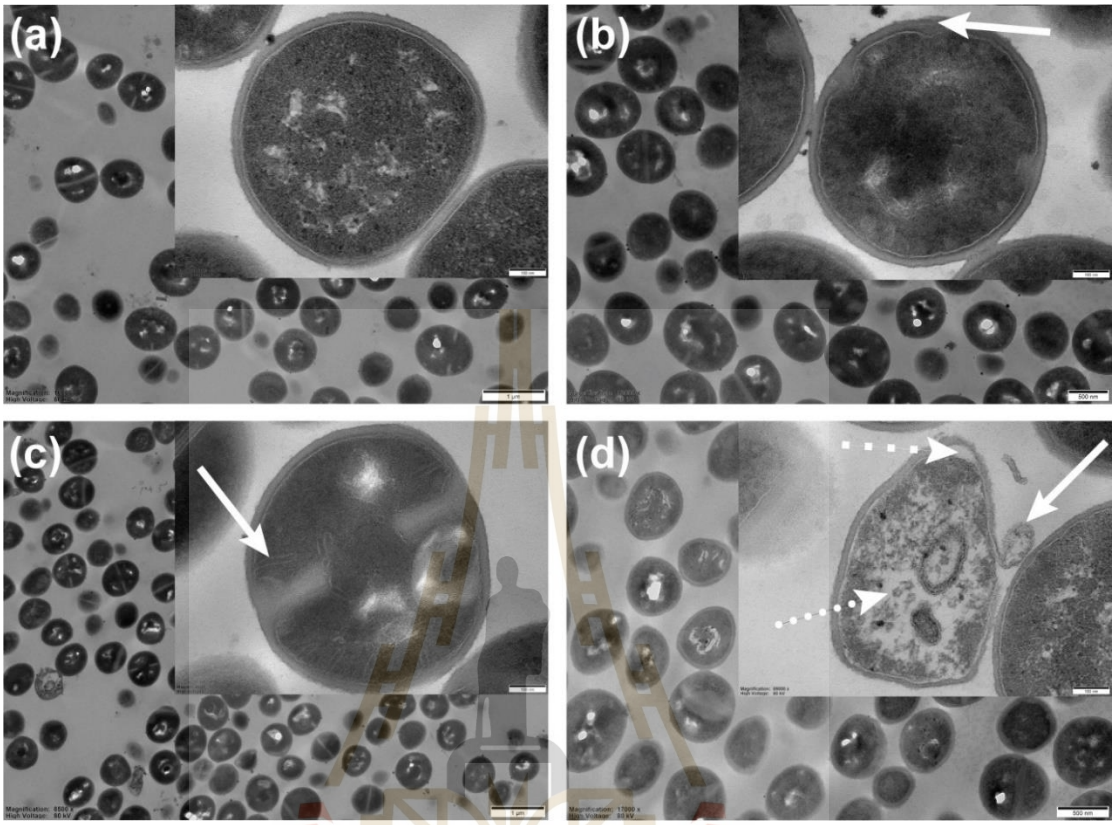

Figure 4.5 The results of TEM, presenting TEM images of MRSA DMST 20651 after treatment with BREO, CLX, and the combination of BREO and CLX. The treatment groups include CTR (untreated cells incubated in CAMHB, picture a), CLX (256 µg/mL of CLX, picture b), BREO (2 mg/mL of BREO, picture c), and BREO + CLX (1 mg/mL of BREO combined with 16 µg/mL of CLX, picture d). The scale bars and magnifications for pictures a, b, c, and d were 1 µm and 10000x, 500 nm and 13000x, 1 µm and 8500x, and 500 nm and 17000x, respectively. The scale bars and magnifications of the inset pictures in pictures a, b, c, and d were 100 nm and 89000x for all.

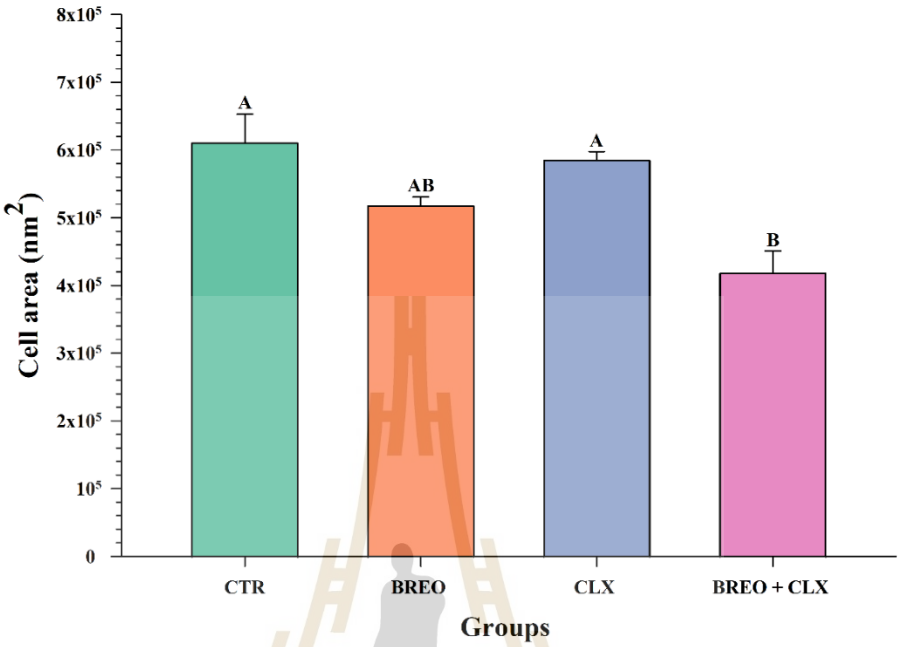

Figure 4.6 The results of the cell area of MRSA DMST 20651 after exposure to BREO, CLX, and the combination of BREO and CLX. The treatment groups include CTR (control, n = 6), CLX (256 µg/mL of CLX, n = 6), BREO (2 mg/mL of BREO, n = 6), and CLX + BREO (16 µg/mL of CLX combined with 1 mg/mL of BREO, n = 6). The cell area (nm^2) was calculated by multiplying the cell width (nm) and the cell length (nm). The results were presented as the mean and standard error of the mean (SEM). Significant differences between groups were determined using one-way ANOVA and Tukey's HSD Post-hoc tests, which are represented by different alphabetic symbols ($p < 0.01$).

CHAPTER V
CONCLUSION

In investigating the antibacterial activity of BREO against MRSA strains, this oil demonstrated moderate effectiveness, with a MIC value exceeding 1000 µg/mL. Despite this, BREO exhibited some antibacterial properties, although it was insufficient against MRSA.

Interestingly, BREO showed enhanced antibacterial activity when combined with CLX compared to each used individually. The synergistic effect observed in this combination proves promising for clinical use, especially in treating MRSA infections. To combat MRSA, the combination of BREO and CLX demonstrated at least a bacteriostatic effect, inhibiting bacterial growth.

Furthermore, BREO exhibited anti-biofilm properties. The combination of BREO and CLX showed a synergistic effect in antibiofilm activity. In this study, we investigated the mode of action of both BREO and CLX. BREO was found to increase cellular membrane (CM) permeability, while CLX affected bacterial cells by breaking down the cell wall. Both compounds demonstrated anti-biofilm formation activity.

These findings suggest a perspective on combining essential oils, including BREO, with conventional antibiotics such as CLX. This synergistic approach may be essential in solving the issue of antibiotic-resistant bacterial strains due to the multiple modes of action of each compound. This approach can increase the effectiveness of antibacterial activity and reduce the chance of developing antibiotic resistance in MRSA strains.

Research on the BREO-CLX combination highlights the potential of such approaches in the antibacterial research field, offering new insights into combating resistant strains through multifaceted interventions. However, further investigation is necessary to explore the potential adverse effects of this essential oil on human cells and ensure its suitability for developing novel innovations for human use. Cytotoxicity testing, as well as evaluations of antioxidant and anti-inflammatory activities, must be

conducted. These investigations are particularly relevant to the lipid properties of BREO, which facilitate effective absorption through the human skin composed of phospholipid bilayers. It is important to note that bacterial cell membrane (CM) components also consist of phospholipid compounds.

5.1 Conclusion findings

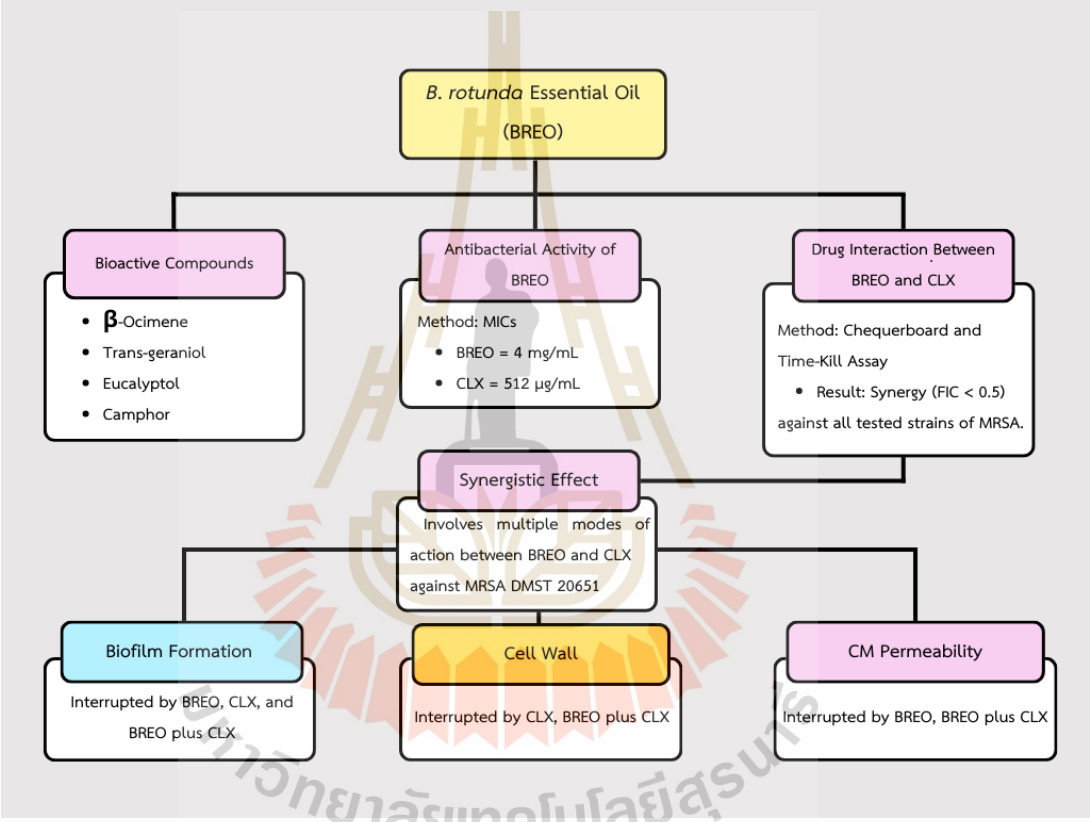

Figure 5.1 Schematic of conclusion findings.

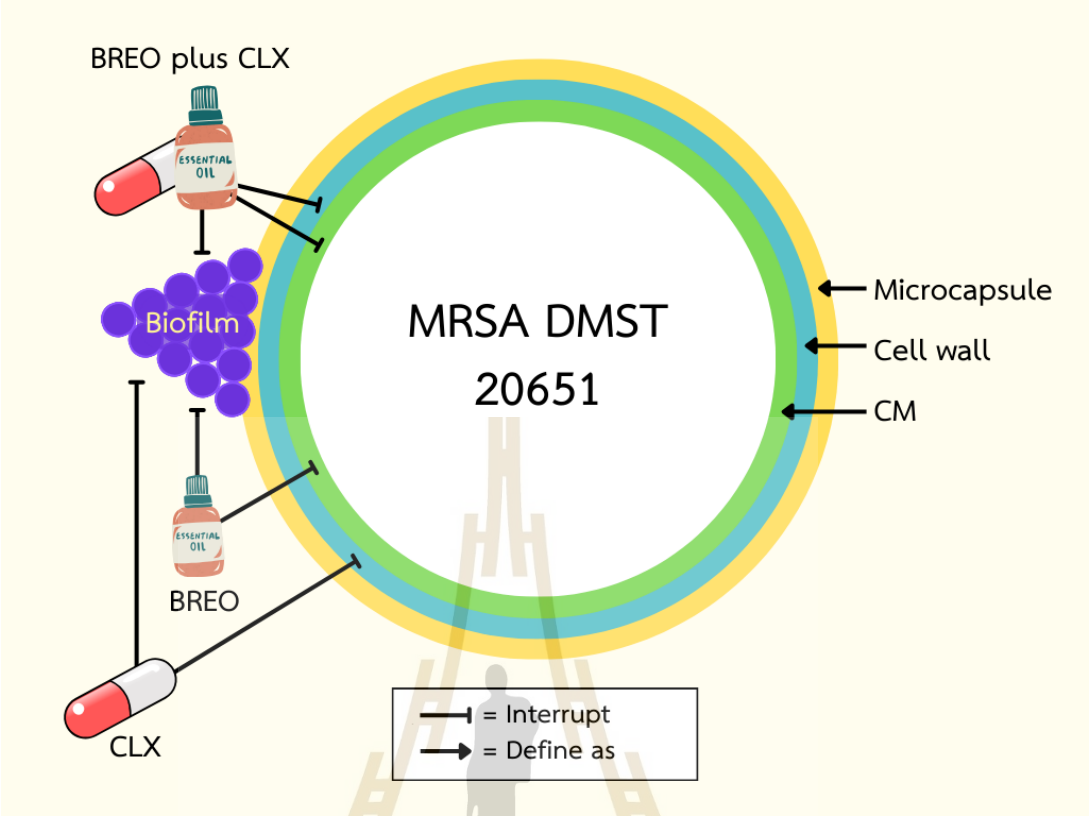

Figure 5.2 Schematic of the mode of action of BREO, CLX, and BREO plus CLX.

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APPENDICES

APPENDIX A

BACTERIAL MEDIA FORMULA

A.1 Mueller Hinton Broth No.2 Control Cations (CA-MHB)

- Composition g/L
  - Beef extract 3.000
  - Casein acid hydrolysate 17.500
  - Starch 1.500
  - Final pH (at 25°C) 7.3±0.1
- Organism
  - *Escherichia coli* ATCC 25922
  - *Pseudomonas aeruginosa* ATCC 27853
  - *Staphylococcus aureus* ATCC 25923
  - *Enterococcus faecalis* ATCC 29212

A.2 Mueller Hinton Agar (MHA)

- Composition g/L
  - HM infusion B from # 300.00
  - Acicase ## 17.500
  - Starch 1.500
  - Agar 17.000
  - Final pH (at 25°C) 7.3±0.1

****Formula adjusted, standardized to suit performance parameters**

 # - Equivalent to Beef infusion from

- Equivalent to Casein acid hydrolysate

- Organism
    - *Escherichia coli* ATCC 25922
    - *Pseudomonas aeruginosa* ATCC 27853
    - *Staphylococcus aureus* ATCC 25923
    - *Staphylococcus aureus* subsp. Aureus ATCC 43300 (MRSA)
    - *Enterococcus faecalis* ATCC 29212

Source of appendix A: https://www.himedialabs.com/us/

APPENDIX B

PUBLICATION

Apinundecha, C., Teethaisong, Y., Suknasang, S., Ayamuang, I., and Eumkeb, G. (2023). Synergistic Interaction between *Boesenbergia rotunda* (L.) Mansf. Essential Oil and Cloxacillin on Methicillin-Resistant *Staphylococcus aureus* (MRSA) Inhibition. Evidence-Based Complementary and Alternative Medicine. 2023, 3453273. (SCOPUS SJR 2022 = Q2).

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Research Article

Synergistic Interaction between *Boesenbergia rotunda* (L.) Mansf. Essential Oil and Cloxacillin on Methicillin-Resistant *Staphylococcus aureus* (MRSA) Inhibition

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Currently, antibiotic resistance is widespread among bacteria. This problem requires greater awareness because bacterial resistance increases, reducing antibiotic use effectiveness. Consequently, new alternative treatments are needed because the treatment options for these bacteria are limited. This work aims to determine the synergistic interaction and mechanism of action of *Boesenbergia rotunda* essential oil (BREO) against methicillin-resistant *Staphylococcus aureus* (MRSA). Gas chromatography-mass spectrometry identified 24 BREO chemicals (GC-MS). The main components of BREO were β-ocimene (36.73%), trans-geraniol (25.29%), camphor (14.98%), and eucalyptol (8.99%). BREO and CLX inhibited MRSA DMST 20649, 20651, and 20652 with a minimum inhibitory concentration (MIC) of 4 mg/mL and 512 μg/mL, respectively. The checkerboard method and the time-kill assay revealed synergy between BREO and CLX with fractional inhibitory concentration (FIC) <0.5 and log reduction >2log10 CFU/mL at 24 hours compared to the most effective chemical. BREO inhibited biofilm formation and increased membrane permeability. Exposure alone to BREO or in combination with CLX inhibited biofilm formation and increased cytoplasmic membrane (CM) permeability. The scanning electron microscopy (SEM) and transmission electron microscopy (TEM) results revealed that alterations in the cell walls, cytoplasmic membrane, and leakage of intracellular components of MRSA DMST 20651 after treatment with BREO alone and in combination with CLX were observed. These results indicate that BREO synergizes and could reverse the antibacterial activity of CLX against MRSA strains. The synergy of BREO may lead to novel drug combinations that increase the effectiveness of antibiotics against MRSA.

1. Introduction

Antimicrobial resistance (AMR) in bacteria has been rapidly increasing worldwide through various resistance mechanisms, resulting in the reduced antibacterial capability of conventional antibiotics to inhibit the growth of these resistant bacteria [1]. The high incidences have created health risks to patients and caregivers, and they have posed huge healthcare burdens globally. Patients with antibiotic-resistant infections have high medical expenses, extended hospital stays, and a high mortality rate compared to infections caused by susceptible bacteria [2]. The possible causes of AMR include excessive antibiotic use in humans and animals, lack of sanitation and hygiene, and discharge of antimicrobial residues toward the environment through fertilizer and animal waste [3].

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a strain of *Staphylococcus aureus*, Gram-positive commensal bacteria that have the potential to develop resistance to several subclasses of β-lactams such as penicillins, cephalosporins,

monobactams, and carbapenems. Furthermore, MRSA is resistant to other antibiotics, including fosfomycin, daptomycin, linezolid, chloramphenicol, gentamicin, tetracycline, fusidic acid, ciprofloxacin, and rifampicin. Correspondingly, this pathogen may cause various infectious diseases, such as bacteremia, pneumonia, osteomyelitis, prosthetic joint infection, and skin infection [4].

Methicillin resistance in MRSA is often associated with the PBP2a protein encoded by the *mec*A gene, resulting in the deletion and insertion of the *mec* element on the bacterial chromosome [5]. In addition, PBP2a facilitates cell wall synthesis while exposed to β-lactams due to poor β-lactam binding activity [6].

MRSA may produce biofilms on the surface of their habitats to protect themselves and survive in the environment. Biofilm is also one of the crucial protection mechanisms of bacteria that can disrupt or prevent the mechanisms of action of some antibacterial agents [7].

To cope with AMR in MRSA, which is resistant to several drugs, antimicrobials derived from plants are one of the treatment options due to the mechanism of action of secondary metabolites produced by plants to defend themselves against pathogens such as bacteria, fungi, and pests [8].

Essential oils are considered an interesting source of coping with these bacteria as they contain several bioactive constituents. Previous studies [9, 10] reported that some essential oils exhibited various pharmacological properties, including antibacterial, antifungal, and anti-inflammatory activity.

Boesenbergia rotunda (syn. *Kaempferia pandurata* Roxb or *Boesenbergia pandurata* Roxb), locally referred to in Thai as "Krachai or Krachai-Dang" and referred to in English as fingerroot, has been used as the traditional medicine in Southeast Asia due to its pharmacological properties, such as antimicrobial activities, biofilm inhibition, and antioxidant, antifungal, and anticancer activities [11]. Furthermore, this plant has also been used to combat drug-resistant bacteria, especially in the staphylococci group described by Teethaisong et al. [12].

Recently, combining conventional antibiotics with medicinal plant chemicals has become a popular strategy for reversing the antibacterial activity of failed antibiotics to treat antibiotic-resistant infections via drug interaction, which involves the effect of multiple antibacterial mechanisms. These strategies might prevent bacteria from developing novel resistance mechanisms, minimize antibiotic use while maintaining current antibiotic classes for therapeutic benefit, and mitigate undesirable effects [13].

Although there are many reports on the antimicrobial activity and synergistic interaction of *B. rotunda* against MRSA, little is available on the antimicrobial activity and synergistic interaction of the essential oil of this plant, either taken alone or in combination with conventional antibiotics, against MRSA. Therefore, this study aimed to investigate the antibacterial activity, synergistic interaction, and some mechanisms of action of BREO. Furthermore, this investigation might provide information on the therapeutic potential of BREO on MRSA inhibition, whether taken alone or in combination with conventional antibiotics such as cloxacillin.

2. Methods

2.1. Bacterial Strains and Antibacterial Agents. The Department of Medical Sciences provided MRSA isolates, including MRSA DMST 20649, 20651, and 20652. In addition, *S. aureus* ATCC 29213 was acquired from the American Type Culture Collection (ATCC) and used as the quality control strain. Sigma-Aldrich provided all antibiotics employed in this experiment, including cloxacillin (CLX) and nisin (NIS).

2.2. Plant Specimen and Essential Oil Preparation. *B. rotunda* rhizomes were obtained at the Suranakhon local market in the Mueang district of Nakhon Ratchasima province, Thailand. Dr. Santi Wattana from Suranaree University of Technology, Thailand, identified and verified the plant specimen. The Forest Herbarium of Thailand deposited a voucher specimen (BKF No. 192160). In this experiment, hydrodistillation was performed to extract the essential oils using a Clevenger-type apparatus [14]. 200 g of dried *B. rotunda* rhizomes was extracted by hydrodistillation of 500 mL of distilled water for 4 hours. The essential oil was dehydrated with anhydrous sodium sulfate and kept at 4°C.

2.3. Chemical Constituents of the Essential Oil. The constituent of BREO was determined using gas chromatography-mass spectrometry (GC-MS) following the approach described by Adams [15] with minor modifications. The essential oil was analyzed using a Bruker 450 gas chromatograph attached to a Bruker 320 mass-selective detector with the Rtx-5MS fused silica capillary column (30 m length × 0.25 mm diameter x 0.25 μm film thickness). The column oven temperature was set at 40°C for 2 minutes, increased to 220°C at 30°C/min, and held at 220°C for 3 minutes. Helium was chosen as the carrier gas and injected at 1 L. Identifying the composition of this oil requires comparing its retention time to that of standard chemicals.

2.4. Minimum Inhibitory Concentration (MIC). The MIC for BREO and CLX was determined following the conditions of the Clinical Laboratory Standards Institute protocol [16]. In brief, cloxacillin was prepared, and BREO was dissolved with 5% DMSO. CLX and BREO were serially diluted by a factor of 2 in the 96-well microplate containing cation-adjusted Mueller–Hinton broth (CAMHB). After 18 hours of incubation, the quantity of MRSA strains was adjusted by measuring the optical density (OD), and 10^8 CFU/mL was obtained and diluted with normal saline to a 5×10^6 CFU/mL, and 20 mL of inoculum was added to the well which included BREO or CLX and CAMHB resulting in a final concentration of 5×10^5 CFU/mL and final volume of 200 μL. As negative controls, wells free of antibacterial agents and bacteria were employed. The 96-well microplate was incubated at 37°C for 18 hours. The MIC value was determined as the lowest concentration that exhibited no turbidity. The MICs of antimicrobial agents were evaluated and compared to the CLSI guideline for drug resistance interpretation.

2.5. Checkerboard Method. The checkerboard method was carried out to determine the synergistic interaction between BREO and CLX, as previously described by Odds [17]. In the same manner as the MIC determination, BREO and CLX were prepared, and their combinatorial effect was evaluated by combining them at 37°C for 18 hours. The fractional inhibitory concentration (FIC) was determined by combining the concentrations of each antibacterial agent that inhibited the growth of the observed bacteria. The following formula was used to calculate and interpret the FIC index

$$\text{FIC index} = \text{FICA} + \text{FICB} = \frac{\text{Conc. of A in MICs of A + B}}{\text{MIC of A alone}} + \frac{\text{Conc. of B in MICs of A + B}}{\text{MIC of B alone}}. \quad (1)$$

The FICI can be described as synergism, no interaction, and antagonism whenever the FIC value is ≤0.5, >0.5–4.0, and >4.0, respectively.

2.6. Time-Kill Assays. Time-kill assays were performed to illustrate antibacterial and synergistic activity against MRSA 20651, with minor adjustments to the procedure described by Teethaisong et al. [12]. In brief, the inoculum (5×10^6 CFU/mL) was exposed to BREO, CLX alone, or in combination for 6 exposure periods (0, 2, 4, 6, 8, and 24 hours). 0.1 mL of aliquots from each treatment interval was diluted with 0.9 mL of normal saline. 10 μL of each dilution was dropped on Mueller–Hinton agar (MHA). After 18 hours of incubation at 37°C, the nutrient plate containing 3–50 colonies were chosen to count using the surface drop method or the Miles and Misra method, which was tested to be as accurate and time-saving as described by Hedges et al. [18, 19]. Then, time-kill curves were plotted. CFU/mL was calculated using the following formula:

$$\frac{\text{CFC}}{\text{mL}} = \text{average number of colonies in each dilution} \times 100 \times \text{dilution factor}. \quad (2)$$

At 24 hours, the synergistic interaction was considered as ≥2 $\log_{10}$ reductions in CFU/mL of the combined agent-treated group compared to the single most effective agent-treated group [20]. In addition, the bactericidal effect was described as a reduction ≥3$\log_{10}$ CFU/mL, and the bacteriostatic effect was determined to be a decrease less than 3$\log_{10}$ CFU/mL of each treatment group at 24 hours compared to the starting inoculum [21].

2.7. Cytoplasmic Membrane (CM) Permeability. This experiment was performed as previously described by Siriwong et al. [22] with a slight modification. The CM damage was determined by measuring the intensity of the OD_{260}-absorbing materials using a UV-VIS spectrophotometer. After incubation for 18 hours, the MRSA was collected and adjusted to a concentration of 5×10^6 CFU/mL in normal saline. Modified cultures (5 mL) were added to 45 mL of CAMHB supplemented with BREO, CLX alone at half-MIC, or BREO plus CLX at FIC concentrations. As a negative control, a flask without an antibacterial agent was utilized. Nisin was applied as a positive control to increase CM permeability in this experiment because it can damage the cytoplasmic membranes of Gram-positive bacteria. These bacterial cultures were incubated at 37°C in a shaking incubator. The CM permeability was determined at 0, 1, 2, 3, and 4 hours. In addition, the OD_{260} intensity of UV-absorbing materials leaked from the cells was evaluated in the supernatant, as described by Paul et al. [23]. All tests were done in triplicate with a Varian Cary 1E UV/VIS spectrophotometer.

2.8. Biofilm Formation Inhibition. Biofilms are formed when a bacterial colony generates polysaccharides, proteins, nucleic acids, and lipids. Bacteria are held together by the extracellular matrix, which creates a three-dimensional film-like structure. Biofilm formation is an antibiotic resistance mechanism that contributes to persistent infections by decreasing the penetration of the drug through these films. Consequently, the inhibitory effect of BREO on biofilm growth was evaluated using the methodology described by He et al. [24] with some modifications. First, the bacterial culture was counted to 5×10^6 CFU/mL in saline solution after incubation for 18 hours. In 96-well microtiter plates, 20 μL of bacterial inoculum was added to 180 μL of CAMHB supplemented with 0.2% glucose and a half-MIC or FIC of BREO, depending on whether it was used alone or in combination with CLX. These solutions were incubated at 37°C for 48 hours. Then, the bacterial medium was removed, and adhering cells on the surface of each well were rinsed with distilled water and stained for 30 minutes with a crystal violet solution containing 0.4% (w/v) crystal violet. After dyeing, the crystal violet solution was washed with distilled water. After that, air-dried adherent cells stained with crystal violet were washed in 100% ethanol in each well. A microplate reader set to 595 nm was used to determine the optical density of the solution in each well. Antibiofilm formation was evaluated through the percentage of inhibition calculated following the formula presented in the study by Gómez-Sequeda et al. [25].

$$\% \text{ inhibition} = \frac{\text{OD negative control} - \text{OD treatment group}}{\text{OD negative control}} \times 100. \quad (3)$$

2.9. Scanning Electron Microscopy (SEM). The SEM sample preparation procedure was performed following the method of Hartmann et al. [26] with slight adjustments. In brief, MRSA DMST 20651 strain was cultured in CAMHB at 37°C for 18 hours and then adjusted to a final concentration of 5×10^5 CFU/mL. This strain was treated with CLX and BREO alone at half-MIC and CLX plus BREO at FIC at 37°C for 4 hours. The positive control was chosen and incubated in an antibiotic-free medium. Next, this strain was fixed with 2% glutaraldehyde in 0.15 M sodium phosphate buffer (pH 7.2), rinsed, and resuspended in distilled water. Then, samples were incubated with 0.5% osmium tetroxide (OsO_4) for 2 hours. The samples were dehydrated using a graded acetone solution (20%, 40%, 60%, 80%, and 100%,respectively), dried in the air, mounted on a carbon stub, and sputtered with gold. Finally, images of the cell morphology of these samples were captured using a scanning electron microscope.

2.10. Transmission Electron Microscopy (TEM). The TEM was used to evaluate the structural damage of bacteria produced by BREO or BREO plus CLX. The procedure for sample preparation was performed following the method of Richards et al. [27] with minor modifications. In brief, after 18 hours of incubation, the final concentration of bacterial suspension was adjusted to 5×10^5 CFU/mL, then incubated for 3 hours at 37°C, and shaken at 110 rpm in a shaking incubator. This suspension was centrifuged at $6000 \times g$ for 15 minutes at 4°C, the supernatant was removed, and the pellet was fixed for 12 hours in 2.5% glutaraldehyde-containing 0.1 M phosphate buffer (pH: 7.2). The samples were rinsed twice in 0.1% phosphate buffer and incubated for 2 hours in 1% OsO_4 at room temperature. This sample was dehydrated for 15 minutes using a graded concentration of acetone solution (20%, 40%, 60%, 80%, and 100%, respectively). The epoxy resin was used to embed these samples. Afterward, these samples were counterstained for 3 minutes with 2% (w/v) uranyl acetate and 2 minutes with 0.25% (w/v) lead citrate. The specimens were then observed and photographed using a 80kV electron microscope. Note that the bacteria growing without CLX served as the control treatment. Cell area was computed to examine the impact of BREO alone and combined with CLX on cell size by multiplying cell width by cell length in TEM images (nm^2).

2.11. Statistical Analysis. The statistical method was performed using IBM SPSS Statistics 22. The data were represented by mean ± standard error of the mean (SEM). The significant differences in CM permeability, biofilm density, and cell area between treated groups were determined using a one-way analysis of variance (ANOVA). Post hoc Tukey's HSD (honestly significant difference) test was compared at a P value<0.01.

3. Results

3.1. Gas Chromatography-Mass Spectrometry (GC-MS). The chemical identification of essential oils was determined by comparing their retention times and mass spectra to a database of reference compounds. There were 24 compounds found in BREO, representing 100% of the overall constitution. The main constituents of BREO were β-ocimene (36.73%), trans-geraniol (25.29%), camphor (14.98%), and eucalyptol (8.99%) (Table 1).

3.2. Minimum Inhibitory Concentration (MIC). The result of MIC determination demonstrated that BREO inhibited MRSA DMST 20649, 20651, and 20652 at a MIC of 4 mg/mL. In *S. aureus* ATCC 29213, the BREO showed a MIC of 2 mg/mL for this strain. CLX resistance was observed in MRSA DMST 20649, 20651, and 20652 strains with MIC of 512 μg/mL. *S. aureus* ATCC 29213 was sensitive to CLX with MIC of 0.125 μg/mL (Table 2).

3.3. Checkerboard Method. The synergistic interaction between BREO and CLX was found in MRSA DMST 20649, 20651, and 20652 with an FIC index<0.5. The concentration of BREO and CLX in the combined test was much lower than that of the single chemical concentration (Table 3).

3.4. Time-Kill Assay. The inhibitory action of BREO alone and in combination with CLX against MRSA DMST 20651 is shown in Figure 1. The findings demonstrated that the growth of the untreated control group was normal. Cell viability after exposure to half-MIC of CLX (256 μg/mL) and BREO (2 mg/mL) revealed growth reduction during the first 6 hours of incubation, after which bacteria started to multiply. After being treated with BREO (1 mg/mL) plus CLX (16 μg/mL), the growth of the bacteria displayed a steady decrease in viable cell count after the first 4 hours of incubation until 24 hours. The group treated with the combined chemical revealed >2$\log_{10}$ reduction in viable cells compared to the single most effective group (BREO-treated group). Therefore, this combined chemical exhibited a synergistic interaction against MRSA DMST 20651. Additionally, the combined group also exhibited a <3$\log_{10}$ decrease in CFU at 24 hours relative to the initial CFU, indicating a bacteriostatic effect.

3.5. Cytoplasmic Membrane (CM) Permeability. Figure 2 shows the findings of CM permeability tests. Compared to the control group, the CLX-treated group did not exhibit any OD_{260}-absorbing material leakage after 1 hour of treatment ($p > 0.01$). OD_{260} of cells treated with BREO, nisin, or BREO plus CLX considerably increased compared to cells treated with CLX alone or a control ($p < 0.01$). There was no statistically

Table 1: Chemical constituents present in *B. rotunda* essential oil.

Retention time (RT) (min)	Peak name	% area
9.502	Tricyclene	0.16
9.872	alpha-Thujene	0.01
10.164	alpha-Pinene	0.65
10.969	Camphene	4.45
12.565	beta-Pinene	0.11
13.745	beta-Myrcene	1.00
14.380	alpha-Phellandrene	0.04
15.180	alpha-Terpinene	0.05
16.113	**Eucalyptol**	**8.99**
16.877	trans-beta-Ocimene	3.92
17.783	**beta-Ocimene**	**36.73**
18.108	gamma-Terpinene	0.06
19.987	Terpinolene	0.15
21.166	beta-Linalool	1.01
23.807	**Camphor**	**14.98**
24.000	Camphene hydrate	0.36
24.665	Isoborneol	0.04
25.320	endo-Borneol	0.28
26.091	4-Terpineol	0.13
27.126	alpha-Terpineol	0.43
32.157	**trans-Geraniol**	**25.29**
32.716	alpha-Citral	0.15
39.831	Methyl cinnamate	1.01
51.294	Caryophyllene oxide	0.01
Total		100.00

The high value of chemical constituents is represented by bold values.

Table 2: Minimum inhibitory concentrations (MICs) of cloxacillin (CLX) and *B. rotunda* essential oil (BREO) against *S. aureus* strains.

Bacterial strains	Minimum inhibitory concentration (MIC)	
	CLX (μg/mL)	BREO (mg/mL)
MRSA DMST 20649	512^{R}	4^{ND}
MRSA DMST 20651	512^{R}	4^{ND}
MRSA DMST 20652	512^{R}	4^{ND}
S. aureus ATTC 29213*	0.125^{S}	2^{ND}

*A reference strain. ^{R}Resistant; ^{S}susceptible; NDno data available.

Table 3: Fraction inhibitory concentration (FIC) index of cloxacillin (CLX) plus *B. rotunda* essential oil (BREO) against *S. aureus* strains.

Bacterial strains	MIC(a)		MIC(c)		FIC		FICI
	BREO (mg/mL)	CLX (μg/mL)	BREO (mg/mL)	CLX (μg/mL)	BREO	CLX	
MRSA DMST 20649	4^{ND}	512^{R}	1^{ND}	16^{R}	0.25	0.03	0.28*
MRSA DMST 20651	4^{ND}	512^{R}	1^{ND}	16^{R}	0.25	0.03	0.28*
MRSA DMST 20652	4^{ND}	512^{R}	1^{ND}	32^{R}	0.25	0.06	0.31*

*Synergistic interaction (FIC index ≤0.5). FICI = FIC index; MIC(a) = MIC value of chemical alone; MIC(c) = MIC value of chemical in combined drug. ^{R}Resistant; ^{S}susceptible; NDno data available.

significant difference throughout the experiment between cells treated with BREO and those treated with BREO plus CLX ($p > 0.01$), except for the 3 hours in which BREO plus CLX exhibited significantly higher cytoplasmic membrane permeability.

3.6. Biofilm Formation Inhibition. A typical quantitative biofilm result exhibited that CLX and BREO, either alone or in combination, prevented the development of biofilms against MRSA 20651, as seen in Figure 3. After 48 h of treatment, MRSA 20651 cultured without antibacterial drug exhibited the highest biofilm biomass. The percentage inhibition of the BREO-treated group, the CLX-treated group, and the combined group was 67.58 ± 0.67, 72.61 ± 0.61, and 80.25 ± 0.60, respectively. All treated groups substantially reduced the biofilm of MRSA 20651 compared to the control group ($p < 0.01$). CLX also showed more significant biofilm inhibition activity than BREO ($p < 0.01$). Besides, the combination of BREO (1 mg/mL) and CLX (16 μg/mL) also revealed the highest biofilm inhibition activity compared to BREO and CLX alone ($p < 0.01$).

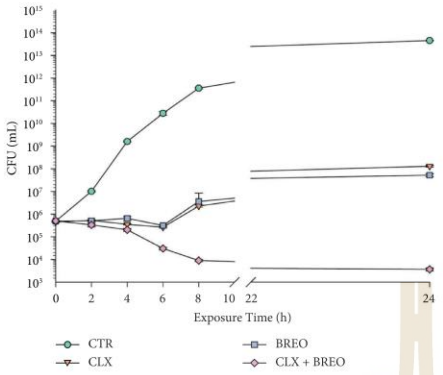

Figure 1: Cell viability of MRSA DMST 20651 cells after exposure to BREO and CLX, whether alone or in combination. CTR = control, CLX = CLX at 256 μg/mL, BREO = BREO at 2 mg/mL, and CLX + BREO = CLX at 16 μg/mL plus BREO at 1 mg/mL. The values were represented by the mean and standard error of the mean (SEM) from triplicates.

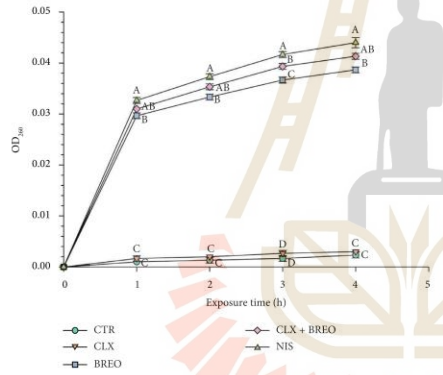

Figure 2: Change in quantity of OD_{260}-absorbing material of MRSA DMST 20651 after treatment with BREO and CLX, whether taken alone or in combination. CTR = control, CLX = CLX at 256 μg/mL, BREO = BREO at 2 mg/mL, and CLX + BREO = CLX at 16 μg/mL plus BRE at 1 mg/mL. NIS = nisin at 8 μg/mL. The values were represented by the mean and standard error of the mean (SEM) from triplicates. The one-way ANOVA and Tukey's HSD post hoc tests were compared between results. The different alphabetical symbols indicate a statistically significant difference between groups ($p < 0.01$).

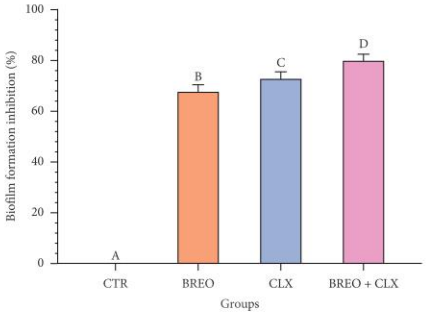

Figure 3: The inhibition of MRSA 20651 biofilms was quantified after treatment with BREO, or CLX, either alone or in combination. CTR = control, CLX = CLX at 256 μg/mL, BREO = BREO at 2 mg/mL, and CLX + BREO = CLX at 16 μg/mL and BREO at 1 mg/mL. The values were represented by the mean and standard error of the mean (SEM) for six replicates. The one-way ANOVA and Tukey's HSD post hoc tests were compared between results. The different alphabetical symbols indicate a statistically significant difference between groups ($p < 0.01$).

3.7. Scanning Electron Microscopy (SEM). MRSA DMST 20651 cells were treated with BREO, CLX, and BREO plus CLX. Untreated cells displayed a cluster of berry-shaped cells with smooth cell surfaces (Figure 4(a)). After treatment with 256 μg/mL of CLX, most damaged cells revealed dents (squared arrow). Additionally, some cells with a rough surface (dotted arrow) and others with withered cells (arrow) were observed (Figure 4(b)). These cells, after exposure to 2 mg/mL of BREO, displayed clefts (dotted arrow), a bleb-like structure on the cell surface (squared arrow), and cell lysis with large debris (arrow) (Figure 4(c)). Dents (arrows), clefts (squared arrows), bleb-like structures on the cell surface (dotted arrows), and significant cell lysis (rectangle arrows) were displayed in cells treated with 1 mg/mL of BREO and 16 μg/mL of CLX (Figure 4(d)).

3.8. Transmission Electron Microscopy (TEM). TEM was applied to assess the cell damage caused by exposure to BREO, CLX, and BREO plus CLX. The MRSA DMST 20651 cells were investigated by TEM treated with BREO (2 mg/mL), CLX (256 μg/mL), BREO (1 mg/mL) plus CLX (16 μg/mL), and untreated cells. In addition, the effect of such compounds on the cell sizes of each group was compared. Figure 5(a) illustrates the structure of untreated, antibacterial-free control. These cells were allowed to grow normally. Untreated cells have a spherical and clear appearance. The cytoplasmic membrane and cell wall were

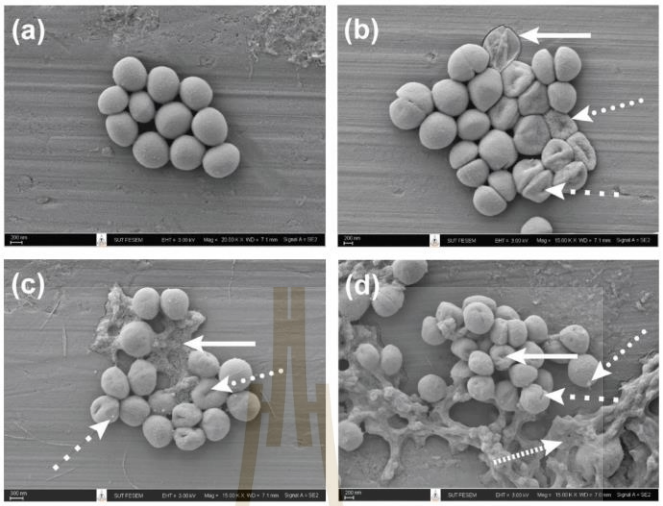

Figure 4: MRSA DMST 20651 SEM images after incubation in CAMHB (100 nm and 20000x) (a) and after exposure to 256 μg/mL of CLX (200 nm and 15000x) (b), 2 mg/mL of BREO (300 nm and 15000x) (c), and 1 mg/mL of BREO plus 16 μg/mL of CLX (200 nm and 15000x) (d).

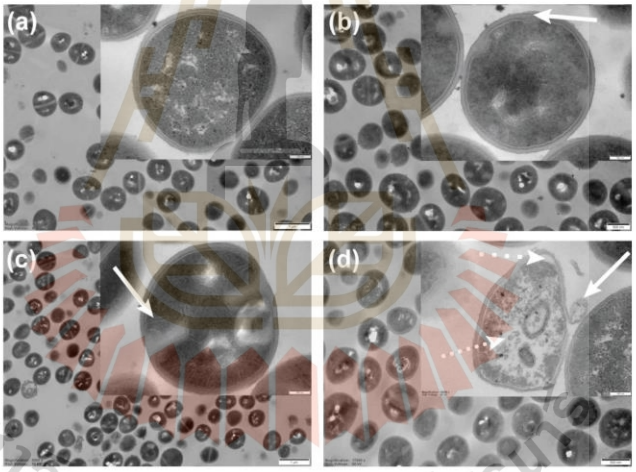

Figure 5: MRSA DMST 20651 TEM images after incubation in CAMHB (original: 1 μm and 10000x; inset: 100 nm and 89000x) (a) and after exposure to 256 μg/mL of CLX (original: 500 nm and 13000x; inset: 100 nm and 89000x) (b), 2 mg/mL of BREO (original: 1 μm and 8500x; inset: 100 nm and 89000x) (c), and 1 mg/mL of BREO plus 16 μg/mL of CLX (original: 500 nm and 17000x; inset: 100 nm and 89000x) (d).

distinguishable from cells treated with other treatment groups. After exposure to CLX at a concentration of 256 μg/mL, the cell wall of the MRSA 20651 strain exhibited morphological changes between the cell wall and the cytoplasmic membrane (arrow) (Figure 5(b)). Figure 5(c) displays cells treated with 2 mg/mL of BREO. The hairpin-

like structure of the cytoplasmic membrane indicated the presence of alterations of the cytoplasmic membrane (arrow). Therefore, these results suggest that BREO suppresses MRSA 20651 by interacting with the cytoplasmic membrane. After exposure to the combination of 1 mg/mL of BREO and 16 μg/mL of CLX, the cell wall in this group was peeled off (arrow). Additionally, the cytoplasmic membrane (squared arrow) and intracellular components (dotted arrow) were lost after exposure to this combination (Figure 5(d)). As shown in Figure 6, the cell areas of the untreated control (CTR) and cells treated with BREO, CLX, and BREO plus CLX were calculated to determine the impact of each treatment on cell size. The untreated control had a cell area of approximately $6.18 \times 10^5 \pm 3.51 \times 10^4$ nm^2. This finding did not reveal statistically significant variation in cell area between the CTR, CLX-treated ($5.85 \times 10^5 \pm 1.31 \times 10^4$ nm^2), and BREO-treated groups ($5.20 \times 10^5 \pm 1.07 \times 10^4$ nm^2) ($p > 0.01$). Additionally, only the CLX plus BREO ($4.34 \times 10^5 \pm 1.76 \times 10^4$ nm^2) group revealed a significant reduction in cell area compared to the untreated control group ($p < 0.01$). However, this combination did not show significant differences in cell area from the BREO-treated group. These findings demonstrate that exposure of MRSA DMST 20651 to BREO plus CLX can reduce cell area.

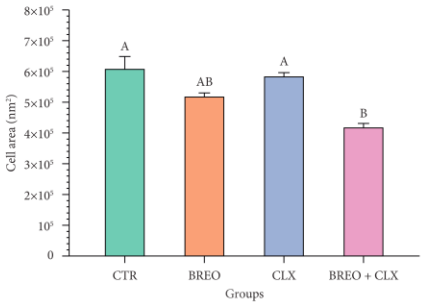

Figure 6: The effect of BREO, CLX, and BREO plus CLX on the cell area of MRSA 20651. CTR = untreated control, BREO = *B. rotunda* essential oil at a concentration of 2 mg/mL ($n = 6$), CLX = cloxacillin at a concentration of 256 μg/mL ($n = 6$), and BREO + CLX = *B. rotunda* essential oil at a concentration of 1 mg/mL and cloxacillin at a concentration of 16 μg/mL ($n = 6$). The cell area was calculated by the cell width multiplied by the cell length (nm^2). Using one-way ANOVA with Tukey's HSD, different alphabets indicate a statistically significant difference ($p < 0.01$). The data were presented as the mean and standard deviation of the mean (SEM).

4. Discussion

Due to their accessibility, affordability, and safety, medicinal plant-derived chemicals are recognized as fascinating sources of antimicrobial agents against various infections [28]. In recent years, people have tended to rely on herbal plants as sources of novel therapeutics, including for treating bacterial infections. This approach provides the way for the development of modern medicine.

B. rotunda is also an exciting alternative to treat antibiotic-resistant bacterial infections. This plant has shown a synergistic interaction with various antibiotics through various antibacterial mechanisms derived from secondary metabolites such as alkaloids, flavonoids, and essential oils [11, 12]. Since the evaluation of *B. rotunda*, the effectiveness of essential oils, both as single and combined therapies, identifying the main active constituent and the mechanism of action still needs to be investigated. In this work, the constituents of BREO were evaluated using the GC-MS technique. The findings found that the main components of BREO were β-ocimene, trans-geraniol, camphor, and eucalyptol.

Chi et al. [29] reported that essential oils extracted from citrus leaves, including *Citrus sinensis*, *Citrus grandis*, and *Citrus aurantifolia* that contained β-ocimene as a major constituent, demonstrated antibacterial activity against *S. aureus*, *Bacillus cereus*, and *Salmonella typhimurium* with inhibition zones and MIC values ranging from 20.1 ± 0.1 to 24.3 ± 0.1 mm and 5.25 to 21 mg/mL, respectively. Due to the antibacterial potential against *S. aureus*, *C. sinensis*, with the highest β-ocimene content compared to the other two species, revealed the highest inhibition effect on MRSA with a zone of 23.2 ± 0.2 mm and the lowest MIC value at a concentration of 5.25 mg/mL. Furthermore, Jaradat et al. [30] found that *Thymus bovei* essential oil containing trans-geraniol exhibited antibacterial activity against *S. aureus* and *Escherichia coli*, with MIC values of 0.25 mg/mL and 0.5 mg/mL, respectively. Camphor oil extracted from *Cinnamomum camphora* had antibacterial activity against *Streptococcus* mutants and *Enterococcus faecalis*, Gram-positive bacteria [31]. Similarly, Lopes-Lutz et al. [32] demonstrated that camphor-enriched essential oils extracted from plants in the genus *Artemisia* had antibacterial action against *S. aureus*, with inhibition zones between 10 ± 0.0 and 25 ± 1.4 mm, compared to 8 ± 0.5 and 18 ± 1.0 mm for methicillin and vancomycin, respectively. These oils were also found to have antifungal efficacy with an inhibition zone ranging from 15 ± 1.4 to 40 ± 2.1 mm against *Microsporum canis*, while amphotericin B exhibited an inhibition zone of 19 ± 1.0 mm. Hamad Al-Mijalli et al. [33] provided the details of *Lavandula multifida* essential oils (LMEOs) containing eucalyptol inhibiting the growth of *S. aureus*, *Listeria monocytogenes*, *Bacillus subtilis*, and *E. coli*, with MIC values ranging from 0.78 to 1.56 mg/mL. Regarding the antibacterial activity determined in this study, the MIC value of BREO was 4 mg/mL. This antibacterial action against MRSA strains may be attributable to the activity of β-ocimene, trans-geraniol, camphor, and eucalyptol.

Although using BREO or CLX monotherapy to inhibit MRSA had low activity, the combination of BREO and CLX showed higher antibacterial action from the synergistic interaction. This finding is in accordance with a previous investigation that the combination of geraniol and norfloxacin had a synergistic interaction (FIC <0.5) against *B. cereus* and *S. aureus* isolates; besides, the combination of geraniol and chloramphenicol synergistically inhibited

E. coli, *Klebsiella pneumoniae*, *Proteus mirabilis*, and *P. aeruginosa* [34]. Similar to a study by Bekka-Hadji et al. [35], *Artemisia herba-alba* essential oil containing camphor exhibited a synergistic interaction with cephalosporins against *S. aureus*. Requena et al. [36] reported that the synergistic interaction occurred when 1 mg/mL of eucalyptol was combined with thymol and cinnamaldehyde at a concentration of 0.05 and 0.1 mg/mL, respectively, against *Listeria innocua*. Our work determined the synergistic interaction using a checkerboard assay, and FIC <0.5 was found, which ODD theory interpreted as a synergistic interaction [17]. In the time-kill experiment, the concentration of each chemical was selected depending on the MIC value. The half-MIC concentrations of BREO and CLX were chosen because these concentrations did not inhibit bacteria growth at around 24 hours of incubation. Usually, the synergistic interaction of the combined chemical can inhibit bacterial growth at doses of each chemical that are less than half the MIC of a single chemical. The finding of this assay demonstrates that the combined drug reduces the growth of MRSA DMST 20651 >2log$_{10}$ CFU/mL compared to the most effective chemical (BREO) at 24 hours. This growth reduction was observed from the start and throughout 24 hours after exposure to the half-MIC of BREO and CLX. The findings of the time-kill assay support the checkerboard method that two chemicals show a synergistic interaction. This synergistic interaction may be influenced by the main constituents of BREO mentioned above. Therefore, the synergistic approach must be verified by investigating the mechanism of action of BREO and CLX, which inhibits cell wall synthesis.

According to the investigation of the mechanism of action, the concentration of chemicals was selected from those used in time-kill tests, ensuring consistency throughout all experiments, following the pattern of Siriwong et al. [22] with some modifications. Furthermore, using concentrations below the MIC value of a single chemical enables a better understanding of the mechanism of action, especially the morphological alteration observed by SEM and TEM, because these concentrations are insufficient to kill all bacteria. The mechanism of action in this work was focused on determining the synergistic interaction of BREO on the alteration of CM permeability and the antibiofilm formation activity.

Regarding the permeability of CM, Asker et al. [37] indicated that the mechanism of β-ocimene in the inhibition of *S. aureus* and *Pseudomonas aeruginosa* was by inhibiting the biosynthesis of lipids of these bacteria in a concentration-dependent manner. According to Tang et al. [38], the essential oil of *Amomum villosum* Lour., containing 20.94% camphor, induced increasing membrane permeability, resulting in leakage of intracellular components, especially DNA and RNA. Furthermore, it was proposed that the mechanism of action of geraniol in inhibiting several pathogens was by adhering and interacting with the membrane lipid of the microbe, resulting in increased membrane permeability [34]. This work determined the permeability of CM by measuring the intensity of OD_{260}, which was explicitly related to the number of intracellular components that leaked out of the cell, such as DNA and RNA. The result demonstrated that the BREO-treated and combined groups increased the CM permeability more than the untreated control and CLX-treated groups. Furthermore, at 4 hours of incubation, the permeability of CM after exposure to the combined drug was not significantly different from that of nisin, which was used as a positive control in previous studies [12, 39]. These results imply that the combination of BREO and CLX could increase the permeability of bacterial CM and may be acted by the main components of BREO, such as β-ocimene, camphor, and trans-geraniol. The antibacterial action of nisin is well known to destroy the bacterial cell membrane. It was employed as a positive control agent for a cell membrane permeability assay in this study. Although nisin substantially increased CM permeability, it is an alternative drug for treating MRSA infection [40]. This study focused on combining BREO and CLX to reverse practically used antibiotics that have lost their antibacterial activity. Hence, using nisin in combination with BREO was not considered in this work.

For antibiofilm formation activity, geraniol and camphor exhibited antibiofilm activity by inhibiting *S. aureus* biofilm with the inhibition percentage of 86.13 ± 5.22 and 81.25 ± 1.63 after exposure to 256 μg/mL of geraniol and 10 mg/mL of camphor, respectively [41, 42]. Furthermore, Vijayakumar et al. [43] found that eucalyptol showed a concentration-dependent manner for inhibition of biofilm formation of *Streptococcus pyogenes* by 89% inhibition after exposure to this chemical at a concentration of 300 μg/mL. Therefore, in our work, the antibiofilm activity of BREO may be due to these compounds mentionedearlier. Adeyemo et al. [44] reported the criteria that good antibiofilm formation occurred when the percentage inhibition was more significant than 50%. These results provide evidence that CLX, BREO, and its combination demonstrate a good effective inhibition of biofilm formation with percentage inhibition of 67.58 ± 0.67, 72.61 ± 0.61, and 80.25 ± 0.60, respectively. In addition, the combined drug exhibited the highest biofilm inhibition activity compared to other groups ($p < 0.01$). Therefore, the combination with a lower concentration of each compound exhibits significantly higher antibiofilm activity than a single one with a higher concentration. This finding is consistent with previous studies that the main constituents of BREO revealed antibiofilm formation activity.

TEM and SEM analyses were performed on cells exposed to BREO, CLX, and their combinations to determine the position of action based on morphological changes. After exposure to BREO, hairpin-shaped coiling of the cytoplasmic membrane could be indicated as an alteration. CLX was responsible for cell wall deformation. However, damage to the cell wall and the cytoplasmic membrane was observed when BREO was combined with CLX. Furthermore, this combination of BREO and CLX displayed the action through several mechanisms, such as inhibition of cell wall synthesis, increased CM permeability, and antibiofilm formation activity, consistent with the purpose of the study. These findings are consistent with Teethaisong et al. [12] and Siriwong et al. [22] that synergistic interaction could occur

when two chemicals with different mechanisms of action inhibit bacteria concurrently.

To fully understand BREO's antibacterial and synergistic properties, additional modes of action associated with resistance mechanisms, cytotoxicity tests, safe dosage for human and animal cells, and the total content of active constituents must be considered in a future perspective. This state of knowledge could lead to additional learning on issues relevant to the synergistic interaction of natural chemicals and conventional antibiotics. Taken together, BREO could synergistically restore the activity of CLX that failed to treat MRSA infections. This could lead to the development of novel treatment options for infections caused by multidrug-resistant MRSA.

5. Conclusions

In summary, BREO demonstrates an inhibitory effect on β-lactam-resistant staphylococci. The mechanism of action of this essential oil is most likely to act at the cytoplasmic membrane, resulting in alterations in the cytoplasmic membrane. Furthermore, another mode of action is biofilm formation inhibitory activity. The synergistic activity of BREO and CLX against MRSA DMST 20651 strains can be attributed to the reduction in biofilm production and increase in cytoplasmic membrane damage resulting in the leakage of cellular components.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Scholarships

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Publications

Apinundecha, C., Teethaisong, Y., Suknasang, S., Ayamuang, I.-o., and Eumkeb, G. (2023). Synergistic Interaction between *Boesenbergia rotunda* (L.) Mansf. Essential Oil and Cloxacillin on Methicillin-Resistant *Staphylococcus aureus* (MRSA) Inhibition. Evidence-Based Complementary and Alternative Medicine. 2023, 3453273. (SCOPUS SJR 2022 = Q2).