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An Investigation on the Antibacterial and Antibiofilm Efficacy of a Traditional Thai Herbal Recipe (THR 01) against Clinical Isolates of Methicillin Resistant *Staphylococcus Aureus* (MRSA) and *Staphylococcus epidermidis*

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Abstract Natural compound from herbal medicine are important for the source of drug discovery and in this study selected on the basis of folklore medicinal reports practiced in Thung Song district, Nakhon Si Thammarat province, southern of Thailand. This study reports about at investigating the effect of six ethanolic extracts from traditional Thai herbal recipe (THR 01) and it components, *Ocimum sanctum* , *Rhinacanthus nasutus* , *Quisqualis indica*, *Vitex glabrata* and *Stemona tuberosa* against clinical isolates of methicillin resistant *Staphylococcus aureus* (MRSA) NPRC S001-S005, *Staphylococcus aureus* ATCC 25923 and *Staphylococcus epidermidis* ATCC 35984 by broth microdilution method. The results indicated that ethanolic extracts of *S. tuberosa* showed strong antibacterial activity against *S. aureus* ATCC 25923 and *S. epidermidis* ATCC 35984 were 250 µg/ML in both bacteria tested. The biofilm inhibitory concentration of 500-1,000 µg/ML of ethanolic extracts were found to be significant, the most efficiency extracts is *S. tuberosa* as compared to other ethanolic extracts. The experiment was conducted at the laboratory of Science and Technology Faculty, Rajamangala University of Technology Srivijaya, Nakhon Si Thammarat Campus during October, 2016 to Desember, 2017. However, this study also required test in vivo and toxicity for develop Thai herbal in the further.

Keywords: recipe, methicillin resistant *Staphylococcus aureus* (MRSA), antibacterial, antibiofilm

Introduction

Staphylococcus aureus is considered as a main pathogen of causing nosocomial infections (Tohidpour *et al.*, 2010) and clinical isolates of MRSA are often encountered, especially in patients of intensive care units

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(ICU) admission and of those who are elderly and repeatedly hospitalized. (Zuo *et al*., 2008) Thus is cause of a serious problem world wide.

In *S. aureus*, biofilm formation is a important problem because bacterial cells encase themselves and escape from host defense systems as well as antimicrobial agents, which leads to chronic infections and difficult to cure (Harris *et al.*, 2016, Sivaranjani *et al.*, 2017). Many studies try to find alternative ways to develop medicinal plant extract as an effective agent for the reduction of antibiotic residue due to extensive use of antibiotics in treatment.

Traditional herbal medicine have the longest history and plays an important role in health systems for the peoples of the world. Nowadays, natural compound from herbal medicine are important for the source of drug discovery, especially true of anti-infectious agents (Ru *et al.*, 2014). Traditional healer namely Mr. Saturn Homkat, He use herbal formulation to treat patient with wound infections for the long time. The herbal formulation was prepared from four herbal component include *Ocimum sanctum* , *Rhinacanthus nasutus* , *Quisqualis indica*, *Vitex glabrata* and *Stemona tuberosa*. Therefore, we interested to study the antibacterial activity of Thai herbal formulation which should be consulted for more information on this approach to drug discovery.

Materials and methods

Preparation of crude extracts

Traditional Thai herbal recipe 01 (THR-01) consist of equal amounts (100g) of their medicinal plant components, *Ocimum sanctum*, *Rhinacanthus nasutus*, *Quisqualis indica*, *Vitex glabrata* and *Stemona tuberosa* Single herbal component was used 100 g. The herbal powder was extracted (1:2, w/v) with 95% ethanol at room temperature for 7 days. After filtration, 95% ethanol was removed with a rotatory evaporator, kept at 55 ℃ untilthey were completely dry and stored in a sterile eppendorf at 4 ℃. Extraction yield (%, w/w) was calculated as the ratioof the weight of the extract to the weight of the crude herb powder. Stock solutions (40 mg/mL) were prepared in methanol and stored at 4 ℃ in the dark for further experiments (Thongrod and Yincharoen, 2017).

Tested Bacterial Strains

Bacterial used in this study were clinical isolates of methicillin resistant *Staphylococcus aureus* (MRSA) NPRC S001-S005, *Staphylococcus aureus* ATCC 25923 and *Staphylococcus epidermidis* ATCC 35984 obtained from the Natural Products Research Center, Faculty of Science, Prince of Songkla University, were used throughout the study.

Evaluation of antibacterial activity

Antibacterial activity was evaluated by broth microdilution method according to Clinical and Laboratory Standard Institute (CLSI, 2012). A broth microdilution method was used to determine the minimum inhibitory concentration (MIC) of the ethanol extract. Serial twofold dilution of the plant extract were mixed with Mueller-Hinton broth in 96-well sterile microtiter plates to give final concentration of 15.625 to 1,000 µg/ml. After that were then incubated at 37 ℃ for 18 h. MIC were observed at least in duplicate as the lowest concentration of plant extract that produced a complete suppression of bacterial growth.

Anti-biofilm with Crystal Violet Assay

Fresh bacterial suspensions were prepared in tryptic soy broth from overnight cultures. 200 µL aliquots of bacterial suspension were inoculated into each wells and incubated for 18–24 h at 37 ℃ to enable adhesion of cells to wells. Following incubation, the medium was remove and add fresh medium supplemented with herbal extracts at concentration 15.625-1,000 µg/mL and incubated for 18–24 h at 37 ℃. Treat cells in triplicate wells for each condition. For well serve as negative control add only fresh medium. After incubation, plates were gently washed two times with sterile water and stained with 50 µL of 0.5% crystal violet for 20 min at room temperature. Excess crystal violet was removed by four times washing with sterile water and dried the plate at least 2 h at room temperature. Biofilm was quantified by measuring the corresponding OD_{570} nm of the supernatant following the solubilization of crystal violet in 200 µL of 95% ethanol (Feoktistova *et al.*, 2016).

Results

Antibacterial activity of plant

THR-01 extracts and its herbal components which are *Ocimum sanctum* , *Rhinacanthus nasutus* , *Quisqualis indica*, *Vitex glabrata* and *Stemona tuberosa* and exhibited different inhibition levels against clinical isolates of MRSA NPRC S001-S005, *Staphylococcus aureus* ATCC 25923 and *Staphylococcus epidermidis* ATCC 35984 as shown in Tables 1 The ratio of MIC to the microdilution method was 15.62 to 1,000 µg/ml. The MIC for the ethanolic extracts present *S.tuberosa* against *S.aureus* ATCC 25923 and *S. epidermidis* ATCC 35984 showed the strongest antibacterial activity with MIC values of 250 µg/ml secondly *R. nasutus* and *Q. india* showed activity of almost all tested bacteria with MIC values of 500 mg/ml while *V.glabrata* weakest activity against bacteria tested with MIC values of

1,000 mg/ml. For results of anti-biofilm activity present, ethanolic extract of *Q. indica* at the concentration of 1,000 µg / ml showed significantly reduced biofilm efficacy of MRSA NPRC R001 and *S. aureus* ATCC 25923 (Fig. 1-2) while at the concentration of 500-1,000 µg / ml showed significantly reduced biofilm efficacy of MRSA NPRC R005 (Fig. 3), *R. nasutus* at the concentration of 1,000 µg / ml showed significantly reduced biofilm efficacy of MRSA NPRC R002-R003 (Fig. 4-5) while at the concentration of 500-1,000 µg / ml showed significantly reduced biofilm efficacy of MRSA NPRC R001 and *S. aureus* ATCC 25923 (Fig. 6-7), *O. sanctum* at the concentration of 1,000 µg / ml showed significantly reduced biofilm efficacy of MRSA NPRC R001-R002 (Fig. 8-9) while at the concentration of 500-1,000 µg / ml showed significantly reduced biofilm efficacy of MRSA NPRC R003 (Fig. 10), *S. tuberosa* at the concentration of 1,000 µg / ml showed significantly reduced biofilm efficacy of MRSA NPRC R002 and MRSA NPRC R005 (Fig. 11-12) while at the concentration of 500-1,000 µg / ml showed significantly reduced biofilm efficacy of MRSA NPRC R001 and MRSA NPRC R003 (Fig. 13-14) *V. glabrata* at the concentration of 1,000 µg / ml showed significantly reduced biofilm efficacy of MRSA NPRC R004-R005 (Fig. 15-16) while at the concentration of 500-1,000 µg / ml showed significantly reduced biofilm efficacy of MRSA NPRC R001 (Fig. 17) *THR 01* at the concentration of 1,000 µg / ml showed significantly reduced biofilm efficacy of MRSA NPRC R003-R005 and *S. aureus* ATCC 25923 (Fig. 18-21).

Table 1. Minimum inhibitory concentrations (MIC) of ethanol extracts of *THR 01*, its herbal components and active constituents against clinical isolates of MRSA NPRC S001-S005, *Staphylococcus aureus* ATCC 25923 and *Staphylococcus epidermidis* ATCC 35984

Bacterial strains	Minimum inhibitory concentration (MIC) (µg/ml)					
	THR 01	*O.sanctum*	*V.glabrata*	*R.nasutus*	*Q.india*	*S.tuberosa*
MRSA NPRC R001	1000	1000	1000	500	500	500
MRSA NPRC R002	1000	500	1000	500	500	1000
MRSA NPRC R003	1000	1000	1000	1000	500	500
MRSA NPRC R004	1000	1000	1000	500	1000	500
MRSA NPRC R005	1000	500	1000	500	500	1000
S. aureus ATCC 25923	1000	1000	1000	500	500	250
S. epidermidis ATCC 35984	500	1000	1000	500	500	250

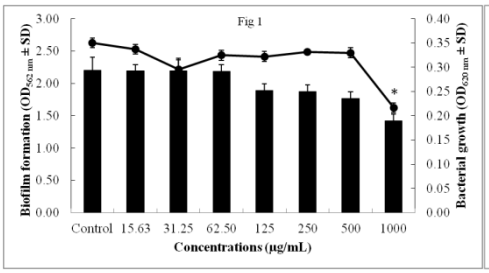
Fig 1
Biofilm formation ($OD_{562\,nm}$ ± SD)
Bacterial growth ($OD_{620\,nm}$ ± SD)
Control 15.63 31.25 62.50 125 250 500 1000
Concentrations (µg/mL)

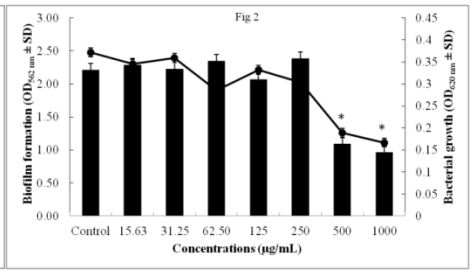
Fig 2
Biofilm formation ($OD_{562\,nm}$ ± SD)
Bacterial growth ($OD_{620\,nm}$ ± SD)
Control 15.63 31.25 62.50 125 250 500 1000
Concentrations (µg/mL)

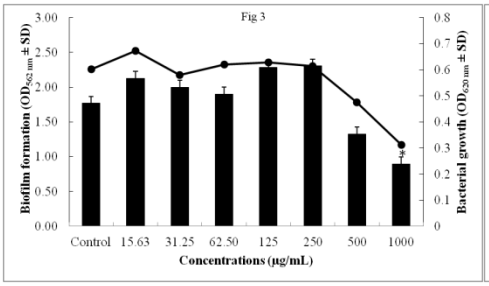
Fig 3
Biofilm formation ($OD_{562\,nm}$ ± SD)
Bacterial growth ($OD_{620\,nm}$ ± SD)
Control 15.63 31.25 62.50 125 250 500 1000
Concentrations (µg/mL)

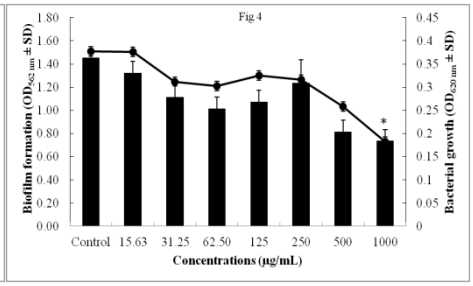
Fig 4
Biofilm formation ($OD_{562\,nm}$ ± SD)
Bacterial growth ($OD_{620\,nm}$ ± SD)
Control 15.63 31.25 62.50 125 250 500 1000
Concentrations (µg/mL)

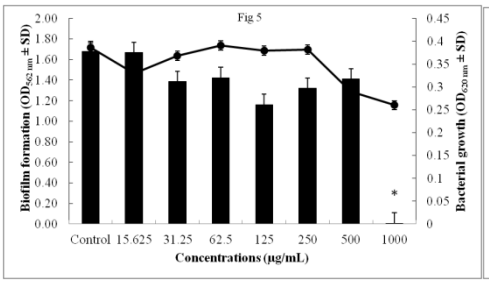
Fig 5
Biofilm formation ($OD_{562\,nm}$ ± SD)
Bacterial growth ($OD_{620\,nm}$ ± SD)
Control 15.625 31.25 62.5 125 250 500 1000
Concentrations (µg/mL)

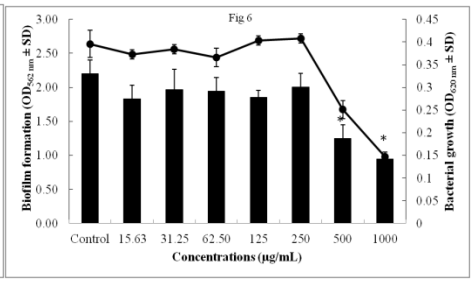
Fig 6
Biofilm formation ($OD_{562\,nm}$ ± SD)
Bacterial growth ($OD_{620\,nm}$ ± SD)
Control 15.63 31.25 62.50 125 250 500 1000
Concentrations (µg/mL)

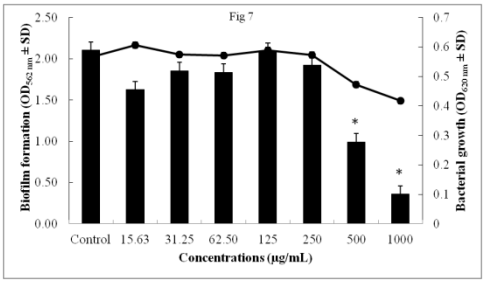
Fig 7
Biofilm formation ($OD_{562\,nm}$ ± SD)
Bacterial growth ($OD_{620\,nm}$ ± SD)
Control 15.63 31.25 62.50 125 250 500 1000
Concentrations (µg/mL)

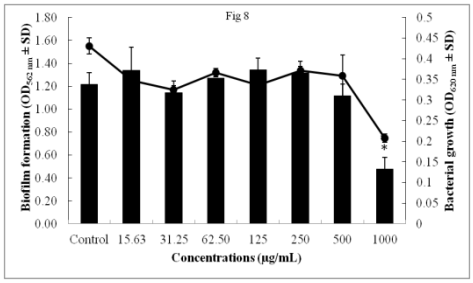
Fig 8
Biofilm formation ($OD_{562\,nm}$ ± SD)
Bacterial growth ($OD_{620\,nm}$ ± SD)
Control 15.63 31.25 62.50 125 250 500 1000
Concentrations (µg/mL)

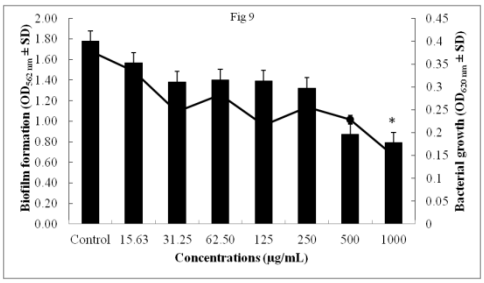
Fig 9
Biofilm formation ($OD_{562\,nm}$ ± SD)
Bacterial growth ($OD_{620\,nm}$ ± SD)
Control 15.63 31.25 62.50 125 250 500 1000
Concentrations (µg/mL)

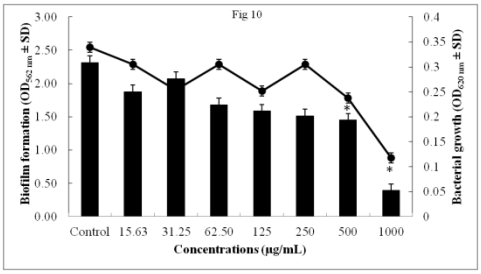
Fig 10
Biofilm formation ($OD_{562\,nm}$ ± SD)
Bacterial growth ($OD_{620\,nm}$ ± SD)
Control 15.63 31.25 62.50 125 250 500 1000
Concentrations (µg/mL)

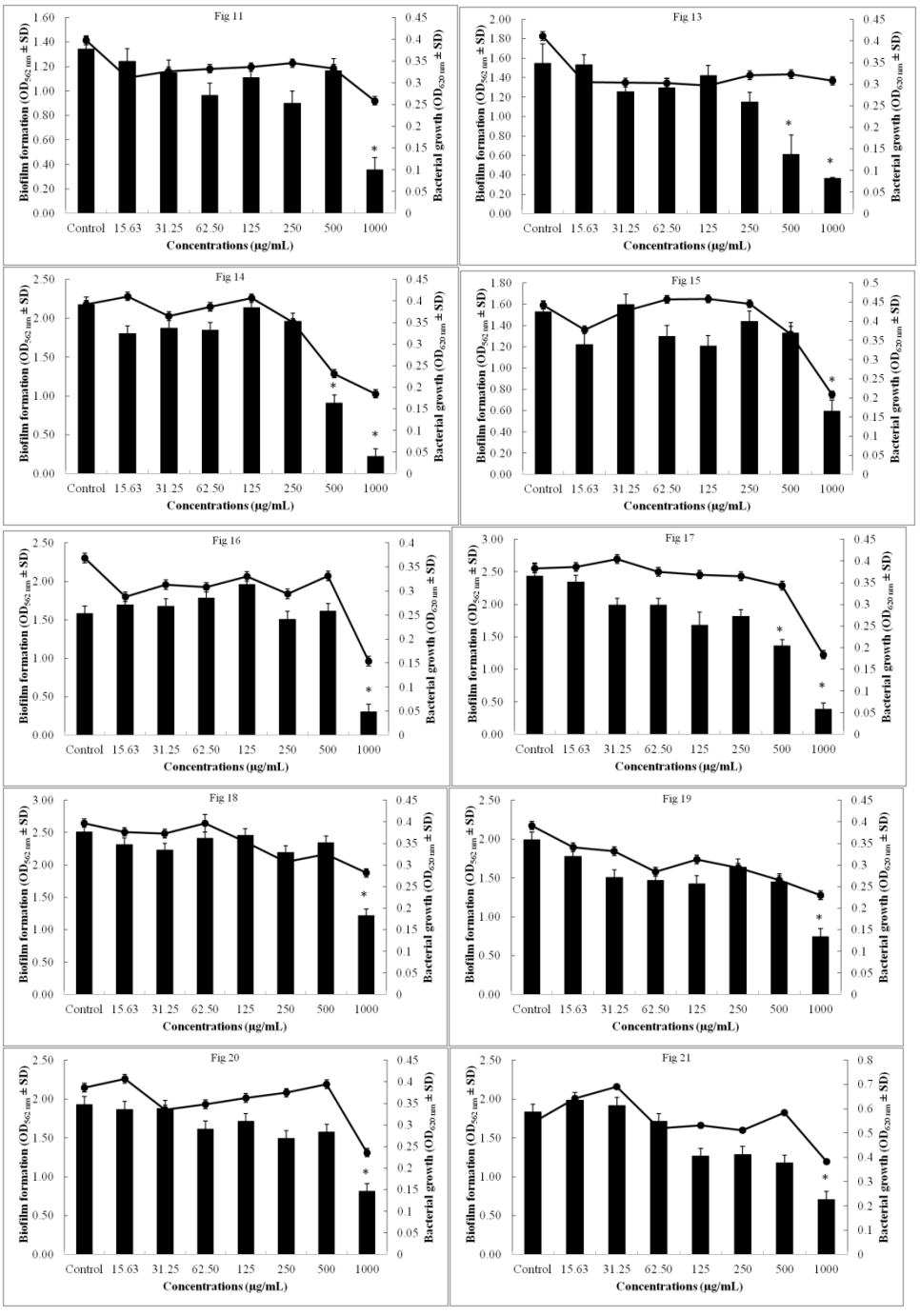

Figure 1 - Figure 21. Effect of different concentrations of *THR 01*, *O. sanctum* , *R. nasutus* , *Q. indica*, *V. glabrata* and *S. tuberosa*. On (linere charts) and biofilm formation (column charts) of methicillin resistant *Staphylococcus aureus* (MRSA) NPRC S001-S005, *S. aureus* ATCC 25923 and *S. epidermidis* ATCC 35984

Discussion

Methicillin-resistant *Staphylococcus aureus* (MRSA) infections are a leading cause of death among all fatalities caused by antibiotic-resistant bacteria (Mohammad *et al.*, 2015). Some studies present the sensitivity of MRSA isolates to trimethoprim-sulfamethoxazole, clindamycin, ciprofloxacin, gentamicin, and erythromycin was 70.66, 66.53, 42.4, 38.05, and 29.35%, respectively. (Sadeghi *et al.*, 2014) and biofilm is factor that makes treatment more difficult.

Many studies try to find alternative ways to develop medicinal plant extract as an effective agent for antibacterial. Such as previous studies, obtained results show MIC of green tea extracts against *S. aureus* ATCC 25923 and MRSA were 400 µg/mL (Radji *et al.*, 2013) nearby with ethanolic extracts of *R. nasutus*, *Q. india* and *S. tuberose* mostly were 500 µg/mL. For in this study revealed that the extracts of *S. tuberosa* showed the most promising antimicrobial properties with the MIC of 250 µg/mL against *S. epidermidis* ATCC 35984 and *S. aureus* ATCC 25923 and weakest effective tested agents were *V. glabrata* against all bacteria tested.

Addition in this study investigate the effects of ethanolic extracts by anti-biofilm assay. Previous studies present results of the biofilm formation revealed that selenium nanoparticles inhibited the biofilm of *S. aureus*, *P. aeruginosa*, and *P. mirabilis* by 42%, 34.3%, and 53.4%, respectively, compared to that of the non-treated samples. (Shakibaie *et al.*, 2015) For results of the anti-biofilm fond that *S. tuberose* showed the most effective inhibited the biofilm were 500 µg/mL against MRSA NPRC R001, MRSA NPRC R003 and *S. epidermidis* ATCC 35984 significant at the 0.05 level while other extract show similar results.

Conclusion

The results of this study showed that a Thai traditional herbal recipe THR 01 and its major constituent, *Ocimum sanctum* , *Rhinacanthus nasutus*, *Quisqualis indica*, *Vitex glabrata* and *Stemona tuberosa*. have antibacterial activity and showed effective inhibited the biofilm. Further studies on their toxicity, and in vivo efficacy are advocated.

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