SERRULATANES FROM EREMOPHILA NEGLECTA: THEIR SPECTRUM OF ANTIBACTERIAL ACTIVITY, CYTOTOXICITY AND MODE OF ACTION.

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A thesis submitted for the degree of

Masters by Research

School of Pharmacy and Medical Sciences

University of South Australia

September 2010

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Abbreviations

The following abbreviations have been used throughout this thesis:

ATCC	American Type Culture Collection
CO_2	Carbon dioxide
CDC	Centers for Disease Control and Prevention
CTAB	Cetyltrimethylammonium bromide
CLSI	Clinical and Laboatory Standard Institute
CFU	Colony Forming Units
CI	Confidence interval
DMAPP	Dimethylallyl Pyrophosphate
DMSO	Dimethylsulfoxide
HDMEM	Dulbecco's Modified Eagle Medium
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal Bovine Serum
IDSA	Infectious Diseases Society of America
IMVS	Institute of Medical and Veterinary Sciences
IPP	Isopentenyl Pyrophosphate
LPS	Lipopolysaccharides
l	Litre
m	Metr**e**
MRSA	Methicillin Resistant *Staphylococcus aureus*
µg	Micrograms
µl	Microlitres
µmol	Micromoles
mg	Milligrams
ml	Millilitres

MBC	Minimum Bactericidal Concentration
MIC	Minimum Inhibitory Concentration
min	Minutes
MH	Mueller Hinton
MDR	Multi Drug Resistant
mMRSA	Multi-drug-resistant Methicillin Resistant *Staph. aureus*
nm	Nanometres
NMR	Nuclear Magnetic Resonance
PMS	Phenazine Methosulfate
TTO	Tea tree oil
VRE	Vancomycin Resistant *Enterococcus*
v/v	Volume per volume

Note on abbreviations for bacterial genera. The conventions used by the *Journal of Applied Microbiology* for bacterial genera are used in this thesis due to the number of different genera tested and discussed. The abbreviations used are as follows:

B., *Bacillus*; Ent., *Enterococcus*; E., *Escherichia*; H., *Haemophilus*; Kl., *Klebsiella*; Mor., *Moraxella*; Myco., *Mycobacterium*; Ps., *Pseudomonas*; Salm., *Salmonella*; Staph., *Staphylococcus*; Strep., *Streptococcus*

Summary

Purpose: Serrulatane diterpenoid compounds from plants in the Australian genus *Eremophila* have previously been shown to have antibacterial activity against a limited range of Gram positive bacteria. This study was undertaken to further examine the spectrum of antibacterial activity of these compounds and the antimicrobial mechanism of action.

Methods: Two serrulatane compounds isolated from *Eremophila neglecta* were used to examine the spectrum of activity, namely 8,19-dihydroxyserrulat-14-ene (**EN1**), and 8-hydroxyserrulat-14-en-19-oic acid (**EN2**). Broth micro dilution assays were used to determine the minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) of the compounds against a range of Gram positive and Gram negative bacteria of medical and veterinary importance including some clinical isolates and multidrug resistant isolates.

Cytotoxic activities of the two compounds were also tested against mammalian cells African green monkey kidney cells (CCL-81 Vero) and The U937 Human leukamic monocyte lymphoma cells.

Compound **EN2** was used in experiments to examine the antibacterial mode of action. Experiments were undertaken to determine the effects of the compound on the bacterial membrane of *Staphylococcus aureus* ATCC 29213. These included a salt tolerance assay, bacterial time killing, bacteriolysis, propidium iodide uptake assays. Transmission electron microscopy of *Staph. aureus* cells treated with both test compounds and control agents were done.

Results: The two serrulatanes **EN1** and **EN2** exhibited antibacterial activity against a range of Gram-positive bacteria. Although both compounds showed antibacterial activity against Gram-positives, **EN1** exhibited greater activity. Minimum inhibitory concentrations for **EN1** against standard strains and multi-drug resistant clinical isolates of *Staphylococcus aureus* ranged from 3.1 to 6.2 µg/ml, with MBCs of 6.2 µg/ml. Compound **EN1** also had activity against *Enterococcus* and *Mycobacterium* species, and moderate activity against the veterinary

pathogen *Erysipelothrix rhusiopathiae*. Of the five Gram-negative bacteria tested, only *Moraxella catarrhalis* showed sensitivity to the two compounds (MIC 6.25 µg/ml).

The salt tolerance assay result shows that the test compound killed the bacteria to 80-95 % at 2xMIC concentration respectively when the bacteria plated in nutrient agar supplemented with 5-7.5 % NaCl.

The bacteriolysis test result indicated that serrulatane compounds have no lysic effect on *S. aureus* cells when compared with control agent terpinen-4-ol.

The propidium iodide (PI) uptake assay was used to investigate the membrane permeability of *Staph. aureus* cells treated with serrulatane compound as nucleic acid binding molecule the propidium iodide is membrane impermeable. The increase in background fluorescence is indicative of membrane damage. Hexadecyltrimethylammonium bromide (CTAB) is a well known membrane permeabilising agent, used as the control agent in the test and an increase in fluorescence proves membrane damage when *Staph. aureus* cells treated with CATB. The result showed that serrulatane compound has similar effect on cell membranes like CTAB.

Electron microscopy of *Staph. aureus* cells treated with serrulatane compounds (**EN1&EN2**), and terpinen-4-ol indicates that the test compounds membrane effects are different from the control agents. Although terpinen-4-ol exhibited a clear damage on cell walls, there was no obvious damage on cell walls treated with the both serrulatane compounds like the bacteriolysis test results.

Conclusions

Serrulatane diterpenoids display activity against a range of Gram-positive bacteria of medical importance. It appears the compounds can act on bacterial membranes to increase permeability although they do not cause direct bacteriolysis. Mode of action test results indicates that the compounds affect the cell membranes but directly cause damage on cell walls like the control agent.

This study has provided an important data for the spectrum of activities of the two serrulatane compounds with a wide range of test strains. The results of cytotoxic activity tests need to be tested with further in-vivo studies to understand the use of serrulatane compounds can be use as a medicine.

Declaration

This thesis presents work carried out by myself and does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; to the best of my knowledge it does not contain any materials previously published or written by another person except where due reference is made in the text; and all substantive contributions by others to the work presented, including jointly authored publications, are clearly acknowledged.

Signature ..... Date .....

Omer Faruk Anakok

Acknowledgments

Firstly, I would like to give my special thanks to my principal supervisor, Dr. Susan Semple for all her support and help throughout my study period and having faith in my abilities. This study would not have been possible without her support.

Secondly, I would like to thank my supervisor Professor Mary Barton for all her help, kindness, understanding and support.

I am grateful to Dr. Chi Ndi from Ian Wark Research Institute for the isolation of the compounds investigated in this study. Special thanks to Associate Professor Enzo Palombo from Swinburne University for his support and Ruth Ellen Williams from Adelaide Microscopy Centre for her help in electron microscopy technique.

I also like to express my appreciation to our laboratory manager Lynda Kutek and other technicians in microbiology laboratory for the support they have given me in this study.

I would like to acknowledge and thank the University of South Australia for giving me the unique opportunity to undertake this Master by research program in the School of Pharmacy and Biomedical Sciences.

My appreciation also goes to my family in Turkey for all their love support and prayers.

Finally to my beautiful and loving wife for sharing the all difficulties of this study with me. For believing in me and giving me hope and courage at all the times.

CHAPTER ONE

GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1. Introduction

The aim of the research described in this thesis was to investigate the cytotoxicity, spectrum of activity and mode of action of some serrulatane diterpenoid compounds isolated from the Australian medicinal plant *Eremophila neglecta*. This project was undertaken as part of a broader project at the University of South Australia examining the antibacterial activity of compounds isolated from *Eremophila* species and their possible use to make antibacterial material coatings to combat biomedical device infections.

The first section of this introductory chapter provides an introduction to antimicrobial resistance and the need to search for new antimicrobial agents, including the need for novel solutions to the problem of device-related infections. This is followed by a brief overview of the role of plant secondary compounds as a source of new antimicrobial agents. A review of the literature pertaining to plants in the genus *Eremophila*, the source of the compounds used in this study, is then undertaken. This includes the traditional use of *Eremophila* species by Australian Indigenous peoples, investigations of crude *Eremophila* extracts for antimicrobial effects and studies to isolate the active components, particularly the serrulatane diterpenoids. This is followed by a discussion of the actions of biocidal antimicrobial agents. The aims of this study are then presented.

1.2. Literature Review

1.2.1 The problems of antimicrobial resistance and medical device-related infections

Resistance of Gram-positive and Gram-negative bacteria to antimicrobial agents is a significant problem of medicine. Over the last two decades, bacterial infectious disease among patients has spread considerably worldwide because of the high increase in antibiotic resistance (Dagan 2003). Indiscriminate use of antibiotics has caused the emergence of antibiotic resistant microorganisms. This has led to serious problems in the treatment of infectious diseases (Nimmo et al. 2003). Additionally, the proportion of social health expenditures caused by antibiotic resistant bacteria has been increasing significantly in the economies of developed countries (Collignon and Turnidge 2000). According to data in a report to Australia's National Health and Medical Research Council, the total economic effect of this problem on Australia's economy will be $250 million per year (Webber 2006).

An recent review from the Infectious Diseases Society of America (IDSA) reports that the number of infections caused by antibiotic-resistant bacteria are significantly growing (Boucher et al. 2009). Of particular concern are the Gram-positive and Gram-negative pathogens recently reported by Boucher et al. (2009) as the "ESKAPE" pathogens namely: *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumanii*, *Pseudomonas aeruginosa*, and *Enterobacter* species. These pathogens cause the majority of US hospital infections and have strong resistance to antibacterial drugs. A report from the Centers for Disease Control and Prevention (CDC) showed that the rates of infection due to methicillin-resistant *Staph. aureus* (MRSA), vancomycin-resistant *Ent. faecium* (VRE), and fluoroquinolone-resistant *Ps. aeruginosa* are rapidly increasing (Cardo et al. 2004). The number of deaths caused by MRSA infection is more than of HIV/AIDS and tuberculosis combined in US hospitals (Klevens et al. 2006; Boucher and Corey 2008). Additionally, panantibiotic-resistant infections caused by Gram-negative pathogens namely *Acinetobacter* species, multidrug-resistant (MDR) *P. aeruginosa*, carbapanem-resistant *Klebsiella* species and *Escherichia coli* are occuring in both the US and other countries (Cardo et al. 2004;

Falagas and Bliziotis 2007). Therapeutic options for infections caused by these organisms are extremely limited.

Staph. aureus is a major hospital-acquired pathogen (Perl 1999; Rotun et al. 1999). *Staph. aureus* strains are increasingly resistant to β-lactams, notably methicillin-resistant *Staph. aureus* (MRSA) strains, have been investigated from clinical sources for decades (Jevons 1961; Jevons and Parker 1964). The ability of these Gram-positive bacteria to acquire resistance to all useful antibiotics is a cause for a considerable concern.

A fully vancomycin resistant strain of MRSA first occurred in the US in 2002 (Diekema et al. 2004) and indicated that the treatment of these strains by the glycopeptide antibiotic is not guaranteed. Linezolid (Zyvox®) and the streptogramin quinupristin/dalfopristin mixture (Synercid®) are the newest anti-staphylococcal agents and have been heralded as a solution to MRSA infections (Diekema et al. 2004). However, a report of resistance to linezolid (Tsiodras et al. 2001) in a clinical isolate of *Staph. aureus* indicates that there is a continual need to search for new agents with activity against this organism (Gibbons 2004).

Another burden of antibiotic resistance is the medical problems caused by bacterial infections on biomedical devices. Some species of human pathogenic bacteria, in particular *Staphylococcus* and *Pseudomonas* species can colonize biomedical device surfaces and these infections present a serious problem for human implant surgery. Colonisation leads to biofilm formation, and when this occurs, combating these bacteria with antibiotics is difficult. The exocellular matrix contains various polysaccharides that form slime, protecting bacterial colonies against antibiotics and host defenses (Costerton et al. 1999). Such biomedical device infections occur with considerable frequency on the surfaces of catheters, pacemaker leads, artificial hips, knee prostheses, stents, and other biomedical implants and devices (Al-Bataineh et al. 2006). The enormous costs associated with these infections on implants and biomedical devices make research on the development of strategies to combat biofilm formation more important. The US market for antimicrobial coatings is estimated to grow to $488.9 million in 2012, with a compound annual growth rate of 15.8% (Frost and Sullivan 2006). A solution for this problem can bring important benefits to patients, the health system and economy. As a

result, there has been considerable interest in the new approaches to combating biomedical infections on medical devices (Siow et al. 2006).

Medicinal plants have been used by traditional peoples to treat the human diseases for centuries. Compounds isolated from plants and other natural sources are one potential source of novel compounds to control infections caused by bacteria resistant to currently available antimicrobial agents. The following section provides a brief overview of the literature investigating plants as a source of novel antimicrobial agents.

1.2.2 Antimicrobial plant products

1.2.2.1 Introduction to the main groups of antimicrobial compounds from plants

Plant species produce a wide range of what are termed "secondary compounds" or "secondary metabolites" in their various structural parts. These compounds are thought to have ecological roles in plants such as protection from microbial attack, interactions with other plants and insects and feeding deterrence. It has been estimated that over 100,000 secondary metabolites are made by plants (Dixon 2001). Many of these compounds isolated from plant species have been shown to exhibit antimicrobial activity. The active compounds belong to a variety of different classes of secondary metabolites. Some of the main classes of these compounds include phenolics and polyphenols, flavonoids, quinones, terpenoids, alkaloids and lignans, (Cowan 1999; Gibbons 2004; Cushnie and Lamb 2005; Rukachaisirikul et al. 2007).

While there are numerous examples of antimicrobial compounds isolated from plants, what follows is a brief overview of some of the more important classes of plant antimicrobial agents and examples of the activities demonstrated by these compounds. It has been pointed out previously (Gibbons 2004) that while large numbers of plant-derived compounds are reported to have antimicrobial in the literature, many have minimum inhibitory concentrations (MICs) of 1 mg ml^{-1} (1000 μg ml^{-1}) or above which are unlikely to be clinically relevant. It is been suggested that inhibitory activity for pure compounds at concentrations below 100 μg ml^{-1} (Gibbons 2004) or 25 μmol l^{-1} (Cos et al. 2006) are more meaningful if compounds are to be

considered true lead compounds. Therefore the review that follows focuses on some compounds with these lower MIC values.

1.2.2.2 Simple phenols and phenolic acids

The ability of plants to synthesize an enormous variety of aromatic substances presents researchers a huge area to investigate. These aromatic substances are phenols or their oxygen-substituted derivatives (Swain and Geissman 1962).

Cinnamic and caffeic acids are common metabolites of a wide group of phenylpropane-derived compounds. The herbs tarragon and thyme contains caffeic acid, which has been determined to be an effective agent *in vitro* against viruses (Wild 1994), bacteria (Thomson and Schultes 1980; Brantner et al. 1996), and fungi (Duke 2002). Catechol and pyrogallol metabolites are hydroxylated phenols which exhibit toxicity to microorganisms. Eugenol is a phenolic substance possessing a C_3 side chain found in the essential oil of clove, it inhibits the growth of both fungi (Duke 2002) and bacteria (Thomson and Schultes 1980).

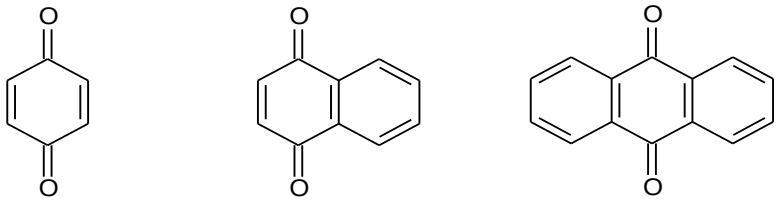
Increased hydroxylation in these compounds has been determined to result in increased toxicity (Geissman and Crout 1969), and additionally some researchers have found that more highly oxidized phenols are more inhibitory (Urs and Dunleavy 1975; Scalbert 1991). Phenolic toxicity mechanisms to microorganisms include enzyme inhibition by the oxidized compounds, possibly through reaction with sulfhydryl groups or nonspecific interactions with protein (Mason and Bruce 1987).

1.2.2.3 Tannins

Tannins are a group of polymeric phenolic metabolites which are found in almost every plant part (Scalbert 1991). There is significant data about the antimicrobial effects of tannins in the literature. In the review of Scalbert (1991), the activities of tannins against filamentous fungi, yeasts, and bacteria have been reported. One of these studies showed that condensed tannins bind to cell walls of ruminal bacteria and prevent bacterial growth (Jones et al. 1994).

1.2.2.4 Quinones

Quinones are highly colored secondary metabolites with two ketone groups in an aromatic ring. They commonly exist in nature and are characteristically highly reactive (Cowan 1999). Common types of quinones include benzoquinones, naphthoquinones and anthraquinones (Figure 1.1). They are responsible for the browning seen when fruits and vegetables are cut or damaged. Some of these compounds have been found to have activity against both Gram-positive and Gram-negative bacteria. Some examples of antimicrobial quinones that have been reported in literature are described below.



Benzoquinone **Naphthoquinone** **Anthraquinone**

Figure 1.1 General groups of quinones

An anthraquinone from *Stereospermum zenkeri* has shown activity against the Gram-negative bacteria *Ps. aeruginosa* with an MIC of 9.5 µg ml^{-1} (Lenta et al. 2007). Another anthraquinone from *Newboudia laevis* has shown activity against *Escherichia coli*, *Kl. pneumonia*, *Proteus mirabilis* and *Enterobacter freundii* with an MIC of 0.31 µg ml^{-1}, and this value was lower than that of gentamicin for the same strains (Eyong et al. 2006). An anthraquinone from *Cassia italic*, a Pakistani tree, has been reported as bacteriostatic for *Bacillus anthracis*, *Corynebacterium pseudodiphthericum*, and *Ps. aeruginosa* and bactericidal for *Burkholderia* (formerly *Pseudomonas*) *pseudomallei* (Kazmi et al. 1994).

Naphthoquinone metabolites are widespread throughout the plant, fungi, bacteria and animal kingdoms and these compounds play defensive roles for many organisms (Thomson 1997).

A naphthoquinone from *Ulmus davidiana* has been reported to show activity against *Staph. aureus*, *Staph. epidermidis*, *Bacillus subtilis*, and *Kl. pneumoniae* with MICs of 0.5 – 8 μg ml^{-1} (Dong-Yun et al. 2000).

Two naphthoquinone compounds, diospyrin and methyljuglone, have been shown to inhibit antibiotic-susceptible and antibiotic resistant strains of *Mycobacterium tuberculosis* and have been patented for this application (Koyama 2006).

Quinones have an important role in energy metabolism in cells, such as the redox cycling role of ubiquinone (coenzyme Q) in mammalian electron transport systems. However, the exact molecular mechanism of the antibacterial activity of secondary quinone compounds is largely unknown (Koyama 2006). Quinones are also known to complex irreversibly with nucleophilic amino acids in proteins (Stern et al. 1996), resulting in inactivation of the protein and loss of function. Potential target regions in the microbial cell are surface-exposed adhesions, cell wall polypeptides, and membrane-bound enzymes (Cowan 1999). Redox-cycling may also play a role in antibacterial mechanisms through the generation of reactive oxygen species that damage macromolecular structures (Cape et al. 2006).

1.2.2.5 Flavonoids

Flavonoids are hydroxylated phenolic substances with a basic structure composed of two benzene rings linked together through a 3 carbon unit (Figure 1.2) (Cowan 1999; Cushnie and Lamb 2005). Phenolic hydroxyl groups may occur at various positions on the two aromatic rings.

These compounds are mostly found in fruits, seeds, stems, leaves and flowers. Some of these metabolites are known to be synthesized by plants in response to microbial infection (Dixon et al. 1983; Harborne and Williams 2000). Some compounds in this class have antimicrobial

activity against a wide array of microorganisms and many structurally diverse flavonoids have antimicrobial activity against human pathogens (Cowan 1999; Cushnie and Lamb 2005).

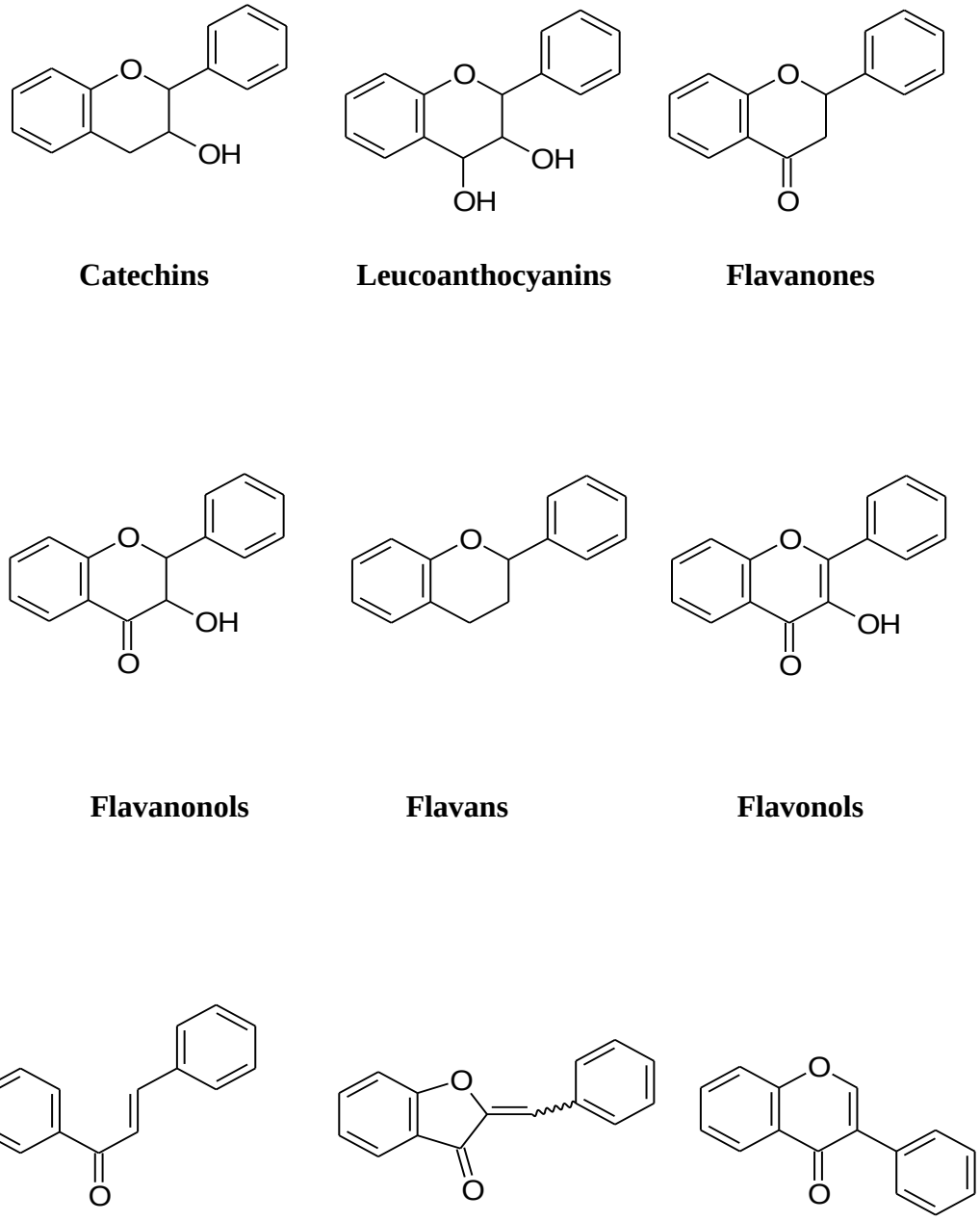


Figure 1.2 Common classes of flavonoids

The majority of antibacterial flavonoids described in the literature have shown activity against Gram-positive organisms such as *Staph. aureus*, with some showing good activity against MRSA. However, some have also shown activity against Gram-negative bacteria such as *Salmonella typhimurium* and *Ps. aeruginosa*. Some examples of antimicrobial activities of flavonoids of different structural types are described below.

Three isoflavone compounds isolated from *Uraria picta* have shown antimicrobial activities. One compound showed activity against *Staph. aureus* and *Proteus vulgaris* with MICs of 12.5 and 25 µg ml^{-1}, respectively. A second compound showed activity against *Staph. aureus, Pr. vulgaris, B. subtilis* and the filamentous fungus *Aspergillus niger*, with MICs in the range of 12.5-25 µg ml^{-1}.The third compound had activity against *Pr. vulgaris, B. subtilis*, *A. niger*, *E. coli* and the yeast *Candida albicans* with MICs of 12.5-25 µg ml^{-1} (Mukhlesur Rahman et al. 2007).

A chalcone group flavonoid from *Glycyrrhiza inflata* has shown activity against *B. subtilis*, *B. coagulans*, *B. cereus*, and *Staph. aureus* with MICs of 2 – 3 µg ml^{-1}. This compound was also active against *Ent. faecalis* and *Ent. faecium* with MIC of 6 µg ml^{-1} (Tsukiyama et al. 2002). A dihydrochalcone from *Piper longicaudatum* has shown activity against *Staph. aureus* and MRSA with MIC values of 10 and 4.5 µg ml^{-1}, respectively (Joshi et al. 2001).

A flavonone from *Dalea scandens* has shown activity against *Staph. aureus* and MRSA strains with an MIC of 1.56 µg ml^{-1} (Nanayakkara et al. 2002). A flavonone from *Physena madagascariensis* has been reported that has activity against *Staph. aureus*, *Staph. epidermidis*, *Enterococcus* sp., and *Listeria monocytogenes* with MICs of 1.8 – 7.5 µg ml^{-1} (Deng et al. 2000). Another flavonone from *Scrutellaria barbata* has shown selective activity against *Staph. aureus* and MRSA with MICs of 3.9 – 15.6 µg ml^{-1} (Sato et al. 2000).

Three pterocarpan flavonoids from *Erythrina subumbrans* have been reported to have antibacterial activities. These compounds showed activity against *Staph. aureus*, MRSA and vancomycin resistant *Staph. aureus* (VRSA) with MICs of 0.78 - 6.25 µg ml^{-1} (Rukachaisirikul et al. 2007).

Three xanthone group flavonoids from *Cratoxylum formosum* have been reported to have antibacterial activities. These compounds had MICs in the range of 1.1 – 9.3 μg ml^{-1} against organisms including *Ps. aeruginosa B. subtilis, Staph. aureus, Strep. faecalis* and *Salm. typhimurium* (Boonsri et al. 2006).

Two isoflavan group flavonoids from *Erythrina zeyheri* have been reported active against MRSA with MICs of 3.13 – 6.25 μg ml^{-1} (Tanaka et al. 2003).

Catechins are a common group of flavonoid metabolites that have been investigated due to their occurrence in oolong green teas. It was reported that green tea exhibited antimicrobial activity and the active compounds were catechins which inhibited *Vibrio cholera* O1 (Borris 1996), *Strep. mutans* (Sakanaka et al. 1989; Batista et al. 1994; Tsuchiya et al. 1994), *Shigella* (Vijaya et al. 1995), and other bacteria and microorganisms (Thomson and Schultes 1980) *in vitro*. The activity of the catechins against *Strep. mutans* has also been tested *in vivo*, with rats fed a diet containing 0.1% tea catechins. Fissure caries (caused by *Strep. mutans*) were reduced by 40% in treated rats compared to controls without treatment (Ooshima et al. 1993).

Studies on the structure-activity relationships of flavones and other flavonoids show conflicting findings about the hydroxyl groups of flavonoids. One study showed that flavonoids lacking hydroxyl groups were more active against microorganisms (Chabot et al. 1992). However, several studies have also reported the opposite effect, that is, the more hydroxylation on the molecule, the greater the antimicrobial activity (Sato et al. 1996).

1.2.2.6 Alkaloids

Alkaloids are nitrogen-containing plant secondary metabolites. Many different groups of alkaloids have been used as drugs, such as quinine which is used as an anti-malarial drug, and the opioid analgesic morphine. The common groups of alkaloids are true alkaloids, protoalkaloids and pseudoalkaloids (Cordell 1981; Dewick 2009). Some examples of antimicrobial activities of alkaloids from plants are described below.

An alkaloid called liriodenin from *Guatteria multivenia* has shown activity against *Staph. aureus* and MRSA with MIC of 2 μg ml^{-1} (Zhang et al. 2002). An alkaloid berberine isolated

from *Hydratis canadensis* has shown anti-staphylococcal activity with an MIC of 31 µg ml^{-1} (Scazzocchio et al. 2001).

Another alkaloid from *Micromelum minutum* has been reported to inhibit the growth of *B. cereus* and *Staph. aureus* with MICs of 6.25 and 12.5 µg ml^{-1} (Nakahara et al. 2002).

A carbazole alkaloid from *Clausena heptaphylla* (Rutaceae) has shown a significant spectrum of activity against *Staph. aureus*, *E. coli* and *Ps. aeruginosa* with MIC values of 3, 6 and 20 µg ml^{-1}, respectively (Chakraborty et al. 1995).

Quaternary alkaloids identified as the major anti-staphylococcal agent from antibacterial extracts of *Zanthoxylum clava-herculis* (Rutaceae), have shown activity against a standard *Staph. aureus* ATCC 25923 strain with an MIC of 4 µg ml^{-1} (Gibbons et al. 2003).

1.2.2.7 Terpenoids

1.2.2.7.1 General introduction to terpenoids

Terpenes are a large class of secondary compounds based on the basic five-carbon isoprene structure (Figure 1.3). They are named according to the number of five carbon units they contain. For example, monoterpenes contain two five carbon units (C10), sesquiterpenes contain three units (C15), diterpenes contain four units (C20) and triterpenes contain six units (C30). In plants the building blocks for these five carbon units are isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) (Figure 1.4).

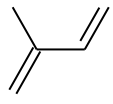


Figure 1.3 Isoprene structure

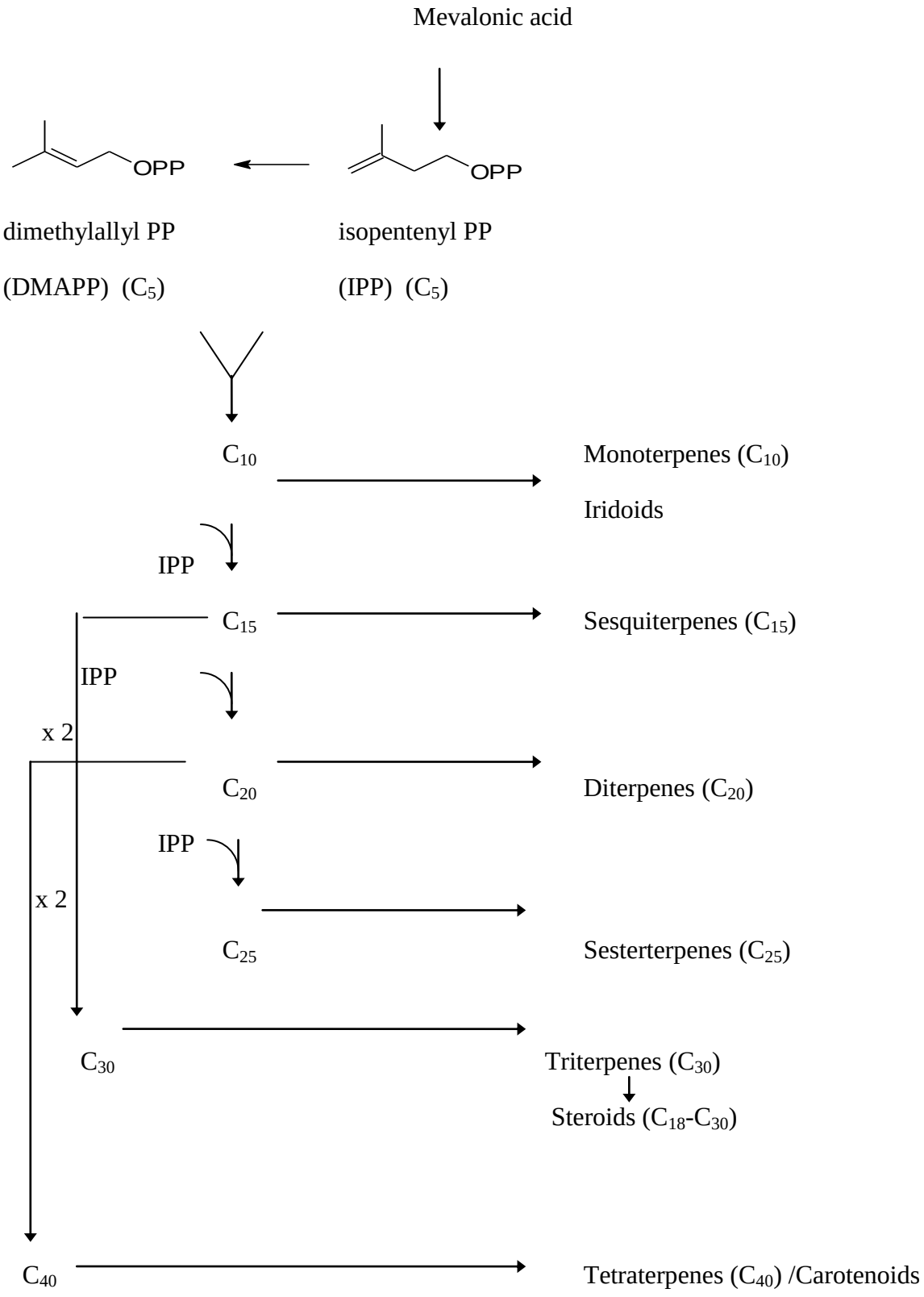


Figure 1.4 Biosynthesis of terpenoids (**adapted from: Dewick (1997))**

Among the secondary metabolites of plant kingdom terpenoids are a structurally most diverse group; they play a significant role in plant direct defence, or as signals in indirect defense responses which involves herbivores and their natural enemies. In recent years, more and more attention has been paid to the investigation of the ecological role of plant terpenoids (Cheng, 2007). Many terpenoid compounds serve as defense molecules against microbes and herbivores or are signal molecules to attract pollinating insects, fruit-dispersing animals or predators which can destroy insect herbivores. As a consequence, many terpenoids have pronounced pharmacological activities and are therefore interesting for medicine and biotechnology (Ashour, 2010). Terpene compounds containing additional elements, usually oxygen, are called terpenoids (Cowan, 1999). A large variety of terpenoids have antibacterial activities (Himejima et al. 1992; Kubo et al. 1992; Ahmed et al. 1993; Habtemariam et al. 1993; Barre et al. 1997; Amaral et al. 1998; Urzua et al. 1998; Dorman et al. 2000; Oliveira et al. 2007; Liu et al. 2007; Lo Cantore et al. 2009; Mathabe et al. 2008), antifungal activities (Harrigan et al. 1993; Kubo et al. 1993; Rao et al. 1993; Ayafor et al. 1994; Rana et al. 1997), and antiviral activities (Fujioka et al. 1994; Hasegawa et al. 1994; Pengsuparp et al. 1994; Sun et al. 1996; Xu et al. 1996).The mechanism of action of terpenoids can vary but lipophilic terpenoids can be involved in membrane disruption (Cowan 1999).

1.2.2.7.2 Monoterpenoids

Terpenoids based on a ten carbon skeleton (two five-carbon units) are termed monoterpenoids. These are often volatile compounds and common components of plant essential oils.

The essential oil of the Australian tea tree (*Melaleuca alternifolia*, Myrtaceae) is a useful antiseptic agent which has been the subject of a number of antimicrobial investigations (Carson and Riley 1995; Carson et al. 2002; Gibbons 2004). The essential oil and its isolated components have activity against both Gram-positive and Gram-negative bacteria (Carson and Riley 1995). The active purified compounds from tea tree oil include γ-terpinene, α-terpineol, terpinen-4-ol and linalool which have been reported with MICs in the range of 0.125-0.25% v/v against *Candida albicans, E. coli, Staph. aureus, Staph. epidermidis* and *Propionibacterium acnes* (Carson and Riley 1995; Raman et al. 1995; Cox et al. 2001).

Another example of antibacterial monoterpenoid is 1,8-cineole. It is found in various essential oils including Artemisia *asiatica*. It was was found to be the major anti-staphylococcal agent of the essential oil of this plant with an MIC of 2 µl ml^{-1}, and was also active against Gram-negative bacteria including *E. coli* and *Ps. aeruginosa* (D Kalemba 2002).

1.2.2.7.3 Sesquiterpenoids

Terpenoids based on a 15 carbon skeleton (three five-carbon units) are termed sesquiterpenoids. These are also common components of plant essential oils.

The guaianolide sesquiterpene lactone 6-*O*-isobutyroplenolin (arnicolide C) isolated from a Nepalese medicinal plant was determined to be active against a methicillin-sensitive *Staph. aureus* (MSSA) strain with the MIC at 38 µg ml^{-1} (Taylor and Towers 1998). A similar guaianolide compound from *Artemisia gilvescens* showed a significant activity against a clinical strain of MRSA with the MIC at 1.95 µg ml^{-1} (Kawazoe et al. 2003). A sesquiterpene lactone, xanthatin, was reported to exhibit specific activity against Gram-positive bacteria. It was profiled against twenty MRSA and seven MSSA strains with MIC values being comparable for resistant and sensitive strains (Sato et al. 1997).

Studies on antimicrobial properties of myrrh (*Commiphora molmol*) have reported that the furano sesquiterpene curzerenone isolated from the plant is responsible for anti-staphylococcal activity with an MIC 0.7 µg ml^{-1} (Dolara et al. 2000). This result may explain the use of myrrh in antiquity for treating wounds and as a local eye medication (Tucker 1986).

Another sesquiterpene named Mansinone F from *Ulmus davidiana* var. *japonica* was reported to exhibit activity against 19 MRSA strains with MIC values in the range of 0.39-3.13 µg ml^{-1}, These results were comparable with vancomycin (MIC range 0.39-1.56 µg ml^{-1}), the most widely used anti-MRSA antibiotic (Shin et al. 2000). The aromatic sesquiterpene phenol xanthorrizol showed activity against MRSA in the concentration range of 16-32 µg ml^{-1} (Aguilar et al. 2001; Mata et al. 2001).

1.2.2.7.4 Diterpenoids

Diterpenoids are the largest groups of plant derived natural products with antimicrobial and anti-staphylococcal activities (Gibbons 2004). These compounds are based on a 20-carbon skeleton and are common components of plant resins.

The genus *Salvia* from the Lamiaceae or mint plant family is known to have important antibacterial and cardioactive properties (Ulubelen 2003). The diterpenes horminone and 7-acetylhorminone from *Salvia blepharochlaena* have been reported to have activity against *Staph. aureus* and *Staph. epidermidis* with an MIC range of 1.5-10 µg ml^{-1} (Ulubelen et al. 2001) and another diterpene 1-oxoferruginol from *Salvia viridis* exhibited a similar level of activity (Ulubelen et al. 2000). Additionally, isopimarane type diterpenes from *Salvia* have comparable activity (MICs of 9 µg ml^{-1}) to the known antibiotics amikacin (16 µg ml^{-1}), ampicillin (8 µg ml^{-1}) and cefoperazone (16 µg ml^{-1}) which are commonly used to treat infections caused by Gram-positive bacteria (Gören et al. 2002). A diterpenoid abietane quinone, sanigerone, from *Salvia prionotis* and *S. lanigera,* exhibited activity against *Staph. aureus* with an MIC at 13 µg ml^{-1} (Chen et al. 2002; El-Lakany 2003).

An acetylated abietane quinone from *Plectranthus hereroensis* (also from the mint family) has been reported that to have anti-staphylococcal activity with an MIC at 31.2 µg ml^{-1} and inhibition of fungal spore germination (Dellar et al. 1996b). A methoxylated abietic acid from *Dauphinia brevilabra* (Lamiaceae) showed activity against a standard laboratory *Staph. aureus* strain with the potency of 1µg ml^{-1} (Dellar et al. 1996a). Another report on the antibacterial diterpenes from the Lamiaceae family showed that the furano-labdane diterpene from *Ballota saxatilis* subsp. *saxatilis* has activity against *Staph. aureus* and *Ent. faecalis* with an MIC of 25 µg ml^{-1} (Çito lu et al. 1998).

Coniferous plants (Pinopsida) are important source antibacterial metabolites including abietane type diterpenes. The MICs of some diterpenes of this class from *Cephalotaxus* and *Chamaecyparis* species were determined to be below 15 µg ml^{-1} (Xiao et al. 2001; Politi et al. 2003).

Another study (Woldemichael et al. 2003) reported that an isopimarane diterpenoid showed activity against *Staph. aureus* and MRSA strains and also against *Bacillus subtilis* with the MICs in range of 2 and 4 µg ml^{-1}. Mechanism of action studies suggested the compound had a damaging effect on bacterial membranes. It was also tested to identify the cytotoxic concentration against human red blood cells and had no haemolytic effect at concentrations up to 32 µg ml^{-1}. The compound was also tested in an *in vivo* murine model of *Staph. aureus* infection, but no protection was observed (Woldemichael et al. 2003).

The totarane diterpene totarol from a coniferous plant *Podocarpus nagi* was shown to have activity against a range of Gram-positive bacteria including MRSA (Muroi and Kubo 1994) and the activity was reported to be improved when tested in combination with other compounds (Kubo et al. 1992). The compound also has activity against *Ps. aeruginosa*. Using this organism, the research group showed that the compound inhibited oxygen consumption and cellular respiration in whole cells and oxidation of NADH in a membrane system. The researchers postulated that the compound caused respiratory inhibition in the bacterial electron transport chain (Haraguchi et al. 1996).

A trachylobane class diterpene from *Mitrephora celebica* was reported to have activity against *Staph. aureus* and *Mycobacterium smegmatis* (a model for assessment of anti-tubercular drugs) with the MICs at 6.25 µg ml^{-1} for both organisms (Zgoda-Pols et al. 2002). The compound has structural similarity to beyerenoic acid from the roots of *Viguiera hypargyrea* (Asteraceae), a compound with activity against *Staph. aureus* and *Ent. faecalis* with MICs of 12 µg ml^{-1}. These results have been suggested to support the use of these roots as a treatment of gastrointestinal disorders in Mexico (Zamilpa et al. 2002).

A South American medicinal plant study reported that a diterpenoid compound from *Fabiana densa* var. *ramulosa* (a plant used in traditional medicine in Chile to treat coughs and lung diseases), had anti-staphylococcal activity with an MIC of < 10 µg ml^{-1} (Erazo et al. 2002). Another South American medicinal plant study showed that an oleoresin from Peruvian *Copaifera pauper* (Leguminosae) had activity against *Staph. aureus* and *Staph. epidermidis* at 5 and 10 µg ml^{-1}, respectively (Tincusi et al. 2002).

Recently, diterpenoids from the Australian medicinal plant genus *Eremophila* called serrulatanes have been identified as antibacterial compounds with activity against Gram-positive bacteria (Liu et al. 2006; Ndi et al. 2007b).These compounds are the focus of the study described in this thesis and will be discussed in more detail in the following section.

1.2.3 Traditional use and antimicrobial activity of plants in the genus *Eremophila*

1.2.3.1 The genus *Eremophila* and its traditional use by Australian Aboriginal People

The genus *Eremophila* (family Myoporaceae) is endemic to Australia. Plants in this genus commonly grow in semi-arid and arid regions (Chinnock 2007). The genus name relates to this preferred habitat (*eremos* = desert and *philus* = love) (Richmond 1993).

The genus *Eremophila* was first described by Robert Brown in 1810 and included in the Myoporaceae family by later Australian botanical researchers. *Eremophila* species are generally shrubs or small trees and have resinous leaves, flowers and woody fruits (Chinnock 2007). These plants have mostly adapted to arid habitats and as part of their tolerance to drought, they produce large quantities of resin (up to 20% of the dry weight) on their leaves and terminal branches (Ghisalberti 1994b).

It is known that the Australian Indigenous people have used various *Eremophila* species for medicinal and ceremonial purposes. The resins and gums produced by these species, were used as natural adhesives and sealants (Maiden 1889; Barr 1988). *Eremophila* species have been used as traditional medicines for colds, fever, sores, wounds, headaches, scabies, and general malaise probably for centuries (Cribb 1981; Barr 1988; Low 1990; Latz and Green 1995). The reported uses of some of the individual species in this genus are described below.

Eremophila alternifolia is small bush which grows on rocky slopes of hills and ranges. It was one of the few plants which the Aboriginal people dried, stored and carried with them. It was

reported to be used for alleviate colds, influenza, fever, and to treat septic wounds and induce sleep (Barr 1993; Latz and Green 1995). This plant has a significant amount of essential oil which has been detected in the leaf extracts (Sutherland and Rodwell 1989).

Eremophila bignoniiflora usually grows in inland areas. Infusions of the plant were used as laxative and the fruits eaten in cases of extreme sickness (Richmond 1993).

Eremophila cuneifolia grows in the loamy and rocky soil of the Gibson Desert. It has resin on the leaves and stems. Aboriginal people made infusions from the leaves to relieve colds. The leaf resin contains diterpenoid metabolites (Ghisalberti 1994a)

Eremophila duttonii is a slow growing bush, which grows in red sands and loamy plains. Infusions of the plant were used primarily as an antiseptic wash for sores and cuts. It is also reputed to have insect repelling properties (Smith et al. 2007). The leaf resin contains serrulatane diterpenes (Tippett and Massy-Westropp 1993).

Eremophila longifolia is a widespread species which grows on heavy clay soils. It is used by Aboriginal people in the smoke treatment of newborn babies and their mothers. The leaves, twigs and bark preparations were also used for headaches, sore eyes and skin infections such as boils (Richmond 1993).

Eremophila sturtii grows on a range of soil types. This plant was used to relieve backaches and infusions have been used to treat sores and cuts, colds and diarrhoea. The leaves and branches also have fly-repellant properties (Silberbauer 1971).

Eremophila neglecta is a shrub that grows 1-2.5 m tall, commonly growing in inland South Australia and the Northern Territory of Australia. This plant has highly resinous leaves and they have been used by Australian Aboriginal people for general well-being (Latz 1982; Ndi et al. 2007b).

1.2.3.2 Pharmacological studies of extracts from *Eremophila* species

The traditional use of *Eremophila* species has led a number of groups to examine the activity of extracts from these species for pharmacological activity in the laboratory. Studies of some *Eremophila* species have reported antibacterial, antiviral, neurological, cardioactive and anti-inflammatory activities (Semple et al. 1998; Rogers et al. 2000; Palombo and Semple 2001; Rogers et al. 2001; Sweeney et al. 2001; Palombo and Semple 2002; Rogers et al. 2002; Pennacchio et al. 2005). Extracts have also been shown to have toxic effects to cancer cells grown in culture (Mijajlovic et al. 2006).

Testing of the antibacterial activity of a range of traditional medicinal Australian plants study showed that *Eremophila duttonii* exhibited the greatest activity against Gram-positive bacteria. It also exhibited rapid bactericidal action in cultures incubated in the presence of the extract within 1 hour for *B. cereus*, *Ent. faecalis* and *Staph. aureus* and 2 hours for *Strep. pyogenes* (Palombo and Semple 2001).

A study on the leaf extracts of some *Eremophila* species which produce large quantities of resin showed antibacterial activity against standard strains of *Staph. aureus* and *Strep. pneumoniae* and *Strep. pyogenes* (Ndi et al. 2007a). Amongst the most active extracts were those of *Eremophila neglecta* (Figure 1.5 and 1.6), *Eremophila serrulata* and *Eremophila virens.*

Figure 1.5 *Eremophila neglecta* habitat (Photo M.O`Leary, northern South Australia, 119 km north of Marla)

Figure 1.6 *Eremophila neglecta* leaves and flowers (Photo M.O`Leary, northern South Australia, 119 km north of Marla)

Another antibacterial research study with extracts of *Eremophila alternifolia* and *Eremophila duttonii* leaves showed that extracts of these plants exhibited antibacterial activity against clinical isolates of methicillin-resistant *Staph. aureus* (MRSA) and vancomycin-resistant enterococci (VRE). The extract from the leaves of *Eremophila duttonii* exhibited rapid bactericidal activity by reducing the number of viable cells to an undetectable level within 1 hour (Palombo and Semple 2002).

An extract of *Eremophila duttonii* has also been shown to have antibacterial activity against some *Clostridium* species and *Listeria monocytogenes* (Shah et al. 2004). Further study with ethanolic extracts of the two traditional medicinal plants, *Eremophila alternifolia* and *Eremophila duttonii* examined the anti-listerial activity of these plant extracts. A foodborne pathogen, *Listeria monocytogenes* is responsible for the disease listerosis. Crude ethanolic extracts from *E. alternifolia* and *E. duttonii*, have been found to inhibit the growth of *Listeria monocytogenes* (Owen and Palombo 2007).

Another study investigating the anti-mycobacterial activity of *Eremophila* species has reported that crude extracts of *E. alternifolia* and *E. longifolia* have shown some activity against *Mycobacterium* species including *Myco. smegmatis* and *Myco. fortuitum* (Meilak and Palombo 2008). However, active components were not identified.

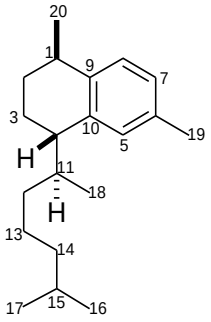
1.2.4 Chemistry of the genus *Eremophila* and antibacterial compounds isolated from *Eremophila* species

1.2.4.1 Studies on the chemistry of *Eremophila* species

Systematic studies on the chemical constituents of *Eremophila* species have been undertaken by a several research groups over a number of years and have shown that these species produce a large number of different secondary metabolites including flavonoids, monoterpenoids, sesquiterpenoids, and diterpenoids (Ghisalberti 1994b). Many of these compounds are found only in the genus *Eremophila*. While a large amount of work has been

performed on the chemistry of *Eremophila* species, it is only in more recent years that research to isolate pharmacologically active compounds, such as antibacterial compounds, has been undertaken.

The most studied secondary metabolite class of *Eremophila* is the diterpenoids. The serrulatanes are the most common group among the nine different types of diterpenoids isolated from *Eremophila* species and are isolated from the resins of the plants (Ghisalberti 1994b). The bicarbocyclic structure of serrulatanes (**1**) is represented in (Figure 1.7).



(1)

Figure 1.7 General structure of the serrulatanes

1.2.4.2 Studies on the antibacterial components of *Eremophila* species

Recent investigations have shown that serrulatanes are responsible for the antibacterial activity of some *Eremophila* extracts. A chemical investigation of the traditional medicinal species *Eremophila sturtii* found two serrulatane diterpenoids, 3,8-dihydroxyserrulatic acid and serrulatic acid. These were found to exhibit bactericidal activity against *Staph. aureus* with minimum bactericidal concentrations (MBCs) of 15 - 200 μg ml^{-1}. The compounds also

showed some moderate inhibition of enzymes cyclooxygenase 1 and 2 which play an important role in inflammation pathways (Liu et al. 2006).

Ndi et al. (Ndi et al. 2007b) reported the isolation of serrulatane type diterpenoids 8,19-dihydroxyserrulat-14-ene (**2**), and 8-hydroxyserrulat-14-en-19-oic acid (**3**) and a structurally-related known *o*-naphthoquinone called biflorin (**4**) (Figure 1.8) from *Eremophila neglecta.* These compounds had antimicrobial activity against standard strains of the Gram-positive bacteria including *Staph. aureus, Strep. pyogenes, and Strep. pneumoniae* with MICs of 6.5 to 101.6 μmol l^{-1}. However, there was no inhibitory activity for these compounds against the Gram-negative bacteria tested including *E. coli* and *Ps. aeruginosa*.

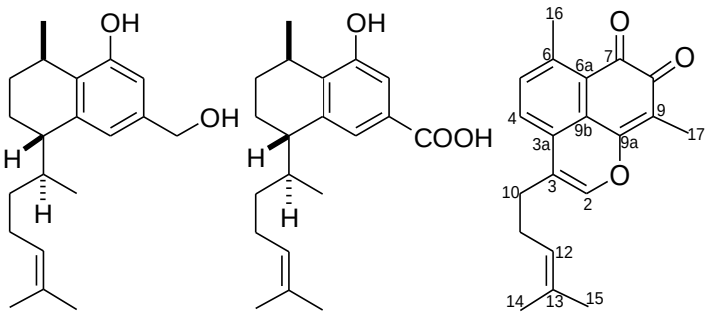


Figure 1.8 Compounds from *Eremophila neglecta*

Another study on *Eremophila serrulata* reported the activity guided isolation of an *o*-naphthoquinone, 9-methyl-3-(4-methyl-3-pentenyl)-2,3-dihydronaphtho[1,8-bc]pyran-7,8-dione (**5**), and serrulatane diterpenoids, 20-acetoxy-8-hydroxyserrulat-14-en-19-oic acid (**6**), and 8,20-dihydroxyserrulat-14-en-19-oic acid (**7**) and 8,20-diacetoxyserrulat-14-en-19-oic acid (**8**) (Figure 1.9). The compounds **5-7** showed antimicrobial activity against *Staph. aureus*. (Ndi et al. 2007a).

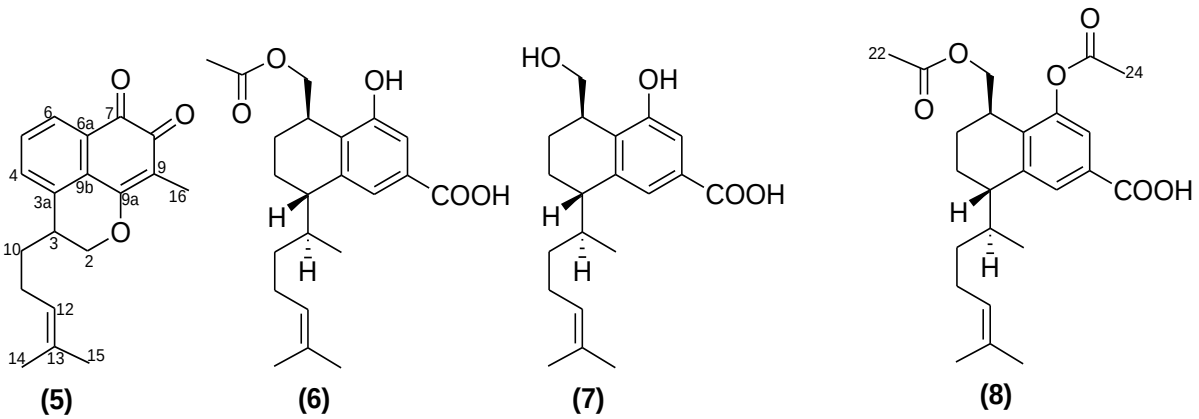


Figure 1.9 Compounds from *Eremophila serrulata*

Serrulatanes have also been isolated from the traditional medicinal species *Eremophila duttonii* in another antibacterial compound identification study. The compounds: serrulat-14-en-7,8,20-triol and serrulat-14-en-3,7,8,20-tetraol from this species were found to have antibacterial activity against the Gram-positive bacteria *Staph. aureus*, *Strep. pyogenes*, *and Strep. pneumoniae* (Smith et al. 2007). A furanosesquiterpenoid with some antibacterial activity was also tentatively identified from the extract of the plant (Smith et al. 2007).

There is structural similarity between the serrulatanes and some other antibacterial compounds called pseudopterosins from *Pseudopterogorgia elisabethae* a West Indian sea whip. Some of these compounds have been reported to exhibit potent anti-mycobacterial activity (Rodríguez et al. 1999; Rodríguez and Ramírez 2001; Smith et al. 2007). *Mycobacterium tuberculosis* is the causative agent of tuberculosis. This may suggest the serrulatanes also have activity against *Mycobacterium* species. However, this has not yet been tested. Although, as reported above, crude extracts of *Eremophila* species have recently been shown to exhibit some antibacterial activity against some bacteria in this group at higher concentrations (Meilak and Palombo 2008). Therefore, there is a need to further examine the spectrum of activity of the purified serrulatanes against other bacteria such as *Mycobacteria* which are important human pathogens.

In addition to the species described above, a number of other *Eremophila* species have been shown to produce serrulatanes. However, the antibacterial activities of most of these compounds have not yet been studied. The other *Eremophila* species from which different serrulatanes have been isolated include: *E. biserrata*, *E. decipens*, *E. denticulata*, *E. drummondii*, *E. drummondii* var *brevis*, *E. falcata*, *E. flaccida*, *E. gibsonii*, *E. glabra*, *E. granitica*, *E. hughesii*, *E. latrobei*, *E. linearis*, *E. paisley*i, *E. philipsii*, *E. purpurascens*, *E. rotundifolia*, *E. subfloccosa*, *E. subteretifolia*, *E. virens*, *E. wilsii*, *E. woolsiana*, *E. clarkei*, *E. decipiens*, and *E. georgei* (Ghisalberti 1994b).

1.2.5 Studies on toxicity of *Eremophila* species and serrulatane compounds

In the search for new antimicrobial agents from plants, an important consideration is also the toxicity of plant extracts and plant compounds to other cell types, particularly mammalian cells (Cos et al. 2006). This is important to establish whether toxic effects are specific to bacteria or to a range of cell types.

There is currently no significant published data for cytotoxic effects of extracts from *Eremophila* species or the serrulatane compounds. The only data has been given in a paper describing the preliminary identification of the antibacterial component of *Eremophila duttonii* (Shah et al. 2004). This paper mentioned that preliminary studies indicated the semi-purified active component was not cytotoxic *in-vitro.* However, the actual data from these preliminary studies was not published.

A study on the activity of extracts of different *Eremophila* species against a number of cancer cell lines has reported that *E. duttonii* and *E. sturtii* have shown significant activities. The activity of the extracts of *E. duttonii* was 26 times higher against cancer cell lines than normal cells suggesting some selectivity in its action (Mijajlovic et al. 2006). While both of these *Eremophila* species are known to contain serrulatane compounds as described above, only crude extracts containing a mixture of components were tested in the study.

There is a need to investigate the toxicity of purified serrulatanes from *Eremophila* species to mammalian cells. One aim of the study described in this thesis, therefore, was to further investigate the effects of purified serrulatane compounds on mammalian cell lines in culture.

1.2.6 Studies on the mechanism of antibacterial action of *Eremophila* extracts

Most of the serrulatanes isolated from *Eremophila* species are phenolic compounds. It is widely known that some phenolic compounds can have direct effects on bacterial membranes and act as biocides. However, the most active of the two serrulatane compounds with antibacterial activity from *Eremophila sturtii*, serrulatic acid, lacks a phenolic hydroxyl group.

To date, the only work published undertaken to examine the mechanism of the antibacterial action of compounds from *Eremophila* species has examined a crude extract of one species. The antibacterial mechanism of action study on a crude ethanolic extract of the leaves of *Eremophila duttonii* showed that it exhibited effects on the integrity of the cytoplasmic membrane of *Staph. aureus*, leading to increased membrane permeability indicated by uptake of propidium iodide and a decrease in ability to exclude NaCl (Tomlinson and Palombo 2005). As described above, *Eremophila duttonii* has subsequently been found to contain antibacterial serrulatanes and a furanosequiterpenoid compound (Smith et al. 2007). However, the mechanism of action work was only performed with a crude extract containing a mixture of compounds, not just serrulatanes. Therefore, further study is required to understand the mechanism of the antibacterial activity of the serrulatanes.

One aim of the research described in this thesis was to therefore investigate the antibacterial mode of action of purified serrulatanes. Specifically, the study sought to examine whether a purified serrulatane compound had direct effects on bacterial cells to act as a "biocide".

1.2.7 Possible applications of serrulatane compounds combating infections at biomedical implants and devices with antibacterial coatings

The project " Development and evaluation of novel antibacterial coatings for the prevention of infection of biomedical implants and devices" conducted at the University of South Australia and led by Professor Hans Griesser (Ian Wark Research Institute) aims to prevent device

related infections caused by bacterial biofilms with use of antibacterial compounds isolated from native plants in the genus *Eremophila*. The enormous costs of infections on biomedical implants and devices make this project of importance. Device infections often involve infections by Gram-positive bacteria including *Staph. epidermidis* and *Staph. aureus* which form biofilm on the surface that can become the resistant to standart antibiotic treatment and most immune defense mechanisms (Griesser et al. 2008). The current unpublished results of the project show that the antibacterial serrulatanes from *Eremophila serrulata,* and *Eremophila neglecta* can successfully prevent the bacterial biofilm formation on material surfaces when they are covalently bound onto the surface (Griesser et al. 2008).

Further development of this technology will require a more complete understanding of the antibacterial actions of the serrulatanes both in solution and attached to surfaces, as well as potential toxic and mutagenic effects. The project described in this thesis will contribute, in part, to this body of research.

1.2.8 Antibacterial biocides and their mechanisms of action

The term ‘biocide’ is mostly used to describe compounds with antiseptic, disinfectant or preservative activity. A biocidal compound may be used for one of these properties or even all of them. Russell (2002) has indicated that there were two general opinions in the literature about biocides: ‘The first was that, as long as they were effective, there was little reason to determine how they achieved their inhibitory or lethal effects. The second view was that antiseptics and disinfectants acted as general protoplasmic poisons and, as such, merited little attention” (Russell 2002).

Antibacterial biocides are a structurally diverse range of chemical agents which have to the ability to have damaging interactions with the bacterial cell. These agents affect the cells by combining specific targets or target regions according to their morphology and physiology. Mechanism of action studies of chemical biocides are now recognized as being important because they inform the future design and development of biocides and can guide for their correct usage (Denyer 1995).

Chemical biocides can exhibit both bacteriostatic and bactericidal effects, although the mechanisms by which they do this are different. Bacteriostatic events usually occur because of metabolic injuries which are reversible upon removal or neutralization of the biocide whereas bactericidal effects can cause irreparable and irreversible damage to the cell structure or functions (Fitzgerald et al. 1989).

The steps of interaction between biocide and bacteria cells take place in the following sequence: 1. Uptake of the biocide by the cell, 2. Partitioning of the biocide to the target, 3. Concentration of the biocide at its targets, 4. Damage to targets (Denyer and Hugo 1991). This mechanism of action process is regulated by the physicochemical properties of both the cell and biocide (Jones et al. 1991).

The antibacterial effect of biocides depends on the successful interaction of the biocide and targets at the applied concentration. The interaction of biocides with the target regions varies according to the sensitivities of individual bacterial species (Russell 1991). For instance, Gram-negative bacteria have an outer membrane on the cell wall containing phospholipids, lipoproteins, lipopolysaccharides (LPS), and proteins. This acts as a supplementary barrier in Gram-negative bacteria whereas Gram-positive cells do not possess this layer. This barrier can be damaged by the treatment of agents such as ethylenediaminetetraacetic acid (EDTA), which increases the permeability of the Gram-negative outer membrane (Russell 1991).

Biocidal agents can act by a variety of mechanisms and some act by more than one mechanism. The target regions of biocides are often classified as the cell wall, cytoplasmic membrane, and cytoplasm (Denyer 1995). Some of the examples of the targets and consequences of biocide actions are given in Table 1.1. according to their target classification.

Target region	Damaging event	Consequence	Example biocides
Cell wall	Structure/functional changes, release of wall components, initiation of autolysis	Abnormal morphology and construction; non- specific increase in cell permeability; lysis	Phenol, Sodium hypochlorite (Pulvertaft and Lumb 2009), Formaldehyde (Pulvertaft and Lumb 2009); cetyltrimethylammonium bromide (CTAB) (Salton and Phenomena 1957); 2-Phenylethanol (Halegoua and Inouye 1979), EDTA (Leive 1974)
Cytoplasmic membrane	1. Loss of structural organization and integrity	Progressive leakage of intracellular material (e.g. potassium ions, inorganic phosphate, amino acids, nucleotides, protein); initiation of autolysis	Sodium dodecyl suphate (El-Falaha et al. 1989); Phenol (Kroll and Anagnostopoulos 1981); Ethanol (Salton 1963); Chlorhexidine (Hugo and Longworth 1964b)
	2. Inhibition of membrane-bound enzymes	Inhibition of respiration and energy transfer; inhibition of ATP synthesis	Chlorhexidine (Chopra et al. 1987); CTAB (Rosenthal and Buchanan 1974)
Cytoplasm	1. Inhibition of cytoplasmic enzymes; interaction with biomolecules (e.g. DNA, RNA)	Inhibition of catabolic and anabolic processes	Formaldehyde (Hugo 1999); Chloroacetamide
	2. Coagulation and precipitation of cytoplasmic constituents	Denaturation of enzymes; destruction of biomolecules	Chlorhexidine and other biguanides (Hugo and Longworth 1964a); some phenolics and heavy metals (Hugo 1999)

Table 1 1 Targets and effects of some example biocides (adapted from Denyer, 1995)

1.3. AIMS

As identified in this literature review, antibacterial serrulatane compounds now have been isolated from four *Eremophila* species. These studies have included testing of serrulatane compounds against a limited number of standard strains of Gram-positive and Gram-negative bacteria and one yeast (*Candida albicans*). None of these studies have examined the toxicity of these purified compounds to mammalian cells or the mechanism of antibacterial action. This project was undertaken to provide further data on the toxicity, mechanism of action and spectrum of serrulatane diterpenoids. Two serrulatane compounds isolated from the species *Eremophila neglecta* were used for these studies due to the availability of sufficient purified compound to undertake these studies.

Specifically the aims of this project were to:

- examine the spectrum of activity of serrulatanes against a range of bacterial species including some clinical isolates;
- examine the *in vitro* cytotoxicity of serrulatanes with mammalian cells;
- investigate the mode of action of serrulatanes through an examination of effects on bacterial cell walls and cell membranes.

CHAPTER TWO

ANTIBACTERIAL SCREENING OF SERRULATANE COMPOUNDS

2.1. Introduction

As described in the previous chapter, a number of plants in the Australian genus *Eremophila* (Myoporaceae) are known as important medicines for Australian Aboriginal peoples (Low 1990; Ghisalberti 1994b; a)

The interesting antibacterial activity of crude *Eremophila* extracts against Gram-positive bacteria, including antibiotic resistant strains (Palombo and Semple, 2002; Ndi et al., 2007a), has led different groups to examine the active antibacterial components of these extracts. Chemical investigations of *Eremophila sturtii,* (Liu et al. 2006), *Eremophila serrulata* (Ndi et al., 2007b), *Eremophila duttonii* (Smith et al., 2007) and *Eremophila neglecta* (Ndi et al., 2007c) have identified various serrulatane diterpenoids as active antibacterial components. The purified serrulatane compounds isolated from these plants were tested for activity against a limited range of laboratory strains of Gram-positive and Gram-negative bacteria and the yeast *Candida albicans.* Activity was displayed specifically against the tested Gram-positives organisms including standard strains of *Staph. aureus*, *Strep. pyogenes*, and *Strep. pneumoniae*. (Liu et al., 2006; Ndi et al., 2007b; Ndi et al., 2007c; Smith et al., 2007).

The study described in this chapter was undertaken to further examine the spectrum of activity of the purified serrulatane compounds isolated from *Eremophila neglecta* against a range of Gram-positive and Gram-negative bacteria that are important human and veterinary

pathogens. This included testing against clinical isolates and some multi-drug resistant isolates. Compounds from this plant were chosen for study due to the availability of sufficient quantities of purified compounds for testing.

As described in Chapter 1 *Eremophila neglecta* is been used by particular groups of Indigenous Australians for their general well-being (Latz and Green 1995). The two bioactive serrulatane compounds previously isolated from *E. neglecta* (Ndi et al. 2007b), which are the subject of the study described in this and subsequent chapters, are 8,19-dihydroxyserrulat-14-ene (for simplicity denoted as **EN1** in the remainder of this thesis), and 8-hydroxyserrulat-14-en-19-oic acid (denoted as **EN2** in the remainder of this thesis).

2.2. Materials and methods

2.2.1 Serrulatane Compounds

Purified serrulatane compounds, 8,19-dihydroxyserrulat-14-ene (**EN1**), and 8-hydroxyserrulat-14-en-19-oic acid (**EN2**) were isolated from the leaves of *Eremophila neglecta* based on methods described previously (Ndi et al., 2007c). Compound identity and purity was confirmed by thin layer chromatography and Nuclear Magnetic Resonance (NMR) spectroscopy, and the purity of the compounds was estimated at greater than 99% by NMR. Compound isolation and identification was undertaken by Dr Chi Ndi, Ian Wark Research Institute, University of South Australia.

2.2.2 Antimicrobial assays

Materials

The culture media used for antibacterial assaying were the following: Mueller-Hinton II broth (BBL^{TM}; Becton Dickinson, France) and brain-heart infusion (BHI; CM 225, Oxoid).

Colombia agar (CM331-Oxoid, Basingstoke, England) and tryptone soya agar (CM0131-Oxoid, Basingstoke, England) were used as solid medium. Control antibiotics vancomycin hydrochloride, ampicillin sodium salt, gentamicin sulfate salt, amoxycillin and clindamycin hydrochloride were purchased from Sigma (USA). Dimethylsulfoxide (DMSO) was purchased from Univar (USA).

Bacterial strains and culture conditions

All bacterial strains were obtained from stock cultures preserved at -80 ^{0}C at the Sansom Institute, University of South Australia. Standard strains used to test minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of the serrulatane compounds were *Staphylococcus aureus* ATCC 29213, *Staphylococcus aureus* ATCC 25923, *Staphylococcus aureus* ATCC 43300, *Staphylococcus epidermidis* ATCC 35984, *Streptococcus pyogenes* ATCC 10389, *Streptococcus pneumoniae* ATCC 49619, *Streptococcus mutans* ATCC 25175, *Bacillus subtilis* ATCC 11774 and 6633, *Bacillus cereus* ATCC 14579, *Enterococcus faecium* ATCC 19434, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922, *Haemophilus influenzae* ATCC 49247, *Moraxella catarrhalis* ATCC 49143, *Salmonella enterica* subsp. *enterica* ATCC 13311, and *Pseudomonas aeruginosa* ATCC 15442. Clinical isolates of *Erysipelothrix rhusiopathiae* (Vetlab, Adelaide, South Australia), *Mycobacterium fortuitum* and *Mycobacterium chelonea* (Richard Lumb, SA Pathology, South Australia) were also used for MIC and MBC determinations. Additionally five clinical isolates of multi-drug-resistant methicillin resistant *Staph. aureus* (mMRSA), two clinical isolates of *Strep. pyogenes* and two clinical isolates of *Strep. pneumoniae* were obtained from Mr Lance Mickan, Infectious Disease Laboratory, Institute of Medical and Veterinary Sciences (IMVS), Adelaide, South Australia. Antibiotic sensitivities of these isolates had been previously examined at the IMVS and are detailed in Appendix 1.

The bacteria, with the exception of *H. influenzae* and the *Mycobacterium* species were grown up from frozen stocks on blood agar plates (Colombia agar supplemented with 5% v/v horse blood) at 37 ^{0}C. *H. influenzae* was grown on chocolate agar (Colombia agar including lysed horse blood by heating) and mycobacteria were grown on tryptic soy agar with 5% (v/v) horse

blood at 30 ^{0}C. The *Streptococcus* species were also incubated at 37 ^{0}C in the presence of 5% carbon dioxide (CO_2).

In the broth microdilution assays, brain heart infusion broth was used for the *Streptococcus* species while cation adjusted MH II broth was used for the *Staphylococcus* species and the Gram-negative organisms *E. coli*, *Mor. catarrhalis*, *Salm. typhimurium*, and *Ps. aeruginosa* and cation adjusted MH II broth with lysed 5% v/v horse blood was used for *Erysipelothrix rhusiopathiae* and *H. influenza*e.

Broth micro dilution assay for determination of MIC and MBC

Both test compounds were firstly dissolved in 1 ml dimethyl sulfoxide (DMSO) as a stock solution. The concentrations of the test compounds in the stock solutions were 50 mg ml^{-1} for EN1 and 31 mg ml^{-1} for EN2.

A broth micro dilution assay was used to determine the MICs of serrulatane compounds against Gram-negative and Gram-positive strains based on previously published methods (Ndi et al., 2007c; CLSI 2009a; b; c). Duplicate twofold serial dilutions of test compound (100 µL/well) to cover the concentration range 3.12 µg ml^{-1}to 400 µg ml^{-1} were prepared in sterile round-bottom 96-well plates (Sarstedt, Technology Park, South Australia), in the appropriate broth containing a final concentration of 1.28% DMSO. The bacterial cell suspension (100 µL/well) corresponding to 1× 10^6 CFU/mL was added in all wells except the control wells of the plate which served as saline, test compounds and media sterility controls. Controls for bacterial growth without test compound and including DMSO were also included on each plate. The final density of bacteria in the assay was 5×10^5 CFU/mL. The final concentration of the test compound was 1.56 µg ml^{-1} to 200 µg ml^{-1}and that of DMSO was 0.64% v/v. Plates were then placed on a shaker for 15 min and incubated in a humidified atmosphere at 37^{0}C overnight with the exception *Streptococcus* species, which were incubated in the presence of 5% CO_2 at 37 ^{0}C. Vancomycin, ampicillin (Gram-positive assays) and gentamicin (Gram-negative assays), were used as positive controls (known antibiotics) in the assays.

Amoxycillin, clindamycin and gentamicin were used as positive controls for assays of *Mor. catarrhalis* (CLSI 2009a; b; c).

After incubation, plates were examined and the MICs of the compounds were determined as the lowest concentration at which no growth was observed in the duplicate wells. The MIC values of the test compounds on each strain were determined visually with the naked eye. Following the determination of the MIC, the MBC was determined by transferring a 10 µL aliquot from each of the wells at the concentration corresponding to the MIC and those concentrations above into 190 µL of appropriate broth in a sterile 96-well plate. The plates were incubated under the same conditions as in the MIC experiment and the *Streptococcus* species incubated in the presence of 5% CO_2. The presence or absence of bacterial growth was determined by visual inspection. The MBC was considered to be the lowest concentration of the compound at which no growth occurred. The entire experiment was performed in duplicate for each compound and each bacterial strain.

2.3. Results

2.3.1 Antibacterial activity results

The two serrulatane-type compounds **EN1** and **EN2** exhibited antibacterial activity against all Gram-positive bacteria tested. Results of the broth dilution assays with the Gram-positive bacteria are summarized in Tables 2.1 and 2.2. Although both compounds showed antibacterial activity against Gram-positive bacteria, **EN1** exhibited greater activity. The MIC values of both compounds showed no differences between duplicate tests.

Against standard strains of *Staph. aureus* (including methicillin resistant strain ATCC 43300) and *Staph. epidermidis* (Table 2.1) MIC values were 6.2 µg $ml^{-1}$and 25.0 µg $ml^{-1}$for compounds **EN1** and **EN2**, respectively. The MBC values for the compounds were the same as or double the MIC concentrations. The compounds also showed activity against clinical isolates of multi-drug resistant of MRSA (mMRSA) with MICs ranging from 3.1 to 6.2 µg $ml^{-1}$for **EN1** and 12.5 to 25.0 µg ml^{-1} for **EN2**.

Microorganism	Compound **EN1** MIC (MBC) µg ml^{-1} and **µmol l^{-1}**	Compound **EN2** MIC (MBC) µg ml^{-1} and **µmol l^{-1}**
Staph. aureus ATCC 25923	6.2 (6.2) **20.7 (20.7)**	25.0 (50.0) **79.1 (158.1)**
Staph. aureus ATCC 29213	6.2 (6.2) **20.7 (20.7)**	25.0 (50.0) **79.1 (158.1)**
Staph. aureus ATCC 43300	6.2 (6.2) **20.7 (20.7)**	25.0 (50.0) **79.1 (158.1)**
mMRSA Clin. Iso. 1	6.2 (6.2) **20.7 (20.7)**	25 (50) **79.1 (158.1)**
mMRSA Clin. Iso. 2	6.2 (6.2) **20.7 (20.7)**	25 (50) **79.1 (158.1)**
mMRSA Clin. Iso. 3	6.2 (6.2) **20.7 (20.7**)	12.5 (25.0) **39.5 (79.1)**
mMRSA Clin. Iso. 4	6.2 (6.2) **20.7 (20.7)**	25.0 (50.0) **79.1 (158.1)**
mMRSA Clin. Iso. 5	3.1 (6.2) **10.3 (20.7)**	25.0 (50.0) **79.1 (158.1)**
Staph. epidermidis ATCC 35984	6.2 (12.5) **20.7 (41.4)**	25.0 (50.0) **79.1 (158.1)**

Clin. Iso.: clinical isolate

Table 2. 1 Antimicrobial activity of compounds EN1 and EN2 against *Staphylococcus* species

Microorganism	Compound **EN1** MIC (MBC) µg ml^{-1} and **µmol l^{-1}**	Compound **EN2** MIC (MBC) µg ml^{-1} and **µmol l^{-1}**
Streptococcus pneumoniae ATCC 49619	3.1 (6.2) **10.3 (20.7)**	6.2 (6.2) **19.8 (19.8)**
Strep. pneumoniae Clin. Iso. 1	12.5 (12.5) **41.4 (41.4)**	50.0 (50.0) **158.1 (158.1)**
Strep. pneumoniae Clin. Iso. 2	3.1 (3.1) **10.3 (10.3)**	6.2 (6.2) **19.8 (19.8)**
Streptococcus pyogenes ATCC 10389	6.2 (6.2) **20.7 (20.7)**	12.5 (25.0) **39.5 (79.0)**
Strep. pyogenes Clin. Iso. 1	3.1 (3.1) **10.3 (10.3)**	6.2 (12.5) **19.8 (39.5)**
Strep. pyogenes Clin. Iso. 2	3.1 (3.1) **10.3 (10.3)**	6.2 (12.5) **19.8 (39.5)**
Streptococcus mutans ATCC 25175	6.2 (6.2) **20.7 (20.7)**	25.0 (50.0) **79.0 (158.1)**
Bacillus subtilis ATCC 11774	3.1 (6.2) **10.3 (20.7)**	6.2 (12.5) **19.8 (39.5)**
B. subtilis ATCC 6633	3.1 (3.1) **10.3 (10.3)**	3.1 (6.2) **9.8 (19.8)**
Bacillus cereus ATCC 14579	6.2 (6.2) **20.7 (20.7)**	6.2 (12.5) **19.8 (39.5)**
Enterococcus faecium ATCC 19434	6.2 (6.2) **20.7 (20.7)**	50.0 (50.0) **158.1 (158.1)**
Ent. faecalis ATCC 29212	6.2 (6.2) **20.7 (20.7)**	50.0 (100.0) **158.1 (316.2)**
Erysipelothrix rhusiopathiae Clin. Iso.	50.0 (100.0) **165.4 (330.8)**	100.0 (200.0) **316.2 (632.5)**
Mycobacterium fortuitum Clin. Iso.	12.5 (12.5) **41.4 (41.4)**	100.0 (200.0) **316.2 (632.5)**
Mycobacterium chelonae Clin. Iso.	12.5 (12.5) **41.4 (41.4)**	100.0 (200.0) **316.2 (632.5)**

Clin. Iso.: clinical isolate

Table 2. 2 Antimicrobial activity of compounds EN1 and EN2 against different Gram-positive microorganisms

The MIC values of **EN2** against standard strains and clinical isolates of *Streptococcus* species ranged from 6.25 to 25 μg ml^{-1}while the **EN1** exhibited greater activity with 3.12-12.5 μg ml^{-1} values respectively (Table 2.2).

The MIC and MBC values of the two compounds were similar for the *Bacillus* spp. tested, ranging from 3.12 to 12.5 μg ml^{-1}. **EN1** showed more activity against *Enterococcus* spp and *Mycobacterium* spp. tested.

The only activity for the two compounds against the five Gram-negative bacteria tested was observed with *Moraxella catarrhalis* (Table 2.3). Compounds 1 and 2 showed MICs of 3.1 and 6.2 μg ml^{-1}, respectively, against this organism.

Microorganism	Compound **EN1** MIC(MBC) μg ml^{-1} and **μmol l^{-1}**	Compound **EN2** MIC(MBC) μg ml^{-1} and **μmol l^{-1}**
Moraxella catarrhalis ATCC 49143	3.1 (6.2) **10.3 (20.7)**	6.2 (12.5) **19.8 (39.5)**
Escherichia coli ATCC 25922	>200	>200
Salmonella enterica ATCC 13311	>200	>200
Haemophilus influenzae ATCC 49247	>200	>200
Pseudomonas aeruginosa ATCC 15442	>200	>200

Table 2. 3 Antimicrobial activity of compounds EN1 and EN2 against different Gram-negative microorganisms

Ampicillin, gentamicin, and vancomycin were used as the controls in the assays. The MIC of ampicillin against *Staph. aureus* (ATCC 29213) was 0.5-2 μg ml^{-1}, against *Strep. pneumoniae*

(ATCC 49619) was 0.125 µg ml^{-1} and against *E. coli* ATCC 25922 was 2-8 µg ml^{-1}. The MBC of ampicillin against *Staph. aureus* (ATCC 29213) was ≥2 µg ml^{-1}, against *Strep. pneumoniae* (ATCC 49619) was ≥0.25 µg ml^{-1} and against *E. coli* ATCC 25922 was ≥4 µg ml^{-1}. The MIC of gentamicin against *E. coli* ATCC 25922 was 0.25-1 µg ml^{-1} and the MIC of vancomycin against *Staph. aureus* (ATCC 29213) was 0.5-2 µg ml^{-1} (CLSI Standards, Antimicrobial Susceptibility testing, 2009a). The MBC of gentamicin against *E. coli* ATCC 25922 was ≥1 µg ml^{-1} and the MBC of vancomycin against *Staph. aureus* (ATCC 29213) was ≥2 µg ml^{-1}. The MICs of clindamycin, ampicillin and amoxillin against *Mor. catarrhalis* ATCC 49143 were 12.5-25 µg ml^{-1} for clindamycin and < 1.6 µg ml^{-1} for ampicillin and amoxillin. The MBCs of clindamycin, ampicillin and amoxillin against *Mor. catarrhalis* ATCC 49143 were ≥25 µg ml^{-1} for clindamycin and ≥ 3.2 µg ml^{-1} for ampicillin and amoxillin. These values were in line with those previously reported (Bell et al. 2009).

2.4. Discussion

Previous research by various research groups (Ndi et al., 2007b; Ndi et al., 2007c; Palombo et al., 2002; Liu et al., 2006; Smith et al., 2007) has only examined a limited range of bacteria with the purified serrulatane compounds. The research described in this chapter has now examined a wider range of bacteria including some clinical isolates. In agreement with previous published research the serrulatanes showed activity against a range of Gram-positive organisms with most Gram-negatives tested not being susceptible at the maximum concentration used.

Compound **EN1** has showed greater activity than the compound **EN2** against Gram-positive bacteria. The MBC values of **EN2** were mostly double the MIC values while for **EN1** MBC values were generally the same as the MIC.

Ndi et al (2007c) reported the MIC and MBC values of the pure serrulatane compounds **EN1** and **EN2** from *Eremophila neglecta* on some standard staphylococcal and streptococcal strains. The MIC (and MBC) values of **EN1** were 25.8 (25.8) µmol l^{-1} for *Staph. aureus* ATCC 29213 and 25923, and 12.9 (12.9) µmol l^{-1} for *Strep. pyogenes* ATCC 10389 and *Strep. pneumoniae* ATCC 49619. The reported MICs for **EN2** were two-fold higher than **EN1**. These

results are comparable with the results obtained in this study (Tables 2.1 and 2.2), although the MIC and MBC for **EN2** against *Staph. aureus* were found to be slightly higher. However, the results were within one two-fold dilution of the published results.

The results of this research show that the activity against streptococci and staphylococci also extends to methicillin-resistant and multi-drug resistant strains of *Staph. aureus* isolated from patients and clinical isolates of *Strep. pneumoniae* and *Strep. pyogenes*. **EN1** showed good activity against the biofilm-producing strain of *Staph. epidermidis* (ATCC 35984), an organism of importance in medical device-related infections (O'Gara and Humphreys, 2001) and *Strep. mutans* (ATCC 25175) an organism which plays an important role in dental decay (Zambon and Kasprzak, 1995). **EN1** also showed good activity against *Enterococcus* species which are important causes of nosocomial infections. *Enterococcus faecium* is one of the "ESKAPE" group pathogens for which strains that have multi-drug resistance have emerged (Boucher et al. 2009).

Both compounds displayed only moderate activity against the veterinary pathogen *Erysipelothrix rhusiopathiae*. This is a pleomorphic, nonsporulating, Gram-positive pathogen. The infections caused by this bacteria are worldwide in distribution and they occur in a wide variety of vertebrate and invertebrate species, including swine, sheep, cattle, horses, dogs, wild bears, kangaroos, reindeer, mice, wild rodents, saltwater fish, crocodiles, eagles, pigeons, chickens and turkeys (Woodbine 1950; Sneath et al. 1951; Conklin and Steele 1979; Wood and Steele 1994).

The two serrulatane compounds also showed significant anti-mycobacterial activity against the clinical isolates of *Mycobacterium fortuitum* and *Mycobacterium chelonae*. The compound **EN1** exhibited the greater activity with an MIC of 12.5 μg ml^{-1} whereas the MIC for compound **EN2** was 100 µg ml^{-1}. A previous study reported that crude extracts of *Eremophila* species *Eremophila alternifolia* and *Eremophila longifolia* exhibited activity against *Mycobacterium smegmatis* and *Myco. fortuitum* (Meilak and Palombo, 2008), although the active components were not identified. The terpenoid alkaloids pseudopteroxazole and *seco*-pseudopteroxazole isolated from the West Indian Sea whip *Pseudopterogorgia elisabethae* are

structurally related to the serrulatanes and have been reported to have antimycobacterial activity against *Myco. tuberculosis* H37Rv at 12.5 μg ml^{-1}. (Rodríguez et al., 1999). These results support the anti-mycobacterial activity of serrulatane compounds and the need for further anti-mycobacterial studies against *Myco. tuberculosis* strains.

The only activity observed against the five different Gram-negative organisms tested was against *Mor. catarrhalis.* This is the first report of antibacterial activity for serrulatane compounds against a Gram-negative organism. Previous studies on serrulatanes and crude extracts of *Eremophila* species have reported a lack of activity against Gram-negatives such as *Ps. aeruginosa and E. coli* (Palombo and Semple, 2001; Liu et al., 2006, Ndi et al., 2007c). The reason of the activity probably comes from the difference of cell wall structure of *Mor. catarrhalis* compared these other Gram-negative organisms. Although classified as Gram-negative, Fenollar (2000) has reported that members of the genus *Moraxella* 'may appear to be Gram-positive when Gram-stained.' *Mor. catarrhalis* has also been reported to resist destaining in the Gram-stain (Verduin et al., 2002). In another study on the susceptibility of β-Lactamase-negative strains to β-Lactam agents, there were 60-fold differences between ampicillin MICs and penicillin G MICs for *Mor. catarrhalis* strains. This could be due to the presence of lipooligosaccharides (LOS) rather than lipopolysaccharides (LPS) in the outer cell wall lipopolysaccharide layer of *Mor. catarrhalis* resulting in changes to the outer membrane permeability which could also affect the susceptibility of *Mor. catarrhalis* to serrulatanes.

The reason for the lack of activity of serrulatanes against Gram-negative strains such as *Ps. aeruginosa*, *Salm. enterica* and *E. coli* is most likely due to the difference in the Gram-negative cell wall structure compared to that of the Gram-positives (Figure 2.1). As other authors have pointed out previously (Smith et al. 2007) the relatively large molecule size and bicyclic conformation of the serrulatanes may prevent entrance of these molecules by size selective porin channels of the outer membrane of the Gram-negative cell wall. This would prevent the compounds from reaching the inner membrane and cytoplasm of Gram-negative species. This is thought to be a major factor in the lack of activity against this group of bacteria by many medicinal plant extracts in general (Cowan 1999; Smith et al. 2007).

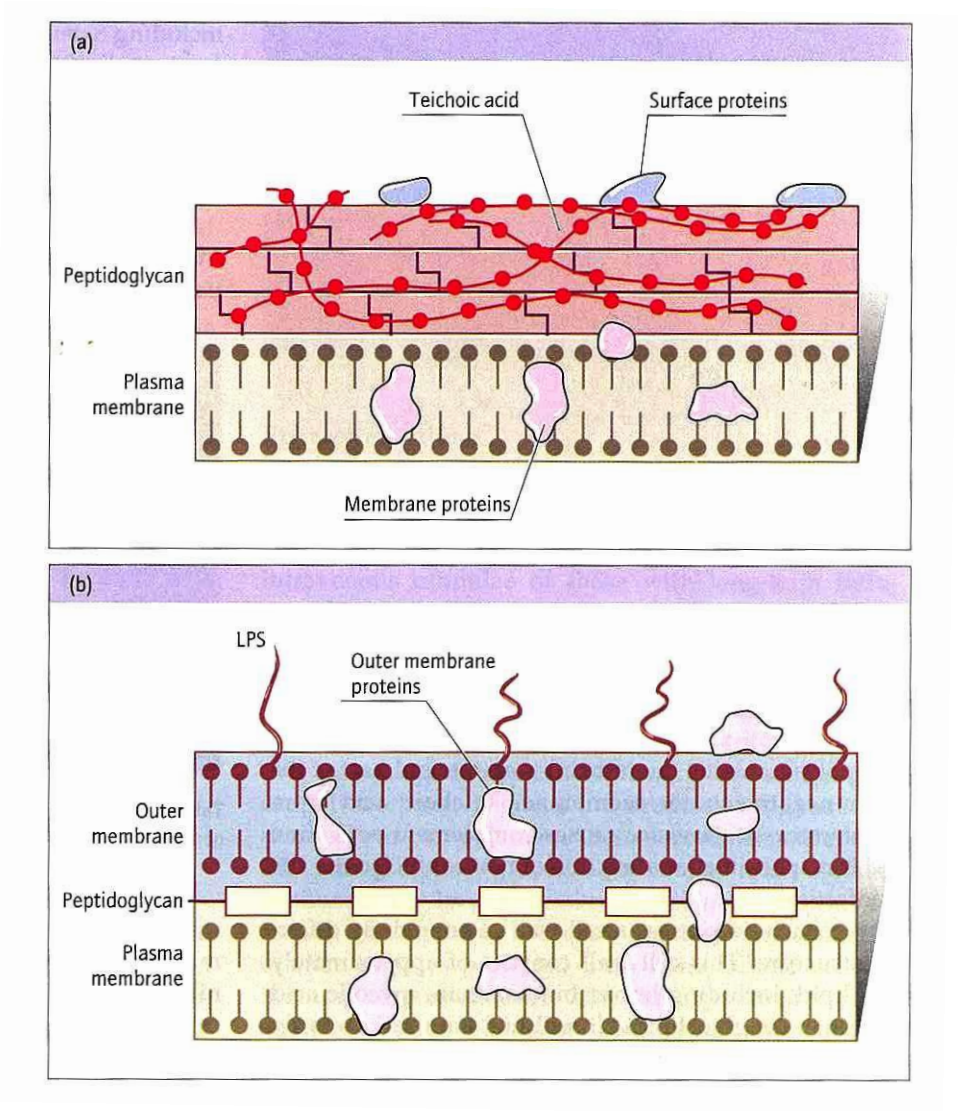


Figure 2.1 Structures of bacterial cell walls.

(a) Gram-positive cell wall. A gram-positive bacterial cell wall has many layers of peptidoglygan that retain the crystal of violet dye when the cell is Gram-stained. This gives the cell a purple color when seen under a microscope.

(b) Gram-negative cell wall. A gram-negative cell wall has a thin peptidoglycan layer. It has an outer membrane which is composed of phospholipids, outer membrane proteins and lipopolysaccharides (LPS).

(Diagram from: Bannister, B.A., Begg, N.T., Gillespie, S.H. (1996) *Infectious Disease*. Blackwell Science, Oxford, UK)

In determination of an anti-infective potential natural product for new drug leads, there are a number of pivotal quality standards need to be set at the point of initial evaluation in pharmacological screening models (Cos et al., 2006). Amongst these is a consideration of what actually constitutes a potentially clinically relevant antibacterial concentration. Cos et al. (2006) have suggested that pure compounds isolated from natural sources such as plants should have 50% inhibitory concentration below 25 µmol l^{-1} to be considered potential lead compounds. Thus, this study indicates that serrulatane compounds have potential as new anti-infective lead compounds against some clinically important bacteria including *Staphylococcus*, *Streptococcus*, *Enterococcus*, *Mycobacterium* and *Moraxella* species.

2.5. Conclusion

This study has provided important data on the spectrum of activities of the two serrulatanes tested with a wider range of test strains than previously examined. Both test compounds showed significant activity against medically important bacteria, however compound **EN1** exhibited more antibacterial activity. The results of this study confirm the activity against streptococci and staphylococci and also show that this activity extends to methicillin-resistant and multi-drug resistant strains of *Staph. aureus* and clinical isolates of *Strep. pneumoniae* and *Strep. pyogenes*. Activity against a Gram-negative species *Mor. catarrhalis* was demonstrated for the first time. The compounds also showed interesting activity against two *Mycobacterium* species (as a model for *Myco. tuberculosis*) and *Staph. epidermidis* an important cause of biofilm infections on medical devices.

CHAPTER THREE

EFFECT OF SERRULATANE COMPOUNDS ON MAMMALIAN CELL VIABILITY

3.1. Introduction

3.1.1 Background

Another important consideration in assessing anti-infective potential is the toxicity of any new antibacterial compounds to other cell types besides bacteria. In other words, whether the compound has selective toxicity to bacterial cells compared to other cell types such as mammalian cells. As described in Chapter 1 there has been no previous examination of the cytotoxicity of serrulatane compounds published in the literature. If these compounds are to be used in a clinical setting, an understanding of their toxic effects is critical. Therefore the next chapter of this thesis examines the cytotoxicity of compounds **EN1** and **EN2** to mammalian cells in culture.

3.1.2 Methods used to examine the cytotoxic effects of antibacterial natural products

In recent years there has been important progress in testing of bioactive agents that has changed the emphasis from *in vivo* animal methods to *in vitro* toxicity methods at least in initial pre-clinical testing. Many researchers have tested the efficiency of the *in vitro* methods (Shrivastava et al. 1992; Barile et al. 1994; Clemedson et al. 1996). Some of the common methods have been used to examine cytotoxic effects of natural products, particularly antibacterials, are tetrazolium-based methods such as MTS, XTT and MTT methods.

The tetrazolium method based on the salt [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) has advantages of ease of use, precision, and rapid determination of toxicity when compared with the MTT assay, an older tetrazolium method (Malich et al. 1997). The MTS assay method is available as a kit form from Promega (CellTiter 96® AQ_{ueous} Non-Radioactive Cell Proliferation Assay).

3.1.3 Principles of the MTS cell proliferation assay

The MTS cell proliferation assay is a colorimetric method for determining the number of viable cells in *in vitro* proliferation or chemosensitivity assays. The assay requires two reagents, the tetrazolium compound MTS and an electron coupling reagent phenazine methosulfate (PMS). MTS is bioreduced by cells into a coloured formazan product that is soluble in tissue culture medium (Barltrop and Owen 1991). The absorbance of the formazan at 490 nm can be measured directly from 96-well assay plates without additional processing (Cory et al. 1991; Riss and Moravec 1992). The conversion of MTS into aqueous, soluble formazan is accomplished by dehydrogenase enzymes found in metabolically active cells (Figure 3.1). The quantity of formazan product as measured by the amount of 490nm absorbance, is directly proportional to the number of living cells in culture (Riss and Moravec 1996).

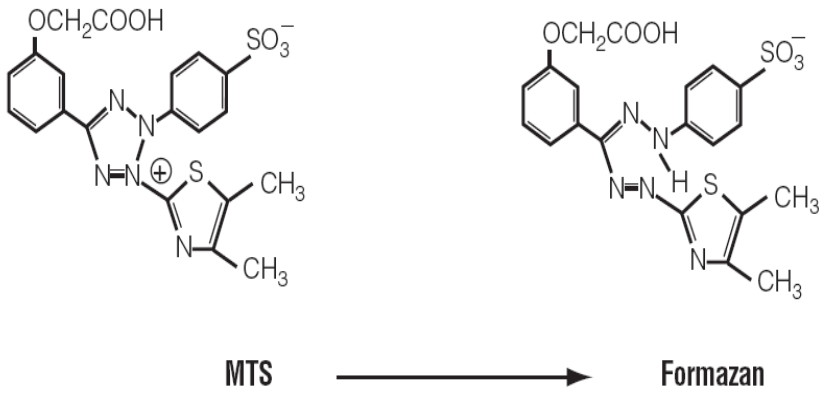


Figure 3.1 Structures of MTS tetrazolium salt and its formazan product (from CellTiter 96® AQueous Assay Kit booklet, Promega, Madison, WI, USA)

3.2. Materials and Methods

3.2.1 Materials

Serrulatane compounds **EN1** and **EN2** were obtained as described in Chapter 2 (section 2.2.1). The culture media used were Dulbecco's Modified Eagle Medium (HDMEM) with 20 mmol l^{-1} 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (Sigma-Aldrich, Australia or Institute of Medical and Veterinary Science (IMVS) Media Production, Adelaide, South Australia) with 10% (v/v) fetal bovine serum (FBS) (GIBCO, Invitrogen, Australia), and RPMI 1640 medium (GIBCO, Invitrogen, Australia) with 10% (v/v) heat-inactivated FBS, 2 mmol l^{-1} L-glutamine, and 20 mmol l^{-1} HEPES. The antibiotic polymyxin B sulfate used as a positive control (known substance) in the cytotoxicity assay and was purchased from Sigma (USA). The CellTiter 96® AQueous One Solution Cell Proliferation Assay kit was purchased from Promega (Madison, WI, USA) (containing [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) and phenazine methosulfate; (PMS). Dimethyl sulfoxide (DMSO) was cell culture grade and obtained from Sigma (USA). Trypan blue was obtained from Sigma (USA).

Cell culture plates (96-well, flat bottom) were used in cytotoxicity experiments. Plates for adherent cells (Vero) were obtained from IWAKI (Japan). Plates for non-adherent cells were obtained from Sarstedt (Technology Park, South Australia).

3.2.2 Cells and culture conditions

Cytotoxicity experiments were conducted using two different cell types. One cell line (Vero) was chosen as an adherent cell line of epithelial-type cells. This cell line is commonly used in cytotoxicity evaluations (Lindgren et al. 1994; Vijaya et al. 1995; Arpornsuwan and Punjanon 2006). The second (U937) was chosen as a non-adherent cell line which is blood cell (human monocyte) derived (Sundström and Nilsson 1976; Matsuda et al. 1991).

Vero cells (ATCC CCL-81 African Green Monkey kidney) were obtained from the Infectious Diseases Laboratory Institute of Medical and Veterinary Science (IMVS), Adelaide, South

Australia. Cells were grown in HDMEM media with 10% v/v FBS and incubated at 37 ^{0}C in a humidified atmosphere containing 5% CO_2. Cells were grown in standard flasks for adherent cell lines (Corning, USA). Cells were routinely passage every 3-4 days when 80-90% confluent.

U937 cells (human leukaemic monocyte lymphoma) were obtained from the Department of Rheumatology, Royal Adelaide Hospital, Adelaide, South Australia and were grown in RPMI 1640 medium with 10% (v/v) heat-inactivated FBS and 2 mmol l^{-1} L-glutamine 2 mmol l^{-1} and HEPES 20 mmol l^{-1}. Cells were incubated at 37 ^{0}C in a humidified atmosphere containing 5% CO_2. Cells were routinely passage every 5-6 days, with a 1 to 5 dilution in fresh cell culture medium. U937 cells were passage for a maximum seven times.

Frozen stocks of both cell lines were stored in liquid nitrogen in a freezing medium consisting of media with 20% v/v FBS and 10% v/v DMSO.

3.2.3 Examination of the relationship between absorbance and cell number in the MTS assay

The MTS/PMS solution was prepared according to the manufacturer's instructions for the CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay (Promega Madison, WI, USA) and aliquots stored at –20°C and protected from light.

Before the cytotoxicity testing with the serrulatane compounds was performed, experiments were undertaken to examine if there was a linear relationship between the cell number and absorbance at 492nm for the CellTiter96Aqueous (MTS) assay for the particular cell lines to be used. Experiments with each cell line were performed to obtain the correlation between the average cell number and absorbance at 492nm.

Cells were counted in the presence of trypan blue solution (0.1% v/v in saline) in a haemocytometer (Improved Neubauer) to obtain a viable cell count. For cell counting, 100 µl of cell suspension and 100 µl trypan blue solution were mixed in a sterile Eppendorf tube, and the solution was transferred to the haemocytometer using a glass pipette. Cells were counted

on an inverted microscope (Olympus CK2) in at least five chambers of the haemocytometer. The average number of viable cells per ml was calculated and the original cell suspension was diluted with the appropriate media. A series of samples were prepared to contain 0 to 1.5 x 10^6 cells per ml. Aliquots of 100 µl were added to the wells of a 96-well cell culture plate to give a series of cell densities from 0 to 1.5 x 10^5 cells per well. Six replicates were performed for each cell density. Cells were incubated in the plates for two hours at 37 ^{0}C in a humidified atmosphere containing 5% CO_2. to allow adherent cells to attach. The MTS assay was then performed.

CellTiter 96® AQueous One Solution Cell Proliferation Assay MTS solution 20µl/well was added to the cells and the plates were incubated for a further 2 hours. Absorbance was then measured at 492 nm using an ELISA reader (Multiscan, Ascent model no: 251). The entire experiment for each cell line was repeated three times, and the mean and standard deviation values were calculated.

Results were entered into Graph Pad Prism Software (Graph Pad Prism 5) and the correlation coefficient (r) calculated.

3.2.4 Cytotoxicity testing of serrulatane compounds

Viable cell counts were performed as described in the previous section. Cells were seeded into 96-well flat bottom tissue culture plates at a density of 7.5x10^4 cells per well (volume 50 µl per well) and incubated for 2 h before test compounds were added.

For the cytotoxicity testing, both serrulatane compounds were firstly dissolved in DMSO and mixed with test media. Controls for DMSO were also tested at different concentrations and it was determined that the maximum test DMSO concentration (0.5% v/v) had no cytotoxic effect on the cell lines which was in agreement with values reported in the literature (Prashanth Kumar et al. 2001). Serial dilutions of the serrulatane compounds in test medium were added ranging from 3.9 to 500 µg ml^{-1} (final concentration range 1.95 to 250 µg ml^{-1} with a final volume of 100 µl per well). Six replicates were performed for each concentration. Plates were incubated at 37 ^{0}C in a humidified atmosphere containing 5% CO_2 for 24h.

The known antibiotic polymyxin B sulfate was also tested in the assay with a final concentration range from 1.95 to 250 μg ml^{-1}.

After 24 h incubation of cells with the serrulatane compounds cells were visually inspected on an inverted microscope (Olympus CK2) at 100x and 200x magnifications for signs of cytotoxicity by examination of general morphology, detachment (for Vero cells), and cell lysis. Cells were scored according to published standards (AAMI, 1999) as 0 (non-cytotoxic), 1(mildly cytotoxic), 2 (moderately cyotoxic) and 3 (severely cytotoxic).

A tetrazolium-based method quantifying the bioreduction of the MTS tetrazolium salt by living cells was then used to assess cell viability in the presence of various concentrations of the test compounds. CellTiter 96® AQueous One Solution Cell Proliferation Assay MTS solution 20μl/well was added to the cells and the plates were incubated for a further 2 hours. Absorbance was then measured at 492 nm using an ELISA reader (Multiscan, Ascent model no: 251). The entire experiment for each compound and each cell line was repeated three times and the mean and standard deviation values were calculated.

The 50% cytotoxic concentrations (CC50) in the MTS assay were determined for each compound using a non-linear regression analysis (variable slope model) in Graph Pad Prism software (GraphPad Prism 5).

3.3. Results

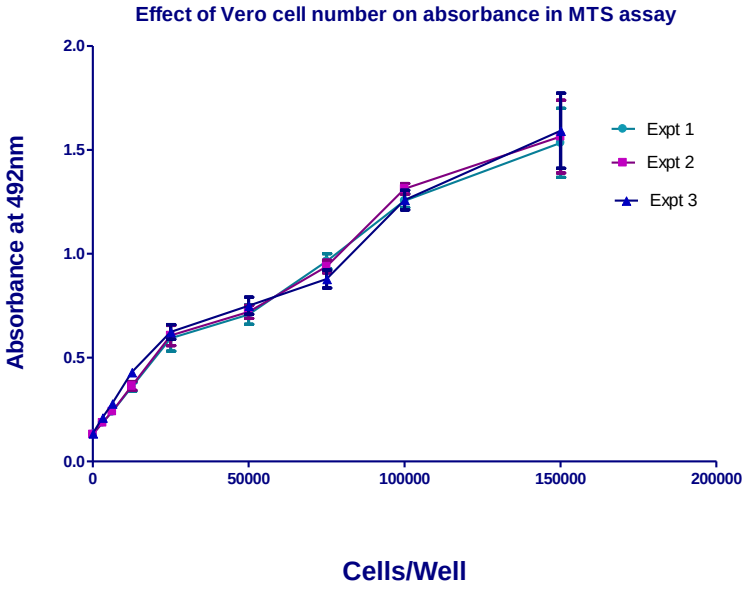
3.3.1 Examination of the relationship between absorbance and cell number in the MTS assay

The effect of cell density for the two cell lines on the absorbance in the MTS assay at 492nm is shown in Figure 3.2.

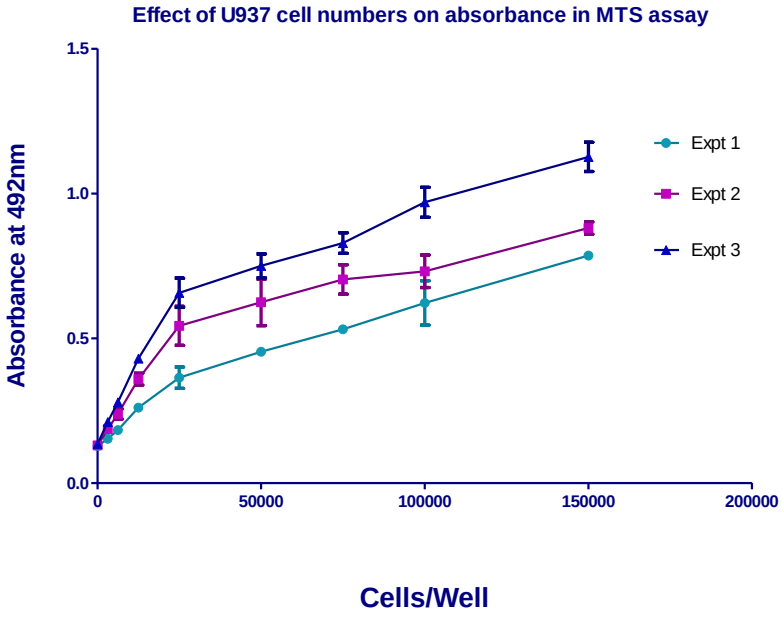
For the Vero cell line there was good correlation between the cell density and absorbance (Figure 3.2A) with correlation coefficients (r values) of 0.99 [95% Confidence interval (CI)

0.94-1.00] (experiment 1), 0.98 [95% CI 0.93-1.00] (experiment 2) and 0.98 [95% CI 0.93-1.00] (experiment 3).

For the U937 reasonable correlation between the cell density and absorbance (Figure 3.2B) was found, however data were more variable. Correlation coefficients (r values) of 0.98 [95% Confidence interval (CI) 0.90-1.00], 0.92 [95% CI 0.66-0.98] and 0.94 [95% CI 0.72-0.99] were obtained for the three replicate experiments.



A)



B)

Figure 3.2 Effect of cell number on absorbance at 492 nm measured using MTS assay.

Vero cells (A) and U937 cells (B). Results for 3 separate experiments are presented. The data for each experiment are the mean+/- SD for 6 replicates

3.3.2 Effect of serrulatanes on cell viability

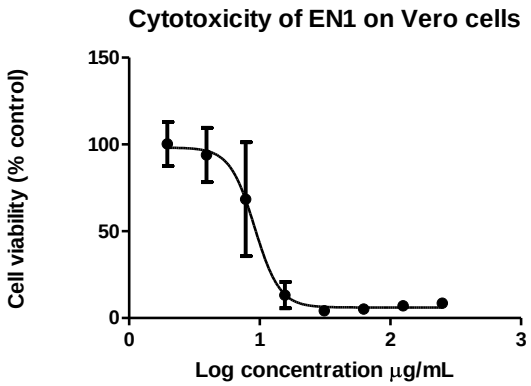
The relationship between concentration of the serrulatane compounds and their cytotoxic effect on CCL-81 (Vero) African green monkey kidney cells using the MTS assay is shown in Figure 3.3 (a and b). Non-linear regression curves were fitted to the data to allow calculation of the 50% cytototoxic concentration (CC_{50}) as summarised in Table 3.1. Both compounds showed moderately high cytotoxic effects on the cells. The CC_{50} of compound **EN1** was calculated as 9.2 µg ml^{-1} [95% confidence interval 6.5 to 12.8]. The goodness of curve fit (measured as the R^2) was 0.80. For compound **EN2** the CC_{50} was 20.0 µg ml^{-1} [95% confidence interval 16.8 to 23.7 µg ml^{-1}] with an R^2 value of 0.98.

By microscopic inspection the maximum non-cytotoxic concentration of the compounds on Vero cells was determined to be 15 µg $ml^{-1}$for **EN1** and 31 µg $ml^{-1}$for **EN2.**

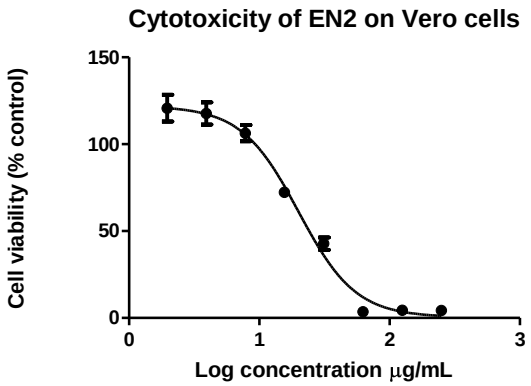
The cytotoxic effects of both compounds on U937 human leukemic monocyte lymphoma cells are shown in Figure 3.3 (c and d). The 50% cytotoxic concentration (CC_{50}) of compound **EN1** (Table 3.1) was not able to be determined accurately in this model but was estimated at 3.6 µg ml^{-1} due to some issues of variability in the data while for compound **EN2** it was 36.3 µg ml^{-1} [95% confidence interval 23.18 to 56.74 µg ml^{-1}], with an R^2 value of 0.73.

By microscopic inspection the maximum non-cytotoxic concentration of the compounds on U937 cells was determined to be 7.5 µg $ml^{-1}$for **EN1** and 62.5 µg ml^{-1} for **EN2**.

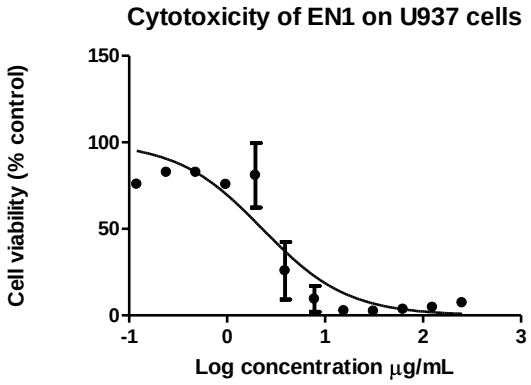
The cells were also tested with the control antibiotic polymyxin B sulfate, with CC_{50} of this compound found to be 90.6 µg ml^{-1} [95% confidence interval 45.8 to 179.3 µg ml^{-1}] for Vero cells and 118 µg ml^{-1} [95% confidence interval 107.8 to 128.9 µg ml^{-1}] for U937 cells.



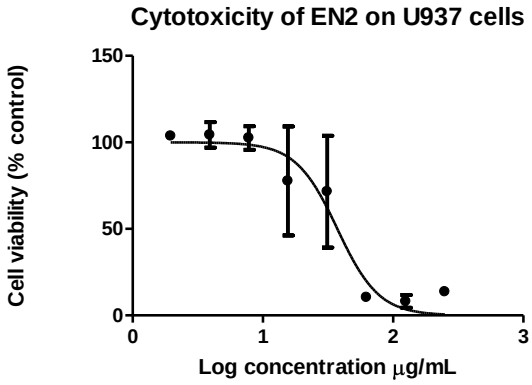
a)



b)



c)



d)

Figure 3.3 Concentration-dependent cytotoxic effect of serrulatane compounds

Non-linear regression for serrulatane compounds EN1 and EN2 on Vero cells (A – B) and U937 cells (C – D) in the MTS assay. Absorbance in the MTS assay was measured at 492 nm. Compounds were tested at concentrations ranging from 1.95 to 250 μg ml^{-1} and incubated with cells for 24 hours. Data represents mean ± S.D. from three independent replicate experiments.

	CC_{50} values	95% confidence interval	Non cytotoxic concentration (by microscopic inspection)
VERO CELLS			
EN1	9.2 µg ml^{-1}	6.5 to 12.8 µg ml^{-1}	15 µg ml^{-1}
EN2	20.0 µg ml^{-1}	16.8 to 23.7 µg ml^{-1}	31 µg ml^{-1}
Polymyxin B sulfate	90.6 µg ml^{-1}	45.8 to 179.3 µg ml^{-1}	125 µg ml^{-1}
U937 CELLS			
EN1	3.6 µg ml^{-1}	not able to be determined	7.5 µg ml^{-1}
EN2	36.3 µg ml^{-1}	23.18 to 56.74 µg ml^{-1}	62.5 µg ml^{-1}
Polymyxin B sulfate	118 µg ml^{-1}	107.8 to 128.9 µg ml^{-1}	250 µg ml^{-1}

Table 3.1 Summary of cytotoxicity data

3.4. Discussion

This is the first study assessing the cytotoxicity of serrulatanes from *Eremophila neglecta* on mammalian cells *in vitro*. Reflecting the antibacterial activity results, compound **EN1** showed slightly more cytotoxicity with CC_{50} concentration of 9.2 µg ml^{-1}compared with 20.0 µg $ml^{-}1$ for compound **EN2** in Vero cells. These results suggest that the compounds have mammalian cell toxicity at concentrations similar to the MIC concentrations against Gram-positive bacteria. Further testing with a range of different cell types and for different exposure times (e.g. longer than 24 hours) is needed to confirm these findings. However, the results suggest

that application of these compounds in a clinical setting may need to be restricted to topical applications.

The Vero cell line, a widely used cell line for examining cytotoxic effects, was found to be a useful for assessing the cytotoxic effects of the serrulatanes using the MTS assay. It showed good correlation between cell numbers and absorbance measured as the endpoint of the assay and consistent results were able to be obtained. Results for the U937 cell line were less consistent and it was not possible to accurately determine a 50% cytotoxic concentration for compound **EN1** with these cells. Compound **EN1** produced steep dose-response curves in both cell lines (from close to 100% cell viability to close to 0% cell viability in one or two two-fold dilutions). This also meant that determination of an accurate 50% cytotoxic concentration was difficult.

The cytotoxicity of the known antibiotic polymyxin B was assessed for comparison with the serrulatanes in the assay. This antibiotic is known to have effects on membrane permeability of bacteria and to be relatively toxic compared to other many antibiotics in clinical use (Fuchs et al., 1998). However, it is well tolerated enough to be used topically in humans including for ocular applications (Sweetman, 2010). The cytotoxicity result of the test control antibiotic polymyxin B sulfate showed that it has less toxicity against the tested cell line when compared with serrulatane compounds. The 50% cytotoxic concentration of polymyxin B sulfate was 90.6 µg ml^{-1} on Vero cells and 118 µg ml^{-1} on U937 cells. This was comparable with an 50% cytotoxic concentration of approximately 100 µg ml^{-1} reported for the compound by Duwe et al. (1986) although this study used a different methodology to assess cytotoxicity and a different cell line (K562 cells, human erythroleukemia).

While the toxicity of the serrulatanes may prevent their use in systemic applications, they may still have potential to be used in topical applications. Some other plant derived compounds and extracts that have toxic effects when applied directly to cells in culture are used safely as topical antiseptics. For example *Melaleuca alternifolia* (tea tree) oil is used widely in topical applications. Studies examining the cytotoxicity of tea tree oil and its components have reported 50% cytotoxic concentrations against a range of fibroblast and epithelial cell lines from 20 to 2700 µg ml^{-1} (Söderberg et al. 1996; Hayes et al. 1997; Mikus et al. 2000;

Schnitzler et al. 2001). Additionally tea tree oil has been demonstrated to have toxic activity against human monocytes at concentrations of ≥ 0.004% (Hart et al. 2000). To compare the cytotoxic activities of serrulatane compounds with other antimicrobial compounds from medicinal plants, further studies need to be undertaken with a range of different cell lines. Cell culture models that more closely reflect the structure of the skin (rather than direct application of compounds to cells) may also be useful in assessing whether the serrulatane compounds may be tolerated in topical applications. For example the Epiderm Skin Irritation Test (EPI-200-SIT) is a three dimensional skin model which provides a way to understand the skin irritating effects of chemicals *in vitro* (MatTek, 2009 Test) .

3.5. Conclusion

In this study, the cytotoxicity of serrulatanes from *Eremophila neglecta* to mammalian cells *in vitro* has been assessed for the first time. These results suggest that the compounds have mammalian cell toxicity at concentrations similar to the MIC concentrations against Gram-positive bacteria. Further testing with a range of different cell types and for different exposure times is needed to confirm these findings.

CHAPTER FOUR

INVESTIGATION OF THE ANTIBACTERIAL MODE OF ACTION OF SERRULATANE COMPOUNDS THROUGH EXAMINATION OF THEIR EFFECTS ON BACTERIAL CELL WALLS AND CELL MEMBRANES.

4.1. Introduction

4.1.1 Previous research on *Eremophila* extracts

As described in the literature review in Chapter 1, there has been no published research examining the mechanism of the antibacterial effects of the serrulatanes. One study examined the effects of a crude extract of *Eremophila duttonii* and showed that it exhibited effects on the integrity of the cytoplasmic membrane of *Staph. aureus*, leading to increased membrane permeability (Tomlinson and Palombo 2005). However, this extract contained a mixture of plant compounds not just serrulatanes.

Many of the antibacterial serrulatanes that have been isolated, including **EN1** and **EN2**, are phenolic compounds (see figures 1.8-1.9, Chapter 1). As such it may be postulated that they have biocidal effects similar to some other phenolic compounds. However, interestingly the bactericidal compound serrulatic acid isolated from *Eremophila sturtii* lacks a phenolic hydroxyl group (Figure 4.1).

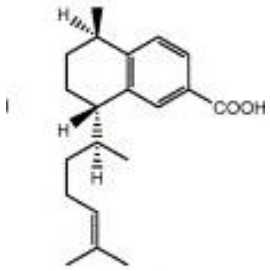


Figure 4.1 Serrulatic acid from *Eremophila sturtii* (from Liu et al. 2006)

Based on the structural characteristics of the serrulatanes as well as previous research suggesting that *E. duttonii* extracts had effects on membrane permeability, investigation of the effects of the serrulatanes on bacterial cell walls and membranes was considered a reasonable initial approach to investigate their mode of action for the study described in this chapter.

An understanding of the mechanism of action of these compounds is important to understanding their likely toxicity and whether bacteria are likely to develop resistance to them. As indicated in Chapter 1, other research at the University of South Australia is examining the covalent attachment of these compounds onto surfaces to deter microbial growth on plastics (such as catheters). Having an understanding of the way the compounds work in solution will be critical to understanding their actions on surfaces as well.

Various approaches have been used to study the mode of action of antibacterial compounds including those from natural sources. An overview of some of these approaches is given in the next section.

4.1.2 Approaches used to study effects on bacterial cytoplasmic membrane function

4.1.2.1 Propidium Iodide (PI) uptake assay:

Propidium iodide (PI) is a fluorescent marker which intercalates with DNA. It is excluded by intact cells but can permeate damaged cells. Propidium iodide uptake has been used to

examine effects on membrane permeability for bacteria. The PI uptake assay was used in the study of the mechanism of antibacterial action of the *Eremophila duttonii* crude extracts (Shah et al. 2004). This study suggested that compounds from this species may be membrane active. The method has also been used by other authors for investigations of other plant extracts such as essential oils (Carson et al. 2002).

The basis of the PI uptake assay is that this nucleic acid-binding molecule is membrane impermeable. Sub-lethal injury of bacterial cells by a compound can affect the membrane permeability, allowing the PI to enter the cell and intercalate with nucleic acids. The PI uptake assay provides a means to determine whether membrane damage is the result of biocidal action or in consequence of some other cellular event (Maillard 2002; Tomlinson and Palombo 2005). The results of study with the *E. duttonii* extract (Shah et al. 2004) showed that bacteria exposed to the extract had decreased membrane stability. The increase in the bacterial culture fluorescence level when exposed to the *E. duttonii* extract was similar to that observed when exposed to CTAB, a known membrane permeabilising agent (Shah et al. 2004).

Therefore, the PI uptake assay was used as one approach in the research described in this chapter to investigate the antibacterial mechanism of action of purified serrulatanes.

4.1.2.2 Salt tolerance assay:

The salt tolerance assay is another method used to investigate the effect of compounds on bacterial membrane permeability in antibacterial mechanism of action studies (Carson et al. 2002; Tomlinson and Palombo 2005). Sublethal injury of bacterial cytoplasmic membranes can result in increased permeability, therefore, affecting the cell membrane's ability to regulate the passage of substances such as inorganic materials (Carson et al. 2002). A loss of tolerance to salts following exposure to an investigated compound may be used to show membrane damage in bacteria (Carson et al. 2002). In a study examining the mechanism of action of *Melaleuca alternifolia* (tea tree) oil on *Staph. aureus,* Carson et al. (2002) examined the ability of *Staph. aureus* cells treated with tea tree oil to grow on nutrient agar supplemented with NaCl. Treatment of *Staph. aureus* with tea tree oil or its components significantly reduced the number of the colonies on media containing NaCl.

4.1.2.3 Adenosine triphosphate (ATP) measurement:

The adenosine triphosphate (ATP) measurement assay is another possible method for detecting bacterial membrane damage in the presence of a compound. This method was used, for example, by Simoes et al. to examine the mechanism of the dialdehyde biocide ortho-phtalaldehyde (OPA), on bacteria, using the Gram-negative species *Pseudomonas fluorescens* (Simoes et al. 2007). Destabilisation of bacterial membranes by an investigational compound results in release of ATP from bacterial cells. The amount of ATP released can be measured using a bioluminescence assay in which the enzyme luciferase catalyzes a reaction between the substrate luciferine and ATP. This results in the emission of photons of light with the intensity of light produced being proportional to the ATP content. (Simoes et al. 2007).

4.1.2.4 Leakage of intracellular components

The leakage of intracellular components is another way that is used to demonstrate a loss of bacterial membrane structure. The method which is used in leakage of intracellular components is called ‘loss of 260-nm-absorbing material’. For example, leakage of cells treated with tea tree oil was used by (Cox et al. 1998) and (Carson et al. 2002) in their investigation of membrane-activity. In this method, treated bacterial cultures are filtered and the absorbance measured at 260 nm. This wavelength is known to be strongly absorbed by nucleic acids, and the appearance of 260nm-absorbing material in filtrates is indicative of damage in the membrane structure (Carson et al. 2002). Marked leakage of a bacterial cell`s cytoplasmic material is considered indicative of gross and irreversible damage to the cytoplasmic membrane (Hugo and Longworth 1964). Carson et al. (2002) reported the loss of 260-nm-absorbing material following treatment of bacteria with TTO and some of its components suggesting that the cytoplasmic membrane was compromised. There are many studies showing that compounds that act on the bacterial cytoplasmic membrane induce the loss of 260-nm-absorbing material, including chlorhexidine (Hugo and Longworth 1964), hexachlorophene (Joswick et al. 1971), phenethyl alcohol (Silver and Wendt 1967), tetracyclines, polymyxin (Corry et al. 1977), α-pinene (Andrews et al. 1980), and lemongrass oil (Onawunmi and Ogunlana 1985).

4.1.2.5 Confocal laser scanning microscopy (CLSM):

The CLSM technique is another method that is used in antimicrobial mechanism of action studies. In this technique, bacteria cells can be treated with the antimicrobial compound labelled with 4-sulfo-2,3,5,6-tetrafluorophenol sodium salt (STP), and incubated. Then cells are harvested by centrifugation and washed with PBS. Visualisation and localization of the labelled compound is examined by using a confocal microscope (Kim et al. 2002). A study using the CLSM technique examined the antimicrobial mechanism of β-Glycyrrhetinic acid isolated from the licorice plant *Glycyrrhiza glabra* against *Bacillus subtilis* and *Staph. epidermidis* (Kim et al. 2002). The compound was labelled with STP which allowed the location of the compound in the bacterial cell to be examined with CLSM. This showed that β-Glycyrrhetinic acid was located within the bacteria and had not caused membrane disruption (Kim et al. 2002).

4.1.2.6 Scanning electron microscopy (SEM)

The scanning electron microscopy (SEM) technique is used in antibacterial mechanism of action studies to detect the morphological changes of bacteria cells. This technique images the sample surface and allows detailed three-dimensional images of bacterial cells to be visualised. Simoes et al. (2007) examined the influence of the dialdehyde biocide ortho-phtalaldehyde (OPA) on bacterial structure by SEM to determine morphological comparison between untreated and treated cells. These scanning electron micrographs revealed a morphological alteration induced by the biocide, where the treated cells appeared elongated.

A natural product (snake venom protein component) called omwaprin has also been examined for its effects on bacterial membranes using SEM (Nair et al. 2007). The antibacterial activity of omwaprin was examined and the protein showed a concentration-dependent antibacterial activity against selected Gram-positive strains including *Bacillus megaterium* and *Staph. warneri*. Morphological changes induced by the protein on these strains were studied by SEM. The micrographs of treated bacterial cells showed membrane blabbing, pore formation and

leakage of cellular contents indicating membrane disruption was the mechanism by which this compound produced its antimicrobial activity (Nair et al. 2007).

4.1.2.7 Transmission electron microscopy (TEM)

Transmission electron microscopy (TEM) is a widely used method in antimicrobial mode of action studies to observe directly the effects of the bioactive compounds on bacterial cells. The use of TEM has generally the advantage of higher magnification resolution compared with SEM and allows observation of cross-sections of the cells.

A study examining the mechanism of action of *Melaleuca alternifolia* (Tea tree) oil on *Staph. aureus* included TEM studies to assess effects on bacteria treated with terpinen-4-ol (one of the active components of TTO). Treated bacteria cells showed multilamellar, mesosomelike structures suggesting cell membrane damage (Carson et al. 2002).

Recently, another mode of antibacterial action study examined the anti-staphylococcal action of a crude extract from *Eleutherine americana* against methicillin-resistant *Staph. aureus* (MRSA) (Ifesan et al. 2009). Transmission electron microscopy was used in conjunction with other mode of action methods, including salt tolerance tests, bacteriolysis and loss of 260-nm-absorbing material, to show that the extract of *Eleutherine americana* caused damage to bacterial membrane morphology.

In the study described in this chapter, TEM was used to examine the antibacterial mechanism of action of serrulatane compounds from *E. neglecta*.

4.1.3. Objectives of the study described in this chapter

The study described in this chapter was undertaken to investigate the antibacterial mode of action of the serrulatane compounds EN1 and EN2 through investigation of the effects of these compounds on bacterial cell walls and cell membranes. Investigations included time kill experiments, bacteriolysis, salt tolerance, and propidium iodide uptake (PI) assays and electron microscopy. A standard strain of *Staph. aureus* which had been shown to be sensitive to both compounds was used as the test organism for these studies. Due to the quantities of

pure compound available, EN2 was used for most of the studies described in this chapter. Both EN1 and EN2 were tested in the electron microscopy studies.

4.2. Materials and methods

4.2.1 Materials

The culture media used for antibacterial assaying were the following: Mueller-Hinton II broth (BBL^{TM}; Becton Dickinson, France), Colombia agar (CM331-Oxoid, Basingstoke, England), Nutrient agar (CM0003-OXOID, Basingstoke, England), Nutrient broth (CM1-OXOID, Basingstoke, England), Heart Infusion broth (CM1032-OXOID, Basingstoke, England) and Sodium chloride (A465-UNIVAR). Phosphate buffer saline (PBS) was purchased from OXOID (BR0014G) and control agents terpinen-4-ol, hexadecyltrimethylammonium bromide (CTAB) was purchased from Sigma (USA). Propidium iodide (PI) was also purchased from Sigma (USA). Serrulatane compounds EN1 and EN2 were isolated from *Eremophila neglecta* as described in Chapter 2 section 2.1.1.

4.2.2 Preparation of bacterial suspension

Staph. aureus (ATCC 29213) was obtained from stock cultures preserved at -80 ^{0}C at the Sansom Institute, University of South Australia. For the salt tolerance and propidium iodide (PI) tests bacteria were grown for 18 hours at 37 ^{0}C on nutrient agar (NA) and then one to two colonies were placed in to 3 ml nutrient broth and incubated for 4 hours with shaking (Tomlinson and Palombo 2005). After incubation the bacterial suspension was centrifuged at 10,000 g for 12 min at 4 ^{0}C, separated from the medium and washed twice with phosphate-buffer saline (PBS; pH 7.4). For the time killing and bacteriolysis tests bacteria were grown overnight at 37 ^{0}C on blood agar and then one to two colonies were placed into 400 ml Mueller-Hinton broth incubated for 18 hours with shaking at 37 ^{0}C, then centrifuged and washed with PBS. After all preparations of the bacterial suspension, the bacterial pellet was resuspended in PBS. The test solutions with bacteria, the compounds and the control agents

were also incubated with shaking to mix well and to minimize the technical difficulties of mixing high concentrations of the control agents.

4.2.3 Salt tolerance

The salt tolerance assay method was based on that from Carson et al (2002) and Tomlinson et al (2005) with modifications. In this test, *Staph. aureus* ATCC 29213 was treated with the test compound **EN2** or positive control compound terpinen-4-ol then tested for its ability to grow on Nutrient Agar (NA) supplemented with NaCl 5.0 or 7.5%.

Before the mechanism of action tests, the MIC of terpinen-4-ol on *Staph. aureus* ATCC 29213 was determined by a broth micro dilution test based on that described in Chapter 2 section 2.1.2 (Carson et al. 1995; Carson and Riley 1995). The MICs of terpinen-4-ol on test strain *Staph. aureus* ATCC 29213 was determined as 0.25% v/v which was in agreement with previous findings (Carson et al., 2002).

Suspensions of *Staph. aureus* ATCC 29213 were prepared as described above (Section 4.2.2). The bacteria cells then treated with the serrulatane compound EN2 or terpinen-4-ol at 0.5x the minimum inhibitory concentration (MIC), 1x MIC, and 2x MIC. The MIC concentration for EN2 was that determined in Chapter 2. After 30 min, samples were removed, serially diluted and 10 µl of bacteria suspension was plated on NA only and NA supplemented with 5% or 7.5% NaCl. Controls without the test compound were also performed. Then the plates were incubated at 37 ^{0}C for 24 h and the number of colony forming units (CFUs) were counted. The resulting colony numbers per ml from the NA plates supplemented with NaCl; were then calculated as a percentage of the colony numbers per ml from the NA-only plate. The entire experiment was performed in triplicate. The methods used in this test those of Carson (2002), and the SEM values were used in statistical analysis of the results rather than standard deviation to follow these authors. Results were analysed using two-way analysis of variance (ANOVA) with Bonferroni posttests using Graph Pad Prism software (Graph Prism 5). A p value of less than 0.05 was considered significant.

4.2.4 Time Killing

A bacterial killing assay was modified from Carson et al (2002). The antimicrobial activities of serrulatane compounds against *Staph. aureus* ATCC 29213 were evaluated by measuring the reduction in the numbers of CFU per milliliter over time. *Staph. aureus* suspensions were prepared as described above in Section 4.2.2. The test suspension was 5 ml and after pre-treatment sample (0.5 ml) was taken, the compound **EN2** and terpinen-4-ol suspensions were added to yield final concentrations of 0.5x, 1x, and 2x the MICs. The test suspensions then were mixed for 20 s with a vortex mixer. After vortexing 0.5 ml of sample was removed at 30 s, serially diluted, and plated onto NA. subsequent samples were taken at 30, 60, and 90 and 120 min and diluted, plated onto NA, and incubated overnight at 37 ^{0}C. The mean number of surviving colonies from duplicate plates per experiment was determined by counting the number of CFUs. The entire experiment was performed in triplicate.

4.2.5 Bacteriolysis

A bacteriolysis test was modified from Carson et al (2002). *Staph. aureus* ATCC 29213 suspensions were prepared as described above. The test suspension was prepared as a volume of 5 ml and after pre-treatment sample (0.5 ml) was taken, the compound **EN2** and terpinen-4ol suspensions were added to yield final concentrations of 0.5x, 1x, and 2x the MICs. The test suspensions then were mixed for 20 s with a vortex mixer. After vortex the OD_{620} was measured at 30 s. The difficulty of solubility when working with higher concentrations of terpinenol was able to be solved by using a shaker incubator for the experiments. Additional test suspensions were measured at 30, 60, 90, and 120 min. The OD_{620} values of serrulatane extract and terpinen-4-ol without bacteria also premeasured before the test. In the third test, the OD_{620} was remeasured 6.5 and 23 h later.

4.2.6 Propidium iodide uptake assay

4.2.6.1 Optimisation of assay conditions

Propidium iodide (PI) uptake assay was modified from Niven & Mulholland (1998) and Tomlinson et al (2005). Fluorescence was measured using a fluorescence spectrophotometer (VARIAN, Cary Eclipse). The maximum fluorescence points of propidium iodide emission wavelength (EM) and excitation wavelength (EX) were determined as described previously (Niven & Mulholland (1998) and Tomlinson et al (2005)). The bacterial suspension of *Staph. aureus* was prepared as described in Section 4.2.2., then 30 µmol l^{-1} PI and 0.2 mmol l^{-1} hexadecyltrimethylammonium bromide (CTAB) were added to the suspension (Niven and Mulholland 1998). The EM was firstly set to 500 nm on spectrophotometer, and the EX value then gradually increased from 550 to 650 nm. The maximum fluorescence point gave the value of EX maximum (EX_{max}). And then the emission wavelength maximum (EM_{max}) was also determined at with EX held constant. The emission wavelength increased from 450 to 550 nm. The slit width used was 10 nm.

For determination of optimum concentration of the PI to use in the assay, a method was used as described by Niven and Mulholland (1998). Suspensions of *Staph. aureus* were prepared and then 0.2 mmol l^{-1} CTAB and PI in concentrations ranging from 10 to 80 µmol l^{-1} were added. Triplicate samples were prepared for each PI concentration. The test solutions were then mixed in a vortex mixer for 20 seconds, and their fluorescence measured after 20 mins.

The measurement of the relationship between absorbance and fluorescence of bacteria suspensions treated with CTAB and PI was also investigated. Triplicate samples of eight different concentration dilutions of PI were prepared, and their absorbance and fluorescence measured.

The fluorescence of the serrulatane compound EN2 and PBS buffer were also investigated. Their solutions were prepared both with and without PI added, and the fluorescence then measured.

4.2.6.2 Testing of EN2 and control compound in the PI assay

Once the optimal conditions for the test had been determined as described above, the propidium iodide uptake assay was conducted in the presence of the EN2 or the control compound CTAB. Bacterial suspensions were prepared as described in Section 4.2.2 and 50 μmol l^{-1} PI added. The MIC amount 25 μg ml^{-1} of the test compound or 0.2 mmol $l^{-1}$of CTAB was then added to one of these solutions, with another being the control. Reagent blanks containing PBS, PI and serrulatane extract were also prepared. The solutions were then mixed with a vortex mixer for 20 seconds and their fluorescence was measured after adjusting the reagent blanks to zero on the spectrophotometer (VARIAN-Cary Eclipse). The process was repeated every 30 mins for 1.5 hrs. Results comparing fluorescence intensity between no treatment control, CTAB and EN2 were analysed using one-way ANOVA with Bonferroni's Post Test using Graph Pad Prism software (Graph Pad Prism 5), p value of less than 0.05 was considered significant. The means and standard deviations from three replicate experiments were calculated.

4.2.7 Transmission electron microscopy (TEM)

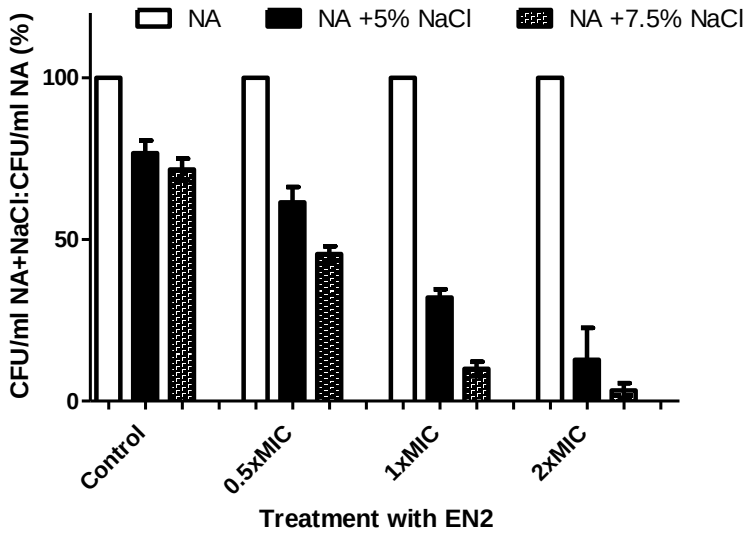
Sample preparation for TEM was based on methods described previously (Carson et al., 2002). Suspensions of *Staph. aureus* ATCC 29213 were prepared as described previously by inoculating and incubating 30 ml of heart infusion broth (HIB). The bacteria cell suspension were then centrifuged at 1.500 x *g* for 10 min, and the pellet was resuspended in HIB supplemented with Tween 80 (0.5% [vol/vol]) (HIB-T). The cell suspensions were then treated with test compounds **EN1, EN2** or terpinen-4-ol at 1x MIC levels for 30 min at 37^0C in an incubator with shaking. The control suspension of untreated cells was also incubated in HIB-T for 30 min in same conditions. After 30 min incubation test samples were centrifuged again at 1500 x *g* for 5 min, the pellets were fixed overnight in 2.5% glutaraldehyde in 0.1 M cacodylate buffer at room temperature. The test samples were then processed in a graded series of ethanol from 70% to 100% with three changes of 20 min each. The pellets were put in 50% ethanol/50% resin for 3 hrs and then left in 100% resin with three changes of at least 8

hrs each and left on a rotator between changes. In the final step, the test samples were embed in fresh resin and polymerised in oven for 24 hrs at 70 ^{0}C. Ultrathin sections were cut by Ms Ruth Williams, microscopist, Adelaide Microscopy at a thickness of 90 nm (Reichert Ultracut S) and stained in uranyl acetate & lead citrate for 5 min in each. The samples were examined with a transmission electron microscope (Philips CM100 TEM) at 80 kV.

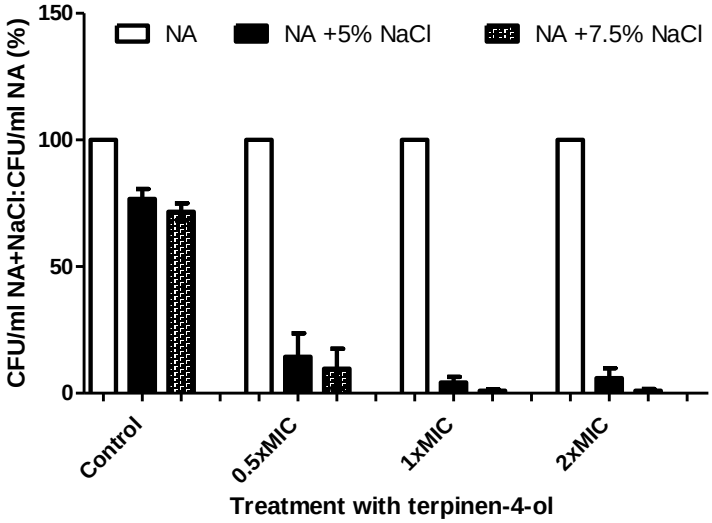
4.3. Results

4.3.1 Loss of salt tolerance

Fig. 4.2 shows the relationship between the salt tolerance of *Staph. aureus* and the concentration of the compound **EN2** (Fig 4.2 A) and control compound terpinen-4-ol (Fig 4.2B). The results are reported as the percentage in CFU/ml on the NA supplemented with NaCl 5 or 7.5% compared to NA-only control plates. Treatment of *Staph. aureus* cells with compound **EN2** at concentrations from 0.5 times to 2 times the MICs reduced the surviving colony numbers on the NaCl containing agar plates indicating a loss of salt tolerance. Results were significantly different for the each of the tested concentrations of EN2 at each of the salt concentrations when compared to the NA alone. At the 5% NaCl concentration: $p<0.001$ for EN2 at 2x MIC and 1x MIC concentration, and $p< 0.01$ at 0.5x MIC. At the 7.5% NaCl concentration: $p<0.001$ for each of the three EN2 concentrations tested. The control compound terpinen-4-ol also caused a loss of salt tolerance with significantly lower colony numbers at both the 5% and 7.5% NaCl concentration ($p<0.001$ for each of the three concentrations tested).



A)



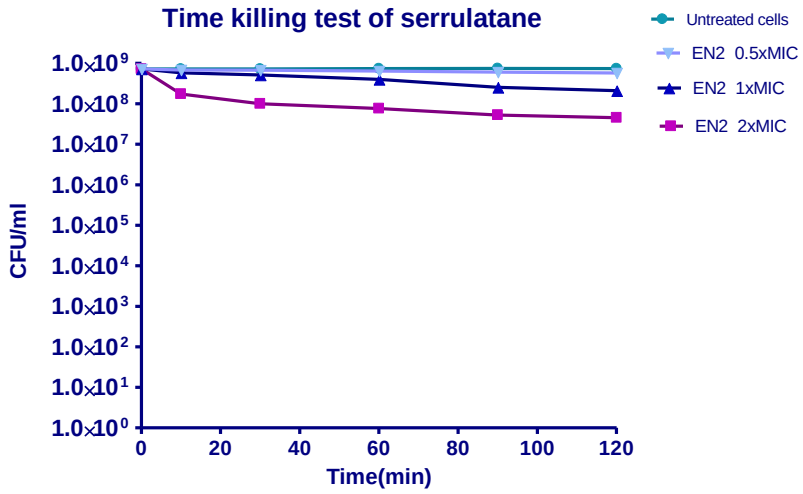
B)

Figure 4.2 Loss of salt tolerance of *Staph. aureus* treated with EN2 and terpinen-4-ol.

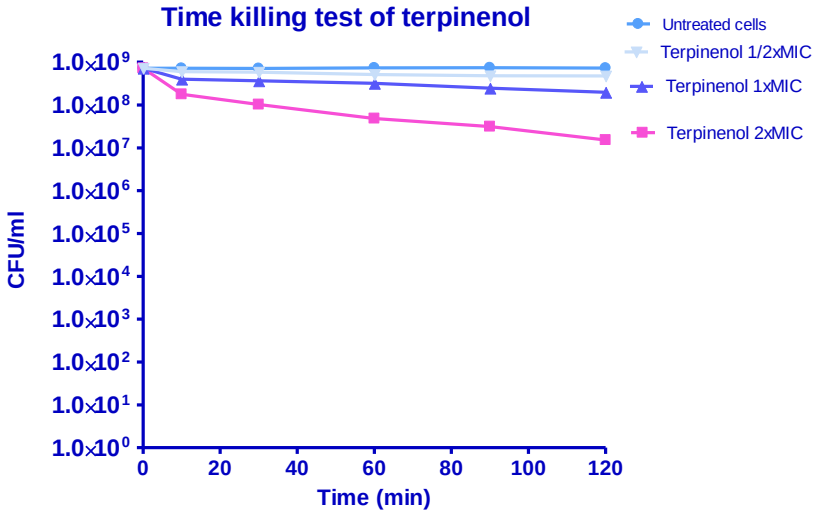
Proportion of *Staph. aureus* cells able to form colonies on NA (white bars), NA supplemented with 5% NaCl (black bars), and NA supplemented with 7.5% NaCl (grey bars) after 30 min of treatment with serrulatane compound EN2 (A) or terpinen-4-ol (B) at 0.5x the MICs, 1x the MICs, and 2x the MIC. The organisms were suspended in PBS. The means ± SEM for at least three replicate experiments are presented. At the 5% NaCl concentration: $p<0.001$ for EN2 at 2x MIC and 1x MIC concentration, and $p< 0.01$ at 0.5x MIC. At the 7.5% NaCl concentration: $p<0.001$ for each of the three EN2 concentrations tested.

4.3.2 Bacterial killing assays

Treatment of *Staph. aureus* with compound EN2 at two times the MIC reduced the viability of *Staph. aureus* cells by ~1 $\log_{10}$ over 2h, while the 1x MIC and 0.5xMIC treatments resulted in ~1 $\log_{10}$ and less than ~0.5 $\log_{10}$ reduction, respectively (Figure 4.3. A). Treatment with terpinen-4-ol at two times the MIC effected the reductions of greater than 2 $\log_{10}$ and reduced the cell viability by ~2 $\log_{10}$ over 120 min (Figure 4.3. B). Terpinen-4-ol and the serrulatane compound EN2 at one-half the MIC had little effect on the viability of *Staph. aureus* cells.



A)



B)

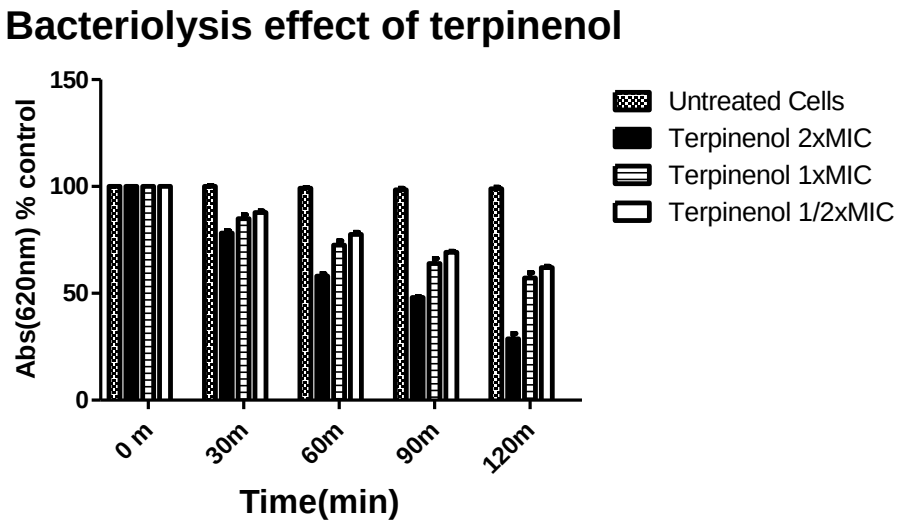
Figure 4.3 Time killing test of serrulatane and terpinen-4-ol

Time-kill curves of *Staph. aureus* ATCC 29213 in control suspensions (●) and after treatment with serrulatane EN2 (A) or terpinen-4-ol (B) at 0.5x MIC (▼), 1x MIC (▲), and 2x MIC (■).The means ± SEM for at least three replicate experiments are presented.

4.3.3 Bacteriolysis

The OD_{620} of suspensions of *Staph. aureus* treated with serrulatane compound **EN2** and terpinen-4-ol is shown in Figure 4.4. The mean value of untreated *Staph. aureus* cells at 620 nm is given as 100% in this figure. Treatment of bacteria cells with terpinen-4-ol at 2 times MIC reduced the OD_{620} from 100 to less than 50% over the two hour period while EN2 had a only a small effect of approximately 20% reduction at two times MIC.

The results the OD_{620}s of *Staph. aureus* cell suspensions at 6.5 or 23 h after the initial OD_{620}s was measured are also shown in Figure 4.4. Similarly treatment of bacteria cells with terpinen-4-ol at 2x MIC reduced the OD_{620} from 100 to less than 20% over the 6.5 hour period and to less than 5% over 23 h. However, EN2 at 2x MIC has just reduced the OD_{620} from 100 to 60-70% in the same period.



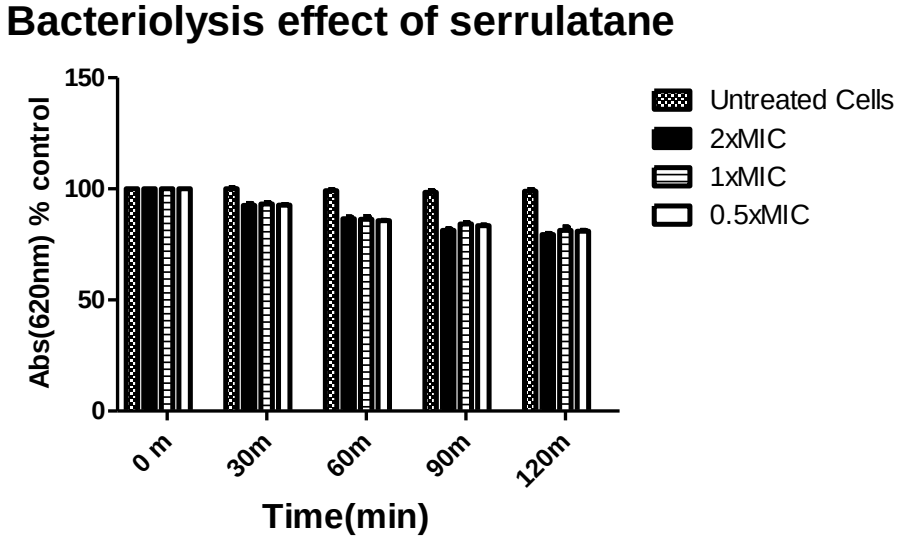


Figure 4.4.A Bacteriolysis test of serrulatanes and terpinen-4-ol

Proportion of the OD_{620} of suspensions *Staph. aureus* after treatment with serrulatane compound EN2 and terpinen-4-ol for 30 min intervals up to 120 min. The means ± SEM for at least three replicate experiments are presented.

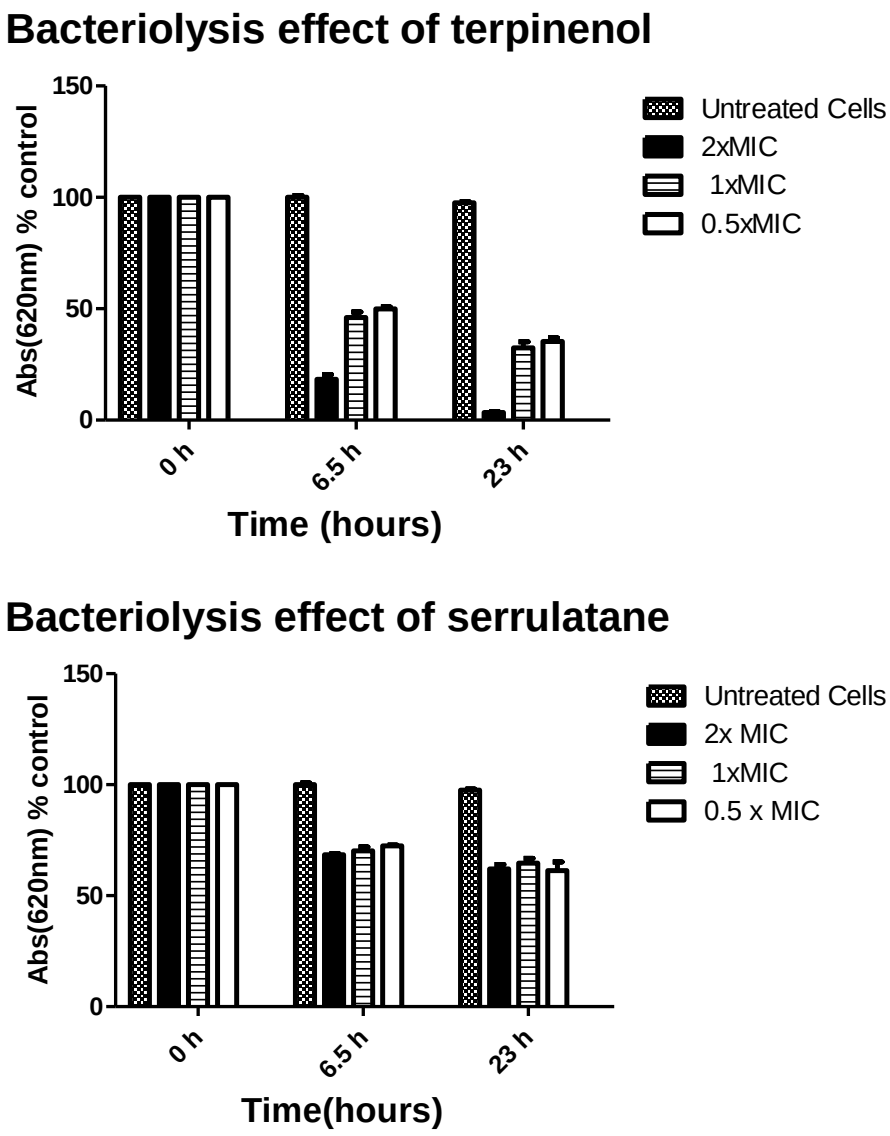


Figure 4.4.B Bacteriolysis test of serrulatanes and terpinen-4-ol

Proportion of the OD_{620} of suspensions *Staph. aureus* after treatment with serrulatane compound EN2 and terpinen-4-ol after 6.5 and 23 hours. The means ± SEM for at least three replicate experiments are presented.

4.3.4 Propidium iodide uptake assay

4.3.4.1 Assay optimization

The emission wavelength (EM) and excitation wavelength (EX) of maximum fluorescence of propidium iodide (PI) were determined. The maximum fluorescence result gave the EM maximum (EM_{max}) value at 634 nm. Additionally the EM maximum (EM_{max}) value was determined as 500nm as described in section 4.2.7.

The effect of PI concentrations on fluorescence of *Staph. aureus* cells in the presence of CTAB was determined before the test. The results are shown in Figure 4.5. Based on these results a PI concentration of 50 µmol l-1 was used in the assay, as higher concentrations did not result in increased in absorbance.

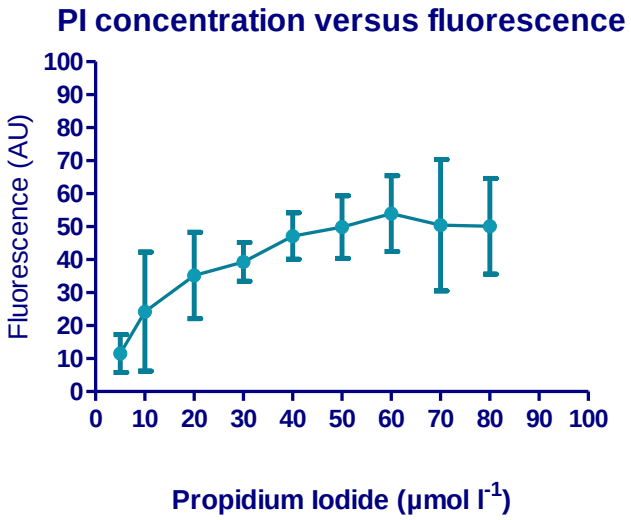


Figure 4.5 PI concentration versus fluorescence intensity

The effect of propidium iodide concentration on the fluorescence of *Staph. aureus* cells in the presence of CTAB. The means ± 95% CI for three replicate experiments is presented.

Before the PI test, the fluorescence of serrulatane compound EN2 was measured and it was found that it did not cause any measurable fluorescence in its own. Therefore, it was determined that serrulatane compound would not interfere with the assay.

4.3.4.2 Testing of EN2 and CTAB

Figure 4.6 shows fluorescence increase of test solutions in which bacteria cells were exposed to the serrulatane compound EN2 or CTAB. The fluorescence of the test cultures with addition of the serrulatane EN2 or CTAB showed significant differences from the untreated PI control ($p < 0.0001$). The results for the CTAB and EN2 treated cultures were not significantly different ($p > 0.05$).

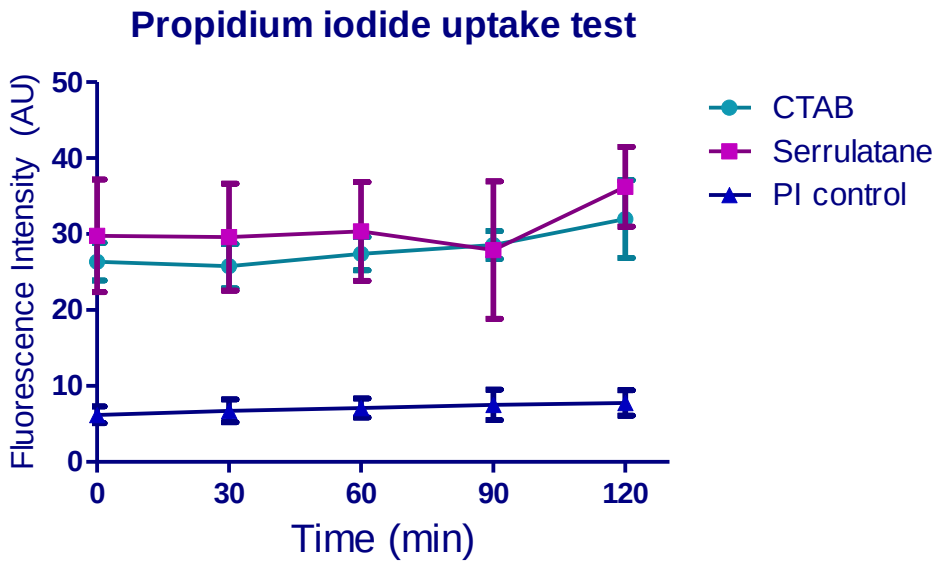


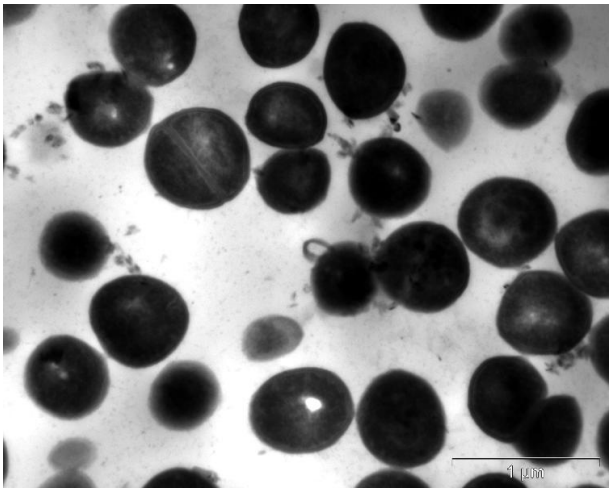
Figure 4.6 Propidium iodide uptake test of serrulatanes and CTAB

Fluorescence of *Staph. aureus* cultures in the presence of serrulatane compound EN2 at MIC level (■), CTAB at 0.2 mmol l^{-1} (●) or with no treatment (▲). Data presented the mean +/- SEM for three replicate experiments. The time zero reading was taken 20 seconds after mixing of reagents.

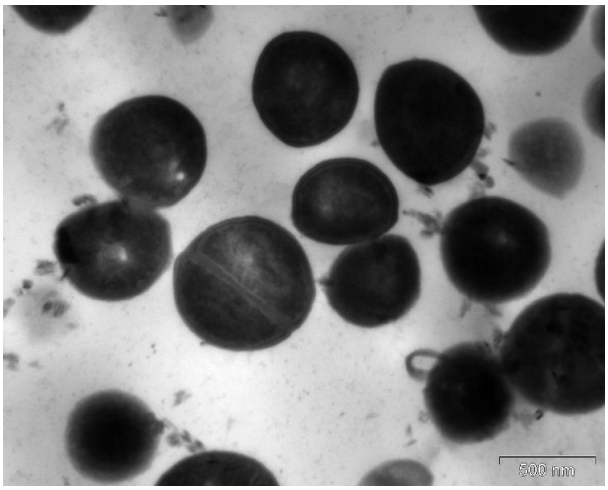
4.3.5 Transmission electron microscopy of bacterial cells treated with serrulatane compounds

The transmission electron microscopy micrographs of *Staph. aureus* cells are represented in Figures 4.7 (untreated cells), 4.8 (treated with EN1), 4.9 (treated with EN2) and 4.10 (treated with terpinen-4-ol).

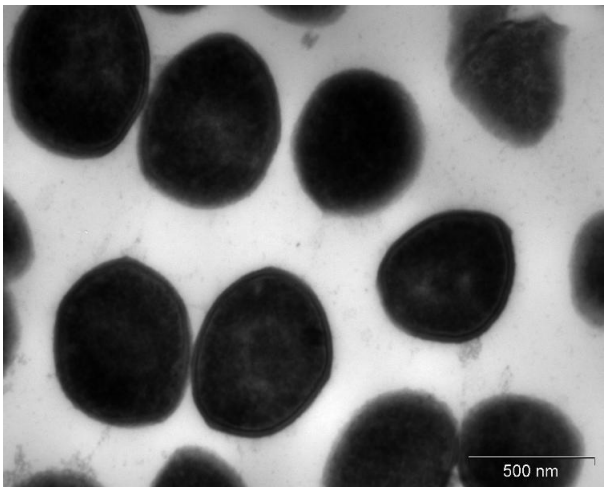
When compared to the control untreated cells, the terpinen-4-ol treated cells (Fig. 4.10) appeared similar to that shown previously for *Staph. aureus* cells treated with the compound (Carson et al., 2002). The bacterial cells contained multilamellar, mesosome-like structures and also there were a greater number of small cells or cell fragments seen in the culture compared to untreated cells. However in the EN1 and EN2 treated cells (figures 4.8 and 4.9) the damage was not as clearly apparent when the serrulatane treated cells were compared, the effects of EN1 appeared to be greater than that of EN2. EN1 treated cells showed some mesosome-like structures and there were some smaller cells and cell fragments present. Neither of the treatments caused conspicuous damage to the cell wall or cytoplasmic membrane.



A)



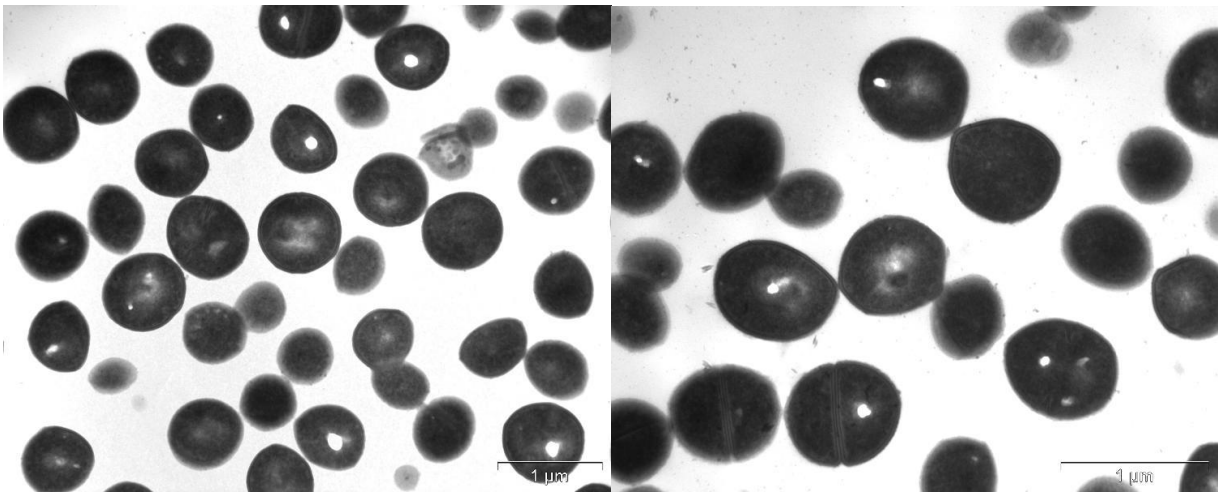
B)



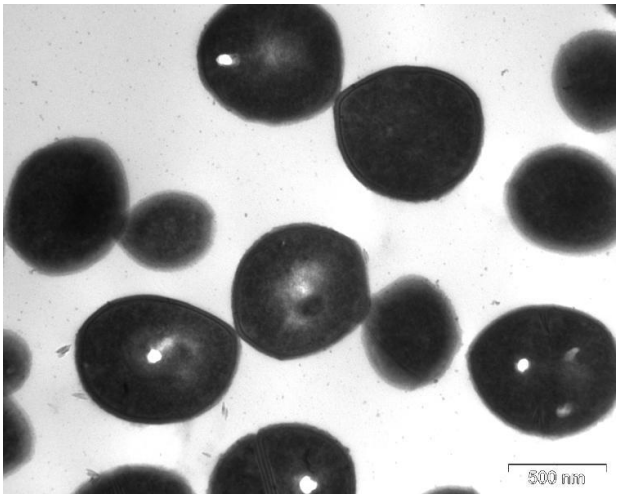
C)

Figure 4.7 Electron microscopy of untreated *Staph. aureus* cells

Electron micrographs of *Staph. aureus* cells stained with uranyl acetate after no treatment. Magnifications: x 13,500 (A), x19,000 (B) and x 25,000 (C).



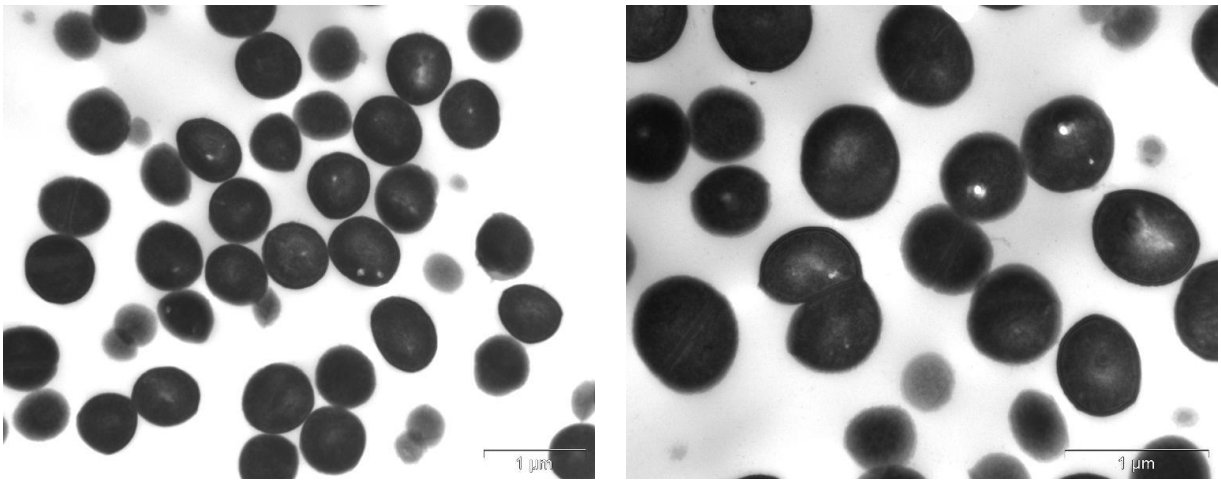
A) **B)**



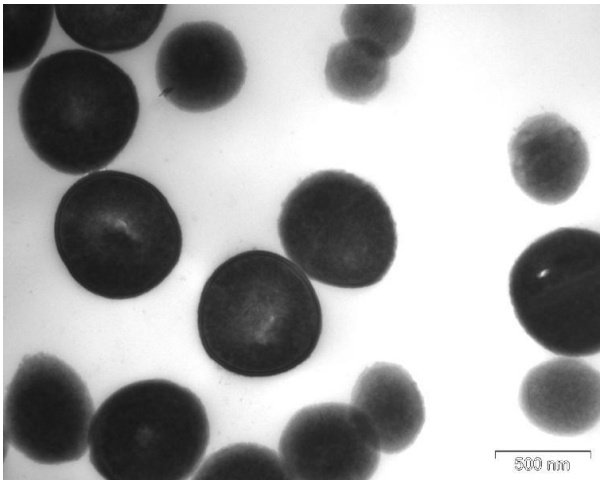
C)

Figure 4.8 Electron microscopy of *Staph. aureus* cells treated with EN1

Electron micrographs of *Staph. aureus* cells stained with uranyl acetate after treatment with serrulatane compound EN1 at 1x MIC. Magnifications: x 13,500 (A), x 19,000 (B) and x 25,000 (C)



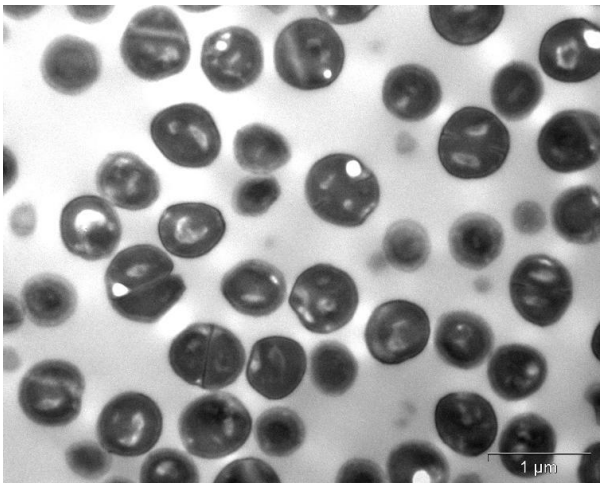
A) **B)**



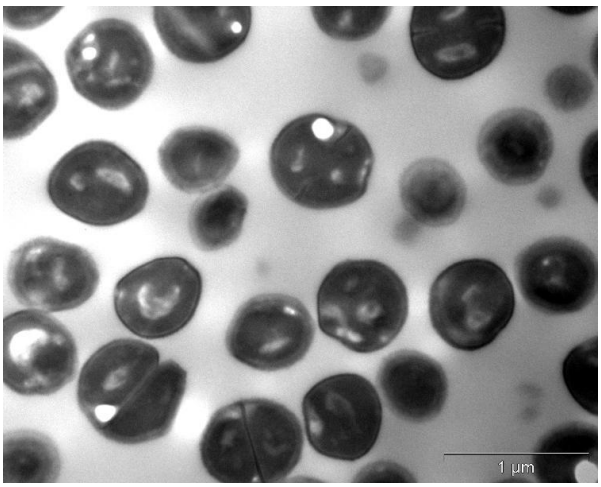
C)

Figure 4.9 Electron microscopy of *Staph. aureus* cells treated with EN2

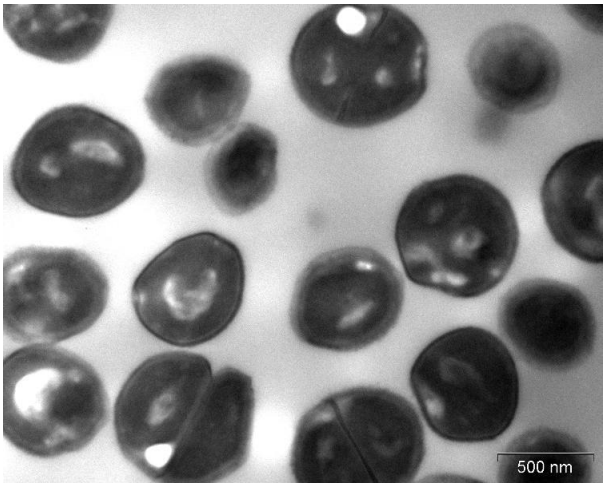
Electron micrographs of *Staph. aureus* cells stained with uranyl acetate after treatment with serrulatane compound EN2 at 1x MIC. Magnifications: x 13,500 (A), x 19,000 (B) and x 25,000 (C)



A)



B)



C)

Figure 4.10 Electron microscopy of *Staph. aureus* cells treated with terpinen-4-ol

Electron micrographs of *Staph. aureus* cells stained with uranyl acetate after treatment with terpinen-4-ol at 1x MIC. Magnifications: x 13,500 (A), x 19,000 (B) and x 25,000 (C)

4.4. Discussion

The results obtained from the mode of action assays suggested that the antibacterial activities of the serrulatane compounds are at least in part due to effects on bacterial cell membranes. The failure of test compound EN2 to cause substantial lysis of *Staph. aureus* cells suggested that the primary mechanism of action of the serrulatane compound was not gross cell wall damage. Similarly results with TEM did not show any clear damage to the cytoplasmic membrane or cell wall when cells were treated with EN1 or EN2.

The PI uptake assay was used to investigate whether the serrulatane compound increased membrane permeability of. *Staph. aureus* cells. In this assay, an increase in fluorescence was observed at a concentration of serrulatane compound that was similar to control agent CTAB, a compound known to permeabilise bacterial cell membranes. The findings of this study were in close agreement with those reported previously by Tomlinson and Palombo (2005) when testing a crude ethanolic extract of *Eremophila duttonii* in the PI uptake assay. *E. duttonii* is also known to contain serrulatane compounds as described in Chapter 1. Therefore the results indicate that some cell membrane damage occurred in the presence of serrulatane compound resulting in increased bacterial cell permeability.

Sub lethal injury of bacterial cell membranes can affect the membrane's ability to osmoregulate the cell adequately or to exclude toxic materials (Gilbert 1984). Thus the loss of salt tolerance can be exploited to reveal membrane damage in sub lethally injured *Staph. aureus* cells (Carson et al. 2002). Loss of salt tolerance was used to investigate the mode of action of serrulatane compound EN2 in *Staph. aureus* cells. Treatment of *Staph. aureus* cells with the compound significantly reduced the number of colony forming units on media containing NaCl. The MIC levels of the test compound showed that it affects the number of survivor colonies. At the two times MICs the colony numbers reduced to fewer than 5% on NA+ 7.5% NaCl and under 10% on NA+ 5% NaCl plates after 30 min. incubation. However, the number of survivor colonies at one-half the MIC were 50% and 70%. The results obtained from salt tolerance test supported the data from PI uptake test that the bacterial cells exposed to serrulatane compound showed decreased membrane stability. These results were also in

agreement with what had previously been reported for a crude extract of *Eremophila duttonii* tested in the salt tolerance assay (Tomlinson and Palombo, 2005)

The results of bacteriolysis test indicate that the serrulatane compound EN2 does not have a strong lytic effect on *Staph. aureus* cells. At two-times the MIC the control compound terpinen-4-ol reduced the OD_{620} of *Staph. aureus* cells less than 50 % over 2 h and less than 5% after 23 h while EN2 could only reduced the value to 60-70% even after 23 h. These findings were also in line with those reported previously by Tomlinson and Palombo (2005) for the crude *E. duttonii* extract were the absorbance of *Staph. aureus* cultures treated with the extract remained constant over a 90 minute period suggesting that cell lysis had not occurred.

The bacteriolysis results for terpinen-4-ol are similar to the results obtained in the previous study of the mechanism of action of tea tree oil and its three major active components (1,8-cineole, terpinen-4-ol and α-terpineol) (Carson et al. 2002). However the terpinen-4-ol was found to have a slightly greater lytic effect in the present study than that reported previously. For example at two-times the terpinen-4-ol MIC the OD_{620} was found to be 53.4% at 6.5 hours and 28.5% after 23 hours in the previous study (Carson et al. 2002). As pointed out previously by Carson et al. (2002), some antibacterial components can cause a huge membrane damage and this causes a whole-cell lysis (Denyer and Hugo 1991). This includes some plant essential oils from oregano, rosewood and thyme and some plant terpenoids (Horne et al. 2001). Carson et al. (2002) postulated that TTO and its components do not cause this type of gross membrane damage but may instead result in the release of autolytic enzymes from the cell membrane that induce eventual cell lysis or a more gradual weakening of the cell wall and loss of osmotic regulation. This may also account for some of the action of the serrulatane compound, however the bacteriolysis seen with EN2 was much less than that seen with terpinen-4-ol suggesting other mechanisms may be involved. The failure of the serrulatane compound, to cause substantial lysis of bacterial cells suggests the compound does not cause whole-cell wall damage.

Transmission electron microscopy of serrulatane compounds EN1, EN2 and terpinen-4 ol indicates that there is an obvious difference between the effect of the serrulatanes and terpinen-4-ol. When compared with untreated cells, terpinen-4-ol showed clear differences in

cell appearance. The study of Carson et al (2002) has reported similar results for the tea tree oil component terpinen-4-ol using TEM as seen with the compound in this study. The multilamellar, mesosome-like structures in the bacteria cells indicated that terpinen-4-ol had damaged the cell walls. Although the effects of serrulatane compounds were not same as terpinen-4-ol, there was a slight difference between the micrographs of EN1 and EN2 Treated bacteria Further TEM studies, perhaps with longer exposure times to the compounds are needed to clarify these effects. However, the results obtained indicate that the serrulatane compounds do not cause a rapid gross cytoplasmic membrane or cell wall damage.

Bacterial time-killing experiments showed some reduction in the number of viable cells in the presence of EN2 and the control compound terpinen-4-ol over a 2 hour period. However, the reductions for the control compound were less marked than those reported previously by Carson et al. (2002). These authors reported an approximate 6 log reduction in the number of viable *Staph. aureus* cells treated with terpinen-4-ol at its MIC concentration. It is not clear what caused the difference in the results in the current study although a different stain of *Staph. aureus* used in the testing may account for some variation. It has been shown in Chapter 2 and in previously published studies, that the serrulatane compounds do have bactericidal effects. It is worth noting that the bacteria tested in the time-kill experiments were stationary phase cultures and it may be that bacteria need to be actively dividing for the serrulatane compounds to have their full bactericidal effect. Further experiments are required to clarify the effects on the serrulatanes on actively growing cultures. Furthermore these experiments used high initial bacterial cell densities (of approximately $1x\ 10^{9}\ ml^{-1}$) in line with that previously used by Carson et al (2002). Use of a lower initial cell density similar to that used in the MIC experiments may yield more meaningful results.

4.5. Conclusion

Sub lethal injury of *Staph*. aureus bacterial cell membranes with the serrulatane compound EN2 resulted in a loss of salt tolerance test and increased PI uptake as indicated by an increased fluorescence in PI assay. The failure of test compound EN2 to cause substantial lysis of *Staph. aureus* cells suggested that the primary mechanism of action of the serrulatane compound was not gross cell wall damage. Similarly results with TEM did not show any clear

damage to the cytoplasmic membrane or cell wall when cells were treated with EN1 or EN2. The results obtained from the mode of action assays suggested that the antibacterial activities of the serrulatane compounds are at least in part due to effects on bacterial cell membranes. However, other sites of action besides the cytoplasmic membrane cannot be excluded.

CHAPTER FIVE

GENERAL DISCUSSION

5.1. Antimicrobial screening of serrulatane compounds

A review of the published literature indicated that previous studies have provided limited information about the spectrum of antibacterial activities of purified serrulatane compounds (Ndi et al., 2007b; Ndi et al., 2007c; Palombo et al., 2002; Liu et al., 2006; Smith et al., 2007). The first aim of this study was to examine the spectrum of activity of purified serrulatane compounds 8,19-dihydroxyserrulat-14-ene (**EN1**), and 8-hydroxyserrulat-14-en-19-oic acid (**EN2**) from *Eremophila neglecta* with a wide range of different Gram-positive and Gram-negative bacteria that are important human and veterinary pathogens. This included testing against clinical isolates and some multi-drug resistant isolates.

In agreement with previous published research the serrulatanes showed activity against a range of Gram-positive organisms with most Gram-negatives tested not being susceptible at the maximum concentration used. Additionally, compound EN1 has showed more activity than compound EN2 which supports the previous study results of Ndi et al. (2007c) with the same compounds. The antibacterial activity results of this study have shown that the serrulatane compounds are very effective against methicillin resistant and multi-drug resistant strains of *Staph. aureus* isolated from patients and clinical isolates of *Strep. pneumoniae* and *Strep. pyogenes*. The compound **EN1** exhibited good activity against the biofilm-producing strain of *Staph. epidermidis* (ATCC 35984), an organism of importance in medical device-related infections (O'Gara and Humphreys, 2001). This result is of importance to ongoing research examining whether serrulatane compounds may be attached to material surfaces to reduce

bacterial biofilm formation. The compound also displayed activity against *Strep. mutans* (ATCC 25175) an organism which can also form biofilms and which plays an important role in dental decay (Zambon and Kasprzak, 1995). The demonstrated activity of **EN1** against *Enterococcus* species was also important as this organism is an important cause of nosocomial infections. *Enterococcus faecium* is one of the "ESKAPE" group pathogens for which multi drug resistance is an increasing problem (Boucher et al. 2009).

Another important antibacterial result of the two serrulatane compounds was anti-mycobacterial activity against the clinical isolates of *Mycobacterium fortuitum* and *Mycobacterium chelonea* that demostrated that EN1 had an MIC of 12.5 μg ml^{-1}. A previous study has reported that the crude extracts of *Eremophila* species *Eremophila alternifolia* and *Eremophila longifolia* exhibited activity against *Mycobacterium smegmatis* and *Myco. fortuitum* (Meilak and Palombo, 2008), although the active components were not identified. These results, in addition to the fact that the serrulatanes are structurally related to terpenoid alkaloids pseudopteroxazole and *seco*-pseudopteroxazole which have been reported to have activity against *Mycobacterium tuberculosis*, suggest the need for further anti-mycobacterial studies with the serrulatanes against *Myco. tuberculosis* strains.

The only gram-negative activity was observed against *Mor. Catarrhalis* which is the first report of antibacterial activity for serrulatane compounds against a Gram-negative organism. Previous studies on serrulatanes and crude extracts of *Eremophila* species have reported a lack of activity against Gram-negatives such as *Ps. aeruginosa and E.coli* (Palombo and Semple, 2001; Liu et al., 2006, Ndi et al., 2007c). The reason of the activity probably comes from the difference of cell wall structure of *Mor. catarrhalis* compared these other Gram-negative organisms.

The reason of the lack of activity of the serrulatanes against Gram-negative organisms such as *Ps. aeruginosa*, *Salm. enterica* and *E. coli* is most likely due to the large molecule size and bicyclic conformation of these compounds. The entrance of these relatively large molecules may be barred by size selective porin channels in the outer membrane of Gram-negative species (Cowan 1999; Smith et al. 2007).

To summarize, this study has provided important information about the spectrum of activities of the two serrulatane compounds. These data include the activity of the compounds against a wide range of different Gram-positive and Gram-negative bacteria that are important human and veterinary pathogens. There is still a need to investigate the antibacterial activities of serrulatane compounds with further studies. One of the potential uses of serrulatanes might be in the production of antibacterial-coated materials for biomedical devices. This study also indicates that serrulatane compounds have potential as new anti-infective lead compounds against some clinically important bacteria including *Staphylococcus*, *Streptococcus*, *Enterococcus*, *Mycobacterium* and *Moraxella* species

5.2. Effect of serrulatanes on cell viability

The second aim of this study was to examine the effects of the two serrulatane compounds on different types of mammalian cell lines. There had been no previous examination of the cytotoxicity of serrulatane compounds published in the literature. Thus, this study was the first report of cytotoxic activities of serrulatane compounds. If these compounds are to be used in a clinical setting, an understanding of their toxic effects is critical.

Two different mammalian cell lines were used to examine the cytotoxicity of the serrulatane compounds **EN1** and **EN2**. Reflecting the antibacterial activity results, compound **EN1** showed slightly more cytotoxicity with a CC_{50} concentration of 9.2 µg ml^{-1}compared with 20.0 µg ml^{-1} for compound **EN2** on Vero cells and 3.6 µg ml^{-1} for **EN1** and 36.3 µg ml^{-1} for **EN2** on U937 cells. These results suggest that the compounds have mammalian cell toxicity at concentrations similar to the MIC concentrations. Further testing with a range of different cell types is needed to confirm these findings. However, the results suggest that application of these compounds in a clinical setting may need to be restricted to applications such as topical antibacterial creams. Evaluation in further cell models that more closely reflect the structure of the skin (such as the Epiderm Skin Irritation Test three-dimensional skin model) may indicate whether the compounds would be able to be used in such applications prior to evaluation in *in vivo* animal skin test models.

Another possible application would be the use of the serrulatane compounds to produce antibacterial coated materials that could be used in biomedical devices or implants to prevent bacterial biofilm formation. Ongoing research at the University of South Australia is using novel coating technologies that allow these compounds to be covalently attached to surfaces (Griesser et al., 2008). The covalent attachment would prevent the compounds from diffusing from the surface to cause systemic toxic effects. However, ongoing evaluations are required to examine whether materials coated with these compounds would cause local irritation or toxicity at the site of implantation.

In future studies it would also be of interest to examine the structure-activity relationships for the serrulatanes. Structural modifications may allow optimisation of the antibacterial effects of these compounds while reducing toxic effects to mammalian cells.

5.3. Investigation of the antibacterial mode of action of serrulatane compounds

The third aim of this study was to investigate the antibacterial mode of action of the serrulatane compounds from *Eremophila neglecta*. To understand their effects on bacterial cell walls and cell membranes a number of mechanism of action methods were used. These included loss of salt tolerance, bacterial time killing, bacteriolysis, propidium iodide (PI) uptake assays and transmission electron microscopy.

The results obtained from mode of action assays showed that the serrulatane compound **EN2** does have effects on bacterial cell membranes. This was shown through effects on salt tolerance of *Staph. aureus* cells and on propidium iodide uptake in the presence of **EN2**. Treatment of *Staph. aureus* cells with serrulatane compound **EN2** significantly reduced the number of colonies on media containing NaCl. At the two times MICs the colony numbers reduced to fewer than 5% on NA plates with 7.5% NaCl and to less than 10% on NA with 5% NaCl after 30 min. Incubation. The proportion of survivor colonies at one-half the MIC resulted were 50% and 70%. The results obtained from salt tolerance test were supported with data from PI uptake test. In this test that the bacteria cells exposed to the serrulatane

compound showed increased membrane permeability to propidium iodide. The results obtained in these two studies were in close agreement with a previous study which had examined a crude extract of *Eremophila duttonii* using the same tests (Tomlinson and Palombo, 2005). The results of this study confirm that the purified serrulatane compound **EN2** has similar effects.

The results of bacteriolysis test indicated that the serrulatane compound **EN2** does not have a strong lytic effect. As such it does not appear that **EN2** causes a gross membrane damage and whole-cell lysis as has been reported previously for some essential oils and plant terpenoid components (Carson et al. 2002). Results of the time-killing assay did not show similar results of Carson's paper for tea tree oil components. As demonstrated in Chapter 2 and in previously published studies, the serrulatane compounds do have bactericidal effects. It is worth noting that the bacteria tested in the time-kill experiments were stationary phase cultures, following methods used by Carson et al. (2002) and it may be that bacteria need to be actively dividing for the serrulatane compounds to have their full cell-killing effect. Further experiments to clarify time-killing effects on actively growing cultures are therefore required. Furthermore, as discussed in Chapter 4, these experiments used high initial bacterial cell densities in line with that previously used by Carson et al (2002). In future experiments, use of a lower initial cell density similar to that used in the MIC experiments may yield more meaningful results.

Transmission electron microscopy of *Staph. aureus* cells treated for 30 minutes with serrulatane compounds **EN1**, **EN2** also indicated that the compounds did not cause gross damage to the bacterial cell membrane or cell wall.

The results obtained from mode of action assays showed that the serrulatane compound **EN2** does have effects on bacterial cell membranes without causing gross cell damage. However, the results of this study to not exclude the potential for other mechanisms of action. There is also a need to further probe the mechanism by which these compounds produce changes in membrane permeability. The antibacterial mechanism of action still needs to be further investigated by further mode of action methods and with different bacterial species. Another project of interest would be an examination of multi-drug resistant strains of bacteria with

serrulatane compounds and there is also a need to look at whether resistance may develop to these agents.

5.4. Conclusion

In this study, the in-vitro spectrum of antibacterial activity of the two serrulatane compounds 8,19-dihydroxyserrulat-14-ene (**EN1**), and 8-hydroxyserrulat-14-en-19-oic acid (**EN2**) was investigated with a range of different Gram-positive and Gram-negative bacteria including clinical isolates. Toxicity to two mammalian cell lines was also investigated. The serrulatane diterpenoids were found to display activity against a range of Gram-positive bacteria of medical importance including clinical isolates. However, the compounds tested were also found to have cytotoxic effects on mammalian cells in culture. This study has provided important data on the spectrum of activities of the two serrulatanes with a wider range of test strains than previously examined. The cytotoxic activity of these compounds needs to be further examined to assess the potential antibacterial applications of this class of compounds.

The antibacterial mode of action of the serrulatane compounds was investigated using *Staph. aureus* as a test organism. A series of tests were conducted to examine the effects on the bacterial cytoplasmic membrane and cell wall including loss of salt tolerance, bacterial time killing, bacteriolysis, propidium iodide (PI) uptake assays and transmission electron microscopy. These assays showed that the serrulatane compound EN2 does have effects on bacterial cell membranes without causing gross cell damage. However, the results of this study to not exclude the potential for other mechanisms of action.

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APPENDIX 1. Antibiotic resistance profile of all clinical isolates used

Clinical Isolates	***Species***	***Pen.***	***Amox.***	***Oxa.***	***Ery.***	***Clin.***	***Van.***	***Rif.***	***Fuc.***	***Tet.***	***Norfloxa.***	***Cip.***	***Gen.***	***Chlor.***	***Trimeth.***	***Mupirocin***
MRSA-1	MRSA	R	R	R	R	S	S	S	S	R	R	R	S	S	R	S
MRSA-2	MRSA	R	R	R	R	S	S	S	S	R	R	R	R	S	R	R
MRSA-3	MRSA	R	R	R	S	S	S	S	R	S	S	S	S	S	S	S
MRSA-4	MRSA	R	R	R	R	S	S	S	S	S	S	S	S	S	S	S
MRSA-5	MRSA	R	R	S	S	S	S	S	S	S	S	S	S	S	S	S
S. pn-1	*Strep. pneumonia*	S			R	S				S				S		
S. pn-2	*Strep. pneumonia*	S			S	S				R				S		
S. pyo-1	*Strep. pyogenes*	S			S	S				S						
S. pyo-2	*Strep. pyogenes*	S			S	S				S						

- MRSA means Methicillin-resistant *Staphylococcus aureus*
- Pen.: Penicillin, Amox.: Amoxicillin, Oxa.: Oxacillin, Ery.: Erythromycin, Clin.: Clindamycin, Van.: Vancomycin, Rif.: Rifampin, Fuc.: Fucidin, Tet.: Tetracycline, Norfloxa.: Norfloxacin, Cip.: Ciproxin, Gen.: Gentamicin, Chlor.: Chloromphenicol, Trimeth.: Trimethoprim