การศึกษาการต้านเชื้อแบคทีเรียของพืชสมุนไพรบางชนิด ในตระกูล LAMIACEAE

นางสาวกานตลักษณ์ พันธุ์โอภาส

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

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

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree

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ปรากฏการณ์ดื้อต่อยาต้านจุลชีพหลายชนิดไม่ได้ทำให้เกิดการขาดประสิทธิภาพในการรักษาโรคติดเชื้อเท่านั้น แต่ยังทำให้เพิ่มค่าใช้จ่ายในการรักษาโรคติดเชื้อด้วย ดังนั้นจึงเกิดความต้องการพัฒนายาต้านจุลชีพชนิดใหม่เพื่อใช้ในการรักษาโรคติดเชื้อ ในการศึกษานี้ใช้พืช 4 ชนิดในตระกูล Lamiaceae คือ *Mentha cordifolia* Opiz ex Fresen *Ocimum basilicum* L. *O. basilicum* L. forma *citratum* Back และ *Hyptis suaveolens* (L.) Poit นำมาทดสอบเดี่ยวๆ และการออกฤทธิ์เสริมกันในการต้านเชื้อแบคทีเรียที่ได้จากการคัดแยกทางคลินิกทั้งชนิดที่ไวและดื้อต่อยาปฏิชีวนะ พืชสมุนไพรทั้ง 4 ชนิดมีฤทธิ์ต้านเชื้อแบคทีเรียทั้งหมดที่ใช้ในการทดสอบ โดย *H. suaveolens* (L.) Poit เดี่ยวๆ แสดงการต้านเชื้อ *S. aureus* ที่ดื้อต่อยา Methicillin ได้สูงสุด และ *H. suaveolens* (L.) Poit เมื่อใช้ร่วมกับ *O.basilicum* L. แสดงการเสริมฤทธิ์กันในการต้านเชื้อ Ciprofloxacin-resistant *P. aeruginosa* จำเป็นที่จะต้องมีการค้นคว้าเพิ่มเติมในห้องปฏิบัติการและการศึกษาทางคลินิก เพื่อให้ได้ข้อมูลสำหรับการพัฒนาการใช้สมุนไพรเหล่านี้เป็นแหล่งใหม่ของยาต้านจุลชีพ

สาขาวิชาชีววิทยา ลายมือชื่อนักศึกษา …… ……..
ปีการศึกษา 2545 ลายมือชื่ออาจารย์ที่ปรึกษา ……..
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม ……
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม ……

KANTALAK PUNOPAS: THE STUDY OF ANTIBACTERIAL ACTIVITY OF SOME MEDICINAL PLANTS IN LAMIACEAE FAMILY

THESIS ADVISOR: Griangsak Eumkeb, Ph.D., 91p. ISBN 974-533-225-9

The presence of drugs-resistant bacteria not only hampered the effective treatment of infectious diseases, but it 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., *Ocimum 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 bacteria tested. *H. suaveolens* L. Poit individually displayed the best antibacterial activity against Methicillin-resistant *S. aureus*. *H. suaveolens* L. Poit in combination with *O. basilicum* L showed synergistic effect against Ciprofloxacin-resistant *P. 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.

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Kantalak Punopas

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LIST OF ABBREVIATIONS

Abbreviation	
1. DNA	deoxyribonucleic acid
2. DMSO	dimethylsulfoxide
3. FIC	Fractional Inhibitory Concentration
4. g	gram
5. h	hour
6. i.e.	id est, that is
7. IM	intramusculary
8. IV	intravenous
9. LPS	lipopolysaccharide
10. mg	milligram
11. mg/ml	milligram per millilitre
12. ml	millilitre
13. MIC	Minimal Inhibitory Concentration
14. MRSA	Methicillin-resistant *Staphylococcus aureus*
15. MSSA	Methicillin-susceptible *Staphylococcus aureus*
16. NaCl	sodium chloride
17. PMF	protein motive force
18. RNA	ribonucleic acid
19. PO	per os

LIST OF ABBREVIATIONS (CONTINUED)

20. ppm	part per million
21. UTI	urinary tract infection
22. WHO	World Health Organization
23. w/w	weight by weight

CHAPTER I

INTRODUCTION

A large portion of the world population, especially in developing countries, depends on the traditional systems of medicine to treat a variety of diseases (McGaw, Jager and Staden, 2000). Several hundred genera of plants are used medicinally. The World Health Organization (WHO) reported that 80% of the world population relies chiefly on traditional medicine and a major part of the traditional therapies, which involve the use of plant extracts or their active constituents (Ahmad, Mehmood and Mohammad, 1998). Due to indiscriminate use of antimicrobial drugs, microorganisms have developed resistance to many antibiotics and that has created immense clinical problems in the treatment of infectious diseases (Davis, 1994). Strains of β-lactam resistant *S. aureus*, methicillin-resistant *S. aureus* (MRSA) has posing a serious problem to hospitalized patients and their care providers (Liu, Durhamand and Richards, 2000). In addition, antibiotics are sometimes associated with adverse effects on host, which include depletion of beneficial gut and mucosal microorganisms, immunosuppression, hypersensitivity and allergic reaction. The drug-resistant bacteria have further complicated the treatment of infectious diseases in immunocompromised, AIDS and cancer patients, especially in the case of nosocomial infections (McGaw, Jager and Staden, 2000). There is not only the lost of an effective of antibiotics against multi-drug resistant bacteria, but also global problem for the lost of budget for treating infectious diseases. In the present scenario of 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 that will be able to act for longer periods before resistance sets in. In recent years, antimicrobial properties of medicinal plants have been reported from different parts of the world including Thailand, in which indigenous cultures possess a rich heritage of healing with medicinal plants (Ahmad and Beg, 2001; Ahmad, Mehmood and Mohammad, 1998; Brantner and Grein, 1994; Samy, Ignacimuthu and Raja, 1999; Sokmen, Jones and Erturk, 1999; Roengsumran, Petsom, Thaniyavarn, Pornpakakul and Khantahiran, 1997).

One of the plant families that is commonly known and found in Thailand is Lamiaceae or Labiatae. The famous plants belonging to this family such as peppermint and basil have been known as kitchen, edible and aromatic perennial herbs, which are cultivated throughout the country. The aromatic leaves are widely used for flavoring foods and beverages. The importance of this family is tied up with the properties of essential oils (Catherine, Githinji and Kokwaro, 1993). About 45 indigenous species in the family Labiatae have been found to be medicinal that indicates the great potential of the species of Lamiaceae in the preparation of drugs in modern medicine (Catherine, Githinji and Kokwaro, 1993). 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 other species are antihelmintic (Catherine, Githinji and Kokwaro, 1993). For this study, four species of Lamiaceae were selected by using references to their traditional usage and previous

antibacterial activity studies (Caceres, Alvarez, Ovando and Samayoa, 1991; Lentz, et al., 1998; Navarro, Villarreal, Rojas and Lozoya, 1996; Rojas, Hernandez, Miranda and Mata, 1992), three of which are kitchen herbs: *Mentha cordifolia* Opiz ex Fresen or Saranae, *Ocimum basilicum* L. or Horaphaa and *Ocimum basilicum* L. forma *citratum* Back or Maenglak. *Hyptis suaveolens* (L.) Poit or Maeng lak khaa is the only weed selected for this study. Many biological activities of the family have been studied as well as an antibacterial activity property (Caceres, Alvarez, Ovando and Samayoa, 1991; Lentz, et al., 1998; Navarro, Villarreal, Rojas and Lozoya, 1996; Okonogi, Pongpaibul, Murakoshi and Sekine, 1993; Rojas, Hernandez, Miranda and Mata, 1992). However, most of these plants were not previously screened against multi-drugs 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 isolated in Thailand should be valuable for Thai clinical practices in Thailand.

Maharat Nakhon Ratchasima Hospital is the largest hospital in Nakhon Ratchasima Province, northeast of Thailand. The problem of drug-resistant bacteria in many sections of the hospital has been previously reported (Maharat Nakhon Ratchasimal Hospital, 2000). To extend the studies by other researchers, these four medicinal plants were tested against four clinical isolates of bacteria that have caused nosocomial infection in the hospital. Eight clinical isolated bacteria, which have been used, are Methicillin-susceptible and resistant *Staphylococcus aureus*, 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 admitted at Maharat Nakhon Ratchasima

Hospital, Nakhon Ratchasima Province. The antibiotics that selected for the study were referenced to the antibiotic resistant profiles and the sample collecting limitation (Maharat Nakhon Ratchasimal Hospital, 2000).

Even though most previous researchers used agar diffusion assay to determine the antibacterial activity of extracts (Ahmad and Beg, 2001; Awadh Ali, Julich, Kusnick and Lindequist, 2001; Elgayyar, Draughon, Golden and Mount, 2001;Rojas, Hernandez, Miranda and Mata, 1992), the most widely used alternative technique to determine the lowest concentration of crude extracts that can inhibit the growing of bacteria or the minimal inhibitory concentration (MIC) is the dilution method (Eloff, 1998; Rojas, Hernandez, Miranda and Mata, 1992). Previous research has shown that some medicinal plants of this study have antimicrobial activities but there is little quantitative data (MIC) on the antimicrobial activities of them. Therefore, this study was undertaken to investigate the effectiveness of the selected herbal crude extracts against drugs-susceptible and resistant bacteria. To determine 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. The purpose of this study was to investigate antibacterial activity of some Thai medicinal plants in the family of Lamiaceae against the clinical isolates bacteria and to provide alternative sources of antibacterial agents.

1.1 Objectives of Study

1. To study the antibacterial activity of each crude extract from leaves of four medicinal plants in Lamiaceae family against a) drug–susceptible bacteria and b) drug-resistant bacteria, which are both clinical isolates.
2. To study the antibacterial activities of combined crude extracts (two species) from leaves of four medicinal plants in Lamiaceae family against a) drug-susceptible bacteria and b) drug-resistant bacteria, which are both clinical isolates.

1.2 Research Hypothesis

1. The crude extracts from leaves of four medicinal plants in Lamiaceae family could show antibacterial activities against a) drug-susceptible bacteria and b) drug-resistant bacteria, which are both clinical isolates.
2. The crude extracts from leaves of four medicinal plants in Lamiaceae family could show combination effect against a) drug–susceptible bacteria and b) drug-resistant bacteria, which are both clinical isolates.

1.3 Scope and Limitation of the Study

The antibacterial activities of the crude extracts from four species of Lamiaceae family that are *H. suaveolens* (L.) Poit, *M. cordifolia* Opiz., *O. basilicum* L. and *O. basilicum* L. forma *citratum* Back were collected from Muang District, Nakhon Ratchasima Province. Dried powdered plants were extracted by 95% ethanol. Then the crude extracts were assessed *in vitro* to determine a) the MIC in

liquid media by broth macrodilution method and b) the antimicrobial combinations of medicinal crude extracts by Checkerboard assay, against eight of the drug-susceptible and drug-resistant isolated bacteria that caused infectious diseases.

1.4 Expected Results

The outcomes from this study should be:

1. Providing information on antibacterial activities of kitchen herbs, to be used as a basic pharmacological data for the clinical treatment in the future.
2. Providing new sources of antimicrobial agents, which can be used against drug-susceptible and resistant bacteria that caused infectious diseases.
3. Providing additional scientific data on antimicrobial activity from combination of kitchen herbs, which has been used broadly as vegetables.
4. Encouraging Thai people to realize and appreciate the value of Thai medicinal plants.
5. Enhancing the development of practicing in Thai traditional medicine.

CHAPTER II

LITERATURE REVIEW

1. MEDICINAL PLANTS

1.1 Medicinal plants used

Family : LAMIACEAE Lindl. (Labiatae Juss.) or Mint family (Hickey and King, 1997).

Distribution

A Lamiaceae family is composed of 224 genera and 5600 species distributed worldwide, especially the Mediterranean region to central of Asia.

Four species of Lamiaceae family; *Mentha cordifolia* Opiz ex Fresen , *Ocimum basilicum* L., *Ocimum basilicum* L. forma *citratum* Back and *Hyptis suaveolens* (L.) Poit (Maeng lak khaa) were collected from Muang District, Nakhon Ratchasima province and evaluated for their antibacterial activities.

1.1.1 *H. suaveolens* (L.) Poit.

Figure 1

H. suaveolens (L.) Poit

Common name: Wild spikenard

Vernacular name: Kaaraa (Surat Thani), Maeng lak khaa (Chum-phon)

(เต็ม สมิตินันทน์, 2523; นันทวัน บุณยะประภัศรและ อรนุช โชคชัยเจริญพร, 2539, เล่มที่ 3)

Traditional medicinal usage:

Leaves of Maeng lak khaa have been traditionally used to treat cough or expectorant, cold, and fever (Caceres, Alvarez, Ovando and Samayoa, 1991). Mexican has locally used them for wound healing and skin infection. (Lentz, et al., 1998).

Antibacterial activity studies:

Alcoholic extracts of leaves and the whole plant of the Maeng lak khaa inhibited growth of some positive and negative bacteria by agar diffusion method (Rojas, Hernandez, Miranda and Mata, 1992). Essential oil of the plant inhibited growth of both gram-positive and gram-negative bacteria (Iwu, Ezeugwu and Okunji, 1990). Acetone extract of the whole plant strongly inhibited the growth of *Staphylococcus aureus, Staphylococcus epidermidis, Staphylococcus albus* and *Baccillus subtilis* (Hussain and Deeni, 1991).

Chemical constituents:

Table 1: Some chemicals in *H. suaveolens* (L.)Poit. from Phytochemical database (Duke, www, 2001; นันทวัน บุณยะประภัศร และอรนุช โชคชัยเจริญพร, 2539, เล่มที่ 3).

Compounds	**Plant part**	**Quantity**
1,8-cineol	plant	130 – 4,555 ppm
P - cymene	Not specified	Not specified
Eugenol	Not specified	Not specified
Limonene	plant	390 ppm
Menthol	plant	Not specified
Myrcene	Not specified	Not specified
α-pinene	plant	215 ppm
α-terpinene	plant	130 ppm
α-terpineol	plant	110 ppm
Thymol	Not specified	Not specified

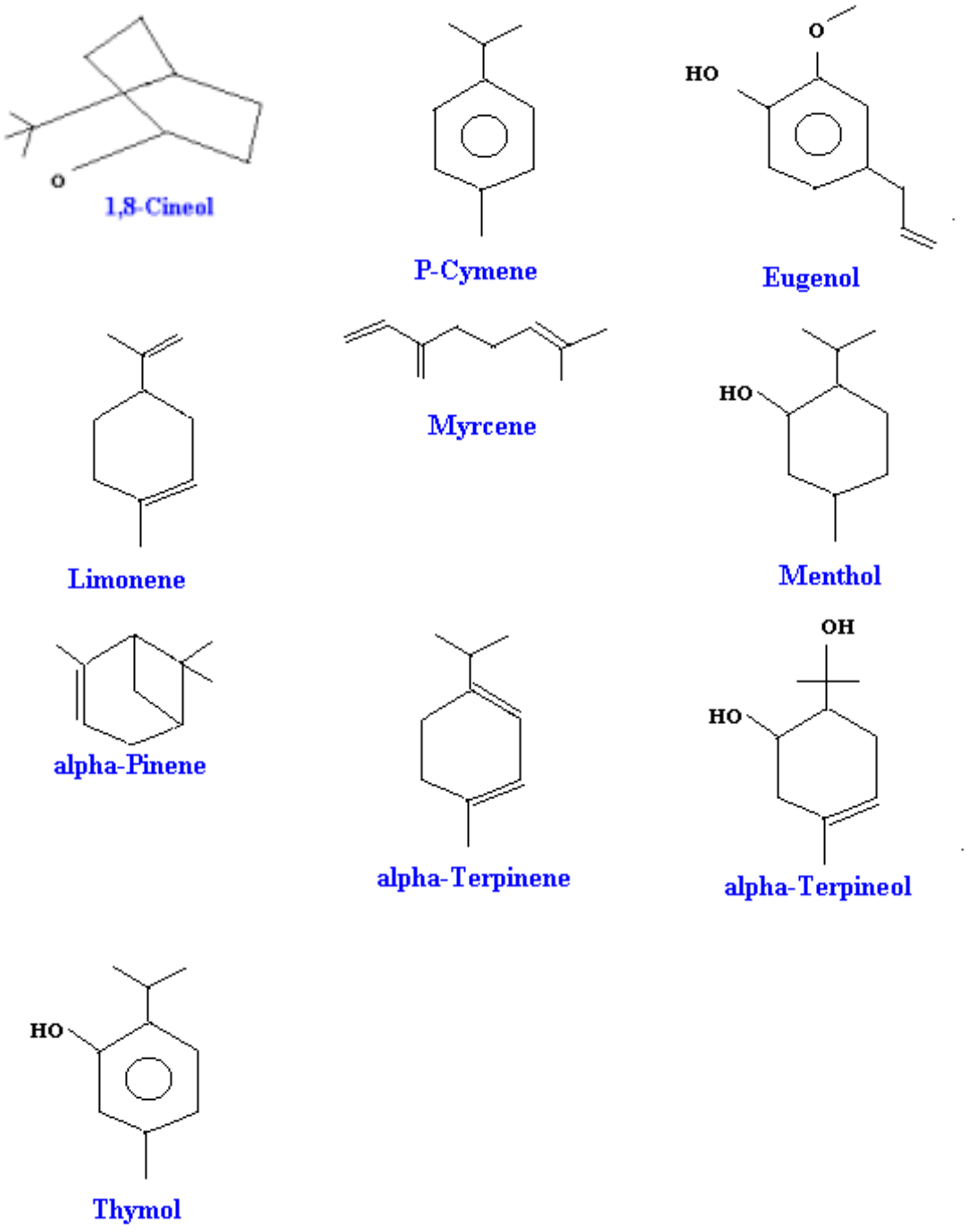


Figure 2 Chemical structures of constituents in *H. suaveolens* L. Poit

(Lide, D.R. and Milne, G.W.A.,1995)

1.1.2 *M. cordifolia* Opiz ex Fresen

Figure 3 *M. cordifolia* Opiz ex Fresen

Common name: Kitchen mint

Vernacular name: Mak ngoh, Sanae (Peninsular), Saranae, Saranae Suan (Central), Hom duan (Northern)

(เต็ม สมิตินันทน์, 2523; นันทวัน บุณยะประภัศร และอรนุช โชคชัยเจริญพร, 2539, เล่มที่ 4)

Traditional medicinal usage:

Whole plant of kitchen mint has been traditionally used as a carminative and an antiflatulence. It has been used locally to temper or relieve headache and used for sprain.

(มหาวิทยาลัยมหิดล, 2537; นันทวัน บุณยะประภัศร และอรนุช โชคชัยเจริญพร, 2539, เล่มที่ 4)

Antibacterial activity studies:

A dried saranae extract caused the decrease in growth rates of *E. coli* and *S. aureus* (อัญชัญ ชุณหะหิรัณย์ และอดิเรก เอกโสวรรณ, 2545).

Chemical constituents:

Table 2: Some chemicals in *M. cordifolia* Opiz ex Fresen.

(นันทวัน บุณยะประภัศร และอรนุช โชคชัยเจริญพร, 2539, เล่มที่ 4)

Compounds	Plant parts	Quantity
Cadinene	Not specified	Not specified
Carvone	Not specified	Not specified
P – cymene	Not specified	Not specified
Limonene	Not specified	Not specified
L – menthol	Not specified	Not specified
Menthone	Not specified	Not specified
Neral	Not specified	Not specified
Ocimene	Not specified	Not specified
Piperitone	Not specified	Not specified
α-pinene	Not specified	Not specified
piperitol	Not specified	Not specified
Trans-carveol	Not specified	Not specified
Trans-carvyl acetate	Not specified	Not specified
coumarin	Not specified	Not specified
Methyl acetate	Not specified	Not specified
Dihydrocarvone	Not specified	Not specified
β - pinene	Not specified	Not specified

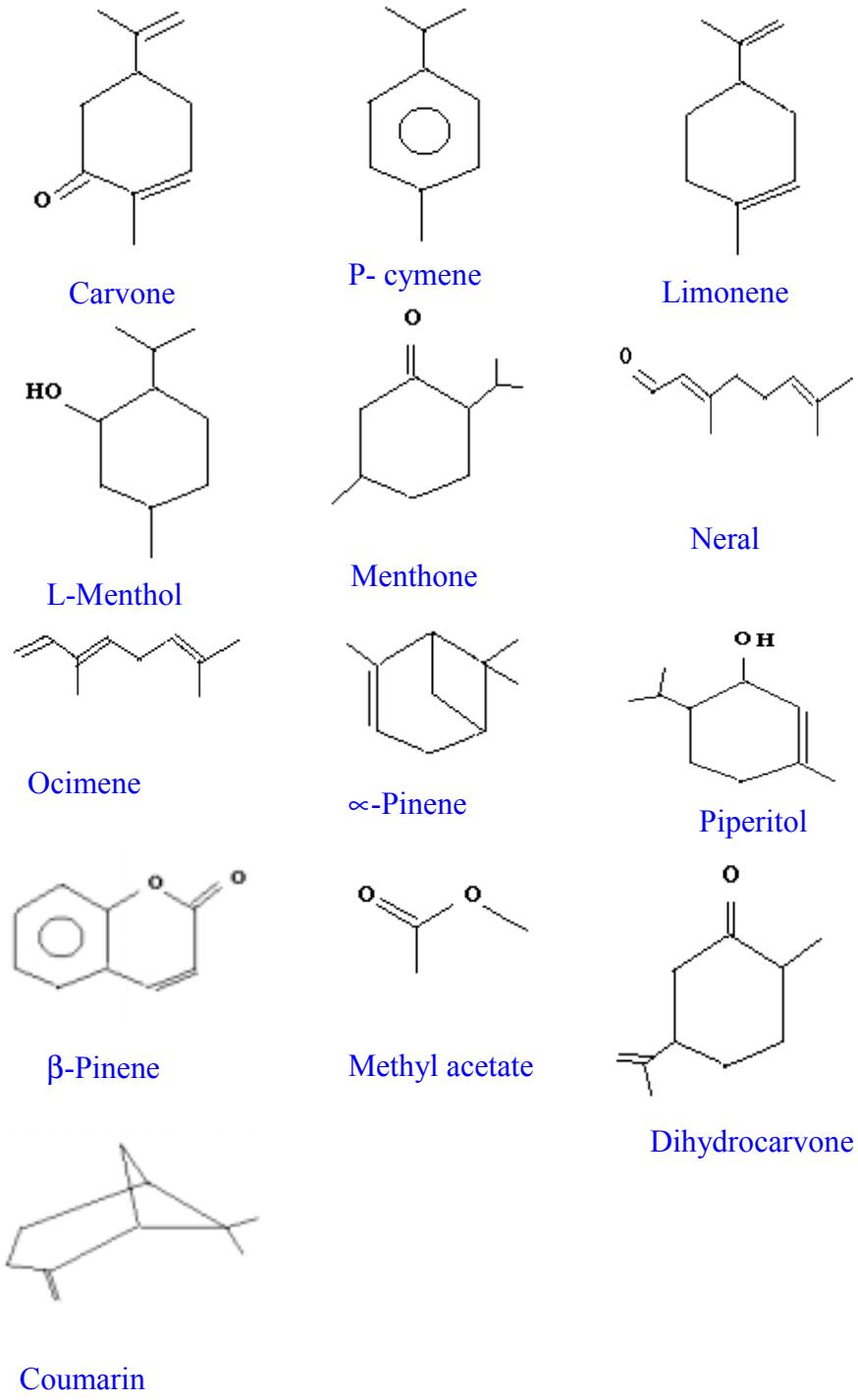


Figure 4 Chemical structures of constituents in M. cordifolia Opiz ex Fresen

(Lide, D.R. and Milne, G.W.A., 1995)

1.1.3 *O. basilicum* L.

Figure 5 *O. basilicum* L.

Common name: Basil, common basil, sweet basil

Vernacular name: Ho-kuai-suai, Ho-wo-su (Karen-Mae Hong Son), Horaphaa (General), Im-khim-Khaao (Sham-Mae Hong Son)

(เต็ม สมิตินันทน์, 2523; นันทวัน บุณยะประภัศร และอรนุช โชคชัยเจริญพร, 2539, เล่มที่ 5)

Traditional medicinal usage:

Fresh sweet basil leaves have been traditionally used as a carminative. A sweet basil fruit has been used as a laxative (มหาวิทยาลัยมหิดล, 2537). In Guatemala, its leaves has been used to treat cough, cold and sore throat (Caceres, Alvarez, Ovando and Samayoa, 1991).

Antibacterial activity studies:

A 50 % w/w ethanolic extract of basil leaves and its juice showed inhibition against growth of *Escherichia coli* and *Salmonella enterilidis* (Caceres, Cano, Samayoa and Aguilar, 1991). Its 95% ethanolic extract and volatile oil could inhibit

Escherichia coli and *Staphylococcus aureus* (Dimayuga and Garcia, 1991). Its acetone and n-hexane of leaf extracts inhibited growth of *Staphylococcus pneumoniae* and *Staphylococcus pyogenes* (Caceres, Figueroa, Taracena and Samayoa, 1993). The methanolic extract of its whole plant showed ability to inhibit *Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa* (Navarro, Villarreal, Rojas and Lozoya, 1996). By agar well diffusion, its essential oil inhibited growth of psychrotrophic pathogen, *Aeromonas hydrophila* (Wan, Wilcock and Coventry, 1998).

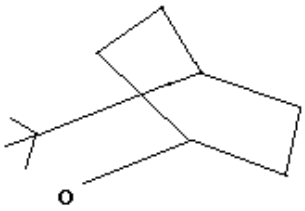
Chemical constituents:

Table 3: Some chemicals in *O. basilicum* L. from Phytochemical database and secondary data.

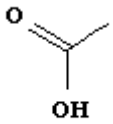
(Duke, www, 2001; นันทวัน บุณยะประภัศร และอรนุช โชคชัยเจริญพร, 2539, เล่มที่ 5)

Compounds	**Plant part**	**Quantity**
1,8-cineol	plant	776 ppm
Acetic acid	Essential oil	Not specified
Alanine	leaf	7,470 ppm
Anethole	plant	Not specified
Apigenin	plant	Not specified
α-terpineol	plant	36-239 ppm
Caffeic acid	leaf	19,000 ppm
Camphor	plant	2-31 ppm
caryophyllene	plant	18-3,196 ppm
Citral	plant	560- 7,000ppm

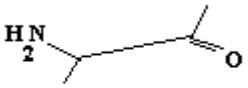
Citronellol	plant	0.2-2,419 ppm
Eriodictyol	leaf	Not specific
Eugenol	leaf	14-8,575 ppm
Geranial	plant	5-3,750 ppm
Geraniol	plant	1-1,000 ppm
Menthol	plant	4-32 ppm
Myrcene	leaf	2-80 ppm
Methyl-eugenol	plant	13-1,400 ppm
Nerolidol	Essential oil	Not specified
Limonene	plant	2-934 ppm
Luteolin	plant	Not specified
Oleanolic-acid	flower	1,300 ppm
p-cymene	plant	1-16 ppm
Rutin	leaf	Not specified
α-thujone	plant	Not specified
Quercetin	leaf	Not specified
Rosmarinic-acid	plant	1,000-19,000 ppm
β - sitosterol	Leaf,flower, root, spout seedling, stem	863-1,705, 1,051, 408, 230 and 230 ppm respectively
Safrole	plant	60-400 ppm
Thymol	leaf	1,415 ppm



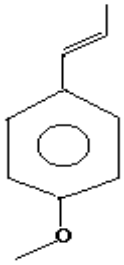
1,8-Cineol



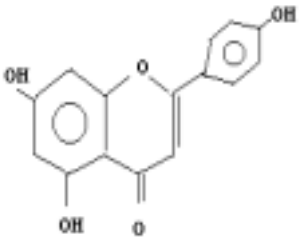
Acetic acid



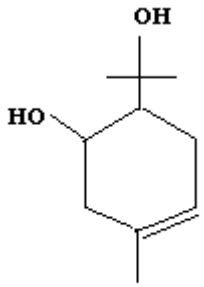
Alanine



Anethole

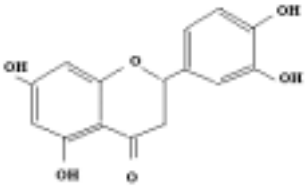


Apigenin

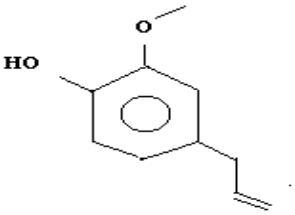


α-Terpineol

Camphor

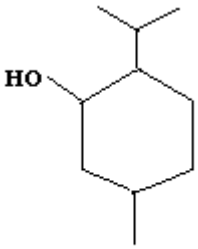


Eriodictyol



Eugenol

Geraniol

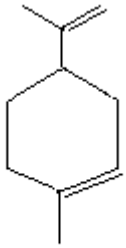


Menthol

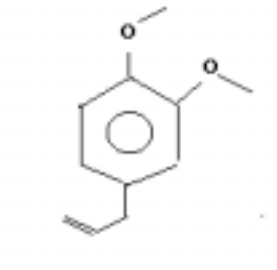
Myrcene

Figure 6 Chemical structures of constituents in *O. basilicum* L.

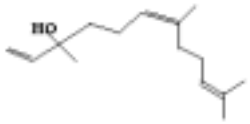
(Lide, D.R. and Milne, G.W.A., 1995)



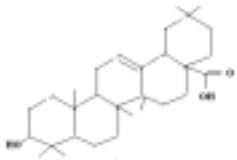
Limonene



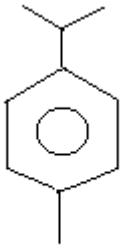
Methyleugenol



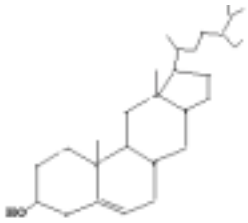
Nerolidol



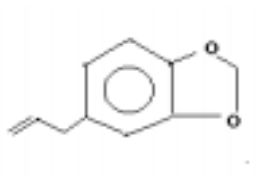
Olenolic acid



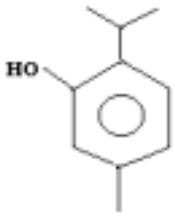
P-Cymene



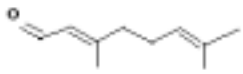
β-sitosterol



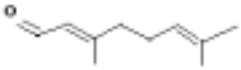
Safrole



Thymol



Citral



Geranial

Figure 6 (cont.) Chemical structures of constituents in *O. basilicum* L.

(Lide, D.R. and Milne, G.W.A., 1995)

1.1.4 *O. basilicum* L. forma *citratum* Back

Figure 7 *O. basilicum* L. forma *citratum* Back

Common name: Hairy basil

Vernacular name: Komko khaao (Northern), Manglak, Maeng lak (Central)

(นันทวัน บุณยะประภัศร และอรนุช โชคชัยเจริญพร, 2539, เล่มที่ 3)

Traditional medicinal usage:

The whole plant of hairy basil has been traditionally used for the treatment of cough, digestive disease, toothache, indigestion and chronic gastrointestinal ailments of children, and used as a carminative, antiflatulence, and diaphoretic. Its leaves have been used to treat cold, bronchitis, skin diseases, carminative and diaphoretic. Its seeds have been used as a laxative, antiamoebic and diuretic.

(มหาวิทยาลัยมหิดล, 2537; นันทวัน บุณยะประภัศร และอรนุช โชคชัยเจริญพร, 2539, เล่มที่ 3)

Antibacterial activity studies:

An alcohol extract of not specified part of hairy basil showed bactericidal effects on both Gram- positive and Gram-negative bacteria. An ether extract of

steam-distillate of hairy basil inhibited growth of *Mycobacterium tuberculosis* (Fransworth and Bunyapraphatsara, 1992).

Chemical constituents:

Table 4: Some chemicals in *O. basilicum* L. forma *citratum* Back.

(นันทวัน บุณยะประภัศร และ อรนุช โชคชัยเจริญพร, 2539 เล่มที่ 3)

Compounds	**Plant part**	**Quantity**
β - caryophyllene	Not specified	Not specified
1- 8, cineole	Not specified	Not specified
citral	Not specified	Not specified
eugenol	Not specified	Not specified
geraniol	Not specified	Not specified
Hept-5-en-2-one,6-methyl	Not specified	Not specified
limonene	Not specified	Not specified
linalool	Not specified	Not specified

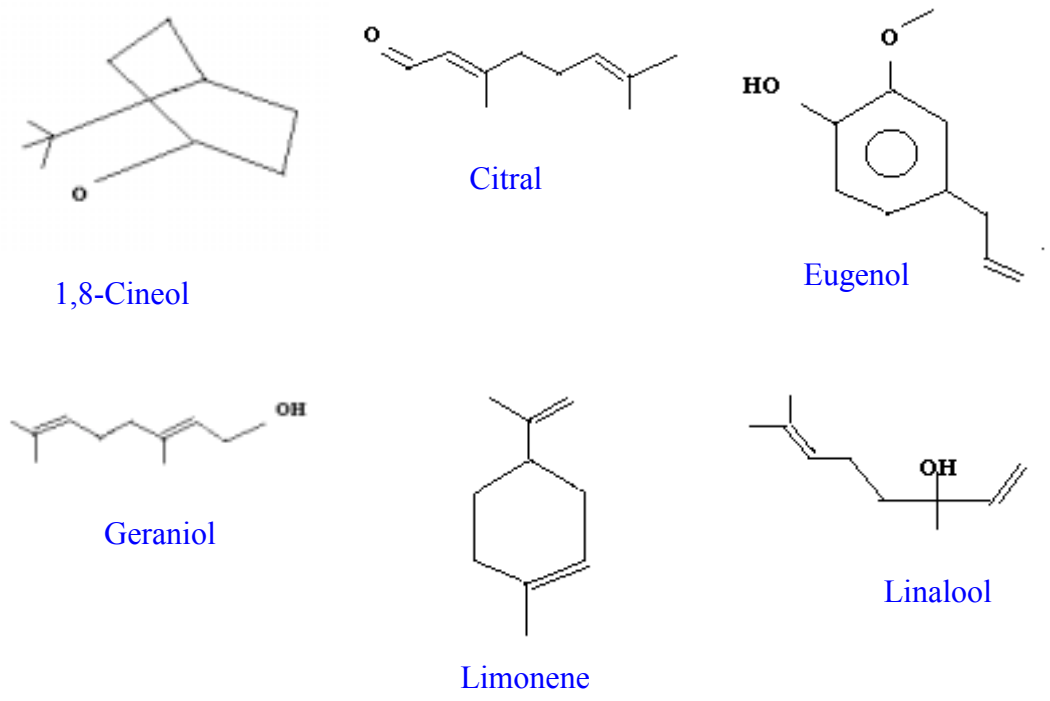


Figure 8 Chemical structures of constituents in O. basilicum L. forma citratum Back (Lide, D.R. and Milne, G.W.A., 1995)

2. EXTRACTION

Extraction is the process of moving one or more compounds of interest from the sample or matrix to an extraction solvent. The sample may be a solid, liquid or gas.

Soxhlet techniques

The Soxhlet extractor (Figure 9) is one of the extraction systems. A solid sample is placed in an extraction thimble inside the middle chamber. Upon boiling, the solvent vapour from the bottom flask travel up to the condenser and then drip through the sample. The sample is soaked in the solvent, which then returns to the

flask when the liquid reaches the top of the siphon. The sample is exposed to fresh solvent after every siphon cycle, usually at a rate of about six cycles per hour. Typical extraction time is 6-24 h (Wison, 2000).

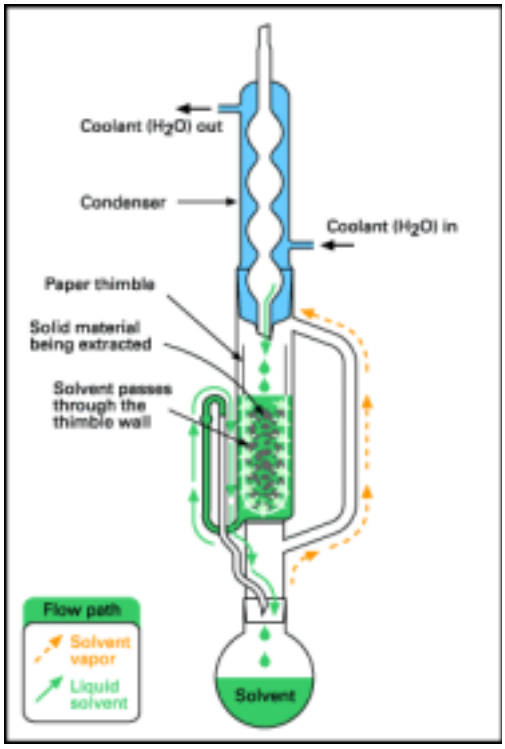


Figure 9 The Soxhlet extractor apparatus

(www.anl.gov/OPA/logos16-2/ extractor2.htm)

3. MICROORGANISMS

3.1 Bacterial structure (Walker, 1999)

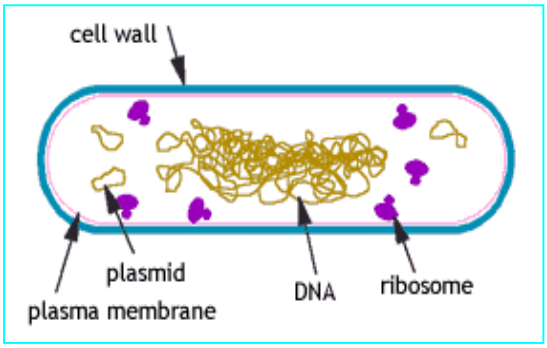


Figure 10 A typical prokaryotic cell

(www.microbeworld.org/img/aboutmmicro/ bacteria/bactdiag.gif)

3.1.1 Components of the bacterial cell envelope

A. The cytoplasmic membrane

The cytoplasmic membranes of Gram-positive and Gram-negative bacteria are indistinguishable. Each is composed of protein, lipids, phospholipids and a small amount of carbohydrate (figure 11 and 12).

It has five principal functions;

1. To act as an osmotic barrier.
2. To serve as the site of selective permeability and carrier-mediated transport.
3. To serve as the site of cytochrome activity and generation of proton motive force (PMF).
4. To synthesize the cell wall.
5. To provide a site to implant the chromosome.

B. The periplasm

The periplasm is the space between the inner and outer membranes of a Gram-negative bacterium, and the cell wall lies within it. The periplasm contains enzymes that hydrolyze large molecules, contains enzymes that hydrolyze antibiotics, and binding protein that facilitate transport.

C. The cell wall

The cell wall is a web-like structure that is sometimes called the murien sacculus. It is composed of peptidoglycan. The cell wall provides the cell with its shape and osmotic stability. The cell wall constituents are peptidoglycan, teichoic acids and lipoteichoic acids.

D. The outer membrane

Only Gram-negative bacteria have an outer membrane. Porins and porin-like protiens in the outer membrane allow the membrane to act as a molecular sieve, restricting the access of some molecules to the cell wall and periplasm. The most clinically significant component of the outer membrane is a phospholipid like molecule called lipopolysaccharide (LPS) that shown in figure 11.

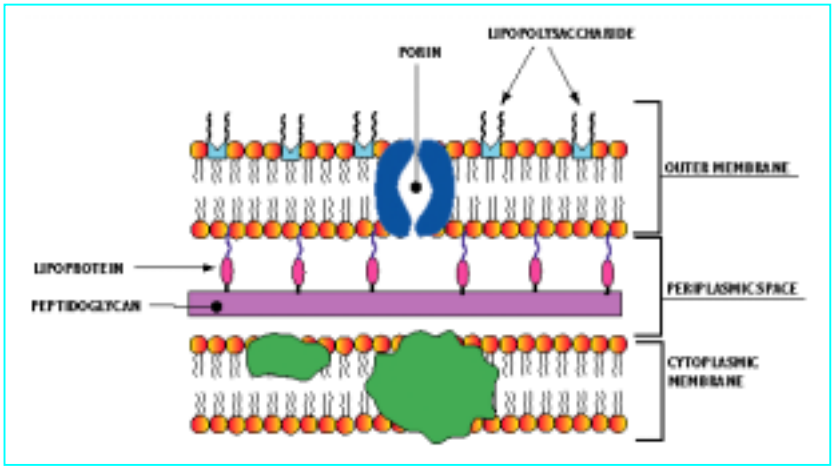


Figure 11 The cell wall of Gram-negative bacteria

(www.liu.edu/.../WebClass/micro-web/ images/gm-ve.gif)

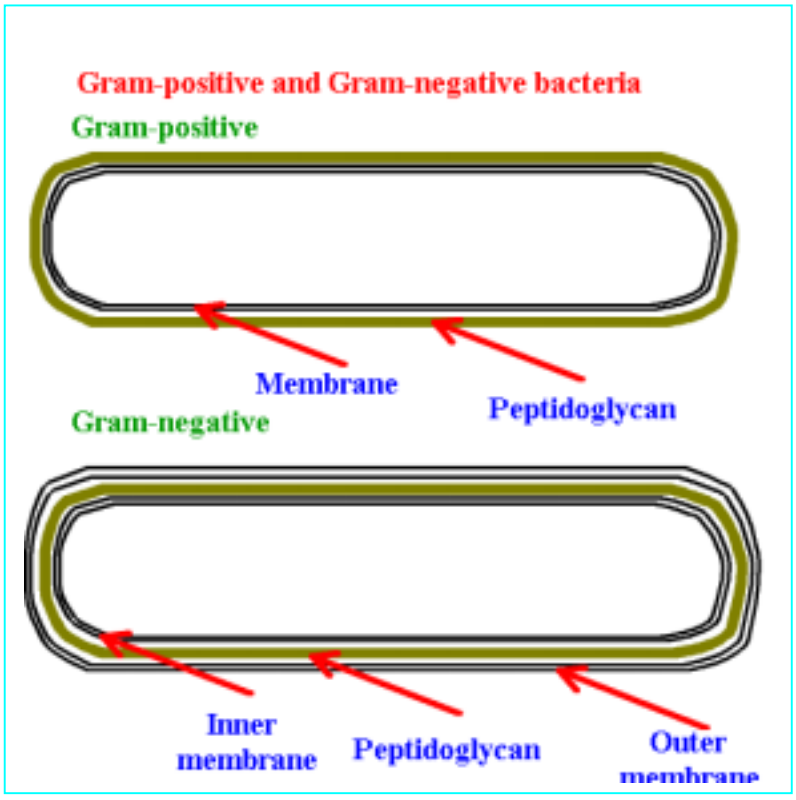


Figure 12 Comparison of the structure of cell walls between gram-positive and gram-negative bacteria. (www.biosite.dk/staabi/ images/gram.gif)

3.2 Bacteria used

3.2.1 *Staphylococcus aureus*

Family: Micrococcaceae

General characteristics

The *S. aureus* is Gram-positive cocci. It is spherical cells (0.5 to 1.5 μm) that appear singly, in pairs, and in irregular clusters that has been described as looking like " branches of grapes". This organisms is non-motile, non-spore forming, facultative anaerobe and chemoorganotroph (both respiratory and fermentative metabolism). Colonies appear creamy, white or light gold and sometimes yellow to orange. The optimum temperature is 30-37 °C (Holt, Krieg, Sneath, Staley and Williams, 1994).

Clinical significance

S. aureus is reponsible for a wide variety of infections and disease due to toxins (see Table 5).

Antibiotic susceptibility characteristics

Penicillin became more widely available and used. By the 1950s, isolated strain of *S. aureus* was resistant to penicillin by producing an enzyme that cleaves its β -lactam ring. The penicillinase-resistant penicillins, which were nafcillin, methicillin and oxacillin, were used to treat the more resistant isolates. The 1970s, resistance developed to these compounds. The MRSA have become a costly problem in hospitals. The rest anti-infective therapy against MRSA is vancomycin (Shimeld and Rodgers, 1999).

Table 5 Diseases associated with *S. aureus* (Mahon and Manuselis, 2000; Shimeld and Rodgers, 1999)

Diseases associated with *S. aureus*
1. Localized infections *Skin infections (folliculitis, impetigo, furuncles, carbuncles) and wound infections* Infection caused by *S. aureus* are supparative and pyogenic. Some of the common skin infections are boils, carbuncles, folicullitis and bullous impetigo. These opportunistic infections occur usually as a result of previous skin injuries.
2. Systemic infections *Bacteremia, septicemia*
3. Toxin production *Food poisoning* *S. aureus* produces enterotoxins that have been identified and associated with gastrointestinal upset. Symptoms appear rapidly, after ingestion of the food, and resolve within 6 to 8 hours. Nausea, vomiting, abdominal pain, and severe cramping are common. *Scaled skin syndrome* Scalded skin syndrome or Ritter's disease, is an extensive exfoliative dermatitis that occurs primarily in newborns and previously healthy young children. *Toxic shock syndrome (TSS)* TSS is a multisystem disease charcterized by high fever, hypotension, and shock. The initial clinical presentation of TSS consists of high fever, rash and signs of dehydration. In extreme cases, patients may be severely hypotensive and in shock.

3.2.2 *Enterobacter cloacae*

Family: Enterobacteriaceae

General characteristics

Ent. cloacae is a Gram-negative, straight rods (0.6-1.0 μm wide× 1.2-3.0 μm long). It is facultative anaerobes and chemoorganotrophs. The optimal temperature is 30-37°C. It wildly distributed in nature. It can be found in the soil, dairy products, water and sewage. It may also be present in the intestinal tract of humans and animals (Holt, Krieg, Sneath, Staley and Williams, 1994; Shimeld and Rodgers, 1999).

Clinical significance

Ent. cloacae generally do not cause disease in healthy individuals but significant cause the infections in immunocompromised or otherwise debilitated patients. This species is an opportunistic pathogen causing burn, wound and urinary tract infection and occasionally septicemia and meningitis (Mahon and Manuselis, 2000).

Antibiotic susceptibility characteristics

Most isolates of *Enterobacter* are resistant to ampicillin and first-generation cephalosporins. Second- and third-generation cephalosporins may be effective (Shimeld and Rodgers, 1999).

3.2.3 *Escherichia coli*

Family: Enterobacteriaceae

General characteristics

E. coli is a Gram- negative, straight rods (1.1-1.5 μm × 2.0-6.0 μm) that occur singly or in pairs. It is facultatively anaerobes and chemoorganotrophs. The optimal temperature is 37°C. It occurs as normal flora in the lower part of the intestine of warm-blooded animals (Holt, Krieg, Sneath, Staley, and Williams, 1994).

Clinical significance

The various types of infections associated with *E. coli* are summarized in Table 6.

Table 6: Characteristics of *E. Coli* associated with diseases. (Shimeld and Rodgers, 1999, p.190)

Group	**Type of infection**	**Comments**
Nephropathogenic E.coli (NPEC)	Urinary tract infection (UT) (pyelonephritis)	Common in woman, young children in diapers and catheterized patients.
Enteropathogenic E.coli (EPEC)	Watery diarrhea	Common in infants, outbreaks in nurseries
Enterotoxigenic E.coli (ETEC)	"Traveler's diarrhea"	Common in travelers to endemic areas.
Enterohemorrhagic E.coli (EHEC)	Hemorrhagic colitis, hemolytic uremic syndrome	Associated with undercooked ground beef, raw milk, other foods, acute renal failure, maybe fatal

Enteroinvasive E.coli (EIEC)	Bloody diarrhea	Dysentery-like diseases, most common in young children in developing countries
Enteroaggregative E.coli (EAEC)	Watery diarrhea	Most common in young children in developing countries, diarrhea may be acute or chronic

Antibiotic susceptibility characteristics

Antibiotics that generally have strong activity against *E. coli* include the sulfonamides, ampicillin, cephalosporins, chloramphemicol, tetracyclines, and aminoglycosides. Sulfamethoxazole-trimethoprim (e.g., Bactrim®, Spectra®) and ciprofloxacin are usually very effective when treatment is started early (Shimeld and Rodgers, 1999).

3.2.4 *Pseudomonas aeruginosa*

Family: Pseudomonadaceae

General characteristics

P. aeruginosa is a Gram negative, straight or slightly curved rods (0.5-1.0 μm × 1.5-5.0 μm). It wildly distributed in nature (Holt, Krieg, Sneath, Staley, and Williams, 1994). It is extremely hardy organism, surviving under conditions that would kill most other bacteria (Shimeld and Rodgers, 1999).

Clinical significance

This organism can occasionally cause disease in healthy individuals. The infections in debilitated or immunocompromised hosts are significantly more common and more serious. This species is a very important opportunistic pathogen in

hospitalized patients. The types of infections associated with *P. aeruginosa* are summarized in Table 7.

Table 7: Types of infection associated with *P. aeruginosa.* (Shimeld and Rodgers, 1999, p.211)

Infections	Comments
Burns and wounds	Wounds may be due to accidental or surgical trauma, infection is often accompanies with blue-green pus due to pigment production.
Bacteremia/septicemia	Result of progressive infection seen in immunocom-promised individuals.
Ecthyma gangrenosum	Syndrome with painful and occur in association with bacteremia.
Osteomyelitis	Inflammation of bone, associated with deep wounds and compound fractures, may be local or spreading.
Otitis externa	"Swimmer's ear" in children who spend prolonged time in swimming pools.
Pneumonia and lung abscesses	Associated with neutropenia, immunosuppression and cytotoxic drugs.
Meningitis	Seen mostly in the immunocompromised.
UTI	Associated with catheters and medical procedures.
Endocarditis	Seen mostly in drug addicts, occasionally seen in patients with prosthetic heart valves.

Antibiotic susceptibility characteristics

P. aeruginosa is one of the most highly resistant organisms encountered in clinical laboratories. Usually a penicillin is used together with an aminoglycoside. The newer quinolones (ciprofloxacin), aztreonam, imipenem, and other third-generation cephalosporins are also active against the organisms (Shimeld and Rodgers, 1999).

4. ANTIBIOTICS

Antibiotics are chemical substances produced form various microorganisms (bacterial and fungus) that kill or suppress the growth of other microorganisms. The term is also uses for synthetic antimicrobial agents such as sulfonamides and quinolones (Salerno,1999).

4.1 Mechanisms of action of clinically used antimicrobial drugs (Brooks, Butel and Ornston, 1995)

Selective toxicity is a drug that harmful to a parasite without being harmful to the host.

The mechanisms of action can be placed under four headings:

a) Inhibition of cell wall synthesis.
b) Inhibition of cell membrane function.
c) Inhibition of protein synthesis (ie, inhibition of translation and transcription of genetic material)
d) Inhibition of nucleic acid synthesis.

Antibiotics that inhibit bacterial cell wall synthesis

(Page, Curtis, Sutter, Walker, and Hoffman, 1997)

The two most important classes of antibiotics that inhibit bacterial cell wall synthesis are β Lactams and glycopeptides. In this literature review describe only β Lactams that is one of antibiotic group associated with the study.

4.2 β Lactams

β Lactam antibiotics possess a four-member nitrogen-containing β lactam ring. It interferes with bacterial cell wall synthesis by inhibiting the cross-linkage of the peptide side chains of the bacterial cell wall. β lactams are mainly bactericidal and exhibit time-dependent killing.

4.2.1 ***Penicillin*** They are a byproduct of *Penicillium notatum*. It consist of a β lactam ring fused to a five-member, sulfur-containing thiazolidine ring. Modification of the side chain at position six of the β lactams ring results in drugs with different antibacterial and pharmacologic properties. There are four classes of penicillins: standard penicillins, antistapylococcal penicillins, aminopenicillins and pseudomonal penicillins.

4.2.2 ***Cephalosporins*** The first cephalosporin was discovered from *Cephalosporium acremonium*. It consists of a β lactam ring fused to a six-member sulfur- containing dihydrothiazine ring. Individual cephalosporins are created by side-chain substitutions at position seven of the β lactam ring and position three of the dihydrothiazine ring. Cephalosporins are traditionally classified into first-, second- and third-generation drugs based on their spectrum against aerobic Gram-negative bacilli, which incerases from first to third generation.

Antibiotic that inhibit bacterial deoxyribonucleic acid synthesis

4.3 Quinolones

They are synthetic antibiotics that consist of a nucleus of two fused six-membered rings. The addition of a fluorine atom at position six of the quinolone nucleus enhances activity against Gram-negative bacteria and led to a new generation of drugs known as fluoroquinolones. Quinolones inhibit bacterial deoxyribonucleic acid (DNA) gyrase, the enzyme responsible for supercoiling, nicking and sealing bacterial DNA. Aquired resistance may develop through either decreased permeability or alterations in DNA gyrase. They are highly active against aerobic Gram-negative bacilli including Enterobacteriaceae (Page, Curtis, Sutter, Walker, and Hoffman, 1997).

Each year, more than 500 metric tons of chemotherapeutic agents of various types are manufactured and used. In 1994, 37% cephalosporin, 17% penicillin and 14% quinolone are produced and used world wide (Madigan, Martinko and Parker, 2000). Three antibiotics were selected to study,

(a) Ceftazidime or Fortum® is a third generation cepharosporin that active against aerobic Gram-negative bacilli particularly Enterobacteriaceae and *P.aeruginosa*. Usual adult dose for this antibiotics is 200 mg every 12 h for IM and 0.5-2 g every 8-12 h for IV(Salerno, 1999). A cost per vial (1g) of ceftazidime is 400 baht.

(b) Methicillin or Staphcillin® is a penicillinase resistant penicillin, which is the usual treatment for *S. aureus*. Over the past 30 years, most strains of *S. aureus* have become resistant to commonly used antibiotics. This makes infections caused by

these organisms more difficult and expensive to treat. Usual adult dose for the methicillin is 1 g every 4-6 h for IM and 1 g every 6 h for IV(Salerno, 1999).

(c) Ciprofloxacin or Cifoxin®is the commonly used of fluoroquinolone. It is useful in the treatment of infections due to aerobic Gram-negative bacilli and particularly *P. aeruginosa* that are not susceptible to less expensive agent (Page, 1997). Usual adult doses for ciprofloxacin is 500 to 750 mg PO every 12 h and 400 mg IV every 12 h (Salerno, 1999).

4.4 Major groups of antimicrobial compounds from plants

Plants are rich in wide variety of secondary metabolites. Useful antimicrobial phytochemicals can be divided into several categories (Cowan, 1999; Kaufman, Cseke, Warer, Duke and Brielmann, 1999).

4.4.1 Phenolics and polyphenols

1) Simple phenols and phenolic acid

Most of the simple phenols are monomeric components of the polymeric polyphenols and acids which make up plant tissues, including lignin, melanin, flavolan and tannins. The sites and number of hydroxyl groups on the phenol group are though to be related to their relative toxicity to microorganisms, with evidence that increased hydroxylation results in increased toxicity. The mechanisms thought to be responsible for phenolic toxicity to microorganisms include enzyme inhibition by the oxidized compounds, possibly through reaction with sulfhydryl groups or through more nonspecific interactions with the proteins.

Phenolic compounds possessing a C_3 side chain at a lower level of oxidation and containing no oxygen are classified as essential oils and often cited as antimicrobial as well.

2) Quinones

The quinones are aromatic rings with two ketones substitution, typically form colored pigments covering the entire visible spectrum. Generally, they are derived from benzoquinone, naphthoquinone or anthroquinone structures. Propable targets in the microbial cell are surface-exposed adhesins, cell wall polypeptides and membrane-bound enzymes.

3) Flavones, flavonoids and flavonols

Flavones are phenolic structures containing one carbonyl group. The flavonoids have two benzene rings separated by a propane unit and are derived from flavone. They are generally found in plants as their glycosides. Their activity is probably due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls. More lipophilic flavonoids may also disrupt microbial membrane.

4) Tannin

The tannins are common to vascular plants existing primarily within woody tissues. Plant tissues that are high in tannin content have a highly bitter taste and are avoided by most feeders. Previous report reviewed the antimicrobial properties of tannins. According to these studies tannin can be toxic to filamentous fungi, yeast and bacteria. Their mode of antimicrobial action may related to their ability to inactivate microbial adhesins, enzymes, cell envelop transport proteins, etc.

5) Coumarin

Coumarins are phenolic substance made of fused benzene and ∝-pyrone rings. They are responsible for the characteristic odor of hay. Coumarin was found in vitro to inhibit *Candida albicans*. They have been found to stimulate macrophages, which could have an indirect negative effect on infection.

4. 4.2 Terpenoids and Essential Oils

The oils are secondary metabolites, which are highly enriched in compounds based on an isoprene structure. They are called terpenes. Their general chemical structure is $C_{10}H_{16}$, and they occur as diterpenes, triterpenes, and tetraterpenes (C_{20}, C_{30} and C_{40}), as well as hemiterpenes (C_5) and sesquiterpenes (C_{15}). When the compounds contain additional elements usually oxygen, they are termed terpenoids. Terpenes or terpenoids are active against bacteria, viruses and protozoa. The mechanism of action of terpenes is not fully understood but it speculated to involve membrane disruption by the lipophilic compounds.

4.4.3 Alkaloids

Heterocyclic nitrogen compounds are called alkaloids. The mechanism of action of highly aromatic planar quaternary alkaloids is attributed to their ability to intercalate with DNA.

4.4.4 Lectins and polypeptides

Peptides are often positively charged and contain disulfide bonds. Their mechanism of action may be the formation of ion channels in the microbial membrane or competitive inhibition of adhesion of microbial proteins to host polysaccharide receptors.

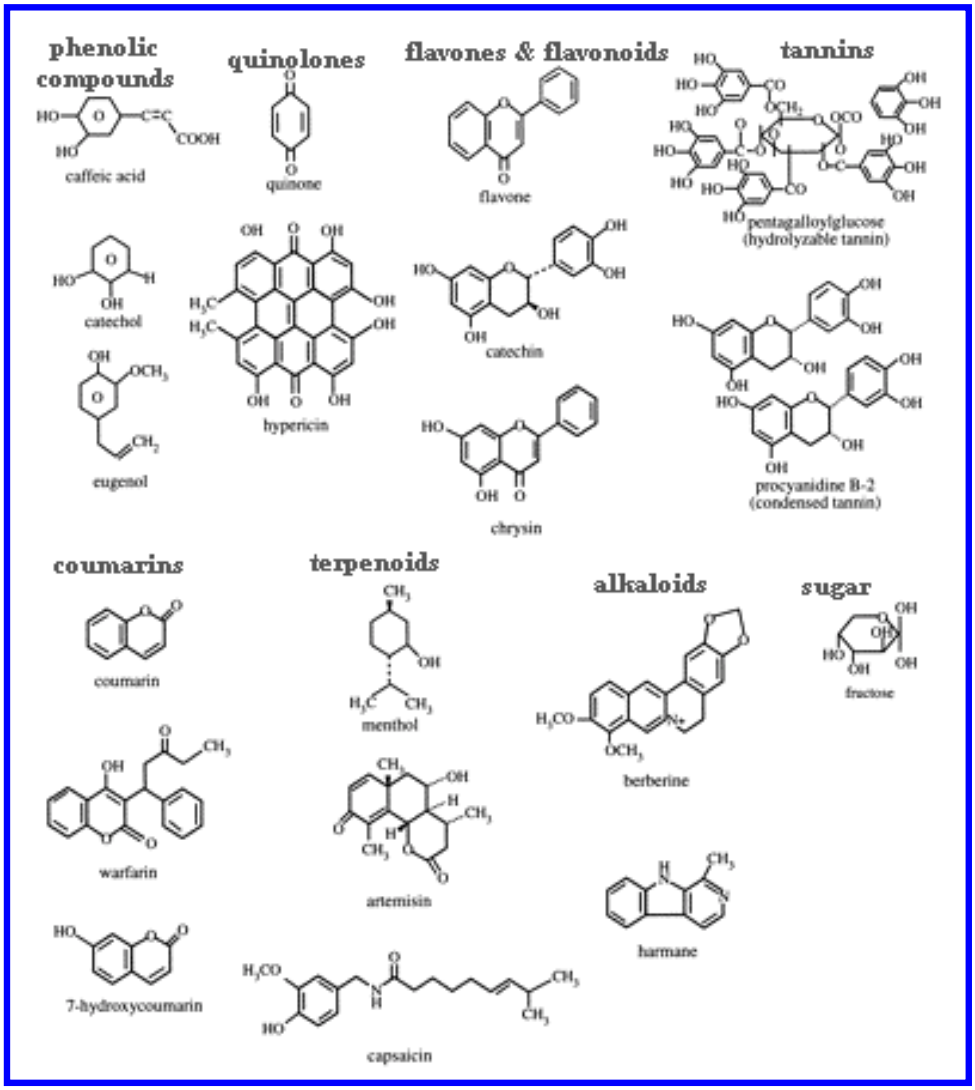


Figure 13 Structure of common antimicrobial plant chemicals. (Cowan, 1999)

5. RESISTANCE TO ANTIBACTERIAL AGENTS

A resistant organism is an organism that will not be inhibited or killed by an antibacterial agent at concentrations of the drug achievable in the body after normal dosage (Mims, Playfair, Roitt, Wakelin and Rosamund, 1998).

5.1 Genetic mechanism of resistant (Mims, Playfair, Roitt, Wakelin and Rosamund, 1998).

5.1.1 Resistance may result from a chromosomal mutation

(a) A single chromosomal mutation in one bacterial cell resulting in the synthesis of an altered protein.

(b) A series of mutations

In the presence of antibiotic, these spontaneous mutants have a selective advantage, survive and out grow the susceptible population. They can also spread to other sites in the same patient of by cross-infection to other patients and therefore become disseminated.

5.1.2 Resistance may be acquired from genes on transmissible plasmids

Bacteria are also able to acquire resistance genes on transmissible plasmids. The plasmids often code for resistance determinants to several unrelated families of antibacterial agents. Therefore a cell may acquire resistance to many different drugs (figure 14).

5.1.3 Resistance may be acquired from "jumping genes"

Resistance genes may also occur on transposons, the so-called " jumping genes", which are capable of integration into the chromosome or into plasmids. The

chromosome provides a more secure position for the genes, but they will be disseminated only as rapidly as the bacteria divide. Transposons moving from the chromosome to plasmids allow chromosomal genes to be disseminated more rapidly. Transposons can also move between plasmids.

5.2 Nongenetic mechanisms of resistance

Nongenetic resistance mechanisms can be classified into three main types (Mims, Playfair, Roitt, Wakelin and Rosamund, 1998; Walker, 1999).

5.2.1 The target site may be altered

The target enzyme may be altered so that it has a lowered affinity for the antibacterial, but still functions adequately for normal metabolism to proceed.

***5.2.2 Access to the target site may be altered (altered uptake*)**

This mechanism involves decreasing the amount of drug that reaches the target by either:

(a) Altering entry, for example by increasing the impermeability to the cell wall. This may be due to changes in porins or LPS molecules.

(b) Pumping the drug out of the cell (known as an efflux mechanism).

5.2.3 Enzymes that modify or destroy the antibacterial agent may be produced (drug inactivation) Some bacteria produce an enzyme that inactivates an antibiotic by attaching a group (methyl, acethyl or phosphate) or by cleaving a key bond (see figure 14).

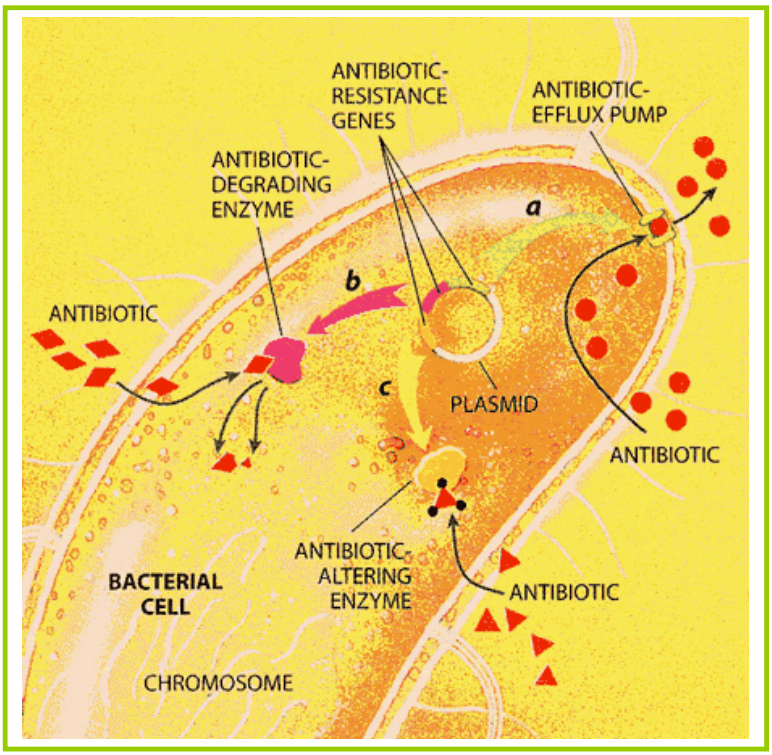


Figure 14 Mechanisms of antibiotics resistance

(www.fda.gov/fdac/features/ 2002/402_bugsIll.html)

6. LABORATORY METHODS USED FOR ANTIMICROBIAL SUSCEPTIBILITY TESTING

The inhibitory activity of an antimicrobial agent is determined either by dilution testing, which produces a quantitative result, or disk diffusion testing, which produces a qualitiative result. The decision concerning which method to use is based on several factors including cost, ease of use, flexibility, and degree of automation.

6.1 Susceptibility test methods

6.1.1 *Disk Diffusion*

Disk diffusion can categorize bacterial isolates into susceptible, resistant, or intermediate to several antimicrobial agents. To do the test, dried-filter paper disks impregnated with a specified amount of drug are applied to the surface of an agar

medium inoculated with the test organism. The antimicrobial agent in the disk diffuses through the agar, creating a gradient of drug concentrations in the agar medium surrounding each disk. The zone size is inversely proportional to the MIC; the larger the zone diameter, the lower the MIC. Zone diameters associated with different drugs, however, cannot be directly compared because the size is influenced by the rate at which the antimicrobial agent diffuses through the agar, and this differs for different drugs.

6.1.2 *Dilution Testing*

Dilution susceptibility tests determine the minimal concentration of an antimicrobial agent needed to inhibit growth of the microorganism being tested. For most dilution tests, antimicrobial agents are tested at $\log_2$ (two-fold) serial dilutions. The lowest concentration at which there is no visible growth is called the MIC.

One advantage of dilution tests is flexibility. Standard media that provide reliable testing for the rapidly growing aerobic, facultative, fastidious, and anaerobic bacteria are available from many vendors. Dilution systems also are adaptable to automation, and several such systems are commercially available (Sawan and Manivannan, 2000).

6.1.2.1 *Agar incorporation test*

In this method the antibiotic dilutions are made in solid agar medium by adding antibiotics to molten agar (at 45°C) and pouring into Petri dishes. The test strains are inoculated on to the surface of the medium. The inoculum is applied with a multipoint inoculator which transfers a small drop of broth culture to each of the desired number of antibiotic-containing plates. A suitable inoculum yields a barely

confluent spot of culture after overnight incubation on an antibiotic- free control plate (Greenwood, 2000).

6.1.2.2 *Broth dilution tests*

Conventional broth dilution test are used when only a few strains of bacteria need to be tested or when an accurate MIC estimation is required. A series of twofold dilutions of the antibiotic under study is prepared in a volume of a suitable broth medium and a standard inoculum of the test strain (commonly 10^5 bacteria) is introduced into each tube. The test is incubated at 37 °C overnight and the end-point is read as that concentration of antibiotic in which no turbidity can be seen. Uninoculated tubes containing broth plus antibiotic and broth alone act as sterility controls (an antibiotic-free tube inoculated with the test organism serves to indicate that the organism is viable in case the end-point is missed (Greenwood, 2000).

Most previous researchers used agar diffusion assay to determine the antibacterial activity of extracts (Ahmad and Beg, 2001; Awadh Ali, Julich, Kusnick and Lindequist, 2001; Elgayyar, Draughon, Golden and Mount, 2001;Rojas, Hernandez, Miranda and Mata, 1992). This technique works well with defined inhibitors but when examining extracts containing unknown components, there are problems leading to the false positive and false negative results (Eloff, 1998). The antimicrobial effect may be inhibited or increased by extrinsic factors or contaminants. The agar type, salt concentration, incubation temperature, and molecular size of the antimicrobial component influence results obtained with agar diffusion assays. The most widely used alternative technique in general microbial assay is serial dilution of the extract in a number of test tubes followed by the addition of the test organism to determine the MIC for the test organism using turbidity as an

indication of growth that is dilution method (Eloff, 1998; Rojas, Hernandez, Miranda and Mata, 1992). To determine the MIC of the medicinal plants in present study, the macrobroth dilution method was used.

6.2 Testing Antimicrobial Combinations

Antimicrobial combinations are selected for various reasons (Sawan and Manivannan, 2000):

1) To attain broad-spectrum activity for empiric therapy in critically ill patients or when polymicrobial infection is suspected.
2) To minimize drug toxicity by using the lowest possible doses of two or more agents that have additive efficacies but independent toxicities, or to reduce the potential for development of resistance to one agent.

Mechanisms

When two antimicrobial agents act simultaneously on a homogeneous microbial population, the effect may be one of the following (Brook, Butel, Ornston, Jawetz, Melnick and Aldelberg, 1995):

(a) Addition The combined action is equivalent to the sum of the actions of each drug when used alone.

(b) Synergism The combined action is significantly greater than the sum of both effects.

(c) Antagonism The combined action is less than that of the more effective agent when used alone

The most popular method used to detect antimicrobial interaction is checkerboard (or chessboard) titration, in which two drugs are cross-titrated against each other (Sawan and Manivannan, 2000). After incubation an isobologram is constructed by plotting the inhibition of growth observed at each drug concentration on an arithmetic scale. The line of additivity joins the MICs of the individual drugs acting alone; a deviaton of this line towards the axes of the graph suggests synergy; a deviation away from the axes is often taken to indicate antagonism, although indifference may also produce this result. Alternatively, the sum of the Fractional Inhibitory Concentrations (ΣFIC) can be calculated (Sawan and Manivannan, 2000).

The **FIC index** are determined as follows (Richard and Xing, 1991):

$$= \quad FIC_A \quad + \quad FIC_B$$

$$= \quad \frac{\text{Conc. of A in MIC of A+B}}{\text{MIC of A alone}} \quad + \quad \frac{\text{Conc. of A in MIC of A+B}}{\text{MIC of B alone}}$$

FIC (A+B)	≤	0.7	Synergy
FIC (A+B)	>	1.3	Antagonism
FIC (A+B)	>	0.7 and ≤ 1.3	Addition

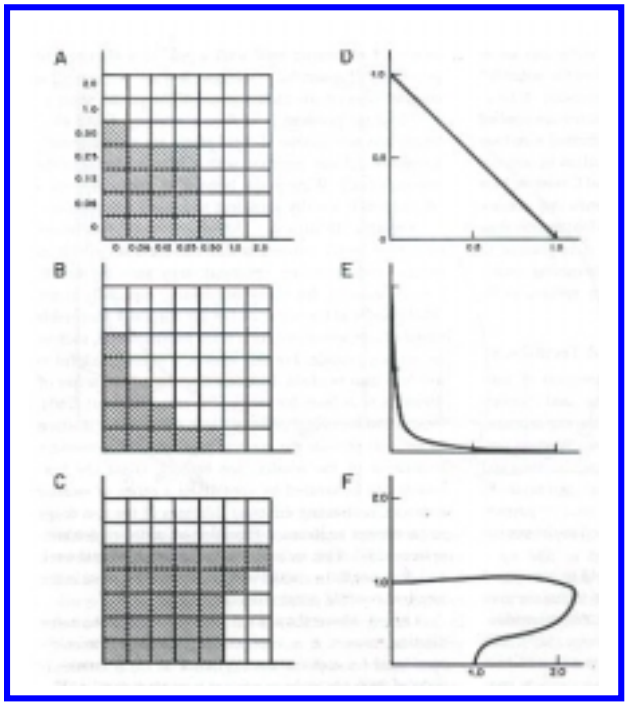


Figure 15 Assessment of antimicrobial combination with the checkerboard method (A, B and C). Results of testing combinations of two drugs (diluted in geometric twofold increments along the x and y axes) are shading, show visible growth. Concentrations are expressed as multiples of the MIC (D, E, and F). Isobolograms (plotted on an arithmetic scale) that represent the results of checkerboards shown in A, B and C, respectively. A and D are additive effect, B and E are synergism, C and D are antagonism (Lorian, 1999).

Checkerboard titrations are relatively simple to perform and allow the assessment of a wide range of drug concentrations. The test assesses synergy at 24 hours only. Dilution of the antimicrobial agents may reduce the concentrations tested to a level at which synergy cannot be detected.

CHAPTER III

MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Plant species

The medicinal plants used were *H. suaveolens* (L.) Poit, *M. cordifolia* Opiz ex Fresen, *O. basilicum* L. and *O. basilicum* L. forma *citratum* Back. All of these four species were collected from home-gardens in Muang district, Nakhon Ratchasima province. Plant samples were washed three times by water, dried and powdered for further used.

3.1.2 Test organisms

3.1.2.1 Bacterial strains

Clinical isolates of the following organisms: Methicillin-sensitive and resistant *S. aureus,* Ciprofloxacin-susceptible and resistant *P. aeruginosa* , and two Ceftazidime-susceptible and resistant *Ent. cloacae* and *E. coli*. All bacteria were obtained from Clinical Microbiology Laboratory, Maharat Nakhon Ratchasima Hospital, Nakhon Ratchasima province.

3.1.2.2 Preparation and maintenance of stock cultures

The clinical isolates of bacteria were inoculated on nutrient agar slopes and incubated overnight at 37 °C. These cultures were stored in a refrigerator at 4 °C. Fresh slope cultures were prepared every 3-4 weeks (Griangsak Eumkeb, 1999).

3.1.3 Culture media

Approximate formula per liter of each medium was as following:

Nutrient agar

HiMedia nutrient agar was used for preparation of stock cultures on agar slopes and the basic agar culture of bacterial cells for colony counting.

The formula was:	Peptic digest of animal tissue	5.0	g
	Sodium chloride	5.0	g
	Beef extract	1.5	g
	Yeast extract	1.5	g
	Agar	1.5	g
	pH (at 25 °C) 7.4 ± 0.2		

Nutrient broth

Difco nutrient broth was used as the basic liquid culture medium for growing the overnight cultures.

The formula was	:	Beef extract	3.0	g
		Peptone	5.0	g
		pH 6.8 ± 0.2		

Mueller Hinton broth (MHB)

Difco Mueller Hinton broth was the medium used for determining the antimicrobial susceptibility testing.

The formula was	:	Beef extract powder	2.0	g
		Acid digest of casein	17.5	g

Soluble starch	1.5	g
pH 7.3 ± 0.1		

*All culture media were dissolved by water.

3.14 Chemicals

All chemicals used were laboratory grade, or otherwise specified.

95% Ethanol	Laboratory reagent
Dimethylsulfoxide (DMSO)	AR grade
Sodium chloride (NaCl)	Laboratory reagent
Augmentin®	Antibiotic

3.15 Equipment

A. Apparatus

Cellulose nitrate	0.45 micron pore size	Whatman
Membrane filter	47 mm diameter	Whatman
Clean air Woerder	type CA/REV6	Woerden
Spectronic 21		Milton Roy
Whirli mixer		Labinco BV
Labofuge	400R	Heraeus
Micropipettors (10-100 microlitre)		Brand
Micropipettors (1000 microlitre)		Witeg
Refrigerated Incubator		VELP scientifica
Retsch Muhle		Rheinische Strabe
Soxhlet extractor apparatus		Buchi

Rotary evaporator	Buchi
Vacuum pump	Buchi

B. Glassware equipment

Beakers (50,100,250,500,1000,3000 ml)	Pyrex
Conical flask (100 and 250 ml)	Pyrex
Pipettes (1,5,10 ml)	Brand
Measuring cylinders (25, 50,100,250,500 ml)	Brand
Petri dishes	Pyrex
Test tube (6" X ¾")	Pyrex
Centrifuge tube	Pyrex

3.2 METHODS

3.2.1 Medicinal plant 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 and completely dried in hot air oven (at 40 °C). The dried leaves were then be powdered by hammer mill and stored in zip-lock bags (at room temperature) until an extraction. Dried powdered leaves were extracted with 95% w/w ethanol using a Soxhlet extractor apparatus. The 50g powder was put in Soxhlet thimble and put into a Soxhlet thimble tube. 500 ml of ethanol was added to a Soxhlet flask, then extracted at 60 °C until the extract was clear or about 12 h (Okemo, Mwatha, Chhabra and Fably, 2001). The ethanol was removed under

pressure using a rotary evaporator. Then dried residue crude extracts were resuspended in 20% at a concentration of DMSO 500 mg/ml and stored in a dark bottle at 4 °C.

3.2.2 Bacterial suspension standard curve

To select bacterial suspensions with a known viable count the following steps were followed (Griangsak Eumkeb, 1999; Richards and Xing, 1993):

2.2.1 A separate loopful of each bacterium was used to inoculate in 100 ml of nutrient broth.

2.2.2 The cultures were incubated at 37 °C for 18 h.

2.2.3 The bacterial cells were pelletted by centrifugation at 4000 r.p.m. for 10 min. Cells were washed twice by resuspending and centrifuging at 4000 r.p.m. for 5 min in 10 ml of 0.9% NaCl.

2.2.4 The cells were resuspended in 50 ml of sterile 0.9% NaCl.

2.2.5 The cell suspensions were diluted so that 5-6 spectrophotometer readings could be obtained over the absorbance range of approximately 0.05-0.25 at a wavelength of 500 nm. Viable counts for each absorbance reading were determined in triplicates using overdried agar plate counting method.

3.2.3 Preparation of crude extracts and inoculum

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

Test organisms were incubated in 100 ml nutrient broth for 18 h at 37 °C. The cultures were centrifuged at 4000 r.p.m. 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-formig units (CFU)/ml using a predetermined calibration curve of absorbance at 500 nm against viable count (Liu, Durham and Richards, 2000).

3.2.4 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 (18h 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 controls 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 h. The MICs were determined for the three replicates. The MIC is defined as the lowest concentration of crude extract at which there is no visible growth in the triplicate tubes (Liu, Durham and Richards, 2000).

3.2.5 Checkerboard determination

Checkerboard determinations in antimicrobial combinations were performed as previously described (Lorian, 1991) with slight modification (Griangsak Eumkeb, 1999). A crude extract “A” and crude extract “B” were diluted to 1/10 of their MICs along the ordinate and abcissa respectively.

An 18h culture of each of the test bacteria was prepared. 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. Tubes of broth with 20% DMSO but without the test crude extracts were used as the controls for each of the test bacteria. The culture was incubated for 24 h 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 as previously described in chapter 2.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 The percentage of extractives obtained from each plant

Table 8 % w/w of four 95% ethanolic plant extracts calculated by using weight of dried residue extract per weight of dried plant.

Plants	**95% Ethanolic extracts (% w/w)**
H. suaveolens L. Poit (Mang lak khaa)	20.8
M. cordifolia Opiz ex Fresen (Saranae)	24.4
O. basilicum L. (Horaphaa)	22.7
O. basilicum L. forma *citratum* Back (Manglak)	28.2

4.2 Bacterial suspensions viable count absorption standard curve

The results of the bacterial suspensions viable counts standard curves for Methicillin-susceptible and resistant *S. aureus,* Ceftacidime-susceptible and resistant *E. coli*, Ceftazidime-susceptible and resistant *Ent. cloacae* and Ciprofloxacin-susceptible and resistant *P. aeruginosa* are shown in Figure 16 to 23.

Figure 16, 18, 19, 21 and 22 indicate that approximately 5×10^8 CFU/ml of clinical isolates of Methicillin-susceptible *S. aureus* (MSSA), Ceftazidime-susceptible *E. coli,* Ciftazidime-resistant *E. coli*, Ceftazidime–resistant *Ent. Cloacae* and Ciprofloxacin–susceptible *P. aeruginosa* have absorption at 500 nm of 0.25, 0.2,0.15, 0.17 and 0.22 respectively.

Figure 17, 20 and 23 indicate approximately 1×10^8 CFU/ml of clinical isolates of Methicillin-resistant *S. aureus* (MRSA), Ceftazidime-susceptible *Ent. cloacae,* and Ciproflaxacin–resistant *P.aeruginosa* have absorption at 500 nm of 0.06, 0.09 and 0.13 respectively.

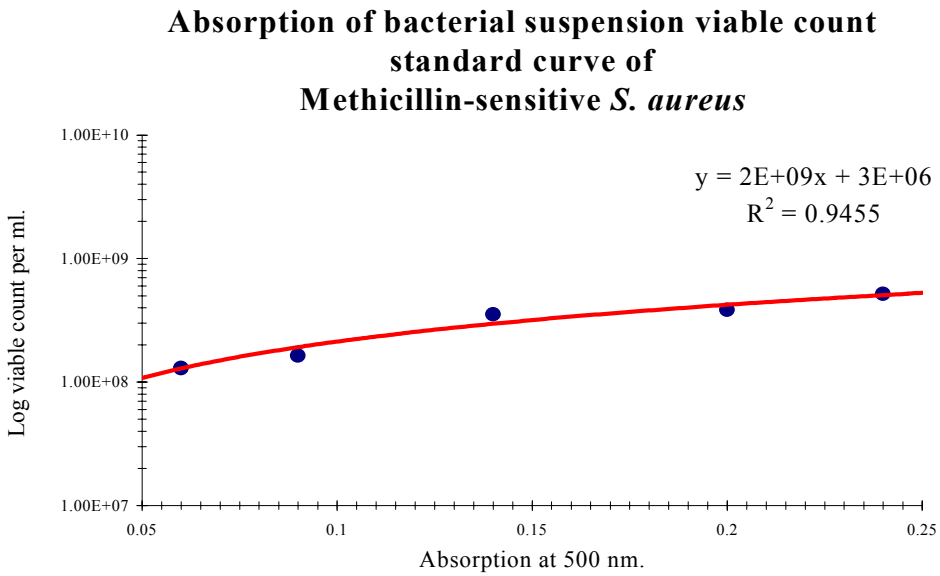


Figure 16 The standard curve of suspensions of

Methicillin susceptible – *S. aureus* (MSSA)

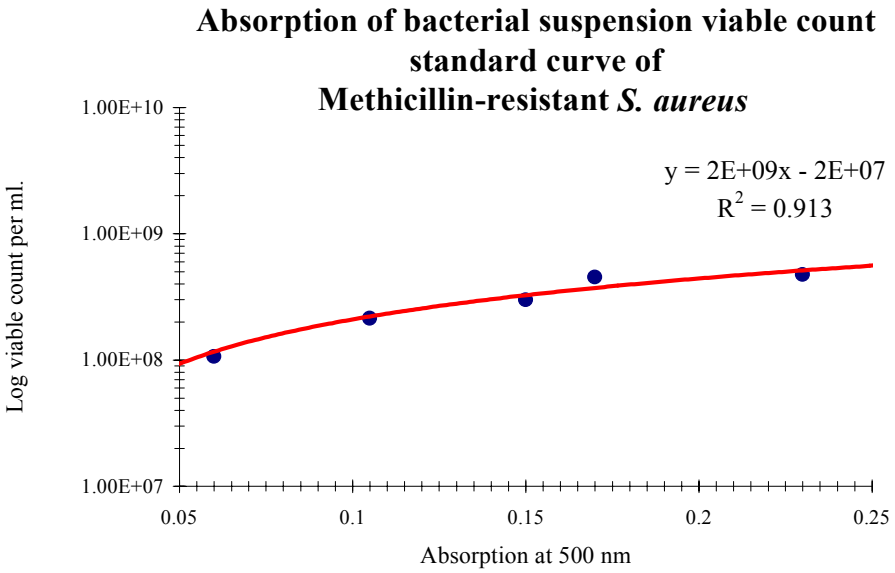


Figure 17 The standard curve of suspension of Methicillin-resistant *S. aureus* (MRSA)

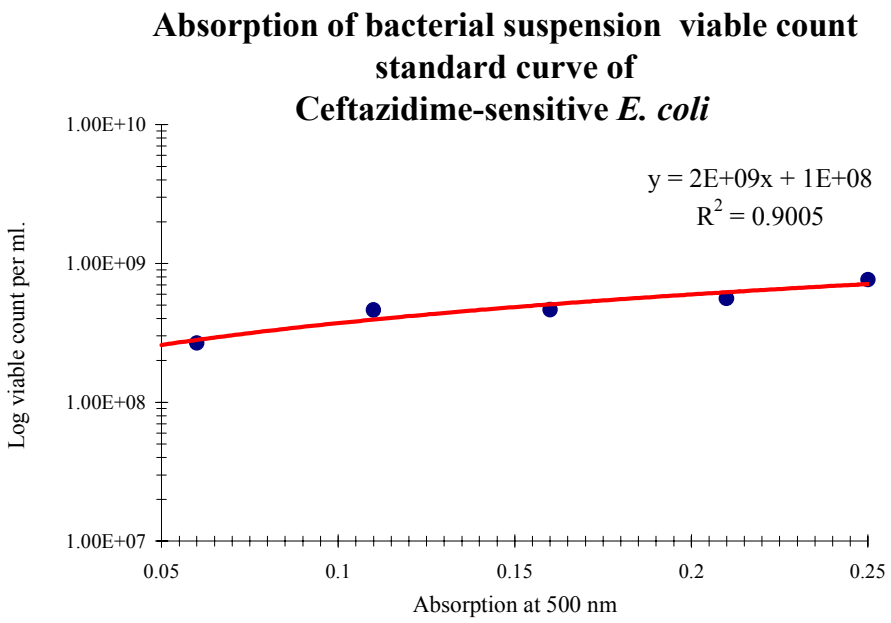


Figure 18 The standard curve of suspensions of Ceftazidime- susceptible *E. coli*

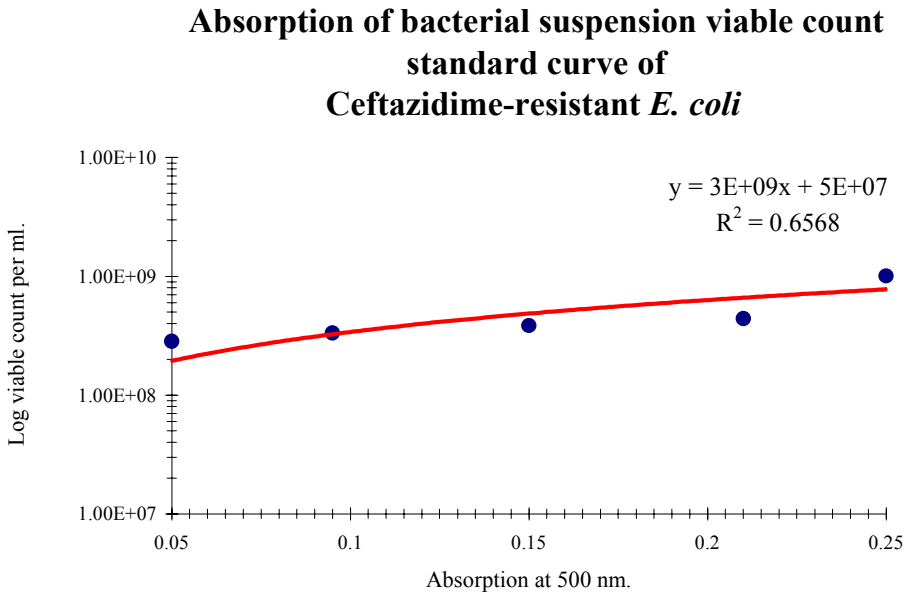


Figure 19 The standard curve of suspensions of Ceftazidime - resistant *E. coli*

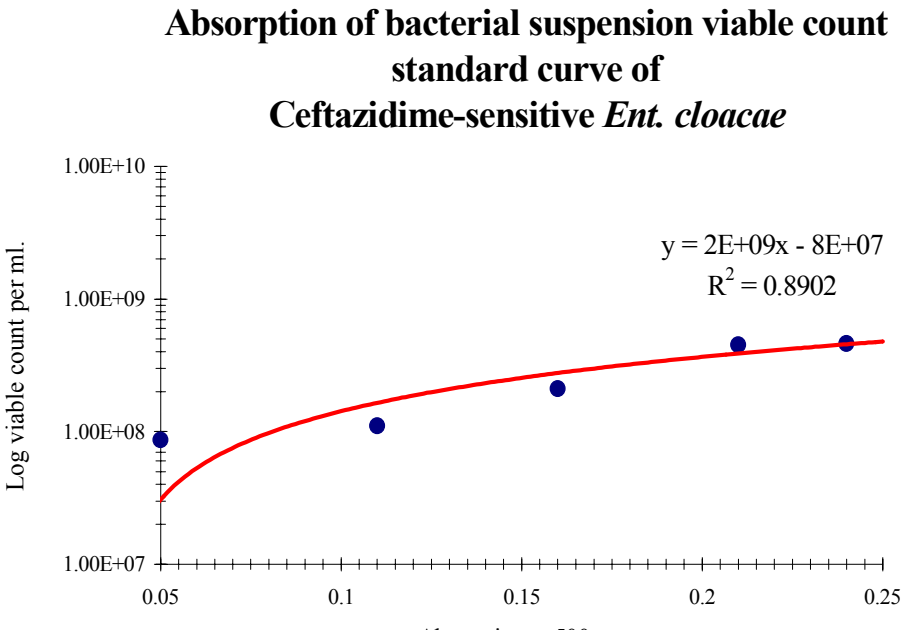


Figure 20 The standard curve of suspensions of

Ceftazidime - susceptible *Ent. cloacae*

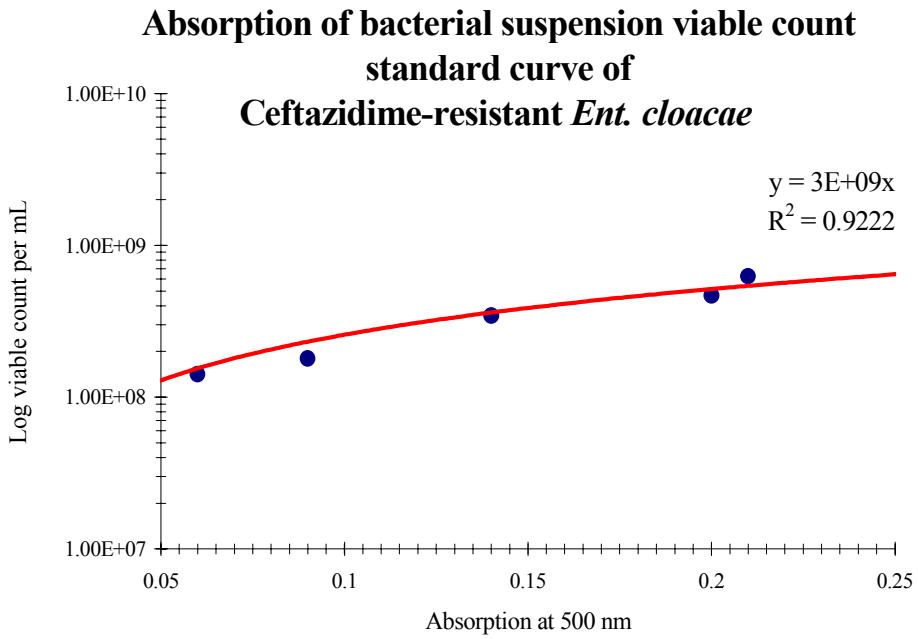


Figure 21 The standard curve of suspensions of

Ceftazidime - resistant *Ent. cloacae*

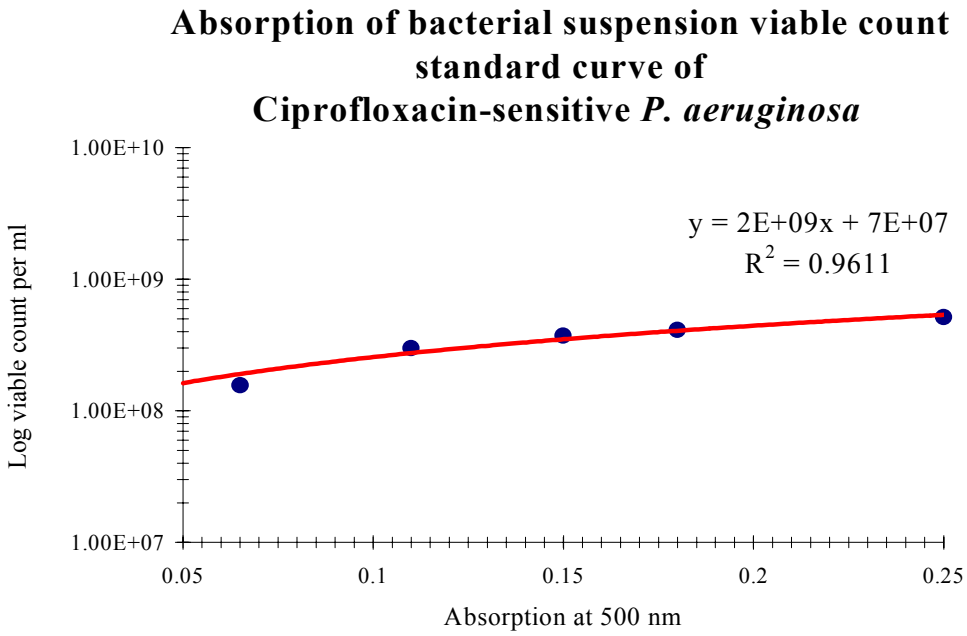


Figure 22 The standard curve of suspensions of

Ciprofloxacin–susceptible *P. aeruginosa*

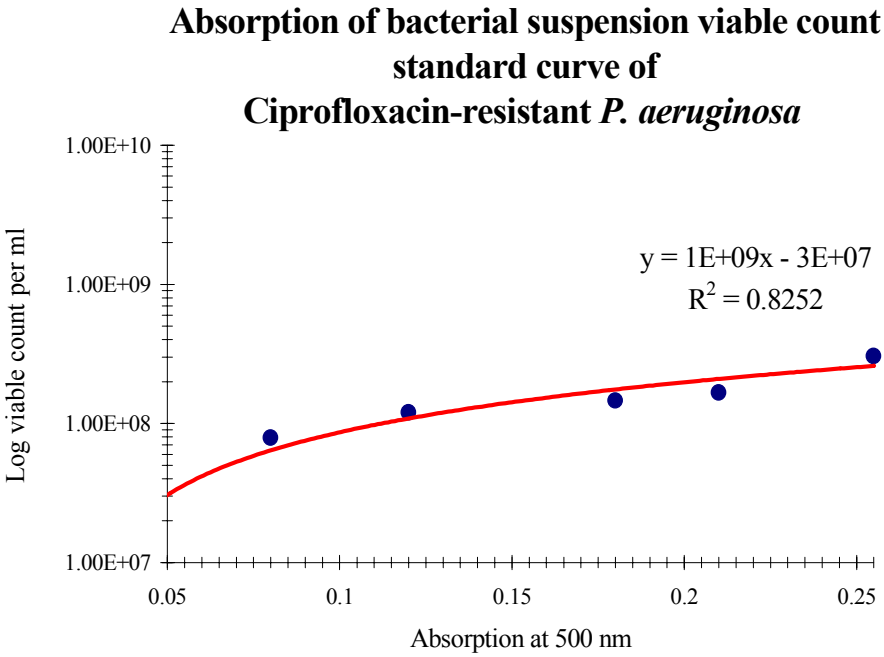


Figure 23 The standard curve of suspensions of

Ciprofloxacin – resistant *P. aeruginosa*

4.2 MIC determination

In present study, a total of four alcoholic leave extracts from Lamiaceae family, *O. basilicum* L., *O. basilicum* L. forma *citratum* Back, *M. cordifolia* Opiz ex Fresen and *H. suaveolens* (L.) Poit, were tested against eight drug–susceptible and resistant clinical isolates of bacteria. The MIC values of the extracts were quantitatively assessed by a macrobroth dilution method as described previously (Liu, Durham and Richards, 2000). The MICs of four medicinal plants in the present study are summarized in Table 9 to 12. The results of present study are encouraging as the four 95% ethanolic leaves extracts of the medicinal plants tested, all showed antibacterial activity against drugs-susceptible and resistant isolates of bacteria tested, both Gram-positive and Gram-negative bacteria. However, tested plants signficantly differ in their activity against test microorganisms. Some of the MICs were more than 100 mg / ml. Only the alcoholic extract was tested, as alcohol was found to be a better solvent for extraction of antimicrobially active substances compared to water and hexane (Ahmad, Mehmood and Mohammad, 1998; Essawi and Srour, 2000). Most of the previous researchers used agar diffusion assay to determine the antibacterial activity of extracts (Ahmad and Beg, 2001; Awadh Ali, Julich, Kusnick and Lindequist, 2001; Elgayyar, Draughon, Golden and Mount, 2001;Rojas, Hernandez, Miranda and Mata, 1992). This technique works well with defined inhibitors but when examining extracts containing unknown components, there are problems leading to the false positive and false negative results (Eloff, 1998). The antimicrobial effect may be inhibited or increased by extrinsic factors or contaminants. The most widely used alternative technique is the dilution method (Eloff, 1998; Rojas, Hernandez, Miranda and Mata, 1992).

Table 9 *S.aureus* – Methicillin sensitive and Methicillin resistant

In vitro MIC of plant extracts against Methicillin-susceptible and resistant clinical isolates of ***S. aureus*** and comparative sensitivity of both isolates to all plant extracts.

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

Table 10 *E.coli* – Ceftazidime sensitive and Ceftazidime resistant

In *vitro* MIC of plant extracts against Ceftazidime-susceptible and resistant clinical isolates of ***E. coli*** and comparative sensitivity of both isolates to all plant extracts.

Medicinal plants	MIC (mg / ml)	
	Ceftazidime sensitive	Ceftazidime resistant
Horaphaa	50	50
Manglak	50	100
Saranae	50	50
Maeng lak khaa	50	100

Table 9 shows the *In vitro* MICs of four medicinal plants extracts tested against methicillin-susceptible and resistant clinical isolatd *S. aureus* and a comparative study between the MICs of the drug-sensitive and resistant isolates. The results showed that *H. suaveolens* (L.) Poit displayed the lowest MIC against both clinical isolates. The growth of methicillin-susceptible and resistant *S. aureus* isolates were inhibited by this extracts at the concentration of 25 and 6.25 mg/ml, respectively. *O. basilicum* L. showed antibacterial activity against methicillin-resistant isolates of *S. aurues* at MIC of 30 mg/ml and methicillin-sensitive at MIC of 50 mg/ml. This concentration (50 mg/ml) was equal to the MICs of *O. basilicum* L forma *citratum* Back against both clinical isolates. *M. cordifolia* Opiz ex Fresen had a highest MIC against both clinical isolates bacteria at concentration of 100 mg/ml. Not only *H. suaveolens* L. Poit showed MICs of the methicillin-resistant isolates *S. aureus* lower than the susceptible isolates but *O. basilicum* L. as well.

Table 10 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 at the concentration of 50 mg/ml. An equal concentration of the extracts of *O. basilicum* L. and *M. cordifolia* Opiz ex Frezen inhibited growth of both drug-susceptible and resistant isolates *E. coli*. *O. basilicum* L forma *citratum* Back as well as *H. suaveolens* L Poit showed antibacterial activity against the resistant isolates at MIC as high as 100 mg/ml.

Table 11 *Ent. cloacae*– Ceftazidime sensitive and Ceftazidime resistant

In vitro MIC of medicinal plant extracts against Ceftazidime-susceptible and resistant clinical isolates of *Ent. cloacae* and comparative sensitivity of both isolates to all plant extracts.

Medicinal plants	MIC (mg /ml)	
	Ceftazidime sensitive	Ceftazidime resistant
Horaphaa	50	50
Manglak	100	100
Saranae	50	50
Maeng lak khaa	100	50

Table 12 *P. aeruginosa* – Ciprofloxacin sensitive and Ciprofloxacin resistant

In vitro MIC of medicinal plant extracts against Ciprofloxacin-susceptible and resistant clinical isolated of ***P. aeruginosa*** and comparative sensitivity of both isolates to all plant extracts.

Medicinal plants	MIC (mg /ml)	
	Ciprofloxacin sensitive	Ciprofloxacin resistant
Horaphaa	25	25
Manglak	50	50
Saranae	25	50
Maeng lak khaa	50	50

Table 11 reports MICs of the tested medicinal plant extracts against Ceftazidime-susceptible and resistant clinical isolated *Ent. Cloacae*. *O. basilicum* L. and *M. cordifolia* Opiz. They displayed an inhibitory effect against both drug-susceptible and resistant isolated *Ent. Cloacae* at the concentration of 50 mg/ml. The highest concentration of extracts at 100 mg/ml that inhibited both isolates was obtained from *O. basilicum* L. forma *citratum* Back. The MIC of *H. suaveolens* L Poit against ceftazidime- resistant isolated 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). Results from Table 9 showed that this plant extract and *O. basilicum* L. displayed lower MIC against methicillin-resistant isolates of *S. aureus.*

Table 12 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 at a concentration of 25 mg/ml . *O. basilicum* L. forma *citratum* Back as well as *H. suaveolens* L. Poit inhibited the growth of ciprofloxaxin-susceptible and resistant *P. aeruginosa* at MIC of 50 mg/ml. *M. cordifolia* Opiz Ex Fresen displayed the growth inhibition of ciprofloxacin-susceptible and resistant *P. aeruginosa* at concentrations of 25 mg/ml and 50 mg/ml, respectively.

Many previous researchers reported the antibacterial activity of *H. suaveolens* L Poit and *O. basilicum* L. The chemical investigation of *H. suaveolens* L Poit reported the presence of diterpenoids, steroids and triterpenes in solvent extracts of the aerial part of the species (Iwu, Ezeugwu and Okunji, 1990). Essential oil from it displayed good antimicrobial activity against *S. aureus, P. aeruginosa* and *E.coli* at

various concentrations (Iwu, Ezeugwu and Okunji, 1990). The methanolic extract from aerial part and leaf at 20 mg /ml (Rojas, Hernandez, Miranda and Mata, 1992) as well as from leaves at 2 mg /ml (Hussain and Deeni, 1991) do not inhibit Gram-negative bacteria, *E. coli* and *P. aeruginosa*. But they showd activities against Gram-positive bacteria, *S. aureus*. The 80% ethanolic extract (Lentz et al, 1998) and 88% ethanolic extract (Caceres, Cano, Samayoa and Aguilar, 1991) do not inhibit growth of *S. aureus* (Lentz et al, 1998), *P. aeruginosa* and *E.coli* (Caceres, Cano, Samayoa and Aguilar, 1991; Lentz et al, 1998). Aqueous extracts did not have activity against *P. aeruginosa*, *E.coli* either (Samy, Ignacimuthu and Raja, 1999). The previous studies in *O. basilicum* L reported that 50% ethanolic extract inhibited growth of *S. aureus* and *E. coli* (Okonogi, Pongpaibul, Murakoshi, And Sekine, 1993), while 88% ethanolic extract of leaves and flowers had no activity against *S. aureus* (Caceres, Cano, Samayoa and Aguilar, 1991). Essential oil of *O. basilicum* showed antibacterial activity against *S. aureus* and *E. coli* (Elgayyar, Draughon, Golden, and Mount, 2001). An aqueous extract inhibited *E. coli* but not *S. aureus* (Okonogi, Pongpaibul, Murakoshi, And Sekine, 1993). Methanolic extract of whole plant at 10 mg/ml inhibited growth of *S. aureus* and 40 mg/ml for *E. coli* and *P. aeruginosa* (Navarro, Villarreal, Rojas, and Lozoya, 1996). 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 (Hussain and Deeni, 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 does not interfere with the antimicrobial action of plant extracts and these extracts might have different mode of action on test organisms (Ahmad and Beg, 2001). All these extracts were active against clinical isolates, both Gram-positive and Gram-negative bacteria. The range of concentrations that most of crude extracts used to inhibit growth of the tested microorganisms were between 50 to 100 mg/ml. The MIC values of all tested extracts are similar because of the similar active constituents present in the Lamiaceae family. However, one of the tested plants significantly differs in its activity against tested microorganisms. *H. suaveolens* L Poit was also highly active against Gram-positive, clinical isolates of *S. aureus,* in the test. That was indicated by the lower concentration of MIC (at 6.25 and 25 mg/ml) when compared with Gram-negative clinical isolates bacteria (at 50 and 100 mg/ml). This result was supported by earlier studies that *S. aureus* was the most easily inhibited of all the bacteria exposed to plant extracts (Grosvenor, Supriono and Gray, 1995). The greater resistance of Gram-negative bacteria to plant extracts has been document previously (Essawi and Srour, 2000). These observations are likely to be the result of the differences in cell wall structure 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). So the previous studies were supported by the results obtained from this study as described above. Commonly, weeds produce many secondary metabolites to kill insects, microorganisms and other predators, which infect them. Therefore, the other probable reason is may be that *H. suaveolens* (L.) Poit produces some secondary metabolites to protect itself from the predators. Although a comparative study of MIC of plant extracts against drugs-susceptible and

resistant clinical isolated bacteria have not been previously reported. Results from the present study indicated the MICs of drugs-susceptible clinical isolates were higher than the MICs of drugs-resistant clinical isolates. According to previous investigators, they reported the effectiveness of a medicinal plant may not result from one main active compound but it is 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 is likely due to the different mechanisms of action 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 provide it with such a potent antibacterial activity. The MICs obtained from this study are rather high (mg /ml) compared to previous reported values (μg /ml). This might be caused by the plant extracts used in the present study being crude, whereas others used rather pure active constituents/ or antibiotic.

4.2 Checkerboard assays

There are several reasons to support the use of antimicrobial combinations. Firstly, decreased emergence of resistant strain. Secondly, decreased dose-related toxicity as a result of reduce dosage and thirdly, polymicrobial infection (Lorian, 1999). Some previous researchers reported antibacterial activities of plant material combination with antibiotics against both Gram-negative and Gram- positive bacteria (Darwish, Aburjai, Al-Khalil and Mahafzah, 2002; Liu, Durham and Richards, 2000). The present study was carried out to screen the combination effect of two medicinal plants against drug-resistant clinical isolated bacteria, MRSA and Cirprofloxacin-resistant *P. aeruginosa,* which are common cause of nosocomial infection. This combination effect might be used advantageously to improve the efficiency to treat infectious diseases. Although, all medicinal plants in the present study used individually showed antibacterial activity against MRSA and ciprofloxacin-resistant *P. aeruginosa* at various degrees, 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 24 to 26.

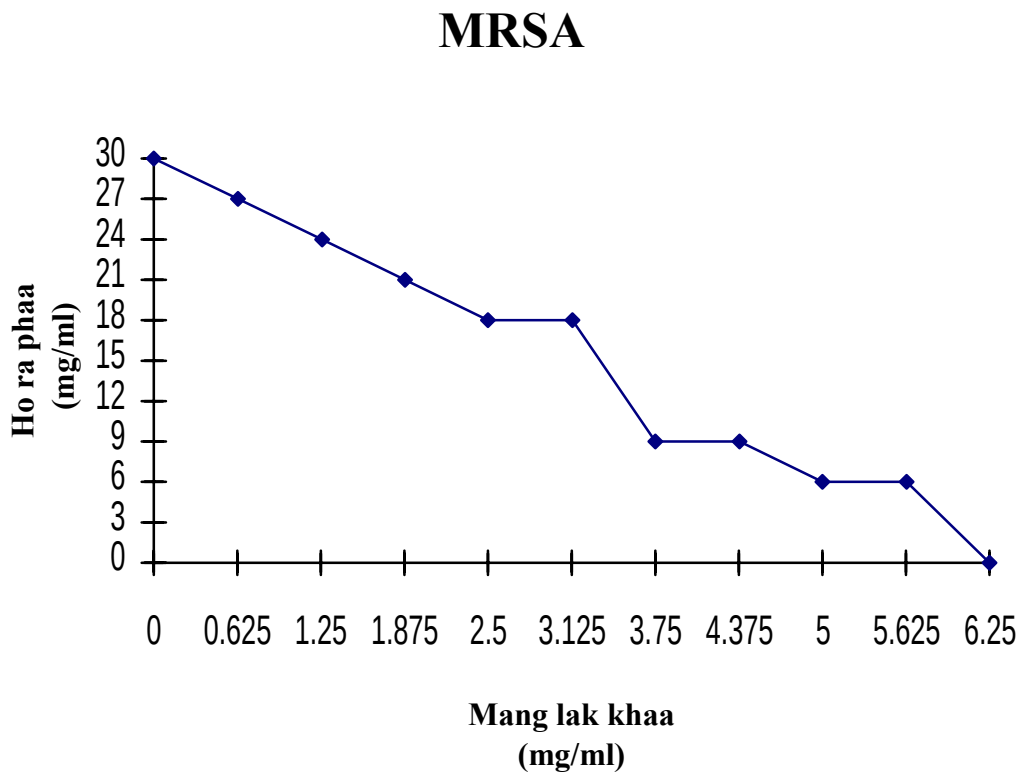


Figure 24 Isobologram constructed from checkerboard MIC data showing antibacterial combination of Horaphaa plus Mang lak khaa against MRSA

In checkerboard test, results from the isobologram represents that Ho ra phaa. and Mang lak khaa combination showed additive activity against MRSA.

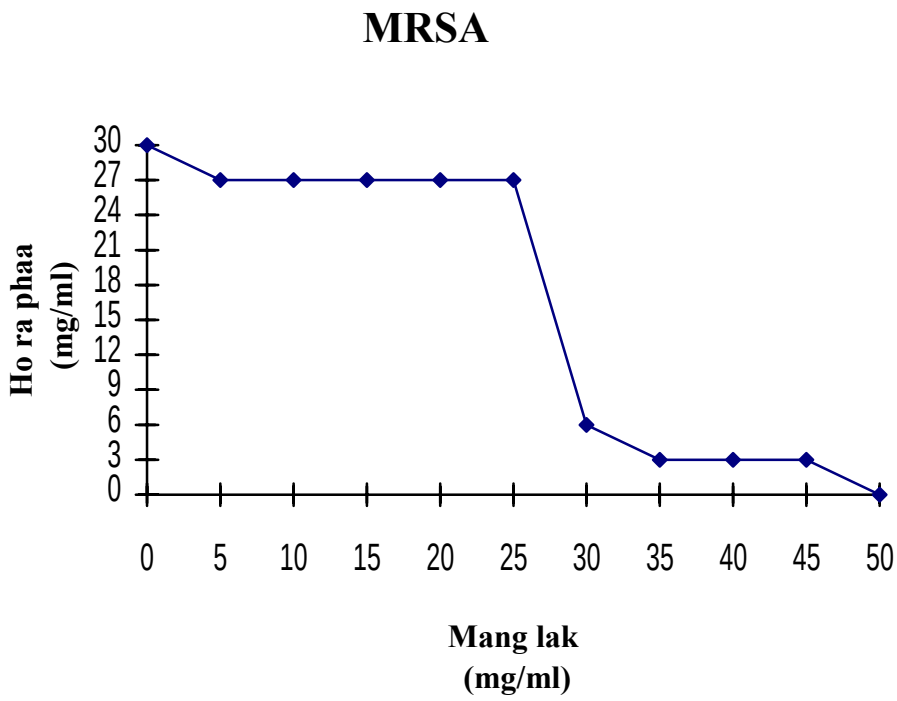


Figure 25 Isobologram constructed from checkerboard MIC data showingantibacterial combination of Horaphaa plus Mang lak against MRSA

The isobologram shows Ho ra phaa. plus Mang lak khaa combination had additive activity against MRSA.

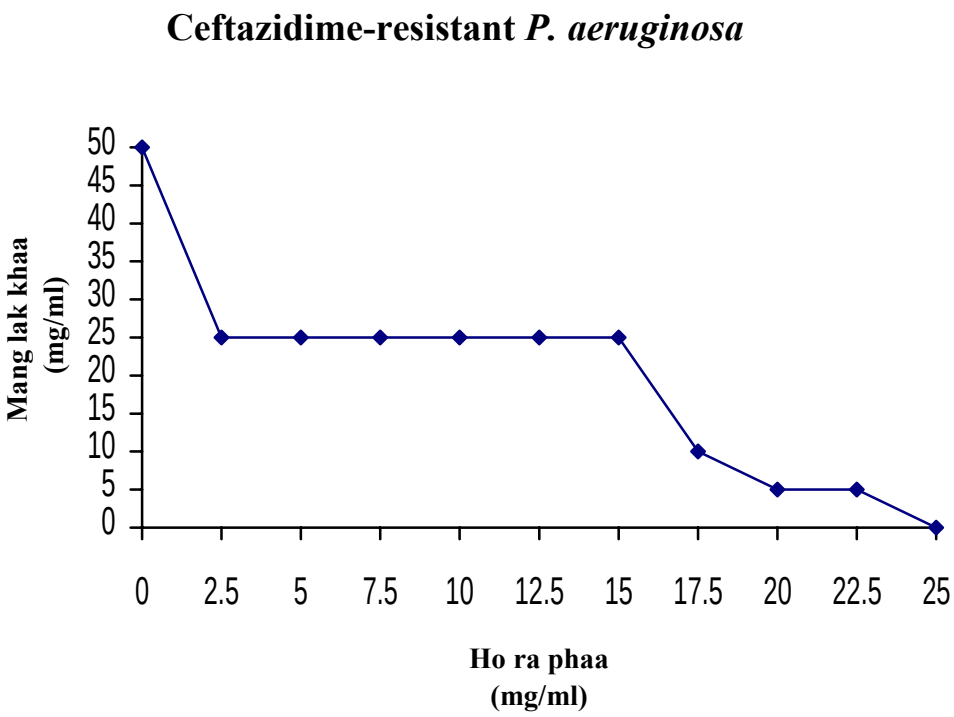


Figure 26 Isobologram constructed from checkerboard MIC data showing antibacterial combination of Horaphaa plus Mang lak khaa against Ciprofloxacin – resistant *P. aeruginosa*

The result from isobologram shows that Ho ra phaa. plus Mang lak combination had synergistic effect against the drug-resistant *P. aeruginosa.*

Table 13 Summary the FICs for checkerboard assays of Horaphaa, Manglak and Maeng lak khaa against the drug resistant bacteria

Crude extract combination	**Test bacteria**	**MIC (mg/ml)**	**MIC (A+B) (mg/ml)**	**FIC (A+B)**	**Type of interation**
Horaphaa Mang lak khaa	MRSA	30 6.25	9 3.75	0.9	addition
Horaphaa Mang lak	MRSA	30 50	3 35	0.8	addition
Horaphaa Maeng lak-khaa	Ciprofloxacin - resistant *P. aeruginosa*	25 50	2.5 25	0.6	synergism

A summary of data from all the isobolograms is given in Table 13. In checkerboard test, results showed that *O. basilicum l.* and *H. suaveolens* L Poit combination and *O. basilicum* L plus *O.basilicum* L forma *citratum* Back combination showed additive activity against MRSA respectively. The FIC index of *O. basilicum* L plus.*H. suaveolens* L Poit combination is 0.9, which indicated an addition property against the isolates bacteria. On the other hand, MIC of *O. basilicum* L individually was reduced from 30 mg/ml to 9 mg/ml and *H. suaveolens* L Poit individually was reduced from 6.25 mg/ml to 3.75 mg/ml (Table 13). *O. basilicum* L plus *O.basilicum* L forma *citratum* Back combination showed FIC index at 0.8 , in addition MIC of both medicinal plants were reduced from 30 mg/ml to 3 mg/ml (*O. basilicum* L.) and from 50 mg/ml to 35 mg/ml (*O.basilicum* L forma *citratum* Back). Interestingly, *O. basilicum* L. plus *H. suaveolens* L Poit combination

showed synergistic effect against the drug-resistant *P. aeruginosa,* which is indicated by 0.6 for FIC index (Table 13). The MIC of *O. basilicum* L. in combinaition against this Gram-negative was reduced ten-fold from 25 mg/ml to 2.5 mg/ml, while the MIC of *H. suaveolens* L Poit in combination was reduced two-fold from 50 mg/ml to 25 mg/ml. The greater resistance of Gram-negative bacteria to plant extracts has been document previously, which difference from the result obtained in this combination effect study (Essawi and Srour, 2000). The combination of *O. basilicum* L. and *H. suaveolens* L Poit showed synergistic effect against Gram-negative bacteria and additive effect against Gram-positive bacteria. This effect is more pronounced on the Gram-negative resistant microorganism, this is probably due to some structural change of the resistant microorganism. The activity against Gram-negative bacteria might also indicated that the plant material acts 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 is probably due to the accumulation of inhibitory concentration at the target sites or due to the additional inhibitory effect of the plant material (Darwish, Aburjai, Al-Khalil and Mahafzah, 2002). Further investigation has to be done to verify this point.

In literature, it has been indicated that the antibacterial activity is due to different chemical agents in the extract, including essential oils (especially thymol), flavonoids and triterpenoids and other compounds of phenolic nature or free hydroxyl-group, which are classified as active antimicrobial compounds (Cowan, 1999). Traditional remedies used by Thai healers are often composed of several plants that belong to different taxonomic groups. Therefore, it is difficult to know the precise contribution of every plants to the property of a given remedy mixture.

Nevertheless, all of the selected plant in this study showed interesting antibacterial properties. Thus, there is now preliminary scientific validation for the use of some of these medicinal plants for antibacterial activity. Although, previous studies reported that the purified active constituent while used individually may not show the same biological activity like presented in the crude extract. The active phytocompounds of these plants used against multidrug-resistant bacteria has to be characterized in further studies. However, the development of any active constituent into medicine will require further toxicity and safety assessment, including the efficacy of the extract evaluated *in vivo*. The study of the synergistic interaction of active phytocompounds is required to exploit these potential plant extracts in the combination therapy of infectious diseases caused by multidrugs- resistant organism. Laboratory and clinical studies of these plants are required in order to understand better antibacterial principle which will allow the scientific community to recommend their use as an accessible alternative to synthetic antibiotics (Darwish, Aburjai, Al-Khalil and Mahafzah, 2002).

CHAPTER V

CONCLUSIONS

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 searh for new, inexpensive and effective drugs from plants, for possible antimicrobial properties. In present study, four 95% ethanolic extracts from plants in Lamiaceae family, including *Ocimum basilicum* L, *Ocimum basilicum* L. forma *citratum* Back, *Mentha cordifolia* Opiz ex Fresen and *Hyptis suaveolens* (L.) Poit, were tested against eight drugs–susceptible and drugs-resistant clinical isolated bacteria. The clinical isolated bacteria in this study were Methicillin-susceptible and resistant *S. aureus*, Ciprofloxacin-susceptible and resistant *P. aeruginosa* and Ceftazidime– susceptible and resistant bacteria of *E. coli* and *Ent. cloacae*. These clinical isolates of bacteria caused high percentage of drugs resistance in many sections of the local hospitals and worldwide, as a result, the cost of treating these infectious diseases is increasing. The MIC value of the extracts were quantitatively assessed by a macrobroth dilution method. Results from the present study showed that all the 95% ethanolic leave extracts from the medicinal plants had antibacterial activity against drug-susceptible and drug-resistant isolates in both Gram-positive and Gram-negative bacteria. *H. suaveolens* L. Poit alone showed the lowest MIC against MRSA at MIC of 6.25 mg/ml. The present study also determined the combination effect of two medicinal plants against MRSA

and drug-resistant *P. aeruginosa*, which are the common causes of nosocomial infection. The results might be used advantageously to improve the efficiency to treat infectious diseases. The synergistic activities 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 by checkerboard or chessboard assay. FIC indices and isobolograms were used as the indication for antimicrobial combination. The results showed that *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, indicated by FIC at 0.9 and 0.8 respectively. Interestingly, the combination of *O. basilicum* L./*H. suaveolens* L Poit showed synergistic effect against the Gram-negative drug-resistant *P. aeruginosa,* indicated by 0.6 for FIC index. The same combination (*O. basilicum* L./*H. suaveolens* L Poit) also showed synergistic effect against isolates of Gram-negative bacteria and additive effect against isolates of Gram-positive bacteria. The effect is more pronounced on the Gram-negative resistant microorganism. This is probably due to some structures change of the resistant microorganism. This might also indicate that the plant material acts by another mechanism like blocking the inhibitory effect of the enzymes or affecting the efflux system. The improvement in the activity of these two plant extracts is probably due to the accumulation of inhibitory concentration at the target sites or due to the additional inhibitory effect of the plant material. Further studies have to be done to characterize the active phytocompounds of these plants. Toxicity issues have to be addressed and the efficacy of non-toxic extracts have to be evaluated *in vivo*. Laboratory and clinical studies of these plants are required in order to better

understand the antibacterial properties so as to allow the scientific community to recommend their uses as an accessible alternative to synthetic antibiotics.

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