SYNERGISTIC INTERACTION BETWEEN ANTIBIOTICS AND NATURAL PRODUCT EXTRACTS TO INHIBIT METHICILLIN-RESISTANT *Staphylococcus aureus* (MRSA)

BY

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การเสริมฤทธิ์ระหว่างยาปฏิชีวนะและสารสกัดธรรมชาติในการยับยั้ง *Staphylococcus aureus* ที่ดื้อต่อยา Methicillin (MRSA)

โดย

กีรติ จ้อยจำรัส

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Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the *Staphylococcus aureus* strains that resists to a variety of antibiotics, especially β–lactam antibiotics. MRSA infections are difficult to treat because very few antibiotics are effective against MRSA. Therefore, the objectives of this research were to search for potential antimicrobial agents from native Thai plant extracts and to study the synergism between these plant extracts and antibiotics against MRSA.

Eleven native Thai plants, i.e., *Acanthus ebracteatus* Vahl., *Cissus quadrangularis* Lin, *Kaempferia parviflora*, *Barleria lupulina* Lindl, *Orthosiphon aristatus* (Blume) Miq, *Glycyrrhiza glabra* L., *Stevia rebaudiana* Bertoni, *Clitoria ternatea* Linn., *Curcuma longa* L., *Curcuma zedoaria* (Berg.) Rosc. and *Zingiber cassumunar* Roxb were extracted with water and 95% ethanol. These extracts were determined for antimicrobial activities against *S.aureus* by agar-well diffusion method. Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) were also determined. The synergistic effect between crude plant extracts and antibiotics was assayed using disc diffusion technique. In addition, the interaction between crude plant extracts and antimicrobial agents was estimated by calculating the fractional inhibitory concentration (FIC index) of the combination.

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The results showed that *Kaempferia parviflora* ethanol extract possessed antibacterial activity against MRSA but not MSSA with inhibition zones of 15±3 mm. The MIC and MBC of *Kaempferia parviflora* ethanol extract against MRSA was 46.87 µg/mL and 96.75 µg/mL, respectively. Study of synergism between plant extracts and antibiotics revealed that when *Kaempferia parviflora* ethanol extract was combined with cefoxitin, the MIC and MBC of cefoxitin against MRSA reduced from 30.47±11.32 µg/mL to 1.76±0.62 µg/mL and from 89.06±14.82 µg/mL to 3.74±1.21 µg/mL, respectively. In addition, the FIC of this combination indicated that *Kaempferia parviflora* ethanol extract and cefoxitin could be synergistic in antibacterial activities against MRSA.

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INTRODUCTION

1.1 Background

The staphylococci are a group of gram positive bacteria that cause a variety of diseases in humans. *Staphylococcus aureus* is the most virulent staphylococcal species. *S.aureus* causes disease through both toxin-mediated and non toxin-mediated mechanisms. *S.aureus* produces numerous human diseases including abscesses, bacteremia, central nervous system infections, endocarditis, osteomyelitis, urinary tract infections, pneumonia, and various syndromes caused by exotoxin such as bullous impetigo, food poisoning, scaled skin syndrome, and toxic shock syndrome (Stephen, et al., 2006).

The treatment of choice for *S.aureus* infections is penicillin. However, some strains of *S.aureus* have developed resistance to penicillin due to the production of an enzyme by the bacteria called penicillinase. The introduction of the semi-synthetic β-lactamase-resistant penicillins such as methicillin and oxacillin in 1959 brought about a general decline in the penicillin resistant *S.aureus* during early 1960. In 1961 Methicillin-resistant *Staphylococcus aureus* (MRSA) were first detected in a hospital in the United Kingdom and the first reported case of MRSA in the United States was in 1968. MRSA is a mutated form of *Staphylococcus aureus* resistant to antibiotics, known as β-lactams such as methicillin, oxacillin, penicillin, and amoxicillin and cephalosporins such as cephalexin and ceflactor (Vardi, et al., 2012).

The emergence of MRSA has led to the development of novel antibiotics. Enormous efforts are being made to synthesize and develop new compounds which can be used as potential antimicrobial agents. Among the potential sources of new

antimicrobial agents, natural products could be an interesting alternative. Important sources of natural products are plants or herbs which are rich in a wide variety of active compounds such as tannin, terpenoids, alkaloids and flavonoids. These compounds have been found in vitro to have antimicrobial properties with less side effects and toxicity when compared to synthetic agents. Therefore, attention has been focused on natural products because they contain complex compound and novel modes of action (Yang, et al., 2011).

Many natural products have a variety of biological activities; therefore, they could exhibit the important role in the combination therapy. The use of drug combinations between natural products and antibiotics may improve the efficacy of antibiotics against resistant bacteria (Li, et al., 2010). In addition, the synergistic interactions between antibiotics and natural products may increase efficiency, reduce undesirable effects, increase the stability or bioavailability of the reactive agents and obtain an adequate therapeutic effect with relatively small doses (Chanda, et al., 2011).

Therefore, the efficiency of natural products to inhibit MRSA will be determined in this study. In addition, the synergism between natural products and some antibiotics to inhibit MRSA will also be evaluated in order to reduce the adverse effects and increase the antibiotic activities to inhibit the resistant bacteria.

1.2 Objectives of research

1. To evaluate the antibacterial activities of natural product extracts against Methicillin-susceptible *Staphylococcus aureus* (MSSA) and Methicillin-resistant *Staphylococcus aureus* (MRSA)

2. To study the synergistic activities between natural product extracts and antibiotics against Methicillin-susceptible *Staphylococcus aureus* (MSSA) and Methicillin-resistant *Staphylococcus aureus* (MRSA)

1.3 Research hypothesis

1. The crude extract from natural plants could show antibacterial activities against clinical isolates of Methicillin susceptible *Staphylococcus aureus* and Methicillin resistant *Staphylococcus aureus*

2. The crude extract from natural plants combined with certain antibiotics could show synergistic effects on antibacterial activity against clinical isolates of Methicillin susceptible *Staphylococcus aureus* and Methicillin resistant *Staphylococcus aureus*

1.4 Scope of the research

The antibacterial activities of natural product extracts against Methicillin-resistant *Staphylococcus aureus* (MRSA) and Methicillin susceptible *Staphylococcus aureus* (MSSA) are investigated in this study. The plants used in this study are *Acanthus ebracteatus* Vahl, *Cissus quadrangularis* L., *Kaempferia parviflora* Wall. ex Baker, *Barleri alupulina* Lindl., *Orthosiphon aristatus* (Blume) Miq., *Glycyrrhiza glabra* L., *Stevia rebaudiana* (Bertoni) Bertoni., *Clitoria bracteata* Poir., *Curcuma longa* L., *Curcuma zerumbet* (Burg) Roxb., and *Zingiber cassumunar* Roxb. All plants are extracted with water and 95% ethanol. The effective plant crude extracts are further investigated for minimal inhibitory concentration and minimal bactericidal concentration. In addition, the synergistic activity between crude plant extracts and antibiotics are also evaluated.

CHAPTER 2

LITERATURE REVIEWS

2.1 General characteristics of *Staphylococcus aureus*

Staphylococcus aureus is Gram-positive cocci, with diameters of 0.5-1.5 µm. The bacteria divide in more than one plane to form grape-like clusters. *S.aureus* is non-motile, non-spore forming facultative anaerobe that grows by aerobic respiration or by fermentation. *S.aureus* can grow in the presence of 10% sodium chloride and at 18-40 °C. *S.aureus* is able to grow on Nutrient agar, Tryptic soy agar and Brain heart infusion agar, and the bacterial colonies are observed within 24 h under incubated at 35-37 °C. Colonies of *S.aureus* on nutrient agar are pigmented, ranging from pale-yellow to orange. Colonies of *S.aureus* are 1-3 mm in diameter, smooth, entire, and show β-hemolysis on blood agar. Members of the genus *Staphylococcus* are catalase-positive and oxidase-negative, distinguishing them from the genus *Streptococcus*, which are catalase-negative. (Stephen, et al., 2006).

Staphylococcus aureus can produce coagulase which is a useful criteria for *S.aureus* characterization. *S.aureus* can ferment mannitol and tolerate high concentration of salt. Therefore, *S.aureus* cultured on Mannitol salt agar produce yellow colonies and a yellow color in surrounding medium as a result of acidification of mannitol in the presence of phenol red indicator. The fermentation of mannitol by *S.aureus* is helpful in its differentiation from *S.epidermidis*. *S.aureus* can produce energy by using a variety of carbohydrates as carbon and energy sources (Watson, et al., 1998). *S. aureus* has two main components of the cell wall, namely lipoteichoic acid and peptidoglycan. The hydrophobic domain of lipoteichoic acid has a role in adherence, whereas peptidoglycan covalently links adhesive proteins (O'Riordan, et al., 2004).

Cell wall-associated virulence factors of *S. aureus* include adhesins, exopolysaccharides and cell wall components such as peptidoglycan and teichoic acid. *S. aureus* synthesizes two broad categories of adhesins, microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) and secreted expanded-repertoire adhesive molecules (SERAMs). Exopolysaccharides, capsule polysaccharides and polysaccharide intercellular adhesin (PIA) are important virulence factors in *S. aureus*. There are a total of 11 capsule serotypes identified in *S. aureus*, which serotype 5 and 8 are the most common. *S.aureus* capsule enhances resistance to phagocytosis (Ferry, et al., 2005).

Staphylococcus aureus expresses enzymes and exotoxins which help the bacteria capable of lysing and invading host tissues, and some strains produce superantigenic toxins that interact directly with the immune system (Ferry, et al., 2005). Many enzymes are produced by *S.aureus*, such as coagulase, protease and hyaluronidase. Coagulase coagulates plasma and blood and also causes fibrin to deposit around bacterial cells. Proteases produced by *S. aureus* are involved in tissue invasion and immunomodulation (Langley, et al., 2003). In addition, proteases can degrade or activate extracellular proteins such as host immunoglobulin, antimicrobial peptides and host tissues (Dinges, et al., 2000). Hyaluronidase hydrolyses hyaluronic acid in intracellular matrix of connective tissue and facilitates the spread of *S.aureus* in tissue during infection.

Toxins produced by *Staphylococcus aureus* include cytotoxins, enterotoxin, and superantigens. Cytotoxins such as α-toxin, β-toxin, γ-toxin, and leukocidins not only lyse host cells but also alter the host immune response, for example, inducing caspase-dependent and caspase-independent apoptosis.

Superantigens are a class of protein toxins that can cause nonspecific T-cell activation and massive cytokines release. Superantigens produced by *S. aureus* include enterotoxins, toxic shock syndrome toxin-1 (TSST-1) and exfoliative toxins (ETs). Enterotoxins cause food poisoning, whereas toxic shock syndrome toxin is responsible for toxic shock syndrome. Exfoliative toxins cause degradation,

exfoliation and also alteration desmoglein-1, a cell-cell adhesin in the epidermis (Bhatia, et al., 2007). .

2.2 Clinical significance of *S.aureus*

Staphylococcus aureus causes a wide range of infections including community and hospital acquired infections. The most common infections are skin and soft tissue infections that include abscesses, cellulitis, impetigo and postoperative surgical wounds with osteomyelitis (Maor, et al., 2009).

Skin infections caused by the coagulase-positive *S.aureus* through the production of toxins and enzymes. The most frequent lesion is a cutaneous abscess or furuncles which are the infections of sebaceous glands or hair follicle. Furuncles were connected abscess to multiple foci and extended into the deeper layer of tissue. *S.aureus* is a major cause of impetigo, either alone or in conjunction with streptococci. The infections are seen in school children, often around the face.

Toxic shock syndrome (TSS), a common community-acquired diseases, has also been attributed to infection or colonization with *S.aureus*. Toxic shock syndrome toxin (TSST-1) and the staphylococcal enterotoxins are known as pyrogenic toxin superantigens (PTSAgs). (Todd J., et al., 1988) Toxic shock syndrome (TSS) is an acute and potentially fatal illness, multisystem disease characterized by high fever, accompanied by low blood pressure, malaise and confusion, which can rapidly progress to stupor, coma, and multiple organs failure (Parsonnet, et al., 2010).

Staphylococcal scalded skin syndrome (SSSS), known as Pemphigus-neonatorum or Ritter's disease, is triggered by epidermolytic exotoxins (exfoliatin) A and B, which are released by *S.aureus* and cause detachment within the epidermal layer by breaking down tight-junctions. Patients with SSSS may have fever and malaise developing a few days after localized staphylococcal infection. They may develop tender diffuse erythematous eruption with flaccid bullae (Ladhani, et al., 1998).

Bacteremia may occur following any localized *S.aureus* infections of the skin, lung and genitourinary tract. The presence of *S.aureus* in blood stream causing endocarditis is commonly seen in patients with cardiovascular disease, diabetes mellitus, and immunologic deficiency. (Maor, et al., 2009)

2.3 Treatment of *Staphylococcus aureus* infections

Infections due to some strains of *S.aureus* can be treated with many anti-microbial agents. Penicillin is the most active antimicrobial agents, but many *S.aureus* strains now resist to penicillins. The resistance to penicillin dues to the production of penicillinase (β-lactamase), which hydrolyzes β-lactam ring of penicillin forming penicillonic acid, which has no antimicrobial activity. (Lowy, 2003)

Because of the problem with penicillin-resistant *S.aureus*, methicillin the first semisynthetic penicillin-resistant to penicillinase was introduced in 1961. At this time the methicillin-resistant strains were isolated in a hospital in the United Kingdom. In 1970, strains of Methicillin resistance *Staphylococcus aureus* (MRSA) were reported in many hospitals in United Kingdom. Since then, MRSA associated with epidemic spread infections have been described throughout the world. The penicillinase-stable penicillins, for example, oxacillin and flucloxacillin are still the main treatment of *S.aureus*.

2.4 Methicillin resistance *Staphylococcus aureus* (MRSA)

Infections due to some strains of *S.aureus* can be treated by many antimicrobial agents such as beta-lactam antibiotics. Beta-lactam antibiotics act by inhibiting enzymes involved in assembling the bacterial cell wall. These enzymes are found in the membrane and catalyze the cross-linking reaction between the peptidoglycan polymers. This cross-linking gives the wall additional rigidity, which is essential to the cell. Many enzymes covalently bind beta-lactam antibiotics at their active site and have been termed penicillin-binding proteins (PBPs).

The fundamental difference between susceptible staphylococci and methicillin resistant staphylococci is in their PBPs. Five PBPs (1, 2, 3, 3', and 4) have been described for susceptible strains of *Staphylococcus aureus*. The physiologic functions of the individual staphylococcal PBPs as transpeptidases, endopeptidases, or carboxypeptidases have not been defined completely. PBPs 1, 2, and 3 appear to be necessary for cell growth and survival.

Methicillin resistance is associated with production of a unique PBP by the *mecA* gene, which is not present in Methicillin susceptible staphylococci. Unlike the other PBPs, PBP2a or PBP2' has a low binding affinity for beta-lactam antibiotics (Hartman, et al., 1984). It is presumed that PBP2a can perform the functions of the other high-affinity PBPs at antibiotic concentrations which inactivate the other PBPs and would therefore otherwise be lethal. Methicillin resistant strains of coagulase-negative staphylococci also produce PBP2a (Tomasz, et al., 1989). Despite the presence of inhibitory concentrations of beta-lactam antibiotics, MRSA can continue cell wall synthesis depending on the uninhibited activity of PBP2a (Katayama, et al., 2000). Although *S.aureus* strains carry the *mecA* gene, only a few strains will express this gene. The expression of *mecA* gene can be constitutive or inducible. This gene is carried on a mobile genetic element, the staphylococcal cassette chromosome mec (SCCmec).

MRSA strains are frequently resistant to common antibiotics including penicillins, cephalosporins, erythromycin, streptomycin, tetracycline, aminoglycoside, fluoroquinolone and other β-lactam antibiotics. The problem of resistance to almost all clinically available antibiotics makes the treatment of MRSA become increasingly difficult and is a serious global problem. Moreover, sometimes antibiotics used for the treatment the infectious diseases are associated with side effects on the host including nausea, vomiting, headache, hypersensitivity, immune-suppression or allergic reactions and may cause severe damage to the liver and cause bone marrow depression. Enormous efforts have been made to synthesize and develop new compounds which can be used as potential antimicrobial agents to combat MRSA infections. Therefore, the search for new effective antimicrobial agents is interesting.

Among the potential sources of new antimicrobial agents, natural products could be interesting alternatives**.**

2.5 Natural products and their antimicrobial activities

Natural products have been used in traditional medicine in many countries. In addition, natural products are used for the development of novel drugs to treat various microbial infections. The extracts of *Senna alata, Eupatorium odoratum, Garcinia mangostana* and *Barleria lupulina* showed strong inhibit the growth of *Propionibacterium acnes* (zone of inhibition >15 mm) (Chomnawang, et al., 2005).

Since many available antimicrobial agents are failure to treat *Staphylococcus aureus* infection, some researchers have focused on the investigation of natural products as source of new bioactive molecules. Suffredini et al., (2004) reported that the plant extracted from Brazilian Amazon rain forest were screened for antibacterial activity against *Staphylococcus aureus*, using a microdilution broth assay, One extract of Brazilian Amazon rain forest was active against *Staphylococcus aureus*. The result show that minimum inhibitory concentration (MIC) was 140 µg/ml and minimal bactericidal concentration (MBC) was 160 µg/ml. The extract of *Camellia sinensis* (leaves), *Delonix regia* (flowers), *Holarrhena antidysenterica* (bark), *Lawsonia inermis* (leaves), *Punica granatum* (rind), *Terminalia chebula* (fruits) and *Terminalia belerica* (fruits) showed broad-spectrum antibacterial activity against all MRSA and MSSA strains with inhibition zone size of 11–27 mm (Aqil, et al., 2005). Essential oils distilled from plants such as lemongrass, lemon myrtle, mountain savory, cinnamon, Melissa and myrtle, exhibited zones of inhibition against MRSA. In addition, these essential oils combined with *Eucalyptus globulus, Eucalyptus australiana, Eucalyptus radiata,* marjoram, pine, cypress, lavender, spruce, peppermint and *Eucalyptus citriodora* oils exhibited inhibitory activity against MRSA (Chao, et al., 2008).

Many researches on the natural products are extended with the identification of the bioactive compound of antibacterial activity in the plants. Plant based natural

constituents can be derived from any part of the plant like bark, leaves, flowers, roots, fruits, seeds, etc. Some parts of the plant may contain active components. The beneficial medicinal effects of plant materials typically result from the combinations of secondary products present in the plant.

2.5.1 *Acanthus ebracteatus* Vahl.

Acanthus ebracteatus Vahl. is a member of the *Acanthaceae* family. *Acanthus ebracteatus*Vahl. has been used as folk medicine for a wide range of tropical diseases. This plant is used as a purgative and anti-inflammatory in arthritis. The whole plant boiled in water can be used to heal rash and skin diseases. The fresh plant is crushed and applied as a poultice in boils or taken orally as depurative. The fruits are taken orally to ease menstrual disorder (Somchaichana, et al., 2012).

*Acanthus ebracteatus*Vahl. is a spiny mangrove herb that is used in an Ayurvedic folk medicine and is commonly distributed in Southeast Asia. It is composed of several chemical constituents, including alkaloids, flavonoids, triterpenoids, sterols, aliphatic glycosides, lignans, phenolic glycosides, quaternary amino acids, and polysaccharides (Mahasiripanth, et al., 2012).

Figure 2.1 *Acanthus ebracteatus* Vahl.
Source: http://home.generalprempark.com, 2 May 2013

2.5.2 *Cissus quadrangularis* Linn.

Cissus quadrangularis Linn. (Syn. *Vitis quadranglularis* L.) is a member of *Vitaceae* family. It is a rambling shrub usually found in hotter parts of India, Sri Lanka, Malaysia, Java and West Asia (Rao, et al., 2011).The stem is useful in piles. The juice of the plant is used to control irregular menstruation, diseases of the ear and in nose bleeding. A paste of the stem is given in asthma and may be useful for muscular pains, burns, and wounds.

Phytochemical studies of *Cissus quadrangularis* Linn. showed the presence of various versatile constituents such as lipids, stilbenoids, triterpenoids, steroids, iridoids and flavonoids (Mishra, et al., 2010).

Figure 2.2 *Cissus quadrangularis* Linn.
Source: http://www.thaicrudedrug.com, 2 May 2013

2.5.3 *Kaempferia parviflora*

Kaempferia parviflora belongs to the *Zingiberaceae* family and is locally known in Thai as Kra-chai-dam. This plant has been used for the treatment of inflammation, diarrhea, vertigo and heart disease (Chaturapanich, et al., 2012). In Thai traditional medicine, the decoction of *K. parviflora* powder with ethanol has been reported to cure fungal infection, allergy, asthma, peptic ulcer and diabetes. This plant extract contains flavonoids, borneol and sylvestrene (Kummee, et al., 2008).

Figure 2.3 *Kaempferia parviflora*

Source: http://64.33.68.172/siamnatural/Krachaidum_info.htm, 2 May 2013

2.5.4 *Barleria lupulina* Lindl.

Barleria lupulina Lindl. (Acanthaceae) is a well-known historical herb used as traditional medicine in India, Thailand and Pakistan. The plant is used for several aliments such as mental disorders, diabetes, snake bites and rheumatoid arthritis. This plant used for general health promoting agents, anti-inflammatory agents, anti-gastric ulcer, antimutagenic activities, antimalarial, antiviral and antimycobacterial infections as well as for the treatment of gastrointestinal disorders, (Suba, et al., 2005).

Figure 2.4 *Barleria lupulina* Lindl.

Source: http://keyserver.lucidcentral.org/weeds, 2 May 2013

2.5.5 *Orthosiphon aristatus* (Blume) Miq.

Orthosiphon aristatus (Blume) Miq. belongs to the family *Lamiaceae*. It has many local names in Thai, such as Yaa-nuat-maeo, Phayap-mek and Bangrak-pa (Pattamadilok, et al., 2003). Many studies indicate that the leaves of *O. aristatus* contain several compounds, including neoorthosiphols A, neoorthosiphols B, ursolic acid, oleanolic acid, acetovanilla- chromene, orthochromene A, orthosiphol A, orthosipholl B, orthosiphonone A, orthosiphonone B, lipophilic flavones, flavonol glycosides and caffeic acid derivatives (Hsu, et al., 2010). Indinesian traditional folk medicine has been used for alternative medicine of various diseases; gout, diabetes mellitus, hypertension, rheumatism, tonsillitis and menstrual disorder and especially those affecting the urinary tract, that is for treating kidney ailments and bladder related diseases (Hunaefi, et al., 2013).

Figure 2.5 *Orthosiphon aristatus* (Blume) Miq
Source: http://saintlucianplants.com, 2 May 2013

2.5.6 *Glycyrrhiza glabra* L.

Glycyrrhiza glabra L., known as licorice and sweetwood, is native to the Mediterranean and certain areas of Asia. Historically, the dried rhizome and root of this plant were employed medicinally by the Egyptian, Chinese, Greek, Indian, and Roman civilizations as an expectorant and carminative (Kondo, et al., 2007). *Glycyrrhiza glabra* L. is composed of triterpene, saponins, flavonoids, poly-saccharides, pectins, simple sugars, amino acids, mineral salts, and various other

substances. This plant has been proved for pharmacological activities such as anti-ulcer activity, anti-asthmatic activity, anti-diuretic activity and anti-herpes simplex (Anil, et al., 2012).

Figure 2.6 *Glycyrrhiza glabra* L.
Source: http://wonderherbs.org/gg.html, 2 May 2013

2.5.7 *Stevia rebaudiana* Bertoni

*Stevia rebaudiana*Bertoni is a perennial herb of the *Asteraceae* family, native to the Amambay region of the North Eastern Paraguay. This plant is also found in Brazil and Argentina (Tavarini, et al., 2012). The leaves of *Stevia rebaudiana* Bertoni contain diterpene derivative steviol (ent-13-hydroxykaur-16-en-19-oic acid). The effects of *S. rebaudiana* Bertoni extracts showed anti-hyperglycemic effects, anti-hypertensive effects, antioxidant activity, and currently they are used as an animal feed supplement (Puri, et al., 2011).

Figure 2.7 *Stevia rebaudiana* Bertoni
Source: http://www.henriettesherbal.com, 2 May 2013

2.5.8 *Clitoria ternatea* Linn.

Clitoria ternatea Linn. belonging to the family *Fabaceae* is aperient twining herb, found in Indo-China, Philippines and Madagascar. Since the flowers of the plant resemble a conch shell, it is commonly called “Shankpushpi” (Daisy, et al., 2009). This plant is an important source of sugar, protein, free amino acids, oil, aglycones, unsaturated sterols, triterpenes, cardiac glycosides and saponin (Subramanian, et al., 2011). This plant has been used for anti-hypoglycemic and anti-helmintic activity.

Figure 2.8 *Clitoria ternatea* Linn.
Source: http://enchantingkerala.org/ayurveda, 2 May 2013

2.5.9 *Curcuma longa* L.

Turmeric is a spice derived from the rhizomes of *Curcuma longa* L., which is a member of the ginger family (*Zingiberaceae)* (Kumar, et al., 2006). Rhizomes are horizontal underground stems that send out shoots as well as roots. The bright yellow color of turmeric comes mainly from fat-soluble, polyphenolic pigments known as curcuminoids. Studies have shown that curcumin is not toxic to humans. Curcumin exerts anti-inflammatory activity by inhibition of a number of different molecules that play an important role in inflammation. Turmeric is effective in reducing post-surgical inflammation (Akram, et al., 2010).

Figure 2.9 *Curcuma longa* L.

Source: http://www.tramil.net/fototeca, 2 May 2013

2.5.10 *Curcuma zedoaria* (Berg.) Rosc.

Curcuma zedoaria (Berg.) Rosc. is also known as round zedoary in English. The rhizome of *C.zedoaria* is used as appetizer and tonic, particularly prescribed to ladies after child birth. Rhizomes yield sesquiterpenes such as curcumol, curcolone, procureumenol, isocurcumenol, furadiene and its iso-derivative, curcumadiol, dehydrocurdione and zederone. Steam distillation of the dried rhizomes yield essential oil containing α-pinene, camphor and sesquiterphenes (Joy, et al., 2002).

Figure 2.10 *Curcuma zedoaria* (Berg.) Rosc.

Source: http://www.aopdh06.doae.go.th, 2 May 2013

2.5.11 *Zingiber cassumunar Roxb.*

Zingiber cassumunar Roxb., commonly known as plai, is widely used in folklore remedies as a single plant or as component of herbal recipes in Thailand and many Asian countries for the treatments of certain conditions, such as inflammation, sprains and strains, rheumatism, muscular pain, wounds, and asthma, cough and respiratory problems, and as a mosquito repellant, acarminative, a mild laxative and an anti-dysenteric agent (Sukatta, et al., 2009).

The main active chemical constituents of the rhizome oil are sabinene, γ-terpinene, α-terpinene, terpinen-4-ol, and (E)-1-(3,4-dimethoxyphenyl)butadiene (DMPBD) (Bhuiyan, et al., 2008).

Figure 2.11 *Zingiber cassumunar* Roxb.
Source: http://www.tistr.or.th/pharma, 2 May 2013

2.6 Synergistic effects of combination of antibiotics and natural products

An alternative approach to treat infectious diseases is the use of plant extracts in the combination with antibiotics. Several studies have proposed that natural products in combination with antibiotics are a new strategy for developing therapies for bacterial infections. The synergistic effect from association of antibiotics with plant extracts against antibiotic resistant bacteria leads to a new choice for the treatment of the infectious diseases. Yang, et al. investigated the synergistic activity of 11 antibiotics with ethanol extract of *Cinnamomum cassia* against *A. baumannii* (ATCC19606), *P. aeruginosa* (ATCC29260) and *S. aureus* (335). The extracts showed synergistic effects with many tested antibiotics, including tetracycline, clindamycin, amikacin, trimethoprim-sulfamethoxazole and carboxyethylpyrrole against *S. aureus* (335) (Yang, et al., 2011).

Piperine was tested in combination with mupirocin against *Staphylococcus aureus* and Methicillin resistance *Staphylococcus aureus*. The result showed that the combination of 50 µg/mL of piperine and mupirocin reduced the MIC of mupirocin from 0.25 to 0.06 µg/mL when tested against *Staphylococcus aureus* ATCC 25923. In addition, the MIC of mupirocin reduced from 0.25 to 0.06 µg/mL when tested against MRSA (Mirza, et al., 2011).

Zhao, et al. (2001) studied the synergistic activity of Epigallocatechin Gallate (EGCg) against MRSA and MSSA. It was showed that the FIC indices of benzyl-penicillin and oxacillin in combination with 6.25, 12.5 or 25 µg/mL of EGCg were 0.126 to 0.625. Adikwu et al. (2010) investigated the synergistic effects of erythromycin and methanol extracts of leaves of *Euphorbia hirta* against *Staphylococcus aureus*.. The MIC of the extracts and erythromycin were 25 mg/mL and 0.005 mg/mL, respectively.. Synergistic effects of erythromycin and *E.hirta* extract against *Staphylococcus aureus* occurred when the ratios of the two compounds

were 9:1, 8:2, 7:3, 6:4, 3:7, 2:8 and 1:9 while the other ratios of these compounds such as 5:5 and 4:6 showed indifference.

Adwan, et al., (2009) studied the synergistic effects of ethanolic extract of *Rus coriaria*, *Sacropoterium spinosum* and *Rosa damascene* and antimicrobial drugs including oxytetracyclin, penicillin G, cephalexin, sulfadimethoxine and enrofloxacin against MRSA. The results showed that the extract was a competitive inhibitor in the protein synthesis process and also revealed high synergism with oxytetracyclin and gentamicin (Adwan, et al., 2009).

CHAPTER 3

MATERIALS AND METHODS

3.1 Bacterial strains

Staphylococcus aureus were isolated from clinical specimens sent to the Clinical Microbiology Laboratory at Thammasat University Hospital. These isolates were identified as *Staphylococcus aureus* by standard laboratory practices including Gram's stain, catalase test, growth on Mannitol salt agar and coagulase test (Brown, et al., 2005)

3.1.1 Gram staining

Each organism was placed onto a microscope slide. Crystal violet solution were added onto the smear and left for one minute. Then the slide was washed off briefly with tap water. After that the smear was flooded with Gram's Iodine solution, left for one minute and washed off with tap water. The smear was decolorized with 95%Ethanol and washed off with tap water. Finally the smear was flooded with safranin solution for one minute and then washed off with tap water. After blotting, the smear was examined under the oil immersion lens.

3.1.2 Catalase test

A small amount of bacterial colonies was placed onto a microscope slide. A drop of 3% H_2O_2 was added onto the smear.

A positive result was the rapid evolution of O_2 as evidenced by bubbling while a negative result was no O_2 bubbles. All Staphylococci were catalase positive.

3.1.3 Growth on Mannitol Salt Agar

Each organism was streaked on Mannitol salt agar plate. The plate was incubated at 35°C for 24-48 hours. All *S.aureus* could grow and produce yellow colonies.

3.1.4 Coagulase test

One or two bacterial colonies cultured on Tryptic soy agar (TSA) plate were suspended in a tube of plasma and incubated at 37°C for 24 hours. If the coagulase is produced by the bacterium, a clot will appear.

3.2 Determination of MRSA and MSSA

All *S.aureus* isolates obtained were screened for methicillin resistance by disc diffusion test using 1 µg oxacillin disc. *S.aureus* ATCC25923 and *S.aureus* ATCC 43300 were also included in this study as a reference strains for MSSA and MRSA, respectively. In addition, polymerase chain reaction (PCR) was performed to detect the *mecA* gene in MRSA isolates. (Murakami, et al., 1991)

3.2.1 Oxacillin disc screening test

A single colony of *S.aureus* was transferred to Tryptic soy broth (TSB) and incubated at 35ºC for 18-24 hours. The bacterial suspension was adjusted to 0.5 Mc Farland standard with TSB to achieve a concentration of approximately 10^8 CFU/mL and inoculated onto TSA plate by three ways swab technique. Then 1 µg oxacillin disc was applied on the surface of the TSA plate. The plate was incubated at 35°C for 24 hours. Resistance was determined according to CLSI where a diameter of the inhibition zone less than10 mm was considered resistant for oxacillin.

3.2.2 Detection of *mecA* gene by Polymerase Chain Reaction (PCR)

Determination *of mecA* gene was performed using the polymerase chain reaction (PCR) method. Primers were selected using the gene sequence deposited in Genbank.

The forward primer was 5'-AAAATCGATGGTAAAGGTTGGC-3'
The reverse primer was 5'-AGTTCTGCAGTACCGGATTTTGC-3'

Extracted DNA was added to a 2 µL PCR mixture containing, 5.0 µL of 10XPCR 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.

DNA Amplifications were performed using the following cycling parameters;

Pre-denaturation	94 °C	3 min	
Denaturation	94 °C	30 seconds	40 cycles
Annealing	55 °C	30 seconds	
Extension	72 °C	1 min	
Final extension	72 °C	5 min	

The amplicons were sized by electrophoresis in 1.5% agarose gel in the presence of ethidium bromide and visualized under UV light. The size of the PCR products is approximately 533 bp.

3.3 Antibiotic susceptibility test

Antibiotic susceptibility test was determined by modified Kirby-Bauer disc diffusion methods according to the Clinical Laboratory Standards Institute, CLSI guidelines (Brown, et al., 2005). One to two colonies were picked by using inoculating loop and suspended in Tryptic soy broth (TSB) and adjusted to match the 0.5 McFarland standard. The bacterial cell suspension was spread on Tryptic soy agar

plate by three-way swab technique. The plate was air dried before antibiotic discs were applied onto the surface of the inoculated plates with sterile forceps. The antibiotic discs used in this study were oxacillin, vancomycin, ampicillin, tetracyclin, ciprofloxacin and cefoxcitin, etc. After 24 hours of incubation period, diameters of inhibition zones were measured and interpreted with a criteria specified by CLSI.

3.4 Preparation of plants materials

Thai plants used in this study consisted of *Acanthus ebracteatus* Vahl, *Cissus quadrangularis* L., *Kaempferia parviflora* Wall. ex Baker, *Barleri alupulina* Lindl., *Orthosiphon aristatus* (Blume) Miq., *Glycyrrhiza glabra* L., *Stevia rebaudiana* (Bertoni) Bertoni., *Clitoria bracteata* Poir., *Curcuma longa* L., *Curcuma zerumbet* (Burg) Roxb., *Zingiber cassumunar* Roxb. Plants samples were dried in oven set at 60°C for 48 hours. After drying, the plants were grounded using blender for 5 minutes.

3.5 Preparation of plants extracts

Thirty grams of dried ground plant materials was added to 500 mL conical flasks. Two-hundred mL of each solvent (water or 95% Ethanol) was added to each flask. Each flask was shaked at a speed of 120 rpm at 25°C for 48 hours. The aqueous extracts were filtered through filter paper and evaporated by freeze drying for 24 hours at -60°C at 200 millitorr vacuum. The ethanol extracts were evaporated by rotary evaporator. All extracts were stored at 4°C.

3.6 Screening of Antibacterial activity of plant extracts

3.6.1 Preparation of inoculum

Methicillin susceptible *Staphylococcus aureus* isolates, Methicillin resistant *Staphylococcus aureus* isolates, reference strains, ATCC 43300 and ATCC 25923 were grown on a TSA plate at 35°C for 24 hours. One or two isolated colonies were

cultured in 5 mL of TSB at 35°C for 24 hours. and then adjusted to match the 0.5 McFarland standard

3.6.2 Agar well diffusion method

Antimicrobial activity was determined by agar well diffusion method. A suspension of any tested bacteria containing about 10^8 CFU/mL was spread on TSA by three-way swab technique using sterile cotton swabs. TSA plate was pouched by cork borer (Ø 6mm) and 80 µl of natural product extracts (5 mg/mL) were added in each well. Standard antibiotic discs were also used as reference.

The plates were then incubated at 35°C for 24 hours and the diameter of the inhibition zone was measured. Zone of inhibition of natural product extracts were compared with controls containing corresponding solvent. Triplicates of each plate have been done.

3.7 Determination of Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC)

3.7.1 Preparation of inoculum

Methicillin susceptible *Staphylococcus aureus* isolates, Methicillin resistant *Staphylococcus aureus* isolates, and reference strains MRSA ATCC 43300 and MSSA ATCC 25923 were grown on a TSA plate at 35°C for 24 hours. One or two isolated colonies were cultured in 5 mL of TSB at 35°C for 24 hours. and then adjusted to match the 0.5 McFarland standard The working inoculum was obtained by diluting to 1:100 in TSB to give a concentration of 10^6 CFU/mL.

3.7.2 Minimal inhibitory concentration (MIC)

The Minimal inhibitory concentration (MIC) was determined by the broth microdilution method, as recommended by CLSI (CLSI, 2014). *Kaempferia parviflora* extract was two-fold serial diluted in 100 µL of TSB into 96 well plates. One-hundred µL of bacteria were added in each dilution and then the plate was incubated at 35°C for 18-24 hours. Each dilution was repeated in duplicates. The MIC was taken as the minimum concentration of the test reagent that inhibited the growth of bacteria.

3.7.3 Minimal bactericidal concentration (MBC)

The Minimal bactericidal concentration (MBC) was defined as the lowest concentration of the compound to kill bacteria. All the dilutions that did not show any turbidity of the bacteria in the MIC studies were determined for MBC. The suspension was streaked onto TSA and incubated at 35˚C for 18-24hours. The lowest concentration that showed no growth on TSA was taken as MBC.

3.8 Synergistic activity between *Kaempferia parviflora* extracts and antibiotics

Preliminary synergistic effect was assayed using disc diffusion technique. The antibiotics used in this assay were ampicillin, tetracycline, vancomycin, oxacillin ciprofloxacin and cefoxcitin. The filter paper disc containing the *Kaempferia parviflora* extract (20 µL/disc), antibiotics disc and mixture of 20 µL *Kaempferia parviflora* extract on antibiotic disc were placed on the TSA plates. The distance between each disc is 30 mm. The plates were incubated at 35˚C for 24 hours. The subsequent zones of inhibition around each disc were measured in three different directions and mean diameters were recorded.

Synergistic activity was determined by the broth microdilution method for detect minimal inhibitory concentration (MIC), as recommended by CLSI (CLSI,

2014). Cefoxicitin was two-fold serial diluted in 50 µL of TSB into 96 well plates and then *Kaempferia parviflora* extract concentration of 11.72-23.44 µg/mL was added in 50 µL into each well. One-hundred µL of bacteria were added in each dilution and then the plate was incubated at 35°C for 18-24 hours. Each dilution was repeated in duplicates. The MIC was taken as the minimum concentration of the test reagent that inhibited the growth of bacteria.

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

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

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

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

Synergism was defined as an FIC index of 0.5 or less, additivity as a FIC index of more than 0.5 and less than 4, and antagonism as FIC index of more than 4 (Ncube, et al., 2012) .

CHAPTER 4

RESULTS

4.1 Bacterial strains

Eighty-one *Staphylococcus aureus* isolates were obtained from clinical specimens sent to the Clinical Microbiology Laboratory at Thammasat University Hospital. All isolates were identified as *Staphylococcus aureus* according to colony morphology, cell morphology, growth and fermentation on Mannitol salt agar, catalase and coagulase production by standard laboratory practices. The results were shown in Figure 4.1-4.3. Fifty-six isolates show all positive results with catalase, coagulase growth on MSA and ferment mannitol were obtained and used for the determination of oxacillin susceptibility test and *mecA* gene.

Figure 4.1 *Staphylococcus aureus* on Tryptic soy agar (TSA)

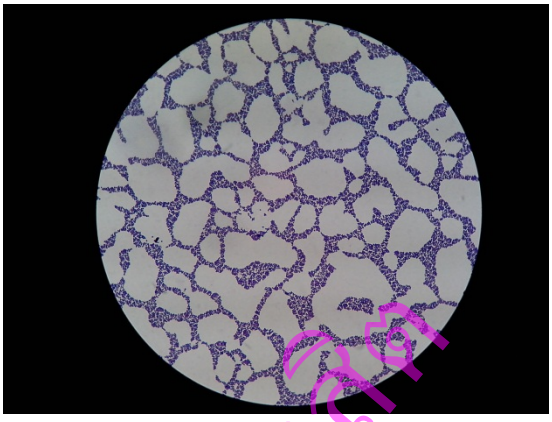

Figure 4.2 Gram stain of *Staphylococcus aureus*

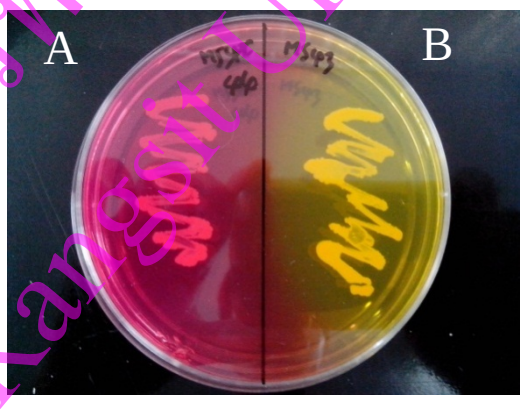

Figure 4.3 Growth of *Staphylococcus aureus* on Mannitol salt agar (MSA)

A : *Staphylococcus epidermidis*

B : *Staphylococcus aureus*

4.2 Screening for MRSA and MSSA

Staphylococcus aureus strains after screened by standard laboratory practices were tested for susceptibility to oxacillin by agar disc diffusion method. The inhibition zone were measured and interpreted with a criteria specified by CLSI.

MRSA isolates exhibited resistance to oxacillin while MSSA isolates showed oxacillin susceptibility. Detection of *mecA* gene revealed that isolates of MRSA showed *mecA* gene while MSSA did not show *mecA* gene (Table 4.1)

The results of *Staphylococcus aureus* identification and MRSA screening are summarized in Table 4.2

Table 4.1 Screening for MRSA and MSSA by oxacillin susceptibility test and determination of *mecA* gene

Strain	Oxacillin susceptibility (Inhibition zone : mm)	Interpretation	*mecA* gene
MSSA1	18	S	-
MSSA2	17	S	-
MSSA3	14	S	-
MSSA4	16	S	-
MSSA5	16	S	-
MSSA6	16	S	-
MSSA7	15	S	-
MSSA8	15	S	-
MSSA9	16	S	-
MSSA10	16	S	-
MSSA11	20	S	-
MSSA12	19	S	-
MSSA13	16	S	-
MSSA14	17	S	-

Table 4.1 Screening for MRSA and MSSA by oxacillin susceptibility test and determination of *mecA* gene (Cont.)

Strain	Oxacillin susceptibility (Inhibition zone : mm)	Interpretation	*mecA* gene
MSSA15	17	S	-
MSSA16	20	S	-
MSSA17	16	S	-
MSSA18	21	S	-
MSSA19	17	S	-
MSSA20	17	S	-
MSSA21	18	S	-
MSSA22	17	S	-
MSSA23	22	S	-
MSSA24	14	S	-
MSSA25	22	S	-
MRSA1	No inhibition zone	R	+
MRSA2	No inhibition zone	R	+
MRSA3	No inhibition zone	R	+
MRSA4	No inhibition zone	R	+
MRSA5	No inhibition zone	R	+
MRSA6	No inhibition zone	R	+
MRSA7	No inhibition zone	R	+
MRSA8	No inhibition zone	R	+
MRSA9	No inhibition zone	R	+
MRSA10	No inhibition zone	R	+
MRSA11	No inhibition zone	R	+
MRSA12	No inhibition zone	R	+
MRSA13	No inhibition zone	R	+
MRSA14	12	I	+
MRSA15	No inhibition zone	R	+
MRSA16	No inhibition zone	R	+
MRSA17	No inhibition zone	R	+
MRSA18	No inhibition zone	R	+

Table 4.1 Screening for MRSA and MSSA by oxacillin susceptibility test and determination of *mecA* gene (Cont.)

Strain	Oxacillin susceptibility (Inhibition zone : mm)	Interpretation	*mecA* gene
MRSA19	No inhibition zone	R	+
MRSA20	No inhibition zone	R	+
MRSA21	No inhibition zone	R	+
MRSA22	No inhibition zone	R	+
MRSA23	No inhibition zone	R	+
MRSA24	No inhibition zone	R	+
MRSA25	No inhibition zone	R	+
MRSA26	No inhibition zone	R	+
MRSA27	No inhibition zone	R	+
MRSA28	No inhibition zone	R	+
MRSA29	No inhibition zone	R	+
MRSA30	No inhibition zone	R	+
MRSA31	No inhibition zone	R	+

Table 4.2 *Staphylococcus aureus* identification results

Type	No. of Strains					Total
	Oxacillin			*mecA*		
	S	I	R	Positive	Negative	
MRSA	-	1	30	31	-	31
MSSA	25	-	-	-	25	25

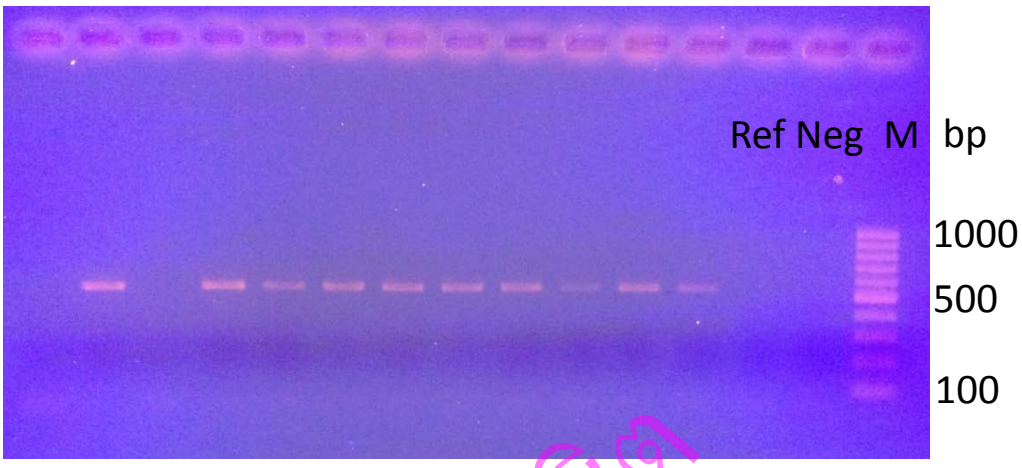

Figure 4.4 Detection of *mecA* gene (The size of PCR product was 533 bp.)

4.3 Screening of Antibacterial activities of plant extracts

The antibacterial activities of eleven plant extracts were tested against all Methicillin resistant *Staphylococcus aureus*, Methicillin susceptible *Staphylococcus aureus Staphylococcus aureus* ATCC 25923 and *Staphylococcus aureus* ATCC 43300 by agar well diffusion method. The results of this study showed that *Kaempferia parviflora* ethanol extract possessed antibacterial activity against only MRSA with inhibition zones of 15±3 mm showed in Table 4.3 and Figure 4.5. From the screening result of the antimicrobial activity, *Kaempferia parviflora* ethanol extract was evaluated for MIC and MBC. The MIC and MBC of *K.parviflora* ethanol extracts against MRSA and MSSA showed in Table 4.4.

Table 4.3 Inhibition zone of plant crude extracts against MRSA and MSSA

Plant extracts	Zone of Inhibition (mm)			
	MSSA		MRSA	
	Aqueous Extract (5mg/ml)	Ethanol extract (5mg/ml)	Aqueous Extract (5mg/ml)	Ethanol extract (5mg/ml)
Acanthus ebracteatus Vahl	-	-	-	-
Cissus quadrangularis Lin	-	-	-	-
Kaempferia parviflora	-	-	-	15±3
Barleria lupulina Lindl	-	-	-	-
Orthosiphon aristatus (Blume) Miq	-	-	-	-
Glycyrrhiza glabra L	-	-	-	-
Stevia rebaudiana Bertoni	-	-	-	-
Clitoria ternatea Linn	-	-	-	-
Curcuma longa L	-	-	-	-
Curcuma zedoaria (Berg.) Rosc	-	-	-	-
Zingiber cassumunar Roxb	-	-	-	-

Table 4.4 Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) of the *K. parviflora* ethanol extracts against MRSA (ATCC 43300) and MSSA (ATCC 25923)

Strain	MIC (µg/ml)	MBC (µg/ml)
MRSA	46.87	96.75
MSSA	750	750

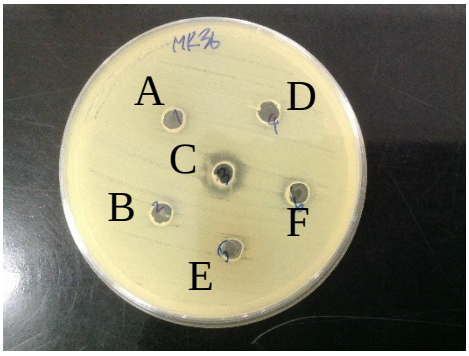

Figure 4.5 Antibacterial activity of *Kaempferia parviflora* ethanol extract

A : Solvent
B : *C. quadrangularis* ethanol extract
C : *K.parviflora* ethanol extract
D : *A.ebracteatus* ethanol extract
E : *B.lupulina* ethanol extract
F : *O.aristatus* ethanol extract

4.4 Antibiotic susceptibility test

The antibiotic susceptibility test of 25 MSSA and 31 MRSA isolates shown in Table 4.5

Table 4.5 Antibiotic susceptibility test

Strain	Antibiotic susceptibility test (mm)					
	Oxacillin	Vancomycin	Ampicillin	Tetracyclin	Ciprofoxacin	Cefoxitin
MSSA1	18	16	18	9	25	24
MSSA2	17	17	16	8	23	24
MSSA3	14	16	14	9	23	24
MSSA4	16	16	14	9	21	24
MSSA5	16	16	13	8	27	23
MSSA6	16	16	13	8	25	23
MSSA7	15	16	13	8	26	22
MSSA8	15	16	13	8	24	24
MSSA9	16	17	15	11	22	24
MSSA10	16	17	16	11	24	21
MSSA11	20	15	41	24	23	24

Table 4.5 Antibiotic susceptibility test (Cont.)

Strain	Antibiotic susceptibility test (mm)					
	Oxacillin	Vancomycin	Ampicillin	Tetracyclin	Ciprofoxacin	Cefoxitin
MSSA12	19	15	35	8	23	23
MSSA13	16	16	12	24	27	23
MSSA14	17	16	13	24	25	24
MSSA15	17	15	11	24	25	25
MSSA16	20	16	38	23	26	23
MSSA17	16	16	12	8	25	22
MSSA18	21	15	23	23	26	22
MSSA19	17	15	12	22	25	24
MSSA20	17	15	14	23	25	23
MSSA21	18	15	12	24	26	23
MSSA22	17	17	8	26	-	-
MSSA23	22	15	39	9	23	24
MSSA24	14	15	12	8	22	24
MSSA25	22	19	19	28	22	-
MRSA1	-	16	13	-	-	20
MRSA2	-	17	9	-	-	-
MRSA3	-	16	13	-	-	11
MRSA4	-	18	14	-	-	9
MRSA5	-	17	14	-	-	13
MRSA6	-	17	14	-	8	9
MRSA7	-	16	12	-	-	8
MRSA8	-	18	8	22	8	-
MRSA9	-	16	12	-	-	9
MRSA10	-	17	13	-	-	10
MRSA11	-	18	9	-	-	14
MRSA12	-	18	14	-	9	9
MRSA13	-	19	12	-	-	-
MRSA14	12	16	10	-	9	18
MRSA15	-	17	9	-	-	15

Table 4.5 Antibiotic susceptibility test (Cont.)

Strain	Antibiotic susceptibility test (mm)					
	Oxacillin	Vancomycin	Ampicillin	Tetracyclin	Ciprofoxacin	Cefoxitin
MRSA16	-	17	R	-	-	9
MRSA17	-	18	12	-	-	-
MRSA18	-	18	12	-	-	-
MRSA19	-	20	9	29	-	-
MRSA20	-	18	12	-	-	-
MRSA21	-	19	14	-	-	-
MRSA22	-	19	15	-	-	-
MRSA23	-	20	9	28	-	-
MRSA24	-	19	10	-	-	-
MRSA25	-	19	15	-	-	-
MRSA26	-	19	15	-	-	-
MRSA27	-	20	9	28	-	-
MRSA28	-	19	13	-	-	-
MRSA29	-	19	15	-	-	-
MRSA30	-	19	15	-	-	-
MRSA31	-	16	17	25	-	-

- : No inhibition zone

4.5 Synergistic activity between *Kaempferia parviflora* ethanol extract and antibiotics

The synergistic effects of *K.parviflora* ethanol extract and antibiotics such as oxacillin, ampicillin, and cefoxitin were tested. The results showed that inhibition zone of the combination of *K.parviflora* and cefoxitin to MRSA was significantly greater than inhibition zone of the extract or antibiotic alone (Table 4.6). In addition, the combination of *K. parviflora* ethanol extract and cefoxitin reduced the MIC and MBC of cefoxitin against MRSA significantly (Table 4.7).

The interaction between *K.parviflora* ethanol extract and antimicrobial agents was estimated by calculating the fractional inhibitory concentration (FIC index) of the combination. The FIC index of the combination of *K.parviflora* ethanol extract and cefoxitin was 0.43. The synergism was defined as an FIC index of 0.5 or less, additivity as a FIC index of more than 0.5 and less than 4, and antagonism as FIC index of more than 4. The result indicated that *K.parviflora* ethanol extract combined with cefoxitin showed synergistic capacity (Table 4.8).

Table 4.6 Synergistic activity between *K. parviflora* ethanol extract and antibiotics

Compound	Zone of inhibition (mm.)	
	MSSA	MRSA
K. parviflora Ethanol extract	No inhibition zone	13.5±1.5
Oxacillin	16.1±1.3	No inhibition zone
Cefoxitin	23.6±0.7	No inhibition zone
Ampicillin	14.5±1.7	No inhibition zone
mixture of *K. parviflora* Ethanol extract (5 mg/ml) and Oxacillin	16.1±1	13.2±1.2
mixture of *K. parviflora* Ethanol extract (5 mg/ml) and Cefoxitin	23.6±0.7	16.3±1.3
mixture of *K. parviflora* Ethanol extract (5 mg/ml) and Ampicillin	14.9±1.6	13.9±1.7

Table 4.7 Synergistic activity between *K. parviflora* ethanol extract and cefoxitin against MRSA and MSSA

Strain	Compound	MIC (µg/ml)	MBC (µg/ml)
MRSA	Cefoxitin	30.47±11.32	89.06±14.82
	K. parviflora ethanol extract	32.81±12.10	70.31±24.71
	Cefoxitin (in combination of *K. parviflora* extract)	1.76±0.62	3.74±1.21
MSSA	Cefoxitin	1.58±1.27	3.04±2.77
	K. parviflora ethanol extract	675±158	750±0
	Cefoxitin (in combination of *K. parviflora* extract)	1.81±1.18	2.48±1.29

Table 4.8 Interaction effect of *K.parviflora* ethanol extracts and Cefoxitin against MRSA

Strains	*K.parviflora*		Cefoxitin		Mean FIC index	Interpretation
	MICs (µg/ml)	MICs combination (µg/ml)	MICs (µg/ml)	MICs combination (µg/ml)		
MRSA	32.81±12.10	12.30±6.45	30.47±11.32	1.76±0.62	0.43	Synergism

CHAPTER 5

DISCUSSIONS AND CONCLUSIONS

Infections from Methicillin-resistant *Staphylococcus aureus* (MRSA) are very difficult to cure because MRSA strains resist almost all clinically available antibiotics. Therefore, searching for new antimicrobial agents from various sources is required. Many compounds found in plants have been shown to possess antimicrobial functions, so they can be served as a source of antimicrobial agents against pathogens. New strategies including the use of natural products and combination of antibiotic and natural product extracts have been considered for treatment of MRSA (Gibbons, 2004). In this study eleven Thai plants, i.e., *Acanthus ebracteatus* Vahl, *Cissus quadrangularis* L., *Kaempferia parviflora* Wall. ex Baker, *Barleri alupulina* Lindl., *Orthosiphon aristatus* (Blume) Miq., *Glycyrrhiza glabra* L., *Stevia rebaudiana* (Bertoni) Bertoni., *Clitoria bracteata* Poir., *Curcuma longa* L., *Curcuma zerumbet* (Burg) Roxb. and *Zingiber cassumunar* Roxb. were extracted with water and 95% ethanol, and the extracts were determined for antimicrobial activities. The *Kaempferia parviflora* ethanol extract demonstrated antimicrobial activity against MRSA while the aqueous extract of this plant did not show antimicrobial activity.

Kaempferia parviflora are widely used in folk medicine for different types of infectious diseases. Kummee, et al. (2008) reported that the ethanol extract of *K. parviflora* exhibited strong anti-fungal activity against *Trichophyton rubrum, Trichophyton mentagrophytes* and *Microsporum gypseum* with MIC values of 62.5, 125 and 250 mg/ml, respectively In addition, Chaichanawongsaroj, et al., (2010) demonstrated that *Kaempferia parviflora* could be one of the effective herbs used for prevention and treatment of *H. pylori* infection since *Kaempferia parviflora* ethyl acetate extract inhibited the invasion of both *H. pylori* virulent strains (cagA+) and non-virulent strains (cagA-) in HEp-2 cells. In addition, Butkhup, et al., (2011)

demonstrated that *Kaempferia parviflora* methanol extract had the inhibitory effect against *Bacillus cereus, Staphylococcus aureus, Streptococcus faecalis, Shigella dysenteriae* and *Salmonella Typhi*. It is remarkable that the *Kaempferia parviflora* methanol extract showed antibacterial activity against *Salmonella Typhi* with a MIC to 4 µg/mL.

In this study *Kaempferia parviflora* ethanol extract showed antibacterial activity against MRSA but the aqueous extract of this plant did not show antibacterial activity. This result indicated that the active antimicrobial compounds in this plant should be non-polar compounds which can be dissolved in organic solvent such as ethanol. Wachirawadee determined the composition of *Kaempferia parviflora* ethanol extract using HPLC, it was showed that the major compound of this extract was 5,7-dimethoxyflavone (Malakul, et al., 2011). Therefore, flavones may be the active compound in *Kaempferia parviflora* ethanol extract that shows antibacterial activity against MRSA.

The modes of action of natural product extracts to inhibit microorganisms have been proposed in many previously reports, i.e. inhibition of the synthesis of PBP2a, β-lactamase enzyme (Yam, et al., 1998), inhibition of the efflux pump (Mirza, et al., 2011), or destruction of cell wall. Ifesan, et al. (2009) demonstrated that the *Eleutherine americana* extracts disrupted cell membrane of *Staphylococcus aureus* cells. In this study, the *Kaempferia parviflora* ethanol extract showed antibacterial activity against only Methicillin resistant *S.aureus* not Methicillin susceptible bacteria. This indicates that antibiotic resistance interferes with the antimicrobial action of the plant extract. Shimizu, et al., (2001) studied the composition of *Arctostaphylos uva-ursi* extract and antibacterial activity of this extract against MSRA and MSSA. In addition, the interaction between antibiotics and *Arctostaphylos uva-ursi* extract was also studied in this research. They demonstrated that corilagin was a major compound found in this extract. Corilagin showed antibacterial activity against MRSA but not MSSA. Moreover, the combination of corilagin and oxacillin reduced the MIC of oxacillin when tested with MRSA but not with MSSA. They described that corilagin might interact only with PBP2a present in MRSA. This data support the present study

that *Kaempferia parviflora* ethanol extract may inhibit the synthesis of PBP2a, β-lactamase enzyme as described by Yam, et al. (1998).

The present study indicates that the MIC of *Kaempferia parviflora* ethanol extract against MRSA was higher than the MIC of this extract against MSSA. This result may be due to the different mechanism of actions between many components in the plant and the main active compound in this agent on the bacteria. Essawi and Srour (2000) reported that the effectiveness of the medicinal plant may not result from one main active compound but from a mixture of various constituents in the plant. In addition, the lower MIC of this extracts against MRSA isolates than MSSA isolates might be due to the synergistic effect from some components in the plant extract that provide it with such a potent antibacterial activity.

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 be used to yield new products with broad spectrum biological activity (Rani, et al., 2009). Therefore, many plant extracts are expected that they will increase the synergism to several antibiotics that bacteria resist. In this study the synergistic effects of *Kaempferia parviflora* ethanol extract and antibiotics such as ampicillin, oxacillin and cefoxitin were tested. The results showed that inhibition zone of the combination of *Kaempferia parviflora* ethanol extract and cefoxitin against MRSA is greater than inhibition zone of the extract or antibiotic alone. In addition, the combination of *Kaempferia parviflora* ethanol extract and cefoxitin reduced the MIC of cefoxitin against MRSA. The results of the combination studies revealed that *Kaempferia parviflora* ethanol extract showed the synergistic effect when combined with cefoxitin against MRSA. However, further study should be performed to demonstrate the mechanism of the synergistic effect. Cefoxitin, a β-lactam antibiotic, acts by covalently binding with penicillin binding proteins (PBPs) in process of cell wall synthesis. In experiment of synergistic effect between *Kaempferia parviflora* ethanol extract and cefoxitin against MRSA can be clarified of the possible mechanism, The results indicate that reduced MIC of cefoxitin when combined with *Kaempferia parviflora* ethanol extract may be mainly due to the alteration of cefoxitin

activity. *Kaempferia parviflora* ethanol extract and cefoxitin directly or indirectly attack the same site, PBP2a on cell wall of MRSA while no effect on the activity of MSSA because MSSA did not have PBP2a on cell wall. The previous study from Zhao et al. reported that the Epigallocatechin Gallate (EGCg) from Tea (*Camellia sinensis*) extract may be major reasons for synergistic effects to inhibit MRSA. EGCg induced damage of MRSA cell wall and possible interfere cell wall biosynthesis (Zhao, et al., 2001).

This report might provide alternative methods to reduce the resistance of *S.aureus* to cefoxitin. Further studies have to be done to characterize the active compounds of *Kaempferia parviflora*. In addition, the toxicity of this plant should be evaluated in vivo. Furthermore, the study of the mode of synergistic interaction of active compounds is required to exploit this extract in the combination therapy of infectious disease caused by multidrug-resistant organism.

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APPENDIX

Research Presentation

1. Joyjamras, K., Sukplang, P., Thongmee, A. “Antimicrobial Activity of Native Thai Plant Extracts against Methicillin-Resistant *Staphylococcus aureus* (MRSA)” Proceeding and poster presentation. The 2nd ASEAN plus three graduate research congress (2nd AGRC). February 5-7, 2014, S31 Sukhumvit Hotel, Bangkok, Thailand.

2. Joyjamras, K., Sukplang, P., Thongmee, A. “Antimicrobial activity of selected native Thai plant extracts against *Staphylococcus aureus*.” Oral presentation. The Annual Medical Sciences Conference 21st. June 16-18, 2013, Centra Government Complex Hotel & Convention Centre Chaeng Watthana, Bangkok, Thailand.

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