THE STUDY OF ANTIBACTERIAL ACTIVITY OF SOME MEDICINAL PLANTS IN LAMIACEAE FAMILY

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Abstract

The presence of drugs-resistant bacteria did not only hamper the effective treatment of infectious diseases, but also increased the cost of treatment. Therefore, there is a need to develop alternative antimicrobial drugs for the treatment of infectious diseases. In this study, four species of Lamiaceae, namely, *Mentha cordifolia* Opiz ex Fresen, *Ocimum basilicum* L., *O. basilicum* L. forma *citratum* Back and *Hyptis suaveolens* (L.) Poit were examined individually for the antibacterial study and the synergistic effect against drugs-susceptible and drugs-resistant clinical isolates of bacteria. All of these four medicinal plants showed antibacterial activities against all clinical isolated test bacteria. *H. suaveolens* (L.) Poit individually displayed the best antibacterial activity against Methicillin-resistant *Staphylococcus aureus. H. suaveolens* (L.) Poit in combination with *O. basilicum* L. showed synergistic effect against Ciprofloxacin-resistant *Pseudomonas aeruginosa.* Further investigation on laboratory and clinical studies are required to elucidate the data for the development of their uses as alternative sources of antimicrobial agent.

Keywords: Lamiaceae, drug-resistant bacteria, MIC, FIC, macrodilution test, checkerboard assay

Introduction

Due to indiscriminate use of antimicrobial drugs, microorganisms have developed resistance to many antibiotics and created immense clinical problems in the treatment of infectious diseases (Davos, 1994). The drug-resistant bacteria have further complicated the treatment of infectious diseases in immunocompromised, AIDS, especially in the case of nosocomial infections (McGrow *et al.*, 2000). There is not only the lost of an effective of antibiotics against multi-drug resistant bacteria, but also the global problem for the lost of budget for infectious diseases treatment. In the emergence of drugs resistance in human pathogenic organisms, there is a need to develop alternative antimicrobial drugs for the treatment of infectious diseases. One approach is to screen new, inexpensive and effective drugs from other sources, including plants, for possible antimicrobial properties. One of the plant families that is commonly known and found in Thailand is Lamiaceae or Labiatae. The famous plants belong to this family such as peppermint and basil have been known as kitchen, edible and aromatic perennial herbs. Many plants have antiseptic properties and are used on cuts and festering wounds. Most members of *Ocimum* are used as expectorants, while a large number of

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other species are antihelmintic (Catherine and Kokwaro, 1993). In this study, four species of Lamiaceae were selected by using references to their traditional usage and previous antibacterial activity studies (Caceres *et al.*, 1991; Lentz *et al.*, 1998; Navarro *et al.*, 1996; Rojas *et al.*, 1992), three of which were kitchen herbs: *Mentha cordifolia* Opiz ex Fresen (*M. cordifolia* Opiz or Saranae), *Ocimum basilicum* L. (*O. basilicum* L. or Horaphaa) and *Ocimum basilicum* L. forma *citratum* Back (*O. basilicum* L. forma or Maenglak). *Hyptis suaveolens* (L.) Poit (*H. suaveolens* (L.) Poit or Maeng lak khaa) was the only weed selected for this study. Many biological activities of the family were studied as well as their antibacterial activities property (Caceres *et al.*, 1991; Lentz *et al.*, 1992; Navarro *et al.*, 1996; Okonogi *et al.*, 1993; Rojas *et al.*, 1992). However, most of these plants were not previously screened against multi-drug resistant pathogenic organisms, especially the clinical isolated bacteria from the specimen of Thai patients. Therefore, the study of Thai medicinal plants against drug-susceptible and resistant bacteria isolates in Thailand should be valuable for the clinical practices in Thailand. The problem of drug-resistant bacteria in many sections of Maharat Nakhon Ratchasima hospital was previously reported (Maharat Nakhon Ratchasima, Clinical Microbiogoly and Pathology, 2000). These four medicinal plants were tested against four clinical isolates of bacteria that caused nosocomial infection in the hospital. Eight clinical isolated bacteria were Methicillin-susceptible and resistant *Staphylococcus aureus* (MSSA and MRSA), Ciprofloxacin-susceptible and resistant *Pseudomonas aeruginosa* and Ceftazidime-susceptible and resistant bacteria of *Escherichia coli* and *Enterobacter cloacae*. All of the tested bacteria were isolated from patients who were admitted at Maharat Nakhon Ratchasima hospital. Some medicinal plants of this study had antimicrobial activities but there was little quantitative data on their antimicrobial activities. Therefore, the purpose of this study was undertaken to investigate the effectiveness of the selected herbal crude extracts against drugs-susceptible and resistant bacteria. To determine minimal inhibitory concentration (MIC), the macrobroth dilution method was used in this study. A checkerboard assay was used to investigate a combination effect between two extracts against the drug-resistant bacteria.

Materials and Methods

Medicinal Plants Extraction

Fresh medicinal plants, which were *H. suaveolens* (L.) Poit, *M. cordifolia* Opiz ex Fresen, *O. basilicum* L. and *O. basilicum* L. forma *citratum* Back, were collected from home-gardens in Muang district, Naknon Ratchasima province. Leaves of each plant were separated, washed and completely dried in hot air oven (at 40^{o}C), then extracted with 95%(w/w) ethanol using a Soxhlet extractor apparatus. The ethanol was removed under pressure using a rotary evaporator. The dried residue crude extracts were resuspended in 20% dimethylsulfoxide (DMSO) at a concentration of 500 mg/ml and stored in a dark bottle at 4^{o}C.

Bacterial Strains

Clinical isolates of the following bacteria: Methicillin-sensitive and resistant *S. aureus*, Ciprofloxacin-susceptible and resistant *P. aeruginosa*, and two Ceftazidime-susceptible and resistant *Ent. cloacae* and *E. coli* were obtained from Clinical Microbiology Laboratory, Maharat Nakhon Ratchasima Hospital, Nakhon Ratchasima province. Nutrient agar (Difco) were used to maintain the clinical isolates of the bacteria.

Preparation of Crude Extracts and Inoculum

Each crude extract (500 mg/ml) was dissolved in 20% DMSO and diluted with steriled water to the required test concentrations.

Test organisms were incubated in 100 ml nutrient broth for 18 hrs at 37^{o}C. The cultures were centrifuged at 4,000 rpm for 10 min, the cell pellets were washed with saline, recentrifuged, and resuspended in saline. The cell concentrations were adjusted with saline to give 5 x 10^{8} colony-forming units (CFU)/ml using a predetermined calibration curve of absorbance at 500 nm against viable count (Liu *et al.*, 2000).

Minimal Inhibitory Concentration (MIC) Determination

The density of the bacterial suspension in normal saline was adjusted to approximately 1 x 10^8 CFU/ml by using the absorption of bacterial suspension viable count standard curve. The inoculum of 0.05 ml of standard suspension (18 hrs culture) of each strain of the test bacteria was added to triplicate tubes containing 4.95 ml Mueller Hinton Broth, plus serial dilutions of the crude extracts, to give approximately 5 x 10^6 CFU/ml. Tubes of broth with 20% DMSO but without crude extracts were used as the negative control and tubes of broth with Augmentin® antibiotic were used as the positive control for each of the test bacteria. Incubation was at 37°C for 24 hrs. The MIC is defined as the lowest concentration of crude extract at which there is no visible growth in the triplicate tubes (Liu *et al.*, 2000).

Checkerboard Determination

Checkerboard determinations in antimicrobial combinations were performed as previously described (Lorian, 1999) with slight modification (Eumkeb, 1999). The test bacterial suspensions were adjusted to 1 x 10^8 CFU/ml using the absorption of bacterial suspension from the previously determined standard curve. 0.05 ml of the bacterial suspension was added to a series of 4.95 ml Mueller Hinton broth, plus 10% serial dilutions of the crude extract combinations, to give 5 x 10^6 CFU/ml. The culture was incubated for 24 hrs at 37°C. The test was carried out in triplicates. MICs were determined for each crude extract combination and the isobolograms were plotted. The alternative way to determine types of combination effect was FIC calculation.

Results and Discussion

The Percentage of Extractives Obtained from Each Plant

The percentage (w/w) of four 95% ethanolic plant extracts calculated by using weight of dried residue extract per weight of dried plant are summarized in Table 1. The results indicated *O. basilicum* L. forma *citratum* Back provided the best quantity crude extract followed by *M. cordifolia* Opiz ex Fresen, *O. basilicum* L. and *H. suaveolens* (L.) Poit, respectively. These percentages of extractives obtained were appropriate values for this method when compare with other researchers.

MIC Determination

The MICs of four medicinal plants in the present study are summarized in Table 2 to 5.

Table 2 shows MICs of plants extracts tested against methicillin-susceptible and resistant clinical isolated *S. aureus.* The results indicated that *H. suaveolens* (L.) Poit displayed the lowest MIC against both clinical isolates. *H. suaveolens* (L.) Poit and *O. basilicum* L. showed the lower MICs against methicillin-resistant isolates than the susceptible isolates.

Table 3 represents the MICs of these plant extracts against Ceftazidime-susceptible and resistant clinical isolates of *E. coli*. All four ethanolic extracts showed a same MIC concentration against the drug-susceptible isolates. *O. basilicum* L. forma *citratum* Back as well as *H. suaveolens* (L.) Poit showed a poor

Table 1. Calculated Ethanolic plant extracts.

Medicinal plants	95% Ethanolic extracts (% w/w)*
H. suaveolens (L.) Poit	20.8
M. cordifolia Opiz ex Fresen	24.4
O. basilicum L.	22.7
O. basilicum L. forma *citratum* Back	28.2

* Calculated from dried residue extract weight per dried plant weight in percent.

antibacterial activity against the resistant isolates at MIC as high as 100 mg/ml.

Table 4 reports MICs of the tested medicinal plant extracts against Ceftazidime-susceptible and resistant clinical isolated *Ent. cloacae*. The MIC of *H. suaveolens* (L.) Poit against ceftazidime- resistant isolates of *Ent. cloacae* was at a concentration of 50 mg/ml. It was lower than those obtained from the drug-susceptible isolates (at a concentration of 100 mg/ml).

Table 5 represents the MIC results from the plant extracts against *P. aeruginosa*, both Ciprofloxacin sensitive and resistant isolates. *O. basilicum* L. showed the lowest MICs against both clinical isolated bacteria.

Many previous researchers reported the antibacterial activity of the medicinal plants but many results were slightly different from this study. Variation between the results reported by previous workers and our studies could be due to differences in the plants physiological state of development, diurnal and seasonal variation, environmental condition, part of the plants, extraction procedure, concentration of the crude extracts and strains of test microorganism. For example, essential oil from *H. suaveolens* (L.) Poit displayed good antimicrobial activity against *S. aureus, P. aeruginosa* and *E. coli* at various concentrations. The methanolic extract from leaves at 2 mg/ml did not inhibit Gram-negative bacteria, *E. coli* and *P. aeruginosa* but showed activities against Gram-positive bacteria, *S. aureus* (Hussain and Decni, 1991). From this study, the plant extracts were found to have antibacterial activity against drug-susceptible and resistant isolated bacteria. This has clearly indicated that antibiotic resistance did not interfere with the antimicrobial action of plant extracts and these extracts might have different mode of action on test bacteria (Ahmad and Beg, 2001). *H. suaveolens* L. Poit was also highly active against Gram-positive, clinical isolates of *S. aureus*, in the test. This result was supported by earlier studies that *S. aureus* was the most easily inhibited when exposed to plant extracts (Grosvenor *et al.*, 1995). From the study, Gram-negative bacteria were greater resistant to plant extracts. These observations were likely to be the result of the differences in cell wall structure

Table 2. *In vitro* MIC of plant extracts against Methicillin-susceptible and resistant clinical isolates of *Staphylococcus aureus.*

Medicinal plants	MIC (mg/ml)	
	Methicillin sensitive	Methicillin resistant
O. basilicum L.	50	30
O. basilicum L. forma *citratum* Back	50	50
M. cordifolia Opiz	100	100
H. suaveolens (L.) Poit	25	6.25

Table 3. *In vitro* MIC of plant extracts against Ceftazidime-susceptible and resistant clinical isolates of *Escherichia coli.*

Medicinal plants	MIC (mg/ml)	
	Ceftazidime sensitive	Ceftazidime resistant
O. basilicum L.	50	50
O. basilicum L. forma *citratum* Back	50	100
M. cordifolia Opiz	50	50
H. suaveolens (L.) Poit	50	100

between Gram-positive and Gram-negative bacteria. The Gram-negative has a multi-layered and complex structure, The outer membrane can act as a barrier to many environmental substances, including antibiotics (Essawi and Srour, 2000). Although a comparative study of the MIC of plant extracts against drugs-susceptible and resistant clinical isolated bacteria have not been previously reported. Results from the present study indicated that the MICs of drugs-susceptible clinical isolates were higher than the MICs of drugs-resistant clinical isolates. According to previous investigators who reported the effectiveness of a medicinal plant that it might not result from one main active compound but it was the mixture of various constituents in the plants (Essawi and Srour, 2000). The lower MIC of drug-resistant than drug-susceptible isolated bacteria observed in this study was likely due to the different mechanisms of actions between many constituents in a medicinal plant and a main active compound in an antibacterial agent on the microorganisms. Otherwise, it might be due to a synergistic effect derived from some constituents in the indigenous leaf extracts that provided it with such a potent antibacterial activity.

Checkerboard Assay

The synergistic activity against both MRSA and ciprofloxacin-resistant *P. aeruginosa* were observed in *O. basilicum* L. plus *H. suaveolens* (L.) Poit combination and *O. basilicum* L. plus *O.basilicum* L. forma *citratum* Back combination. The isobolograms obtained from plotting of checkerboard MIC determinations are shown in Figure 1. Results from the isobologram represented that *O. basilicum* L. and *H. suaveolens* (L.) Poit combination and *O. basilicum* L. plus *O. basilicum* L. forma *citratum* Back combination had additive activity against MRSA. Additionally, *O. basilicum* L. plus *H. Sauveolens* (L.) Poit combination had synergistic effect against the drug-resistant *P. aeruginosa* as well. Summary the FICs for checkerboard assays are shown in Table 6. The combination of *O. basilicum L.* and *H. suaveolens* (L.) Poit showed synergistic effect against Gram-negative bacteria and additive

Table 4. *In vitro* MIC of plant extracts against Ceftazidime-susceptible and resistant clinical isolates of *Enterobacter cloacae.*

Medicinal plants	MIC (mg/ml)	
	Ceftazidime sensitive	Ceftazidime resistant
O. basilicum L.	50	50
O. basilicum L. forma *citratum* Back	100	100
M. cordifolia Opiz	50	50
H. suaveolens (L.) Poit	100	50

Table 5. *In vitro* MIC of medicinal plant extracts against Ciprofloxacin-susceptible and resistant clinical isolates of *Pseudomonas aeruginosa.*

Medicinal plants	MIC (mg/ml)	
	Ciprofloxacin sensitive	Ciprofloxacin resistant
O. basilicum L.	25	25
O. basilicum L. forma *citratum* Back	50	50
M. cordifolia Opiz	25	50
H. suaveolens (L.) Poit	50	50

effect against Gram-positive bacteria. This was probably due to the plant material acted by another mechanism presumably by blocking the inhibitory effect of the enzymes or affecting the efflux system. In other cases, the improvement in the activity of these two plant extracts was probably due to the accumulation of inhibitory concentration at the target sites or due to the additional inhibitory effect of the plant material. (Darwish *et al.,* 2002)

Conclusion

At presently, there is an emergence of multiple drug resistance to human pathogenic organisms, so there is an urgent need to develop alternative antimicrobial drugs for the treatment of infectious diseases. One approach is to search for new, inexpensive and effective drugs from plants, for possible antimicrobial properties. In

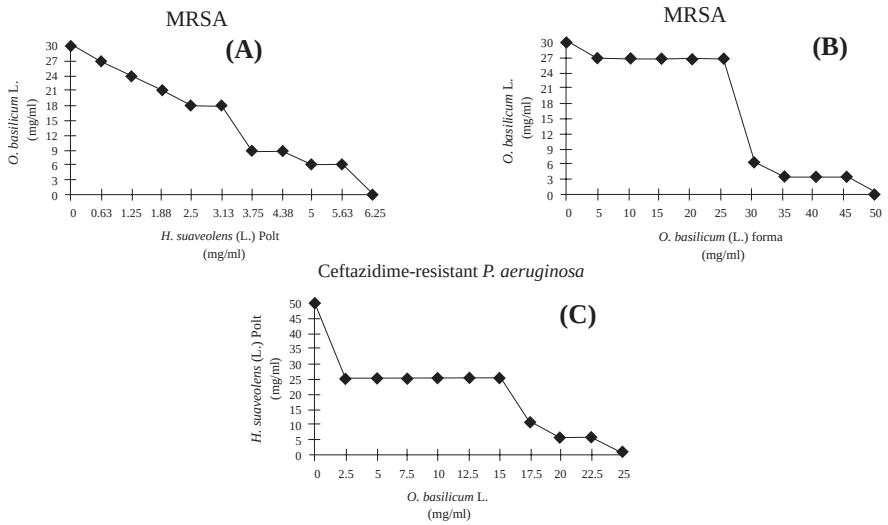

Figure 1. Isobologram constructed from checkerboard MIC data showing antibacterial combination of: (A) *O. basilicum* L. plus *H. suaveolens* (L.) Poit against MRSA; (B) *O. basilicum* L. plus *O. basilicum* L. forma against MRSA; (C) *O. basilicum* L. plus *H. suaveolens* (L.) Poit against Ciprofloxacin - resistant *P. aeruginosa.*

Table 6. Summary the FICs for checkerboard assays of *Ocimum basilicum* L., *O. basilicum* L. forma and *Hyptis suaveolens* (L.) Poit against drug resistant bacteria.

Crude extract combination	Test bacteria	MIC (mg/ml)	MIC (A+B) (mg/ml)	FIC (A+B)	Type of interaction
O. basilicum	MRSA	30	9	0.9	addition
H. suaveolens		6.25	3.75		
O. basilicum	MRSA	30	3	0.8	addition
O. basilicum L. forma		50	35		
O. basilicum	Ciprofloxacin-	25	2.5	0.6	synergism
H. suaveolens	resistant	50	25		
	P. aeruginosa				

this study, four 95% ethanolic extracts from plants in Lamiaceae family were tested against eight drugs-susceptible and resistant clinical isolated bacteria. The MIC values of the extracts were quantitatively assessed by a macrobroth dilution method. *H. suaveolens* (L.) Poit alone showed the lowest MIC against MRSA at MIC of 6.25 mg/ml. The study also determined the combination effect of two medicinal plants against MRSA and drug-resistant *P. aeruginosa*. The combination of *O. basilicum* L./ *H. suaveolens* (L.) Poit showed synergistic effect against the Gram-negative drug-resistant *P. aeruginosa*. *O. basilicum* L. plus *H. suaveolens* (L.) Poit combination and *O. basilicum* L. plus *O. basilicum* L. forma *citratum* Back combination showed additive activity against Gram-positive MRSA. These results indicated that crude extract from *H. suaveolens* (L.) Poit. had potential to against MRSA. This may result from it has terpenes or terpenoids which are active against bacteria, viruses and protozoa. There is now preliminary scientific validation for the use of some of these medicinal plants for antibacterial activity. The active phytocompounds of these plants used against multidrug-resistant bacteria and their toxicity have to be characterized in further studies.

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