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Synergism between natural product extracts and antibiotics against Methicillin-resistant *Staphylococcus aureus* (MRSA)

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Abstract

Staphylococcus aureus is recognized as one of the major infective agents causing both community and hospital acquired infections. Methicillin-resistant *S. aureus* (MRSA) is one of the multidrug resistant strains. Therefore, very few antibiotics are effective in treating infections caused by MRSA. Many compounds were synthesized and developed for the treatment of MRSA infections. In this study, twenty-five natural products, such as *Capsicum flutescens* L., *Piper nigerum*, *Gracinia mangostana*, *Curcuma zanthorrhiza* Roxb. , *Aloe vera* L., etc., were determined for antimicrobial activities. In addition, synergisms between these natural products and antibiotics were also studied. The results showed that bergamot oil demonstrated antibacterial activity against MRSA and Methicillin-susceptible *S. aureus* (MSSA). The MIC and MBC of bergamot oil against both strains were 0.39 μg/ml and 0.78 μg/ml, respectively. It was found that a combination of bergamot oil and tetracycline reduced the MIC and MBC of tetracycline which indicated that they worked synergistically against MRSA and MSSA.

***Keywords*:** methicillin-resistant *S. aureus*, *natural product extracts*, *synergism*, MRSA

บทคัดย่อ

เชื้อสแตปฟิโลคอคคัส ออเรียส (*Staphylococcus aureus, S. aureus*) มีบทบาทสำคัญในการก่อให้เกิดโรคติดเชื้อได้ทั้งในโรงพยาบาลและชุมชน เชื้อสแตปฟิโลคอคคัส ออเรียส ที่ดื้อต่อยาเมธิซิลลิน (Methicillin-resistant *S. aureus*, MRSA) เป็นสายพันธุ์ของ *S. aureus* ที่ดื้อต่อยาปฏิชีวนะได้หลายชนิดโดยเฉพาะกลุ่มเพนนิซิลลิน (penicillin) รวมทั้งเมธิซิลลิน (methicillin) การดื้อต่อยาปฏิชีวนะนี้มีบทบาทสำคัญต่อการรักษาโรคติดเชื้อ สารประกอบและสารสังเคราะห์หลายชนิดถูกนำมาใช้เพื่อยับยั้งเชื้อ MRSA ในการศึกษานี้ได้ทำการตรวจกรองฤทธิ์ต้าน *S. aureus* จากสารสกัดจากพืช 25 ชนิด เช่น มะกรูด (*Citrus hystrix*), พริก (*Capsicum flutescens* L.), พริกไทย (*Piper nigerum*), มังคุด (*Gracinia mangostana*), ว่านชักมดลูก (*Curcuma zanthorrhiza* Roxb) และ ว่านหางจระเข้ (*Aloe vera* L.) เป็นต้น และยังทำการศึกษาการเสริมฤทธิ์ของสารสกัดกับยาปฏิชีวนะบางชนิด จากการทดลองพบว่า น้ำมันสกัดจากมะกรูด (bergamot oil) มีฤทธิ์ในการยับยั้งทั้ง MRSA และ *S. aureus* ที่ตอบสนองต่อยาเมธิซิลลิน (Methicillin-susceptible *S. aureus*, MSSA) โดยค่า MIC และ MBC ของน้ำมันสกัดจากมะกรูดต่อเชื้อทั้งสองสายพันธุ์มีค่าเท่ากับ 0.39 μg/ml and 0.78 μg/ml ตามลำดับ นอกจากนี้พบว่าเมื่อใช้น้ำมันมะกรูดร่วมกับ ยาเตตราซัยคลิน (tetracycline) มีผลให้ค่า MIC และ MBC ของยาเตตราซัยคลินลดลง ซึ่งแสดงให้เห็นว่า น้ำมันมะกรูดมีการเสริมฤทธิ์กับยาเตตราซัยคลินในการยับยั้ง MRSA และ MSSA

คำสำคัญ: *สารสกัดธรรมชาติ, การเสริมฤทธิ์, เชื้อสแตปฟิโลคอคคัส ออเรียส ที่ดื้อต่อยาเมธิซิลลิน (MRSA)*

1. Introduction

Staphylococcus aureus is recognized as one of the major causes of infections in humans occurring in both communities and hospitals (Gillespie & Hawkey, 2006). The bacteria commonly cause skin and wound infections and can spread from skin lesions to deep tissues causing infections of bloodstream, bones, joints and deep organs. Infections due to some strains of *S. aureus* can be treated by many antimicrobial agents. Among the most active is Penicillin, but about 90% of strains found in hospitals are now resistant (Jensen, Wright, & Robison, 1997; Murray, Rosenthal, & Pfaller, 2013).

The use of antibiotics in the treatment of infections has led to the emergence of resistant *S. aureus* strains. Methicillin-resistant *S. aureus* (MRSA) is one of the strains that is resistant to a variety of antibiotics including penicillins, cephalosporins, erythromycin, streptomycin,

tetracycline, aminoglycoside, fluoroquinolone, and other β-lactam antibiotics (Grundmann, Aires-de-Sousa, Boyce, & Tiemersma, 2006). Methicillin resistance is associated with production of a unique penicillin-binding proteins (PBPs) named PBP2a or PBP2', by the mecA gene, which is not present in Methicillin susceptible staphylococci. Unlike other PBPs, PBP2a or PBP2' has a low binding affinity for beta-lactam antibiotics. Despite the presence of inhibitory concentrations of beta-lactam antibiotics, MRSA can continue its cell wall synthesis depending on the uninhibited activity of PBP2a. The problem of *S. aureus* being resistant to methicillin and many other antibiotics makes the treatment of MRSA more difficult (Lundstrom & Sobel, 2004). In order to combat MRSA infections enormous efforts are being made to synthesize and develop new compounds which can be used as potential antimicrobial agents. Among the new potential agents, natural products possess remarkable options (Saleem et al., 2010).

Natural products have been used in traditional medicine all over the world and predate the introduction of antibiotics and other modern drugs. Important sources of natural products are plants or herbs which are rich in a wide variety of active compounds such as tannins, terpenoids, alkaloids, and flavonoids. These compounds have been found in vitro to exert antimicrobial properties (McChesney, Venkataraman, & Henri, 2007). Moreover, synergism between natural products and antibiotics is a new strategy for developing therapies for difficult-to-treat infections. The synergistic effect enables the use of some antibiotics when they are no longer effective on their own against resistant bacteria (Aiyegoro & Okoh, 2009).

2. Objectives

The objectives of this research were to search for a potential antimicrobial agent from native Thai plant extracts and to study the synergism between these extracts and antibiotics against Methicillin-resistant *S. aureus* (MRSA) and Methicillin-susceptible *S. aureus* (MSSA).

3. Materials and methods

3.1 Bacterial strains

Fifty isolates of *S. aureus* were collected from clinical specimens sent to the Clinical Microbiology Laboratory at Thammasat University Hospital, Thailand. These isolates were identified as *S. aureus* by standard microbiological methods including gram stain, catalase, coagulase, and growth on Mannitol Salt Agar. *S. aureus* ATCC25923 (MSSA reference strain) and *S. aureus* ATCC43300 (MRSA reference strain) were also included in this study.

All *S. aureus* isolates were screened for MRSA and MSSA by using oxacillin disk diffusion test. The bacterial suspension was adjusted to a 0.5 McFarland standard (10^8 CFU/ml) and inoculated on the surface of Mueller-Hinton agar (MHA) by the three-way swab technique using a sterile cotton swab. oxacillin (1 μg), cefoxitin (30 μg), ampicillin (10 μg), tetracycline (30 μg), and ciprofoxacin (5 μg) disks (Becton Dickinson, Sparks, MD.) were placed on the surface of the inoculated agar plate. The agar plate was then incubated at 35˚C for 24 hours. The inhibition zones were measured to interpret antibiotic susceptibility test results. In addition, all isolates were determined for *mecA* gene using the method described by Kohner, Uhl, Kolbert, Persing, and Cockerill (1999). Briefly, bacterial DNA of the isolates were extracted using a DNA extraction kit. Extracted DNA was added to a 2 µl PCR mixture containing, 5.0 µl of 10X PCR buffer, 0.2 µl of forward primer, 0.2 µl of reversed primer, 2.4 µl of 10 mM dNTP, 0.2 µl of 5 U/µl *Taq* Polymerase and 40 µl of distilled water. The two primers used were Primer *mecA*-1(RSM-2647), 5'-AAA ATC GAT GGT AAA GGT TGG C-3' and Primer *mecA*-2 (RSM-2648), 5'-AGT TCT GCA GTA CCG GAT TTG C-3'. A target region of the *mecA* gene was amplified for 40 cycles in a programmed thermal cycle as follows: denaturation at 94˚C for 30 second, annealing at 55˚C for 30 second and extension at 72˚C for 1 minutes with a final extension at 72˚C for 5 minutes. A 533-bp fragment of the *mecA* gene should be amplified from MRSA.

3.2 Preparation of natural product extracts

Plant crude extracts and *Citrus hystrix* (bergamot) oil extract were kindly given by the Faculty of Oriental Medicine, Rangsit University, Thailand. Fifty milligrams of powdered crude extracts of plants including *Aloe vera* L., *Centella asiatica* L., *Eurycoma longifolia*, *Angle marmelos*, *Morus alba* L., *Stevia rebandiana*, and *Ganoderma lucidum* were dissolved in 5 ml of sterile distilled water. Crude extracts of *Dillenia indica*, *Clitoria ternatea* L., *Tiliacora triandra*, *Gracinia*

mangstana, *Kaempferia parviflora* Wall., *Alpinia galangal*, *Curcuma zanthorrhiza* Roxb., *Ficus pubigera* Wall., *Hibiscus sabdariffa* L., *Curcuma longa* L., *Vitis vinifera* L., *Nelumbo nuclfera* , *Cryptoepis buchanani*, *Anaxagorea luzonensis*, and *Cryperus rotundus* L. were prepared by dissolving 50 mg of each powdered crude extract in 5 ml of 20% Dimethyl sulfoxide (DMSO). Then the suspension was filtered through 0.22 µm pore filter membrane. Fresh juice extracts of *Capsicum flutescens* L. and *Piper nigerum* were prepared by squeezing. Bergamot oil extract obtained was prepared from *Citrus hystrix* using a steam distillation technique as described by Cassel, Vargas, Martines, Lorenzo, and Dellacassa (2009). Each extract was filtered through 0.22 µm pore filter membrane.

3.3 Screening for antimicrobial activity of natural products

Antimicrobial activity was determined by agar disk and well diffusion methods according to the Clinical and Laboratory Standard Institute (CLSI) protocol guideline. A suspension of each test bacterium containing about 10^8 cells/ml was spread on Muller Hinton Agar (MHA) by the three-way swab technique using a sterile cotton swab. Twenty µl of each extract solution was dropped on a 6 mm sterile filter paper disk and placed on the agar surface to conduct the disk diffusion method. When well diffusion method was employed, the inoculated MHA plate was punched by a cork borer with a diameter of 6 mm and 50 µl of each natural product extract was added in each well. The control disks were similarly prepared using distilled water and 20% DMSO instead. Standard antibiotic disks were also used as references. All plates were incubated at 35˚C for 18-24 hours. The diameter of the inhibition zones was measured. Triplicates of each plate were carried out. The natural product extract that showed the largest inhibition zone was then marked as the "chosen" natural product extract for further studies.

3.4 Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC)

The MIC of the chosen natural product extract was determined by broth dilution method. The tests were performed with the type strain of MRSA (ATCC43300) and MSSA (ATCC25923). The suspension of the natural product extract was serially diluted in 100 µl of Tryptic Soy Broth (TSB) into 96-well plates. One hundred µl of bacteria (10^6 CFU/ml) in TSB were added into each well. The plate was incubated at 35˚C for 18-24 hours. Each test was performed in duplicates. The lowest concentration of the extract that inhibited the growth of bacteria was the MIC. Broth from all wells that did not show growth in the MIC studies were streaked onto Tryptic Soy Agar (TSA) and incubated at 35˚C for 18-24 hours. The lowest concentration that did not show growth on TSA was the MBC.

3.5 Determination of synergism between antibiotics and natural product extract

Synergisms between antibiotics and the chosen natural product extract were determined by disk diffusion method. Each bacterial type strain was prepared to a 0.5 McFarland standard (10^8 CFU/ml) in TSB and inoculated on the surface of MHA plate by the 3-way swab technique. Twenty µl of the chosen natural product extract were loaded onto each antibiotic disk including clindamycin, oxacillin, ampicillin, erythromycin, ceforoxime, ciprofloxacin, tetracycline, and sulfamethoxazole. Each antibiotic disk without the chosen natural product extract and a disk containing 20 µl of natural product extract alone were used as controls. After placing all disks on the surface of inoculated MHA the plates were incubated at 35˚C for 18-24 hours. The inhibition zone around each disk was measured. Triplicates of each plate were carried out.

The antibiotic that showed the largest inhibition zone when combined with the chosen natural product extract was then marked as the "chosen" antibiotic for further studies.

The MIC of the combination of the chosen natural product extract and chosen antibiotic was determined by the Checkerboard assay previously described by Ramadia, Kamat and Kamat (2013). Briefly, the chosen natural product extract and antibiotic solution were serially diluted in 100 µl of TSB into 96-well plates. The antibiotic solution was serially diluted along the abscissa, while the chosen natural product extract was diluted along the ordinate. One hundred ml of the test bacteria (10^6 CFU/ml) were added into each well. Plates were incubated at 35˚C for 18-24 hours and then observed for growth of the test organism. The combination of the drugs in which the growth is completely

inhibited was considered as effective MIC for the combination.

3.6 Time-kill study

Kinetics of killing was determined according to the method previously described by Sukplang and Thongmee (2014). Briefly, one ml of the test culture was added to 9 ml of fresh TSB. Kinetics of kill testing was conducted by exposing 10^6 to 10^7 cells of the bacteria to the test agent at 10 times concentration of the MIC for various periods of time. Care was taken to insure that each strain was in "log phase" growth in TSB at 37˚C at the time of the exposure to the test agents; the chosen natural product extract, the chosen antibiotic, and the mixture of both in a 1:1 ratio. At the end of the exposure time (i.e. 5, 15, 30, and 60 minutes), the "treated" cells were serially diluted in 0.85% sterile saline solution. The diluted bacterial suspensions were spread on to Total Plate Count Agar and incubated at 37˚C for 24 hours to determine the surviving colony forming unit (CFU) per ml.

4. Results

MRSA exhibited resistance to oxacillin while MSSA showed susceptibility. Most MSSA showed susceptibility to ampicillin while MRSA showed resistance. All MRSA showed resistance to ciprofloxacin while MSSA showed susceptibility. For the *mecA* gene determination all MRSA isolates showed *mecA* gene while MSSA did not.

The aqueous crude extracts of natural products and fresh juice of *Capsicum flutescens* L. and *Piper nigerum* were not found to be active against all bacteria tested, i.e. MSSA clinical isolates, MRSA clinical isolates, MSSA (ATCC25923), and MRSA (ATCC43300). *Citrus hystrix* (bergamot oil) showed the largest inhibition zone against all bacteria tested (Table 1). As a result, bergamot oil was marked as the chosen natural product extract for the study of synergism with antibiotics.

The study of synergistic effects between bergamot oil and antibiotics tested against MRSA (ATCC43300) and MSSA (ATCC25923) showed that the inhibition zone of the combination of bergamot oil and tetracycline against the ATCC strains of MRSA and MSSA was greater than that of the bergamot or tetracycline alone (Table 2). Therefore, tetracycline was marked as the chosen antibiotic for the synergistic study. In addition, the combination of bergamot oil and tetracycline reduced the MIC and MBC of tetracycline against the ATCC strains of MRSA and MSSA (Table 3). Kinetics of kill study showed that the mixture of bergamot oil and tetracycline was superior to bergamot oil or tetracycline alone in the rate at which it killed both MRSA and MSSA as shown in Table 4. The mixture of bergamot oil and tetracycline killed MRSA and MSSA at least 3 logs in 30 minutes whereas tetracycline and bergamot oil separately took more than 30 minutes to achieve the same kill.

5. Discussion

Resistance of MRSA strains to many antibiotics make it difficult to treat infections caused by this pathogen. Several new strategies to treat MRSA have been considered including the use of natural products and synergistic effects between antibiotics and natural product extracts (Chomnawang, Surassmo, Wongsariya, & Bunyapraphatsara, 2009). The combination may reduce adverse effects from antibiotics and prevent the emergence of resistant strains (Farooqui et al., 2015).

In this study, the antimicrobial screening of twenty-five natural plant product extracts were tested against MSSA and MRSA. Seven powdered crude extracts of plants dissolved in distilled water, fifteen powdered crude extracts dissolved in 20% DMSO, and freshly squeezed juice of *Capsicum flutescens* L. and *Piper nigerum* were not active against the test bacteria. In contrast, *Citrus hystrix* or bergamot oil obtained by distillation technique showed antibacterial activity against all *S. aureus* tested. The result indicated that the active antimicrobial compounds in this plant should be non-polar compounds which could be dissolved in organic solvents. Fisher and Phillips (2006) determined the composition of bergamot oil using gas chromatography. It was showed that the major compound of this extract was linalool. Therefore, linalool may be the active compound in bergamot oil that shows antibacterial activity against *S.aureus*.

The potential effect of combinations of different antibiotics or natural substances has been exhaustively studied because the synergistic interaction between natural products and antibiotic can lead to new products with broad spectrum biological activity (Rani, Jain, Dureja, Kumar, &

Kumar, 2009). In the present study bergamot oil from *Citrus hystrix* was used to study the synergism with antibiotic disks. The results of the combination revealed that *Citrus hystrix* showed synergistic effect against *S.aureus* when combined with tetracycline.

Tetracycline inhibits protein synthesis by blocking the attachment of charged aminoacyl-tRNA to the A site on the ribosome and prevents introduction of new amino acids to the nascent peptide chain. Bacteria usually acquire resistance to tetracycline from horizontal transfer of a gene that either encodes an efflux pump or a ribosomal protection protein. Efflux pumps actively eject tetracycline from the cell, preventing the buildup of an inhibitory concentration of tetracycline in the cytoplasm. Ribosomal protection proteins interact with the ribosome and dislodge tetracycline from the ribosome, allowing for translation to continue (Chopra & Roberts, 2001). The reduced MIC and MBC of tetracycline when combined with bergamot oil presented in this study could be due to either the alteration of tetracycline activity or bacterial antibiotic resistance, or even both. Therefore, further studies should be performed to demonstrate the mechanism of the synergistic effect.

6. Conclusion

The bergamot oil demonstrated antibacterial activity against MRSA and MSSA. The combination of bergamot oil and tetracycline reduced the MIC and MBC of tetracycline against MRSA and MSSA. The results indicated that the combination of bergamot oil and tetracycline could be synergistic in antibacterial effects against MRSA and MSSA. Although bergamot oil itself showed lower MIC, the purpose of this study was to see if natural product extracts would reduce the MIC of antibiotics when used together. In this case the result showed that tetracycline was needed in lower concentration to inhibit MRSA and MSSA when combined with bergamot oil. This would give an option in combatting with MRSA and MSSA if the use of antibiotics is prefer to natural products alone. In addition, the mixture of bergamot oil and tetracycline killed MRSA and MSSA at least 3logs in 30 minutes whereas tetracycline and bergamot oil separately took more than 30 minutes to achieve the same kill. This report might provide alternative methods to treat infections caused by antibiotic resistant strains of *S. aureus*. Synergistic effect enables the use of tetracycline when it is not effective on its own against resistant bacteria.

Table 1 Inhibition zone (mm.) of natural product extracts against *S.aureus* strains

Natural products	*S.aureus* ATCC25923	*S.aureus* ATCC43300	MRSA Clinical isolates	MSSA Clinical isolates
Aloe vera L.	N	N	N	N
Centella asiatica L.	N	N	N	N
Eurycoma longifolia	N	N	N	N
Angle marmelos	N	N	N	N
Morus alba L.	N	N	N	N
Stevia rebandiana	N	N	N	N
Ganoderma lucidum	N	N	N	N
Dillenia indica	N	N	N	N
Clitoria ternatea L.	N	N	N	N
Tiliacora triandra	N	N	N	N
Gracinia mangstana	N	N	N	N
Kaempferia parviflora Wall	N	N	N	N
Alpinia galangal	N	N	N	N
Curcuma zanthorrhiza Roxb.	N	N	N	N
Ficus pubigera Wall.	N	N	N	N
Hibiscus sabdariffa L.	N	N	N	N
Curcuma longa L.	N	N	N	N
Vitis vinifera L.	N	N	N	N
Nelumbo nuclfera	N	N	N	N
Cryptoepis buchanani	N	N	N	N
Anaxagorea luzonensis	N	N	N	N
Cryperus rotundus	N	N	N	N
Citrus hystrix (bergamot oil)	25	27	28±1	24±2
Capsicum flutescens L.	N	N	N	N
Piper nigerum	N	N	N	N

N= no inhibition zone

Table 2 Inhibition zone (mm.) of bergamot oil and combination with antibiotics against *S.aureus* strains

Natural product and antibiotics	*S.aureus* ATCC25923	*S.aureus* ATCC43300
bergamot oil	25	32
clindamycin	24	No inhibition zone
bergamot oil+ clindamycin	25	33
oxacillin	15	No inhibition zone
bergamot oil+ oxacillin	23	24
ampicillin	20	No inhibition zone
bergamot oil+ ampicillin	21	29
erythromycin	27	No inhibition zone
bergamot oil+ erythromycin	26	28
ceforoxime	25	No inhibition zone
bergamot oil+ ceforoxime	27	22
ciprofloxacin	26	No inhibition zone
bergamot oil+ ciprofloxacin	27	29
tetracyclin	8	No inhibition zone
bergamot oil+ tetracyclin	26	39
sulfamethoxazole	30	30
bergamot oil+ sulfamethoxazole	30	30

Table 3 MIC and MBC of tetracyclin and bergamot oil

strain	compound	MIC (µg/ml)	MBC (µg/ml)
MRSA (ATCC43300)	tetracyclin	16.2	32.4
	bergamot oil	0.39	0.78
	tetracyclin (in combination with bergamot oil)	4.05	4.05
MSSA (ATCC25923)	tetracyclin	8.1	8.1
	bergamot oil	0.39	0.78
	tetracyclin (in combination with bergamot oil)	4.05	4.05

Table 4 Time kill of the mixture of tetracyclin and bergamot oil against the ATCC strains of MRSA and MSSA

		Bacterial cell number (CFU/mL)			
	Contact time	Growth control	Tetracyclin	Bergamot oil	Mixture of tetracyclin and bergamot oil
MRSA	0 min	1.6×10^7	1.6×10^7	1.6×10^7	1.6×10^7
	5 min	Not determined	2.0×10^7	2.8×10^5	2.1×10^5
	10 min	Not determined	1.8×10^7	2.1×10^5	1.3×10^5
	30 min	5.6×10^8	1.2×10^7	1.0×10^5	0
	60 min	1.1×10^9	8.0×10^6	0	0
MSSA	0 min	1.9×10^7	1.9×10^7	1.9×10^7	1.9×10^7
	5 min	Not determined	8.2×10^5	3.2×10^5	1.4×10^5
	10 min	Not determined	2.3×10^5	2.0×10^5	0
	30 min	5.6×10^8	3.1×10^5	1.1×10^5	0
	60 min	1.2×10^9	0	0	0

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