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Plant-derived antimicrobial agents and their synergistic interaction against drug-sensitive and -resistant pathogens

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I hereby declare that I have written the submitted dissertation myself and in this process have used no other sources or materials than those expressly indicated.

I hereby declare that I have not applied to be examined at any other institution, nor have I used the dissertation in this or any other form at any other institution as an examination paper, nor submitted it to any other faculty as a dissertation.

List of Publications:

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Abbreviations

α	alpha
β	beta
µ	micro
m	milli
M	molar
h	hour
g	gram
l	liter
A. baumanni	*A. baumannii*
APT	attached proton test
B. subtilis	*Bacillus subtilis*
^{13}C	carbon
C. albicans	*Candida albicans*
$CHCl_3/CDCl_3$	chloroform/deuterated chloroform
CH_2Cl_2	dicholormethane
COSY	correlation spectroscopy
D_6	deuterated pyridine
DEPT	distortionless enhancement proton target
DMSO	dimethyl sulfoxide
E. coli	*Escherichia coli*
E. faecalis	*Enterococcus faecalis*
E. faecium	*Enterococcus faecium*
FICI	Fractional Inhibitory Concentration Index
^{1}H	proton
HMBC	heteronuclear multiple bond correlation
HPLC	high performance liquid chromatography
HSQC	heteronuclear multiple quantum correlation
IR	infra red
MeOH/MeOD	methanol/deuterated methanol
MHB/MH2	Mueller Hinton broth/Mueler Hinton agar
MIC	minimal inhibition concentration
MBC	minimal biocide concentration
MRSA	methicillin-resistant *Staphylococcus aureus*
MS	mass spectroscopy
MTT	3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide
NMR	nuclear magnetic resonance
P. aeruginosa	*Pseudomonas aeruginosa*
SD	standard deviation
S. agalactiae	*Streptoccoccus agalactiae*
S. aureus	*Staphylococcus aureus*
S. epidermidis	*Staphylococcus epidermidis*
S. pyogenes	*Streptococcus pyogenes*
S. saprophyticus	*Staphylococcus saprophyticus*
TLC	thin layer chromatography
VRE	vancomycin-resistant enterococci

Summary

Resistance toward antibiotics has become a problem on a global scale. Methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus faecalis* (VRE) are a major cause of morbidity and mortality in hospitalized patients. To overcome resistance, many antimicrobial agents have been investigated and Traditional Chinese Medicinal (TCM) plants were also examined as source of alternative agents.

Eucalyptus globulus Labill (Myrtaceae) was the most active plant among the 84 TCM plants tested against multidrug-resistant bacteria. The results indicate that non-volatile compounds contributed to the antimicrobial activity, instead of the essential oil. However, the essential oil of fruits of *E. globulus* exerted a strong inhibition against MRSA and VRE, in comparison to the oils of the leaves of *E. globulus*, *E. radiata* and *E. citriodora*. The fruit oil contained mainly aromadendrene, 1,8-cineole and globulol, which was determined by GLC/MS. Aromadendrene might be responsible for the antimicrobial properties, whereas 1,8-cineole and globulol exhibited low activities. Furthermore, the checkerboard assay demonstrated that combinations of 1,8-cineole and aromadendrene reduce the MIC in most cases in an additive way, whereas the time-kill assay indicates a synergistic effect.

Chemical investigation of the essential oil of *Kadsura longipedunculata* Finet et Gagnep (Schisandraceae) detected δ-cadinene, camphene and borneol as the major components. The oil of *K. longipedunculata* exerted good antimicrobial and trypanocidal activities. Additionally, the oil was confirmed to have antioxidant and cytotoxic activities. Lipoxygenase inhibition and prostaglandin E_2 production inhibition accounted for anti-inflammatory activity of the oil. Two major components of the oil, camphene and borneol, might contribute partially to the biological activity. Furthermore, the combination of camphene and borneol act synergistically against the tested bacteria observed by the checkerboard and time-kill methods.

Bioassay-guided fractionation of *Siegesbeckia pubescens* Makino (Asteraceae) substantiated five bioactive compounds identified as daucosterol, pubetallin, siegeskaurolic acid, *ent*-16β,17-dihydroxy-kauran-19-oic acid and 16α-(–)-kauran-17,19-dioic acid. The isolated compounds showed some different degrees of antimicrobial activity. Additionally, the combination of pubetallin and *ent*-16β,17-dihydroxy-kauran-19-oic acid enhanced the inhibitory effect against MRSA observed by the checkerboard method.

Antimicrobial activity of some TCM plants has been confirmed and the results validated the use of plants as infection remedies. The essential oils or isolated compounds either given alone or in combination could be promising agents to combat multidrug-resistant pathogenic microorganisms.

Zusammenfassung

Antibiotikaresistenz stellt ein zunehmendes globales Problem dar. Der Methicillinresistente Keim *Staphylococcus aureus* (MRSA) und der Vancomycinresistente Erreger *Enterococcus faecalis* (VRE) sind die Hauptverursacher von Krankenhausinfektionen, was weltweit zu vielen Todesfällen führt. Um diese Resistenzen zu umgehen wurden bereits viele antimikrobielle Stoffe geprüft. In dieser Studie wurden Pflanzen aus der Traditionellen Chinesischen Medizin (TCM) auf ihre Nutzbarkeit als Quelle alternativer Heilmittel untersucht.

Von den 84 untersuchten Pflanzen zeigte *Eucalyptus globulus* Labill (Myrtaceae) die stärkste Aktivität gegen multiresistente Bakterien. Die Ergebnisse deuten darauf hin, dass nicht das ätherische Öl die Wirksamkeit bedingt, sondern die nicht-flüchtigen Bestandteile des Extraktes. Allerdings wies das ätherische Öl, das aus den Früchten von *E. globulus* gewonnen wurde, eine starke Inhibition gegen MRSA und VRE auf. Dieser Effekt konnte in den ätherischen Ölen aus den Blättern von *E. globulus*, *E. radiata* und *E. citriodora* nicht beobachtet werden. Mittels GLC/MS konnte nachgewiesen werden, dass das Öl aus den Früchten von *E. globulus* überwiegend aus Aromadendren, 1,8-Cineol und Globulol besteht. Aromadendren könnte für den antimikrobiellen Effekt verantwortlich sein, wohingegen 1,8-Cineol und Globulol nur eine geringe Aktivität zeigten. Darüber hinaus konnte im Checkerboard-Assay gezeigt werden, dass eine Kombination aus 1,8-Cineol und Aromadendren den MIC reduziert. Diese Analysemethode legt in den meisten Fällen eine additive Wirkung der beiden Stoffe nahe, wohingegen der Time-Kill-Assay einen synergistischen Effekt vermuten lässt.

Durch die chemische Untersuchung des ätherischen Öles aus *Kadsura longipedunculata* Finet et Gagnep (Schisandraceae) konnten die Hauptinhaltsstoffe δ-Cadinene, Camphen und Borneol identifiziert werden. Das Öl aus *K. longipedunculata* zeigte eine gute antimikrobielle und trypanozide Aktivität. Außerdem konnte nachgewiesen werden, dass das Öl antioxidativ und zytotoxisch wirkt. Der entzündungshemmende Effekt des Öles beruht auf der Inhibition sowohl von Lipoxygenase als auch der Produktion von Prostaglandin E_2.

Zwei Hauptinhaltsstoffe des Öles, Camphen und Borneol, könnten teilweise zur biologischen Aktivität beitragen. Außerdem konnte sowohl im Checkerboard- als auch im Time-Kill-Assay nachgewiesen werden, dass die Kombination aus diesen beiden Stoffen synergistisch gegen die getesteten Bakterien wirkt.

Durch die Bioassay-gelenkte Fraktionierung von *Siegesbeckia pubescens* Makino (Asteraceae) konnten fünf bioaktive Substanzen identifiziert werden: Daucosterol, Pubetallin, Siegeskaurolische Säure, *ent*-16β,17-dihydroxy-kauran-19-oische Säure und 16α-(–)-kauran-17,19-dioische Säure. Die isolierten Substanzen zeigten unterschiedliche antimikrobielle Aktivität. Weiterhin konnte im

Checkerboard-Assay gezeigt werden, dass eine Kombination von Pubetallin und *ent*-16β,17-dihydroxy-kauran-19-oischer Säure den hemmenden Effekt gegen MRSA erhöht.

Hiermit konnte die antimikrobielle Aktivität verschiedener TCM-Pflanzen nachgewiesen werden und die Ergebnisse bestätigen, dass Pflanzen als Heilmittel gegen Infektionen eingesetzt werden können. Sowohl die ätherischen Öle als auch die Reinsubstanzen könnten entweder als Monopräparate oder in Kombination Anwendung finden und scheinen viel versprechende Mittel zu sein, um multiresistente Krankheitskeime zu bekämpfen.

Chapter 1

General Introduction

1.1 Infectious disease and multiple antibiotic resistances in bacteria

Multiple surveillance studies have demonstrated that resistance among prevalent pathogens is increasing at an alarming rate, leading to greater patient morbidity and mortality from nosocomial infections. Among Gram-positive organisms, the most important resistant pathogens are methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE). Important causes of Gram-negative resistance include extended-spectrum β-lactamases (ESBLs) in *Klebsiella pneumoniae*, *Escherichia coli*, and multidrug-resistance genes observed in *Pseudomonas aeruginosa*, and *Acinetobacter* (Jones, 2001; Tenover, 1995). Many drugs that have frequently been used in the past as empiric therapy have become less effective against nosocomial pathogens (Jones, 2001).

1.1.1 Methicillin-resistant *Staphylococcus aureus* (MRSA)

Staphylococcus aureus is one of the major causes of community-acquired and hospital acquired infections. The spectrum of *S. aureus* infections includes toxic shock syndrome, food poisoning, meningitis as well as dermatological disorders ranging from minor infections and eczema to blisters and scalded skin syndrome. The most common life-threatening manifestation of *S. aureus* infection is bacteremia ranging from uncomplicated bacteremia to fixed endovascular infection, such as endocarditis (Haddadin et al., 2002).

Before the introduction of antibiotics, the mortality rate of staphylococcal bacteraemia was ~70%. MRSA has now exerted its own impact upon the mortality rate. The studies quoted MRSA bacteraemia mortality rates over 50-80%. Now the clinical consequences of resistance to vancomycin further complicate the management of MRSA infections. Mortality was 63% in patients who became infected with vancomycin-intermediate *S. aureus* (VISA). The mortality is even higher (78%) in patients with septicaemia caused by VISA additionally resistant to rifampicin, another staphylococcal agent (Dancer, 2008).

Vancomycin-resistant *S. aureus* (VRSA) was first isolated in Japan in 1997 (Hiramatsu et al., 1997). Shortly VRSA isolates were also reported in the U.S.A. and other countries (Smith et al., 1999; Srinivasan et al., 2002). Strains of vancomycin-intermediate *S. aureus* (VISA) with vancomycin MIC of 8 μg/ml have been reported from Japan, United States, France, United Kingdom, and Germany (Fridkin et al., 2003; Hanaki et al., 2007).

This pattern has continued among the newer agents. Linezolid which was introduced clinically in 2000, resulted in the first linezolid-resistant MRSA strains (Tsiodras et al., 2001). Daptomycin was introduced in 2003, and MRSA resistance to it was first reported within 2 years. MRSA has become a worldwide problem, although its prevalence differs widely among countries. The epidemiology of MRSA has been altered, infections are no longer strictly to hospital, but also reveal in community without healthcare-associated risk factors for acquisition of MRSA (Kluytmans-Vandenbergh and Kluytmans, 2006). MRSA which considered as hospital associated- pathogen (HA-MRSA) has been recognized the risk factors such as hospitalization, surgery, lengths of stay, enteral feedings, and the antibiotics use (macrolide and levofloxacin especially) (Graffunder and Venezia, 2002). Community-acquired methicillin-resistant *S. aureus* (CA-MRSA) has now developed as epidemic that causes rapidly progressive, severe disease including necrotizing pneumonia, sepsis and necrotizing fascities. (Boyle-Vavra and Daum, 2006). Differences are found in SCCmec types, the presence of additional antibiotic resistance genes, bacterial growth and the distribution of toxin genes (Groom et al., 2001).

MRSA strains harbour the *mecA* gene, which encodes the low-affinity penicillin-binding protein 2a (PBP2a). The production of PBP2a confers resistance to otherwise inhibitory concentrations of all β-lactam antibiotics. The *mecA* gene is carried on a mobile genetic element, the staphylococcal cassette chromosome *mec* (SCC*mec*), which is integrated in the chromosome of *S. aureus* (Ito et al., 2001; Ito et al., 2004; Ma et al., 2002). Notably, SCC*mec* type IV has been found in twice as many clones as any other SCC*mec* type, and it is this SCC*mec* type that is most commonly found in clones from patients with CA-MRSA infections (Robinson and Enright, 2003).

1.1.2 Vancomycin-resistant enterococci (VRE)

Enterococci have been recognized as an important cause of endocarditis for almost a century. Enterococci are the second to third most important bacterial genus in hospital infections (Schaberg et al., 1991). Two most reliable antienterococcal drugs are now inactive against these strains. This inactivity results in the use of less-effective regimens or, more recently, in the use of newer antienterococcal agents, such as linezolid or quinupristin-dalfopristin. Although both of these newer agents have proved to be effective in treating VRE infections, baseline rates of reduced susceptibility to quinupristin-dalfopristin have been relatively high, and emergence of resistance to linezolid during treatment is increasingly being documented (Marshall et al., 2002). Especially *E. faecium* possesses a broad spectrum of natural and acquired antibiotic resistances. Infection caused by VRE is associated with high morbidity and mortality rates, in particular, to immunosuppressed patients. In addition, there are at least 4 variables that contribute to the spread of VRE in hospitals: poor infection control practices, admission of patients who are already colonized, antibiotic use, and prolonged stays by patients (Cetinkaya et al., 2000).

Vancomycin interdicts bacterial growth primarily by blocking the cross-linking of adjacent peptidoglycan strands by peptide bonds during synthesis of the bacterial cell wall. Without sufficient cross-linking, the cell wall becomes mechanically fragile and the bacteria lyse when subjected to changes in osmotic pressure.

Vancomycin binds to the D-alanine-D-alanine (D-Ala-D-Ala) terminus of the pentapeptide moiety of the peptidoglycan precursor before cross-linking. The D-Ala-D-Ala dipeptide forms complementary hydrogen bonds with the peptide backbone of vancomycin. It is thought that the vancomycin-peptidoglycan complex physically occludes the subsequent action of transpeptidase enzymes (the targets of penicillins) and in so doing blocks formation of the peptide cross-bridges that confer strength on the peptidoglycan (Klare et al., 2003; Pootoolal et al., 2002).

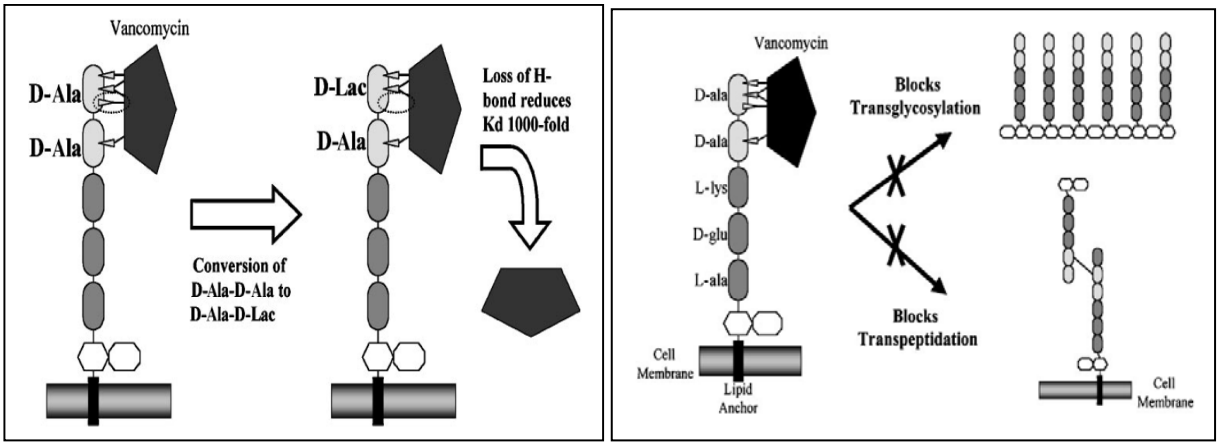


Figure 1.1 The bactericidal effect of vancomycin on gram-positive bacteria (Pootoolal et al., 2002).

The transglycosylation step that connects the disaccharide unit of the pentapeptide precursor to existing glycan strands also appears to be inhibited by vancomycin. The two most prevalent clinical isolates of vancomycin-resistant enterococci, Van A and Van B, reprogram the peptidoglycan synthetic machinery, replacing the D-Ala-D-Ala dipeptide with D-Ala-D-lactate. The loss of a crucial hydrogen bond between vancomycin and the terminal dipeptide results in a decrease (by three orders of magnitude) in the binding affinity of the antibiotic for the peptidoglycan (Pootoolal et al., 2002).

There are five recognized phenotypes of vancomycin resistance, VanA, VanB, VanC, VanD, and VanE. Two of these (VanA and VanB) are mediated by newly acquired gene clusters not previously found in enterococci. VanA and VanB resistance phenotypes were described primarily in *E. faecalis* and *E. faecium*. VanA-resistant strains possess inducible, high-level resistance to vancomycin (MIC 64 µg/ml) and teicoplanin (MIC 16 µg/ml) (Arthur and Courvalin, 1993). VanA is more widely distributed and is by far the predominant type of resistance reported in Europe. While VanB strains are fairly common in the United States, with some hospitals reporting VanB exclusively, VanA still predominates(Clark et

al., 1993). The *vanD*-strain carrying this resistance trait was an *E. faecium* strain that was inhibited by vancomycin at 64 μg/ml and teicoplanin at 4 μg/ml. Partial sequencing of the ligase gene showed that it was distinct from but similar to the *vanA* and *vanB* ligase genes. VanD appears to be located on the chromosome and is not transferable to other enterococci. The *vanE* vancomycin resistance gene has recently been described in *E. faecalis* BM4405, which is resistant to low levels of vancomycin (MIC, 16 μg/ml) and susceptible to teicoplanin (MIC, 0.5 μg/ml) (Cetinkaya et al., 2000; Ostrowsky et al., 1999).

1.1.3 Streptococci

Streptococcus agalactiae bacteremia is common in neonates and sometimes occurs in females after obstetric or gynecologic surgery (Schuchat, 1999). *Streptococcus pyogenes* is responsible for a wide spectrum of human disease, varying from superficial (pharyngitis and erysipelas), invasive (bacteremia and pneumonia), toxin-mediated (toxic shock syndrome) and immunologically-mediated (rheumatic fever and glomerulonephritis) disease (Efstratiou, 2000). In this era of increasing antimicrobial resistance, it is remarkable that *S. pyogenes* are susceptible to penicillin (Macris et al., 1998). Macrolides are prescribed for patients with allergies to β-lactams, and resistance to macrolides has emerged in this organism. The emergence of macrolide-resistance, erythromycin-resistant, clindamycin-resistant strains was recently documented (Alos et al., 2003).

Recent prescription of a macrolide (especially azithromycin) is a predictor of erythromycin resistance, as well as a possible risk factor for resistance at a community level Only 1 strain of *S. pyogenes* with high-level fluoroquinolone resistance has been reported previously (Richter et al., 2003). A study reported that the recovery of a second clinical isolate of *S. pyogenes* with mutations in the *gyrA* and the topoisomerase IV gene *(parC)* genes conferring resistance to multiple fluoroquinolones (Richter et al., 2003). Serotype M-protein gene *(emm)* type 6 *S. pyogenes* has intrinsic reduced susceptibility to fluoroquinolones, as a result of a polymorphism in *parC.* This finding was also demonstrated in erythromycin-resistant M/*emm* type 6 *S. pyogenes,* which raises concern for the emergence of multidrug-resistant *S. pyogenes* (Orcheln, 2005).

1.1.4 Gram-negative bacteria

Recent clinical attention has also focused on the increasing frequency of non-lactose-fermenting gram-negative pathogens responsible for hospital-acquired infections. The National Nosocomial Infection Surveillance System (NNIS) found that, in 2003, gram-negative bacilli were associated with 23.8% of bloodstream infection, 65.2% of pneumonia episodes, 33.8% of surgical site infection, and 71.1% of urinary tract infections. The specific gram-negative pathogens most commonly associated with ICU-acquired infections were *P. aeruginosa* in pneumonia, *K. pneumoniae* and *Enterobacter* species in bloodstream infection, *P. aeruginosa* and *Enterobacter* species in surgical-site infection,

and *E. coli* and *P. aeruginosa* in urinary tract infection. Among gram-negative pathogens, *Acinetobacter* organisms were the only gram-negative pathogens associated with consistently increasing proportions of hospital acquired pneumonias, surgical site infections, and urinary tract infections in nearly all NNIS hospitals during the study period. *Acinetobacter* organisms are intrinsically resistant to many antimicrobials and can acquire resistance to others. It was found that were resistant to amikacin, imipenem, and ceftazidime (Gaynes and Edwards, 2005).

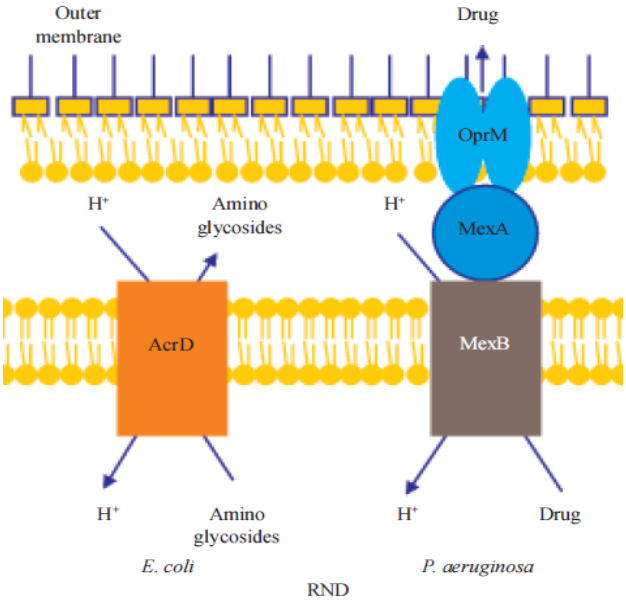


Figure 1.2 Illustration of the main types of bacterial drug efflux pumps shown in *Escherichia coli* and *Pseudomonas aeruginosa*. Illustrated are Acr D and MexAB-OprM, two members of thr resistance-nodulation-division (RND) family (Schweizer, 2003).

Most Gram-negative bacteria are intrinsically less susceptible to many antibiotics, especially amphiphilic and lipophilic ones, than are the average Gram-positive bacteria (with the exception of mycobacteria). It was once thought that this difference can be largely explained by the presence, in the Gram-negative bacteria, of the outer membrane. This additional membrane layer indeed acts as a very effective permeability barrier. Thus the influx of hydrophilic drugs through the porin channels is difficult as they are usually much larger than the typical nutrient molecules. Furthermore, lipophilic drugs cannot penetrate through these channels (owing to the highly ordered nature of water molecules within the channels and diffuse only very slowly across the lipid bilayer domain of the outer membrane, which contains a lipopolysaccharide-containing outer leaflet of unusually low fluidity (Nikaido and Vaara, 1995). However, the discovery of the AcrAB multidrug efflux pump (Ma et al., 1993) destroyed the simplistic and static idea that the resistance of Gram-negative bacteria can be explained entirely by the outer membrane permeability barrier. The AcrAB system of *Escherichia coli* is a multidrug efflux system composed of an RND-type transporter AcrB and a periplasmic accessory protein AcrA, and pumps out a wide variety of lipophilic and amphiphilic inhibitors directly

into the medium, presumably through the TolC outer membrane channel. AcrA, a highly elongated protein, is thought to bring the outer and inner membranes closer. It forms a trimer that interacts with a monomeric AcrB, which was shown by in vitro reconstitution to be a proton antiporter (Nikaido and Zgurskaya, 2001; Schulz, 1993).

Like some Enterobacteriaceae species, *P.aeruginosa* has an inducible AmpC β-lactamase and is inherently resistant to those β-lactams that induce this enzyme and are hydrolyzed by it (e.g., cephalothin and ampicillin). Moreover, many antibiotics are excluded from the pseudomonal cell. This exclusion long was attributed to the cell's impermeability, although evidence of this was scanty and although the belief proved difficult to reconcile with the discovery that *P. aeruginosa* copiously manufactures a porin (OprF) that forms large outer membrane pores. In the early 1990s, it began to be realized that much of this "impermeability-mediated resistance" (as it was widely called at the time) actually reflected efflux by MexAB-OprM, a pump system that removes b-lactams, macrolides, chloramphenicol, fluoroquinolones, novobiocin, sulfonamides, tetracycline, and trimethoprim, as well as various dyes and detergents. The natural role of MexAB-OprM may be to remove amphipathic permeants, which otherwise would disorganize the cytoplasmic membrane (Schweizer, 2003).

The genomic sequence for *P. aeruginosa* suggests the presence of at least 5 further efflux systems, which await even preliminary characterization. Multidrug efflux systems are also being reported in many other gram-negative bacteria, including Enterobacteriaceae as well as other nonfermenters. At a functional level, the role of broad-spectrum efflux pumps may be to remove amphipathic substances (i.e., those with hydrophilic and hydrophobic parts) from membranes, preventing disorganization (Srikumar et al., 2000).

1.1.5 *Candida* spp.

The genus *Candida* consists of more than 100 different species comprising nonpathogenic organisms as well as opportunistic fungal pathogens. In immunocompetent humans, *Candida* spp. can cause superficial infections of the skin (Odds, 1994). Those infections are usually not dangerous for healthy immunocompetent individuals. Importantly, *Candida* spp. can cause systemic life-threatening disease in immunocompromised patients. In case of systemic and invasive Candidiasis, the causative species is able to penetrate epithelial tissues, to enter the blood stream and to disseminate into different organs (Calderone et al., 2009).

Candida albicans was the most common pathogen in candidemic patients, but the proportion of non-*C. albicans* strains showed an increasing trend during 1997-1999 with *C. glabrata and C. parasilopsis* was the second and the third most common species observed overall, respectively. resistance to fluconazole and itraconazole was observed in *C. glabrata* and *C. krusei*, whereas isolates of *C.*

albicans, *C. parapsilosis*, and *C. tropicalis* were all highly susceptible to both fluconazole (98.9 to 100% susceptible) and itraconazole (96.4 to 100% susceptible) (Pfaller et al., 2000). The overall mortality of patients with candidemia was 40-60%. Mortality was highest in the group of patients with multi-organ dysfunction syndrome, especially among those in need of hemodialysis. Risk factors for the development of candidemia reported such as age, malignancy, steroid use, catheterization (Cheng et al., 2005). A particular characteristic phenotype of *C. glabrata* is its reduced susceptibility to clinically used azoles. The inherent resistance against this class of antifungal may be a major virulence factor of *C. glabrata*. This view is supported by the finding that *C. glabrata* is often isolated from patients undergoing azole therapy. Moreover, the prophylactic use of azoles in treatment of *Candida* infections may actually contribute to the clinical prevalence of *Candida* sp.(Pfaller et al., 2007).

The resistance mechanisms of fungal cell comprise reduced uptake, upregulation of drug efflux pumps, sequestration of toxic substances in the vacuole, intracellular drug inactivation, changes in membrane permeability and alteration of drug targets (Cowen and Steinbach, 2008).

1.2 Herbal medicine and Traditional Chinese Medicine (TCM)

Over the past decade, herbal medicine has become a topic of increasing global importance, with both medical and economic implications. In term of medical system, herbal medicines continue to play a central role in the healthcare systems particularly in developing countries. A good example is TCM, that has served the health needs of the Chinese population for almost 5000 years. This system of medicine has its own unique methods of diagnosis and incorporates >7000 species of medicinal plants into clinical practice (Mahady, 2001). In the recent years, there is an increasingly interest in the western world in TCM. Europe has imported TCM products from China resulted in annual sale of US $ 180 million. The considerable attractiveness of TCM raised the motivation of phytochemists and pharmaceutical biologists to investigate the pharmacological basis of TCM (Efferth et al., 2007).

TCM comprises natural product from plants, animals, and minerals, acupuncture and other practices. The practice follows theories of holistic and integrative principles. Therapy is based on understanding relation between part and whole, distinguished symptom and cause and treating each individual case as unique. The foundation of this practice is the Chinese materia medica. More than 6.000 substances are used and their effects are documented (Newman et al., 2000). Considering the immense number of the TCM substances and the amount of resources it will take to study them thoroughly, it is not surprising that there is still much work to be done on basic levels. The active ingredients of most Chinese herbs are still unknown. For those in which some of the active ingredients are known, there is little understanding of their pharmacologic properties.

Chinese herbal medicines have been used as a complex mixture of multi components of different classes or plants called a formula. This mixture gives some advantages to increase or to reinforce the effects of each medicinal substance, to minimize toxicity or side effects, to accommodate complex clinical situations, and to alter the actions of the substances (Efferth et al., 2007; Zhu and Woerdenbag, 1995). These complex mixtures may contain secondary metabolites (SM), which are specific for a single target (monotarget SM). A majority of SM, however, can interfere with several targets (multitarget SM) in a pleiotropic fashion. The composition of such extracts appears to be optimized, since the components are not only additive but apparently synergistic in their bioactivity (Wink, 2008a). It is necessary to understand how and why the herbs are combined as well as some general rules for combining herbs including the compatibility, contraindication, and dosage (Wu, 2005).

Although this mixture of herbs is common in TCM, western pharmacologists are usually skeptical about these prescriptions and doubt their efficacy. However, empirical observations and several controlled clinical studies have shown, that many of these complex mixtures have clear biological activities and several exhibit a significant clinical efficacy in controlled clinical studies (Efferth et al., 2007).

1.2.1 *Eucalyptus globulus* Labill.

Eucalyptus (Myrtaceae) is one of the world's most important and most widely planted genera. Only about 20 *Eucalyptus* species have been exploited commercially, mostly for their essential oils (Coppen, 2002; Ghisalberti, 1996).

1.2.1.1 Essential oil of *Eucalyptus*

Many volatile oils – particularly those of herbs and spices, but including those from *Eucalyptus* – have been used to extend the shelf-life of foods, beverages, pharmaceutical and cosmetic products; their antimicrobial and antioxidant properties have also pointed to a role in plant protection (Deans, 2002). *Eucalyptus globulus*, or Blue Gum, oil was a traditional Australian aboriginal remedy for infections and fevers. It is now used all over the world for relieving coughs and colds, sore throats and other infections. Its main constituent, 1,8-cineole, is mucolytic (i.e. it thins out and relaxes the flow of mucus) and is excreted through the lung surface. *E. radiata* oil is sometimes preferred by aromatherapists for its more pleasant smell while *E. smithii* oil is sometimes preferred due to a perception that it is better tolerated by the skin. *E. radiata* and *E. smithii* oils have also been shown to be useful for treating disorders of the respiratory system, although with some differences in their uses. A steam inhalation with *Eucalyptus* is not only an effective cold treatment because it relieves nasal and bronchial congestion, but because it is also claimed to inhibit proliferation of the cold virus (Davis, 1990).

When applied as a diluted oil on the skin Eucalyptus has a warming and slightly anaesthetic effect. Massaging with such an oil, therefore, will help to relieve respiratory infections, pain caused by rheumatic joints, neuralgia, fibrositis and muscular aches. Burns, blisters, insect bites and skin infections such as abscesses are also claimed to respond positively to the topical application of the essential oil or extract. It is also said to be valuable for easing the symptoms of shingles, chickenpox and cold sores (Beerling et al., 2002).

Of the large and diverse range of compounds found in eucalyptus oils, the most important is the monoterpene ether 1,8-cineole. It is used for medicinal, flavour and fragrance purposes and has significant biological activity (e.g. mosquito repellency (Klocke, 1987)). *E. globulus* remains the chief source of cineole worldwide although the oil contains a lower proportion of it than *E. polybractea* and some other species. However, *E. globulus* is widely grown for its wood and for pulp production and, as a result, the 'waste' leaf is available for production of oil. This and other commercially produced oils with concentrations of cineole less than that in *E. polybractea* (e.g. *E. smithii* and *E. radiata*) are either fractionated to enhance cineole levels to the 70 – 75 per cent or 80 – 85 per cent required by monographs published by ISO and various national pharmacopoeias or sold for uses where cineole content is not so critical (e.g. aromatherapy) (Brophy and Southwell, 2002).

Although cineole-type (medicinal) eucalyptus oils dominate world production, lemon-scented (perfumery) oils are also produced. The worldwide production of citronellal oils from *E. citriodora* and the citral (i.e. neral, geranial) oils from *E. staigeriana* was estimated at 320 and 70 t respectively for 1984. Eucalyptus oil of the type from *E. globulus* and that from *E. citriodora*, have both been the subjects of RIFM monographs also gives a separate monograph on eucalyptol. Such a wide variety of applications, actual or potential, has meant that the antimicrobial properties of volatile oils and their constituents from a large number of plants have been assessed and reviewed (Deans, 2002; Deans and Ritchie, 1987; Dellacassa et al., 1989; Palombo and Semple, 2002; Rios and Recio, 2005). In recent years attempts have been made to identify the component(s) of the oils responsible for such bioactivities (Carson and Riley, 1995; Deans, 2002; Deans and Ritchie, 1987; Dorman and Deans, 2000; Inoue et al., 2004).

The antimicrobial activity of eucalyptus oils and other volatile oils would be expected to reflect their composition, the structural configuration of the constituents and their functional groups, along with potential synergistic interactions between the constituents. Aqueous solubility, and the ability of toxic compounds to penetrate the fungal or bacterial cell wall, is also likely to be an important factor and this, too, will be influenced by the chemical nature of individual compounds within the oil. However, while some general observations can be made about the antimicrobial activity of different classes of terpenes, detailed structure–activity relationships are still not well understood.

Carbonylation of terpenoids, for example, is known to increase their bacteriostatic activity but not necessarily their bactericidal activity (Griffin *et al*. 1999), while alcohols possess bactericidal rather than bacteriostatic activity against vegetative bacterial cells (Deans, 2002).

1.2.1.2 Non-volatile components of *Eucalyptus*

Although earlier research focused on the volatile constituents of eucalypts – their essential oils – and these have largely been the basis upon which the industry exploiting the aromatic and medicinal uses of eucalyptus has been built, research in the last two decades has demonstrated the commercial potential, particularly in the pharmaceutical field, of the non-volatile constituents. Research carried out since the 1970s has indicated that the potential for eucalyptus to be used to combat some of the most serious and widespread illnesses and diseases may rest with some of its non-volatile constituents, rather than with the more familiar volatile oils (Lassak, 2006).

The chemistry of the volatile oils is only one facet of *Eucalyptus* secondary metabolite chemistry. The genus also contains flavonoids, triterpenes, long chain ketones, glycosides, acylphloroglucinol derivatives and adducts combining more than one of these chemical entities. However, the great majority of the other bioactive metabolites so far identified in eucalypts have proved to be acylphloroglucinols (Ghisalberti, 1996; Singh and Bharate, 2006). Perhaps the most exciting advances in this field relate to the discovery of four groups of novel, highly bioactive acylphloroglucinol derivatives peculiar to the genus *Eucalyptus*: the robustadials, sideroxylonals, macrocarpals and euglobals. Perhaps the most exciting advances in this field relate to the discovery of four groups of novel, highly bioactive acylphloroglucinol derivatives peculiar to the genus *Eucalyptus*: the robustadials, sideroxylonals, macrocarpals and euglobals. These have been described earlier in this volume and embrace a number of activities, any or all of which could, in the longer term, be exploited commercially. Robustadials A and B, with a combined phloroglucinol– monoterpene structure, are antimalarial constituents of *E. robusta* leaves (Cheng and Snyder, 1988). The sideroxylonals, dimers of a diformylphloroglu-cinol moiety isolated from *E. sideroxylon* and *E. grandis*, are antibacterial but also show strong attachment-inhibiting activity towards the blue mussel; sideroxylonal A is one of the most powerful antifouling agents known (Satoh et al., 1992). The macrocarpals, present in the leaves of *E. macrocarpa*, *E. globulus* and *E. amplifolia*, have a combined isopentyldiformylphloroglucinol –sesquiterpene structure and exhibit a range of biological activities. They are all strongly antibacterial against Gram-positive bacteria, including ones which cause dental diseases, and some have recently been found to be active against Gram-negative bacteria (Bharate et al., 2007; Murata et al., 1990; Osawa et al., 1995; Osawa et al., 1996). Macrocarpals A, B and C demonstrated antibacterial activity against periodontopathic bacteria. Among tested bacteria, *P. gingivalis* displayed the greatest sensitivity to macrocarpals; additionally,

its trypsin-like proteinase activity and binding to saliva-coated hydroxyapatite beads were inhibited by macrocarpals. These results indicate that eucalyptus leaf extracts may be useful as a potent preventative of periodontal disease (Nagata et al., 2006). The antibacterial and antiviral properties of macrocarpals appear to be due more to the diformylphloroglucinol moiety than to the different sesquiterpenoid groups attached to it, although Osawa *et al*. (1996) have suggested that the antibacterial potency may be regulated by the structure of the sesquiterpene. The euglobals are the largest, and structurally most variable, of these four groups of *Eucalyptus* metabolites. They are formyl phloroglucinol adducts with either a mono- or a sesquiterpenoid moiety attached. Euglobals have been isolated from *E. globulus* (Amano et al., 2008) and other species *Eucalyptus*, but have been detected in many more species. Many euglobals show strong inhibition of Epstein-Barr virus activation induced by 12-*O*-tetradecanoylphorbol-13-acetate, a tumour promoting substance, and this gives rise to hope that they might, in the future, play some role in cancer prevention (Takasaki et al., 1995). Several new euglobal analogues (named as S-euglobals) were synthesized from phloroglucinol via a biomimetic three component reaction involving Knoevenagel condensation followed by [4+2]-Diels–Alder cycloaddition with monoterpene. Few of the euglobal analogues showed antibacterial activity against MRSA (Bharate et al., 2008).

Extracts of *E. globulus, E. maculata* and *E. viminalis* significantly inhibited the growth of six Gram-positive bacteria (*S. aureus*, MRSA, *Bacillus cereus*, *Enterococcus faecalis*, *Alicyclobacillus acidoterrestris*, *Propionibacterium acnes*), and of a fungus (*Trichophyton mentagrophytes*), but they did not show strong antibacterial activity against Gram-negative bacteria (*E. coli*, *P. putida*). Compounds 2',6'-dihydroxy-3'-methyl-4'-methoxy-dihydro-chalcone, eucalyptin and 8-desmethyl-eucalyptin, isolated from *E. maculata* extracts, exhibited potent antimicrobial activities against seven micro-organisms with minimum inhibitory concentrations (MIC) ranging from 1.0 to 31 mg/l (Takahashi et al., 2004).

1.2.2 *Kadsura longipedunculata* Finet et Gagnep

The plant of *K. longipedunculata* (Schisandraceae) is a climbing plant distributed in eastern and south-central China (Li et al., 1989) at 100–1200 m a.s.l., in various habitats including rocky slopes, along streams, and hillsides of forests. It has been used in folk medicine for the treatment of rheumatoid arthritis as well as gastric and duodenal ulcers (Li et al., 1989). Individual plants of KL have male flowers with exclusively red or exclusively yellow androecia, whereas the tepals of both sexes are pale yellow, and the gynoecium is green. All flower types simultaneously produce heat and floral odors (dominated by methyl butyrate) throughout a 4–5 h nocturnal period (Yuan et al., 2008).

Five dibenzocyclooctadiene lignans were isolated from the stems collected in Guangxi province. Three of them are named benzoyl-, isovaleroyl-, and isobutyroyl-binankadsurin A (Li et al., 1991). Six

triterpenoid acids were isolated from the stems and assigned as (24Z)-3-oxo-1 2a-acetoxylanosta-8,24-dien-26-oic acid, (24Z)-3-oxo-1 2a-hydroxylanosta-8,24-dien-26-oic acid, neokadsuranic acids B, and C (Li et al., 1989).

The effects of twelve triterpenoid acids isolated from the ethanolic extract of the stems were examined on cholesterol biosynthesis. (24Z)-3-Oxo-lanosta-8,24-dien-26-oic acid (3-oxo-LA) was most active in depressing cholesterol biosynthesis from [2-^{14}C] mevalonate in a 10,000 x g supernatant fraction of rat liver homogenate (Kangouri et al., 1990). Longipedunins A–C, benzoyl-binankadsurin A, acetyl-binankadsurin A and schisanlactone A, were further isolated. Longipedunins A and schisanlactone A showed inhibitory activity against HIV-1 protease with IC50 values of 50 and 20 μM, respectively (Sun et al., 2006).

Two novel triterpene dilactones, kadlongilactones A and B, were isolated from the leaves and stems of KL. Both compounds exerted significant inhibitory effects against human tumor K562 cells with IC50 1.40 and 1.71 μg/ml, respectively (Pu et al., 2005). In the continuous search for bioactive metabolites from this plant, nine novel triterpene derivatives, longipedlactones A–I, were also isolated (Pu et al., 2006). A triterpenoid, schisanlactone E, and two lignans, 9-(β-D-glucopyranosyloxy)-3'-methoxy-3,4-(methylenedioxy)-7,9'-epoxylignan-4'-ol and 3-methoxy-3',4'-(methylenedioxy)-9,9'-epoxylignan-4,7'-diol were also reported (Pu et al., 2007). Two tetrahydrofuran lignans, Kadlongirins A–B, and a sesquiterpenoid, 2,7-dihydroxy-11,12-dehydrocalamenene, together with seven lignans (grandisin, fragransin B1, vladirol F, kadsuralignan C, otobaphenol, isoanwulignan, and 4-[4-(3,4-dimethoxyphenyl)-2,3-dimethylbutyl]-2-methoxy phenol) were isolated. Kadlongirins B exhibited weak anti-human immunodeficiency virus-1 activity with an EC50 value of 16.0 μg/ml, and therapeutic index value of 6.7 (Pu et al., 2008).

In addition, kadsulignans E–G were isolated from the root bark of KL (Liu and Huang, 1992). The essential oil of the root barks of KL consisted mainly of sesquiterpenoids and monoterpenoids. δ-cadinene (13.8%), nerolidol (11.4%) and δ-cadinol (10.4%) were the main sesquiterpenoids. The oil contained mainly sesquiterpenoids such as α-santalene (10.1%), δ-cadinene (25.6%) and 2,4α,5,6,7,8-hexanhydro-3,5,5,9-tetramethyl-benzocycloheptene (19.8%). The essential oil showed strong activities against Gram-positive bacteria and moderate activities against the yeast, while no significant effect on Gram-negative bacteria were observed (Song et al., 2007).

1.2.3 *Siegesbeckia pubescens* Makino

Plants of the genus *Siegesbeckia* are annual herbs widely distributed in China and they have been used as a traditional medicine, 'Xi-Xian', to treat rheumatic arthritis, hypertension, malaria, neurasthenia, snake-bite and eczema (Jiang et al., 1992). A glycoside named darutoside was isolated from *S. orientalis* and *S. pubescens* (Kim et al., 1979). Isolation and structure determination of

orientalide and darutigenol melampolides was reported from *S orientalis* (Barua et al., 1980; Baruah et al., 1979). A series of *ent*-kaurane (Murakami et al., 1975; Xiong et al., 1992) and *ent*-pimarane diterpenoids and sesquiterpenoids (Barua et al., 1980; Baruah et al., 1979; Xiang et al., 2005; Zdero et al., 1991) from *Siegesbeckia* species have been reported.

From the petrol and diethyl ether extracts of *S. pubescens* used in Yunnan, eight diterpenoids were isolated with sitosterol and daucosterol from *S. pubescens* (Xi-Xian). Their structures were elucidated as ent-16β,17,18-trihydroxy-kauran 18-O-β-D-glucopyranoside, ent-kauran-2α,16β,17-triol, ent-16αH,17,18-dihydroxy-kauran-19-oic acid, ent-kauran-16β,17,18-triol, ent-16β,17-dihydroxy-kauran-19-oic acid, ent-16αH,17-hydroxy-kauran-19-oic acid, grandifloric acid and kirenol (Jiang et al., 1992). Five new sesquiterpenes and diterpenoids were also reported by Xiang (Xiang et al., 2005). From the aerial parts of *S. orientalis***,** in addition to a known sesquiterpene lactone, nine new germacranohdes, three melampolides, three geranylnerol derivatives and three *ent*-pimarenes were isolated.

Methanol extract of the aerial part of *S. glabrescens* was found to inhibit protein tyrosine phosphatase 1B (PTP1B) activity at 30 mg/mL. Bioassay-guided fractionation led to the isolation of two active diterpenes, *ent*-16βH,17-isobutyryloxykauran-19-oic acid and *ent*-16βH,17-acetoxy-18-isobutyryloxy-kauran-19-oic acid, along with *ent*-16βH,17-hydroxy-kauran-19-oic acid. The later compound substituted with a hydroxyl group at C-17 in kaurane-type showed no inhibitory effects towards PTP1B. However, the other compounds were are non-competitive inhibitors of PTP1B with IC50 values of 8.7 and 30.6 μM, respectively (Kim et al., 2006).

The in vitro and in vivo immunosuppressive activity of the ethanol extract of *S. orientalis* on the immune responses in mice showed significantly suppressed concanavalin A (Con A)- and lipopolysaccharide (LPS)-stimulated splenocyte proliferation in vitro in a concentration dependent manner (Sun and Wang, 2006).

1.3 Plants as a source of novel antimicrobial agents

Antibiotics are the most important classes in therapeutic agents and have given an enormous impact on both of life expectancy and improve quality of life (Clark, 1996). After discovery of penicillin, other antibiotics isolated from microorganisms are introduced. However, the emergence of antibiotic resistant microorganism towards synthetic antimicrobial agents, the research in order to find an alternative antimicrobial agent became more extensive.

Plant products play an important role in the health care systems of the remaining 20% of the population, mainly residing in developed countries. Currently, at least 119 chemical substances which is derived from 90 plant species, can be considered as important drugs in many countries. Of these 119 drugs, 74% were discovered as a result of chemical studies directed at the isolation of the active

substances from plants used in traditional medicine (Newman et al., 2000). A multitude of plant compounds is readily available over-the-counter from herbal suppliers and natural-food stores (Cowan, 1999).

There are about 350.000 species of plants growing on the earth and it is estimated that at least 5000 different of chemical compounds are present in a single species of plant. It is apparent that the secondary metabolites of plant origin constitute a tremendous resource for exploring useful drugs (Kuo and King, 2001). Secondary metabolites (SM), which plants employ to defend themselves against bacteria, fungi or viruses, can be used in almost the same way in medicine to treat microbial or viral infections. SM of plants are not compounds with random structures but active metabolites, which have been selected during evolution, we can use them in medicine (Wink, 2008a).

Plants usually produce complex mixtures of SM, whose compositions show substantial differences between developmental stages and organs. These mixtures are regularly composed of SM from different classes. Most plants accumulate phenolic compounds (flavonoids and tannins), that are regularly accompanied by terpenoids (monoterpene, sesquiterpenes, triterpene or saponins (Wink, 1999a; Wink and Schimmer, 1999).

Much research has been done in crude extracts, fractions, essential oils and also isolated compounds to search antimicrobial compounds (Rios and Recio, 2005). The modern microbiological techniques demonstrates that higher plants frequently exhibit significant potency against human bacterial and fungal pathogens (Mitscher et al., 1987). Many paper have been published on antimicrobial extracts and isolated compounds from plants. The chemical structures of the antimicrobial agents found in higher plants belong to most commonly encountered classes of higher plant secondary metabolites (terpenoids, polyphenols, iridoids, saponins, polyenes, anthraquinones and some alkaloids) (Wink and Schimmer, 1999).

Plant antimicrobials are not used as systemic antibiotics at present. The main reason for this is their low level of activity, especially against gram-negative bacteria. The reported MIC is often in the range of 100 to 2.000 µg/ml; higher than those of common antibiotics. Some studies offered a possible explanation for the apparent ineffectiveness of plant antimicrobials. Stermitz have proposed that plants produce compounds that can be effective antimicrobials if they can find their way into the cells of pathogens (Stermitz et al., 2000). Thus production of MDR inhibitors by plants would be one way to ensure delivery of antimicrobial compounds. Berberis plant produce berberine and also synthesizes the MDR pump inhibitor 5'-methoxyhydnocarpin D which facilitates the penetration of berberine into *S. aureus*. Some studies also demonstrated the inhibition of *Nor*A multidrug transporter of *S. aureus* by reserpine (Markham, 1999). Previous study demonstrated that the inhibiting of the MDR pumps in Gram-negative leads to striking increase in antimicrobial activity. The

activity of rhein, the active compound from rhubarb, increased 100 to 2000 fold by disabling the MDR pumps. It seems to be potential to develop different type of antibacterials from plants which can be as MDR inhibitors. There has been a considerable effort to discover plant-derived antibacterial which is active against multiple antibiotics-resistant microorganisms (Hammerschmidt, 1999). Another useful application stemming from knowledge of MDR-based resistance is in drug discovery, including the finding of new plant antimicrobials. It seems that efflux by MDRs does provide a satisfactory explanation for the apparent ineffectiveness of many plant antimicrobials in vitro (Tegos et al., 2002).

1.3 Secondary metabolites of plants: with special reference to terpenoids

Terpenoids constitute a large and widely distributed class of natural compounds whose carbon skeleton is derived from C5 isoprene units. The terpenes are classified by the number of these C5 isoprene units; monoterpenoids (C10), sesquiterpenoids (C15), diterpenoids (C20), triterpenoids steroids/saponins (C30/C27), and tetraterpenoids (C40) with total number of all terpenoids exceed than 22.000 at present (Hanson, 2003; Wink, 1999a; Wink, 1999b; Wink, 2008b). Mono- and sesquiterpenes are often volatile and can be isolated as essential oils. They are especially abundant in Asteraceae, Apiaceae, Lamiaceae, Rutaceae, Lauraceae, Cupressaceae, Pinaceae, Myrtaceae and Zingiberaceae (Wink, 2004). These compounds are usually lipophilic and are stored in specialized oil cells, trichomes, resin channels, or other dead cells (Wink, 1997; Wink, 2004).

In general, mono-, sesqui-, and diterpenes are toxic to herbivores and microbes, and may function as defense compounds against such organisms (Tholl et al., 2004), but volatile terpenes also serve to attract pollinating insects (Wink, 2004). Some sequiterpenes are found in the higher boiling portions of essential oils. Sesquiterpene lactones are common biologically active constituents of plants of the compositae (asteraceae) family. Many diterpenoids are wood resin products. Diterpenes occur in both enantiomeric series. Diterpenes with the *ent*-kaurene skeleton are widespread. ent-Kaurenoic acid is a biosynthetic precursor of the diterpenoid gibberellins plant growth hormons (Hanson, 2003).

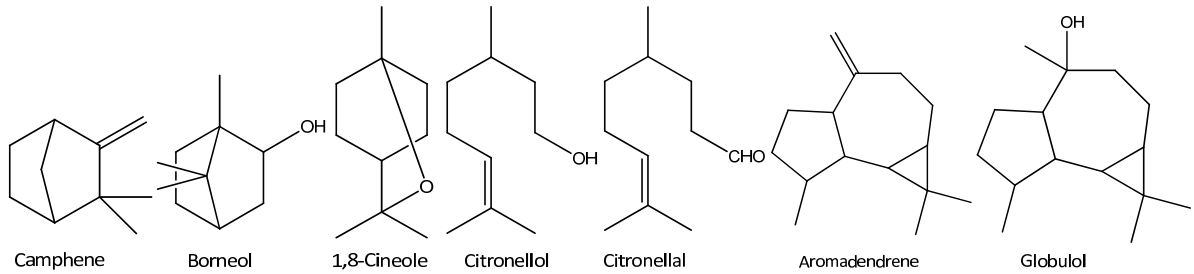


Figure 1.3 The chemical structure of monoterpenes (camphene, borneol, 1,8-cineole, citronellol, citronellal) and sesquiterpenes (aromadendrene, globulol).

Triterpenes and steroids can occur as aglycons but more often occur as saponins. Whereas free triterpenes and steroids are lipophilic molecules, saponins are water soluble and amphipilic molecules that can make biomembrane leaky. Therefore, they show antimicrobial and anti-herbivore activity (Wink, 2004).

Terpenoids can disturbe cells (modulate membrane fluidity, increase membrane permeability or solubilise biomembranes). In an aequous environment, small lipophilic SM will be trapped by biomembranes. The lipophilic SM will dive into the membrane and form hydrophobic interactions with the lipophilic side chains of phospholipids or cholesterol. Higher concentrations can influence membrane fluidity. In addition, these molecules can disturb the interaction of membrane proteins with membrane lipids, which are important for their correct three dimensional conformation. A change in protein confirmation will most likely modulate protein activity (Wink, 2008a).

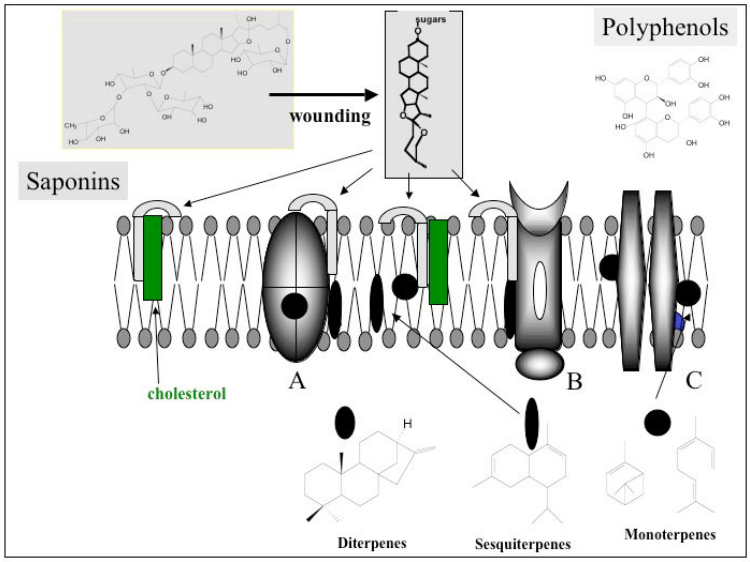


Figure 1.4 Interaction of terpenoids with biomembranes and membrane proteins. Lipophilic terpenoids make hydrophobic interactions with lipids and membrane proteins. Saponins will complex cholesterol (in green) and can permeabilise cells. Membrane proteins (transporters, A; receptors, B; ion channels, C) (Wink, 2008a).

Essential oil (EO) can comprise more than sixty individual components. Major components (Figure 1.4) can constitute up to 85% of the EO, whereas other components are present only as a trace (Russo et al., 1998). There is some evidence that minor components have a critical part to play in antibacterial activity, possibly by producing a synergistic effect between other components. This has been found to be the case for sage (Marino et al., 2001) certain species of *Thymus* (Marino et al., 1999) and oregano (Paster et al., 1995). The composition of EOs from a particular species of plant can differ between harvesting seasons and between geographical sources (Faleiro et al., 2003; Juliano et al., 2000; Marino et al., 1999).

Essential oils have been traditionally used for treatment of infections and diseases all over the world for centuries (Rios and Recio, 2005). The antimicrobial properties of plant volatile oils and their constituents from a wide variety of plants have been assessed (Ashour et al., 2009; Gopanraj et al., 2005; Raman et al., 1995; Smith-Palmer et al., 1998; Suschke et al., 2007; Svoboda and Hampson, 1999) and reviewed (Burt, 2004; Deans and Ritchie, 1987; Dorman and Deans, 2000; Kalemba and Kunicka, 2003). It is clear that these plant secondary metabolites have potential in medical procedures and applications in the cosmetic, food and pharmaceutical industries (Dorman and Deans, 2000).

1.5 Isolation of bioactive compounds using bioassay-guided fractionation

Testing large numbers of extracts or fractions to determine whether they produce a biological effect is usually one of the first steps in the discovery of bioactive compounds. A bioassay is any *in vitro* or *in vivo* system used to detect the biological activity of an extract or a pure substance from a living organism. The application of this assay to monitor the presence of a bioactive compound during the isolation process is called bioassay-guided fractionation (isolation). Thus, all fractions generated are tested for biological activity, and those giving a positive test are further processed until the bioactive agent is obtained in a pure form (Ghisalberti, 2008).

The process of isolation of a pure active compound from a plant is very long and tedious. This approach involves some steps including: collection and identification of plant material, preparation of extracts, biological screening of extracts, separation or fractionation, purification, and structure elucidation. The first step of screening of biologically active compounds is the selection of plant species. Currently collection of plants based on consideration of ethnomedical information and chemotaxonomic relationship, instead of random collection. A correlation between biological activity and use in traditional medicine has been demonstrated (Hamburger and Hostettmann, 1991). The examples of traditional medicines providing leads to bioactive natural products are legion. It is sufficient to point to some confirmations of the wealth of this source. Artemisinin (qinghaosou) is the antimalarial sesquiterpene from a Chinese medicinal herb that has featured in herbal remedies since ancient times (Wright, 2005).

Selection of in vitro antimicrobial tests for the screening of crude extracts considers some factors, including simplicity, accuracy, and reproducibility. Specificity and sensitivity should be taken into ascertain, especially for testing of extracts. Antibacterial assays can be classified into three groups, namely diffusion, dilution and bioautographic methods. Agar diffusion technique is one of the most commonly used in antimicrobial susceptibility testing but there are some problems with this technique. This technique can be used for preliminary screening of antimicrobial, but this method

(disk, cylinder or hole) is not suitable for lipophilic substances such as essential oils or non polar extracts which are not well soluble in water (Rios et al., 1988).

Dilution technique is mainly used to determine the MIC from essential oils, extracts or pure substances. The micro dilution technique using 96 wells plate gives some advantages. The technique is robust, is not expensive, gives reproducible results, is more sensitive, requires a small quantity of sample, can be used for large numbers of samples, and requires little time. One or two of the series of wells should be used with a known antibiotic to provide reference MIC values for the test organism. The observation can be performed by addition of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole (MTT) or p-Iodonitro-tetrazolium chloride (INT) (Canonica et al., 1969; Murakami et al., 1975; Xiang et al., 2004; Xiong et al., 1992).Bioautography is a very useful and relatively simple laboratory technique for the rapid detection of compounds that affect the growth rate of test organisms. Bioautography combines TLC with a bioassay in situ and allows the localization of active compounds in fractions or extracts (Betina, 1973). It can be categorized as direct, agar overlay/immersion and agar diffusion/contact bioautography (Choma, 2005). The most commonly used method is direct bioautography. Agar-overlay technique, a hydrid of direct and contact methods, can be used when the direct bioautography is not possible. The inhibition zones of bioautography are visualized by the detection of dehydrogenase activity with a tetrazolium salt (MTT, INT, etc) (Choma, 2005; Hamburger and Hostettmann, 1991; Rahalison et al., 1991; Rios et al., 1988).

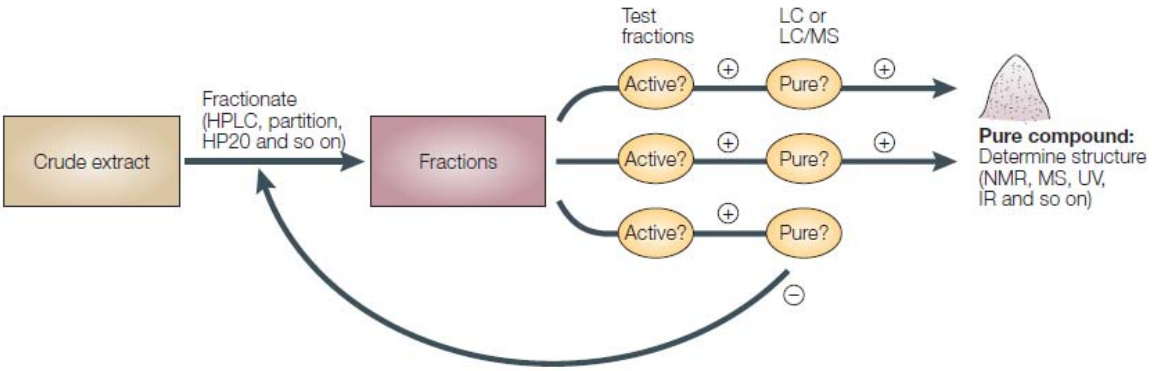


Figure 1.5 Scheme for bioassay guided fractionation. Several cycles of fractionation are usually needed to obtain a pure compound. HP20, a solid-phase adsorber; HPLC, high-performance liquid chromatography; IR, infrared spectroscopy; LC/MS, liquid chromatography; NMR, nuclear magnetic resonance; UV, ultraviolet (Koehn and Carter, 2005)

The separation of one or more substances from a crude extract or fractions of an extract can be a long and expensive process. Obtaining a pure compound often requires several separation steps involving different chromatographic techniques (multistep and multidimensional). This is particularly the case when dealing with bioactive metabolites, where the target compound(s) may be present only in trace quantities in a matrix of dozens of other constituents. There are many cases in which

the isolation of the bioactive metabolite is a relatively straightforward process, especially if the fractionation is guided by a bioassay (Ghisalberti, 2008).

A broad range of chromatographic techniques have emerged, such as HPLC and liquid-liquid partition chromatography, which allow efficient and mild isolation of pure compounds. The characterization of natural products in the past few decades has taken advantage of spectroscopic techniques as well as chemical techniques to determine the structure of natural products (Colegate and Molyneux, 2008).

With the possibilities of modern spectroscopy-NMR, MS and X-ray diffraction, structure elucidation has become rather straightforward. The major challenge today is the discovery of plants with promising activities and the isolation of active principles. There are, however, many obstacles which seriously hamper this type of investigation. First, it is possible that a broad range of structurally diverse compounds contribute to the overall pharmacological activity of a plant extract and synergistic effects between those active principles may exist. This is particularly the case for medicinal plants possessing less specific activities (Hamburger and Hostettmann, 1991).

1.6 Synergy in medicinal plants towards infections

1.6.1 Phytotherapy acts in pleitropic mode

In the early of 21 century two new paradigms in medicine have shifted, that are transition to multitarget therapy and tendency to use multidrug therapy. Phytotherapy has long followed and developed these strategies by using mono-extracts or extract combinations containing mixtures of bioactive compounds and by activating primarily self-healing and protective processes of the human body, rather than attacking and directly destroying the damaging agents. These strategies are based on therapeutic experiences and the consideration that a complex pathophysiological process can be influenced more effectively and with fewer or no severe side-effects by a combination of compounds or extracts than by a single isolated compound (Wagner, 2005).

Many of phytomedicine are available on the market as crude extract of plants and the herbal practitioner have always believed on their therapeutic effect. The mode of action of many phytomedicine is still unclear but there are several instances of a total extract having a better effect than an equivalent dose of a single isolated compound. Speculation as to the reason for this, probably it enhanced bioavailability, cumulative effects or additive properties needs further research (Williamson, 2001). Synergy effects of the mixture of bioactive constituents and their byproducts contained in plant extracts reclaimed to be responsible for the improved effectiveness of many extracts. For a long time, the mechanisms underlying the synergy effects remained unexplained. Based on results of the latest investigations in classic pharmacological, molecular–biological and clinical works, the following four mechanisms can be involved: (1) Synergistic multi-target effects. (2) Pharmacokinetic or physicochemical effects based on improved solubility, resorption rate and

enhanced bioavailability. (3) Interactions of agents with resistance mechanisms of bacteria. (4) The respective elimination or neutralization of adverse effects by agents contained in the extract (Wagner and Ulrich-Merzenich, 2009).

1.6.2 Synergy towards infections

Combination of drugs can be as one strategy employed to combat the resistance of microorganisms. Bacteria gains antibiotic resistance due to three reasons namely: (i) modification of active site of the target resulting in reduction in the efficiency of binding of the drug, (ii) direct destruction or modification of the antibiotic by enzymes produced by the organism or, (iii) efflux of antibiotic from the cell . In recent years, increasing attention has been focused on investigating phytochemicals as possible medicinal agents against MDR bacteria. There are wide range of phytochemicals which act as multidrug resistance modifier as shown in Figure 1.6. Plants are known to produce an enormous variety of chemicals defense such as terpenoids, glycosteroids, flavonoids and polyphenols. The secondary metabolites from plant are good sources for combination therapy (Hemaiswarya et al., 2008). A number of phytochemical exhibiting synergistic interactions with antibiotics have been characterized, whereas investigations of combination of active phytocompounds in this direction are in their infancy. There are a wide range of phytochemicals which act as multidrug resistance modifiers depicted and their mechanism of action as Receptor or active site modification. Studies through reverse transcription-PCR and a semiquantitative PBP2a latex agglutination assays indicted that the synergy between EGCg and BLA was achieved since both directly or indirectly attacked the same target site namely, peptidoglycan present on the cell wall (Zhao et al., 2001).

Synergism can also achieved by decreased outer membrane permeability. Thymol and carvacrol, two main compounds of the essential oil of *Thymus vulgaris*, act as so-called ‘‘membrane permeabilizers’’and that way facilitate the penetration of antibiotics into Gram-negative bacteria (Helander et al., 1998).

Combination of some drugs might also result to reverse the resistance by blocking the efflux pumps. Hence, it becomes apparent that plants adopt a different paradigm – ‘‘synergy’’ – to combat infections. A case in study to reiterate this view is the observation on the combined action of berberine and 5’-methoxyhydnocarpin, both of which are produced by berberry plants. Berberine, a hydrophobic alkaloid that intercalates into DNA, is ineffective as an antibacterial because it is readily extruded by pathogen – encoded multidrug resistance pumps (MDRs). Hence, the plant produces 5’-methoxyhydnocarpin that blocks the MDR pump (Stermitz et al., 2000). The presence of 5’-methoxyhydnocarpinin barberry plants provides support for the hypothesis that, in general, plant antibacterials are individually relatively weak but function in synergy. Tegos and co-workers demonstrated that the activity of rhein, plumbagin, resveratrol, gossypol, coumestrol, and berberine

by disabling the MDRs (Tegos et al., 2002). These findings also suggest that plant antimicrobials might be developed into effective antimicrobial agent against Gram negative and/ or MDR bacteria in combination with inhibitors of MDRs (Lewis and Ausubel, 2006).

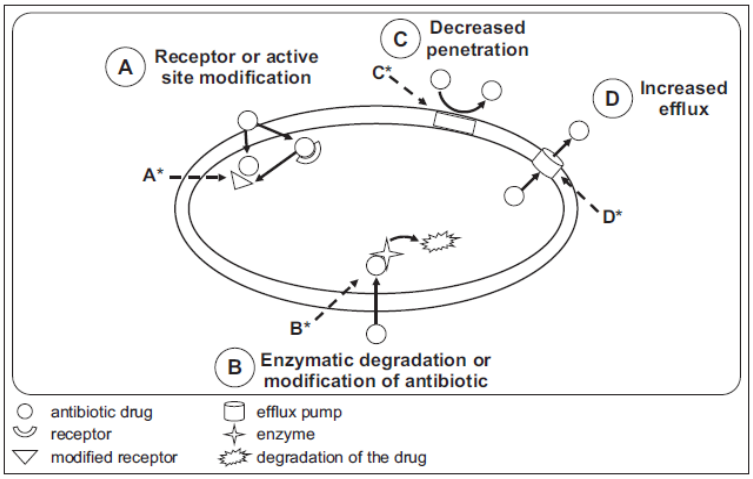


Figure 1.6 Strategies of bacteria to antagonize the effect of antibiotics and natural products which can overcome resistance problems: (A*) Corilagin, tellimagrandin I, diterpene 416 and compound P inhibit PBP2 a, a modified receptor; (B*) EGCg inhibits the b-lactamase; (C*) thymol, carvacrol, gallic acid increase the outer membrane permeability and (D*) EGCg, 5'-methoxyhydnocarpin, reserpine, arnosicacid and isopirmarane derivatives inhibit the efflux pumps (Hemaiswarya et al., 2008; Wagner and Ulrich-Merzenich, 2009)

Interestingly, berberine produced synergy against candidiasis in mice (Han and Lee, 2005). Coleman (2010) investigated some saponins which were combined with photosensitizer compounds. Yeast *C. albicans* displayed increased susceptibility to photodynamic inactivation due to the ability of the saponins to increase cell permeability, thereby facilitating penetration of the photosensitizers. The large proportion of compounds identified as antifungal agents containing saponin structural features suggests it may be a suitable chemical scaffold for a new generation of antifungal compounds (Coleman et al., 2010).

Sato demonstrated a synergistic effect of erybraedin A or eryzerin C was combined with vancomycin, the fractional inhibitory concentration (FIC) index against VRE ranged from 0.5306 to 1.0 and from 0.5153 to 0.75, respectively (Sato et al., 2004). Potentiated effect between flavones isolated from *Sophora exigua* and antibiotics against MRSA had also been reported (Sato et al., 1995).

A large number of essential oils with antimicrobial and antifungal effects have been and still are used internally for the supportive treatment of infections of the respiratory tracts as well as topically for the therapy of skin infections, essential oils in several recent investigations were combined with antibiotics with the aim of improving the antimicrobial effect and at the same time reducing the concentration of antibiotics. In the first in vitro experiments with essential oils of *Origanum vulgare*, *Pelargonium graveolens* and *Melaleuca alternifolia* in combination with norfloxacin and amphotericin B, distinct synergy effects against *Bacillus cereus, B. subtilis, E. coli, S. aureus* and

several Candida strains could be detected at a simultaneous reduction of antibiotic concentrations (Rosato et al., 2008; Rosato et al., 2009). The measured fractional inhibitory concentration indexes (FICI), according to the isobologram criteria established by Berenbaum (1989), showed that in all cases real synergy effects could be measured.

Williamson (2001) use the term polyvalent (multivalent/pleitropic) action to denote an enhanced or and cooperative sort of effect, without qualifying it, in attempt pre-empt some of the critism faced. The general outstanding of synergy is that it is an effect seen by a combination of substances being greater than would have been expected from a consideration of individual compounds. This can be applied to either an increased therapeutics, a reduced side effect or, both (Williamson, 2001).

Controlled clinical studies and traditional experience have confirmed that a number of drugs used in phytomedicine show a good efficacy, clearly indicating that complex mixture work in humans. The utilisation of complex mixtures with pleiotropic agents has successfully been selected by nature and still presents a therapeutic approach, which shows many advantages over mono-target compounds. However, a lot of research is needed to firmly establish the mode of action of such drugs, especially to discovered potential synergists (such as uptake enhancers or MDR inhibitors) that may have a much more general application (Wink, 2008a).

1.5.3 Evaluation of synergy

For antimicrobial drugs, there are many models for experimental designs to measure such combination effects of paired and triple combinations of inhibitory agents with tests *in vitro* that show positive interactions inhibiting the growth of target microorganisms. One of the best known and very simple forms of such tests is the checkerboard experiment in which a two dimensional array of serial concentrations of test compounds is used as the basis for calculation of a fractional inhibitory concentration index (FICI) to demonstrate that paired combinations of agents. For an antibiotic combination to be synergistic by this method, there must be an at least fourfold reduction in the MIC of each antibiotic when the two agents are combined compared with the MIC of each antibiotic tested by itself (Odds, 2003).

The isobole method can be applied under most condition and is independent of the mechanism of action involved (Berenbaum, 1989). The isobole is a curve, constructing by plotting coordinates consisting of doses of the individual agents. If the agents do not interact, the coordinate points form a straight line. If synergism exists, the curve is found to be concave, whereas if antagonism occurs, the convex curve will be obtained (Houghton, 2009).

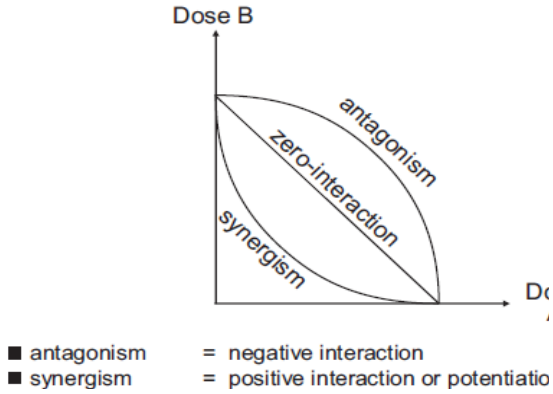


Figure 1.7 Isobole for synergism, a zero interaction and antagonism (Wagner and Ulrich-Merzenich, 2009).

The other method uses time-kill curves to compare differences in colony counts of an organism over a predetermined time interval. Synergy was defined as a 100-fold or 2-log10 decrease in colony count at 24 h by the combination compared with that by the most active single agent (Pillai et al., 2005).

1.7 Objective of the present study

The research was aimed

1. To evaluate the antimicrobial activity of TCM plants used as infection remedies and to find out the promising activity of TCM plants especially to combat multidrug-resistant bacteria;
2. To investigate the chemical composition and the antimicrobial activity of essential oil of some TCM plants;
3. To isolate bioactive compounds using bioassay-guided fractionation; and
4. To study additive or synergistic interactions of the isolated active compounds applied in combination.

Chapter 2

Materials and Methods

2.1. Plants and crude extracts of TCM

2.1.1 Plant materials

2.1.1.1 *Eucalyptus globulus* (Myrtaceae)

The fruits of *E. globulus* were kindly provided by Prof. Thomas Efferth. The identity of the plant has been authenticated by Dr. Wahyono from Department of Pharmacognosy, Gadjah Mada University, Indonesia and the voucher specimen (P6868) was deposited at the Department of Biology, Institute of Pharmacy and Molecular Biotechnology, Heidelberg University, Germany.

2.1.1.2 *Kadsura longipedunculata* (Schisandraceae)

The stem barks of *K. longipedunculata* were commercially obtained from China. The plant material was purchased from a TCM plants shop in January 2007 by Wan Chuangxing. The identity of the plant was confirmed by DNA barcoding. DNA was isolated from the dried crude drug as well as reference plants in the Botanical Garden of Heidelberg. The chloroplast *rbc*L gene was amplified and sequenced. The drug and the authentical reference had identical sequences. Voucher specimens (P6879) of the plant material are deposited at the Department of Biology, Institute of Pharmacy and Molecular Biotechnology, Heidelberg University.

2.1.1.3 *Siegesbeckia pubescens* (Asteraceae)

The aerial parts of *S. pubescens* was purchased from Kräuter Schulte, Germany. The plant was confirmed at the Botanical Garden, Heidelberg University, Germany and a voucher specimen has been stored with code P6906.

2.1.1.4 Other TCM plants

All other TCM plants have each accession number and are deposited as voucher specimen (shown on appendix) at Institute of Pharmacy and Molecular Biotechnology (IPMB), Heidelberg University, Germany.

2.1.2 Preparation of Crude MeOH and CH_2Cl_2 extracts of TCM

Crude MeOH extracts were prepared from 5 g of dried powder of plants by maceration with 100 ml MeOH. The macerates were filtered through Whatman No. 1 filter paper. The filtrates were concentrated under vacuum to a dark gum that constituted the crude extract. The same procedure was followed for preparing CH_2Cl_2 extracts with 20 g of plants in 300 ml CH_2Cl_2 solvent. Crude MeOH and CH_2Cl_2 extracts were stored at 4 ^{o}C for further antimicrobial activity testing.

2.2 Essential oils and monosubstances

2.2.1 Isolation of essential oils

Essential oils of fruits from *E. globulus* and of stem barks from *K. longipedunculata* were obtained by hydrodistillation for 6 h using a Clevenger-type apparatus. After extraction, the essential oils were kept in separated sealed vials at 4 ^{o}C for further analysis. Essential oils of the leaves from *E. globulus* (Bergland-Pharma, Heimertingen, Germany)*, E. radiata* (Primavera Life, Sulzberg, Germany) and *E. citriodora* (Primavera Life, Sulzberg, Germany) were obtained commercially.

2.2.2 Monosubstances

The monoterpenes (–)-camphene (75% purity), (–)-globulol (≥98.5% purity) and 1,8-cineole (99% purity) were purchased from Sigma Aldrich*,* St. Louis, USA. (–)-Borneol (≥95% purity), (±)-citronellal (≥80% purity), (±)-citronellol (90-95% purity) and (+)-aromadendrene (≥97% purity) from Fluka Chemika, Buchs, Switzerland.

2.3 Laboratory chemicals and equipments

2.3.1 Chemicals and solvents

The chemical and solvents used in extraction and isolation were CH_2Cl_2 p.a, *n*-pentane (all from Merck, Darmstadt, Germany) and MeOH (JT Baker, Germany), DMSO (Grüssing, Filsum, Germany). Solvents below were purchased from the chemical store, Heidelberg University as technical degree. Used solvents were: *n*-hexane, acetone, BuOH, chloroform, ethanol 95%, MeOH, ethyl-acetate. For NMR measurement, deuterated solvents such as MeOD, $CDCl_3$ and D_6 were used.

Chemicals for chromatography were pre-coated TLC plates (AluO) silica gel 60 F_{254} with layer thickness 0.2 mm (Merck, Darmstadt, Germany), silica gel 60 with 63-200 µm mesh size (Merck, Darmstadt, Germany), and glasswool (Assistent, Germany).

Media and chemicals for trypanocidal assay were fetal bovine serum, MEM and RPMI 1640 media (Invitrogen, Karlsruhe, Germany), alamar blue (Biosource International, Hamburg, Germany), resazurin and diminazene aceturate (Sigma-Aldrich, Germany).

Chemical of anti-inflammatory testing were lipoxidase enzyme, K_3PO_4 buffer (all from Fluka chemika, Buchs, Switzerland), and linoleic sodium (Sigma-Aldrich, Germany).

Some other chemicals were used such as kanamycin, streptomycin, nystatin (all from Applichem, Darmstadt, Germany), penicillin, ampicillin, rifamycin, rutin (all from Roth, Karlsruhe, Germany), vancomycin (Cell Pharm, Hannover, Germany), Bacto-agar (BD Diskardit, Becton Dickinson, Germany), MTT, DPPH (Sigma Aldrich*,* St. Louis, USA), inhibitor NS-398 (Cayman Chemicals, Ann Arbor, USA.

2.3.2 Equipments

The following equipments were used: Varian NMR spectrometer, gas chromatography (Varian 3400), Hewlett-Packard gas chromatograph (GC 5890 II, Hewlett-Packard GmbH, Bad Homburg, Germany) coupled to a quadropole mass spectrophotometer (SSQ 7000, Thermo-Finnigan, Bremen, Germany), FTIR-Spectrophotometer Tensor 37 HTS-XT (Bruker Optics GmbH, Ettlingen, Germany), Mithras LB 940 instrument (Berthold Technologies, Bad Wildbad, Germany), Tecan® Safire II Reader, balance (Sartorius AG, Göttingen, Germany), centrifuge (Eppendorf), freeze dryer (Osterode, Germany), hot plates (Janke & Junkel, Germany), pH-meter (Mettler Toledo, Schwerbach, Switzerland), rotatory evaporator (Buchi, Switzerland), drying ovens (Memert, Schwabach, Germany), UV lamp 254 and 366 nm (Camag, Muttenz, Switzerland), incubator (Heraeus Instruments, Hanau), shaking incubator (New Brunscwick Sceintific Co, Inc, New Jersey, USA), sonicator (Sonorex super RK102H), refrigerator 4 ^{o}C (Liebherr, Biberach, Germany), sterile hood Type A/B3 Herasafe (Heraeus Instruments, Hanau), vortex-Genie (Scientific Industries, USA), freezer -20 ^{o}C (Liebherr, Biberach), freezer -80 C (Sanyo, Japan), densitometer (Densimat, bioMérieux, France).

2.3.3 Miscellaneous

Caspase-Glo™ 3/7 Assay kit (Promega® Mannheim, Germany), PGE2 Monoclonal EIA Kit® (Cayman® Chemicals, Ann Arbor, USA), 96-well plates (Greiner bio-one, Frickenhausen, Germany), disposable cuvettes (Ratiolab, Dreieich-Buchshlag, Germany), 50 ml and 15 ml falcon blue caps (Greiner Bio-One, Frickenhausen, Germany), parafilm (Pachiney Plastic Packaging), pasteur pipettes (WU Mainz, Germany), petri-dishes (Greiner Bio-One, Frickenhausen, Germany), TLC-micropipettes 5 µl (Brand GmBH, Wertheim, Germany), disposable capillaries (Hirschmann laborgeräte, Germany), test tubes (2 ml, 1.5 ml) (Eppendorf), transfer pippets (Eppendorf), pipettes tip 1-100 µl, 101-1000 µl (Starlab GmBH, Ahrenburg, Germany), Whatman™ paper (Whatman paper company, Germany), sterile loops (Greiner bio-one, Frickenhausen, Germany), medical applicator (Heinz Herrenz, Hamburg, Germany), Bacillol desinfectan (Bode chemie, Hamburg, Germany), polyalcohol antiseptic (Antiseptica, Pulheim, Germany), gloves (Freeform SE, Malaysia), disposable bags (Brand GmBH, Wertheim, Germany).

2.4. Chromatography

2.4.1 Thin layer chromatography (TLC)

TLC experiments were performed on TLC aluminum sheet pre-coated silica gel 60 F254 plates using an appropriate solvent system. Chromatograms were observed under the UV light at wavelength of 245 and 365 nm, followed by spraying with 10% sulfuric acid reagent and subsequent heating at 110 ^{o}C for 2 minutes.

2.4.2 Column chromatography

Colum chromatography was used to further fractionate or isolate some compounds of the mixture. Columns were packed with silica gel (63-200 µm mesh size) using a wet method. The samples which previously prepared by dry method were then placed neatly on the top of silica gel on the column. The *n*-hexane fraction was fractionated by column chromatography using a petroleum benzene/DCM gradient, whereas CH_2Cl_2 fraction was eluted with a dichloromethane/MeOH gradient. The fractions were collected, concentrated and then monitored with TLC. The fractions which showed the same profile of chromatograms were combined together. The combined fractions were placed on the air current to facilitate drying and crystallization. Once drying, the crystals were re-washed again to get the pure compound. The obtained crystals were dried in the desiccators and were then weighed.

2.4.3 Gas liquid chromatography/flame ionization detector (GLC/FID)

The quantitative analysis was carried out by high-resolution GLC using a Varian 3400 gas chromatograph equipped with flame ionization detector (FID) and OV-1 column (30 m × 0.25 mm × 0.25 µm) (Ohio Valley, Marietta, USA). The operating conditions were as follows: carrier gas was helium with a flow rate of 2 ml/min, split ratio 1:20. The oven temperature was programmed with an initial temperature of 40 °C, 2 min isothermal, 300 °C, 4 °C/min, then 10 min isothermal. Injector and detector temperatures were set at 250 and 300 °C, respectively. The PeakSimple® 2000 chromatography data system (SRI Instruments, California, USA) was used for recording and integrating the chromatograms. Areas under peaks of chromatogram were used for calculation the abundance of each component of essential oil.

2.4.4 Gas liquid chromatography/mass spectrometry (GLC/MS) analysis

GLC/MS was carried out on a Hewlett-Packard gas chromatograph (GC 5890 II) equipped with a DB-5 column. Samples (2 µl) were injected with a split mode (split ratio, 1:15) with the carrier gas helium at a flow rate of 2 ml/min. The capillary column was coupled to a quadrupole mass spectrometer (SSQ 7000, Thermo-Finnigan, Bremen, Germany). The injector temperature was 250 ^{o}C. All mass spectra were recorded in the following conditions: electron energy, 70 eV; ion source, 175 °C. The oil components were identified by their retention indices relative to C8–C28 *n*-alkanes, computer matching with the Wiley Registry of Mass Spectral Data, 8th edition, NIST Mass Spectral Library (December 2005) and by comparison of their mass spectra with data already available in the literature and in our own date base (Adams, 2004; Ashour et al., 2009).

2.5 Fractionation of *E. globulus* with *n*-pentane and MeOH

Nine gram of milled *E. globulus* fruits were exhaustively extracted with *n*-pentane (10 h) in a 70 ml Soxhlett-extractor (size 22 x 80 mm). This extraction resulted the pentane fraction (which is also good soluble in CH_2Cl_2). The residue was further extracted with MeOH (10 h) to give MeOH fraction. Both the pentane and MeOH extracts were dried using freeze-dryer and were weighed. A 5 µl of the pentane fraction was analyzed with GC-MS.

The MeOH fraction was further partitioned with acetone and water solvents to give acetone and water subfractions, whereas the pentane fraction was partitioned with methanol aquoues to give ES polar and PH fractions. The PH fraction further separated by TLC preparative with solvent system ethyl acetate 100% showed 3 subfractions. All spots were scratched and re-dissolved with solvent to give subfraction A, B and C. Each obtained fraction and subfraction was tested against microorganisms.

2.6 Isolation of compounds of *S. pubescens* using bioassay guided-fractionation

2.6.1 Bioautography

Bioautography was performed in bioassay-guided fractionation of antimicrobial compounds or fractions. Firstly, samples of fractions were spotted on TLC-plate then developed in the solvent system. Mixture of CH_2Cl_2 (9) : MeOH (1) was used as eluent for n-hexane fraction, whilst mixture of CH_2Cl_2 (7) : MeOH (3) for CH_2Cl_2 fraction. The developed TLC-plates were dried to remove all trace of the solvent system on the plate. The plate was then put in sterile petri dish for bioautography.

In this experiment, bioautography was carried out using agar-overlay method against *Bacillus subtilis* as a test bacterium (Rahalison et al., 1991). An inoculum suspension was prepared in sterile saline and adjusted with 0.5 McFarland standard. The suspension was added into molten-Mueller Hinton agar 0.6% to give final concentration 10^6 cfu/ml. The inoculated medium was then distributed over the TLC plate to give thickness 1-2 mm. After solidification of the medium, the TLC plate was incubated overnight at 35 ^oC. Next, the plate was sprayed with 2 mg/ml of a tetrazolium salt (MTT) solution and then further incubated for 30 minutes. The active compounds/fractions appeared as clear zones with the background of purple color (Beque and Kline, 1972; Rahalison et al., 1991). Clear zones indicated no bacterial growth.

2.6.2 Extraction, fraction ation and isolation of bioactive compounds of *S. pubescens*

Dried bark (600 g) of *S. orientalis* were pulverized and extracted with MeOH (3 x 2 l). The MeOH extract was then evaporated to give a dark green gum. Each macerate was filtered and dried using a rotatory evaporator under reduced pressure and freeze-dried to give the MeOH extract.

The MeOH extract was suspended in MeOH containing 10% water and partitioned with *n*-hexane solvent. The *n*-hexane soluble portion was concentrated in vacuo and freeze-dried to yield the n-hexane fraction. The aqueous layer was then successively partitioned with CH_2Cl_2 and BuOH, and these layers were also evaporated in vacuo and freeze-dried to give the CH_2Cl_2 fraction, the BuOH fraction, and aquoeus fraction, respectively. Each fraction was then screened against microorganisms to know the active fractions.

As the hexane and CH_2Cl_2 fractions were found to be active, the hexane fraction (12.4 g) were applied to column chromatography on silica gel 60 (0.063–0200mm) and eluted with petroleum benzene-CH_2Cl_2 gradient. Fractions with components having same profiles of thin layer chromatography separation were combined. The *n*-hexane fraction afforded 5 fractions and all of them were tested against *Bacillus subtilis* by bioautography.

Ten gram of CH_2Cl_2 fraction was chromatographed by silica gel 60 column chromatography using CH_2Cl_2-MeOH gradient as mobile phase. From CH_2Cl_2 fraction, 6 fractions were obtained and then further tested their antimicrobial activity using bioautography.

After knowing the active spot/fractions, the active fractions were then purified. Purification via crystallization was done by dissolving the active fraction with an appropiate solvent and subsequently subjecting it to air drying for crystallization. The crystals were re-washed again to get the pure compound. The obtained crystals were dried in the desiccators and were then weighed. TLC experiments were performed with differently solvent systems to ensure whether the compound was pure or not. About 5 mg of isolated compounds were analyzed by NMR and mass spectrometry. The rest of samples were kept to be tested for antimicrobial activity.

2.7 Structure elucidation

The identification of the chemical structure of compounds involved a combination of different techniques including one and two dimensional (^{1}H and ^{13}C) nuclear magnetic resonance (NMR) and mass spectrometry (MS). If necessary, additional parameters such as IR properties were also determined.

2.7.1 NMR spectroscopy

Isolated compounds were dried in desiccators and then small amounts of isolated pure compounds were dissolved in suitable solvents e.g. deuterated-solvent for NMR analysis. The solutions were transferred into NMR tubes and analyzed by NMR.

All NMR measurements were recorded at the Department of Chemistry, IPMB, on Varian AM-500 spectrometer operating at 300 MHz or 500 MHz by Tobias Timmermann. Structural assignments were based on spectra resulting from one or more of the following NMR experiments: ^{1}H, ^{13}C, , ^{1}H-^{1}H

correlated spectroscopy (COSY), ^{1}H -^{13}C direct correlation or heteronuclear multiple quantum coherence (HMQC), ^{1}H -^{13}C long-range correlation or heteronuclear multiple connectivity (HMBC), distortionless polarization transfer (DEPT) and attached proton transfer (APT). Multiplicity for ^{13}C was deduced from DEPT experiments; s = C, d = CH, t = CH_2, q = CH_3. Spectra of pure compounds were processed using Bruker 1D WIN-NMR or 2D WIN-NMR software. They were calibrated using solvent signals (^{13}C: $CDCl_3$ 77.00 ppm, CD_3OD 49.00 ppm, pyridine-D_6 39.70 ppm). The structures of the compounds were confirmed by comparison with reference data from available literatures.

2.7.2 Mass spectrometry

Mass spectral analyses were carried out by Rudy Heiko (Department of Chemistry, IPMB) with electron impact module (EI/MS).

2.8 Antimicrobial activity testing

2.8.1 Microbial Strains

Gram-positive bacteria: *Bacillus subtilis* ATCC 6051, *Staphylococcus aureus* ATCC 29213, *Staphylococcus epidermidis* ATCC 14990, *Staphylococcus saprophyticus* ATCC 15305, *Streptococcus pyogenes* ATCC 12344, *Streptococcus agalactieae* ATCC 27956, *Streptococcus oralis* ATCC 3507 and *Enterococcus faecalis* ATCC 29212; Gram-negative bacteria: *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Klebsiella pneumonia* ATCC 700603, *Acinetobacter baumannii* ATCC BAA 747; Fungi: *Candida albicans* ATCC 90028, *Candida glabrata* ATCC MYA 2950, *Candida parasilopsis* ATCC 22019.

Multidrug-resistant bacteria (reference strains): vancomycin-resistant enterococci (VRE) *Enterococcus faecalis* ATCC 51299, methicillin-resistant *Staphylococcus aureus* (MRSA) NCTC 10442. Multi-drug resistant bacteria also involved clinical isolates of MRSA such as MR134/93, MR131/98, MR 2387/00, MR1150/93, MR 1000/93, MR635/93, MR1678/96, BL7127/98, USA300, and clinical isolates of VRE such as VRE902247, VRE902251, VRE902316, VRE 902267. All of the microorganism cultures were obtained from the Department of Infectious Disease, Medical Microbiology and Hygiene, Heidelberg University, Germany. The strains were sub-cultured on an appropriate agar plate 24 h prior to any antimicrobial test.

2.8.2 Culture media

Columbia agar supplemented by 5% sheep blood (Col 5% SB) (Becton & Dickinson Diagnostic, Germany) was used for activation bacterial isolates while CHROMagar Candida medium (BD diagnostic, Germany) was used for fungal isolate. Mueller Hinton broth (MH) (Fluka, Buch, Switzerland) and Mueller Hinton agar (MH2) (bioMérieux, France) were used for MIC and MBC

determination (*B. subtilis, S. aureus, S. epidermidis, S. saprophyticus,* MRSA*, E. coli* and *P. aeruginosa*). Brain heart infusion (BHI) (BD) and Col 5% SB were used for MIC and MBC determination of streptococci and enterococci. Antifungal activity test used the Sabouraud dextrose broth (SDB) (Oxoid) and Sabouraud dextrose agar with chloramphenicol (SDA) (BD, Germany) for MIC and MBC determination.

2.8.3 Diffusion test

Qualitative antimicrobial screening was performed using agar-well diffusion as described by (Clinical Laboratory Standards Institute, 2006). Extracts, fractions or isolated compounds were dissolved in 5% DMSO. The suspension was standardised with 0.5 McFarland turbidity standard using densitometry (bioMérieux, France) and then diluted in broth medium to give a cell suspension of about 10^6 CFU/ml at final concentration. A sterile cotton swab was used to distribute the suspension over the MH2 (for bacteria) and SDA (for yeast). After the agar surface dry, the wells with 6 mm diameter were punched out and filled with 20 µl of the test samples. Inhibition zones were measured after 24 h (for bacteria) and 48 h (for fungi) incubation at 36 ^{o}C. Diffusion assay was also performed for DMSO as negative control, ampicillin, vancomycin, and nystatin 10 µg/ml as positive control. The assay was repeated twice and the results were described as diameter of inhibition zone (unit: mm).

2.8.4 MIC and MBC determination

As solvent for all of the test samples, DMSO was firstly tested against bacteria to ensure that DMSO did not contribute to the antimicrobial activities of the samples. The antimicrobial testing has used DMSO with final concentration 5% due to no inhibition of bacterial growths at this concentration.

The MIC of the samples was determined by broth microdilution methods as recommended by CLSI (Clinical and Laborators Standards Institute, 2006). Antimicrobial testing was performed in duplicate and negative control was also included. Column 1 of microtiter plate was filled with 90 µl of broth medium, whereas column 2 – 11 of plate with 50 µl of medium. Next, 10 µl of the samples were added to the column 1 and 50 µl of the solution was removed to column 2. The two fold dilution was followed for the next column through column 11 to obtain a certain range of concentration. Colum 12 was filled with DMSO as control negative (Figure 2.1).

Inoculum suspension was prepared in sterile saline solution (0.85% NaCl) and the turbidity was adjusted with 0.5 McFarland standard (approximately 10^8 cfu/ml). After adjusting, the suspension should be used not later than 15 minutes.

The prepared inoculum was diluted 1:100 in sterile broth medium and 50 µl of suspension was added to microplate 96-wells to give a final concentration of 5 x 10^5 cfu/ml. The plates were incubated at 36 ^oC for 24 h and be observed for the microbial growth. If necessary, the microbial growth can be observed by adding 20 µl of MTT 2 mg/ml. The minimal inhibitory concentration (MIC) was determined as the lowest concentration which showed no bacterial growth (neither turbidity nor precipitation) or no color change from MTT. To determine minimal biocide concentration (MBC), 3 µl of each culture medium was removed from each well which no growth occured and plated on Col 5% SB agar (for bacteria) or SDA (for fungi). After 20 h of incubation at 36 ^oC, MBC was defined as the lowest concentration at which no viable cell growth on the plates (Figure 2.2). The experiment was performed in duplicate and repeated at least two times.

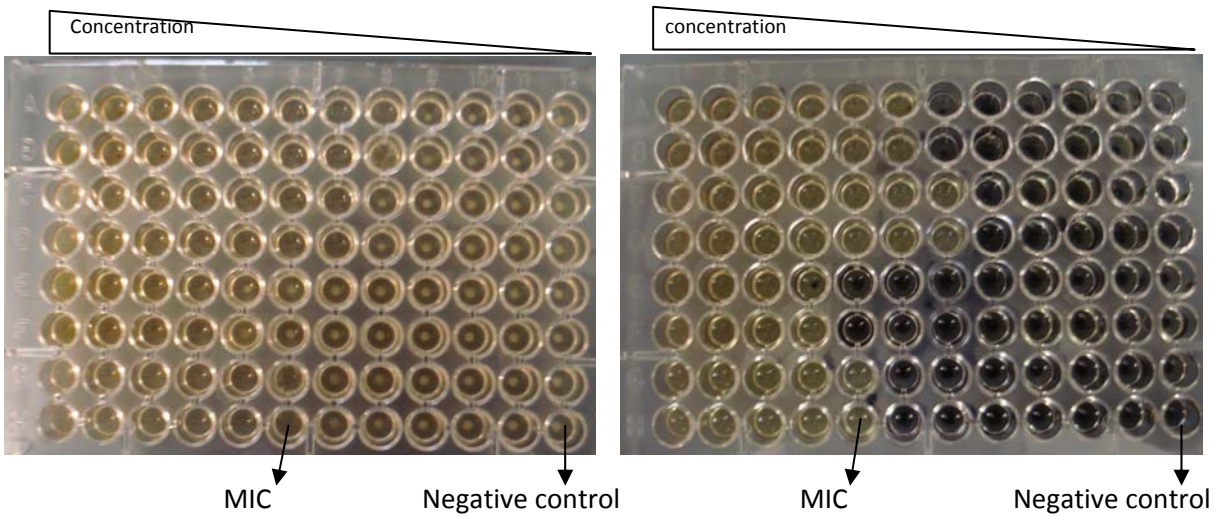


Figure 2.1 Examples of separately antimicrobial activity result determined by broth microdilution method. The experiment was observed by visually (precipitation) (*left*) and by adding MTT (*right*).

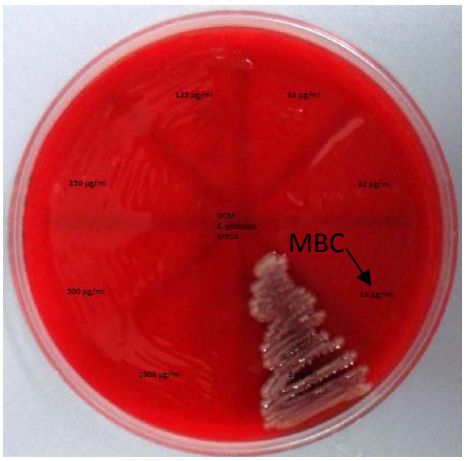


Figure 2.2 Example of minimal bactericidal concentration (MBC) determination.

2.8.5 Checkerboard assay

The checkerboard method was used to determine the effect of combinations of individual compound at different concentrations. In this method, two tested substances were added to a medium in 96-well microtiter plate to give two fold dilutions in the vertical and horizontal direction, respectively (Iten et al., 2009). From checkerboard method, it is possible to create many different combinations. The concentration of each compound were tested ranging from 4×MIC to 1/16×MIC of each single compound. By this ranging, it is possible to observe antagonism as well as synergism. For comparison, both substances have also tested as single compounds at the same plate.

The checkerboard assay was performed using 96-well microtiter plate as previously reported with minor modification (Dougherty et al., 1977). The plate I was prepared by first pipetting 90 µl of medium solution into row B. Row C-H was filled with 50 µl of medium. Next, 10 µl of substance I was added to each well of row B, twofold serial dilutions were then made from row B through row H. The substance I was oriented with the highest concentration in the upper rows.

Column 2 of the plate II was filled with 90 µl of medium solution and column 3-12 with 50 µl of medium. Afterwards, 10 µl of substance II was added to column 2 and two fold serial dilution was further done from column 2 until 12. The plate II was oriented with the highest concentration on the top to the lowest concentration on the bottom.

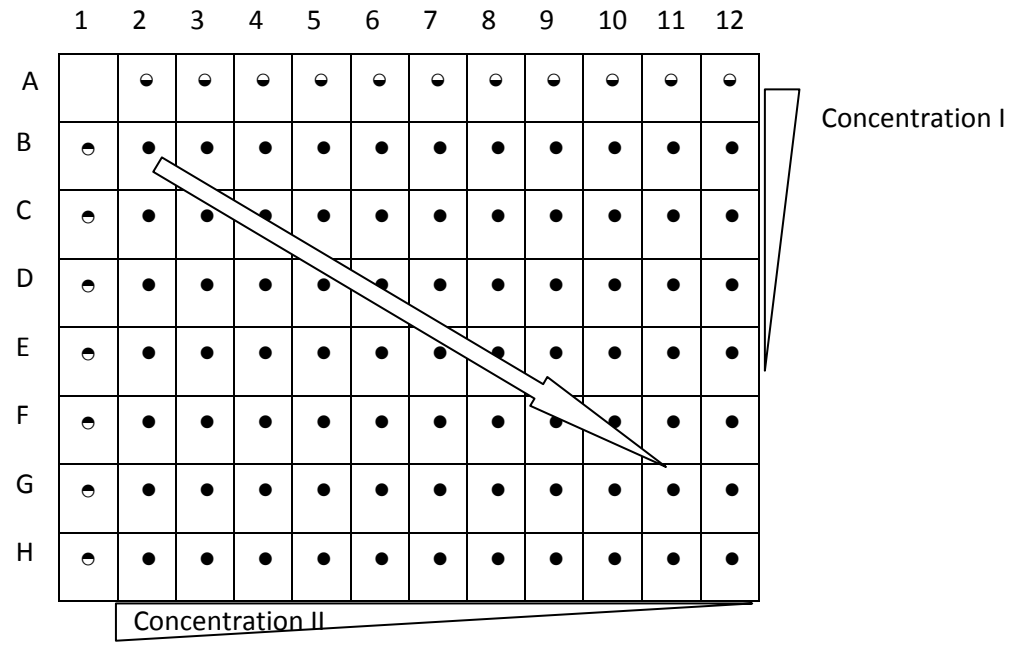


Figure 2.3 Layout of checkerboard assay. ◓: Substance I; ◒: Substance II; ●: Combination of substance I and II; and ⇨: Direction of concentration gradient.

Contents of the plate II were transferred to the plate 2 with the same position so column 1 contained only substance I and row A contained only substance B. This transfer achieved a concentration gradient with the highest in the upper left to the lowest in the lower right. The detailed plate lay out

is presented in Figure 2.3; each well contained a unique combination of substance I and II. Next step, each well in the plate was inoculated with 100 µl of the inoculums suspension (5.10^5 cfu/ml at a final concentration), with exception column 1 and row A with 50 µl volume. Final volume of each well was 200 µl, besides column 1 and row A were 100 µl.

The plate was incubated for 24 h at 36 ^{o}C and observed for no microbial growth. The MIC of substance I and II alone were determined in column 1 and in row A, respectively. A lack of growth in wells below the MIC value of both substances indicated a possible synergistic effect.

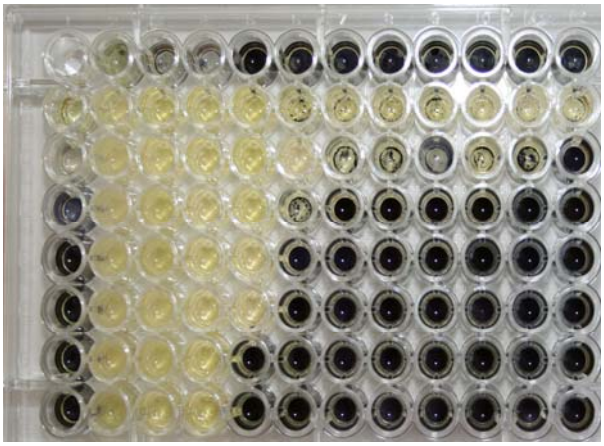


Figure 2.4 Example of a checkerboard result

2.8.6 Fractional inhibitory concentration index (FICI)

To analysis the results of checkerboard assay, the minimum inhibitory concentrations obtained from combinations were converted into the fractional inhibitory concentrations (FIC). This value describes the degree of synergistic effect of combination with a numerical expression (Elion et al., 1954). The more synergistic effect, the lower the FIC index (Singh et al., 2000). The FIC of each substance is calculated from the MIC of combination divided by the MIC of a substance alone. The FIC index (FICI) of the combinations of two substances is the sum of their each individual FIC.

Fractional inhibitory concentrations Index (FICI) were calculated as follows (Elion et al., 1954; Hall et al., 1983):

$$FICI = \frac{\text{MIC of substance A in combination}}{\text{MIC of substance A alone}} + \frac{\text{MIC of substance B in combination}}{\text{MIC of substance B alone}}$$

Based on some literatures (Matsumura et al., 1999; Paluszynski et al., 2008; Pillai et al., 2005), the FICI of combination of two compounds was interpreted as

$FICI \leq 0.5$	synergistic effect
$0.5 < FICI \leq 1$	additive effect
$1 < FICI \leq 4$	indifferent effect, and
$FICI > 4.0$	antagonistic effect.

2.8.7 Isobologram

Isobologram is a curve that ilustrates the result of the checkerboard assay and the FICI values. The axis of the isobologram represents the dose of substance A and the ordinate represents the dose of substance B. The straight line connecting the intercept points representing the individual doses with the same effect as the combination (additivity line). Below this line we find the area of synergistic ($FICI \leq 0.5$) and additive ($0.5 < FICI \leq 1$) interactions. Values above of the straight line represent antagonistic interactions ($FICI > 4$) (Iten et al., 2009). If synergy is generated, the effect of combination is greater than expected from the individual substances. In another word, the dose needed to produce the same effect will be less than the sum of individual substances, hence, the curve formed a concave shape (Williamson, 2001).

2.8.5 Time-kill method

Time-kill method was evaluated to study the kinetic of bacterial growth. Despite that, time-kill assay was used to investigate the synergistic effect of combination two substances. In this investigation, time kill experiments were performed based on the combination obtained from the checkerboard method. Overnight culture was inoculated to the sterile solution, and adjusted with standard turbidity 0.5 Mc Farland and then further diluted in Mueller Hinton broth. The medium contained of tested samples was inoculated with a suspension culture of the test strain to give concentration of bacteria approximate 5 x 10^5 cfu/ml.

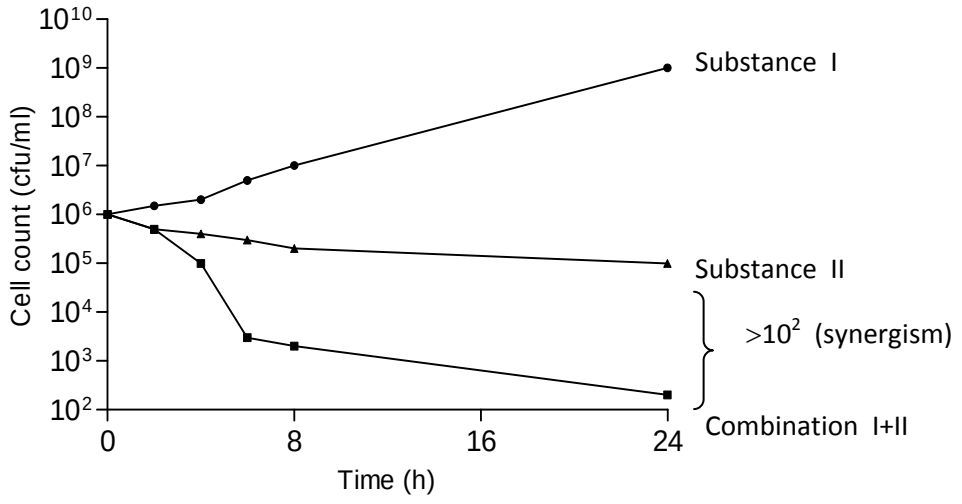


Figure 2.4 Time-kill curve. Synergism was achieved when treatment of the combination caused more than 100 colony counts reduction after 24 h in comparison with the most active single substance.

The combinations and every single compound were tested at sub-inhibitory concentration (not more than one-half of MIC value). At 0, 2, 4, 6, 8 and 24 h of incubation at 36 ^oC, aliquots were withdrawn. At every point time, the aliquots were diluted with sterile saline solution and the dilutions were

spread onto Col 5% SB agar. After overnight incubation at 36 ^{o}C, the colonies were counted. The test was performed in duplicate and the number of colonies expressed as cfu/ml (Suschke et al., 2007).

2.9 Antitrypanosomal activity

Trypanosoma b. brucei TC221 bloodstream forms, the causative agent of Nagana, were grown in Baltz medium supplemented with 20 % inactivated fetal bovine serum and 1 % penicillin-streptomycin. The cells were incubated in a humified atmosphere containing 5 % CO_2 at 37 °C. Trypanocidal activity was determined as described in a previous paper (Nibret et al., 2009). Briefly, the samples were serially diluted with the medium in a two-fold fashion to attain final concentrations ranging from 250 to 3.91 µg/ml in 96-well plates. *T. b. brucei* cells were seeded into 96 wells at a density of 1 x 10^4 cells per 100 µl. The cells were incubated for a total of 48 hours and the antitrypanosomal activity of the extracts was evaluated using resazurin as cell proliferation indicator dye with some modifications from the method that was used by Rolón (Rolón et al., 2006). Furthermore, 10 µl of resazurin was added to trypanosome culture and the culture was incubated with the resazurin for 24 h before measuring the plates after 48 h of incubation. The absorbance of the plates was read using Tecan® Safire II Reader at dual wavelengths of 570 nm and 600 nm. Trypanocidal activity was tested in triplicate and repeated twice.

2.10 Antioxidant Activity

The free radical scavenging capacity of the essential oil and its components were determined in three independent experiments with diphenylpicrylhydrazyl ($DPPH^{\bullet}$) assay. Equal volumes of various sample solutions and 0.2 mM methanolic solution of $DPPH^{\bullet}$ were mixed and the absorbance was measured against a blank at 517 nm using a Tecan® Safire II Reader after incubation in the dark for 30 min at room temperature. Rutin was used as a positive control. The percent inhibition was calculated using the following equation:

Inhibition (%) = 100 x [A517 (control) – A517 (sample)/A517 (control)]

2.11 Antiinflammatory effects

2.11.1 Prostaglandin E_2 inhibition

The ability of the essential oil and its components to inhibit prostaglandin E_2 (PGE_2) production was assessed using PGE2 Monoclonal EIA Kit® (Cayman® Chemicals). MIA PaCa-2 (pancreatic cancer cell) were treated with 50 µl of each sample (25 µg/ml) and incubated at 37 ºC with 5% CO_2. Twenty four hours later, cells were stimulated with 30 µM arachidonic acid. The culture supernatants were collected after 15 min then centrifuged to remove debris. Prostaglandin levels were determined from the supernatant by a competitive enzyme immunoassay. Inhibition of the prostaglandin E_2 level was

calculated relative to the untreated control in three independent experiments; the selective COX-2 inhibitor NS-398 (Cayman) was used as a positive control.

2.11.2 Lipoxygenase Inhibition

The reaction mixtures containing 10 µl enzyme (7.9 U/ml) and 20 µl of the tested samples in 0.1 M phosphate buffer pH 9.0 were incubated at room temperature for 10 min. The reaction was started by addition of the substrate sodium linoleate (Sigma) (62.5 µM) to the reaction mixture. The reaction was monitored with 10 min period, by recording absorbance at 234 nm using a LKB® Biochrom spectrophotometer (Baylac and Racine, 2003). The initial reaction rates were determined from the slope of the straight portion of the curve and inhibition of the enzyme activity was calculated from three independent experiments by comparison with the control (ethanol).

2.12 In vitro Cytotoxicity Assay

HepG2 (human hepatocellular liver carcinoma), MIA PaCa-2 (human pancreatic carcinoma) and HeLa (human cervical carcinoma) cell lines were maintained in DMEM medium, whereas HL60 (Human promyelocytic leukemia), MDA-MB-231 (human breast adenocarcinoma) and SW-480 (human colon adenocarcinoma) cell lines were maintained in RPMI1640 medium. Both media were supplemented with L-glutamine, 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 U /ml streptomycin. Cells were grown at 37 °C in a humidified atmosphere of 5% CO_2.

The cytotoxicity was determined with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, based on cellular conversion of tetrazolium salt into a formazan product (Marks et al., 1992), in three independent experiments. Briefly, 2×10^5 cells of each cell lines were seeded in a 96-well plate. Cells were cultivated for 24 h and then treated with various concentrations of the samples at 37 ^oC for 24 h. Afterwards, 0.5 mg/ml MTT was added to each well and incubated for 4 h. The formed formazan was dissolved in 200 µL DMSO and the absorbance was recorded at 570 nm with a Tecan® Safire II Reader. Cytotoxicity is expressed as the concentration of sample inhibiting cell viability by 50%.

2.13 Caspase-Glo 3/7 assay

The influence of the oil and its components on caspase 3/7 activity Mia PaCa-2 cell line was detected using Caspase-Glo™ 3/7 Assay kit (Promega® Mannheim, Germany). Cells cultured in DMEM were seeded in 96-well plates and treated with the samples (0.125 – 1 mg). After 24 h treatment, 100 µl of caspase 3/7 reagent were added to each well, mixed and incubated for 30 min at room temperature. Luminescence was measured using Mithras LB 940 instrument (Berthold Technologies, Bad Wildbad, Germany). Cellular apoptosis was expressed as percentage of the untreated medium control (Youns et al., 2009) from three independent experiments.

2.14 Statistical analysis

The IC50 value, the drug concentration which resulted in a 50% reduction in cell viability or inhibition of the biological activity, was determined from a four-parameter logistic curve (SigmaPlot 11.0). Statistical analysis of all data was performed using a Student's *t*-test or Kruskal-Wallis test followed by Dunn's post-hoc multiple comparison test (GraphPad Prism® 5.01, GraphPad Software, Inc., CA, USA). A significance level of $P<0.05$ denotes significance in all cases.

Chapter 3

Antimicrobial Evaluation of Some TCM Plants for their Potential against Multidrug-Resistant Bacteria

3.1 Results

3.1.1 The antibiotic susceptibility testing of MRSA and VRE

The susceptibility testing was necessary to corroborate that the test microorganisms were really resistant to multiple-antibiotics. The clinical/patient isolates consisted of 9 MRSA strains, 3 strains VRE *E. faecium* and 1 strain *E. faecalis*. Reference strains of MRSA NCTC 10442 and VRE *E. faecalis* ATCC 51299 were also included for comparison.

Table 3.1
The result of antibiotic susceptibility test of MRSA and VRE (reference strains and clinical isolates)

		Ampicillin		Vancomycin		Kanamycin		Streptomycin		Rifamycin	
Strains	**Sources**	**MIC**	**Int**	**MIC**	**Int**	**MIC**	**Int**	**MIC**	**Int**	**MIC**	**Int**
Methicillin-resistant *Staphylococcus aureus*											
MRSA 10442	National Collection of Type Cultures	6.2	R	0.8	S	3	S	50	R	0.05	S
MR134/93	Northen German epidemic strain	>50	R	0.8	S	>100	R	>100	R	>100	R
MR131/98	Southern German epidemic strain	50	R	0.8/1.6	S	>100	R	>100	R	0.05	S
MR2387/00	Rhein-Hessen epidemic strain	12.5	R	1.6	S	>100	R	3	S	0.05	S
MR1150/93	Berlin epidemic strain	25	R	0.8	S	>100	R	>100	R	0.05	S
MR1000/93	Hannover epidemic strain	>50	R	25	R	>100	R	>100	R	0.05	S
MR635/93	Wien epidemic strain	>50	R	0.8	S	>100	R	>100	R	1.5	S
MR1678/96	Barnim epidemic strain	25	R	0.8	S	3	S	25	R	0.05	S
BL7127/98	Cx-strain, sporadic in Heidelberg	50	R	50	R	>100	R	12	S	0.05	S
USA300	Epidemic of community-acquired	>50	R	0.8/1.6	S	>100	R	12	S	0.05	S
Vancomycin-resistant enterococci											
VRE 51299	American Type Culture collection	0.4	R	50	R	>100	R	>100	R	3	S
VRE902247	VRE *E. faecium* (clinical isolate)	>50	R	50	R	>100	R	>100	R	25	R
VRE902251	VRE *E. faecium* (clinical isolate)	>50	R	>50	R	>100	R	>100	R	25	R
VRE902316	VRE *E. faecium* (clinical isolate)	>50	R	50	R	>100	R	>100	R	25	R
VRE902267	VRE *E. faecalis* (clinical isolate)	1.5	R	>50	R	>100	R	>100	R	1.5	S

Unit of MIC in μg/ml, Int: interpretation; R: resistant; S: susceptible
All MRSA strains were clinical isolates, except MRSA NCTC 10442
All VRE strains were clinical isolates, except VRE ATCC 51299

Antibiotic susceptibility testing showed that all MRSA strains (reference strains and clinical isolates) were resistant to at least two antibiotics as presented Table 3.2. All strains of MRSA were resistant to ampicillin with MIC values ranging from 6.2 to ≥ 50 μg/ml. *Staphylococcus* is categorized as ampicillin-resistant if the MIC value is more than 0.5 μg/ml (Amsterdam, 2005; NCCLS, 2003). In addition, all MRSA strains were resistant to kanamycin with MIC values of ≥ 64 μg/ml, with the exception of MRSA 10442 and MR1678/96 (Amsterdam, 2005). Only 2 MRSA strains (MR1000/93 and

BL7127/98) are categorized as vancomycin-resistant based on MIC breakpoint (≥ 16 μg/ml) as recommended by FDA (FDA, 2008). About 70% of the test MRSA strains showed resistance to streptomycin. On the other hand, rifamycin exhibited a good inhibition against all of MRSA with exception MR134/93 was documented to be resistant.

All VRE strains were confirmed as unsusceptible to vancomycin with MIC values ≥ 50 μg/ml as also shown in Table 3.1. All 5 VRE strains have been documented resistance to ampicillin except VRE *E. faecalis*. Based on NCCLS, enterococci treated by ampicillin with MIC value of ≥ 16 μg/ml are categorized as ampicillin-resistant (NCCLS, 2003). Kanamycin and streptomycin were also not able to inhibit all VRE strains. About 40% strains of VRE were found to be resistant to rifamycin.

3.1.2 Screening of antimicrobial activity of TCM plants

MeOH and CH_2Cl_2 extracts of a total of 84 Chinese medicinal plants were previously screened for antimicrobial agent against some microorganisms representing Gram-positive bacteria, Gram-negative bacteria and yeast. The screening was performed using broth microdilution method and be expressed as minimum inhibitory concentration (MIC) along with minimum biocidal concentration (MBC). Preliminary antimicrobial screening resulted in 20 plants with MIC values of ≤ 500 μg/ml which were considered to be further examined.

The antimicrobial activities of the MeOH and CH_2Cl_2 extracts of the most active 20 TCM plants are listed in Table 3.2. In general, all of the tested extracts were found to be active against one or more test microorganisms (*Staphylococcus aureus, S. epidermidis, S. agalactiae* and *Pseudomonas aeruginosa*). Among 20 plants, CH_2Cl_2 extracts showed higher inhibitory properties than those of MeOH extracts. *Streptococcus agalactiae* was found to be susceptible to all the tested plants with different degrees of susceptibility. Most extracts showed a good activity against *S. epidermidis*. However, *P. aeruginosa* was resistant to most the MeOH and CH_2Cl_2 extracts of TCM plants. The most interesting activity against *P. aeruginosa* was obtained from the CH_2Cl_2 extracts of *Cinnamomum cassia, E. globulus* and *Scutellaria baicalensis* with MIC values ranging from 250–500 μg/ml. The CH_2Cl_2 extract of *Siegesbeckia pubescens* was active against *S. agalactiae* and *S. epidermidis* with MIC value of 16 and 125 μg/ml, respectively.

Kadsura longipedunculata exhibited antibacterial activity against *S. agalactiae* with MIC value of 62 μg/ml. Other TCM plants such as *Erodium stephanianum* and *Coptis chinensis* demonstrated also good activity against Gram-positive bacteria. However, *Chrysanthemum indicum* and *C. morifolium* were the least effective plants among the tested plants.

Table 3.2
Screening of antimicrobial activity of MeOH and CH_2Cl_2 extracts of the most active 20 TCM plants

No	Plants (Family)	Part of plant	Extract (μg/ml)	*S. agalactiae*		*S. epidermidis*		*S. aureus*		*P. aeruginosa*	
				MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
1	*Centella asiatica* (Apiaceae)	Aerial part	MeOH	4000	8000	>8000	>8000	8000	8000	8000	>8000
			CH_2Cl_2	16	31	125	500	250	500	8000	>8000
2	*Panax ginseng* (Araliaceae)	Rhizome	MeOH	62	500	8000	>8000	8000	>8000	8000	>8000
			CH_2Cl_2	62	125	500	2000	500	2000	8000	>8000
3	*Chrysanthemum indicum* (Asteraceae)	Flower	MeOH	1000	2000	8000	>8000	8000	8000	>8000	>8000
			CH_2Cl_2	1000	1000	8000	>8000	8000	>8000	>8000	>8000
4	*Chrysanthemum morifolium* (Asteraceae)	Flower	MeOH	4000	8000	>8000	>8000	8000	>8000	>8000	>8000
			CH_2Cl_2	500	500	4000	8000	4000	8000	>8000	>8000
5	*Siegesbeckia pubescens* Asteraceae	Aerial part	MeOH	62	500	8000	8000	8000	8000	8000	>8000
			CH_2Cl_2	16	16	250	1000	250	1000	8000	8000
6	***Epimedium sp.* (Berberidaceae)**	**Leaf**	**MeOH**	**62**	**125**	**125**	**1000**	**250**	**2000**	**8000**	**8000**
			CH_2Cl_2	**125**	**250**	**250**	**1000**	**250**	**500**	**>8000**	**>8000**
7	*Isatis indigotica (root)* (Brassicaceae)	Root	MeOH	1000	1000	8000	8000	8000	>8000	8000	>8000
			CH_2Cl_2	62	62	1000	4000	2000	4000	>8000	>8000
8	*Isatis indigotica (leaf)* (Brassicaceae)	Leaf	MeOH	2000	4000	16	250	4000	8000	4000	>8000
			CH_2Cl_2	500	1000	16	62	2000	8000	>8000	>8000
9	***Glycyrrhiza glabra* (Fabaceae)**	**Bark**	**MeOH**	**125**	**250**	**125**	**250**	**125**	**500**	**4000**	**8000**
			CH_2Cl_2	**16**	**16**	**60**	**125**	**125**	**250**	**4000**	**>8000**
10	*Erodium stephanianum* (Geraniaceae)	Aerial part	MeOH	1000	1000	60	250	1000	2000	4000	8000
			CH_2Cl_2	16	16	250	500	250	1000	8000	>8000
11	***Hypericum japonicum* (Hypericaceae)**	**Herb**	**MeOH**	**16**	**120**	**125**	**250**	**16**	**62**	**4000**	**8000**
			CH_2Cl_2	**16**	**16**	**16**	**60**	**16**	**16**	**>8000**	**>8000**
12	*Scutellaria baicalensis* (Lamiaceae)	Wood	MeOH	4000	8000	1000	2000	2000	2000	4000	4000
			CH_2Cl_2	62	125	125	500	250	500	500	500
13	*Cinnamomum cassia* (Lauraceae)	Wood	MeOH	4000	4000	8000	8000	4000	8000	8000	8000
			CH_2Cl_2	125	250	250	500	250	1000	500	500
14	***Magnolia officinalis* (Magnoliaceae)**	**Stem bark**	**MeOH**	**62**	**125**	**16**	**62**	**16**	**31**	**8000**	**8000**
			CH_2Cl_2	**16**	**16**	**16**	**16**	**16**	**31**	**>8000**	**>8000**
15	***Eucalyptus globulus* (Myrtaceae)**	**Fruit**	**MeOH**	**62**	**125**	**16**	**16**	**16**	**250**	**500**	**1000**
			CH_2Cl_2	**16**	**16**	**16**	**16**	**16**	**31**	**250**	**500**
16	*Paeonia lactiflora* (Paeoniaceae)	Rhizome	MeOH	2000	2000	62	250	500	500	1000	2000
			CH_2Cl_2	62	62	1000	2000	1000	4000	8000	8000
17	*Rheum palmatum* (Polygonaceae)	Rhizome	MeOH	2000	2000	250	1000	500	2000	4000	8000
			CH_2Cl_2	125	250	16	2000	250	4000	4000	8000
18	*Coptis chinensis* (Ranunculaceae)	Rhizome	MeOH	500	500	500	500	250	500	8000	>8000
			CH_2Cl_2	nt	nt	nt	nt	nt	nt	nt	Nt
19	*Kadsura longipedunculata* (Schisandraceae)	Stem bark	MeOH	62	125	8000	8000	8000	8000	>8000	>8000
			CH_2Cl_2	62	125	>8000	>8000	>8000	>8000	>8000	>8000
20	*Alpinia galangal* (Zingiberaceae)	Fruit	MeOH	1000	2000	>8000	>8000	>8000	>8000	>8000	>8000
			CH_2Cl_2	120	250	1000	4000	1000	4000	>8000	>8000

nt: not tested, sorted by family name
Plants (*E. globulus, Epimedium sp., H. japonicum, G. glabra, and M. officinalis*) which showed a good activity against *S. aureus* were further investigated against MRSA and VRE

Eucalyptus globulus, Epimedium sp., Hypericum japonicum, Glycyrrhiza glabra, and Magnolia officinalis were found to be very active against *S. aureus* with MIC value between 15 and 250 μg/ml. As the investigation was carried out with intention of the most promising antimicrobial activity, thus, these five plants were furthermore investigated for their potential application against multidrug-resistant pathogens.

3.1.3 Anti-MRSA and anti-VRE of 5 TCM plants

Figure 3.1 presents the result of MeOH and CH_2Cl_2 extracts of the 5 most active Chinese medicinal plants tested against MRSA and VRE strains (reference strain and clinical isolates). Regarding the effect of CH_2Cl_2 extracts, all the 5 CH_2Cl_2 extracts have inhibited MRSA strains with MIC values below than 100 μg/ml. MRSA strains were significantly inhibited with MIC value less than 20 μg/ml by CH_2Cl_2 extracts of *E. globulus* (8–16 μg/ml), *M. officinalis* (16 μg/ml), and *H. japonicum* (12 μg/ml) as shown in Figure 3.1. The strong anti-VRE activities were also documented by CH_2Cl_2 extracts of *E. globulus, M. officinalis*, and *H. japonicum* with MIC values of 8–16, 16, and 32 μg/ml, respectively.

In addition, MeOH extracts of *E. globulus* (MIC 16–32 μg/ml) and *M. officinalis* (MIC 24 μg/ml) also exhibited marked inhibitions against MRSA. Against VRE, MeOH extracts of *E. globulus* and *M. officinalis* had MIC values of 12 and 16 μg/ml, respectively.

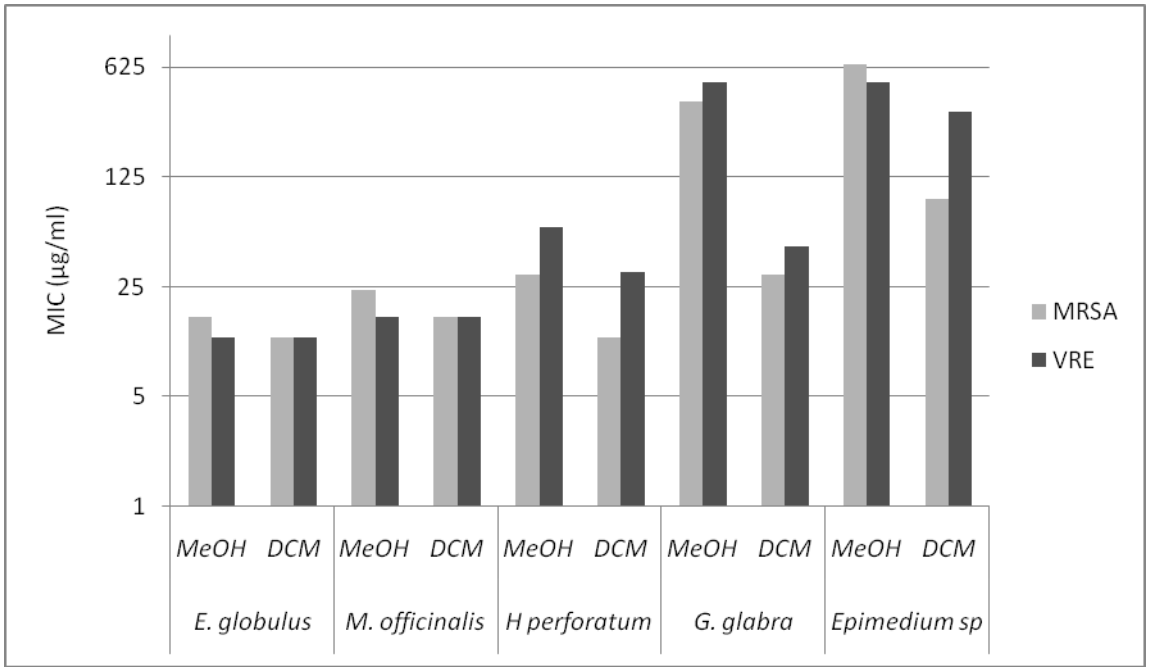


Figure 3.1 Anti-MRSA and anti-VRE of MeOH and CH_2Cl_2 extracts from the most active 5 plants of TCM

In this study, the MeOH and CH_2Cl_2 extracts from *E. globulus* exhibited the best activity against MRSA and VRE compared to the other plants. Regarding the activity of MeOH and CH_2Cl_2 from *E. globulus* as anti-MRSA and anti-VRE, the CH_2Cl_2 extract showed more active than the MeOH extract. The same results were also found in 4 other extracts with varied degrees of activity. In addition, both MeOH and CH_2Cl_2 extracts generally need higher concentration to inhibit VRE compared to those of MRSA.

3.1.4 Fractionation of *E. globulus* and corresponding anti-MRSA activities

Since *E. globulus* exerted the most pronounced antimicrobial activity among the TCM plants tested, it was subjected to fractionation. The obtained fractions were evaluated for their antimicrobial activity followed by GC/MS analysis. The bioassay guided-fractionation (Figure 3.2) resulted the pentane and the methanol fraction. Low activity was exhibited by the MeOH fraction, however the pentane fraction was found to be very active with MIC value of 4 µg/ml. Further fractionation of the pentane fraction produced the fractions ES and PH. Antimicrobial activity revealed that the fraction PH was more active than the ES fraction. Additionally, separation of the PH fraction using preparative TLC generated three subfractions named A (Rf: 0.72), B (Rf: 0.45,) and C (Rf: 0.20). Among the three subfractions, the subfraction A was found to be the most active against MRSA with MIC value of 8 µg/ml as shown in Figure 3.3.

Instead of the fractions mentioned above, the essential oil of *E. globulus* was also investigated for antimicrobial activity. The essential oil of *E. globulus* inhibited MRSA strains at an MIC value of 250 µg/ml as shown in Figure 3.3. In comparison to the activity of the pentane fraction of *E. globulus*, the essential oil showed lower activity than the pentane fraction.

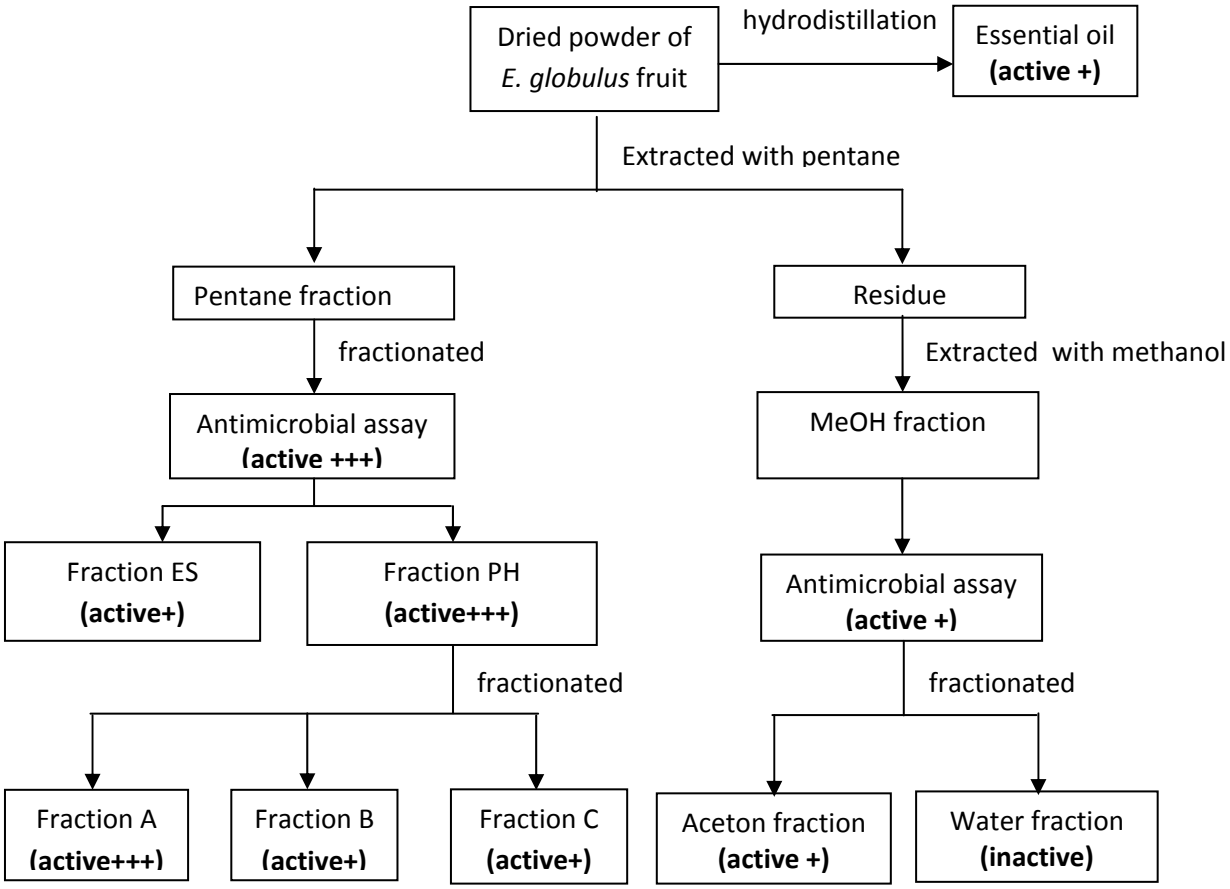


Figure 3.2 Scheme of fractionation of *E. globulus* fruit

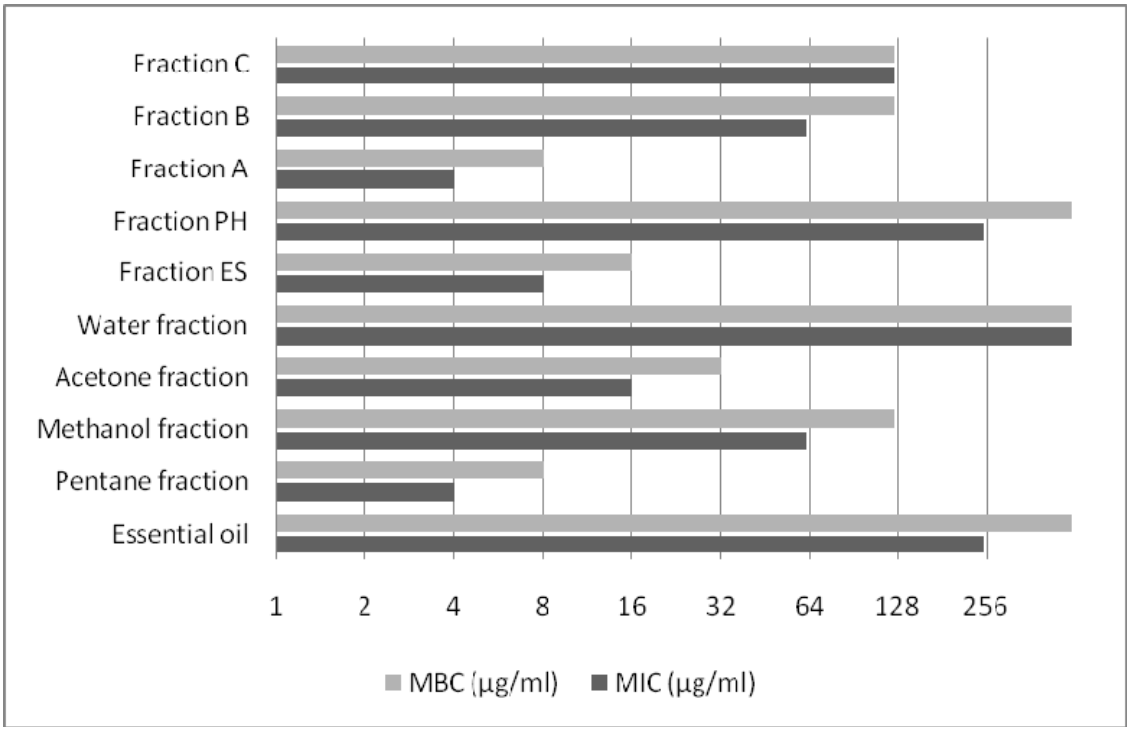


Figure 3.3 MIC and MBC values of fraction of *E. globulus* against MRSA

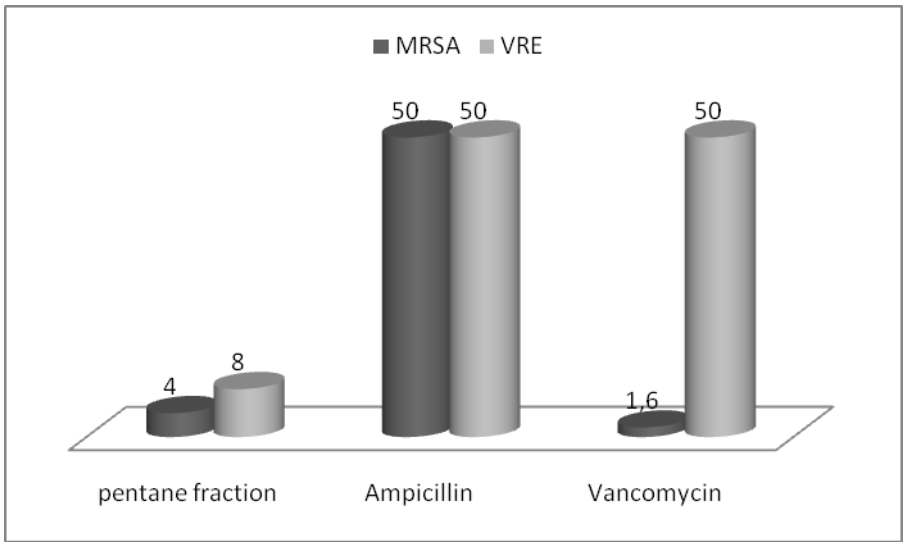


Figure 3.4 Anti-MRSA and anti-VRE activities of the pentane fraction of *Eucalyptus globulus* in comparison to ampicillin and vancomycin.

To know how well the anti-MRSA and anti-VRE activities of the pentane fraction, reference antibiotics such as ampicillin and vancomycin were also tested. All MRSA and VRE strains were inhibited by ampicillin mostly at ≥ 50 µg/ml. Vancomycin has been often found inhibiting MRSA strains at 1.6 µg/ml, whereas VRE strains were inhibited at concentration higher than 50 µg/ml. Against MRSA, activity of pentane fraction was approximately twelve times stronger than ampicillin and one-half time greater than vancomycin. Furthermore, VRE were inhibited by the pentane fraction at concentration about six times lower than those of ampicillin and vancomycin.

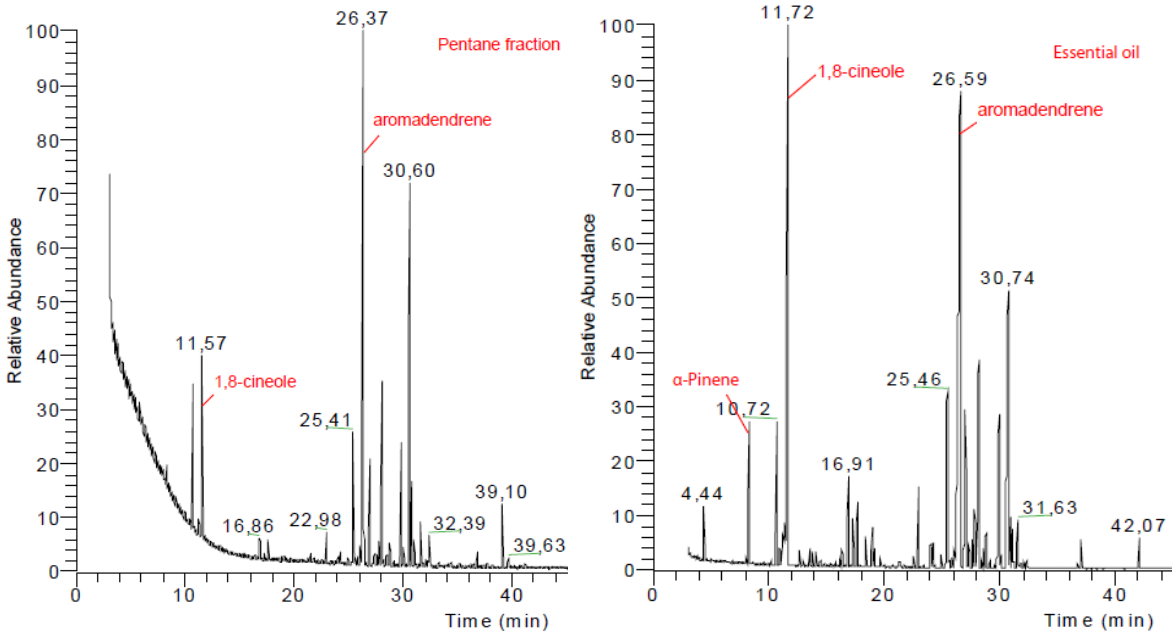


Figure 3.5 The GLC/MS profile of the pentane fraction and the essential oil of *Eucalyptus globulus*.

3.1.5 Chemical investigation of the pentane fraction and the essential oil of *E. globulus* using GLC/MS

The components of the pentane fraction and the essential oil of *E. globulus* is presented in Figure 3.5 and Table 3.2. The GLC/MS analysis of the pentane fraction showed that the fraction contained mainly of aromadendrene (26.32 %), globulol (18.42 %), ledene (9.21 %), 1,8-cineole (8.42 %) and α-phelandrene (7.11 %). On the other hand, the essential oil consisted majority of aromadendrene (31.17 %), 1,8-cineole (14.55 %), globulol (10.69 %), ledene (9.21 %) and α-gurjunene (5.10 %). About 44 components of the essential oil has been identified, whereas by 32 components of the pentane fraction has been investigated. Some components were found in the essential oil but were not in the fraction. 1,8-Cineole of the essential oil was found with higher abundance than those of the pentane fraction. However, the antibacterial activity of the pentane fraction was superior than those of the essential oil.

Table 3.3
Composition of the essential oil and the pentane fraction of fruits of *Eucalyptus globulus* determined by GLC/MS.

Constituents		Relative abundance (%)	
		Essential oil	Pentane fraction
α-Pinene	928	1.53	-
α-Phellandrene	1004	2.61	7.11
p-Cymene	1019	0.49	0.26
1,8-Cineole	1031	14.55	8.42
γ-Terpinene	1057	0.18	Tr
τ-Terpinene	1081	0.18	Tr
Isoterpinolene	1086	0.27	-
Linalool	1095	0.12	-
Carvenone	1151	0.07	-
Borneol	1156	0.41	-
Terpinen-4-ol	1170	1.87	1.05
α-Terpineol	1181	0.85	Tr
Sabinol	1191	1.14	0.53
p-Ment-1(7)-en-2-one	1228	0.62	0.26
Piperiton	1234	0.31	-
Geraniol	1249	Trace	-
Thymol	1301	Trace	-
Exo-2-hydroxycineole acetate	1332	0.14	-
α-Terpinyl acetate	1344	1.27	1.58
Geranyl acetate	1374	0.20	0.26
Isoledene	1380	0.81	0.53
3,3,7,11-tetramethyl-ricyclo (6.3.0.0(2.4))undec-8-ene	1402	0.18	-

α-Gurjunene	1418	5.10	5.79
Aromadendrene	1454	31.17	26.32
allo-Aromadendrene	1469	3.68	5.26
γ-Gurjunen	1480	0.70	Tr
α-Selinene	1489	0.84	Tr
Longifolene	1494	1.75	Tr
Ledene	1505	7.13	9.21
γ-Cadinene	1516	0.24	0.26
Dehydroaromadendrene	1512	0.75	0.53
δ-Cadinene	1526	0.64	Tr
α-Calacorene	1537	0.16	Tr
Epiglobulol	1563	5.17	6.05
Palustrol	1565	0.22	Tr
Viridiflorol	1568	0.24	0.53
Globulol	1589	10.69	18.42
τ-Eudesmol	1593	1.24	Tr
Guaiol	1594	0.79	-
β-Eudesmol	1600	0.31	-
Cubenol	1603	0.11	Tr
Sesquiterpene alcohol	1620	0.55	Tr
τ-Cadinol	1635	0.17	1.58
α-Eudesmol	1648	0.18	1.05

Tr: trace amount, less than 0.05%

3.2 Discussion

Chinese herbal medicines have been employed to cure some illness including infectious diseases such as antibacterial, antifungal, and antiviral agents for treatment of wounds, sores, skin diseases, dermatitis, diarrhea, fever, and cough. Some medicinal plants have been confirmed to have antibacterial properties, for example Zuo (2008) have screened 19 extracts from Chinese herbs for their anti-MRSA activity and found *Dendrobenthamia capitata* as the most active plant with MIC value of 1.25 mg/ml (Zuo et al., 2008). However, some bioactive compounds have not been reported yet and a promising activity of such extracts and compounds against multidrug-resistant microorganisms is still challenging.

In the present study, 84 Chinese medicinal plants have been examined for antibacterial activity with special attention against multidrug-resistant bacteria. Plant extracts as well as isolated plant compounds with MIC in the range 100–1000 µg/ml are categorized as a good activity (Tegos et al., 2002). However, *Eucalyptus globulus, Magnolia officinalis, Hypericum japonicum, Glyzhyrrhiza glabra* and *Epimedium* sp. were found to have a pronounced activity with MIC value below 100 µg/ml.

Magnolia officinalis have exerted a promising activity against all the Gram positive bacteria tested, some of which were MRSA and VRE, with MIC value between 16 and 24 µg/ml. The plant has been traditionally employed for treatment of acute pain, diarrhoea, coughs and urinary problems (Chan et al., 2008). Neolignans piperitylmagnolol, magnolol, and honokiol were reported to be responsible to the antibacterial activity against VRE and MRSA with MIC values in the range of 6.25 – 25 µg/ml (Ho et al., 2001; Syu et al., 2004).

Hypericum japonicum have exhibited MIC values of 12 – 60 µg/ml against MRSA and VRE. Bioactive compounds, phloroglucinol derivatives sarothralen A, B, C, D, and G, were isolated from MeOH and ether extracts of *H. japonicum* using bioactivity- guided fractionation (Ishiguro et al., 1994; Ishiguro et al., 1991; Ishiguro et al., 1986; Ishiguro et al., 1987; Ishiguro et al., 1990) . The antimicrobial activities of these compounds were comparable to or greater than that of streptomycin against the Gram-positive bacteria *S. aureus*, *B. subtilis* and the acid-fast bacterium *Mycobacterium smegmatis* (Singh and Bharate, 2006). *Hypericum* species was also reported have a good antifungal (Fenner et al., 2005).

Glycyrrhiza glabra, well known as licorice, have showed a good inhibition against antibiotic-sensitive and antibiotic-resistant pathogens. The plant contains flavonoids (hispaglabridin A,B, methylglabridin, glabridin, glabrene, glabrol) which might be responsible for antibacterial properties(Mitscher et al., 1978). Another study reported that glycyrrhetinic acid showed antibacterial activities by inhibition of DNA, RNA and protein biosynthesis (Kim et al., 2002).

Additionally, a comparable activity has been shown by *Epimedium* sp. Literature studies reported that *Epimedium* sp contains flavonoids such as hyperoside, epimedin B, C, anhydroicaritin, tricin, icariin and flavonols such as brevicornin, the flavons wushanicariin, baohuoside VI, and ikarisoside (Guo et al., 1996; Li et al., 2005).

In our investigation, *E. globulus* have exhibited the most prominent activity against multidrug-resistant bacteria. Traditionally *E. globulus* has been used to relieve sore throat, bronchitis, pneumonia, and respiratory infection (Beerling et al., 2002). The chemical composition of the pentane fraction and the essential oil of *E. globulus* determined by GLC/MS showed similarity, however, the antimicrobial activity of the pentane fraction was higher than those of the essential oil. It seems that non-volatile compounds might be contributed to the activity but their presence was not detected by GLC/MS. Furthermore, the fractionation of the pentane fraction with methanol aqueous produced the semi polar fraction, in which essential oil is insoluble, exhibited a high antibacterial effect. Hence, the overall activity of the pentane fraction of *E. globulus* was also attributed by non-volatile compounds. Previous papers reported the presence of triterpenoids (betulinic acid, betulonic acid, ursolic acid) and polyphenolic compounds (euschapic acid, ellagic acid, gallic acid) from fruits of

E. globulus (Liu et al., 2007; Yang and Guo, 2007). A number of studies confirmed the antimicrobial activity of those triterpenoids against Gram-positive bacteria (Akiyama et al., 2001; Horiuchi et al., 2007; Zuo et al., 2008). *Eucalyptus globulus* was also reported to have a number of phloroglucinol sesquiterpene- or monoterpene-coupled compounds, namely, the macrocarpals and euglobals (Osawa et al., 1995; Tan et al., 2008). Macrocarpal which has active aldehyde group and cyclopropane rings was reported to have strong antibacterial activity against Gram-positive bacteria (Murata et al., 1990).

In the present study, however, it is difficult to ascribe whether antimicrobial activity of *E. globulus* was caused by specific classes of non-volatile compounds or even their combination between volatile and non-volatile compounds. It is also possible that the mixture of secondary metabolites with several molecular targets might have inhibited pathogenic bacteria in a pleiotropic mode (Wink, 2008a). A similar study reported the synergistic interaction of the non-volatile and volatile fractions on the antimicrobial activity of *Tarchonanthus camphorates* (Van Vuuren and Viljoen, 2009). The apparent mode of action whereby the essential oil and non-volatiles interact to enhance activity is complex and may be attributed to a number of different mechanisms. Essential oils known can disrupt the bacterial cell wall (Burt, 2004) might be enhance entry for selected non-volatile components into the cell and allow them to exert bactericidal activity (Van Vuuren and Viljoen, 2009). However, this postulation needs further investigation.

In conclusion, this study has demonstrated that several extracts of TCM plants exhibited antimicrobial activity against important clinical pathogens including MRSA and VRE. This result has validated the use of some plants as infection remedies. However, in vitro and in vivo toxicity assays are warranted to assess the safety of these extracts. Further investigation is recommended to isolate bioactive compounds and study mode of actions.

Chapter 4

Synergistic Properties of the Terpenoids Aromadendrene and 1,8-Cineole from the Essential Oil of *Eucalyptus globulus* against Antibiotic-Susceptible and Antibiotic-Resistant Pathogens

4.1 Abstract

The aim of the present study was to investigate the chemical composition of the essential oil of the fruits of *Eucalyptus globulus* and to examine the potential application of the fruit oil against multidrug-resistant bacteria. GLC/MS analysis in the fruit oil showed that aromadendrene was the main compound followed by 1,8-cineole and globulol. The three most abundant components of the fruit oil were also tested individually against microorganisms. In addition, the synergistic effects of combinations of the major constituents (aromadendrene and 1,8-cineole) of the fruit oil were also investigated. All Gram-positive bacteria were susceptible to the fruit oil with different degrees of susceptibility as determined by microdilution method. The oil exerted a marked inhibition against multidrug-resistant bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE) *Enterococcus faecalis*. The results indicated that aromadendrene might be responsible for the antimicrobial properties, whereas 1,8-cineole and globulol exhibited low activities. The checkerboard assay demonstrated that combinations of 1,8-cineole and aromadendrene reduce the MIC in most cases in an additive way, whereas the time-kill assay indicates a synergistic effect.

Keywords: *Eucalyptus globulus*; essential oil; aromadendrene; 1,8-cineole; multidrug-resistant bacteria; synergism

4.2 Introduction

Members of the genus *Eucalyptus* (Myrtaceae) originate from Australia but have been naturalized on most continents. *Eucalyptus* has been used in folk medicine throughout the world and the medicinal properties of these plants have been investigated. This family is an important source of essential oils with a wide range of biological activities such as antibacterial, antifungal, analgesic and anti-inflammatory properties (Ramezani et al., 2002; Sartorelli et al., 2007; Silva et al., 2003). Essential oils from *Eucalyptus* species are widely used in modern pharmaceutical, food and cosmetic industries (Lis-Balchin et al., 1998).

The essential oil of leaves of *Eucalyptus globulus* Labill. has been used all over the world as an antiseptic and for relieving symptoms of cough, cold, sore throat and other infections (Kumar et al., 2007; Van Wyk and Wink, 2004). On the other hand, the essential oil of fruits of *E. globulus* has not been much explored yet. The chemical composition of the fruit oil has been studied (Cimanga et al.,

2002; Ghalem and Mohamed, 2008; Pereira et al., 2005), but antimicrobial properties have not been examined. Additionally, the contribution of the major components of the fruit oil to the antimicrobial activity has not been investigated. A previous study reported that the main compound of the fruit oil was 1,8-cineole (Basias and Saxena, 1984), but also a different composition has been reported with aromadendrene as the main constituent (Pereira et al., 2005). In the present study we have reinvestigated the chemical composition of the fruit oil. Furthermore, the three major components of the fruit oil (aromadendrene, 1,8-cineole, and globulol) were examined against both antibiotic-susceptible and antibiotic-resistant microorganisms. Moreover, we have studied whether the two major compounds produce additive or synergistic antibacterial effects, when applied in combination (as is the case in the oils).

4.3 Results

4.3.1 Chemical composition of the essential oils

The essential oil of fruits from *E. globulus* (EGF) yielded 0.71% ($^{w}/_{w}$) with a pale yellow color and a pleasant odor. The chemical composition of EGF was determined by GLC/MS analysis (Table 4.1). The sesquiterpene aromadendrene (31.17%) was the most abundant component followed by 1,8-cineole (14.55%), globulol (10.69%), and ledene (7.13%).

Table 4.1

Composition of the essential oil of the fruits of *Eucalyptus globulus* determined by GLC/MS.

Constituents	RI (DB5)	Relative abundance (%)
		EGF
α-Pinene	925	1.53
α-Phellandrene	1002	2.61
p-Cymene	1024	0.49
1,8-Cineole	1030	14.55
γ-Terpinene	1057	0.18
τ-Terpinene	1087	0.18
Isoterpinolene	1098	0.27
Linalool	1111	0.12
Carvenone	1164	0.07
Borneol	1166	0.41
Terpinen-4-ol	1176	1.87
α-Terpineol	1189	0.85
Sabinol	1199	1.14
p-Ment-1(7)-en-2-one	1231	0.62
Piperiton	1249	0.31
Geraniol	1252	Trace

Thymol	1302	Trace
Exo-2-hydroxycineole acetate	1338	0.14
α-Terpinyl acetate	1348	1.27
Geranyl acetate	1373	0.20
Isoledene	1378	0.81
3,3,7,11-Tetramethyl-tricyclo(6.3.0.0(2.4))undec-8-ene	1396	0.18
α-Gurjunene	1412	5.10
Aromadendrene	1446	31.17
allo-Aromadendrene	1466	3.68
γ-Gurjunen	1476	0.70
α-Selinene	1490	0.84
Longifolene	1493	1.75
Ledene	1504	7.13
γ-Cadinene	1518	0.24
Dehydroaromadendrene	1526	0.75
δ-Cadinene	1543	0.64
α-Calacorene	1555	0.16
Epiglobulol	1566	5.17
Palustrol	1581	0.22
Viridiflorol	1593	0.24
Globulol	**1595**	**10.69**
τ-Eudesmol	1600	1.24
Guaiol	1607	0.79
β-Eudesmol	1611	0.31
Cubenol	1616	0.11
Sesquiterpene alcohol	1627	0.55
τ-Cadinol	1631	0.17
α-Eudesmol	1657	0.18

trace: trace amount, less than 0.05%
EGF: *E. globulus* fruits

4.2.2 Antimicrobial activity of the essential oil, aromadendrene, 1,8-cineole, and globulol

MICs of EGF and the main constituents (aromadendrene, 1,8-cineole, and globulol) tested against 13 microorganisms are shown in Table 4.2. The EGF exerted a good inhibitory activity against all Gram-positive bacteria with MIC values between 0.06 and 1 mg/ml. *Streptococcus pyogenes* was the most sensitive strain of bacteria to EGF (MIC 0.06 mg/ml) and to aromadendrene (MIC 0.12 mg/ml). Of the tested Gram-negative bacteria, EGF did not show a substantial inhibition against *P. aeruginosa, K. pneumonia*, and *E. coli*, except *A. baumannii* (MIC = 1 mg/ml). However, EGF exhibited a moderate activity against yeasts with MIC values of 1–4 mg/ml.

To study the potential application of the oils to treat of multidrug-resistant bacteria, the fruit oil was tested against MRSA and VRE (reference strains and clinical isolates). The results are given in Table

4.3. All antibiotic-resistant bacteria were susceptible to EGF with MIC values between 0.25 and 1 mg/ml. The antimicrobial activity of aromadendrene was slightly lower than EGF except against *S. saprophyticus, S. epidermidis*, MRSA (clinical isolates), VRE *E. faecalis*, and yeasts. All of the tested microorganisms were less affected by 1,8-cineole, except yeasts which were inhibited at 8 mg/ml. It was surprising that globulol which had been reported as the active principle of the ethanol extract of EGF (Tan et al., 2008) showed low antimicrobial activities. In our investigation, globulol inhibited only streptococci, *E. faecalis* and *A. baumannii* with MIC values ranging from 1–4 mg/ml.

Tabel 4.2
The MIC values of essential oil of *Eucalyptus globulus* fruits, aromadendrene, 1,8-cineole, and globulol determined with microdilution method.

Microorganism		*E. globulus* fruits		Aromadendrene		1,8-Cineole		Globulol		Standard Drug (µg/ml)	
		MIC*	MBC	MIC*	MBC	MIC	MBC	MIC	MBC	MIC	MBC
G+	*B. subtilis*	0.25	0.5	1	1	32	NT	> 4	NT	0.2	0.8
G+	*S. saprophyticus*	1	4	1	8	> 8	NT	> 4	NT	1.6	3.1
G+	*S. aureus*	0.25	1	0.5	2	64	NT	> 4	NT	0.4	0.8
G+	*S. epidermidis*	0.5	4	0.5	1	> 8	NT	4	> 4	0.8	1.6
G+	*S. agalactiae*	0.25	0.5	0.5	1	> 8	NT	1	> 4	0.4	0.4
G+	*S. pyogenes*	0.06	0.12	0.12	0.12	16	NT	2	> 4	0.1	0.2
G+	*E. faecalis*	1	2	2	2	> 8	NT	4	> 4	1.6	3.1
G-	*E. coli*	8	NA	> 8	NT	> 8	NT	> 4	NT	NI	NT
G-	*P. aeruginosa*	> 8	NT	> 8	NT	> 8	NT	> 4	NT	NI	NT
G-	*K. pneumoniae*	> 8	NT	> 8	NT	> 8	NT	> 4	NT	NI	NT
G-	*A. baumannii*	1	1	2–4	2	8	8	4	> 4	NI	NT
Yeast	*C. albicans*	4	4	4	4	8	8	> 4	NT	1.6	1.6
Yeast	*C. glabrata*	2	4	2	4	8	8	> 4	NT	1.6	1.6

Concentrations are given in mg/ml
* Significant difference between MIC of the essential oil and of 1,8-cineole; and between aromadendrene and 1,8-cineole ($P < 0.05$)
NT: not tested, NA: not active at the tested concentration
Vancomycin was used as standard drug for bacteria, whereas nystatin for yeasts

Table 4.3
Antibacterial activity of the essential oil of fruits of *Eucalyptus globulus*, aromadendrene, 1,8-cineole, and globulol against MRSA and VRE strains.

	E. globulus fruit		Aromadendrene		1,8-Cineole*		Globulol		Standard drug (µg/ml)	
Microorganism	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
MRSA NCTC 10442	0.25	0.25	0.5	1	64	>64	>4	NT	0.8	1.6
MRSA (clinical isolates)	0.12–1	0.25–2	0.25–1	0.5–4	NA	NT	4 to >4	NT	0.8–50	1.6 to >50
VRE *E. faecalis* ATCC 51299	1	2	1	2	NA	NT	4	NT	50	50
VRE *E. faecalis* (clinical isolates)	1	2	1	2	NA	NT	>4	NT	50 to >50	>50
VRE *E. faecium* (clinical isolates)	0.5–1	0.5–2	1	2–4	NA	NT	>4	NT	>50	>50

Concentrations are given in mg/ml
* Significant difference between MIC of the essential oil and of 1,8-cineole; and between aromadendrene and 1,8-cineole ($P < 0.05$)
NT: not tested, NA: not active at the tested concentration
Vancomycin was used as standard drug

4.2.3 Effect of combinations of aromadendrene and 1,8-cineole

Most antimicrobial activities of aromadendrene and 1,8-cineole as single entities were lower than those of EGF. In order to study whether aromadendrene and 1,8-cineole in combination produce a higher inhibition via an additive or synergistic interaction, checkerboard and time-kill experiments were performed.

Table 4.4

Result of the checkerboard assay: ratio of aromadendrene and 1,8-cineole (Aro/Cin), concentration of aromadendrene (Aro) and 1,8-cineole (Cin) in mg/ml, and FICI values. The interaction as reflected by FICI values is considered to be synergistic at ≤ 0.5, additive at > 0.5–1, indifferent at >1–4.0, and antagonistic at > 4.0.

MRSA				
Aro/Cin	(Aro)	(Cin)	FICI	Interpretation
0.5/0	0.5	0	-	-
1/16	0.25	4	0.6	additive
1/8	0.25	2	0.56	additive
¼	0.25	1	0.52	additive
1/266	0.12	32	0.75	additive
1/133	0.12	16	0.49	synergistic
0/32	0	64	-	-

B. subtilis				
Aro/Cin	(Aro)	(Cin)	FICI	Interpretation
1/0	1	0	-	-
1/4	0.5	2	0.56	additive
1/2	0.5	1	0.53	additive
1/1	0.5	0.5	0.52	additive
1/0.5	0.5	0.25	0.51	additive
1/16	0.25	4	0.38	synergistic
1/8	0.25	2	0.31	synergistic
0/32	0	32	-	-

S. aureus				
Aro/Cin	(Aro)	(Cin)	FICI	Interpretation
1/0	0.5	0	–	–
1/8	0.25	16	0.75	additive
¼	0.25	8	0.63	additive
½	0.12	32	0.75	additive
1/1	0.12	16	0.49	synergistic
1/533	0.06	32	0.56	additive
0/64	0	64	–	–

S. pyogenes				
Aro/Cin	(Aro)	(Cin)	FICI	Interpretation
0.12/0	0.12	0	–	–
1/32	0.06	2	0.63	additive
1/16	0.06	1	0.56	additive
1/8	0.06	0.5	0.53	additive
1/133	0.03	4	0.50	synergistic
0/16	0	16	–	–

Checkerboard assays and isobolograms of all four tested bacteria gave additive or synergistic profiles when aromadendrene was combined with 1,8-cineole at sub-inhibitory concentrations (Table 4.3 and Figure 4.1). Synergistic effects were observed with at least one dose pair of combination against MRSA, *B. subtilis, S. aureus*, and *S. pyogenes*. Synergy was noted at 0.12 mg/ml aromadendrene plus 16 mg/ml 1,8-cineole for MRSA, 0.12 mg/ml aromadendrene plus 16 mg/ml 1,8-cineole for *S. aureus*, and 0.03 mg/ml aromadendrene plus 4 mg/ml 1,8-cineole for *S. pyogenes*. A predominant synergy was also observed against *B. subtilis* when 0.25 mg/ml aromadendrene was combined with either 4 mg/ml or 2 mg/ml 1,8-cineole. All other dose pair combinations resulted in additive effects. Neither indifferent nor antagonistic effects were found in the combinations.

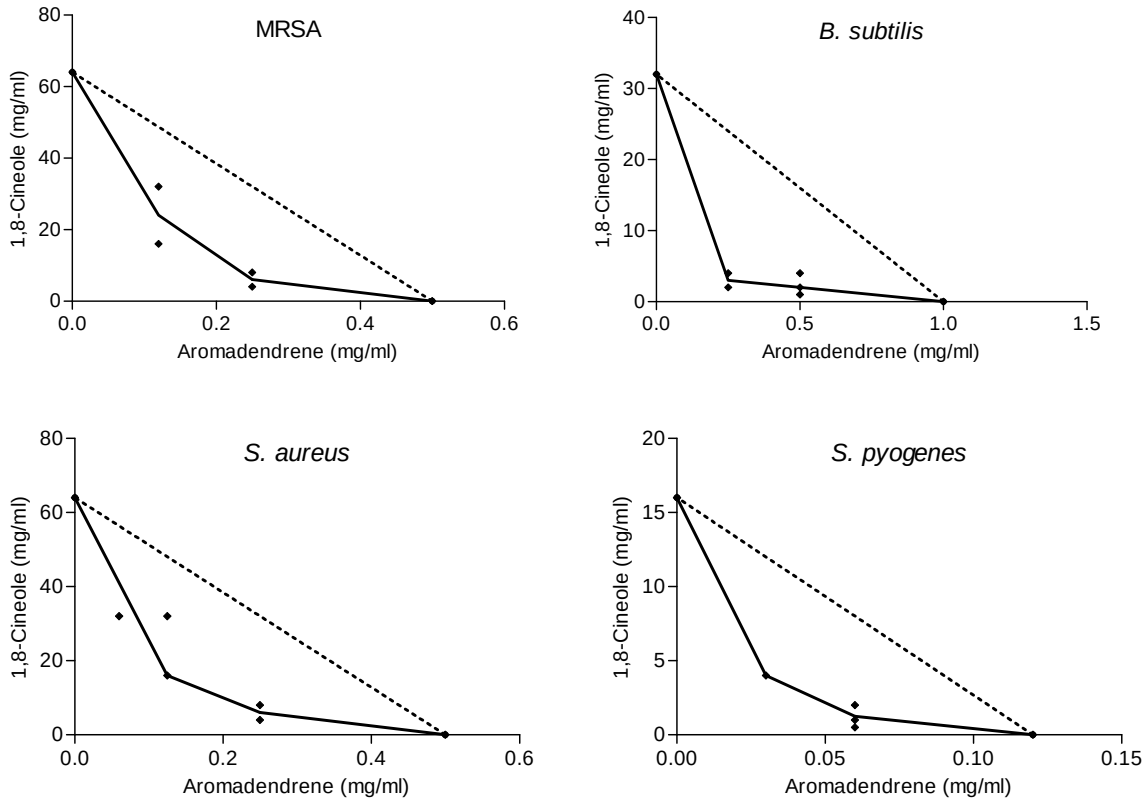


Figure 4.1 Isobologram depicting the effect of aromadendrene and 1,8-cineole against MRSA, *Staphylococcus aureus, Bacillus subtilis*, and *Streptococcus pyogenes*.

Time kill experiments were performed with combinations of aromadendrene and 1,8-cineole against *S. pyogenes* and MRSA (Figs. 2 and 3), of which *S. pyogenes* exhibits the highest susceptibility. Figure 4.2 illustrates the synergistic kinetics of combinations of aromadendrene and 1,8-cineole in *S. pyogenes* in the time kill assay. Combinations of 0.03 mg/ml aromadendrene plus 4 mg/ml 1,8-cineole, and of 0.06 mg/ml aromadendrene plus 4 mg/ml 1,8-cineole revealed a 2 $\log_{10}$ decrease of colony counts after 24 h compared to the single substance (aromadendrene). Particularly, the combination of 0.06 mg/ml aromadendrene and 8 mg/ml 1,8-cineole reduced the viable cell number of *S. pyogenes* approximately 5 $\log_{10}$ after 24 h.

Similarly, synergism was observed in MRSA when 0.25 mg/ml aromadendrene was combined with 32 mg/ml 1,8-cineole as presented in Fig. 3. The combinations significantly reduced the number of MRSA colonies (4$\log_{10}$) compared to aromadendrene.

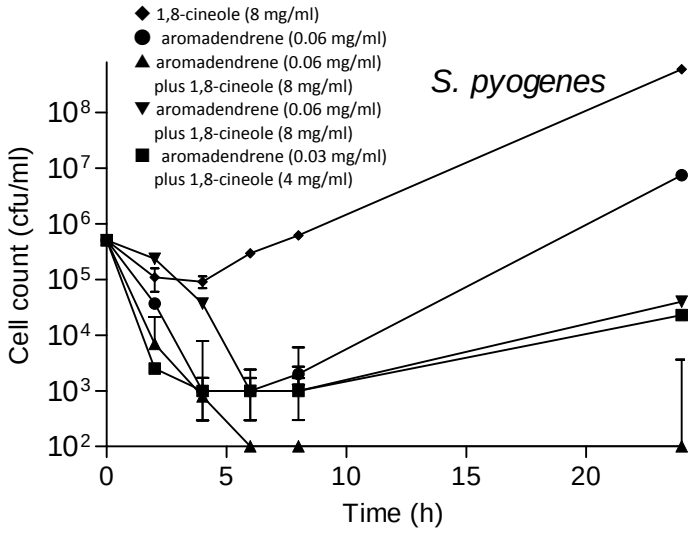


Figure 4.2 Time-kill curve of aromadendrene and 1,8-cineole alone and in combination against *Streptococcus pyogenes*.

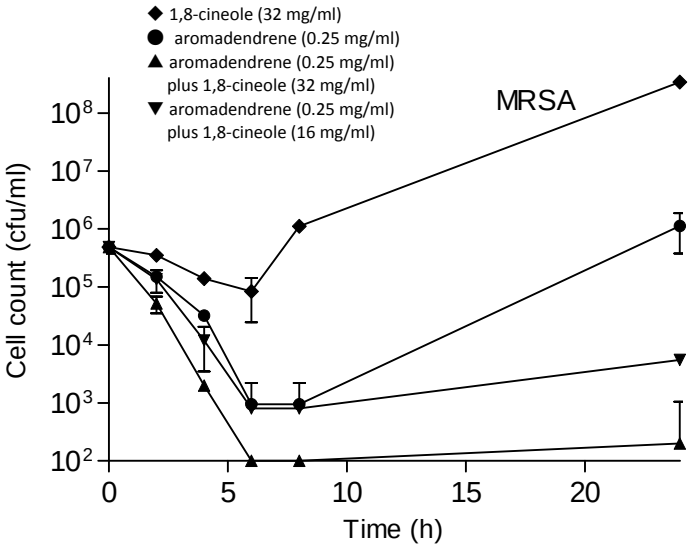


Figure 4.3 Time-kill curve of aromadendrene and 1,8-cineole alone and in combination against MRSA.

4.4 Discussion

Resistance toward antibiotics is increasingly observed in some pathogenic microorganisms including Gram-positive (MRSA and VRE) and Gram-negative bacteria like *P. aeruginosa* and have become a tremendous problem on a global scale. These bacteria are the major causes of nosocomial infections (Coates et al., 2002; Taubes, 2008). To overcome resistance, many antimicrobial agents have been investigated and essential oils were also included as alternative agents.

Eucalyptus oil has been integrated in medical systems all around the world since they possess potent antibacterial properties. Eucalyptus oils commonly found in health care systems are derived from leaves. To our knowledge, this investigation for the first time reports the antimicrobial effect of the

fruit oil of *E. globulus,* particularly against multidrug-resistant bacteria. The results revealed that the essential oil of EGF (MIC values of 0.25 – 1 mg/ml) may serve as a potential drug in this context (Aqil et al., 2006). Furthermore, low MIC against MRSA and VRE might be advantageous in certain therapeutic applications such as inhalations or topical applications with regard to toxicity and stability of formulations (Guba, 2001).

Our GLC/MS study revealed aromadendrene to be the most abundant compound of EGF. A similar composition has been also reported from plants grown in Portugal (Pereira et al., 2005). It is most likely that aromadendrene was the main active principle of the EGF because aromadendrene showed higher antimicrobial properties than 1,8-cineole and globulol. The sesquiterpene aromadendrene bears a reactive exocyclic methylene group and a cyclopropane ring which can alkylate proteins and thereby disturb the conformation of protein. Additionally, since the compound is highly lipophilic, it causes disruption of cellular biomembranes (Sikkema et al., 1994; Wink, 2007; Wink, 2008a; Wink, 2008b). On the other hand, 1,8-cineole (the second major constituent of EGF) exhibited low antimicrobial activities, in agreement with previous reports (Raman et al., 1995; Tzakou et al., 2001).

A previous study corroborated that minor components play a role in antibacterial activity, possibly by producing synergistic effects with other components (Burt, 2004). Some studies had also shown synergistic effects of combinations such as limonene/1,8-cineole (van Vuuren and Viljoen, 2007), cinnamaldehyde/eugenol, thymol/eugenol, carvacrol/eugenol, and thymol/carvacrol (Iten et al., 2009; Pei et al., 2009). In the present study, we could demonstrate that combinations of aromadendrene and 1,8-cineole apparently exhibited synergistic and additive antimicrobial properties. A clear synergistic effect of the aromadendrene and 1,8-cineole pair has been demonstrated in time-kill experiments. Synergistic effects of the combinations appeared only if 1,8-cineole (the weaker substance) was present in high doses. Most dose pair combinations produced additive effects in the checkerboard method. The differences occurred because the time-kill assay records a bactericidal effect, while the checkerboard method reveals inhibition of bacterial growth (Matsumura et al., 1999).

In conclusion, we have identified that aromadendrene apparently contributes significantly to the antimicrobial activity of EGF. Combinations of aromadendrene and 1,8-cineole showed additive effects in most cases, but also synergistic behavior in the time-kill assay. However, the possibility remains that other components of EGF contribute to the observed antimicrobial activity. Importantly, EGF exhibited a pronounced antimicrobial effect towards multidrug-resistant bacteria. Thus, EGF and its components, alone or in combination with other antibacterial agents, may provide a promising new scheme in phytotherapy.

Chapter 5

Inhibitory effect of Four Essential Oils of *Eucalyptus* against Multidrug-Resistant Bacteria and their Relationship with the Chemical Composition

5.1 Abstract

The essential oil of fruits from *E. globulus* (EGF) and of leaves from *E. globulus* (EGL), *E. radiata* (ERL) and *E. citriodora* (ECL) was tested against multidrug-resistant (MDR) bacteria using microdilution method. The result of antimicrobial activity showed that EGF exerted the most pronounced activity among the oils tested against methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant enterococci (reference strains and clinical isolates). The activity of the oils can be ranked as EGF > ECL > ERL ~ EGL. However all the oils and the components were hardly active against MDR Gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia* and *Acinetobacter baumannii*. The chemical composition analyzed by GLC/MS revealed that EGF contained mainly of aromadendrene and 1,8-cineole, whereas ECL consisted of citronellal and citronellol as the major compounds. Furthermore, 1,8-cineole was most abundant compound in EGL and ERL oils. In addition, the major components of the oils (aromadendrene, 1,8-cineole, citronellal, and citronellol) were also examined against all bacteria tested. Among all the tested components of the oils, aromadendrene was found to be the most active compound, followed by citronellol, citronellal and 1,8-cineole. The investigation corroborated the relationship between the chemical composition of the oils and the antimicrobial activity.

Keywords: *Eucalyptus*; essential oil; aromadendrene; 1,8-cineole; multidrug-resistant bacteria; antimicrobial activity.

5.2 Introduction

Increasing antimicrobial resistance presents a major threat to public health since it reduces the effectiveness of antimicrobial treatment as well as increases morbidity and mortality (Smith and Coast, 2002). Methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE) are the most important resistant pathogens among Gram-positive bacteria concerning with nosocomial infections. The emergence of resistance in Gram-negative bacteria (*Klebsiella pneumoniae*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Acinetobacter*) are also complicating the treatment of nosocomial infections (Paterson, 2006). Many drugs that have frequently been used in the past as empiric therapy have become less effective against these pathogens (Jones, 2001). Hence, there is an urgent needed to find an alternative antimicrobial agent for treatment of resistant pathogenic microorganisms (Aksoy and Unal, 2008).

Many essential oils – including those from *Eucalyptus* – have been used in folk medicine throughout the world and the medicinal properties of these plants have been investigated (Deans, 2002). Essential oils from *Eucalyptus* were reported possess antibacterial, antifungal, analgesic and anti-inflammatory properties (Ramezani et al., 2002; Sartorelli et al., 2007; Silva et al., 2003); and have also been widely used in pharmaceutical, food and cosmetics products.

Eucalyptus globulus Labill (Myrtaceae) is the principal source of *Eucalyptus* oil in the world and has been used as an antiseptic and for relieving symptoms of cough, cold, sore throat and other infections (Fleming, 2000; Kumar et al., 2007; Van Wyk and Wink, 2004). The oil, well known as 'eucalyptus oil' commercially, has been produced from the leaves and supposes to have a powerful antibacterial activity (Lis-Balchin et al., 1998). Instead of 'eucalyptus oil', *E. radiata* and *E. citriodora* are also widely used for aromatherapy. *Eucalyptus radiata* oil has also been shown to be useful for treating disorders of the respiratory system (Davis, 1990). *Eucalyptus citriodora* is one of the well known *Eucalyptus* and the antibacterial, antifungal, analgesic and anti-inflammatory properties were also reported (Lis-Balchin et al., 1998; Silva et al., 2003). However, none of these oils have been published about their activity against multidrug-resistant bacteria. On the other hand, the essential oil of fruits of *E. globulus* has not been much explored yet, although the chemical composition of the fruit oil has been studied (Cimanga et al., 2002; Ghalem and Benali, 2008; Pereira et al., 2005).

In this investigation, we have examined antimicrobial activity of the fruit oil of *E. globulus* and the leaves oils of *E. globulus*, *E. radiata* and *E. citriodora* to study their potential application against multidrug-resistant bacteria. The major components of the oils (aromadendrene, 1,8-cineole, citronellal, and citronellol) were also evaluated against all bacteria tested. Furthermore, the chemical composition of the oils was reinvestigated to study a relationship between the chemical compositions and their antimicrobial properties.

5.3 Results and discussion

5.3.1 Chemical composition of the essential oils

The chemical composition of fruits of *E. globulus* (EGF) determined by GLC/MS is presented in Table 5.1 and Figure 5.1, in comparison to the leaves oils from *E. globulus* (EGL), *E. radiata* (ERL), and *E. citriodora* (ECL). About 65 compounds were identified, representing 97.02 – 99.89 % of the total oils. The chemical investigation revealed some similarity in components of the essential oils, however they showed some different abundances. Monoterpenoids were predominantly found in the 3 leaves oils (EGL, ERL, and ECL), whereas sequiterpenoids were more abundant in the fruit oil (EGF).

The essential oil of EGF contained mainly of aromadendrene (31.17%) followed by 1,8-cineole (14.55%), globulol (10.69%), and ledene (7.13%). This result was in agreement with a previous study on the essential oil of EGF grown in Portugal (Pereira et al., 2005), however, another prior work

found 1,8-cineole as the main compound (Basias and Saxena, 1984). There are often some differences found in composition of oils from the same plant species due probably to the difference of genetic variation and environmental factors (climate, harvesting seasons, geographical location) (Magina et al., 2009; Nishimura and Calvin, 1979; Pereira et al., 2005).

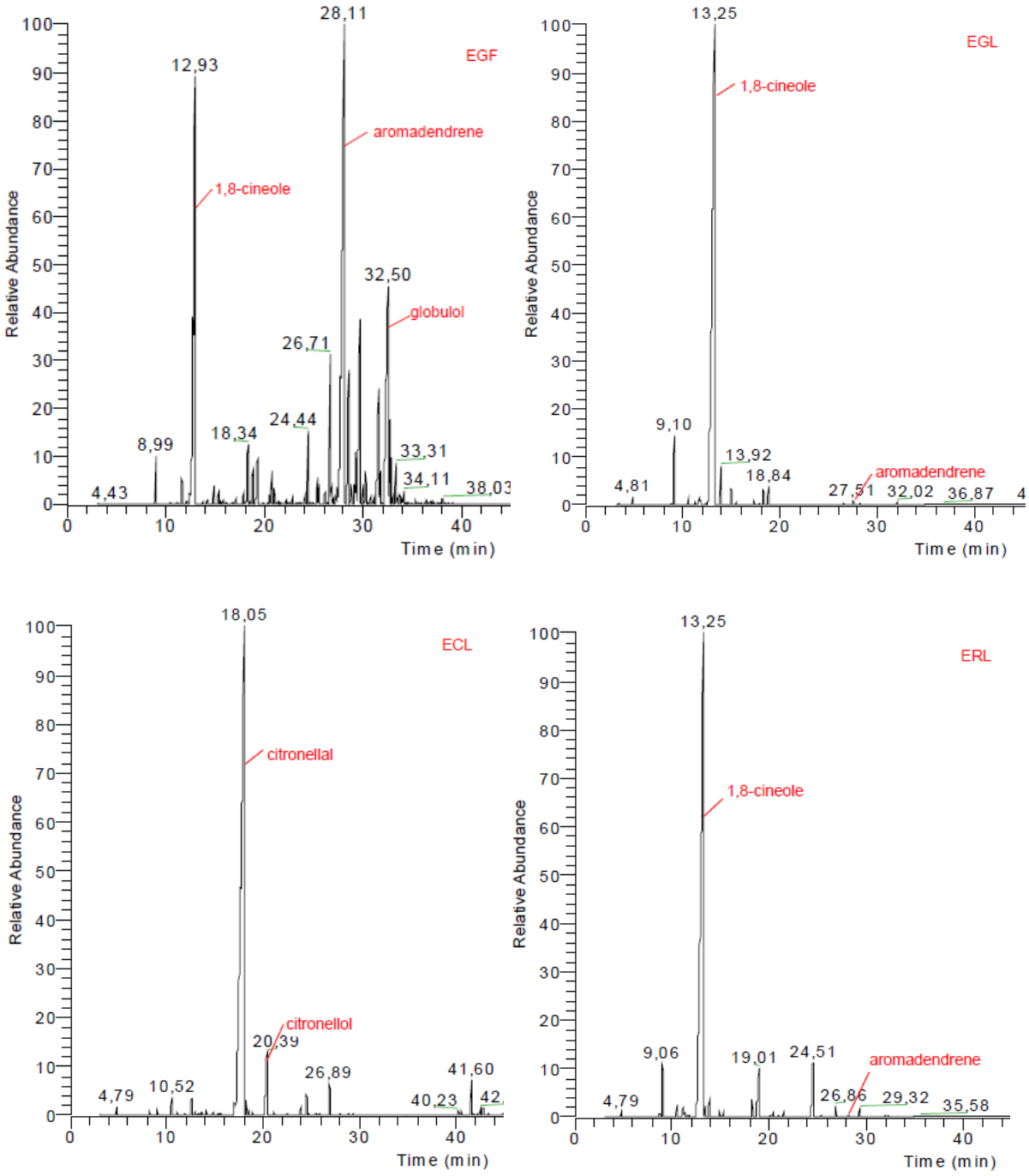


Figure 5.1 The chromatograms of the *Eucalyptus* essential oils determined by GLC-MS on column DB-5. *Eucalyptus globulus* fruits (EGF); *E. globulus* leaves (EGL); *E. citriodora* leaves (ECL); and *E. radiata* leaves (ERL).

Table 5.1
Composition and relative abundance of the essential oil of fruits of *Eucalyptus globulus* and the essential oils from *Eucalyptus* leaves.

No	Constituents	RI (DB-5)	Relative abundance (%)			
			EGF	EGL	ECL	ERL
1	α-Thujene	903	-	-	0.46	-
2	α-Pinene	925	1.53	4.74	0.38	3.68
3	Sabinene	968	-	-	0.58	1.40
4	β-Pinene	970	-	0.88	0.91	-
5	β-Myrcene	989	-	0.88	0.15	-
6	α-Phellandrene	1002	2.61	1.40	-	-
7	*p*-Cymene	1024	0.49	-	-	-
8	Limonene	1027	-	-	0.5	-
9	**1,8-Cineole**	1030	14.55	86.51	-	82.66
10	γ-Terpinene	1057	0.18	2.57	0.25	Tr
11	*p*-Menth-3,8-diene	1069	-	-	0.13	-
12	Terpinolene	1085	-	0.77	0.1	Tr
13	τ-Terpinene	1087	0.18	-	-	-
14	Isoterpinolene	1098	0.27	-	-	-
15	Linalool	1111	0.12	Tr	-	Tr
16	**Citronellal**	1160	-	Tr	90.07	-
17	Isopulegol	1177	-	Tr	tr	-
18	Carvenone	1164	0.07	-	-	-
19	Borneol	1166	0.41	0.09	-	-
20	Terpinen-4-ol	1176	1.87	0.69	-	1.53
21	α-Terpineol	1189	0.85	0.50	-	7.03
22	Sabinol	1199	1.14	-	-	-
23	**Citronellol**	**1227**	**-**	**0.16**	**4.32**	**Tr**
24	*p*-Ment-1(7)-en-2-one	1231	0.62	-	-	-
25	Piperiton	1249	0.31	Tr	-	Tr
26	Geraniol	1252	tr	-	-	-
27	Thymol	1302	tr	-	-	-
28	Exo-2-hydroxycineole acetate	1338	0.14	-	-	-
29	α-Terpinyl acetate	1348	1.27	-	-	Tr
30	Citronellyl acetate	1351	-	-	Tr	-
31	Geranyl acetate	1373	0.20	-	-	-
32	Isoledene	1378	0.81	-	-	-
33	3,3,7,11-tetramethyl tricyclo(6.3.0.0(2.4))undec-8-ene	1396	0.18	-	-	-
34	α-Gurjunene	1412	5.10	0.11	-	-
35	β-Caryophyllene	1418	-	-	1.46	-
36	**Aromadendrene**	1446	31.17	0.41	-	Tr
37	α-Caryophyllene	1452	-	-	Tr	0.04
38	*allo*-Aromadendrene	1466	3.68	0.16	-	Tr
39	γ-Gurjunen	1476	0.70	-	-	-

40	α-Selinene	1490	0.84	-	-	-
41	Longifolene	1493	1.75	-	-	-
42	Ledene	1504	7.13	Tr	-	-
43	γ-Cadinene	1518	0.24	-	-	-
44	Dehydroaromadendrene	1526	0.75	-	-	-
45	δ-Cadinene	1543	0.64	-	Tr	0.55
46	α-Calacorene	1555	0.16	-	-	-
47	Epiglobulol	1566	5.17	Tr	-	-
48	Spathulenol	1567	-	Tr	-	Tr
49	Caryophyllen oxide	1572	-	-	-	-
50	Palustrol	1581	0.22	-	-	-
51	Viridiflorol	1593	0.24	Tr	-	Tr
52	Globulol	1595	10.69	Tr	-	Tr
53	τ-Eudesmol	1600	1.24	-	-	-
54	Guaiol	1607	0.79	-	-	-
55	β-Eudesmol	1611	0.31	-	-	-
56	Cubenol	1616	0.11	-	-	-
57	Sesquiterpene alcohol	1627	0.55	-	-	-
58	τ-cadinol	1631	0.17	-	-	-
59	α-Eudesmol	1657	0.18	-	-	-
	Monoterpene hydrocarbons		4.77	11.24	3.46	5.10
	Oxygenated monoterpenes		21.58	87.96	94.41	91.26
	Sesquiterpene hydrocarbons		52.15	0.68	1.49	0.62
	Oxygenated sesquiterpenes		19.67	0.01	0.01	0.04
	Total identified		98.17	99.89	99.37	97.02

Tr: trace amount, less than 0.05%
EGF: *E. globulus* fruits, EGL: *E. globulus* leaves, ECL: *E. citriodora* leaves, ERL: *E. radiata* leaves

Whereas 1,8-cineole (86.51%), α-pinene (4.74%), γ-terpinene (2.57%) and α-phellandrene (1.40%) was main compounds of EGL, aromadendrene was found to be a minor compound. EGL is well known as 1,8-cineole-rich oil. On this investigation, it was noted that the EGF contained the significantly different main compound with the EGL, since the EGF contained of 1,8-cineole less than 15 %.

1,8-Cineole (82.66%) was also found as a major compound of ERL followed by α-terpineol (7.03%), and α-pinene (3.68%). On the other hand, aromadendrene was a trace element of ERL. *Eucalyptus radiata* oil is sometimes preferred by aromatherapists for its more pleasant smell than EGL. This oil has also been shown to be useful for treating disorders of the respiratory system and is known with a high 1,8-cineole content of 80 % (Lis-Balchin et al., 1998). For medicinal purpose, British Pharmacopoeia specifies that eucalyptus oil must contain 1,8-cineole not less than 70%. The specification is identical to that of the European Pharmacopoeia and Chinese Pharmacopoeia. In the case of *E. citriodora* oil, it should contain at least 65% citronellal (Coppen, 2002).

On the other hand, neither aromadendrene nor 1,8-cineole was detected from essential oil of ECL, but citronellal (90.07%) and citronellol (4.32%) were found to be major constituents. The high abundance of citronellal, the characteristic monoterpene of EGL (the lemon-scented *Eucalyptus*), was reported by previous authors (Batish et al., 2006a; Batish et al., 2006b; Bignell et al., 1997; Rao et al., 2003; Silva et al., 2003). In this investigation, linalool, terpinen-4-ol, α–terpineol, piperiton, viridoflorol, and globulol were common found in the 1,8-cineole-rich oils, whereas the monoterpene α-pinene and γ-terpinene was present in all the oils.

Tabel 5.2
The antimicrobial activity of the essential oils of *Eucalyptus* against multidrug-resistant bacteria

Microorganisms	ECL		ERL		EGL		EGF	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Gram-positive								
MRSA								
MRSA NCTC 10442*	2	4	4	>4	4	>4	0.25	0.06
USA300	2	4	>4	NT	>4	NT	0.5	1
1678/98	4	>4	>4	NT	>4	NT	0.125	0.5
635/93	2	4	>4	NT	4	>4	0.25	0.25
2387/00	2	4	4	>4	4	>4	0.12	0.12
1000/93	1	2	4	>4	2	4	0.12	0.25
BL7127/98	4	>4	>4	NT	4	>4	0.5	0.5
MR131/98	4	>4	>4	NT	>4	NT	0.5	0.5
MR134/93	>4	NT	>4	NT	4	>4	0.5	2
MR1150/93	>4	NT	>4	NT	>4	NT	1	2
VRE								
VRE *E. faecalis* ATCC 51299*	>4	NT	>4	NT	>4	NT	1	2
VR902291	>4	NT	>4	NT	>4	NT	0.5	0.5
VR902316	>4	NT	>4	NT	>4	NT	0.5	0.5
VR902247	>4	NT	>4	NT	>4	NT	1	2
VR902267	>4	NT	>4	NT	>4	NT	1	2
Gram-negative								
*Escherichia coli**	>4	NT	>4	NT	>4	NT	8	NA
*Pseudomonas aeruginosa**	>4	NT	>4	NT	>4	NT	>8	NT
*Klebsiella pneumonia**	>4	NT	>4	NT	>4	NT	>8	NT
*Acinetobacter baumannii**	2	2	1	1	2	2	1	1

Concentrations are given in mg/ml; NT: not tested
EGF: *Eucalyptus globulus* fruits; EGL: *E. globulus* leaves; ECL: *E. citriodora* leaves; ERL: *E. radiata* leaves
* reference strain

5.3.2 Antimicrobial activity of the essential oils and the major components of the oils

The antimicrobial activities of the 4 essential oils and their major components (aromadendrene, 1,8-cineole, citronellal and citronellol) against multidrug-resistant bacteria are shown in Table 5.2 and 5.3. The EGF exerted a powerful activity against MRSA strains with MIC values between 0.25 and 1 mg/ml. All strains of VRE were inhibited by EGF with MIC values of 0.5–1 mg/ml. Aromadendrene was confirmed to be much stronger than 1,8-cineole against MRSA and VRE. It seems that the most pronounced antimicrobial activity of EGF might be attributed by aromadendrene. This compound has a reactive exocyclic methylene group and a cyclopropane ring which can alkylate proteins and

thereby disturb the conformation of protein. Additionally, since the compound is highly lipophilic, it can disrupt the biomembrane (Sikkema et al., 1994; Wink, 2007; Wink, 2008).

In contrast to aromadendrene, all the tested microorganisms was not affected by 1,8-cineole up to concentration 8 mg/ml. Furthermore, the leaves oils with high 1,8-cineole contents (EGL and ERL) were less effective against pathogens. EGL and ERL inhibited MRSA with MIC range 2 to >4 mg/ml and 4 to >4 mg/ml, respectively. 1,8-Cineole as a single substance which observed to have a low antimicrobial effect was in agreement with a previous report (Aridogan et al., 2002; Inouye et al., 2001). However, 1,8-cineole has been used for medicinal, flavor and fragrance purposes. Many studies reported the biological activity of 1,8-cineole was confirmed as mosquito repellency (Klocke, 1987), antitumor in rats (Juergens et al., 2003), and anti-inflammatory activity (Santos et al., 1997).

Tabel 5.3
The antimicrobial activity of the major components of the essential oils of *Eucalyptus* against multidrug-resistant bacteria

Microorganisms	Aromadendrene		1,8-Cineole		Citronellal		Citronellol	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Gram-positive								
MRSA								
MRSA NCTC 10442*	0.5	0.5	>8	NT	2	2	1	2
USA300	1	4	>8	NT	8	>8	2	2
1678/98	0.25	0.5	>8	NT	1–2	2	0.125	0.125
635/93	0.25	0.25	>8	NT	>8	>8	8	8
2387/00	0.25	0.5	>8	NT	8	8–>8	2	4
1000/93	0.25	0.25	>8	NT	>8	>8	4	4
BL7127/98	1	1	>8	NT	8	>8	1–2	4
MR131/98	0.5	1	>8	NT	0.5–1	1–2	0.125	0.5
MR134/93	1	4	>8	NT	8	>8	4	8
MR1150/93	1	2	>8	NT	8	>8	4	8
VRE								
VRE *E. faecalis* ATCC 51299*	1	2	>8	NT	>8	>8	2	4
VR902291	1	2	>8	NT	8–>8	>8	8	4
VR902316	1	2	>8	NT	>8	>8	8	8
VR902247	1	4	>8	NT	>8	>8	8	8
VR902267	1	4	>8	NT	8–>8	>8	8	8
Gram-negative								
Escherichia coli	>8	NT	>8	NT	>8	>8	4	8
Pseudomonas aeruginosa	>8	NT	>8	NT	>8	>8	>8	>8
Klebsiella pneumonia	>8	NT	>8	NT	>8	>8	>8	>8
Acinetobacter Baumannii	2	2	8	8	2–4	4	0.125–0.25	0.25– 0.5

Concentrations are given in mg/ml; NT: not tested
EGF: *Eucalyptus globulus* fruits, EGL: *E. globulus* leaves, ECL: *E. citriodora* leaves, ERL: *E. radiata* leaves
* reference strain

On the other hand, ECL exhibited anti-MRSA activity with MIC range 1–4 mg/ml. ECL exhibited a comparable activity and was confirmed to contain predominantly of citronellal and citronellol. Whereas citronellal inhibited all MRSA strains with the MIC value ranging from 0.5 to >8 mg/ml, citronellol inhibited at concentration 0.125 to 8 mg/ml. Two compounds were active against MRSA as they have an aldehyde group (citronellal) and a hydroxyl group (citronellol). Secondary metabolites with aldehyde normally possess good antibacterial activity through alkylation of amino, carboxyl, and hydroxyl groups of protein and DNA (Wink, 2008). Citronellol which bears a hydroxyl group may act as protein denaturing agents (Pelczar et al., 1988). The low activity of citronellal could be related to its relatively high evaporation rate which decreased the amount of citronellal (Lertsatitthanakorn et al., 2008). A number of studies confirmed citronellal was weakly active monoterpene (Dorman and Deans, 2000; Griffin et al., 1999; Kurita et al., 1981). Also, citronellol was evaluated to be more active than citronellal as antifungal by previous authors (Kordali et al., 2007; Lertsatitthanakorn et al., 2008). It is noteworthy that citronellol was effective to inhibit bacteria although found as minor component of ECL. The result corroborated that minor components of essential oil also contributed to the overall antimicrobial activity. A previous study reported a synergistic effect was shown from combination of citronellol and citronellal.

The 4 essential oils of *Eucalyptus*, however, were hardly active against multidrug-resistant Gram-negative bacteria, except against *A. baumannii* (MIC of EGF, EGL, ERL, ECL was 1, 2, 1, 2 mg/ml respectively). The low susceptibility of Gram-negative is probably due to the presence of an outer membrane which acts a physical barrier to lipophilic compounds including essential oils (Luqman et al., 2008; Salari et al., 2005; Vaara, 1992). Moreover, Tegos and his colleague reported that the low activity of plant compounds due to MDR efflux (Tegos et al., 2002). *Pseudomonas aeruginosa* was reported to have MexAB-OprM pump and also in *E. coli* was discovered the AcrAB multidrug efflux pump (Ma et al., 1993). Multi-drug effluxes were intensely also reported among Enterobacteriaceae including *Klebsiella* sp. These efflux pumps remove antibiotic as well as noxious substances from cells (Schweizer, 2003). *Acinetobacter baumannii* was more susceptible to the essential oils than the other tested bacteria due probably to the difference of outer membrane from enterobacteriaceae (Scott et al., 1976). *Acinetobacter baumannii* is an important cause of nosocomial infections mainly affecting immunocompromised patients. These multidrug resistant (MDR) strains show an outstanding ability to rapidly evolve resistance to new antibiotics (Joly-Guillou, 2005). The result demonstrated that the *Eucalyptus* oils can be a good source of antibacterial agent particularly against *A. baumannii*.

Among the tested 4 oils, the antimicrobial activity of the oils can be ranked as EGF > ECL > ERL ~ EGL. Previous studies demonstrated *E. globulus* oil (91 % of cineole) was less active towards bacteria than *E. radiata* (84% cineole) and *E. citriodora* oil showed much greater antifungal activity (Lis-Balchin et al., 1998; Luqman et al., 2008; Oyedeji et al., 1999; Ramezani et al., 2002). Regarding the activity of

the major component of the oils, aromadendrene has shown the most pronounced activity followed by citronellol, citronellal and 1,8-cineole. This recent study indicated that the chemical composition of oils determines the antimicrobial properties. The major components might be contribute to the antimicrobial activity, however, the contribution of minor components should be considered to produce a synergistic, additive or even antagonistic interaction.

In conclusion, the essential oil from fruits of *E. globulus* might be a potential source for treatment infectious disease particularly caused by multi-drug resistant bacteria, instead of from leaves which is now well established. The result corroborated the relationship between the chemical composition of the essential oil and the antimicrobial activity. Further investigation about the safety and the clinical study are warranted.

Chapter 6

Biological Activities of the Essential Oil of *Kadsura longipedunculata* (Schisandraceae) and its Major Components

6.1 Abstract

Objective: to determine the chemical composition of the essential oil of *Kadsura longipedunculata,* and the biological activities of the oil and its major components.

Methods: The essential oil from stem bark of *Kadsura longipedunculata* was analyzed by capillary gas chromatography (GLC/FID) and gas chromatography–mass spectrometry (GLC/MS). The ability of the oil to reduce diphenylpicrylhydrazine ($DPPH^{\bullet}$) was used to evaluate the antioxidant activity. Inhibition of both lipoxygenase and prostaglandin E_2 was used to assess the anti-inflammatory activity. Antimicrobial activity was studied *in vitro* against a range of bacteria and fungi using diffusion and microdilution methods. Inhibition of trypanosome proliferation was assessed using resazurin as vital stain. The *in-vitro* cytotoxicity of the essential oil on six human cancer cell lines (HepG2, MIA PaCa-2, HeLa, HL-60, MDA-MB-231 and SW-480) was examined using the MTT assay.

Key findings: Fifty compounds, representing 97.63% of total oil were identified. δ-Cadinene (21.79%), camphene (7.27%), borneol (6.05%), cubenol (5.12%) and δ-cadinol (5.11%) were found to be the major components of the oil. The oil exerted a good antimicrobial activity against all Gram-positive bacteria tested including methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococcus (VRE) *Enterococcus faecalis*. *Streptococcus pyogenes* and *S. agalactiae* were the most sensitive bacteria with a minimal inhibitory concentration (MIC) of 60 µg/ml oil. The essential oil showed a moderate fungicidal activity against yeast, but it did not show any activity against Gram-negative bacteria. The essential oil showed a good trypanocidal activity in *Trypanosoma b. brucei* with an IC_{50} value of 50.52 ± 0.029 µg/ml. Radical scavenging activity had an IC_{50} value of 3.06 ± 0.79 mg/ml. 5-Lipoxygenase inhibition (IC_{50}= 38.58 µg/ml) and prostaglandin E_2 production inhibition (28.82% at 25 µg/ml) accounted for anti-inflammatory activity of the oil. The oil exhibited some degree of cytotoxic activities against MIA PaCa-2, HepG-2 and SW-480 cell lines with IC_{50} values of 133.53, 136.96 and 136.62 µg/ml, respectively. The oil increased caspase 3/7 activity (an indicator of apoptosis) 2.5 to 4 fold in MIA Paca-2 cells. Camphene and borneol did not show antioxidant activity. However, both compounds exhibited some degree of antimicrobial, trypanocidal, anti-inflammatory, and cytotoxic activities.

Conclusions: The present investigation provided evidence and confirmed the efficacy of *K. longipedunculata*, a traditionally used Chinese medicinal plant for the treatment of inflammation and infection.

Keywords: *Kadsura longipedunculata*; essential oil; antimicrobial & anti-inflammatory; trypanocidal; cytotoxicit; traditional Chinese medicine (TCM)

6.2 Introduction

Essential oils have been used in traditional medicine all over the world to treat infection and many diseases. Essential oils are a rich source of biologically active compounds and generally possess strong and persistent odors, usually characteristic of the plant in which they are found. Essential oils were shown to possess in vitro antibacterial, antifungal, antioxidant, analgesic, anti-inflammatory, insecticidal and antiviral properties (Ngassoum et al., 2007; Silva et al., 2003; Vardar-Unlu et al., 2003). Some components of essential oils were also shown to exert both in vitro and in vivo antitumor activities against murine leukemia, hepatoma, and melanoma cells. Additionally, the chemotherapeutic values of some essential oil components against cancer cells are under evaluation in Phase I clinical trials (Crowell, 1999). Moreover, some essential oils are directly used in pharmaceutical, food and perfumery industries and are found in market products of these industries.

Kadsura longipedunculata Finet et Gagnep belongs to the medicinally important family Schisandraceae which is widely distributed particularly in southern China. This plant is an evergreen scandent vine and is used in traditional Chinese medicine (TCM) for the treatment of rheumatoid arthritis as well as gastrointestinal diseases such as gastric and duodenal ulcers, traumatic injury, ulcers with pyogenic infection (Chang et al., 1998). Previous findings confirmed that a few lignans and triterpenoids possess some beneficial biological effects, including anti-inflammatory, antioxidant (Choi et al., 2006), antihyperlipidemia (Kangouri et al., 1990), antitumor, and antiviral activities (Sun et al., 2006). A previous study also reported that the essential oil from the roots of *K. longipedunculata* have antimicrobial, antioxidant, and cytotoxic activities and also showed δ-cadinene as the main compound of the oil (Song et al., 2007). However, the contribution of the main components of the essential oil towards its biological activity has not been investigated.

To our knowledge, no reports on the essential oil from stem bark have been published. Therefore, the present study was aimed to investigate the chemical composition of the essential oil of *K. longipedunculata* stem bark and also to examine its biological activities that were based on its ethnopharmacological use for the treatment of inflammations and infections. Additionally, we tested the potential of this plant as an antitumor drug. We report for the first time the contribution of two major compounds (camphene and borneol) of the essential oil to its biological properties.

6.3 Results

6.3.1 Chemical Composition of the Essential Oil

The yield of essential oil from *K. longipedunculata* after hydrodistillation was 0.64%. The chemical composition of the essential oil is listed in Table 6.1. By GLC-MS, 50 components were identified representing 97.63% of the total oil. The most abundant constituents were δ-cadinene (21.79%), camphene (7.27%), borneol (6.05%), cubenol (5.12%), and δ-cadinol (5.11%). The oil contained 22.63% monoterpenes (which include 10.25% oxygenated monoterpenes) and 75% sesquiterpenes (which include 23.77% oxygenated sesquiterpenes).

Table 6.1
Chemical composition of *K. longipedunculata* essential oil.

No	Compounds	RI (OV-1)	Percentage (%)[§]
Monoterpene hydrocarbons			
1	Tricycline	905	0.55
2	α-Thujene	913	0.18
3	α-Pinene	918	1.55
4	Camphene	931	7.27
5	β-Pinene	959	0.81
6	β-Myrcene	980	0.08
7	α-Terpinene	1005	Tr
8	P-Cymene	1009	0.13
9	Limonene	1017	1.56
10	γ-Terpinene	1046	0.18
11	Terpinolene	1076	0.07
Oxygenated monoterpenes			
12	1,8-Cineole	1015	0.50
13	Camphor	1114	0.19
14	Borneol[*]	1145	6.05
15	Terpinen-4-ol[*]	1157	1.26
16	α-Tepineol	1168	0.21
17	Bornyl acetate	1266	2.04
Sesquiterpene hydrocarbons			
18	δ –Elemene	1331	4.03
19	α-Cubebene	1344	1.20
20	α-Copaene	1368	2.83
21	β-Elemene	1381	1.50
22	α-Gurjunene	1405	1.16
23	β-Caryophyllene	1408	0.19
24	β –Copaene	1418	0.11
25	(+)-Aromadendrene	1428	0.33
26	α-Humulene	1441	0.52
27	allo-Aromadendrene	1449	0.90
28	β –Chamigrene	1463	2.42
29	γ –Muurolene	1466	1.41
30	Germacrene D	1468	0.59
31	β –Selinene	1473	1.74
32	*epi*-Bicyclosesquiphellandrene	1477	2.04
33	Viridiflorene	1484	0.48

34	α- Muurolene*	1489	3.45
35	Calamenene	1504	1.39
36	δ-Cadinene*	1512	21.79
37	Cadina-1,4-diene*	1519	0.94
38	α –Calacorene	1523	1.37
39	Cadala-1(10),3,8-triene	1540	0.84
Oxygenated sesquiterpenes			
40	*trans*-Nerolidol*	1547	0.88
41	Spathulenol*	1555	3.07
42	β-Caryophyllene oxide	1559	0.67
43	Viridiflorol	1566	0.75
44	γ-Eudesmol	1604	2.00
45	Cubenol	1607	5.12
46	τ –Cadinol	1619	4.39
47	δ -Cadinol*	1623	5.11
48	τ –Muurolol	1630	1.10
49	Cadalene	1647	0.38
50	α-Bisabolol*	1664	0.30
Monoterpene hydrocarbons			12,38
Oxygenated monoterpenes			10.25
Sesquiterpene hydrocarbons			51.23
Oxygenated sesquiterpenes			23.77
Total			97.63

*Previously reported (Song et al., 2007)
[§]Average of two analysis
tr: trace, the percentage less than 0.1%

6.3.2 Antimicrobial Assay

The antimicrobial activity of the essential oil and the two components (camphene and borneol) were determined with the agar diffusion method (Table 6.2). The crude oil produced inhibition zones ranging from 8.0 to 13.0 mm whereas isolated components of the oil displayed an antimicrobial effect with inhibition zones ranging from 6.7 to 11.0 mm.

All Gram-positive bacteria were susceptible to the oil with MIC values between 0.06 and 2 mg/ml. *Streptococcus agalactiae* and *S. pyogenes* were the most susceptible bacteria with an MIC of 0.06 mg/ml. The oil exhibited a substantial activity against *B. subtilis, S. saprophyticus, S. epidermidis, S. aureus* and *E. faecalis* with an MIC between 0.25 – 0.5 mg/ml, but did not show activity against Gram-negative bacteria such as *E. coli* and *P. aeruginosa* even at the highest concentration. The oil displayed a moderate activity against yeasts such as *C. albicans*, *C. glabrata* and *C. parasilopsis* with MIC values between 1 – 2 mg/ml. The oil exhibited a good activity against antibiotic-resistant strains, both MRSA and VRE, with an MIC value between 0.5 – 2 mg/ml. The statistical analysis revealed significant differences in antimicrobial activities between the oil and the isolated compounds (camphene and borneol) ($P < 0.01$).

Table 6.2

In vitro antimicrobial activity of *K. longipedunculata* oil, components and reference antibiotics determined with the diffusion method.

No	Microorganisms	Diameter of inhibition zone (mm)					
		Essential oil	Borneol[§]	Camphene[♣]	Ampicillin	Vancomycin	Nystatin
	Gram-positive						
1	*Bacillus subtilis* ATCC 6051	8.0 ± 0.0	7.3 ± 0.6	6.7 ± 0.6*	29.7 ± 0.6	20.8 ± 0.8	NT
2	*Staphylococcus saprophyticus* ATCC 15305	11.7 ± 0.6	8.3 ± 0.6*	9.7 ± 1.5	33.7 ± 0.6	17.0 ± 0.0	NT
3	*Staphylococcus epidermidis* ATCC 14990	11.3 ± 0.6	10.3 ± 1.5	9.7 ±0.6*	25.3 ± 1.3	18.0 ± 0.0	NT
4	*Staphylococcus aureus* ATCC 29213	13.0 ± 1.0	NI***	NI***	22.0 ± 0.0	17.3 ± 0.8	NT
5	*Streptococcus agalactieae* ATCC 27956	9.0 ± 0.0	7.0 ± 1.0*	9.3 ± 1.2	30.0 ± 0.0	29.7 ± 0.6	NT
6	*Streptococcus pyogenes* ATCC 12344	9.7 ± 0.6	9.7 ± 0.6	9.7 ± 0.6	30.0 ± 0.0	17.3 ± 0.6	NT
7	*Enterococcus faecalis* ATCC 29212	9.0 ± 1.0	NI***	NI***	24.7 ± 0.6	16.3 ± 0.6	NT
8	VRE *E. faecalis* ATCC 51299	8.3 ± 1.2	NI***	NI***	25.0 ± 0.0	12.7 ± 0.6	NT
9	MRSA NCTC 10442	11.7 ± 0.6	NI***	NI***	0.0 ± 0.0	17.7 ± 0.0	NT
10	MRSA MR 818014 (clinical isolate)	11.3 ± 0.6	NI***	NI***	7.3 ± 1.2	17.3 ± 0.6	NT
11	MRSA MR 818081 (clinical isolate)	12.0 ± 1.0	NI***	NI***	9.6 ± 0.6	16.7 ± 0.6	NT
	Gram-negative						
12	*Escherichia coli* ATCC 25922	NI	NI	NI	10.7 ± 0.6	NI	NT
13	*Pseudomonas aeruginosa* ATCC 27853	NI	NI	NI	NI	NI	NT
	Yeast						
14	*Candida albicans* ATCC 90028	8.6 ± 0.6	10.7 ± 1.2	10.0 ± 0.0	NT	NT	17.3 ± 0.6
15	*Candida glabrata* ATCC MYA 2950	10.3 ± 0.6	10.0 ± 1.1	10.0 ± 0.6	NT	NT	12.7 ± 0.6
16	*Candida parasilopsis* ATCC 22019	8.3 ± 0.6	11.0 ± 1.7	7.3 ± 0.6	NT	NT	12.3 ± 1.2

NI: no inhibition, NT: not tested

The dosage of essential oil and components were 3.2 mg, ampicillin and vancomycin were 10 μg, and nystatin 20 μg

Presented data are mean values ± SD

§ Significant differences between the essential oil and borneol (* $P < 0.05$; ** $P < 0.01$. *** $P < 0.001$)

♣ Significant differences between the essential oil and camphene (* $P < 0.05$; ** $P < 0.01$. *** $P < 0.001$)

Tabel 6.3

Minimal inhibitory concentration (MIC) and minimal biocidal concentration (MBC) of *K. longipedunculata* oil, components and reference antibiotics determined with microdilution method.

		K. longipedunculata		**Borneol**		**Camphene**		**Ampicillin**		**Vancomycin**		**Nystatin**	
No	Microorganisms	**MIC***	**MBC**	**MIC***	**MBC**	**MIC***	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
	Gram-positive												
1	*Bacillus subtilis* ATCC 6051	0.25	0.25	2	4	2	4	0.1	0.8	0.2	0.8	NT	NT
2	*Staphylococcus saprophyticus* ATCC 15305	0.25	2	1	4	1	4	0.2	0.4	1.6	3.1	NT	NT
3	*Staphylococcus epidermidis* ATCC 14990	0.5	2	1	4	2	4	0.4	0.8	0.8	1.6	NT	NT
4	*Staphylococcus aureus* ATCC 29213	0.5	2	4	8	4	>8	0.4	3.1	0.4	0.8	NT	NT
5	*Streptococcus agalactiaeae* ATCC 27956	0.06	0.06	1	2	1	1	0.1	0.2	0.4	0.4	NT	NT
6	*Streptococcus pyogenes* ATCC 12344	0.06	0.06	1	2	1	2	0.1	0.1	0.1	0.2	NT	NT
7	*Enterococcus faecalis* ATCC 29212	2	4	8	>8	>8	>8	0.4	0.8	1.6	3.1	NT	NT
8	VRE *E. faecalis* ATCC 51299	2	4	8	>8	>8	>8	0.4	0.8	25	50.0	NT	NT
9	MRSA NCTC 10442	0.5	2	4	8	8	>8	6.2	12.5	0.8	1.6	NT	NT
10	MRSA MR 818014 (clinical isolate)	1	2	8	8	>8	>8	6.2	6.2	0.8	0.8	NT	NT
11	MRSA MR 818081 (clinical isolate)	1	2	8	8	>8	>8	6.2	50	0.8	0.8	NT	NT
	Gram-negative												
12	*Escherichia col*i ATCC 25922	NI	NI	4	8	8	>8	6.2	12.5	NI	NI	NT	NT
13	*Pseudomonas aeruginosa* ATCC 27853	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NT	NT
	Yeast												
14	*Candida albicans* ATCC 90028	2	4	4	8	8	8	NT	NT	NT	NT	1.6	1.6
15	*Candida glabrata* ATCC MYA 2950	2	4	4	4	4	4	NT	NT	NT	NT	1.6	1.6
16	*Candida parasilopsis* ATCC 22019	1	2	2	4	4	4	NT	NT	NT	NT	1.6	1.6

The unit of oil and components were given in mg/ml, meanwhile of reference antibiotics were in μg/ml

NI: no inhibition, NT: not tested

Presented data are mean values

* Significant differences between the oil and borneol; the oil and camphene ($P < 0.01$)

6.3.3 Trypanocidal Activity

The results of trypanocidal activity are summarized in Table 6.4. The essential oil showed moderate activity with an IC_{50} value of 50.52 ± 0.029 µg/ml, whereas camphene and borneol exhibited trypanocidal activity with a statistically significant IC_{50} values of 80.66 ± 0.87 and 70.00 ± 1.28 µg/ml, respectively ($P < 0.001$).

Table 6.4
Antitrypanosomal and radical scavenging activity of *K. longipedunculata* essential oil, camphene and borneol.

Substances	IC_{50} (µg/ml)	IC_{50}
	Trypanocidal Activity	DPPH Assay
K. longipedunculata oil	50.52 ± 0.029	3.06 ± 0.79 mg/ml
Camphene	80.66 ±0.87♣	>10 mg/ml
Borneol	70.00 ± 1.28§	>10 mg/ml
Positive control	0.17± 0.03	4.10 ± 0.41 µg/ml

Presented data are means ±SD
♣ Significant differences between the oil and camphene ($P < 0.001$)
§ Significant differences between the oil and borneol ($P < 0.001$)

6.4.4 Antioxidant Activity

As shown in Table 6.4, the essential oil was able to reduce the purple-colored DPPH radical to the yellow-colored diphenylpicrylhydrazine with an IC_{50} of 3.06 ± 0.79 mg/ml. On the other hand, camphene and borneol were hardly active; they showed a 10% inhibition up to concentration of 10 mg/ml.

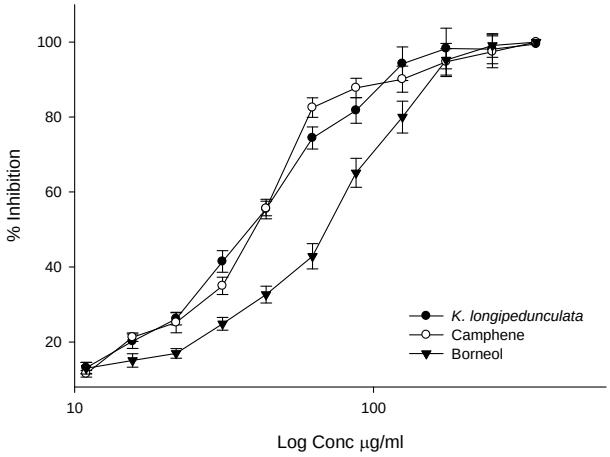


Figure 6.1 Inhibitory effect of *K. longipedunculata* essential oil, camphene and borneol on soybean 5-lipoxygenase from three independent experiments. A significant difference between the oil and borneol ($P < 0.001$).

6.3.5 5-Lipoxygenase Inhibition

The inhibition of 5-lipoxygenase by the essential oil, camphene and borneol is shown in Figure 6.1. The IC_{50} value of oil was 38.58 ± 3.8 µg/ml, whereas the IC_{50} of camphene and borneol were 39.72 ± 2.16 and 69.22 ± 3.66 µg/ml, respectively. The inhibition of 5-lipoxygenase of the oil and camphene showed no significant difference ($P > 0.05$), however a significant difference was found between the oil and borneol ($P < 0.001$).

6.3.6 Inhibition of PGE_2 Production

The amount of prostaglandin E_2 produced by MIA PaCa-2 cells was determined to assess a potential inhibitory effect of the essential oil and its components on cyclooxygenase activity. As shown in Figure 6.2, 25 µg/ml the oil inhibited prostaglandin E_2 formation by 28.82%, whereas borneol and camphene displayed 33.74 and 45.78% inhibitory activity compared to untreated control, respectively. A concentration of 3.14 µg/ml of NS-398 was used as positive control and resulted in 64.11% inhibition of PGE_2 production. The oil and borneol showed no significant difference in the inhibition of prostaglandin ($P > 0.05$), however a significant difference was observed of the oil and camphene ($P < 0.05$).

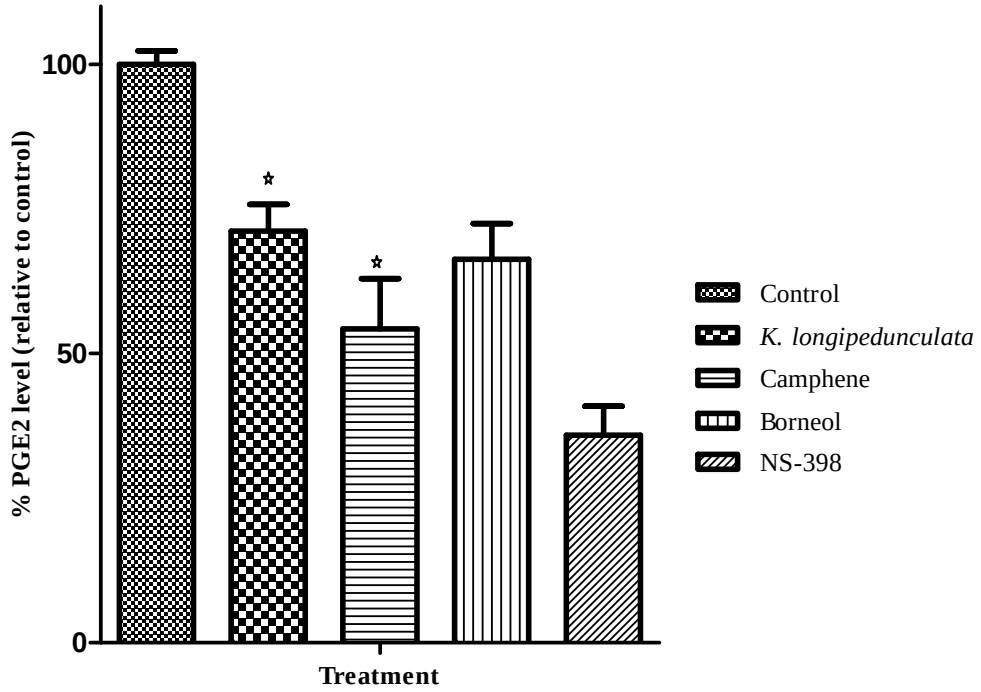


Figure 6.2. Inhibition of PGE2 in MIA PaCa-2 cells with *K. longipedunculata* essential oil, camphene and borneol at concentration 25 µg/ml; NS-398 at concentration 3.14 µg/ml. The graph is presented as mean ± SD. A significant difference between the oil and camphene ($P < 0.05$).

6.3.7 Cytotoxicity

The cytotoxicity of the essential oil, camphene and borneol in HepG2, MIA PaCa-2, HeLa, HL-60, MDA-MB-231 and SW-480 cell lines is illustrated in Figure 6.3. The oil exhibited some degree of cytotoxicity against MIA PaCa-2, HepG-2 and SW-480 cell lines (IC_{50} values were 133.53, 136.96 and 136.62 µg/ml, respectively). Camphene exerted a cytotoxic effect against SW-480 and HL-60 (IC_{50} values were 124.21 and 167.75 µg/ml, respectively). The statistical analysis revealed significant differences in cytotoxicity between the oil and borneol ($P < 0.01$), however no significant differences were observed between the oil and camphene ($P > 0.05$).

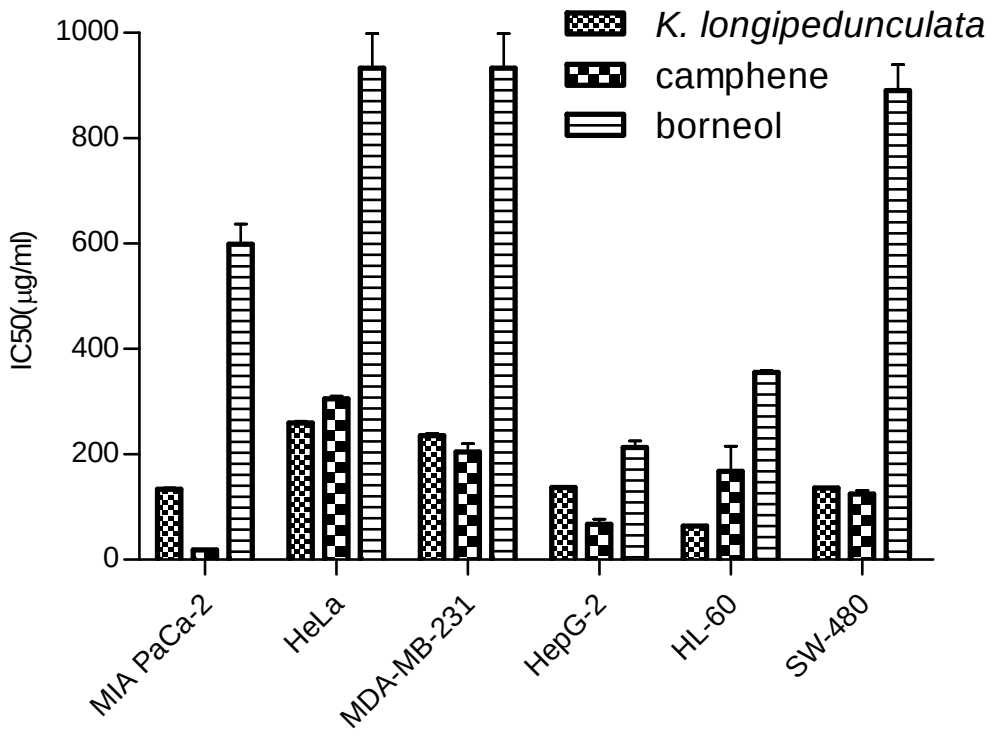


Figure 6.3 Cytotoxic activity of the essential oil from *K. longipedunculata*, of camphene and borneol in mammalians cell lines. The data are shown as mean ± S.D of IC_{50} values (µg/ml). Significant differences between the essential oil and borneol ($P < 0.01$).

6.3.8 Caspase Assay

To gain insights into the mechanism involved in the cytotoxicity induced by the essential oil and its components, caspase 3/7 activity was evaluated as an indicator and measure for apoptosis (Figure 6.4). After 24 h of incubation of MIA PaCa-2 cells with essential oil, the caspase 3/7 activity was increased 2.5 to 4 fold above the control. Camphene and borneol stimulated caspase activity in a dose-dependent manner, from 1.75 to 4.5 fold and 3 to 5 fold, respectively. A significant difference was observed between the oil and the 2 isolated compounds at concentration 0.25 mg/ml ($P < 0.01$).

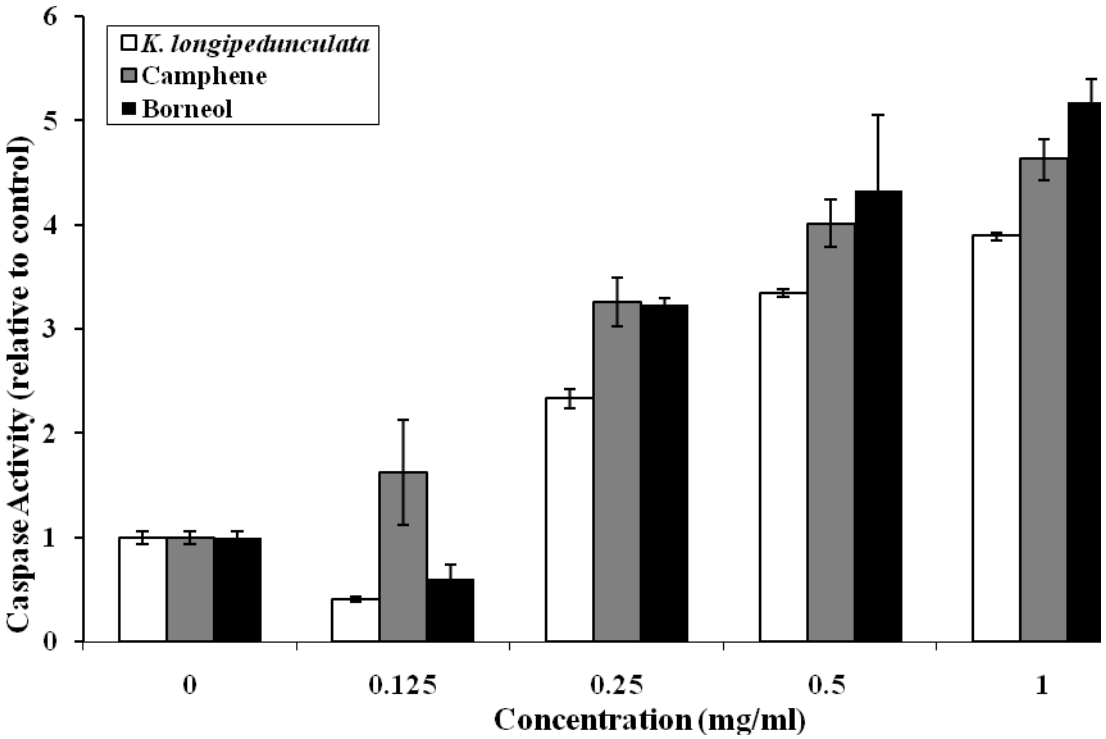


Figure 6.4 Caspase activity of *K. longipedunculata* essential oil, camphene and borneol. The data are shown as mean ± SD from three independent experiments.

6.4 Discussion

The essential oil exerted an inhibitory activity against all Gram-positive bacteria and yeasts but no activity against Gram-negative bacteria. Previous investigations had also shown Gram-positive bacteria to be more susceptible to essential oils than Gram-negative bacteria (Inouye et al., 2001; Smith-Palmer et al., 1998). The low susceptibility of Gram negative-bacteria such as *E. coli* and *P. aeruginosa* to the essential oil is probably due to the fact that these types of bacteria have an outer membrane consisting of a very thick lipopolysaccharide layer which serves as a barrier membrane to lipophilic substances (Inouye et al., 2001). Methicillin-resistant *Staphylococcus aureus* and vancomycin- resistant *Enterococcus faecalis* strains were sensitive to the oil with an MIC value twice as high as in non-resistant strains, indicating that the oil has a different mode of action compared to penicillin (Möller et al., 2006).

The pronounced antimicrobial and trypanocidal activities of the oil could be attributed to camphene and/ or borneol. Both the oil and two isolated compounds are lipophilic substances and might have interacted with membrane lipids and proteins and this would result in cell disruption and finally cell death (Wink, 2006; Wink, 2007).

The good radical scavenging activity and significant inhibition of both cyclooxygenase and lipoxygenase validated the anti-inflammatory activity of the essential oil of *K. longipedunculata*. 5-Lipoxygenase (5-LOX) converts arachidonic acid into leukotrienes, which are mediators of inflammatory and allergic reactions. Low IC_{50} values suggest the better inhibitory actions on 5-LOX and hence a greater anti-inflammatory activity. Additionally, prostaglandin E_2 is the major inflammatory mediator produced by cyclooxygenase from arachidonic acid. COX expression is induced by various stimuli, and the overexpression is closely related to the pathogenesis of some degenerative diseases including cancer. Reduction of prostaglandin E_2 level is directly related to COX inhibition. Inhibition of both 5-LOX and COX by the oil would be very useful for relief of inflammation.

Cytotoxicity of the oil was highest in the HL-60 cell line whereas the other cell lines were less sensitive. The low of cytotoxicity of the oil in HeLa cell may be due to the expression of ABCB7; ABC-transporter which are detected in most organs, increase survival of endothelial cells since they can pump out toxins that have entered the cells by diffusion (Cavadini et al., 2007). Insensitivity of MDA-MB-231 to essential oil may be related to the highly expressed ABCG2 (breast cancer resistance protein, BCRP), and ABCC11 (multidrug resistance protein 8, MRP8), which have been implicated as resistance factors in breast cancer (Kruh et al., 2007). These ABC-transporters play an important role in MDR (multiple drug resistance) and cause a failure of cancer chemotherapy by increasing the efflux of cytotoxic agent and thereby decreasing its efficacy (Wink, 2008a; Wink, 2008b).

Apoptosis can be deduced from activation of caspase 3/7 activity. The assay demonstrated that an increase of caspase activity corresponds to decrease of viability cell (Gurtu et al., 1997). The essential oil, borneol and camphene stimulated caspase activity in MIA PaCa-2 cells at all tested concentrations.

Comparing caspase activity with the cytotoxicity against MIA PaCa-2 cells, camphene was the most active cytotoxic agent, however, the caspase induction was close to that of borneol. This suggests that camphene can induce cell death by more than one mechanism such cell cycle arrest or any other form of cell death beside apoptosis. On the other hand, borneol exhibited lower cytotoxicity, but the caspase activity was relatively high, indicating that apoptosis is the main mechanism of action.

In contrast to the other biological activities, both camphene and borneol showed low radical scavenging activities. The result is in agreement with previous report (Vardar-Unlu et al., 2003). The crude oil was found to be more effective than the isolated compounds indicating possible synergistic interactions of the components of the oil.

6.6 Conclusion

The results of the present investigation provided evidence and confirmed the efficacy of *K. longipedunculata*, a plant traditionally used against inflammation and infection. Moreover, the essential oil of *K. longipedunculata* could be a potential drug against trypanosomes. Our experiments indicated that camphene and borneol, the main compounds of the essential oil, contributed partially to the biological activities of the oil. Further investigation is warranted for possible involvement of other components of the essential oil of *K. longipedunculata*.

Chapter 7

The Monoterpenoids Camphene and Borneol Act Synergistically against Human-Pathogenic Bacteria

7.1. Results

7.1.1 Fractional inhibitory concentration index (FICI)

Table 7.1 shows that camphene possessed the same antibacterial activity as borneol, with the exception of MRSA. Monoterpene camphene inhibited MRSA at MIC value of 8 mg/ml, whereas borneol showed activity at MIC value of 4 mg/ml. However, both compounds inhibited *S. aureus, S. pyogenes* and *B. subtilis* at concentration 4, 1, 2 mg/ml, respectively.

Table 7.1
Minimum Inhibitory concentration (MIC) of camphene and borneol determined by microdilution broth.

Microorganisms	Camphene (mg/ml)		Borneol (mg/ml)	
	MIC	MBC	MIC	MBC
MRSA NCTC 10442	8	>8	4	8
Staphylococcus aureus ATCC 29213	4	>8	4	8
Streptococcus pyogenes ATCC 12344	1	2	1	2
Bacillus subtilis ATCC 6051	2	4	2	4

The combination of camphene and borneol enhanced the microorganism susceptibility. Table 7.1 demonstrates the additive and synergistic interaction of the combinations against MRSA, *S. aureus, S. pyogenes* and *B. subtilis*. The combination of champene and borneol were confirmed to inhibit MRSA with FIC indexes ranging from 0.5 – 0.62, whereas inhibited *S. aureus*, *S. pyogenes* and *B. subtilis* with the same FIC index of 0.5 – 0.75. These FIC indexes indicated the interactions of two compounds seemed to be between additive and synergistic interactions. It is noteworthy, no antagonistic and indifferent interactions were observed. The lowest concentration of the combinations at which inhibited bacteria was observed in the combination of 2 mg/ml camphene plus 1 mg/ml borneol against MRSA, 1 mg/ml camphene plus 1 mg/ml borneol against *S. aureus* and 0.5 mg/ml camphene plus 0.5 mg/ml borneol against *B. subtilis*. These dose-pair combinations revealed the FIC index of 0.5 indicating apparent synergistic interactions (Matsumura et al., 1999). Of the tested bacteria, remarkably, synergisms were found in the 1:1 dose-pair ratio against the tested strains, except toward MRSA with the 1:2 dose-pair ratio.

Table 7.2

Result of the checkerboard assay: ratio of camphene and borneol (Ca/Bo), concentration of camphene [camphene] and borneol [borneol] in mg/ml, and FICI values. The interaction as reflected by FICI values is considered to be synergistic at ≤ 0.5, additive at > 0.5–1, indifferent at >1–4.0, and antagonistic at > 4.0.

MRSA				
Ca/bo	**[Camphene]**	**[Borneol]**	**FICI**	**Int**
8/0	8	0	-	-
4/1	4	1	0.62	Add
2/1	2	1	0.5	Syn
½	1	2	0.62	Add
¼	0.5	2	0.56	Add
0/4	0	4	-	-

S. aureus				
Ca/bo	**[Camphene]**	**[Borneol]**	**FICI**	**Int**
4/0	4	0	-	-
2/1	2	1	0.75	Add
1/1	1	1	0.5	Syn
1/4	0.5	2	0.75	Add
1/4	0.25	2	0.62	Add
0/4	0	4	-	-

S. pyogenes				
Ca/bo	**[Camphene]**	**[Borneol]**	**FICI**	**Int**
1/0	1	0	-	-
2/1	0.5	0.25	0.75	Add
4/1	0.5	0.12	0.62	Add
1/1	0.25	0.25	0.50	Syn
1/4	0.12	0.5	0.62	Add
0/1	0	1	-	-

B. subtilis				
Ca/bo	**[Camphene]**	**[Borneol]**	**FICI**	**Int**
2/0	2	0	-	-
2/1	1	0.5	0.75	Add
1/2	0.5	1	0.62	Add
1/1	0.5	0.5	0.5	Syn
1/4	0.25	1	0.62	Add
0/2	0	2	-	-

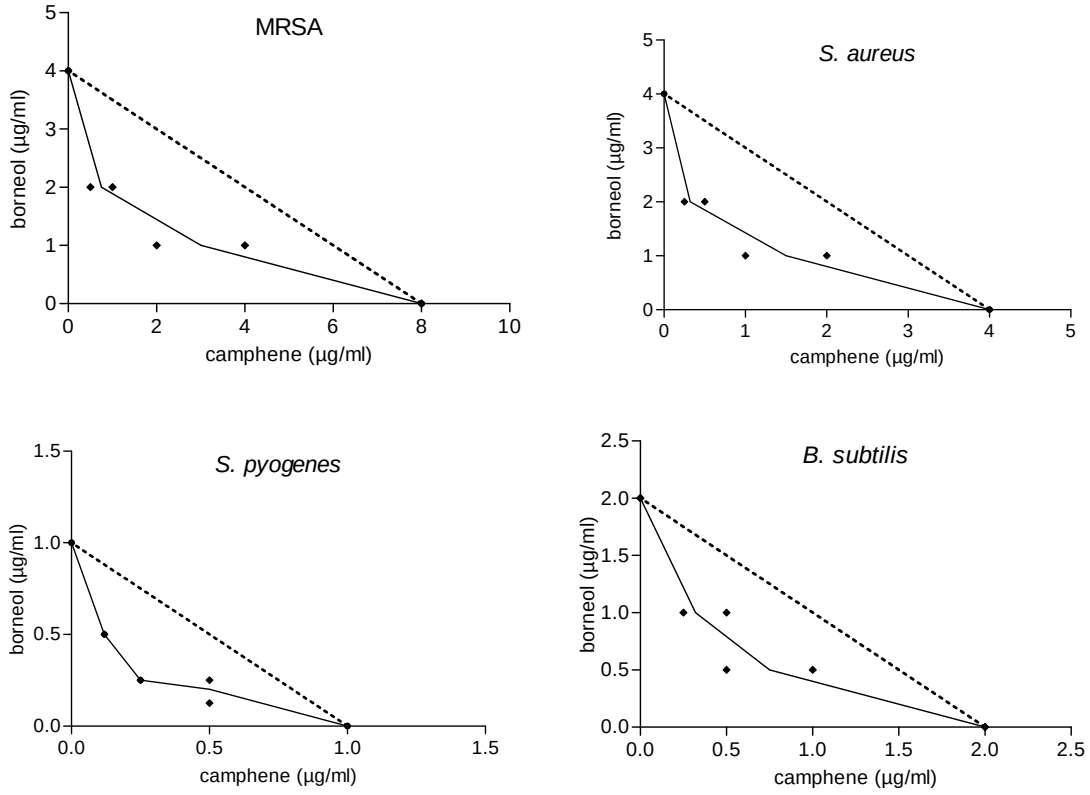


Figure 7.1 Isobologram of the combination of camphene and borneol.

7.1.2 Isobologram

Isobolograms (Figure 7.1) were constructed by plotting the MIC of camphene and borneol in combinations. A concave curve indicates that the MICs were considerable turned down and the synergistic interaction were generated by combination (Berenbaum, 1989).

7.1.3 Time-kill assay

The time-kill curve of the combinations of camphene and borneol against *S. pyogenes* is presented in 5.2. The combination of 0.5 MIC camphene plus 0.5 MIC borneol reduced the cell counts of *S. pyogenes* of >6log 10 after 24 h incubation indicating the synergistic interaction. On the other hand, the synergistic interactions were not found from the combination of 0.5 MIC camphene plus 0.25 MIC borneol and 0.25 MIC camphene plus 0.5 MIC borneol in *S. pyogenes*.

The combination of champene and borneol, in three different dose ratios, showed the synergistic effects against MRSA. As depicted in Figure 7.2, both camphene and borneol given as single substance at 0.5 × MIC decreased the MRSA cells number in the first 2 h, however the cells increasingly grew up to 24 h. Both combinations of 0.5 × MIC camphene and 0.5 MIC borneol and combination of 0.25 × MIC camphene and 0.5 × MIC borneol decreased >6log$_{10}$ the bacterial growth of MRSA (in the curve shown as overlapped). Furthermore, about >4 log10 cell number of MRSA was reduced by the combination of 0.5 MIC Camphene and 0.25 MIC borneol. Additionally, the time-kill curve accounted for the synergistic interaction in dose-dependent manner (Cappelletty and Rybak, 1996).

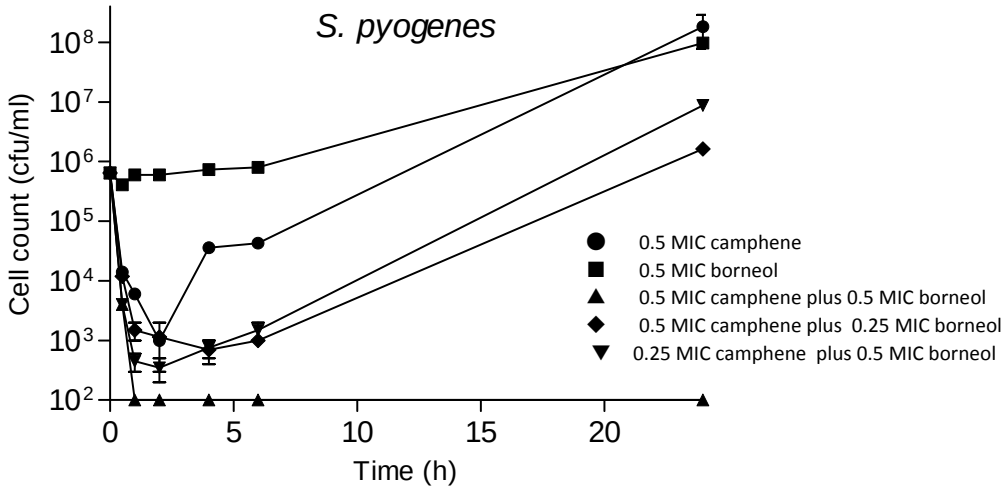


Figure 7.2 Time-kill curve of camphene and borneol alone and in combination against *S. pyogenes*.

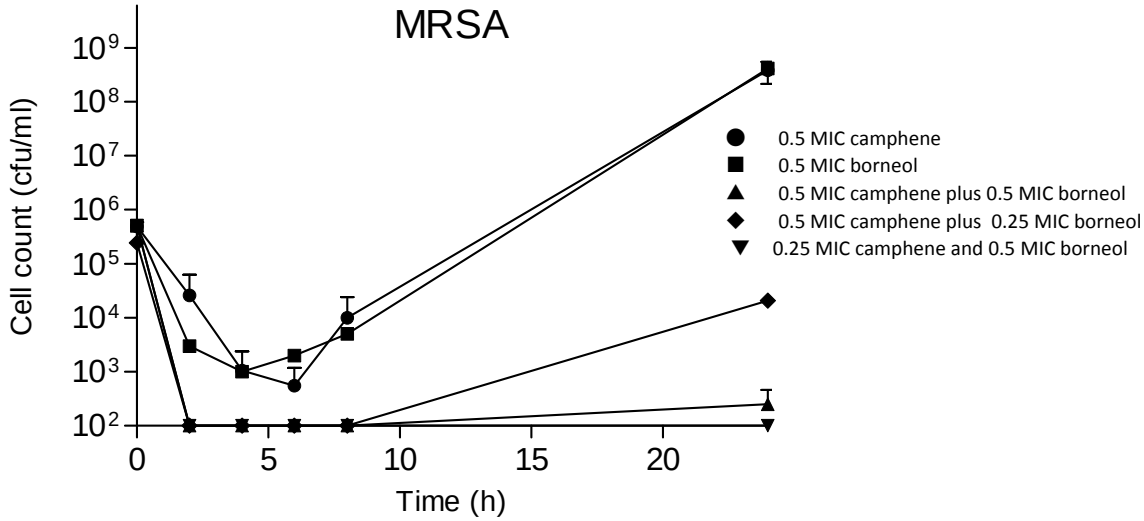


Figure 7.3 Time-kill curve of camphene and borneol alone and in combination against MRSA.

7.2 Discussion

We have investigated the synergistic interaction of the monoterpenoids camphene and borneol carried out by checkerboard and time-kill assay. Checkerboard assay is the most common method used to study synergism, although sometimes has a problem with the reproducibility and only observe the bacteriostatic effect (Cappelletty and Rybak, 1996). Some researchers consider time-kill method to be more reliable to observe the synergistic effect of combinations. But, this method has also disadvantages as it is time consuming, labor-intensive and the reliance on the reading at one time point (usually 24 h) as the sole determinant of the interaction (White et al., 1996). Isobole method of Berenbaum(1989) seems to be demonstrative and be the method choice for the proof of synergy effects (Hemaiswarya et al., 2008).

In the recent study, we demonstrated that the combinations of camphene and borneol produced additive and synergistic interactions. Some researchers conducted preliminarily studies on the mechanism of action of borneol to promote penetration (Wu et al., 2006) the permeability of drugs through the cornea. A previous study suggested that camphene can affect and damage the cell-wall structure or may interfere with its formation (Yamaguchi et al., 2009). The combination effects might generate structural conformation changes which affect reaction with biomembrane thus enhancing uptake by the bacterial cells (D'Arrigo et al., 2010). In addition, single constituents of a mono-extract or a multi-extract combination are able to affect several targets in a synergistic multi-target effect (D'Arrigo et al., 2010; Wagner and Ulrich-Merzenich, 2009).

Many authors noticed the antimicrobial activity of the major components of essential oils and examined the possible additive or synergistic effect of the components of essential oil (Imelouane et

al., 2009; Sonboli et al., 2006; Vardar-Unlu et al., 2003). Iten and his colleagues reported the additive antimicrobial effects of the active components of the essential oil of *Thymus vulgaris* (Iten et al., 2009). A synergistic effect of carvacrol and butylated hydroxyanisol was observed against some *Fusarium* species [197] while anethole and polygodial were also effective against filamentous fungi (Himejima and Kubo, 1993; Kubo and Himejima, 1991).

In conclusion, the result indicated the components of essential oil, camphene and borneol, contribute to the antimicrobial activity in synergistic manner against human-pathogenic bacteria. Investigation of combinations of other constituents is needed to study a possible synergistic action.

Chapter 8

Bioactive Compounds of *Siegesbeckia pubescens* (Asteraceae) and their Antimicrobial Activity Alone and in Combination

8.1 Results

8.1.1 Chemical investigation of the active fractions of *Siegesbeckia pubescens*

The extraction of aerial parts of *S. pubescens* with MeOH yielded a crude MeOH extract (85.8 g). The extract was then suspended with MeOH-water and was successively partitioned with n-hexane, CH_2Cl_2, BuOH to give the fraction of n-hexane (17.3 g), CH_2Cl_2 (17.2 g), butanol (24.5 g) and aqueous (10.2 g).

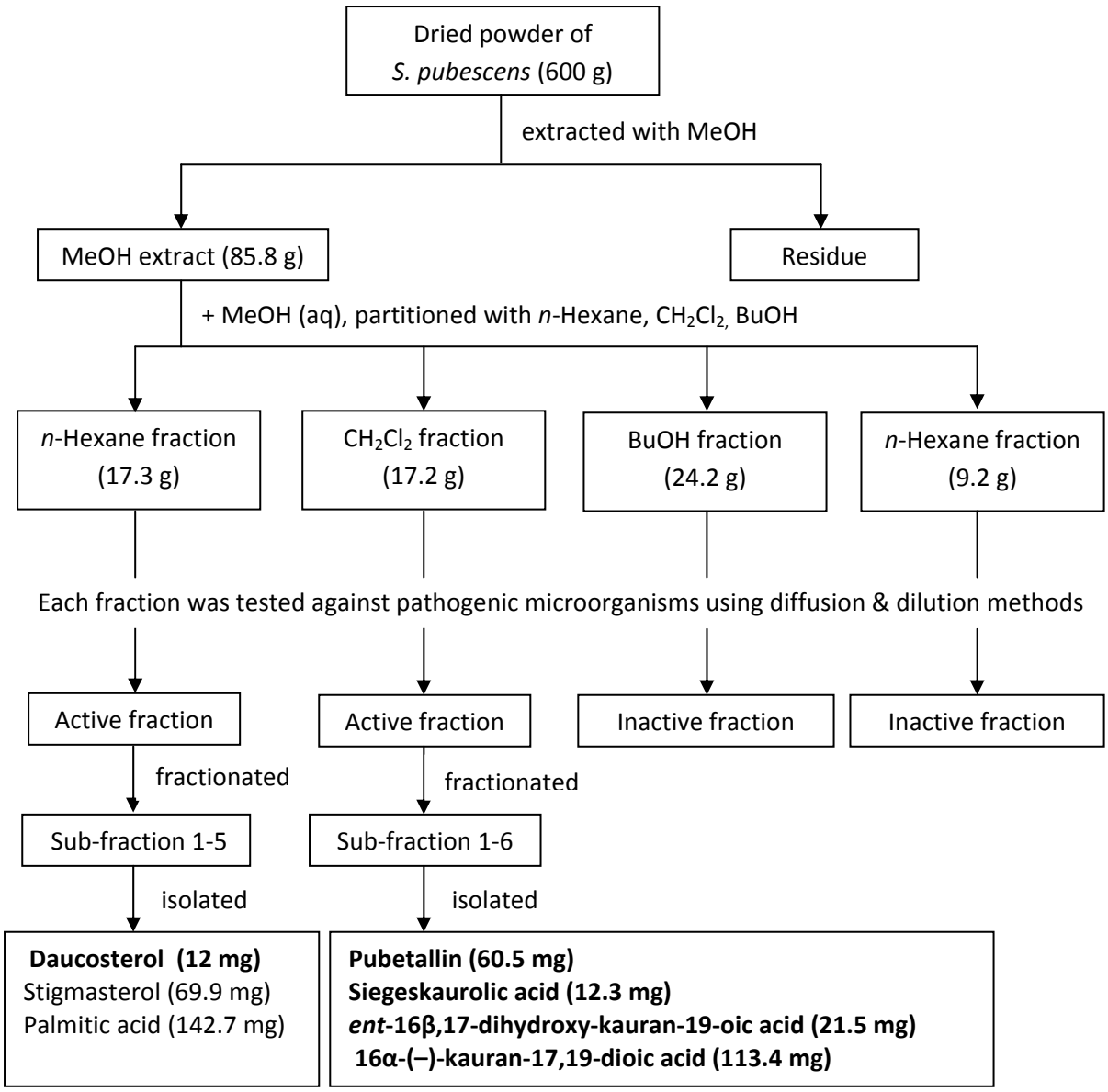


Figure 8.1 Scheme of bioassay guided-fractionation of *Siegesbeckia pubescens.*

Since the hexane and the CH_2Cl_2 fraction were found to be active against bacteria tested, these two fractions were further investigated (Figure 8.1). Fractionation of the hexane fraction (12.4 g) using a normal phase column chromatography (petroleum benzene/CH_2Cl_2 gradient) afforded 3 compounds namely daucosterol (compound 1), stigmasterol, and palmitic acid. Daucosterol was observed to inhibit the bacteria tested, whereas stigmasterol and palmitic acid did not show inhibitory effects.

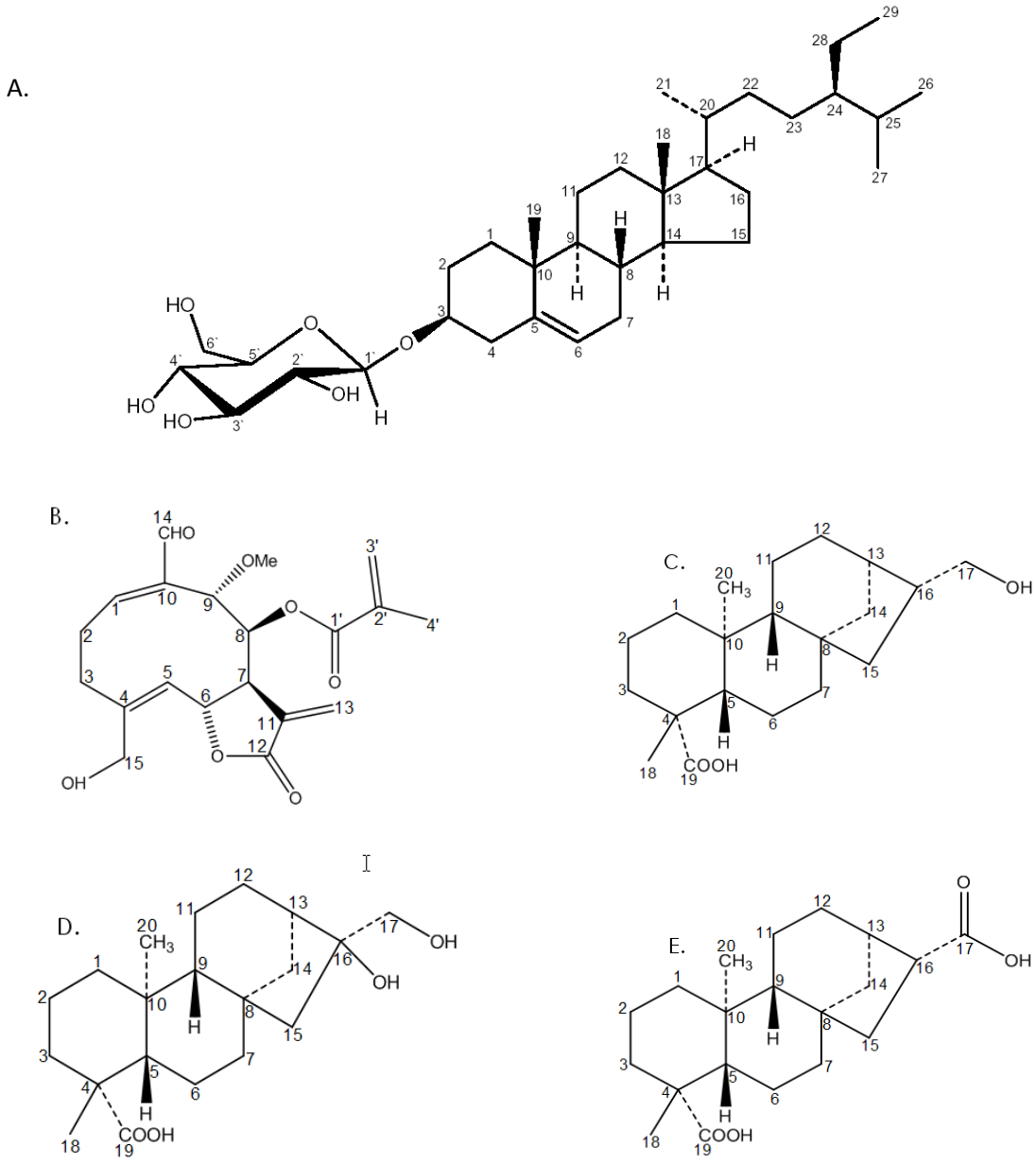


Figure 8.2 The chemical structure of bioactive compounds isolated from *Siegesbeckia pubescens*. A. daucosterol; B. pubetallin; C. siegeskaurolic acid; D. *ent*-16β,17-dihydroxy- kauran-19-oic acid; and E. 16α-(–)-kauran-17,19-dioic acid.

Further fractionation of CH_2Cl_2 fraction using column chromatography with a petroleum benzene/CH_2Cl_2/MeOH gradient as mobile phase confirmed four active compounds. They were identified as pubetallin (compound 2), siegeskaurolic acid (compound 3), *ent*-16β,17-dihydroxy-kauran-19-oic acid (compound 4) and 16α-(-)-kauran-17,19-dioic acid (compound 5). The chemical structures of the isolated compounds are presented in Figure 6.2.

Compound 1, daucosterol or β-sitosterol-3-O-glucoside, has a molecular formula $C_{35}H_{60}O_6$. The EI-MS spectra showed a molecular ion peak at *m/z* at 396.3 (without a glucose moiety). ^{13}C-NMR spectrum revealed that this compound had a double bond at 125 ppm (C-5) and 140 ppm (C-6). The existence of six carbons with chemical shifts of 102.59 ppm (C-1′), 75.36 ppm (C-2′), 78.51 ppm (C-3′), 71.72 ppm (C-4′), 78.11 ppm (C-5′), and 62.86 ppm (C-6′) indicated a glucose molecule attached to β-sitosterol. All of the carbon chemical shifts (^{13}C-NMR and HMBC) were in a close agreement to a previous report (Lendl et al., 2005).

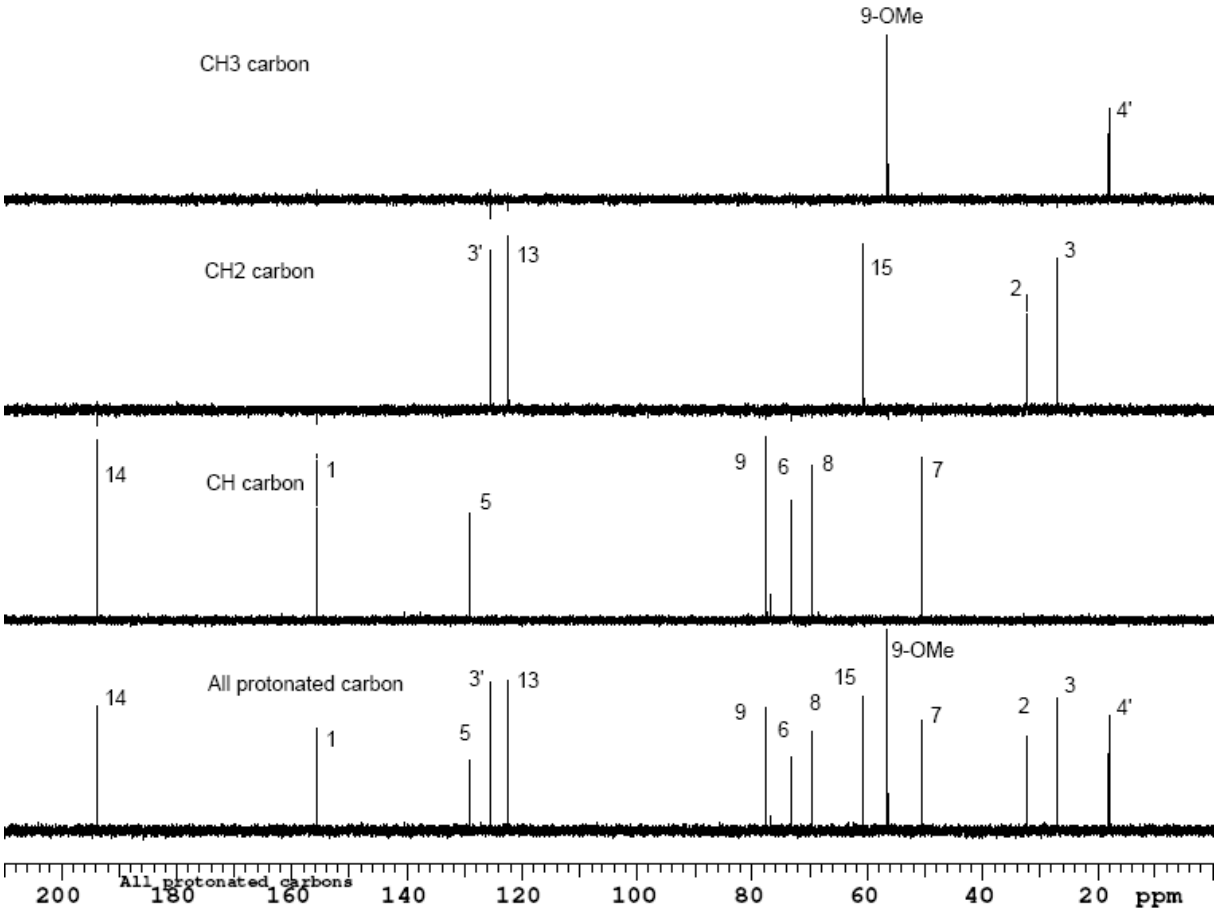


Figure 8.3 Spectra of DEPT experiment of compound 2 (pubetallin).

Compound 2 was isolated as a white crystal. The molecular formula was $C_{20}H_{24}O_7$ with molecular weight 376. The ^{13}C-NMR spectrum (^{1}H decoupled) showed carbon signals for twenty carbon atoms. DEPT experiment (Figure 8.3) confirmed that the compound has one methyl (18.43 ppm, C-4'), one methoxyl group (56.87 ppm, CH3-O-9), two methylene groups (27.50 ppm, C-3 and 32.78 ppm, C-2), one methylene group (61.10 ppm, C-15) adjacent to oxygen, two olefinic methylene groups (122.78 ppm C-13 and 135.96 ppm, C-3´), one methine group (51.08 ppm, C-7), three methine groups (69.96 ppm, C-8; 73.63 ppm, C-6; 78.07 ppm, C-9) adjacent to oxygen, two olefinic methine group (129.60 ppm, C-5 and 156.19 ppm, C-1), and one aldehyde moiety (194.20 ppm, C-14). Additionally, the compound also had six quaternary carbons (four olefinic groups at 125.86, 141.00, 134.00, 139.20 ppm; and two ester carbonyl groups at 166.00 and 169.5 ppm).

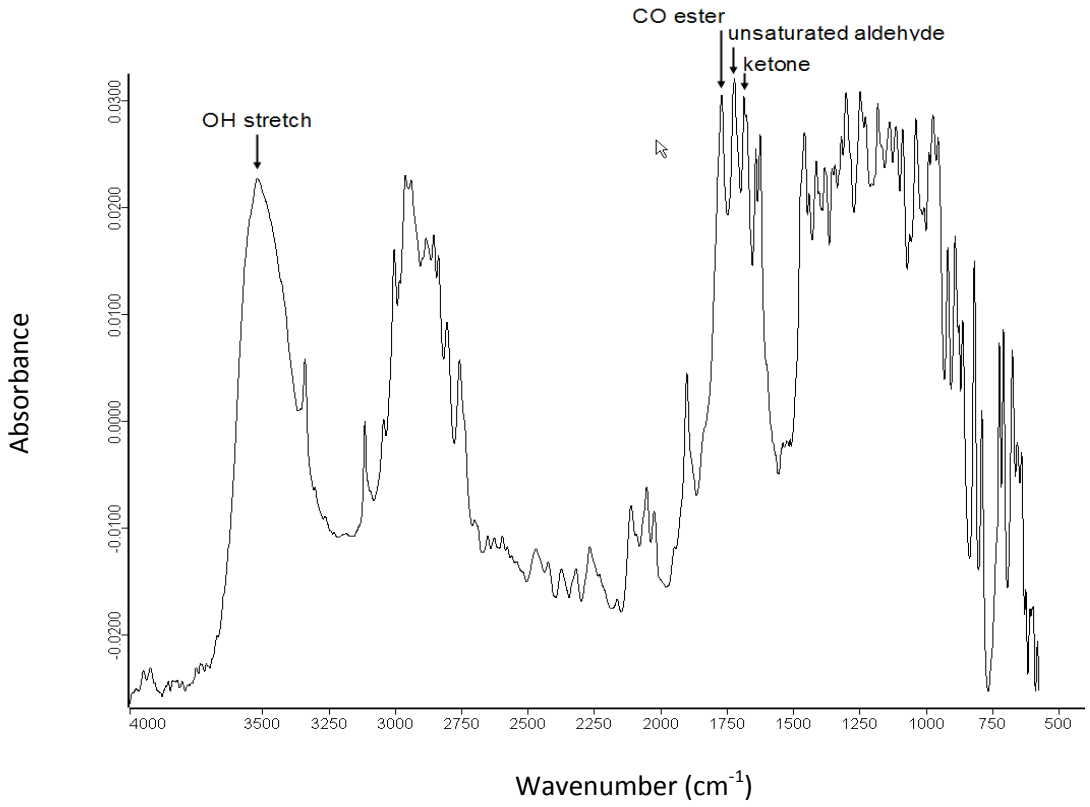


Figure 8.4 Infra red spectrum of compound 2 (pubetallin).

The ^{1}H NMR showed the presence of one methyl (δ 1.60, s), one methoxyl (δ 3.15, s) and four methylene groups of this compound. The downfield chemical shift of H-14 (δ 9.50 ppm) indicated that the 1(10)-double bond was *cis*. The IR spectrum (Figure 8.4) showed the presence of a ketone (1690 cm^{-1}), a hydroxyl (3500 cm^{-1}), ester (1760 cm^{-1}), and an unsaturated aldehyde (C=CCHO, 1713 cm^{-1}). The COSY experiment established the correlation of H1´-H2', H3´-C4', and H8-H9 as shown in Figure 8.6. Based on the data, compound 2 was assigned as pubetallin (Barua et al., 1980; Wang and Hu, 2006).

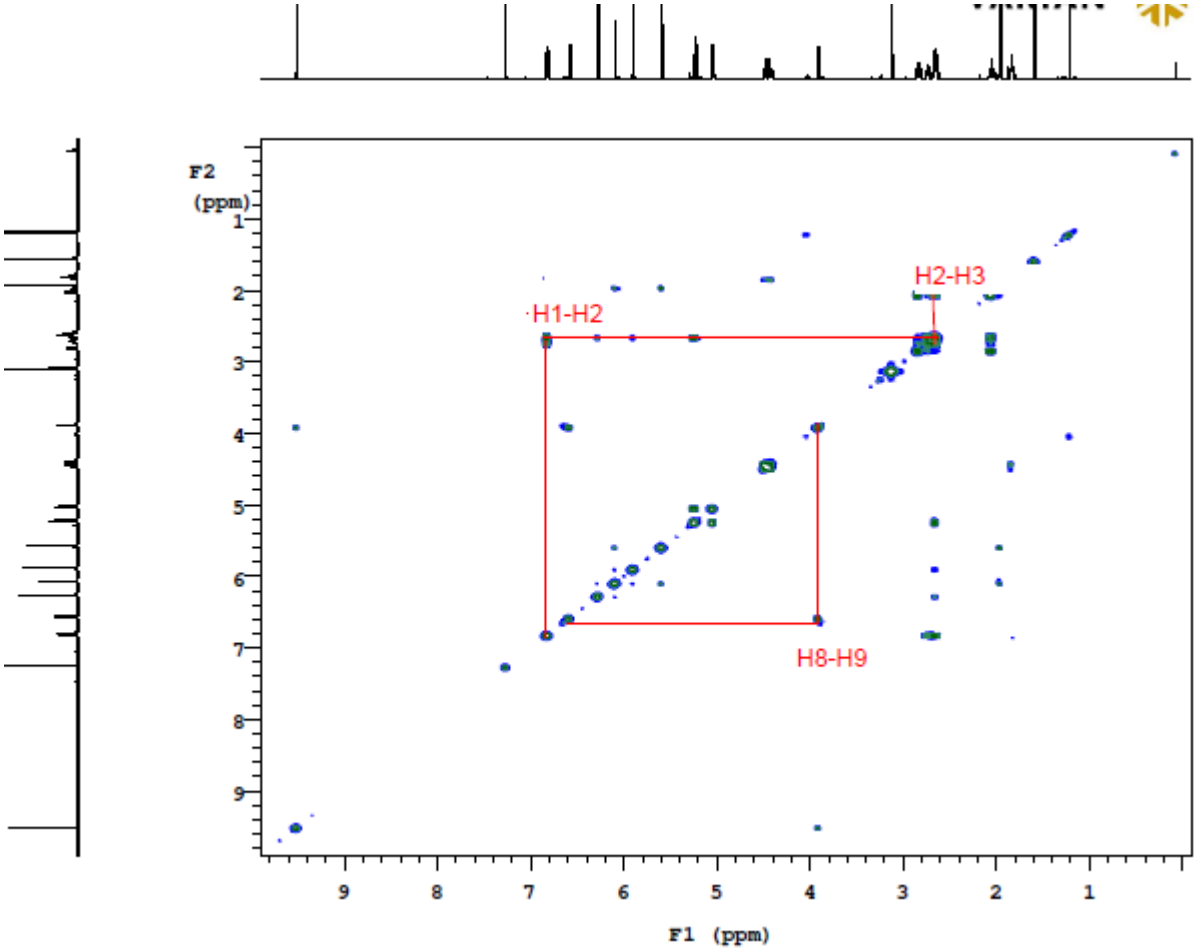


Figure 8.5 COSY experiment of compound 2 (pubetallin).

Compounds 3, 4, and 5 were substantiated as *ent*-kaurane type-diterpenoid compounds. All 3 compounds showed some similarity signals of ^{13}C NMR spectra as presented in Figure 8.3. Based on the spectra, compound 3 showed a quaternary carbon as carboxylic acid (C-19) and one methylene group which is adjacent to oxygen (C-17). Compound 4 had one carboxylic acid (C-19), one methylene groups which is adjacent to oxygen (C-17), and one methine carbon attached to oxygen (C-16). On the other hand compound 5 revealed two carboxyl quaternary carbons as carboxylic acids (C-17 and C-19), but no hydroxyl group was observed.

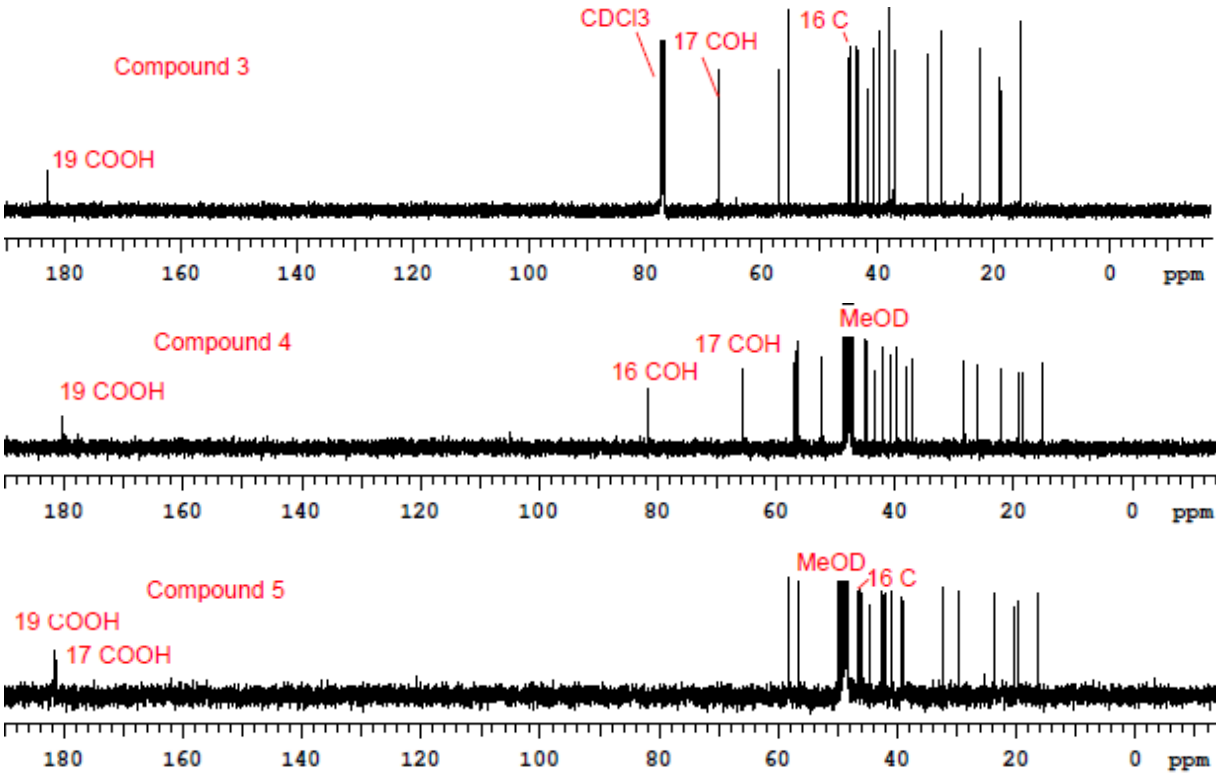


Figure 8.6 ^{13}C NMR spectra of compound 3, 4 and 5.

Compound 3 was isolated as a colorless needle crystal. The mass fragmentation pattern of compound 3 exhibited a molecular ion at *m/z* 320.2 corresponding to its molecular formula $C_{20}H_{32}O_3$. The fragment ions at *m/z* 302 and 289 were observed due to the loss of H_2O and of $–CH_2OH$ group from the molecular ion, respectively. The presence of a fragment ion peak at m/z 274 was characterized as $[M\text{-}HCO_2H]^+$.

^{13}C (DEPT) NMR spectrum revealed two methyl groups (C-18; C-20), nine methylene groups (C-2; C-6; C-11, C-12; C-3; C-14; C-1; C-7; C-15), one methylene group (C-17) adjacent to oxygen, two methyne groups (C-13; C-16, C-5, C-9). The HMBC measurement showed that C-18 coupled to C-19 and C-5. Additionally, COSY experiment (Figure 8.7) confirmed that H-16 coupled to H-17, H-6 coupled to H-7, and H-1 coupled to H-2. On the basis of the obtained data and literatures, compound 3 was identified as *ent*-16αH,17-hydroxy-kauran-19-oic acid which is known as siegeskaurolic acid (Jiang et al., 1992; Park et al., 2007).

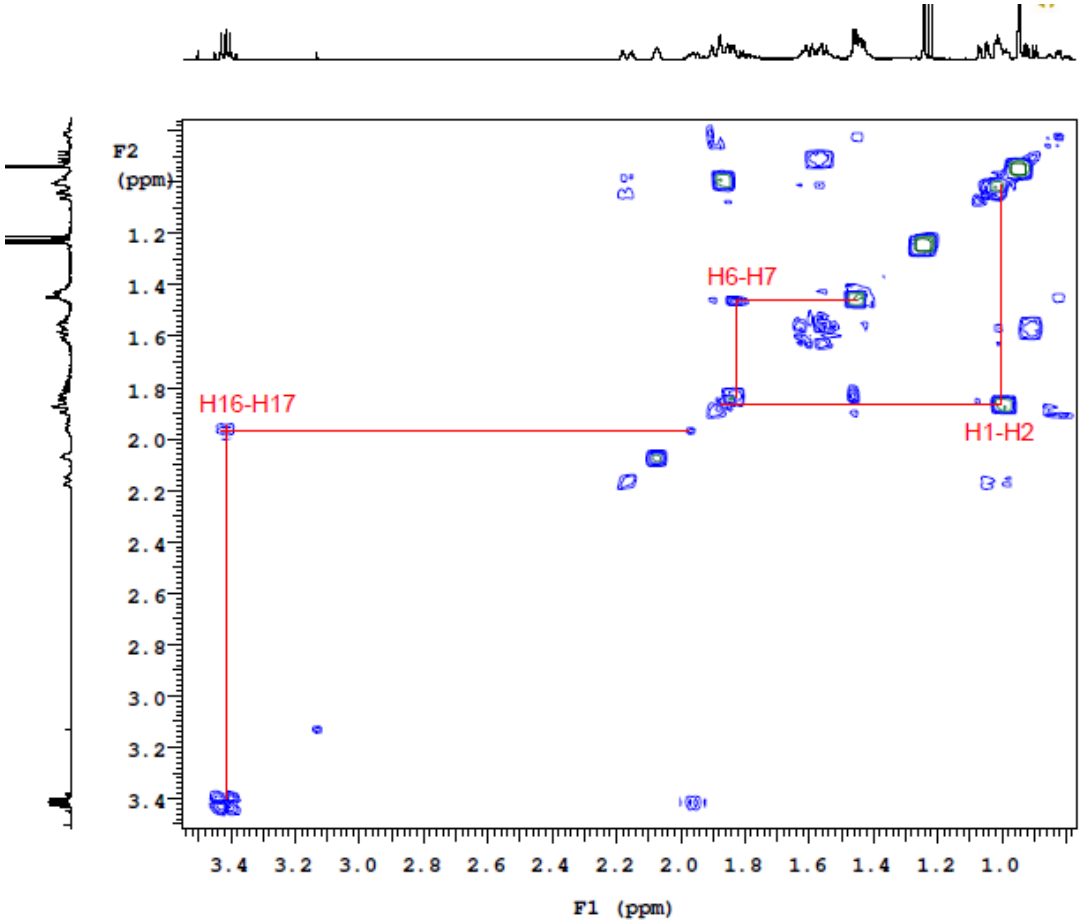


Figure 8.7 COSY experiment of compound 3.

The EI-MS spectrum compound 4 showed a molecular ion peak at *m/z* 336.3 corresponding to the molecular formula $C_{20}H_{32}O_4$. ^{13}C-NMR spectrum of compound 4 (Figure 8.6) revealed twenty carbon signals. A up-shield chemical shift of δ 81.70 ppm (C-16) indicating an oxygenated quaternary carbon. A significant correlation of H-18 and C-19, H-5 and C-18, H-15 and C-16, H-9 and C-10, H-2 and C-3 were confirmed from HMBC experiment. Additionally, COSY experiment (Figure 8.8) established that H-4 and H-5, H-15 and H-16, and H-16 and H-17 were coupled one to another. All obtained data confirmed the substance as *ent*-16β,17-dihydroxy-kauran-19-oic acid (Jiang et al., 1992).

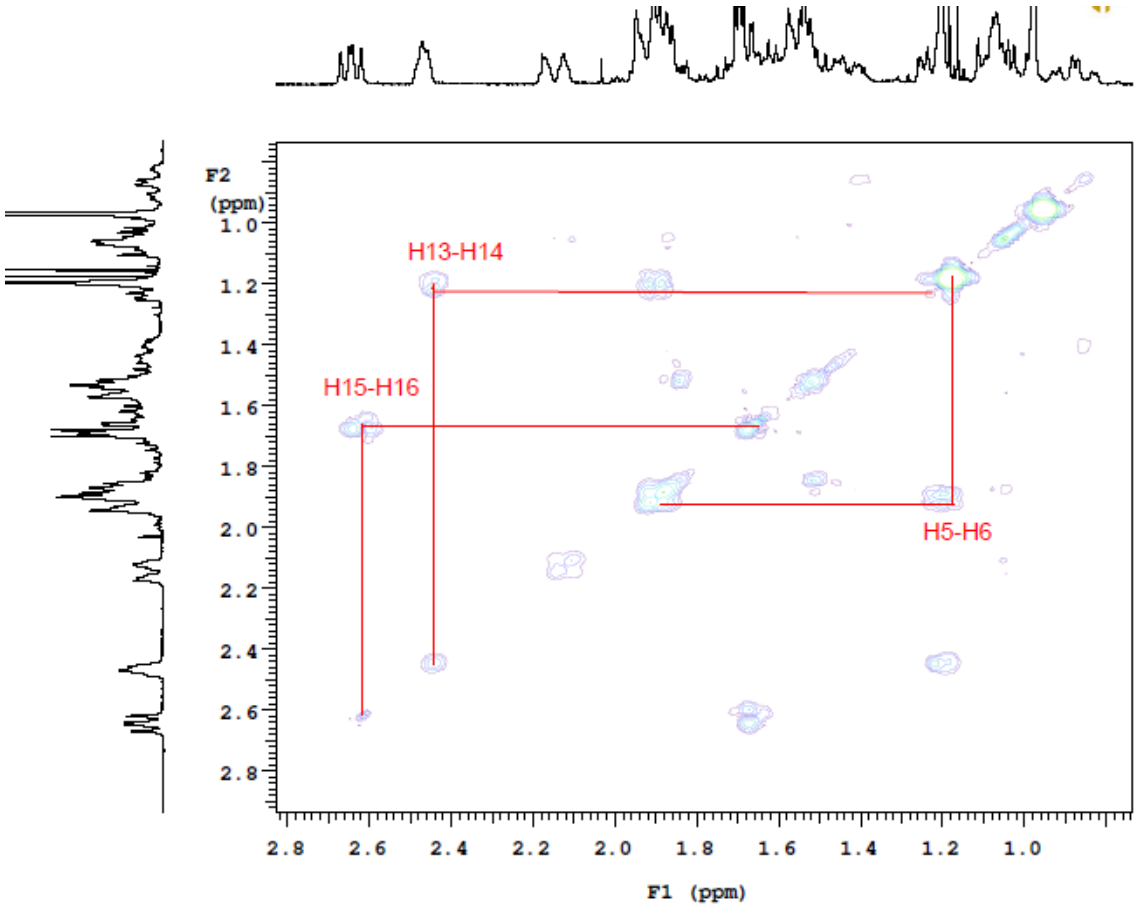


Figure 8.8 COSY experiment of compound 4.

High resolution mass measurement of compound 5 showed a molecular ion at *m/z* 334.23 (M^+) with a molecular formula $C_{20}H_{30}O_4$. The ^{13}C-NMR (Figure 8.6) showed compound 4 possessed 2 carboxylic acids groups that are at C-17 (179.93 ppm) and C-19 (180.46 ppm). Long-range couplings from H-16 to C-17, H-3 and C-19, H-20 and C-9 as well as H-20 and C-1 clearly established. The COSY experiment (Figure 8.9) revealed expected correlations between H-5 and H-6, H-13 and C-14, and between H-15 and H-16. All obtained data was in accordance with a previous report and confirmed compound 5 as 16α-(–)-kauran-17,19-dioic acid (Gao et al., 1985).

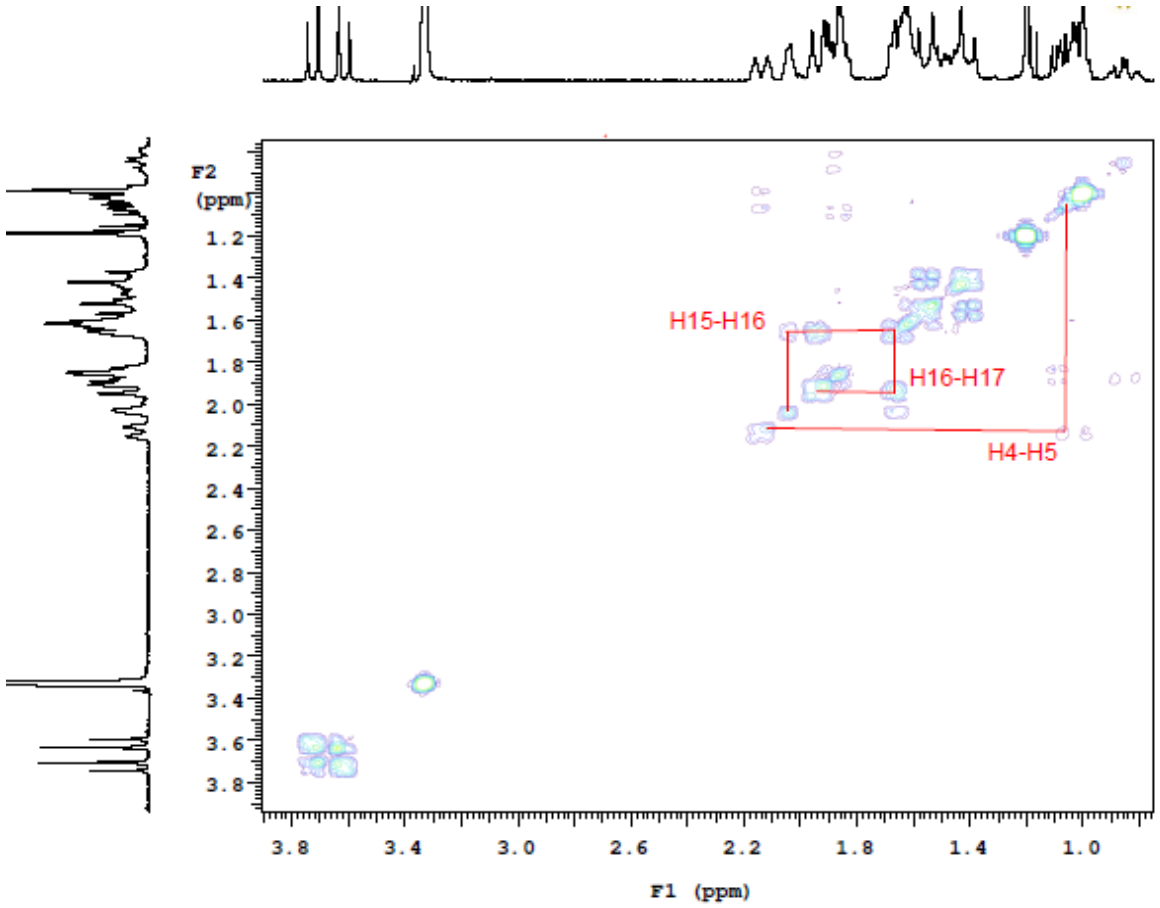


Figure 8.9 COSY experiment of compound 5.

The complete interpretation of ^{13}C-NMR and or ^{1}H-NMR spectra from compound 1-5 are further described as below:

Daucosterol (1) (Lendl et al., 2005). White powder, molecular formula $C_{35}H_{60}O_6$ with EI-MS *m/z* 396.3. ^{13}C-NMR (500 MHz, C_6D_6): 36.96 (C-1), 30.27 (C-2), 78.63 (C-3), 37.49 (C-4), 140.92 (C-5), 121.36 (C-6), 32.07 (C-7), 32.18 (C-8), 50.36 (C-9), 36.94 (C-10), 23.4 (C-11), 39.35 (C-12), 42.49 (C-13), 56.83 (C-14), 23.40 (C-15), 28.54 (C-16), 56.26 (C-17), 11.98 (C-18), 19.98 (C-19), 36.39 (C-20), 19.02 (C-21), 34.22 (C-22), 26.40 (C-23), 46.06 (C-24), 29.48 (C-25), 19.43 (C-26), 21.47 (C-27), 24.52 (C-28), 12.17 (C-29), 102.59 (C-1´), 75.36 (C-2´), 78.51(C-3´), 71.72 (C-4´), 78.11 (C-5´), 62.86 (C-6´).

Pubetallin (2) (Wang and Hu, 2006). Colorless crystal needle, molecular formula $C_{20}H_{24}O_7$ with EI-MS *m/z* 376 $[M]^+$. ^{1}H-NMR (300MHz, $CDCl_3$) δ: 6.60 (H-1), δ: 2.05; 2.85 (H-2), δ: 2.70; 2.80 (H-3), δ: 5.05 (H-5), δ: 5.30 (H-6), δ: 2.70 (H-7), δ: 6.60 (H-8), δ: 3.90 (H-9), δ: 5.90;6.30 (H-13), δ: 9.50 (H-14), δ: 4.45;4.50 (H-15), δ: 6.60 (9-O-Me), δ: 6.80 (H-3'), δ: 1.20 (H-4'). ^{13}C-NMR (300 MHz, $CDCl_3$) δ: 156.19 (C-1), δ: 32.77 (C-2), δ: 27.50 (C-3), δ: 141.00 (C-4), δ: 129.60 (C-5), δ: 73.63 (C-6), δ: 51.08 (C-7), δ: 69.96 (C-8), δ: 78.07 (C-9), δ: 139.20 (C-10), δ: 134.00 (C-11), δ: 169.50 (C-12), δ: 122.78 (C-13), δ: 194.20 (C-14), δ: 61.10 (C-15), δ: 56.87 (9-O-Me), δ: 166 (C-1'), δ: 125.86 (C-2'), δ: 135.96 (C-3'), δ: 18.43 (C-4').

***Ent*-16αH,17-hydroxy-19-kauranoic acid as reported (3)** (Jiang et al., 1992; Park et al., 2007). Colorless needles, $C_{20}H_{32}O_3$ with molecular weight 320.2. EI-MS, *m/z* (rel. int.): 320.2 $[M]^+$ (22), 302 $[M\text{-}H,O]^+$ (75), 289 $[M\text{-}CH,OH]^+$ (7), 284 (17), 274 $[M\text{-}HCO,H]^+$ (80), 261 (20), 243 (20), 123 (78) and 109 (base peak). ^{1}H-NMR (300MHz, $CDCl_3$) δ: 1.90;0.84 (H-1), δ: 1.46; 1.90 (H-2), δ: 1.88; 1.90 (H-3), δ: 1.14 (H-5), δ: 1.82 (H-6), δ: 1.46 (H-7), δ: 1.58 (H-8), δ: 1.12 (H-9), δ: 1.95 (H-10), δ: 1.60 (H-11), δ: 1.42; 1.58 (H-12), δ: 2.10 (H-13), δ: 2.18: 2.20 (H-14), δ: 0.98; 1.58 (H-15), δ: 1.96 (H-16), δ: 3.41 (H-17), δ: 1.24 (H-18), δ: 9.5 (H-19), δ: 0.96 (H-20). ^{13}C-NMR (300 MHz, $CDCl_3$) δ: 40.75 (C-1), δ: 19.10 (C-2), δ: 37.21 (C-3), δ: 43.39 (C-4), δ: 56.99 (C-5), δ: 22.39 (C-6), δ: 41.65 (C-7), δ: 44.76 (C-8), δ: 55.33 (C-9), δ: 39.63 (C-10), δ: 18.88 (C-11), δ: 31.40 (C-12), δ: 38.18 (C-13), δ: 37.89 (C-14), δ: 45.02 (C-15), δ: 43.65 (C-16), δ: 67.56 (C-17), δ: 28.96 (C-18), δ: 182.98 (C-19), δ: 15.57 (C-20).

***Ent*-16β,17-dihydroxy-19-kauranoic acid (4)** (Jiang et al., 1992). This substance is a white amorphous crystal with molecular weight: 336.23 ($C_{20}H_{32}O_4$). ^{13}C-NMR (300 MHz, CD_3OH), δ: 40.75 (C-1), δ: 19.08 (C-2), δ: 37.02 (C-3), δ: 45.04 (C-4), δ: 56.67 (C-5), δ: 22.17 (C-6), δ: 42.13 (C-7), δ: 44.59 (C-8), δ: 56.18 (C-9), δ: 39.66 (C-10), δ: 18.40 (C-11), δ: 26.06 (C-12), δ: 43.47 (C-13), δ: 38.01 (C-14), δ: 52.55 (C-15), δ: 81.70 (C-16), δ: 65.66 (C-17), δ: 28.26 (C-18), δ: 180.44 (C-19), δ: 15.06 (C-20).

16α-(–)-kauran-17,19-dioic acid (5) (Gao et al., 1985)**.** White powder with molecular weight: 334.3 ($C_{20}H_{30}O_4$). EI-MS *m/z* 334, 316, 288. ^{13}C-NMR (300 MHz, $CDCl_3$), δ: 39.59 (C-1), δ: 18.46 (C-2), δ: 37.75 (C-3), δ: 44.55 (C-4), δ: 56.98 (C-5), δ: 22.52 (C-6), δ: 41.23 (C-7), δ: 45.09 (C-8), δ: 55.39 (C-9), δ: 38.02 (C-10), δ: 19.15 (C-11), δ: 28.30 (C-12), δ: 41.49 (C-13), δ: 40.89 (C-14), δ: 43.46 (C-15), δ: 45.51 (C-16), δ: 179.93 (C-17), δ: 31.08 (C-18), δ: 180.46 (C-19), δ: 15.03 (C-20).

8.1.2 Antimicrobial activity of the fractions and the isolated compounds of *S. pubescens*

8.1.2.1 Antimicrobial activity of the fractions of *S. pubescens*

The result of antimicrobial activity of all four fractions of *S. pubescens* determined by diffusion and microdilution methods is summarized in Table 8.1. The hexane and the CH_2Cl_2 fractions were found to be active as antibacterial agents, whereas butanol and aqueous fractions were not effective. The diffusion method exhibited that the hexane fraction inhibited the tested bacteria with diameter of inhibition zones ranging from 7.0 to 14.7 mm, whereas the CH_2Cl_2 fraction resulted inhibition zones between 7.7 and 15.0 mm. The microdilution method confirmed that the hexane fraction showed antimicrobial activity with MIC value of between 15 and 8000 µg/ml and for the CH_2Cl_2 fraction had MIC values ranging from 15 to 4000 µg/ml.

Streptococci were observed to be the most susceptible strains toward the hexane and CH_2Cl_2 fractions. The hexane fraction exhibited inhibition against *S. agalactiae* and *Streptococcus oralis* with the MIC value of 15 µg/ml. Also, the CH_2Cl_2 fraction showed a good activity against *S. pyogenes* (MIC: 15 µg/ml). With exception of *A. baumannii*, however, all fractions showed no inhibition against Gram-negative bacteria (*E. coli* and *P. aeruginosa*) and yeasts (*C. albicans* and *C. glabrata*) up to the highest concentration of 8000 µg/ml.

Table 8.1

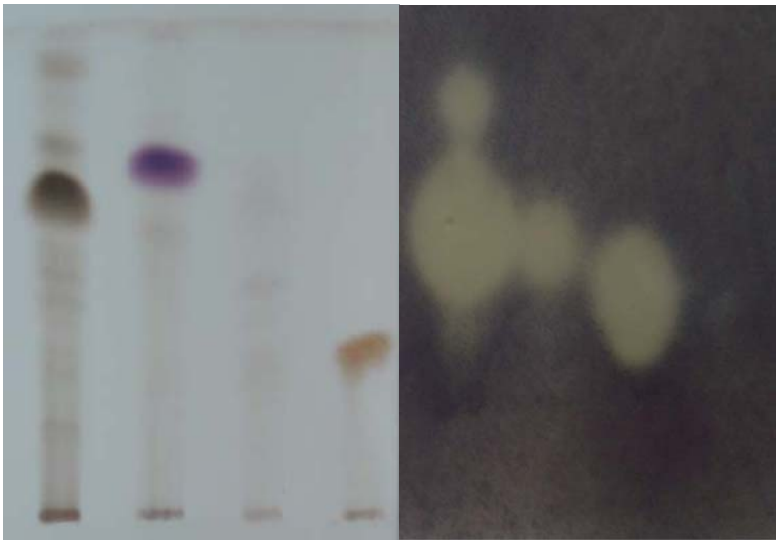
Antimicrobial activities of *n*-hexane, CH_2Cl_2, butanol and aqueous fractions of *S. pubescens*

No	Microorganisms	n-Hexane		CH_2Cl_2		BuOH		aqueous	
		ZI	MIC	ZI	MIC	ZI	MIC	ZI	MIC
	Gram-positive								
1	MRSA NCTC 10442	7.3 ± 0.6	1000	12.7 ± 0.6	500	NI	NT	NI	NT
2	MRSA clincal isolate	NI	8000	13.7 ± 0.6	1000	NI	NT	NI	NT
3	MRSA clinical isolate	8.3 ± 0.0	8000	12.3 ± 0.6	500	NI	NT	NI	NT
4	*Staphylococcus epidermidis* ATCC 14990	9.3 ± 1.2	250	15.3 ± 0.6	250	NI	8000	NI	NT
5	*Staphylococcus aureus* ATCC 29213	9.7 ± 1.2	1000	12.0 ± 0.0	500	NI	NT	NI	NT
6	*Staphylococcus saprophyticus ATCC* 15305	NI	1000	10.0 ± 0.0	1000	NI	NT	NI	NT
7	*Streptococcus oralis ATCC 3507*	10.0 ± 0.0	15	12.0 ± 0.0	62	NI	NT	NI	NT
8	*Streptococcus pyogenes ATCC 12344*	13.0 ± 0.0	30	15.0 ± 0.0	15	NI	NT	NI	NT
9	*Streptococcus agalactiae* ATCC 27956	7.0 ± 0.0	15	12.0 ± 0.0	62	NI	NT	NI	NT
10	*Bacillus subtilis* ATCC 6051	14.7 ± 1.2	30	7.7 ± 0.0	250	NI	NT	NI	NT
	Gram-negative								
11	*Pseudomonas aeruginosa* ATCC 27853	NI	NT	NI	NI	NI	NT	NI	NT
12	*Escherichia coli* ATCC 25922	NI	NT	NI	NI	NI	NT	NI	NT
13	*Acinetobacter baumannii*	NI	4000	NI	4000	NI	4000	NI	8000
	Fungi								
13	*Candida albicans* ATCC 90028	NI	NT	NI	NI	NI	NT	NI	NT
14	*Candida glabrata* ATCC MYA 2950	NI	NT	NI	NT	NI	NT	NI	NT

NI: no inhibition, NT: not tested

Unit of ZI (zone inhibition): mm

Unit of MIC (minimal concentration inhibition): µg/ml



1 2 3 4 1 2 3 4

Figure 8.10 Bioautogram of CH_2Cl_2 fractions of *S. pubescens* developed with MeOH: CH_2Cl_2 (9:1). (*left*: chromatogram; *right*: bioautogram; 1-4: fraction 1-4, respectively). Clear zones indicate an antimicrobial activity of the tested fractions.

Bioautography of the active fractions revealed some clear zones on a purple background indicating the antimicrobial activity of these fractions (Figure 8.10). Bioassay-guided fractionation of the hexane fraction led to isolation of a bioactive compound namely β-sitosterol-glucoside or daucosterol (compound 1); and of CH_2Cl_2 fraction was isolated pubetallin (compound 2), siegeskaurolic acid (compound 3), *ent*-16β,17-dihydroxy- kauran-19-oic acid (compound 4), and 16α-(–)-kauran-17,19-dioic acid (compound 5).

8.1.2.2 Antimicrobial activity of isolated compounds of *S. pubescens*

Table 8.2
Minimum inhibitory concentration (MIC) of isolated compounds of *Siegesbeckia pubescens* determined by microdilution method.

Microorganisms	Compounds [MIC in mM (µg/ml)]				
	1	**2**	**3**	**4**	**5**
Gram positive					
MRSA *S. aureus* NCTC 10442	NI	1.3 (500)	3.2 (1000)	3.0 (1000)	NI
MRSA *S. aureus* (clinical isolate)	NI	2.7 (1000)	3.2 (1000)	3.0 (1000)	NI
MRSA *S. aureus* (clinical isolate)	NI	2.7 (1000)	3.2 (1000)	3.0 (1000)	NI
Staphylococcus aureus ATCC 29213	NI	1.3 (500)	1.6-3.2 (500-1000)	3.0 (1000)	NI
Staphylococcus epidermidis ATCC 14990	NI	2.7 (1000)	3.2 (1000)	3.0 (1000)	NI
Staphylococcus saprophyticus ATCC 15305	NI	1.3 (500)	1.6 (500)	3.0 (1000)	NI
Streptococcus oralis ATCC 3507	0.25 (250)	0.7 (250)	0.2 (60)	1.5-3.0 (500-1000)	NI
Streptococcus pyogenes ATCC 12344	0.25 (250)	1.3 (500)	0.2 (60)	3.0 (1000)	NI
Streptococcus agalactiae ATCC 27956	0.9 (250)	2.7 (1000)	0.4-0.8 (125-250)	1.5 (500)	NI
Bacillus subtilis ATCC 6051	0.9 (1000)	2.7 (1000)	1.6 (500)	1.5-3.0 (500-1000)	NI
Gram-negative					
Pseudomonas aeruginosa ATCC 27853	NI	2.7 (1000)	3.2 (1000)	NI	NI
Escherichia coli ATCC 25922	NI	NI	NI	NI	NI
Acinetobacter baumannii	(0.9) 1000	2.7 (1000)	NT	3.0 (1000)	3.0 (1000)
Fungi					
Candida albicans ATCC 90028	NI	NI	NI	NI	NI
Candida glabrata ATCC MYA 2950	NI	NI	NI	NI	NI

NI: No inhibition, NT: not tested; 1: daucosterol; 2: pubetallin, 3: Siegeskaurolic acid, 4: *ent* 16α,17-dihydroxy-kauran-19-oic acid; 5: 16α-(–)-kauran-17,19-dioic acid

Table 8.2 summarizes the MIC data of compound 1–4 obtained by microdilution method. All the isolated compounds were active against Gram-positive bacteria; and *S. oralis* was the most susceptible strain with a variable degree of susceptibility. However, the compounds did not show inhibition against yeasts (*C. albicans* and *C. glabrata*).

Compound 2 was observed to be the most active compound among the isolated compounds against staphylococci. Compound 2 exerted a good activity against *S. aureus* as well as MRSA NCTC 10442 with the MIC value of 500 µg/ml or 1.3 mM. On the other hand, compound 3 revealed the most pronounced activity against streptococci. It inhibited the growth of *S. oralis* and *S. pyogenes* at MIC value of 60 µg/ml or 0.2 mM. The anti-MRSA activity of compound 3 was slightly lower compared to either compound 2 or compound 4. All strains of MRSA were inhibited by compound 4 at concentration 1000 µg/ml or 3 mM. In addition, compound 4 exhibited an antimicrobial activity against *S. aureus*, *S. epidermidis*, *S. saprophyticus* with MIC value of 1000 µg/ml. Compound 1 exhibited a marked antibacterial activity against streptococci, *B. subtilis* and *A. baumannii* with MIC values between 250 and 1000 µg/ml. To inhibit the other test microorganisms, compound 1 needed higher concentration more than 1000 µg/ml. Compound 5 showed less inhibitory activity compared to the other isolated compounds. No inhibition was observed from compound 5 against all organisms tested up to the highest concentration 1000 µg/ml, with exception of *A. baumannii*. Most the isolated compounds showed an inhibition against *A. baumannii*. Anti-pseudomonas activity was shown by compounds 2 and 3 with MIC value of 2.7 and 3.2 mM, respectively; however, no compounds showed activity against *E. coli*.

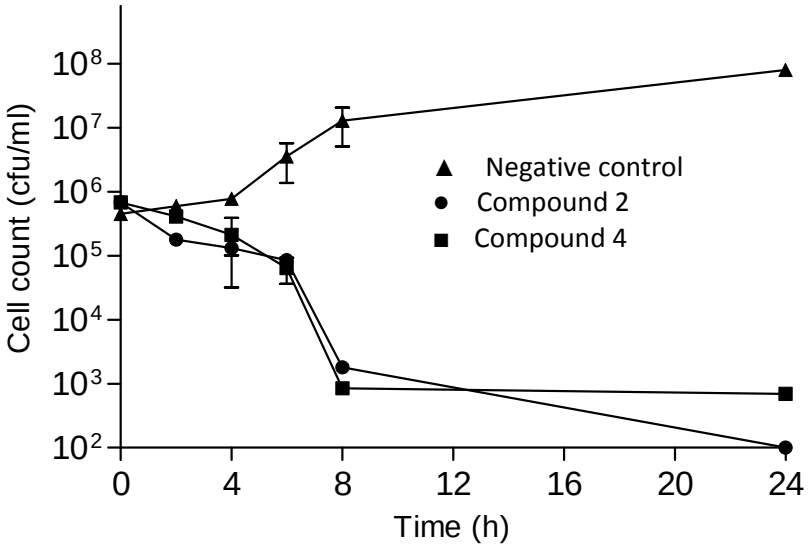


Figure 8.11 Time-kill curve of compound 2 (pubetallin) and compound 4 (16β,17-dihydroxy-kauran-19-oic acid) at MIC against *Streptococcus pyogenes.*

8.1.3 Time-kill experiment

Time kill assay was performed to study the kinetic of bacterial inhibition after contact with isolated compounds at certain time periods. In this experiment, compound 2 and 4 were tested at MIC value against *S. pyogenes* in comparison to the negative control (DMSO). The killing curve (Figure 8.11) showed that the growth of *S. pyogenes* without any treatment (negative control) increased by the time of incubation. Both compounds decreased about $3\log_{10}$ viable cells after 8 h incubation. After 24 h incubation, no viable cell of *S. pyogenes* treated by compound 2 was detected; and about 10^3 cfu/ml was found from the plate treated by compound 4. This result showed that compound 2 exhibited a bactericidal effect and compound 4 showed a bacteriostatic effect.

8.1.4 Effect of combination of isolated bioactive compounds

To study a potential synergistic or additive interaction between the isolated compounds, two isolated compounds were applied in combination. As the amount of isolated compounds was limited, it was only possible to investigate combination of pubetallin (compound 2) and 16β,17-dihydroxy-kauran-19-oic acid (compound 4) against MRSA using the checkerboard assay.

Table 8.3

Result of the checkerboard assay of pubetallin and *ent*-16β,17-dihydroxy-19-kauranoic acid against MRSA. Ratio of pubetallin and *ent*-16β,17-dihydroxy-19-kauranoic acid (C2/C4), concentration of pubetallin (C2) and *ent*-16β,17-dihydroxy-19-kauranoic acid (Cin) in mg/ml, and FICI values. The interaction as reflected by FICI values is considered to be synergistic at ≤ 0.5, additive at > 0.5–1, indifferent at >1–4.0, and antagonistic at > 4.0.

C2/C4	(C2)	(C4)	FIC (C2)	FIC (C4)	FICI	Interaction
1/0	500	-	-	-	-	-
8/1	400	100	0.80	0.10	0.9	Additive
1/1	250	250	0.5	0.25	0.75	Additive
1/2	125	250	0.25	0.25	0.5	Synergistic
1/4	100	400	0.20	0.40	0.6	Additive
1/8	62.5	500	0.12	0.50	0.62	Additive
0/1	-	1000	-	-	-	-

C2: pubetallin, C4: *ent*-16β,17-dihydroxy-kauran-19-oic acid

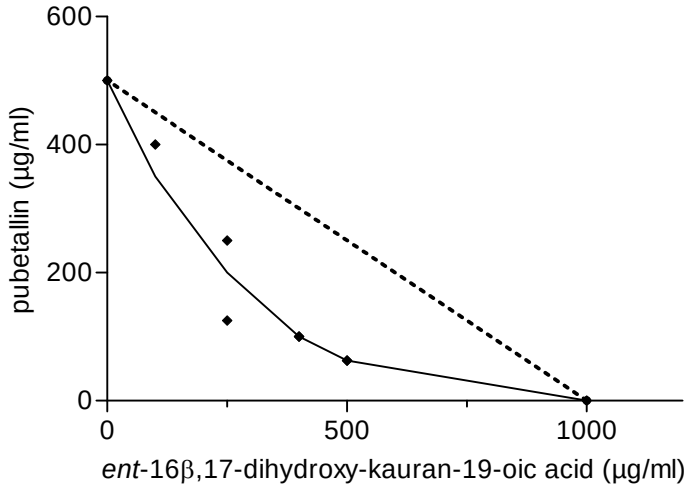


Figure 8.12 Isobologram depicting the effect of pubetallin and *ent*-16β,17-dihydroxy-kauran-19-oic acid against MRSA.

The result of the checkerboard assay showed the combinations between compound 2 and compound 4 resulted FIC indexes ranging from 0.5 to 0.9 against MRSA. With exception of 1:2 dose pair ratio, additive interactions (0.5<FIC<1) were observed in most the dose pair combinations. The combination of the 1:2 dose pair ratio had four-fold reduced the MIC value; and has generated a synergistic interaction (Table 8.3). Furthermore, the isobologram (Figure 8.2) displayed a concave curve indicating the enhancement of antimicrobial effect of the combinations.

8.3 Discussion

Isolation and identification of bioactive compounds of aerial parts from *S. pubescens* afforded 5 compounds with different degrees of antimicrobial activity. All of the isolated compounds are terpenoids specifically sesquiterpene lactone and diterpenes. Terpenoids have well known function as defence compounds, a chemical defence in plants against herbivore and microbial attacks. Prior works have shown that sesquiterpene and kaurane-type diterpene exhibit other biological properties, including anti-HIV, antifungal, antiparasitic, antidiabetic, antihyperlipidemic, anti-inflammatory and antinoniceptive activities (Barrero et al., 2000; Chang et al., 1998; Jiang et al., 1992; Kim et al., 2006; Park et al., 2007; Wu et al., 1996). To the best of our knowledge, the antimicrobial activities of the fractions of *S. pubescens* as well as the isolated compounds are being reported for the first time.

Compound 1, daucosterol, is a steroid saponin which is amphipilic. It had a good activity against *Streptococcus* ssp., but needed higher concentration to inhibit *Staphylococcus* ssp. The possible mode of action of daucosterol is by disturbance of biomembrane. The lipophilic moiety of this compound solubilizes in the membrane bilayer, whereas the hydrophilic sugar part remains outside

and interact with glycoprotein or glycolipid. This interaction generate pores and makes cells leaky (Wink, 2006).

Compound 2, pubetallin, exerted a marked inhibitory activity against the tested microorganisms. As a sesquiterpene lactone, pubetallin has an exocyclic methylen group which is very reactive. The exocyclic methylen group can alkylate protein and then disturb conformation and recognition processes of protein. Additionally, such alkylations may protect proteins from proteolytic activity or can also inhibit the access of substrates or ligands to binding at a protein (Wink, 2008a; Wink, 2008b). The exocyclic methylene group of sesquiterpene lactone seems to be responsible to antimicrobial activity (Rodriguez et al., 1976). However, a chemical structure-antimicrobial activity relationship has not been reported (Barrero et al., 2000).

Compound 3–5 (*ent*-kaurane type diterpenoids) exhibited a varied level of inhibitory effect toward pathogenic bacteria. In this present investigation, compound 3 was found to be active inhibiting bacterial growth. A previous study reported compound 3 had anti-inflammatory and antinoniceptive properties via COX-2 and TNF-α inhibition (Park et al., 2007). Taken together, these activities support treatment of infectious diseases. The antimicrobial effect of compound 5 was lower compared to the other isolated compounds. It seems that the presence of hydroxyl groups of *ent*-kaurane compounds is necessary for the antimicrobial action. In addition, compound 5 has carboxylic acid groups which possibly give very strong negative charges to the molecule so that it is difficult to penetrate the biomembrane (Wink, 2008a). Our finding demonstrates that the more hydrophilic the compounds, the lower the antimicrobial activity. This result was in agreement with a previous paper showing that increasing the hydrophilicity of kaurene diterpenoids reduced their antimicrobial activity (Mendoza et al., 1997).

A membrane disruption could be suggested as one of the likely mode of action of terpenoid such as compound 1–5 (Cowan, 1999). Sesquiterpene and diterpene compounds possess lipophilic properties and are able to be dissolve in biomembranes. They influence the environment of membrane proteins and thus change their conformation and bioactivity. If membrane are exposed at higher concentration to terpenoids, membrane permeability were be disturbed (Wink, 2003).

Our result was in accordance with some previous studies on derivatives of kaurane-type diterpenoid that exhibited activity against Gram-positive, but no interesting effect against Gram-negative bacteria (Mendoza et al., 1997; Urzúa et al., 2006). In this investigation, all isolated compounds were active against Gram-positive bacteria including MRSA. They showed a weak activity against Gram-negative bacteria, however *A. baumannii* was susceptible to all isolated compounds.

In this result, most antimicrobial activities of single isolated compounds were lower than those of the fractions. Thus, all the chemical constituents might be generate the antimicrobial properties of

fraction and or extract by an additive or even synergistic interaction. Some previous studies also reported the superiority of pharmacological effect of some herbal extracts as compared to the single constituents. For example, extracts of *B. aquifolia* and *B. repens* have a better antimicrobial effect as compared with pure berberine alone. The activity of the alkaloid berberine was potentiated by another constituent, 5'-methoxy-hydnocarpin, which does not have antimicrobial effect by itself (Stermitz et al., 2000).

It is interesting that the combination of pubetallin and *ent*-16β,17-dihydroxy-19-kauranoic acid produced a potentiated effect. Most the ratio combinations demonstrated additive interactions (Matsumura et al., 1999) but a synergistic interaction was shown in 1:2 dose pair combination. The lipophilic compounds can submerge into the membrane, interact with the lipophilic side chains of phospholipids or cholesterol, and therefore can disturb three dimensional conformation (Wink, 2008a). Alternatively, the single constituents of a mono-extract or multi-extract combinations are able to affect in multi targets, and act together in an synergistic way (Wagner and Ulrich-Merzenich, 2009).

In conclusion, the antimicrobial action of *S. pubescens* was mainly attributed by the terpenoids. The sesquiterpene pubetallin and the diterpene *ent*-16β,17-dihydroxy-19-kauranoic acid applied in combination demonstrated a potentiated interaction. Further investigation to evaluate the efficacy of these compounds is warranted.

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