

ANTI-STAPHYLOCOCCAL SCREENING OF SELECTED THAI MEDICINAL PLANTS FROM NAKHON RATCHASIMA PROVINCE

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Abstract

To investigate and evaluate the anti-staphylococcal activity of selected plants which used in Thai traditional medicine, twenty-six ethanolic extracts of 24 selected Thai medicinal plant species were prepared. An antibacterial activity of the extracts was evaluated for *Staphylococcus aureus* and Methicillin-resistant *S. aureus* (MRSA) using paper disc agar diffusion method and broth microdilution method. Of the 26 extracts tested, four plant extracts including, whole plants of *Limnophila aromatica* (Lam.) Merr, leaves of *Piper betle* Linn., leaves of *Rhodomyrtus tomentosa* (Aiton) Hassk., and leaves of *Xanthostemon youngii* C.T.White & W.D.Francis produced inhibition zones on *S. aureus* and MRSA. The inhibition zones were ranged from 8 mm to 13 mm. Then, minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) values of all extracts were determined. An ethanolic extract of *R. tomentosa* demonstrated as the most effective plant with MIC and MBC values of 0.31/0.62 and 0.62/0.62 mg/ml against *S. aureus* and MRSA, respectively. In addition, MIC values of *P. betle*, *X. youngii* and *L. aromatica* against *S. aureus* and MRSA ranged from 1.25-2.5 mg/ml whereas the extracts had the MBCs value ranging from 1.25-5.0 mg/ml. The results from this study showed the four plant extracts acted as an alternative source of anti-staphylococcal activity against *S. aureus* and its resistant strain.

Keywords: Methicillin-resistant *S. aureus*, *S. aureus*, anti-staphylococcal activity, plant extract

Introduction

Staphylococcus aureus is a strong clinical pathogenic bacterium with multi-drug resistant isolates. The staphylococcal infections were

increasing not only in the hospitals but also in the community (Laupland *et al.*, 2008; David and Daum, 2010). This bacterium has currently

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developed resistance to penicillin and several β -lactam antibiotics such as methicillin, oxacillin, nafcillin, cloxacillin, and dicloxacillin (David and Daum, 2010). Methicillin-resistant *S. aureus* (MRSA) has been described to be resistance to other antibiotics such as erythromycin, clindamycin, tobramycin (Lindqvist *et al.*, 2009), fluoroquinolones (van der Mee-Marquet *et al.*, 2007), linezolid (Tsiodras *et al.*, 2001), as well as glycopeptides including vancomycin and teicoplanin (Vaudaux *et al.*, 2001). The concomitant bacteria virtually resists to available antibiotics. This issue has raised the specter of untreatable infections caused by this organism (Enoch *et al.*, 2007), which had been thought to be defeated by new drug. This unsolved problem demands new antimicrobial agents to be developed urgently.

Medicinal plants have been used as folk therapies for infectious diseases in many tropical countries, including Thailand. These remedies provide a rationale investigation of the natural products for the treatment of staphylococcal infection. In this research, we have investigated the antimicrobial activity of 24 Thai medicinal plants using disc diffusion method and determination of minimum inhibitory and bactericidal concentrations.

Methodology

Collection of Plant Materials

All plant species were collected in Nakhon Ratchasima province. Each plant material were dried at 60°C for three days, then powdered and extracted by soaking with 95% ethanol. The solvents were evaporated under reduced pressure in a rotary evaporator until complete dryness. The extracts were diluted in dimethyl sulfoxide (DMSO, SRL, India) and used for antibacterial assays.

Bacterial Strains Testing

Staphylococcus aureus ATCC29213 and Methicillin-resistant *S. aureus* (MRSA) NPRC001R obtained from Department of Microbiology and Natural Products Research Center, Faculty of Science, Prince of Songkla University, were used in this study. Both bacterial strains were cultured in tryptic soy

broth (TSB, Himedia, India) at 37°C for 18 h. The bacterial strains were maintained in TSB containing 20% glycerol at -80°C.

Screening for Anti-Staphylococcal Activity

Antibacterial activity of the plant extracts were tested against pathogenic bacteria by paper disc diffusion method (CLSI, 2009). Briefly, the extracts were dissolved in DMSO (250 mg/ml) and then 10 μ l of each extracts were applied to sterile filter paper discs (Whatman No. 1; 6 mm in diameter) to give final concentration of 2.5 mg of the extract per disc and were dried at 37°C for overnight. Dried discs were placed on the surface of Mueller Hinton agar plates seeded with test pathogenic bacteria. The plates were incubated at 37°C for 18-24 h. Vancomycin and DMSO discs were used as controls. The test was determined in triplicate and the average diameter of inhibition zone was calculated.

Determination of Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC)

The MICs of the ethanolic extracts and vancomycin were examined using a broth microdilution method according to CLSI guideline. The extracts and vancomycin were serially diluted twofold in Mueller Hinton broth (MHB, Himedia) in 96-well microtitre plates to obtain final concentrations ranged from 0.15-10 mg/ml and 0.125-8 μ g/ml, respectively. Then, an equal volume of 100 μ l of log phase culture of *S. aureus*, approximately 10^6 CFU/ml, was then added to each well. After incubation at 37°C for 16-18 h, MIC was defined as the lowest concentration of the extracts and antibiotic at which the microorganism does not exhibit visible growth, as indicated by the turbidity of the medium.

The MBCs were recorded as the lowest concentration of the extracts and antibiotic at which the bacteria inoculated on TSA was completely killed. All assays were carried out in triplicate.

Statistical Analyses

In the paper disc diffusion assay, the average size of the inhibition zone ($N = 3$) is reported for those extracts that resulted in a

reliable and clear kill zone compared to the diameter of the filter paper disc (6 mm).

Results and Discussion

In traditional medicine, we believed that an increased consumption of herbal plant is associated with lower risks of degenerative diseases, and infectious diseases. In recent years, many medicinal plants have been widely researched to determine the herbals for their antimicrobial activity.

In this study, screening for antibacterial activity of 24 medicinal plant species against *S. aureus* ATCC25923 and MRSA NPRC001R were shown in Table 1. Along with the plants investigated, *Limnophila aromatica*, *Piper betle*, *Rhodomyrtus tomentosa*, and *Xanthostemon youngii* produced inhibition zones on *S. aureus* and MRSA. The inhibition zones ranged from 8 mm to 13 mm.

Plant extracts were screened for their growth inhibitory property by paper disc diffusion assay. However, using the size of inhibition zone to indicate relative antibacterial activity is not typically quantitative and sufficient. As showed in Table 1, some extracts such as *Andrographis paniculata*, *Feronia limonia*, *Melastoma malabathricum*, bark of *Peltophorum pterocarpum*, *Persicaria odorata*, and *Thunbergia laurifolia* did not give an antibacterial activity whereas using microdilution assay, they possessed MIC value at 2.5 mg/ml. This result suggested that the inhibition zone may be involved by many factors such as the evaporation, solubility, and diffusion rate of the active compounds throughout the medium. This demonstration has been reported by Limsuwan and Voravuthikunchai (2013).

When the MICs of the four extracts were determined by broth dilution method, only *R. tomentosa* gave MIC and MBC values below 1 mg/ml on *S. aureus* and MRSA (Table 1). There are two extracts, *P. betle* and *X. youngii* that could exhibit MIC values 1.25 mg/ml. In addition, *L. aromatica* and six extracts had MIC values 2.5 mg/ml. For another the sixteen extracts, they demonstrated weak antibacterial activity (MIC values 5-10 mg/ml and MBC values 5->10 mg/ml).

Among the plant species tested, the leaf extract of *R. tomentosa* produced good activity against *S. aureus* and MRSA, as indicated by the lower MIC and MBC values. The similar results have been reported in recent years (Sianglum *et al.*, 2011; Visutthi *et al.*, 2011; Saising and Voravuthikunchai, 2012; Limsuwan and Voravuthikunchai, 2013). *Rhodomyrtus tomentosa* or Downy rose myrtle is an evergreen shrub native to Southeast Asia including Thailand. This plant is used as a medicinal plant to treat skin, oral, gastrointestinal, and urinary tract infections. The each extract of flower, fruit, and leaf from this plant clearly exhibited a good antibacterial activity against Gram-positive bacteria (Sianglum *et al.*, 2011; Visutthi *et al.*, 2011; Saising and Voravuthikunchai, 2012; Limsuwan and Voravuthikunchai, 2013). An isolated compound, named rhodomyrtone, from the leaf of this plant presented strong antibacterial activity against many pathogenic Gram-positive bacteria (Sianglum *et al.*, 2011; Visutthi *et al.*, 2011; Saising and Voravuthikunchai, 2012; Limsuwan and Voravuthikunchai, 2013).

Piper betle is a glabrous climbing vine. It is abundantly distributed in many Asian countries. The leaves have been used in traditional medicine as carminative, stimulant, antiseptic, antifungal, and antibacterial agent. The volatile oil known as Betel oil is the chief constituent of the leaves. *Piper betle* can be of great benefit in treating diseases caused by bacteria and fungi. Previous studies on the betel leaves, roots and whole extract (mixture of volatile and non-volatile) of the green variety showed a very strong antimicrobial activity. The extract (400 µg/disc) showed zone of inhibition against *S. aureus* (6.77±0.25 mm) (Akter *et al.*, 2011). On *Streptococcus pyogenes*, the extract gave high inhibition zone with 29 mm, however, MIC/MBC values demonstrated moderate activity (500-1000 µg/ml) (Limsuwan and Voravuthikunchai, 2013). Moreover, recent study has reported a good promising antibacterial activity of *P. betle* leaf extract on clinical isolates of multiple drug resistant Gram positive and Gram negative bacteria (Valle *et al.*, 2016).

Table 1. Antibacterial activity of selected Thai medicinal plants against *Staphylococcus aureus* and MRSA

Medicinal plants	Family	Plant part	Inhibition zone* (mm)		MIC/MBC (mg/ml)	
			SA ¹	MRSA ²	SA	MRSA
<i>Aegle marmelos</i> (L.) Correa ex Roxb.	RUTACEAE	fruit	ND	ND	10/>10	10/>10
<i>Andrographis paniculata</i> (Burm.f.) Wall ex Nees.	ACANTHACEAE	whole plant	ND	ND	2.5/>10	5/10
<i>Azadirachta indica</i> A. Juss	MELIACEAE	leaf	ND	ND	5/>10	5/10
<i>Bauhinia purpurea</i> L.	FABACEAE	leaf	ND	ND	5/5	5/10
<i>Cerbera odollam</i> Gaertn.	APOCYNACEAE	flower	ND	ND	5/10	5/10
<i>Careya arborea</i> Roxb	LECYTHIDACEAE	leaf	ND	ND	5/10	5/5
<i>Centella asiatica</i> (L.) Urban.	APIACEAE	whole plant	ND	ND	10/>10	10/>10
<i>Feronia limonia</i> (L.) Swing	RUTACEAE	leaf	ND	ND	2.5/>10	5/5
<i>Linnophila aromatica</i> (Lam.) Merr	PLANTAGINACEAE	whole plant	13.0 ± 0.5	13.0 ± 0.6	2.5/5	2.5/5
<i>Melastoma malabathricum</i> Linn	MELASTOMATACEAE	leaf	ND	ND	2.5/5	5/5
<i>Metha cordifolia</i> Opiz.	LABIATAE	leaf	ND	ND	10/>10	10/>10
<i>Moringa oleifera</i> Lam.	MORINGACEAE	leaf	ND	ND	5/>10	10/>10
<i>Peltophorum pterocarpum</i> (DC.) Backer ex K. Heyne	LEGUMINOSAE-CAESALPINIOIDEAE	leaf	ND	ND	5/5	5/10
		bark	ND	ND	2.5/2.5	2.5/5
		fruit	ND	ND	10/>10	10/10
<i>Persicaria odorata</i>	POLYGONACEAE	whole plant	ND	ND	2.5/>10	5/10
<i>Phyllanthus acidus</i> (L.) Skeels	PHYLLANTHACEAE	leaf	ND	ND	10/>10	10/>10
<i>Piper betle</i> Linn.	PIPERACEAE	leaf	8.8 ± 0.4	8.0 ± 0.6	1.25/1.25	1.25/1.25
<i>Rhodomerytus tomentosa</i> (Aiton) Hassk.	MYRTACEAE	leaf	9.3 ± 0.8	9.3 ± 0.8	0.31/0.62	0.62/0.62
<i>Schleichera oleosa</i> (Lour.) Oken.	SAPINDACEAE	leaf	ND	ND	5/>10	10/>10
<i>Sesbania grandiflora</i> (L.) Poiret	FABACEAE	leaf	ND	ND	10/>10	10/>10
<i>Syzygium cumini</i> (L.) Skeels	MYRTACEAE	leaf	ND	ND	5/5	5/5
<i>Tamarindus indica</i> L.	FABACEAE	bark	ND	ND	10/>10	10/10
<i>Thunbergia laurifolia</i> Lindl.	ACANTHACEAE	leaf	ND	ND	2.5/>10	10/>10
<i>Tiliacora triandra</i> (Colebr.) Diels	MENISPERMACEAE	leaf	ND	ND	5/>10	5/>10
<i>Xanthostemon youngii</i>	MYRTACEAE	leaf	11.6 ± 0.5	10.6 ± 0.6	1.25/2.50	1.25/2.50
Vancomycin			1.92 ± 0.04	1.95 ± 0.14	0.5/0.5 µg/ml	1.0/1.0 µg/ml

* The results were obtained from 3 separated experiments (±SD values); ¹ *S. aureus* ATCC25923; ² MRSA NPCR001R
 ND: not detected.

Limnophila aromatic (syn. *Limnophila grastissima* Blume) belongs to the PLANTAGINACEAE (figwort, snapdragon) family. Several species of *Limnophila* sp. are found in Southeast Asia including Thailand. It has been used as a side dish in Thai cuisine and a medicinal herb, especially in Nakhon Ratchasima province. An essential oil (0.1 wt.%) from dry leaves of *L. aromatica* that contains main constituents of limonene and perillaldehyde has been reported an antibacterial activity (Venkata *et al.*, 1989). In addition, methanolic extract of *L. aromatica* presented an antibacterial activity against *S. aureus* with MIC value 2.6 mg/ml (Nanasombat and Teckchuen 2009).

Xanthostemon sp. is a genus of at least 45 species, most of which are endemic plant, with a range extending from the Philippines to the Solomon Islands, New Caledonia and Australia. *X. youngii* is a shrub or small tree up to 17 m in height with large heads of showy, brilliant red flowers. Biological activity of this plant has not been investigated. However, the same genus, *X. whitei*, had been showed an antibacterial activity against *S. aureus* (Setzer *et al.*, 2006).

Conclusions

Our results demonstrated that the plant species *R. tomentosa*, *P. betle*, *X. youngii* and *L. aromatica* had a potential to be an antibacterial agent against *S. aureus* and MRSA. Thus, these plants may contain active compounds which might be useful for healing the diseases caused by *S. aureus*. Further studies are necessary for these plants to be investigated the other parameters of antimicrobial efficacy, and their toxicity and therapeutic activity *in vivo* should be evaluated.

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