STUDIES ON SKIN ANTISEPSIS AND ENHANCED PENETRATION OF CHLORHEXIDINE

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SUMMARY

Effective skin antisepsis prior to incision of the skin, for example, during surgery, is essential in preventing subsequent infection. However, infections associated with invasive procedures remain a significant cause of morbidity and mortality and impose additional costs upon the Health Service. Current evidence-based guidelines recommend that 2% (w/v) chlorhexidine digluconate (CHG), preferably in 70% (v/v) isopropyl alcohol (IPA), is used for skin antisepsis prior to incision of the skin. However, many antiseptics poorly permeate the skin and microorganisms residing in the deeper layers and around hair follicles, may survive the procedure and cause infection. In this current study, the antimicrobial efficacy of CHG, six essential oils [tea tree oil (TTO), thymol, eucalyptus oil (EO), juniper oil, lavender oil and citronella] and novel benzylidenecarboxamidrazone and thiosemicarbazone compounds were determined against a panel of microorganisms commonly associated with skin infection (*Staphylococcus epidermidis*, *S. aureus*, meticillin-resistant *S. aureus*, *Propionibacterium acnes*, *Acinetobacter spp.*, *Pseudomonas aeruginosa* and *Candida albicans*) in an *in vitro* suspension assay. In addition, the antimicrobial activity of CHG, TTO, EO and thymol, which all demonstrated a broad spectrum of activity in the suspension assay, were further assessed against biofilm cultures of *S. epidermidis in vitro*. The antimicrobial activity of CHG, a thiosemicarbazone and a carboxamidrazone compound in combination with TTO, EO and thymol was also assessed. The results demonstrated synergistic activity of CHG in combination with EO against biofilm cultures of *S. epidermidis*, with significantly reduced concentrations of CHG and EO required to inhibit biofilm growth compared to CHG or EO alone.

Skin permeation of CHG was subsequently investigated using an *in vitro* human skin model (Franz cell) and the penetration profile was determined by serial sectioning of the full thickness human skin using a cryomicrotome and analysis of CHG with high performance liquid chromatography. Two percent (w/v) CHG in aqueous solution and in 70% (v/v) IPA demonstrated poor skin permeation; however the skin permeation was significantly enhanced in combination with 5% - 50% (v/v) EO. Detectable levels of CHG did not permeate through full thickness skin in 24 h. Skin permeation of 2% (w/v) CHG in 70% (v/v) IPA in the presence of 10% (v/v) EO was subsequently studied. The results demonstrated a significantly enhanced skin penetration of CHG after a 2 min application, with CHG detected at significant levels to a depth of 600 μm with CHG in combination with EO and IPA compared to 100 μm with IPA alone. Combination antisepsis comprising CHG and EO may be beneficial for skin antisepsis prior to invasive procedures to reduce the number of microorganisms on and within the skin due to enhanced skin penetration of CHG and improved efficacy against *S. epidermidis* in a biofilm mode of growth. Further studies are required to establish the efficacy of EO in combination with CHG and IPA *in vivo*, and the further studies to establish the toxicity and skin tolerance of EO alone and in combination with CHG.

Key words: skin permeation, essential oil, biofilm, HPLC, Franz diffusion cell

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LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BA	Blood agar
BHI	Brain heart infusion
BSI	Blood stream infection
°C	Celsius
CDC	Centres for Disease Control and Prevention
cfu	Colony forming units
CHG	Chlorhexidine digluconate
CLSI	Clinical and Laboratory Standards Institute (previously NCCLS)
cm	Centimetre
cm^2	Centimetre squared
CNS	Coagulase-negative *Staphylococcus spp.*
CRI	Catheter related infections
CVC	Central venous catheter
Da	Dalton
DMSO	Dimethylsulphoxide
DoH	Department of Health
EO	Eucalyptus oil
EPIC	Evidence-Based Practice in Infection Control
FFP	Fresh frozen plasma
FIC	Fractional inhibitory concentration
FICI	Fractional inhibitory concentration index
g	Gram
GRE	Glycopeptide-resistant enterococci
h	Hour
HAI	Hospital associated infections
HAI-BSI	Hospital acquired blood stream infection
HCAI	Healthcare associated infections
HICPAC	Healthcare Infection Control Practices Advisory Committee
HIS	Hospital Infection Society
HPA	Health Protection Agency
HPLC	High performance liquid chromatography
IPA	Isopropyl alcohol
L	Litre
LOD	Level of detection
LOQ	Level of quantification
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
M	Molar
MBC	Minimum bactericidal concentration
MFC	Minimum fungicidal concentration
mg	Milligram
Mg^{2+}	Magnesium ion
MHA	Mueller-Hinton agar
MHB	Mueller-Hinton broth

MIC	Minimum inhibitory concentration
min	Minute
mL	Millilitre
mm	Millimeter
mm^2	millimetre squared
MRSA	Meticillin-resistant *S. aureus*
MSSA	Meticillin-sensitive *S. aureus*
n	Number
NAO	National Audit Office
NCCLS	National Committee for National Laboratory Standards (since 2005, CLSI)
NCTC	National Collection of Type Cultures (UK)
NHS	National Health Service
NICE	National Institute of Clinical Excellence
nm	Nanometer
PBS	Phosphate buffered saline
PVP-I	Povidone-iodine
QAC	Quaternary ammonium compound
s	Second
SAB	Sabouraud dextrose broth
SC	Stratum corneum
SD	Standard deviation
SEM	Standard error of mean
spp.	Species
SSI	Surgical site infection
TSA	Tryptic soya agar
TSB	Tryptic soya broth
TTO	Tea tree oil
UHBNHSFT	University Hospitals Birmingham NHS Foundation Trust
v/v	Volume per volume
w/v	Weight per volume
µL	Microlitre
µm	Micrometer

1. CHAPTER: INTRODUCTION

1.1. Skin

1.1.1. Function and physiology of skin

Skin is the largest organ of the body, covering approximately 2 m^2 in the adult human body and accounting for approximately 10% of the total body mass (Mills and Cross, 2006). Its main function is to protect the human body from the outside environment, such as microorganisms, toxins, chemicals and radiation, prevent water loss from the human body and regulate the body temperature. Skin also has metabolic activity containing various enzymes in the epidermal layer (Haftek *et al.*, 1998, Mills and Cross, 2006), such as proteases in stratum corneum (SC) which cause skin desquamation (Haftek *et al.*, 1998). The structure (such as the number of sebaceous glands, hair density and thickness of skin) and physiology of skin (such as metabolic activity, pH and humidity) may have interpersonal and even intrapersonal variability, depending on the body site, age (Waller and Maibach, 2005, Waller and Maibach, 2006) and even circadian rhythm (Yosipovitch *et al.*, 1998). The normal pH of the skin has been estimated as 4.7, however many external factors such as use of water and soap, affect the skin surface pH and therefore it ranges between 4 to 7 (Lambers *et al.*, 2006).

1.1.2. Structure of skin

The skin has a layered structure, which is broadly categorised into the non-viable epidermidis called stratum corneum, viable epidermidis, and dermis (Figure 1.1). The epidermis is composed of stratified squamous epithelium, mainly keratinocytes, and the dermis is a complex of many different cell types and contains vasculature and nerve endings. The skin also includes the skin appendages. The thickness of skin and the density of hair follicles and glands vary amongst the body sites.

Figure 1.1. Schematic structure of mammalian skin (Mills and Cross, 2006).

1.1.2.1. Stratum corneum

The outermost layer of the skin and epidermal layer is the stratum corneum (SC), also called the horny layer, which has been shown to be the main skin permeation barrier. Stratum corneum is only approximately 15-20 μm (approx 15 cell layers) thick; however its composition of closely packed keratinized cells, also called corneocytes, with intercellular spaces filled with lipid bilayers give this layer its effective barrier properties. The corneocytes are tightly packed and flattened (approximately 0.5 μm thick) due to contraction of keratin filaments and loss of intracellular organelles such as nucleus, and are connected by corneodesmomes, enhancing the SC barrier properties (Haftek *et al.*, 1998). Furthermore, the inner surface of the cell membrane of keratinocytes in the SC is covered with a protective cornified envelope, providing strength to the cell membrane. The intercellular spaces in SC are filled with lipid bilayers (lamellae), which are composed of non-polar lipids, including ceramides (47%), free fatty acids (9%) and their esters as well as cholesterol (27%) and its sulphates. The structure of the lipid bilayer demonstrates heterogeneity, for example providing both lipophilic and hydrophilic domains (Haftek *et al.*, 1998).

1.1.2.2. Epidermis

The epidermal layer consists of five distinctive cell strata, including the SC, which are formed by the keratinocytes (squamous epithelium) at the different stages of maturation. In the basal layer epidermal stem cells divide and the cells undergo terminal differentiation into keratinocytes and move upwards during their maturation, forming the distinctive layers of stratum basale, stratum spinosum, stratum granulosum, stratum lucidum and finally SC. During the maturation process the cells synthesize keratin filaments and lipid lamellae, which are transported into the intercellular space in lamellar bodies (vesicles). At the final stages of maturation the

keratinocytes lose the cell organelles and flatten by contracting keratin filaments on the mature SC. The dead keratinocytes are eventually shed (desquamation). The intercellular lipid bilayer changes composition during the transition from the basal layer towards the SC, such as phospholipids are replaced by sphingolipids on the top layers (Mills and Cross, 2006). The epidermal basal layer also contains melanocytes, which produce melanin pigment that protects the skin from the UV radiation.

1.1.2.3. Dermis

The dermis is mainly composed of connective tissue, especially collagen (70%), elastin and glycosaminoglycans, which are synthesized by fibroblasts (Gawkrodger, 2002). The dermal layer can be divided into the papillary dermis, which is a thin layer on the top part of the dermis and interdigitates with epidermal ridges, and the reticular dermis (Gawkrodger, 2002). The dermal layer also contains blood vessels and lymphatic tissue, as well as nerve endings. Other cells found in this layer are dermal dendrocytes, mast cells, macrophages and lymphocytes.

1.1.2.4. Skin appendages

Skin appendages include the hair follicles and sebaceous, apocrine and eccrine glands which are located within the skin. Hair follicles with the associated sebaceous glands as well as erector muscles are called pilosebaceous units. The hair follicle is composed of hair, hair shaft and hair bulb, and are associated with the sebaceous gland which secretes sebum via ducts in the upper parts of the hair canal. The sebaceous gland is formed from the epithelial cells and the hair follicle is lined with an outer root sheath (epidermal keratinocytes); the inner root sheath and basement membrane are only discovered at the lower parts of the hair follicle (Meidan *et al.*,

2005). The pilosebaceous units are complex structures in which physiological changes, such as metabolic activity, are variable (Meidan *et al.*, 2005).

Human skin contains two types of hair: terminal hairs are pigmented, long (>2 cm) and thick (>0.03 mm) and their roots are deeper in the dermis (>3 mm depth) and thinner, shorter vellus hairs which reach to a depth of 1 mm (Meidan *et al.*, 2005). The hair density, size and the total coverage depends on the body site. The highest hair density, apart from on the scalp, is found on the forehead (nearly 300 follicles per cm^2), and there are between 14 and 32 hair follicles per cm^2 on thorax, back, arms, thighs and calves (Otberg *et al.*, 2004). The total skin surface coverage of hair follicle orifices, between 50 to 140 μm in diameter, range from 0.09% to up to 10% however these figures vary greatly amongst the studies (Meidan *et al.*, 2005, Otberg *et al.*, 2004).

Sebaceous glands secrete sebum, which acts as a lubricant and protects the skin; it is a lipophilic substance mainly composed of triglycerides, but it also contains wax esters and other minor components. Its secretion is highly regulated, for example hormonal changes, age as well as circadian rhythm all affect sebum production (Meidan *et al.*, 2006). Sebaceous glands are present all over the body except soles and palms. Apocrine and eccrine glands are sweat glands; the eccrine glands produce sweat and function in body temperature regulation, and the apocrine glands secrete lipids-rich secretions (Mills and Cross, 2006). Apocrine glands, mainly present on parts of the body such as axilla, scalp, face, perineal and suprapubic region, are regulated by hormonal changes after puberty like the sebaceous glands, and secrete solution containing lipids, protein, ammonia, steroids and reducing sugars (Blackburn, 1991). Eccrine glands are present on all parts of the body, and secrete aqueous solutions containing inorganic salts, and small quantities of protein, amino acids, lactic acid and urea (Blackburn, 1991).

1.1.2.5. Skin microbiota

Skin becomes colonised with microorganisms soon after birth, and the resident microorganisms help in maintaining the healthy skin by preventing the pathogenic microorganism from colonising the skin. The skin surface is a hostile environment for growth of many microorganisms due to the limited nutrient availability, low water tension (dryness), high salt concentration, and low pH. However, resident microorganisms, such as *Staphylococcus spp.* and other Gram-positive bacteria, are able to survive and proliferate in these conditions. Transient skin microorganisms, such as Gram-negative rods and yeasts, may be found to colonise the skin surfaces however they are not able to proliferate/ grow on the normal healthy skin surface.

The number and type of microorganisms varies amongst the body sites and amongst individuals, depending on the physicochemical factors on the skin, such as humidity, temperature, pH and the nutrient availability such as lipid-rich sebum, apocrine and eccrine gland secretions; the concentration of natural antimicrobial compounds produced by the skin or skin microorganisms also affects the skin microbiota (Noble, 1993). The most common types of microorganisms residing on the skin includes Gram-positive bacteria, such as coagulase-negative *Staphylococcus spp.* (CNS) (especially *S. epidermidis*), *Micrococcus spp.*, *Corynebacterium spp.*, and *Propionibacterium acnes*; yeast *Candida spp.* and *Malassezia spp.* and Gram-negative bacteria such as *Klebsiella spp.*, *Enterobacter spp.* and *Proteus spp.* can colonise the surface of skin however they are often not able to grow or sustain life on the skin surface environments and are called the transient microbiota (Aly and Maibach, 1976, Noble, 1993).

Few studies have analysed the skin microbiota by using molecular techniques, sequencing bacterial small subunit ribosomal RNA genes (16S rRNA), which mainly demonstrated Gram-

positive bacteria, including *Staphylococcus spp.*, *Streptococcus spp.*, *Propionibacterium spp.*, *Corynebacterium spp.* (Dekio *et al.*, 2005), *Kocuria spp.*, *Micrococcus spp.* (Cove and Eady, 1998, Gao *et al.*, 2007, Grice *et al.*, 2008) and yeast *Malassezia spp.* (*Pityrosporum spp.*) (Cove and Eady, 1998) on the healthy human skins; however, Gram-negative microorganisms, such as *Acinetobacter spp.*, *Brevibacterium spp.* and *Pseudomonas spp.*, were also recovered. The study by Gao *et al.* (2007) evaluated the microorganisms on the skin of forearms of six healthy adults, and showed a great number of microbial species, with 119 genera of microorganisms, of which 30 phylotypes (8% of all clones) were unidentified; they only recovered *Propionibacterium spp.*, *Corynebacterium spp.*, *Staphylococcus spp.* and *Streptococcus spp.* from all the volunteers and in repeated measures 8-10 months later. Another molecular study of microbiota on and within the skin of inner elbow demonstrated 113 taxa of which proteobacteria such as *Pseudomonas spp.* were recovered from all skin depths (recovered by punch biopsies, scraping and swabbing) (Grice *et al.*, 2008); the other species identified included *Corynebacterium spp.*, *Kocuria spp.*, *Propionibacterium spp.*, and *Micrococcus spp.* They only uncovered one unidentified species. Furthermore, the study by Dekio *et al.* (2005) identified skin microbiota from the foreheads of five healthy volunteers using molecular typing techniques, which distinguished 19 microbial species, of which *Propionibacterium spp.* and *Staphylococcus spp.* were found in all four volunteers, and 13 new phylotypes.

Anaerobic *P. acnes* reside deeper in the hair follicles or sebaceous glands and aerobic Gram-positive cocci on the surface layers of skin (Elsner, 2006), however CNS have also been thought to reside in the deeper layers of skin and the pilosebaceous units (Brown *et al.*, 1989). A study investigating the number and type of microorganisms in the pilosebaceous units on the backs of people with acne vulgaris estimated the number of *Propionibacterium spp.*, *Staphylococcus spp.* and *Pityrosporum spp.* in the hair follicle and sebaceous glands as 2.6×10^5, 5.5×10^3 and 1×10^2

per unit respectively (Leeming *et al.*, 1984). Another study evaluated the number and type of microorganisms in the pilosebaceous glands of healthy individuals, and found aerobic cocci in 44 out of the 138 pilosebaceous glands microsectioned from the back, but anaerobic microorganisms were present in most glands with over 1 x 10^4 anaerobic diphteroids per follicle (Puhvel *et al.*, 1975). Both *Staphylococcus spp.* and *P. acnes* have been associated with the colonisation of intravascular catheters (Martin-Rabadan *et al.*, 2008). Another study evaluating the type and number of microorganisms on the skin surface swabs and the deeper skin samples (biopsies) found *P. acnes*, *Staphylococcus spp.*, and smaller numbers of diphteroids and *Micrococcus spp.*, in both the surface and deeper layers of skin (Nielsen *et al.*, 1975).

Antimicrobial compounds such as antibiotics and skin antiseptics can alter the normal microbiota, when normal microbiota are killed, allowing the more pathogenic transient bacteria to grow (Sullivan *et al.*, 2001). A study by Aly and Maibach (1976) assessed the changes in skin microbiota after six months repeated use of chlorhexidine (CHG) based soap compared to non-medicated soap, and demonstrated a reduction in aerobic microorganisms and an increase in Gram-negative rods after the use of CHG. Furthermore, another study looking at the skin microbiota on hospitalized patients found more antimicrobial resistance among the skin microorganisms compared to non-hospitalised patients (Larson *et al.*, 1986).

1.2. Skin antisepsis

Antiseptic agents mainly act on non-specific cellular targets, including the plasma membranes, cell wall proteins and lipids and cytoplasm (Figure 1.2). Microbial sensitivity to antimicrobial agents is affected by the physicochemical characteristics of the antimicrobial agent, the microbial cell wall structures, as well as the physiological status of microorganisms (Denyer and Stewart, 1998). The cell wall of Gram-negative bacteria is more complex compared to Gram-positive bacteria, and often permeation of high molecular weight compounds to its cellular targets is hindered due to its outer cell membrane, which is absent in the Gram-positive bacteria.

Figure 1.2. Schematic structure of Gram-positive and Gram-negative bacterial cell wall and the potential targets for biocides (Denyer, 1995).

1.2.1. Structure of bacterial cell wall

The bacterial cell wall structures vary between Gram-positive and Gram-negative bacteria. Both Gram-positive and –negative bacteria contain the plasma membrane (between the cytoplasm and outside environment) and the cell wall. Gram-negative bacteria also contain the outer cell membrane, and periplasm between the outer membrane and the plasma membrane.

The cytoplasmic membrane is the main permeability barrier of the microbial cell, separating the cytoplasm and environment and maintaining the cell integrity. It is also a site for many proteins that are involved with metabolic activity, transport and energy conservation (Moat *et al.*, 2002). The cytoplasmic membrane is composed of a phospholipid bilayer with embedded protein molecules, and contains hydrophilic and hydrophobic domains. The lipid bilayer is stabilised by hydrogen bonds, hydrophobic interactions and ionic interactions between the negatively charged phospholipids and magnesium and calcium ions (Madigan *et al.*, 2000).

The bacterial cell wall is mainly composed of peptidoglycan, which is formed by glycan chains (N-acetylglucosamine and N-acetylmuramic acid) interconnected by peptide cross-links (Madigan *et al.*, 2000). Gram-positive bacteria have multiple layers of peptidoglycan, which compose up to 90% of the Gram-positive bacterial cell wall, compared to Gram-negative bacteria which are often composed of a single peptidoglycan monolayer (Madigan *et al.*, 2000). Furthermore, peptidoglycan has more cross-bridges between the glycan chains in Gram-positive bacteria compared to Gram-negative bacteria (Moat *et al.*, 2002). The cell wall of Gram-positive bacteria also contains acidic polysaccharides, teichoic acids, imbedded or attached to the peptidoglycan cell wall, cell membranes and capsular polymers. They are often found linked to

lipids, and therefore called lipoteichoic acids. Teichoic acids contribute to the negative charge of the Gram-positive cell surface (Madigan *et al.*, 2000).

The cell wall peptidoglycan in Gram-negative bacteria is thinner compared to Gram-positive cell; however Gram-negative bacteria have an additional lipid bilayer outside the cell wall called the outer membrane. The outer membrane lipid bilayer is mainly composed of phospholipids, polysaccharides and proteins (Madigan *et al.*, 2000). Polysaccharides are often tightly linked to lipids and form lipopolysaccharide complexes (LPS) at the outer layers of the outer membrane; lipoproteins complexes are found in the inner layers of the outer membrane. The outer cell membrane is relatively permeable to low molecular weight hydrophilic substances, and most of the transport occurs through the water-filled porin-channels, proteins embedded in the outer membrane. Periplasm is the space between the cytoplasmic membrane and the outer membrane of Gram-negative bacteria, and contains many proteins which include transport proteins, binding protein, degrading enzymes and chemoreceptors (Moat *et al.*, 2002). In addition to the cell wall and cell membranes, some bacterial cells produce polysaccharide or polypeptide capsule (also called a slime layer) outside the bacterial cell (Moat *et al.*, 2002).

1.2.2. Topical skin antiseptics

Appropriate and effective skin antisepsis is essential in preventing infections which may arise from breach of the skin, for example during surgery or prior to insertion of intravascular devices. A variety of topical antimicrobial agents is currently available for skin antisepsis, and is found in different concentrations and formulations. However, the most commonly used antiseptics used for skin preparation prior to invasive procedures are alcohols, CHG and povidone-iodine (PVP-I),

of which CHG and PVP-I have shown more persistent antimicrobial activity compared to alcohols (McDonnell and Russell, 1999).

Current guidelines for skin antisepsis prior to insertion of intravascular catheters, such as central venous catheters (CVC), recommend 2% (w/v) CHG, preferably in 70% (v/v) isopropyl alcohol (IPA) (O'Grady *et al.*, 2002, Pratt *et al.*, 2007), however guidelines for skin antisepsis prior to surgery is not clear (Lipp, 2006), varying amongst the type of surgery, location and the surgeon's preference (Mangram *et al.*, 1999). The most common antiseptic agents used for patient's skin antisepsis prior to surgery are CHG, PVP-I and IPA, and in addition triclosan and PCMX (para-chloro-meta-xynelol) are used as surgical scrubs (Mangram *et al.*, 1999). A two-step skin antisepsis technique, such as application of 0.5% (w/v) CHG in 70 % (v/v) IPA followed with 10% (w/v) PVP-I (Langgartner *et al.*, 2004), 15% (w/v) CHG scrub followed by 10% (w/v) PVP-I (Guzel *et al.*, 2009) or povidone-iodine or CHG scrub followed by alcoholic povidone-iodine or alcoholic CHG (Traore *et al.*, 2000) has also been suggested in reducing skin colonisation.

1.2.2.1. Chlorhexidine

Chlorhexidine is a biguanide antiseptic (Figure 1.3) with a broad spectrum antimicrobial activity against Gram-positive and -negative bacteria, yeasts and some lipid-enveloped viruses, but has little effect on mycobacteria, spores and most viruses (McDonnell and Russell, 1999). It has been widely used as an antiseptic agent in mouthwashes, hand washes, skin antiseptics, dressings and as a coating in intravascular catheters. Chlorhexidine disrupts cytoplasmic bacterial cell membrane phospholipids and the loss of cell membrane integrity leads to leakage of intracellular components and inhibition of enzyme activity (membrane-bound ATPase), and at higher concentrations CHG causes coagulation of the cytoplasmic components and precipitation of

proteins and nucleic acids (McDonnell and Russell, 1999, Sheppard *et al.*, 1997). Chlorhexidine activity against yeast is similar to its activity against bacterial cells, targeting yeast cell membranes; however the yeast cell wall reduces its activity as there is limited diffusion through the cell wall to the target cell membranes (McDonnell and Russell, 1999).

Figure 1.3. Chemical structure of a water soluble chlorhexidine salt, chlorhexidine digluconate [1,6-Bis(N^5-[p-chlorophenyl]-N^1-biguanido)hexane digluconate] (Farkas *et al.*, 2007).

Two percent (w/v) CHG in 70% IPA is recommended for skin antisepsis prior to insertion of intravascular devices by Evidence-Based Practice in Infection Control (EPIC) guidelines (Pratt *et al.*, 2007) and Centres for Disease Control and Prevention (CDC) guidelines (O'Grady *et al.*, 2002). Chlorhexidine, especially 2% (w/v) in 70% (v/v) IPA, have demonstrated superior antimicrobial activity compared to PVP-I [10% (w/v)] in reducing blood culture contamination (Suwanpimolkul *et al.*, 2008, Mimoz *et al.*, 1999), infections associated with surgical incision (Maki *et al.*, 1991) and intravascular catheter related infections (Balamongkhon and Thamlikitkul, 2007, Chaiyakunapruk *et al.*, 2002). However, CHG has been associated with skin irritation and toxicity against skin fibroblasts *in vitro* (Hidalgo and Dominguez, 2001), and its efficacy is reduced in the presence of organic matter, such as serum (McDonnell and Russell, 1999).

Chlorhexidine demonstrates an intermediate onset of antimicrobial activity, with bactericidal activity demonstrated after 15 s *in vitro* (Adams, 2006), and significant reduction in number of skin microorganisms after 30 s application of 2% (w/v) CHG in 70% (v/v) IPA (Hibbard, 2002). In the study by Hibbard *et al.* (2002) which compared the antimicrobial activity of 2% (w/v) CHG in aqueous solution and in 70% (v/v) IPA, and 70% (v/v) IPA alone in reducing skin colonisation over a 24 h period, it was demonstrated that all the three agents were effective in reducing microbial load on the skin up to 24 h. However CHG in 70% (v/v) IPA demonstrated significantly higher reduction in number of microorganisms at 24 h compared to aqueous CHG although the difference was only seen in abdominal skin and not at the inguinal site (Hibbard *et al.*, 2002). Addition of alcohol to a CHG-based hand scrub formulation also demonstrates superior antimicrobial efficacy compared to an alcohol-free scrub (Kampf *et al.*, 1998). In addition to the antimicrobial activity, chlorhexidine and another biguanine antiseptic, alexidine, have been shown to possess anti-inflammatory properties by binding to lipopolysaccharide (LPS) and lipoteichoic acid (LTA) released from Gram-negative and Gram-positive bacteria respectively (Zorko and Jerala, 2008).

1.2.2.2. Povidone-iodine

The most common iodophor used in the healthcare environment is povidone-iodine (PVP-I; Figure 1.4), which is a complex of iodine and polyvinylpyrrolidone. It is a halogen-releasing agent and has a rapid, broad spectrum of antimicrobial activity against Gram-positive and Gram-negative bacteria, fungi, enveloped viruses and some spores (McDonnell and Russell, 1999). It is thought to act on the cell wall of the microorganism causing oxidation and attacking proteins, nucleotides and fatty acids (McDonnell and Russell, 1999). However, skin irritation and toxicity

against iodophors has been reported and its use is contraindicated in newborns and during pregnancy due to absorption through the skin (McDonnell and Russell, 1999).

Chlorhexidine, especially in alcoholic solution, has been shown to exhibit superior antimicrobial activity compared to PVP-I (Balamongkhon and Thamlikitkul, 2007, Chaiyakunapruk *et al.*, 2002, Maki *et al.*, 1991, Mimoz *et al.*, 1999, Suwanpimolkul *et al.*, 2008); however few studies have demonstrated comparable efficacy of CHG and PVP-I, such as PVP-I and alcoholic 0.5% (w/v) CHG in reducing catheter related blood stream infections (CR-BSI), CVC tip colonisation and CVC skin site colonisation (Humar *et al.*, 2000), tincture of iodine and CHG in reducing blood culture contamination (Trautner *et al.*, 2002) and alcoholic and aqueous CHG and PVP-I against *S. epidermidis* in a planktonic and a biofilm modes of growth *in vitro* (Adams *et al.*, 2005). Povidone-iodine has been shown to improve the efficacy in tincture of iodine [2% (w/v) PVP-I in 47% (v/v) alcohol] compared to the aqueous solution [10% (w/v) PVP-I] in reducing the blood culture contamination rates (Little *et al.*, 1999).

Furthermore, the study by Block and colleagues (2000) found 10% (w/v) PVP-I more effective than aqueous 0.5% (w/v) CHG against meticillin-resistant *S. aureus* (MRSA), meticillin-sensitive *S. aureus* (MSSA) and vancomycin-resistant (VRE) and -sensitive enterococci in a surface test, with PVP-I reducing the microbial load on the dried surfaces within 3 min compared to CHG in ≥10 min. Moreover, they found that MRSA were less susceptible to CHG compared to MSSA, but there were no significant differences in PVP-I activity against MSSA and MRSA (Block *et al.*, 2000).

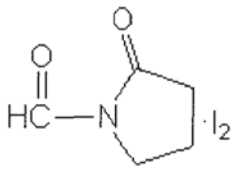

Figure 1.4. Chemical structure of povidone-iodine, the iodine complex with neutral polyvinylpyrrolidone polymer carrier.

1.2.2.3. Alcohols

Alcohols used for skin antisepsis include ethyl alcohol (ethanol) and isopropyl alcohol (IPA; Figure 1.5). Alcohol in aqueous solutions at concentrations of 70% to 92% (v/v) have a rapid antimicrobial activity against Gram-positive and Gram-negative bacteria, fungi and many viruses (McDonnell and Russell, 1999). Isopropyl alcohol is more active against bacteria than ethanol, which has better efficacy against viruses (McDonnell and Russell, 1999). Alcohol's rapid activity is due to the membrane damage, denaturation of proteins, and interference with the metabolism and lysis of the microorganism. Alcohols are volatile compounds and evaporate from the skin, which is ideal for skin preparation prior to invasive procedures due to the drying effect. However, they also cause dryness and irritation of the skin.

Isopropyl alcohol and ethyl alcohol are most commonly used as skin antiseptics, and are often combined with another antimicrobial agent such as chlorhexidine due to the lack of residual antimicrobial activity. Combining CHG with IPA has demonstrated superior activity compared to IPA alone in preventing infections associated with peripheral venous catheters (Small *et al.*, 2008). Furthermore, Kampf and colleagues recommended incorporation of alcohol in CHG hand rubs to improve efficacy against MRSA (Kampf *et al.*, 1998) and VRE (Kampf *et al.*, 1999).

Figure 1.5. Chemical structure of isopropyl alcohol (2-propanol) (McDonnell and Russell, 1999).

1.2.2.4. Essential oils

Many essential oils are used in foods, beverages, cosmetics and healthcare products such as soaps, mouthwashes and toothpastes. They have been used not only as an aromatic substance but also as a natural choice of preservative (due to antioxidant or radical-scavenging properties) as well as an antimicrobial agent. Furthermore, their activity as skin penetration enhancers for delivering drugs through the skin has been studied (discussed further in section 1.3.3.4). They are secondary metabolites derived from plants and flowers, and are complex mixtures of heterogeneous, volatile compounds, such as terpenes. However, their role as potential antiseptic agents has received research interest, especially their activity against hospital pathogens such as meticillin-resistant *S. aureus* (MRSA). Many essential oils, and their terpene constituents, have demonstrated a broad spectrum of activity (Cowan, 1999). Tea tree oil (TTO) (from the leaves of *Melaleuca alternifolia*) has been shown to be efficient in eradicating MRSA from skin (Dryden *et al.*, 2004, Caelli *et al.*, 2000), reducing hand contamination (Messager *et al.*, 2005), treating pulmonary tuberculosis (Sherry *et al.*, 2004) and diabetic foot ulcers (Sherry *et al.*, 2003) and demonstrates an excellent activity against *Staphylococcus spp.* in a biofilm mode of growth (Brady *et al.*, 2006). Eucalyptus oil (EO)(from the leaves of *Eucalyptus globulus*) has been successfully used to treat pulmonary tuberculosis (Sherry and Warnke, 2004) and to clear MRSA from skin (Sherry *et al.*, 2001b). Combinations of essential oils including TTO and EO have demonstrated efficacy against MRSA osteomyelitis (Sherry *et al.*, 2001a). Thymol is known for its excellent antimicrobial activity and has been used in surgical dressings, antiseptic lotions,

mouthwashes and internally as an antiparasitic agent (Trombetta *et al.*, 2005). Although the antimicrobial activity of various natural compounds has been shown in *in vitro* assays, there is only a limited number of clinical trials assessing the efficacy of these compounds (Martin and Ernst, 2003a, Martin and Ernst, 2003b, Martin and Ernst, 2004). Furthermore, variation amongst the test organisms, growth media and methods, and the source or extraction method of the natural compounds cause a great variability in the data obtained from *in vitro* studies (Rios and Recio, 2005). Essential oils are generally regarded as safe when used at low doses, however some reports on toxicity and skin irritation have been reported; skin irritation and toxicity in higher doses of TTO after ingestion has been described, however use of TTO diluted for topical use (Hammer *et al.*, 2006) and protecting TTO from oxidation or other damage by improper storage (Hausen *et al.*, 1999) reduces the risk of adverse effects. Further studies on irritation and toxicity of other essential oils, and terpenes, are needed.

The antimicrobial activity of many of the essential oils is not yet fully understood, however they are thought to act on the plasma membranes of the microorganisms, increasing the permeation of the cell membrane and leakage of intracellular constituents (Cowan, 1999); TTO has been shown to act by interfering with the microbial cytoplasmic membranes, causing a loss of nucleic acids (Carson *et al.*, 2002), inhibition of respiration and increase in the permeability of bacterial cytoplasmic and yeast plasma membranes (Cox *et al.*, 2000) and leakage of potassium ions (Hada *et al.*, 2003). The main constituent of TTO, terpinen-4-ol (Loughlin *et al.*, 2008) and to a lesser degree linalool and α-terpineol, have demonstrated bactericidal activity alone, but p-cymene fraction of TTO did not have antimicrobial activity (Carson and Riley, 1995). Other studies have demonstrated membrane damage by the lipophilic cyclic monoterpenes, such as α-Pinene and Limonene, and their partitioning with the lipid membranes, increasing membrane fluidity, affecting the respiration and function of cell membrane enzyme activity, and increased

permeability to protons and ions (Sikkema *et al.*, 1995). Thymus oil, and its main constituents carvacrol and thymol, have demonstrated a cell membrane damaging effect against fungal cells (Pinto *et al.*, 2006). Another study assessing the antibacterial activity of thymol, methol and linalyl (monoterpenes) showed alteration in the bacterial plasma membrane permeability and leakage of intracellular constituents and proposed that the action is dependent on the physico-chemical characteristics of the compound as well as the lipid composition and surface charge of the bacterial membranes (Trombetta *et al.*, 2005). However, Carson *et al.* (2002) consider that there is more than one component of the essential oil or more than only one target, and that several components and targets may be involved in activity of essential oil.

1.2.2.5. Other novel antimicrobial agents

Search for new antimicrobial agents for treatment and prevention of infection is vital due to new emerging infections and emergence of antimicrobial resistance of many pathogens. Carboxamidrazone compounds have been studied for efficacy against malaria parasites (Gokhale *et al.*, 2001, Gokhale *et al.*, 2003), and a novel carboxamidrazone derivate, a benzylidenecarboxamidrazone compound, developed by a team at Aston University, UK, has demonstrated antimicrobial activity against Gram-positive bacteria, including MRSA (Rathbone *et al.*, 2006) and *Mycobacterium tuberculosis* (Billington *et al.*, 1998, Billington *et al.*, 2000). In addition, a thiosemicarbazone group of antimicrobial compounds have demonstrated anti-parasitic (anti-trypanocidal and anti-amoebic) activity (Aguirre *et al.*, 2004) as well as efficacy against Gram-positive and Gram-negative bacteria and yeast *in vitro* (Kizilcikli *et al.*, 2007, Kasuga *et al.*, 2003). Furthermore, a study in Aston University demonstrated a good antimicrobial activity against *Clostridium difficile* (Costello *et al.*, 2008). Thiosemicarbazones contain a 5-nitrofuryl moiety which is thought to be important for the antimicrobial activity.

However, the mode of activity of carboxamidrazone compounds is not well understood and requires further studies. Both groups of antimicrobial compounds, thiosemicarbazones and carboxamidrazones, have demonstrated a good antimicrobial activity *in vitro*, but their potential in eradicating microorganisms associated with skin needs to be elucidated.

1.3. Skin penetration of chlorhexidine and methods for enhancing skin penetration

1.3.1. Skin penetration of chlorhexidine

The current skin antisepsis procedures cannot totally eradicate the resident microorganisms from the skin, but remove the transient microorganisms from the skin and reduce the resident skin microbial load to low a level. Many antimicrobial agents exhibit restricted permeation of the skin (Hendley and Ashe, 1991) and fail to reach the deeper layers, including the hair follicles (Brown *et al.*, 1989, Hendley and Ashe, 2003, Hendley and Ashe, 1991, Leeming *et al.*, 1984, Malcolm, 1980); estimated 20% of the microorganisms on the skin are not reached by antiseptic agents (Sebben, 1983). Effective permeation of CHG within the skin tissue is essential as microorganisms not only colonise the skin surface but are also found to inhabit the hair follicles and lower skin depths (Hendley and Ashe, 1991).

Skin permeation kinetics of CHG in the human skin is not well understood, and only few studies have assessed skin penetration of chlorhexidine-compounds (Lafforgue *et al.*, 1997, Wang *et al.*, 1990). Chlorhexidine has been shown to bind to the SC intercellular cholesterol with high affinity, without removing the endogenous cholesterol, and accumulate to the SC layer without penetrating through the SC due to its large molecule size (898 Da) (Aki and Kawasaki, 2004).

Lafforgue and colleagues evaluated CHG skin penetration through the excised hairless rat skin over 48 h and demonstrated negligible penetration through the intact skin (0.01% of the applied dose) and skin without SC (0.87% of the applied dose), and showed high amount of CHG deposited in the skin layers of intact skin. Wang *et al.* demonstrated that chlorhexidine phosphanilate did not penetrate through the intact human abdominal skin (with the epidermal and dermal layers) *in vitro* assay over 48 h, however permeated through the skin without SC. However, a study assessing the CHG absorption through vaginal mucosa after repeated washing with 0.2% (w/v) CHG demonstrated small amounts of CHG permeating through to the blood (10-83 ng/ mL) on 35% of the study group but CHG did not accumulate in the blood in the repeated washing (Nilsson *et al.*, 1989).

1.3.2. Routes for skin penetration

Skin penetration is a passive process and affected by the drug solubility and partitioning in the vehicle and in the skin structures and diffusion of the drug in the skin. The stratum corneum, and its tightly packed keratinized cells, lipid bilayers, and corneodesmosomes, is generally regarded as the major penetration barrier. Penetration of substances through the SC is thought mainly to occur through the intercellular route (i.e. between the cells) (Bunge *et al.*, 1999); even though the transcellular route (through the cell membranes and intracellular lipid bilayers) is shorter than the intercellular path, the route requires partitioning of the drug molecule repeatedly between the lipophilic and hydrophilic domains (Haftek *et al.*, 1998, Mills and Cross, 2006). The intercellular route follows the lipid bilayers rather than transversing them. Lipophilic compounds are thought to travel along the lipid domains of the lipid bilayer (hydrocarbon chains) and the hydrophilic compounds alongside the polar head groups regions of the lipid bilayer.

For the systemic drug delivery, the transappendageal route (through the sweat glands and sebaceous glands and the hair follicles) is called the 'shunt route', as there are less lipid membranes to be crossed compared to the trans- and intercellular paths and the hair follicles have a rich vascular supply (Hueber *et al.*, 1994). Yet the contribution of the transappendageal drug delivery route to the skin penetration is regarded as minimal due to their relatively low density and reduced penetration into the hair follicles due to production of lipophilic sebum, and the importance of transfollicular route in drug delivery is under much debate (Meidan *et al.*, 2005, Otberg *et al.*, 2004). However, the potential surface area for absorption through hair follicles depends on the size of the hair follicle as well as the density of the hair follicles, and it is estimated between 14 mm^2 per cm^2 skin (forehead) and approximately 1 mm^2 per cm^2 skin (forearm) (Otberg *et al.*, 2004). Furthermore, in skin antisepsis the follicular penetration is important due to the hair follicles and sebaceous glands harboring microorganisms, which can persist following skin antisepsis and cause infection when the skin barrier is breached (Elliott *et al.*, 1997, Leeming *et al.*, 1984, Langgartner *et al.*, 2004, Safdar and Maki, 2004, Worthington and Elliott, 2005). Effective and rapid permeation of the antiseptic agent into the deeper layers of skin is therefore essential in preventing infections associated with invasive procedures.

1.3.3. Skin penetration enhancers

Methods which interfere with the interaction between the drug and SC, such as disrupting the SC lipid bilayers and tight junctions (desmosomes), enhancing partitioning of the drug into the skin, enhancing the drug solubility (thermodynamic activity) within the skin, or disrupting keratin filaments, will affect the drug skin permeation (Williams and Barry, 2004). Skin penetration

enhancers are often categorised into physical (such as electroporation and sonophoresis), electrical (such as iontophoresis) and chemical skin penetration enhancers, and supersaturated drug delivery systems and drug encapsulation.

1.3.3.1. Physical skin penetration enhancers

Physical skin penetration enhancers include iontophoresis (electrical charge) and sonophoresis (ultrasound waves). Iontophoresis, which involves the application of an electrical current on the skin, has also been studied for enhancing the delivery of drug into the skin (Kalia *et al.*, 2004), such as delivering aciclovir to the basal layers of the epidermis to target herpes simplex lesions (Volpato *et al.*, 1998). The drug penetration in the skin with application of iontophoresis is due to electropulsion and eletroosmosis (connective flow), and therefore the technique is more effective in enhancing skin permeation of ionised drugs (Volpato *et al.*, 1998). Iontophoresis has been shown to be an effective skin penetration enhancer technique, especially in combination a terpene and alcohol, in delivering large molecules, such as insulin (Pillai and Panchagnula, 2003) and methotrexate (Prasad *et al.*, 2007) into the skin. Sonophoresis utilizes low frequency ultrasound (20-100 hHz) to deliver drugs through the skin, such as protein molecules (insulin), oligonucleotides for gene therapy and immunogens for immunisation (Ogura *et al.*, 2008); it is thought that the low frequency ultrasound enhance skin penetration by altering ultrastructure of the SC, causing disorder in the SC lipids by cavitation (Ogura *et al.*, 2008) and inducing convective solution flow (Morimoto *et al.*, 2005). However, the increased permeation of skin is heterogeneous.

1.3.3.2. Chemical skin penetration enhancers and supersaturated drug delivery systems

Chemicals that enhance skin penetration by disruption of the lipid bilayers include sulphoxides [such as dimethylsulphoxide (DMSO) which is an aprotic solvent], Azone, pyrrolidones, fatty acids, alcohols (including glycols and fatty alcohols such as IPA), surfactants (anionic and non-ionic surfactants such as Tween 80), urea, terpenes (essential oils) and phopholipids (Williams and Barry, 2004). Stratum corneum keratinocytes, that normally contain 15-20% water, are able to absorb large amount of water (up to ten times their dry weight) in the intracellular keratin, disrupting the SC structure and therefore acting as a skin penetration enhancer (Mills and Cross, 2006, Williams and Barry, 2004). The skin permeation enhancing property of urea is also thought to be due to its skin hydrating properties, in combination with its keratolytic activity (Williams and Barry, 2004). Supersaturated systems do not alter the skin structures but changes the thermodynamic activity, however using oversaturated drug solutions are unstable and requires cosolvent to increase the drug solubility (Iervolino *et al.*, 2001).

Alcohols, which are widely used as antimicrobial agents, also have rapid skin penetration enhancing properties. However, at high concentrations they have also been shown to extract SC lipids and proteins (Dias *et al.*, 2007), especially longer chain alcohols such as IPA compared to ethanol (Goates and Knutson, 1994). Ethanol can enhance skin penetration in a concentration dependent fashion up to 63% (v/v), after which the permeation decreases (Williams and Barry, 2004). Alcohol functions as a solvent (increasing solubility of the drug in the vehicle), changing thermodynamic activity of the drug in the vehicle and by creating supersaturated systems due to evaporation on the skin, and at the high concentration extracting SC lipids (Williams and Barry, 2004).

1.3.3.3. Liposomes, nanocapsules and emulsions

Drugs can be encased into liposomal or other carriers, which can alter their solubility in the vehicle, penetration into the skin and hair follicles and control their release. Incorporation of currently used antimicrobial agents into micro- or nanoparticles (Constant *et al.*, 2006, Alves *et al.*, 2007) such as CHG in poly(ε-caprolactone) nanocapsules (Lboutounne *et al.*, 2002, Lboutounne *et al.*, 2004), or into liposomes (Betz *et al.*, 2001, Verma and Fahr, 2004), such as triclosan (Catuogno and Jones, 2003) and CHG (Jones *et al.*, 1997), has been investigated for enhancing skin penetration of drugs. These may have advantages over other enhancers or drug carriers by enabling controlled release of the antimicrobial agents/ sustained activity, enhanced skin permeation and enhanced penetration into the hair follicles (El Maghraby *et al.*, 2006), as well as targeting the antiseptics to the site of microorganisms and also may reduce antiseptic compound degradation and skin irritation (Constant *et al.*, 2006). Nanoencapsulated CHG incorporated into a gel formulation demonstrated a sustained antimicrobial activity compared to 60% isopropanol or 62% (v/v) ethanol-based hand rub gel (Nhung *et al.*, 2007), and Lboutounne and colleagues (2002) demonstrated a sustained antimicrobial activity up to 8 h and diffusion of CHG into hair follicles *ex vivo* following treatment with CHG incorporated into poly(ε-caprolactone) nanocapsules. A study evaluating nanoparticle penetration into hair follicles demonstrated that the particles deposit in the hair follicles longer compared to drug without nanaparticle carrier (10 d compared to 4 days) (Lademann *et al.*, 2007). Furthermore, Jones *et al.* (1997) enhanced delivery of triclosan and CHG into staphylococcal and streptococcal biofilms. Other carriers, which have been studied for enhanced skin penetration, include water-in-oil nanoemulsions or microemulsions, which may enhance hydrophilic drug skin permeation through lipophilic SC (Schmalfuss *et al.*, 1997) and hair follicles, which have lipophilic environment due to sebum (Wu *et al.*, 2001).

1.3.3.4. Essential oils and terpenes

Terpenes, the major constituents of essential oils, are composed of carbon, hydrogen and oxygen and may also contain alcohols, esters, or ketones as subgroups. They have been studied for their skin penetration enhancing properties (Table 1.1) (Aqil *et al.*, 2007, Sapra *et al.*, 2008, Cornwell *et al.*, 1996). In addition, the crude essential oil such as EO (Biruss *et al.*, 2007), sweet basil oil (Fang *et al.*, 2004), chenopodium and ylang ylang (Williams and Barry, 2004) has been shown to increase skin penetration of steroids, indomethacin and 5-fluorouracil respectively. Eucalyptus oil in a microemulsion has been studied for enhancing skin penetration of hydrocortisone (El Maghraby, 2008).

1,8-Cineole (Figure 1.6) is the main terpene in EO, estimated 44.3% to 80% of the crude EO extracted from *E. globulus* leaves (Cimanga *et al.*, 2002, De Vincenzi *et al.*, 2002, Sacchetti *et al.*, 2005), and has been widely studied for its skin permeation enhancing properties (Williams *et al.*, 2006). It is a polar, oxygen containing terpene, which has been demonstrated to enhance skin permeation of hydrophilic compounds and to a lesser extent lipophilic compounds (Femenia-Font *et al.*, 2005, Narishetty and Panchagnula, 2005, Yamane *et al.*, 1995); the lipophilic compounds were more effectively transported into the skin with hydrocarbon terpenes such as D-limonene (Williams and Barry, 2004, Aqil *et al.*, 2007). Larger terpenes, such as those containing 15 or more carbons, are more lipophilic and appear be less effective as a skin permeation enhancers than monoterpenes (Aqil *et al.*, 2007) however they may have prolonged skin permeation activity (Williams and Barry, 2004). Terpinen-4-ol, which is the main constituent of TTO (estimated 30-40% of TTO) and is also present in smaller quantities in other essential oils such as lavender oil, juniper oil, and thyme oil, has also been studied for skin penetration enhancing properties (Reichling *et al.*, 2006).

Terpenes, including 1,8-cineole, have been shown to bind in large quantities to SC (Cornwell *et al.*, 1996, Cal *et al.*, 2001) and are thought to act as skin penetration enhancers by increasing drug partitioning into the skin (solvent effect), as well as enhancing drug diffusion and reversibly disrupting lipid bilayers (Aqil *et al.*, 2007, Cal *et al.*, 2001, Cornwell *et al.*, 1996). The study by Williams *et al.* (2006) showed that the 1,8-cineole partitioning in the skin lipids is heterogeneous causing ordered and disordered areas in SC lipids. Furthermore, these volatile compounds may alter the thermodynamic activity of the drug due to evaporation of terpenes like alcohol (Williams and Barry, 2004, Sapra *et al.*, 2008, Cross *et al.*, 2008); Cross *et al.* demonstrated evaporation of TTO from filter paper within hours.

The skin permeation of terpenes themselves is not well understood; however, terpenes, such as terpinen-4-ol, have been shown to bind to SC (Cal *et al.*, 2001, Cornwell *et al.*, 1996) and are thought to deposit in the hydrophilic dermis and epidermis (Cal, 2008). Eucalyptol has been shown not to permeate the skin *in vitro* assays, but retained in the skin (Cal *et al.*, 2006), however terpinen-4-ol has demonstrated limited skin penetration *in vitro* assay (Reichling *et al.*, 2006, Cross, 2007).

Figure 1.6. Chemical structure of cineole, the main terpene compound in the leaves of *Eucalyptus spp.* (dos Anjos and Alonso, 2008).

Table 1.1. The type, formulae and the main source of various terpenes studied for skin penetration enhancing properties (Aqil *et al.*, 2007).

Type of compound	Terpene	Chemical formula	Source
Monoterpene	Limonene	$C_{10}H_{16}$	Lemon peel of *Citrus limon*
	Terpinolene	$C_{10}H_{16}$	Minor constituent on many herbs, spices, and plants such as tea tree (leaf)
Monoterpene alcohol	Menthol	$C_{10}H_{20}O$	Flowers of *Menthe piperita*
	Linalool	$C_{10}H_{18}O$	Fruits of *Coriandrum sativum*
	Geraniol	$C_{10}H_{18}O$	Small quantities in may essential oils such as geranium, lemon, citronella
Monoterpene peroxide	Ascaridole	$C_{10}H_{16}O_2$	*Chenopodium ambrosioides* (Mexican tea)
Monoterpene phenol	Thymol	$C_{10}H_{14}O$	Thyme
	Carvacrol	$C_{10}H_{14}O$	Thyme, pepperwort and bergamot
Ether	Cineole	$C_{10}H_{18}O$	Leaves of *Eucalyptus globulus* and other *Eucalyptus spp.*
Ketone	Carvone	$C_{10}H_{14}O$	Caraway seeds and many other essential oils
Sesquiterpene alcohol	Nerolidol	$C_{15}H_{26}O$	Many types of plants and flowers, such as neroli, ginger, jasmine, lavender, tea tree and lemon grass
	Farnesol	$C_{10}H_{26}O$	Many types of plants and flowers, such as citronella, neroli, cyclamen, lemon grass, tuberose, rose, musk, balsam and tolu

1.4. Healthcare associated infections

Healthcare associated infections (HCAI), the infections which result from exposure to healthcare environment, are a major concern in the clinical environment and are associated with significant morbidity and mortality, increased length of hospital stay and increased costs to the Health Service (HPA, 2008). Estimated 8.2% of the hospital patients in England in 2006 (HIS, 2007) acquired infection whilst in hospital. The National Audit Office (NAO) estimate that HCAI accounts for approximately 300,000 infections a year in England, and contributes to 5,000 deaths a year with a cost to the National Health Service (NHS) estimated at an extra 1 billion pounds a year (NAO, 2004). A significant number of HCAI (15%) are thought to be preventable by complying with the infection control practices and adequate hygiene measures, including appropriate skin antisepsis prior to invasive procedures and hand hygiene (NAO, 2004). However, many factors contribute to acquiring HCAI, such as the underlying health of the patient, immunosuppressive therapies, invasive procedures and medical implants as well as the healthcare environment, including high bed occupancy and poor staffing levels in the health care setting (Vincent, 2003). The hospital environment as a source of infection and the role of cleaning and disinfecting surfaces have acquired great interest, especially with the high number of *Clostridium difficile* and MRSA infections, however their direct influence in the HCAI rate requires further studies (Boyce, 2007, Dancer, 2008, Dettenkofer and Spencer, 2007).

The five main categories of the hospital acquired infections are given in Figure 1.7. Surgical site infections (10.7%) and blood stream infections (6.2%) are often associated with resident or transient skin microorganisms, which could be reduced by adequate hygiene measures including appropriate asepsis during invasive procedures.

Figure 1.7. The main sites of hospital acquired infections in England (NAO, 2000).

The healthcare-associated blood stream infections (HA-BSI) are defined as infections acquired two or more days after admission to the healthcare facility (HPA, 2008). A significant number of HA-BSI are caused by the insertion of medical devices through the skin, for example intravascular catheters (Vincent, 2003), in particular central venous catheters (CVC) which are used in estimated 7% of the hospital patients for treatment and monitoring purposes (DoH, 2007); Estimated 22.3% (non-teaching hospitals) to 38.3% (teaching hospitals) of the all BSI in England are CVC related (Coello *et al.*, 2003). However, many other types of intravascular catheters are also widely used in the management of patients, including peripheral vascular catheters, arterial catheters, pulmonary artery catheters and intravascular catheters for haemofiltration (Mermel *et al.*, 2001). Overall, the insertion and placement of medical devices accounts for approximately half of the HA-BSI (Coello *et al.*, 2003). The main causative microorganisms of HA-BSI is *Escherichia coli*, however, the greatest increase (37% from 2003 to 2007) in HA-BSI is associated with the coagulase-negative *Staphylococcus spp.* (CNS) (HPA, 2008). Other

microorganisms associated with HA-BSI include *S. aureus* (of which 40% are meticillin-resistant), *Candida spp.*, glycopeptides-resistant enterococci (GRE) and *Streptococcus pneumonia* (Figure 1.8; HPA 2008).

Figure 1.8. The distribution of causative microorganisms of blood-stream infections in England in 2003- 2007 by voluntary surveillance (HPA, 2008).

Surgical site infections (SSI) account for 14% of the HCAI with infection rates ranging from 0.5% (knee surgery) to 10.3% (bowel surgery) in 2003-2007 (HPA, 2008). The majority of SSI are superficial affecting the skin and subcutaneous tissue (61.2% to 77.6% of all SSI). Of all the SSI reported between 2003 and 2007 (5,012 voluntary reports of which bowel and coronary artery bypass surgery were the main surgical groups), *S. aureus* counted for 38% of SSI cases, of which 64% were MRSA, and Enterobacteriaceae counted for 21% of the SSI cases (Figure 1.9).

Figure 1.9. The distribution of causative microorganisms of surgical site infections in England in 2003-2007 (all surgical categories; total reported cases 5,178; HPA, 2008).

1.4.1. Microbial biofilms

Microbial biofilms contribute to a large number of HCAI, especially medical implant and CRI, and are associated with persistent infections and resistance to antimicrobial treatment. The microbial biofilm has been defined as a community of microorganisms which are irreversibly attached to surfaces, interfaces or to each other (aggregates), and are embedded in extracellular polymer matrix (Donland and Costerton, 2002). Microorganisms in a biofilm mode of growth have altered growth rate, phenotype and expression of genes compared to growth in a planktonic

mode of growth. Cell-to-cell communication (quorum sensing) with diffusible small molecules (such as pheromones in Gram-positive bacteria and N-acylhomoserine lactone in Gram-negative bacteria) has also been found to regulate gene expression in the community of microorganisms (Morton *et al.*, 1998). For instance, 45 genes of *Ps. aeruginosa* in a biofilm mode of growth have altered expression compared to in a planktonic mode of growth (Donlan and Costerton, 2002). Environmental stress, such as exposure to sub-lethal concentrations of antimicrobial agents, may alter gene expression and enhance biofilm mode of growth (Rachid *et al.*, 2000), such as sub-MIC levels of CHG and benzalkonium chloride (Houari and Di Martino, 2007), tetracycline and quinupristine-dalfopristin (Rachid *et al.*, 2000) and alcohol (Knobloch *et al.*, 2002). Therefore proper use and at adequate level of antimicrobial agents is important in reducing microbial biofilm mode of growth.

1.4.1.1. Formation of microbial biofilms

The microbial biofilms form preferably in a high shear force environments and demonstrate higher resistance to antimicrobial agents than biofilms formed in low shear force environments (Donlan and Costerton, 2002). The most common pathogens associated with formation of biofilm on the surface of intravascular catheters and other medical implants include CNS and *C. albicans.* Attachment of microorganisms onto the surface is initiated by formation of a conditioning film, which contains organic molecules and electrolytes such as fibrin, fibrinogen, collagen, laminin, lipids and polysaccharides (Donlan, 2002, Morton *et al.*, 1998). The microorganisms attach to the surfaces firstly by unspecific interactions such as hydrophobicity and electrochemical interaction (surface charge) as well as cell surface proteins (Morton *et al.*, 1998, Ziebuhr *et al.*, 2006). Following the initial attachment the microorganisms begin to produce an exopolysaccharide matrix, which enhances binding of the microorganisms and also other microorganisms to the

biofilm and protects the cells from the environmental stress factors; furthermore, attached microorganisms begin expression of adhesion molecules or other cell surface molecules such as polysaccharide intercellular adhesion (PIA) molecule and teichoic acid in *S. epidermidis* (Sadovskaya *et al.*, 2005, Ziebuhr *et al.*, 2006) that enhance the binding and formation of the biofilm. Microorganisms may also bind to tissues through specific receptors, flagella, fimbriae or other proteins or polysaccharides on the cell surface (Donlan, 2002, Morton *et al.*, 1998). Microorganisms can also exist on the skin as microbial aggregates (microcolonies), which are similar to microbial biofilm (Akiyama *et al.*, 2002).

1.4.1.2. Structure of microbial biofilm

The exopolymeric (exopolysaccharide) matrix, also called glycocalyx, comprise a large proportion of the microbial biofilm, with only estimated 15% of the volume of the biofilm being microbial cells (Donlan and Costerton, 2002). The composition of the extracellular polymer matrix varies amongst the microorganisms, however it is generally regarded as well hydrated, containing polysaccharides, inorganic and abiotic substances which shows heterogeneity both in the structure of the biofilm and also changes within the biofilm over time. Furthermore, the biofilm microenvironment demonstrates heterogeneity also in levels of nutrients, oxygen, pH and metabolic waste products (Morton *et al.*, 1998). The cells in the bacterial biofilm are also shown to produce channels in the polymer matrix between the cell clusters (Donlan and Costerton, 2002). Furthermore, clusters of microorganisms in the biofilm may break off or detach after their tensile force is exceeded, and pose a risk to health due to possible obstruction in blood supply by the infective emboli, potentially causing stroke and other tissue damage (Donlan and Costerton, 2002).

1.4.1.3. Resistance of microbial biofilms to antimicrobial agents

The biofilm mode of growth protects the community of microorganisms from the environmental factors such as host defences (macrophages and antibodies) and antimicrobial agents, therefore enabling infections to persist despite antimicrobial treatment (Donlan and Costerton, 2002, Gristina *et al.*, 1989, Trafny, 1998, Stewart and Costerton, 2001). Several studies have demonstrated reduced antimicrobial efficacy against microbial biofilms, with higher concentrations required to kill microbial cells in a biofilm mode of growth (Amorena *et al.*, 1999, Saginur *et al.*, 2006). The underlying causes for the biofilm resistance are thought to be poor antimicrobial penetration through biofilm exopolymeric matrix, reduced nutrient availability, slow growth of the microorganisms or other phenotypic changes in the biofilm mode of growth (Stewart, 2002, Donlan and Costerton, 2002). Cell-to-cell signalling (quorum-sensing) in a large cell population has been found to alter expression of genes which control biofilm mode of growth and resistance to environmental stress, for instance production of degrading enzymes by microbial colonies in the presence of antimicrobial agents (such as hydrogen peroxide degraded by catalases). The exopolymeric matrix may hinder the antimicrobial agent diffusion through the biofilm and also interact with the molecules therefore reducing their antimicrobial activity and contact with the microbial cells (Donlan and Costerton, 2002). Moreover, close contact between the microorganisms in a biofilm may contribute to the acquisition of resistance genes by transfer of resistance genes (plasmids) through conjugation (Donlan and Costerton, 2002).

1.5. Aims of this thesis

The aim of this thesis:

- To undertake a qualitative and quantitative assessment of the microorganisms present in excised full thickness donor human skin and the residual CHG in the donor human skin prior to *in vitro* skin permeation studies of CHG.
- To investigate the antimicrobial efficacy of CHG against a panel of Gram-positive and Gram-negative bacteria and fungi commonly associated with catheter related infections (*S. epidermidis, S. aureus,* MRSA, *P. acnes, Acinetobacter spp., Ps aeruginosa* and *C. albicans*) in a planktonic mode of growth and against *S. epidermidis* in a biofilm mode of growth *in vitro*, and assessing the skin penetration of CHG in an *in vitro* human skin model.
- To investigate the antimicrobial efficacy of six essential oils (TTO, thymol, EO, juniper oil, lavender and citronella) alone and in combination with CHG, against a panel of microorganisms associated with catheter-related infections in a planktonic mode of growth and against *S. epidermidis* in a biofilm mode of growth *in vitro*.
- To assess the skin penetration of CHG in combination with IPA and in the presence of various concentrations of EO in an *in vitro* human skin model.
- To evaluate the antimicrobial efficacy of novel benzylidenecarboxamidrazones and thiosemicarbazone compounds alone and in combination with TTO, EO and thymol against a panel of microorganisms associated with catheter-related infections in a planktonic mode of growth

2. CHAPTER: QUALITATIVE AND QUANTITATIVE EVALUATION OF MICROORGANISMS PRESENT IN EXCISED HUMAN SKIN

2.1. Introduction

Invasive procedures such as surgery or insertion of intravascular catheters are a major cause of healthcare associated infections (HCAI) including blood stream infections (BSI) and surgical site infections (SSI) which account for 0.7 to 8.1 infections per 1000 post-operative days in NHS hospitals (HPA, 2007, NAO, 2000). These infections are often caused by normal skin microorganisms, such as *S. epidermidis* and other Gram-positive cocci, present in the patient's skin (Elliott *et al.*, 1997, Safdar and Maki, 2004, Traore *et al.*, 2000). Resident skin microorganisms are not generally harmful to health, and can play a beneficial role in protecting against the pathogenic microorganisms (Cogen *et al.*, 2008). Transient skin microbiota, such as *Candida spp.*, *Enterococcus spp.,* and *Klebsiella spp.*, are not normal skin commensals but are often associated with hospital acquired bacteraemia and wound infections (HPA, 2007). When the natural skin barrier is breached during invasive procedures such as surgery, insertion of intravascular catheters, taking blood or when a patient's immune system is compromised due to illness or chemotherapy, these opportunistic pathogens can enter the body and cause infection (Langgartner *et al.*, 2004, Safdar and Maki, 2004, Worthington and Elliott, 2005). Therefore effective skin antisepsis prior to invasive procedures is of utmost importance in preventing HCAI.

However, microorganisms such as CNS and *Propionibacterium spp.* reside not only on the surface of the skin but also in the deeper layers of skin and hair follicles (Brown *et al.*, 1989,

Hendley and Ashe, 1991, Hendley and Ashe, 2003, Leeming *et al.*, 1984, Malcolm, 1980). Many antimicrobial agents applied topically poorly permeate below the surface layers of skin into deeper layers, therefore these microorganisms are often not eliminated and survive the antisepsis procedure (Hendley and Ashe, 2003, Hendley and Ashe, 1991). Whilst these skin microorganisms cannot be totally eradicated through antisepsis the bioload should be significantly reduced to as low levels as possible to reduce the risk of infection during invasive procedures (Elsner, 2006).

To evaluate the skin permeation of antiseptic agents, *in vitro* skin models are often used in the place of *in vivo* studies. For investigating skin penetration profiles of antiseptic agents the full thickness human skin is preferable (Fasano and McDougal, 2008), as many differences between animal models and human skin absorption have been shown to be permeant-specific (Bronaugh *et al.*, 1986, Harrison *et al.*, 1984). However, to be able to use donor human skin the residual antiseptic agents in the skin have to be evaluated prior to the study.

2.2. Aims of this study

The aim of this part of the study was to undertake a qualitative and quantitative assessment of the microorganisms present in excised full thickness donor human skin which was to be used in subsequent investigations described in this thesis. In addition, the susceptibility of the microorganisms to CHG and a panel of common antibiotics was established. Finally, the concentration of residual CHG in excised human skin obtained from patients undergoing surgery was evaluated.

2.3. Materials

2.3.1. Chemicals and microbiological media

Hydrogen peroxide (6% v/v) was purchased from Thornton & Ross Ltd. (Huddersfield, UK). Fresh frozen human plasma (FFP) was obtained from University Hospital Birmingham NHS Foundation Trust (UHB NHSFT). Methanol, glacial acetic acid and acetonitrile (all HPLC grade) were purchased from Fisher Scientific (Leicestershire, UK). Sodium heptane sulphonate, diethylamine (both HPLC grade), phosphate buffered saline (PBS) and aqueous 20 % (w/v) CHG, were purchased from Sigma-Aldrich (Dorset, UK) and tetramethylparaphenylenediamine was purchased from Oxoid (Basingstoke, UK). Giemsa stain was purchased from BDH (Poole, UK).

Congo red agar was prepared by mixing 0.4 g of heat sterilised Congo red (Hopkins and Williams Ltd, Essex, UK) in 10 mL of distilled water with molten agar containing 490 mL of brain heart infusion (BHI) (Oxoid, Basingstoke, UK), 5 g of agar No.1 (Oxoid) and 25 g sucrose (Fisher Scientific, Leics, UK), sterilised according to the manufacturers' recommendations and cooled down to 45°C. Mueller-Hinton agar (MHA) and Mueller-Hinton broth (MHB) (both from Oxoid) were also prepared and sterilised in line with the manufacturers' recommendations. Five percent blood agar (BA) was prepared by adding 5% (v/v) horse blood (Oxoid) in molten Columbia agar base (Oxoid) sterilised according to manufacturer's instructions and cooled down to 45°C.

2.3.2. Equipment

High performance liquid chromatography (HPLC) was performed using an Agilent 1200 series high performance liquid instrument (Agilent Technologies, UK) with UV detection and with a CPS-2 Hypersil reverse phase chromatography column (150 x 4.6mm, 5μm particle size) supplied by Thermo Electron Corporation (UK). Frozen skin sections were cut using a cryomicrotome purchased from Bright Instruments (Cambs, UK). The skin sections were emulsified using a stomacher (Lab-Blender 400) purchased from Seward medical (London, UK) and the bacteria were identified using the Vitek compact 2 semi-automated bacterial identification system (BioMerieux, Basingstoke, UK). Franz diffusion cells used for skin permeation studies were kindly provided by Dr B. Conway, Aston University (Birmingham, UK). The visualisation of the skin sections were performed with Zeiss microscope (Zeiss ltd, Hertfordshire, UK) with AxioCam HRc (Zeiss ltd) camera.

2.3.3. Microbial cultures

Staphylococcus epidermidis RP62A, *S. aureus* NCTC 9865, *S. hominis* ATCC 35982, and *Pseudomonas aeruginosa* ATCC 15442 were used as control strains in the bacterial identification tests (catalase, coagulase, oxidase and Congo red agar assays) and stored on MicroBank beads (Pro-Lab Diagnostics, Cheshire, UK) at -70°C until required.

2.3.4. Skin samples

Full thickness human skin samples were obtained from patients undergoing breast reduction surgery (The Stephen Kirby Skin Bank, Queen Mary's Hospital, London, UK) and full ethical committee approval was obtained prior to this study (REC 2002/ 169). The full thickness human skin was frozen on the day of excision and stored at -70°C until required.

2.4. Methods

2.4.1. Quantitative evaluation of the microorganisms present in excised human skin

Ten skin samples were allowed to thaw at room temperature and two 2 cm x 2 cm sections from each skin sample were removed using a sterile scalpel. Each sample was placed in a sterile stomacher bag with 1:10 (w/v) of sterile PBS and homogenized for 2 minutes. The homogenized suspensions were then serially diluted with sterile PBS. One millilitre of each undiluted suspension and each of the 10^{-1} to 10^{-6} dilutions were placed into sterile Petri dishes in quadruplicate and mixed with molten blood agar (BA), containing 5% (v/v) defibrinated horse blood, cooled down to 45°C. Once the agar had set, the plates were incubated at 37°C in air for 48 h and in anaerobic conditions (10% carbon dioxide, 10% hydrogen and 80% nitrogen) for 72 h, both in duplicate. Following incubation the microbial colonies were counted and the results expressed as colony forming units (cfu) per 1 cm^2 of skin.

2.4.2. Qualitative evaluation of the microorganisms present in excised human skin

Samples of the excised human skin, homogenized as described above, were cultured by spreading 200 µL of the undiluted homogenate over the surface of a BA plate (in duplicate) and the plates were incubated as described above. Microorganisms recovered on BA were then identified as follows: a single colony of each microorganism with unique colonial morphological characteristics was subcultured onto a fresh BA plate in order to obtain a pure culture.

Preliminary identification of each microorganism was established by standard laboratory techniques (described below). Identification of each microorganism was confirmed by the semi-automated microbial identification system, Vitek 2.

2.4.2.1. Catalase test

Gram-positive cocci from skin isolates, as identified by Gram-staining and inspection under the light microscope (x 100 objective), were assessed for catalase activity; one colony from pure bacterial culture was mixed with one drop of hydrogen peroxide and observed for a typical foaming reaction indicating a positive result. *S. epidermidis* RP62A was used as a positive control.

2.4.2.2. Tube coagulation test

Catalase-positive Gram-positive cocci were further tested for coagulase production by the tube coagulase test. Five millilitres of BHI supplemented with 10% (v/v) FFP was inoculated with five colonies of the test microorganism. The suspensions were incubated at 37°C for 4 h and cultures were examined for fibrin-clot formation. *Staphylococcus aureus* and *S. epidermidis* were used as a positive and negative control respectively.

2.4.2.3. Congo red agar assay

Coagulase-negative *Staphylococcus spp.* were further assessed for slime production, a key characteristic associated with biofilm formation. Ten colonies from a pure bacterial culture were inoculated onto Congo red agar and incubated in air at 37°C for 24 h. *Staphylococcus epidermidis*

RP62A and *S. hominis* were inoculated on separate plates as a positive and negative control respectively. A positive result was indicated by the development of characteristic black, dry, crystalline colonies, surrounded by a small diffuse area of black discolouration in the agar.

2.4.2.4. Oxidase test

The oxidase test was used to differentiate oxidase negative enteric bacteria from oxidase positive Pseudomonads. The assay was performed by smearing three colonies of the pure test microorganism onto sterile filter paper strips soaked with tetramethylparaphenylenediamine (oxidase reagent). *Pseudomonas aeruginosa* and *Escherichia coli* served as positive and negative controls respectively. A positive oxidase reaction was indicated by the development of deep blue/ violet colour on the filter paper strip.

2.4.3. Antimicrobial susceptibility of the microorganisms recovered from excised human skin

2.4.3.1. Preparation of the microbial inocula

Microorganisms recovered from excised human skin were inoculated onto MHA and incubated at 37°C for 24 h. Microbial inoculum for CHG susceptibility assay was prepared by inoculating 10 identical colonies from each fresh microbial culture into sterile PBS. The microbial concentration was adjusted to 1 x 10^8 cfu/ mL by dilution with sterile PBS and measuring the optical density at 570 nm (standard curves cfu versus absorbance were constructed prior to the study, see Appendix

Figures 9.1.-9.4). The suspensions were further diluted with MHB to obtain inocula containing 1 x 10^6 cfu/ mL.

2.4.3.2. Chlorhexidine susceptibility assay

Microbial susceptibility to CHG was assessed by a broth microdilution assay performed in line with NCCLS guidelines (NCCLS, 2000). In brief, serial double dilutions of aqueous CHG with MHB were performed in round-bottom, 96-well sterile microtitre plates. The last column on the microtitre plate served as control and contained MHB with inoculum alone. Each well containing 100 µL of diluted CHG solution was inoculated with 100 µL of the bacterial suspension containing 1 x 10^6 cfu/ mL (as described above). The final concentrations of CHG in the wells ranged from 0.03 µg/ mL to 32 µg/ mL. The microtitre plates were covered and incubated in air at 37°C for 24 h. The lowest concentration of CHG without visible microbial growth was defined as the minimum inhibitory concentration (MIC). Minimum bactericidal concentrations (MBC) were determined by removing the total volume (200 µL) from each of the clear wells onto duplicate Petri dishes, mixed with 20 mL of molten MHA cooled down to 45°C (therefore diluting the antimicrobial activity of CHG by 200-fold) and allowed to set before incubating in air at 37°C for 24 h. The assay was performed in triplicate.

2.4.3.3. Antibiotic sensitivity assay

Antimicrobial susceptibility testing on the selected bacterial isolates was performed by the clinical microbiology laboratory, UHB NHS FT (Birmingham, UK), using Vitek 2. Antimicrobial

susceptibility was determined towards amoxicillin, ampicillin, chloramphenicol, erythromycin, fusidic acid, gentamicin, penicillin, tetracycline, trimethroprim, and vancomycin.

2.4.4. Quantitative evaluation of CHG in excised human skin

2.4.4.1. Validation of CHG quantification method by HPLC

The high performance liquid chromatography method for quantifying CHG was validated by repeating a series of CHG concentrations five times. Chlorhexidine dilutions (128 μg/ mL to 0.0016 μg/ mL) were performed in PBS, filtered through 0.45 μm nylon filter (Kinesis, UK) and the samples were analysed with HPLC as described by Amini (Amini, 2001); The samples were run at room temperature through a reverse phase chromatography column (CPS-2 Hypersil) at a flow rate of 1.2 mL/ min for 9 min, with ultraviolet detection at 254 nm. The isocratic mobile phase consisted of methanol: water mixture (75:25) with 0.005 M sodium heptane sulphonate and 0.1% (v/v) diethylamine adjusted to pH 4 with glacial acetic acid. The samples were analysed in duplicate and the standard curve was drawn from peak area versus concentration of CHG. The level of detection (LOD) and level of quantification (LOQ) were calculated from the standard curve according to the following equation:

LOD= 3 x standard deviation (SD) / slope

LOQ= 10x SD/ slope.

2.4.4.2. Investigation of CHG extraction from excised human skin

The elution of CHG from skin was evaluated by injecting 128 μg of CHG into 30 skin sections and incubating the skin at room temperature for 30 min. Chlorhexidine was eluted from skin by placing 1 mL of PBS, PBS with 10 % (v/v) acetonitrile and mobile phase solutions (75% methanol, 25% distilled water, 0.1% sodium heptane sulphonate, 0.1% diethylamine, pH 4) to ten CHG spiked skin samples each and incubated for 1 h at room temperature. The samples were mixed by vortexing, centrifuged at 6000 g for 10 min and were filtered prior to analysis of CHG content by HPLC.

2.4.4.3. Evaluation of CHG concentration in excised human skin

Punch biopsies (7 mm in diameter) were cut from full thickness human skin samples in triplicate, placed onto a cork disc in embedding compound (Bright Instruments, Cambs, UK), sprayed with cryospray (Bright Instruments) and frozen at -20˚C prior to sectioning. The frozen samples were sectioned horizontally with a cryotome (Bright Instruments) and each section placed into a pre-weighed Eppendorf tube and the total weight measured to determine the weight of each skin sample. Chlorhexidine was eluted from the skin samples and filtered before analysis by HPLC. The results were calculated as μg of CHG per mg of skin.

2.4.4.4. Microscopic evaluation of skin sectioning

The variation in SC thickness amongst the donor skins and the uniformity of the parallel sectioning of skin samples by microtome were evaluated by light microscopy. The skin samples were cut with a cryomicrotome as described above, into 30 μm sections perpendicular to the SC

and the measurement were performed triplicate on each donor skins. Skins were also sectioned parallel to the skin surface and the skin sections were placed onto a microscope slide, fixed with ethanol and stained with 10% Giemsa stain for 10 mins; the slides were flushed with water and air dried before visualising under light microscope x 40 objective.

2.5. Results

2.5.1. Quantitative evaluation of the microorganisms present in excised human skin

All ten donor human skin samples were found to contain microorganisms, with a significant variation amongst the donors (Table 2.1). The mean aerobic count was 643 cfu/ cm^2 (range 2 – 2,205) and mean anaerobic count was 2,611 cfu/ cm^2 (range 4 – 12,021). There was no correlation between the weight of skin sample and the microbial load (see Appendix Figures 9.7. and 9.8).

Table 2.1. The type and number of microorganisms (cfu per cm^2) recovered from excised full thickness human skins (breast reduction surgery, n=10). The frozen human skins were defrosted, homogenized in phosphate buffer saline and cultured on blood agar at 37°C in air and in anaerobic conditions for 48 h and 72 h respectively (performed in duplicate).

Donor	Mean cfu/ cm^2		Microorganisms isolated
	Aerobic culture	Anaerobic culture	
1	142	173	*S.epidermidis*
2	2,205	8,468	*S.epidermidis* *S.lugdunensis* *Micrococcus spp.* *Kocuria kristinae* *Aci.heamolyticus* Unidentified G + rod
3	374	584	*S.epidermidis* *Dermacoccus spp.*
4	10	8	*S.epidermidis* *S.haemolyticus* *S.lentus* *Klebsiella pneumonia* *Aci.heamolyticus* *Rzb.radiobacter*
5	1,241	12,021	*S.epidermidis* *Alloicoccus otitis* *Aer.viridans* *Granulicatella spp.* *Kocuria kristinae* *Bacillus spp.* Unidentified G + rod
6	2	7	*S.epidermidis* *S.lentus*
7	345	2 474	Unidentified G + rod *S.epidermidis* *S.hominis*
8	2,078	2,348	Unidentified G + rod *S.epidermidis*
9	26	20	*S.epidermidis*
10	5	4	Unidentified G + rod *S.epidermidis* *S.hominis* *S.auricularis* *S.aureus*

2.5.2. Qualitative evaluation of the microorganisms present in excised human skin

Gram-positive cocci, Gram-positive rods and Gram-negative rods were isolated from excised full thickness human skins (Table 2.1). The most common microorganisms recovered were CNS, with *S. epidermidis* recovered from all ten donor skin samples. A further six different *Staphylococcus spp.* were also identified from eight samples. Five out of 26 (19%) *S. epidermidis* isolates were positive for slime production as indicated on Congo red agar (Figure 2.1). Gram-negative rods including *Acinetobacter spp., Klebsiella spp.* and *Rhizobium spp.* were also recovered from two out of ten (20%) of the donor skin samples. Ten out of 70 (14%) skin isolates (Gram-positive rods n= 8, Gram-positive cocci n= 2) could not be identified by standard laboratory techniques or the Vitek 2 identification system.

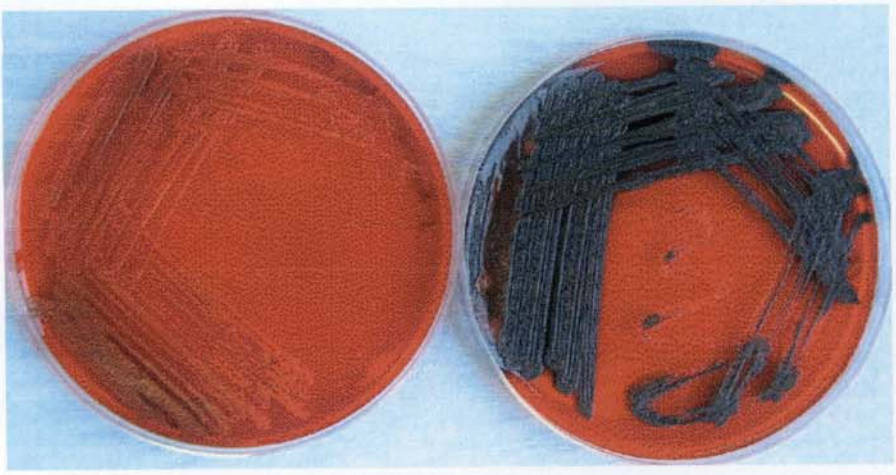

Figure 2.1. The congo red agar assay for assessment of slime production by *Staphylococcus spp.* Non-slime producing culture on the left (*S. hominis*) and positive reaction for slime production showing dry, crystalline black colonies presented on the right (*S. epidermidis* RP62A). The culture plates were incubated at 37°C in air for 24 h.

2.5.3. Antimicrobial susceptibility of the microorganisms recovered from excised human skin

2.5.3.1. Chlorhexidine susceptibility assay

Microorganisms isolated from excised human skin were sensitive to CHG, with an overall median MIC of 0.5 μg/ mL (range 0.06 μg/ mL to 2 μg/ mL) and median MBC of 1 μg/ mL (range 0.06 μg/ mL to 8 μg/ mL) (n= 60) (see Appendix Table 9.1).

2.5.3.2. Antibiotic sensitivity assay

Sixteen isolates of *Staphylococcus spp.* were tested for antibiotic sensitivity; ten isolates of *S. epidermidis* (one from each donor skin), five CNS (other than *S. epidermidis*) and one isolate of *S. aureus*. Nine out of ten (90%) *S. epidermidis* isolates were resistant to ≥ 1 antibiotics. Three out of ten (30%) were resistant to ≥ 6 antibiotics. Furthermore, six out of ten (60%) *S. epidermidis* and two out of five (40%) CNS isolates were positive for beta-lactamase activity. Ampicillin and penicillin were the antibiotics against which the highest number of microorganisms demonstrated resistance (n= 7 and n= 8 respectively). Other antibiotics against which resistance was demonstrated were erythromycin (n= 5), trimethroprim (n= 5), tetracycline (n= 4), chloramphenicol (n= 3), fusidic acid (n= 2), amoxicillin (n= 1), gentamicin (n= 1) and vancomycin (n= 1).

2.5.4. Quantitative evaluation of CHG in excised human skin

2.5.4.1. Validation of CHG quantification method by HPLC

The standard curve for CHG quantification produced a linear line (R^2= 0.999) over a CHG range of 0.00325 µg/ mL to 32 µg/ mL (see Appendix Figure 9.9). The level of detection (LOD) and level of quantification (LOQ) were calculated 0.0157 µg/ mL and 0.0523 µg/ mL respectively. The average retention time was 3.6 min.

2.5.4.2. Investigation of CHG extraction from excised human skin

The recovery of CHG from skin using PBS, PBS with 10 % (v/v) acetonitrile and mobile phase as extractants were 9.55 %, 6.65 % and 50.3% respectively. To improve the extraction of CHG from skin with the mobile phase solution the incubation temperature was increased to 60°C. This extraction method improved the level of recovery to 94.4 ± 1.82% and was adopted in all subsequent experiments.

2.5.4.3. Evaluation of CHG concentration in excised human skin

Skins samples from four donors were analysed for residual CHG. There were no detectable levels of CHG in skin samples from two donors. Skin from the remaining two donors had low levels of CHG, with the highest concentration on the top 100 µm of skin between 0.005 µg/ mg tissue and 0.029 µg/ mg tissue. There were no detectable levels of CHG below 1350 µm depth.

2.5.4.4. Microscopic evaluation of skin sectioning

Representative microscopy images of cross-sections of each donor human skin (n= 4) are presented in Figures 2.2. to 2.5. The images show approximately 20 μm thick intact SC (the sections were evaluated in triplicate, data not shown). Furthermore, cross-sections were taken from frozen, non-treated skin sample and from skin sample following flattening of the skin by a microtome blade prior to sectioning (Figure 2.6); the latter image demonstrates an even skin surface following flattening compared to un-treated skin sample which demonstrated furrowing, and the technique was utilized in the subsequent skin permeation studies (Chapter 5). The parallel sectioning of the donor skins and Giemsa staining demonstrated changes in the skin structures at various skin depths; examples of section 1 (from surface to 20 μm depth), section 4 (skin depth 60-80 μm) and section 60 (skin depth 1470-1500 μm depth) are shown in Figures 2.7. to 2.9.

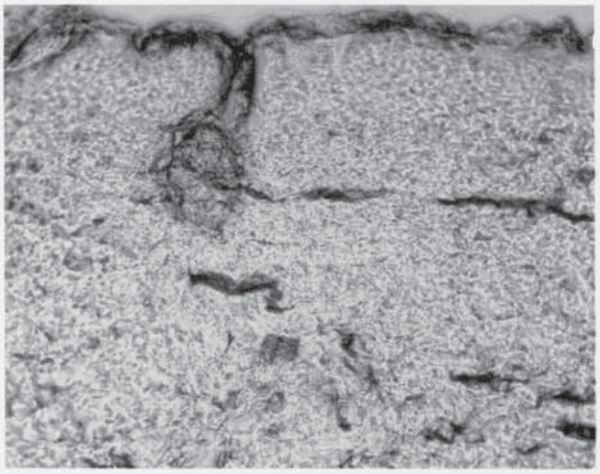

Figure 2.2. Cross-section of donor human skin one. Frozen skin (- 20°C) was cut into a 30-μm section perpendicular to stratum corneum with a cryomicrotome and inspected under x 10 objective of light microscope without staining (the scale bar 20 μm; Zeiss microscope with AxioCam HRc camera, Zeiss ltd, UK).

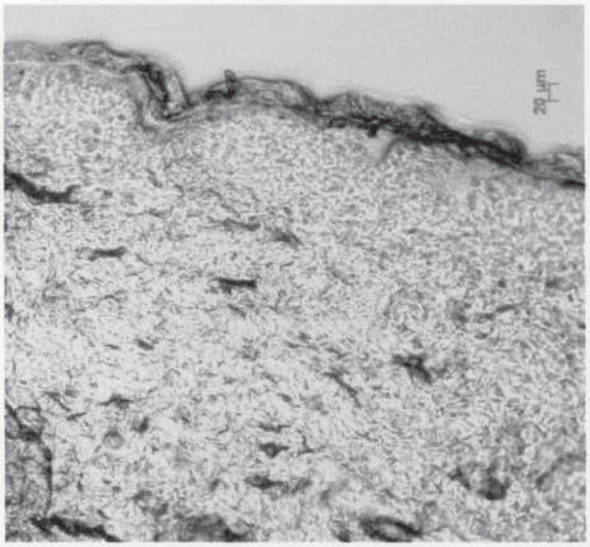

Figure 2.3. Cross-section of donor human skin two. Frozen skin (- 20°C) was cut into a 30-µm section perpendicular to stratum corneum with a cryomicrotome and inspected under x 10 objective of light microscope without staining (the scale bar 20 µm; Zeiss microscope with AxioCam HRc camera, Zeiss ltd, UK).

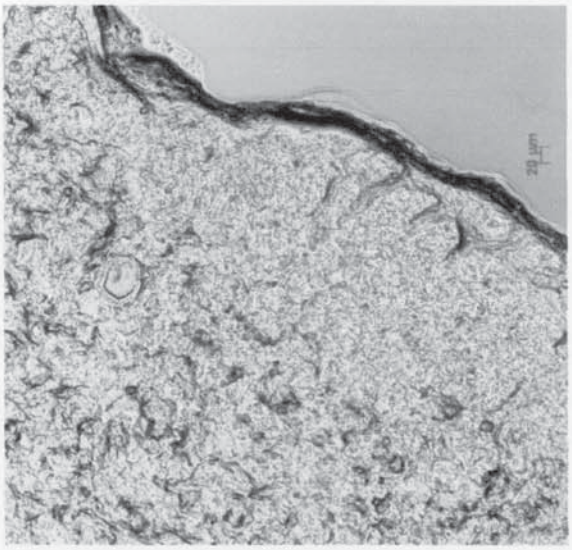

Figure 2.4. Cross-section of donor human skin three. Frozen skin (- 20°C) was cut into a 30-µm section perpendicular to stratum corneum with a cryomicrotome and inspected under x 10 objective of light microscope without staining (the scale bar 20 µm; Zeiss microscope with AxioCam HRc camera, Zeiss ltd, UK).

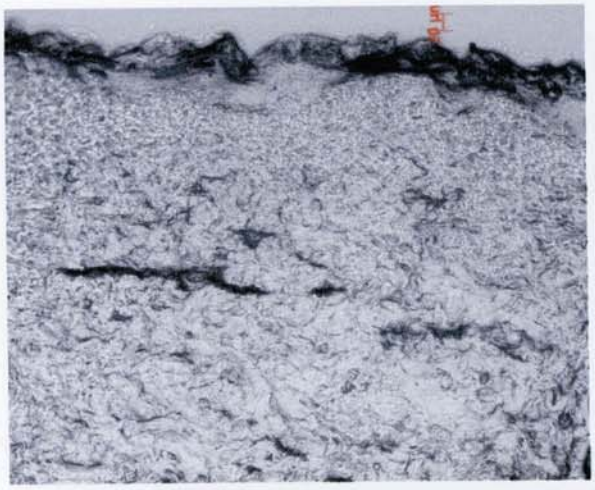

Figure 2.5. Cross-section of donor human skin four. Frozen skin (- 20°C) was cut into a 30-μm section perpendicular to stratum corneum with a cryomicrotome and inspected under x 10 objective of light microscope without staining (the scale bar 20 μm; Zeiss microscope with AxioCam HRc camera, Zeiss ltd, UK).

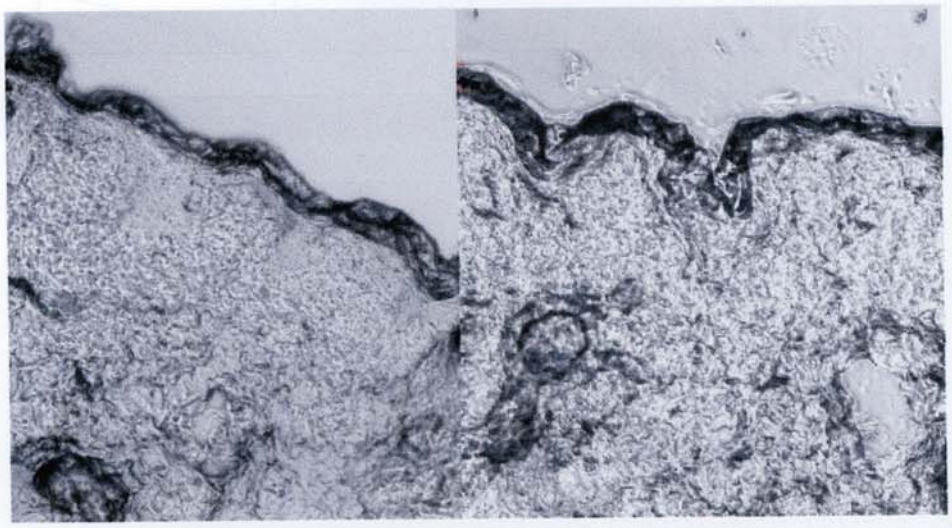

Figure 2.6. Cross-sections of human donor skins following flattening of the skin prior to sectioning (left) and without flattening (right). Frozen skins (- 20°C) were cut into 30-μm sections perpendicular to stratum corneum with a cryomicrotome and inspected under x 10 objective of light microscope without staining (Zeiss microscope with AxioCam HRc camera, Zeiss ltd, UK).

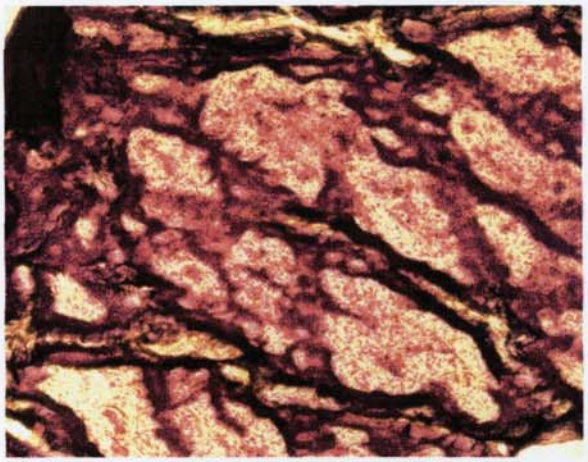

Figure 2.7. Light microscopy image of the first 20-μm section of excised human donor skin cut parallel to stratum corneum. Skin was frozen to -20°C and cut with a cryomicrotome. The skin section was mounted onto a microscope slide, fixed with ethanol and stained with 10% Giemsa stain before inspecting under x 40 objective of light microscope (Zeiss microscope with AxioCam HRc camera, Zeiss ltd, UK).

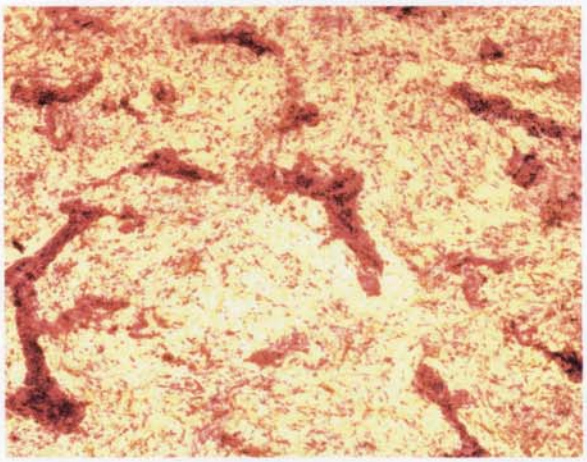

Figure 2.8. Light microscopy image of the fourth 20-μm section of excised human donor skin cut parallel to stratum corneum (approximate skin depth 60- 80 μm). Skin was frozen to -20°C and cut with a cryomicrotome. The skin section was mounted onto a microscope slide, fixed with ethanol and stained with 10% Giemsa stain before inspecting under x 40 objective of light microscope (Zeiss microscope with AxioCam HRc camera, Zeiss ltd, UK).

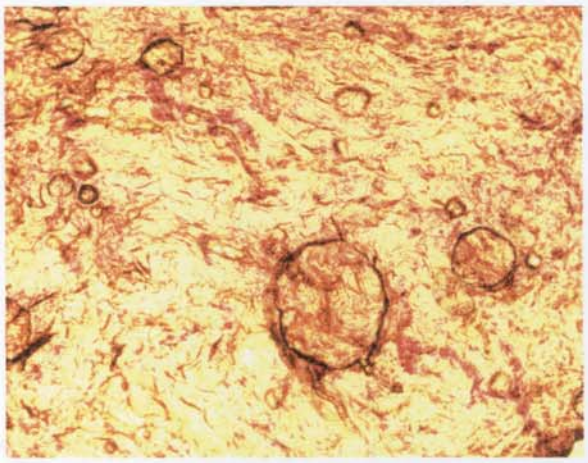

Figure 2.9. Light microscopy image of the 60th section of excised human donor skin cut parallel to stratum corneum (approximate skin depth 1470- 1500 µm; the first 1- 30 skin sections were cut to 20-µm thickness and sections 30-60 with 30-µm thickness). Skin was frozen to -20°C and cut with a cryomicrotome. The skin section was mounted onto a microscope slide, fixed with ethanol and stained with 10% Giemsa stain before inspecting under x 40 objective of light microscope (Zeiss microscope with AxioCam HRc camera, Zeiss ltd, UK).

2.6. Discussion

In addition to determining the residual concentration of CHG contained within the donor skin samples, this study established the number, type and sensitivity of microorganisms contained on, and within, donor human skin samples obtained by excision following antisepsis with CHG. This preliminary study was undertaken in an attempt to determine the level of CHG and microbial load on the donor skin samples prior to use in several subsequent investigations contained within this thesis.

The results from ten donor skins showed a diverse range and quantity of microorganisms. The most common microorganisms recovered from the skin were CNS, especially *S. epidermidis* which was isolated from all ten donors. These Gram-positive cocci are normal skin commensals, and are often not harmful to human health. Indeed, normal human skin microbiota can help to maintain healthy skin partly by preventing transient, potentially pathogenic microorganisms invading the skin. They do so by preventing their attachment to the skin surface and competing for the minimal nutrients available on skin surface (Cogen *et al.*, 2008). *Staphylococcus spp.* and other Gram-positive cocci are normal skin microorganisms, which are frequently resistant to dry conditions and higher salt concentrations that prevail on the skin surface (Cove and Eady, 1998). *Staphylococcus epidermidis* and other CNS have been regarded as skin contaminants when found in clinical specimens; however their role in infections, as a potential pathogen, is emerging. These opportunistic pathogens can carry genes that confer resistance to antimicrobial agents and are often able to colonise foreign surfaces where they can produce bacterial biofilms (Ziebuhr *et al.*, 2006). Other Gram-positive microorganisms found in this study were *Micrococcus spp.* and

its related species *Kocuria spp*. These are often non-harmful skin commensals, however there are few reports of *Kocuria kristinae* causing infections, such as cholecystitis following laparoscopic procedure, and catheter related bacteraemia following long term CVC (Basaglia *et al*., 2002, Ma *et al*., 2005). Concerns about the Vitek 2- system diagnosing CNS wrongly as *Kocuria spp*. has been voiced (Ben-Ami *et al*., 2005), however Boudewijns *et al*. (2005) have shown that *Kocuria spp*. identification can be correct, yet this may be due to skin contamination in the clinical sample rather than a true pathogen (Boudewijns *et al*., 2005).

In this study three Gram-negative bacteria *Acinetobacter spp*., *Klebsiella spp*. and *Rhizobium radiobacter* were isolated from two different donors. *Acinetobacter spp*. is often found in the environment and colonising skin, however in severely ill patients it can cause infections such as BSI and SSI (HPA, 2007). Moreover, bacteraemia caused by *Acinetobacter spp*. is increasing and *Acinetobacter spp*., especially *A. baumannii*, are becoming increasingly resistant to antimicrobial treatment in the hospital environment (HPA, 2007). Also the number of *Klebsiella spp*. bacteraemia and SSI is increasing (NAO, 2000). *Rhizobium radiobacter*, previously known as *Agrobacterium spp*., has also been reported to cause infections such as bacteraemia, however it is thought to be an opportunistic pathogen with low virulence, affecting mainly immunocompromised individuals with intravascular devices (Chen *et al*., 2008). More interestingly, it can produce extracellular slime and can therefore attach to surfaces such as intravascular devices (Chen *et al*., 2008). Microbial biofilms, where microorganisms are encased in extracellular matrix, are more resistant to antimicrobial agents than planktonic microorganisms (Saginur *et al*., 2006). In this study six CNS were positive for slime production, indicating their

ability to grow as a bacterial biofilm or aggregates embedded in extracellular matrix, therefore rendering them less susceptible to antimicrobial treatment.

In this study many microorganisms were recovered from the skin which were not demonstrated in previous studies (Messager *et al.*, 2001, Maillard *et al.*, 1998). This may be in part due to different sampling methods, as this study examined the whole full thickness skin compared to the surface or surface layers of the skin as were examined by other investigators. Also a high degree of variability amongst donor skins, as was demonstrated in this study, may influence the differences in the results. However, many clinical studies investigating skin antiseptic procedures have demonstrated that microorganisms on the skin cannot be totally eradicated (Edmiston *et al.*, 2007, Langgartner *et al.*, 2004, Sebben, 1983) which is also shown in the guidelines for antiseptic compounds used for preoperative skin preparation (O'Grady *et al.*, 2002). This study also showed a higher number of microorganisms in anaerobic cultures compared to aerobic cultures, which suggests the presence of strictly anaerobic microorganisms within the skin. This may have influenced the results as anaerobic or facultative anaerobic bacteria reside in the deeper layers of skin and skin appendages, which are not reached by antimicrobial agents during skin antisepsis. In this study *Propionibacterium spp.*, an anaerobic Gram-positive cocci, was not recovered from the ten donor human skins. *Propionibacterium acnes* is a normal skin microorganism which has been shown to colonise the surface of skin and sebaceous glands (Till *et al.*, 2000). Moreover, it has been demonstrated to colonise wounds during spinal surgery (McLorinan *et al.*, 2005) and cardiac surgery (Kuhme *et al.*, 2007). It may be that the storage conditions and the processing of skin samples prior to incubation in anaerobic environment have influenced the lack of recovery of *P. acnes* in this study.

Sensitivity of the skin isolates to CHG, one of the most common skin antiseptics used prior to surgery and other invasive procedures, was evaluated. All of the skin isolates tested were susceptible to CHG, with an overall median MIC and MBC of 0.5 μg/ mL and 1 μg/ mL respectively, which are in line with other studies (Koljalg *et al.*, 2002). In addition, the level of residual CHG contained within the donor skin samples in this study was very low: two of the donor skins had no detectable level of CHG (LOD 0.016 μg/ mL), and two donor skins had low level of CHG (≤ 0.027 μg/ mg tissue). As one millilitre of full thickness human skin equals approximately one gram (see Appendix Figure 9.10), the CHG concentration of 0.027 μg/ mg tissue is estimated 27 μg/ mL, which is higher than the CHG bactericidal concentration required to eliminate the microorganisms isolated from skin in this study *in vitro*. However, donor skin number one had a high number of microorganisms (142 and 173 cfu/ cm^2 for aerobic and anaerobic cultures respectively), which may suggest that in *in vivo* conditions the antimicrobial efficacy of CHG was reduced. This may be due to interfering endogenous agents, such as organic compounds, and poor permeation of CHG to the deeper layers of skin. Furthermore, some microorganisms, such as *Staphylococcus spp.*, can live as aggregates on the skin surface rendering them less susceptible to antimicrobial agents (Edwards and Harding, 2004). Minimum inhibitory concentrations and MBCs are often increased in microorganisms growing in a biofilm compared to free living (planktonic) cells (Saginur *et al.*, 2006). In this study all isolates were sensitive to CHG when in a suspension as free floating planktonic cells; however their sensitivity to CHG was not tested whilst in a biofilm mode of growth.

In addition to assessing the sensitivity of isolates to CHG, this study determined the sensitivity patterns of selected CNS isolates to a panel of common antibiotics. In contrast to the sensitivity

demonstrated towards CHG, many of the isolates were resistant to antibiotics tested. Indeed, many isolates were resistant to more than five common antimicrobial agents, in particular the beta-lactam antibiotics including penicillin and related compounds. Therefore the prevention of infections associated with invasive procedures, often caused by microorganisms residing within the skin, is of utmost importance. Chlorhexidine demonstrated excellent antimicrobial efficacy against the skin isolates, and resistance or reduced susceptibility towards CHG is low (Cookson *et al.*, 1991, Thomas *et al.*, 2005). In contrary, resistance towards other widely used skin antiseptics, such as triclosan has been reported (Yazdankhah *et al.*, 2006, Schweizer, 2001), and more importantly, its cross-resistance to antibiotics (Chuanchuen *et al.*, 2001).

2.7. Conclusion

A wide range of microorganisms was found within the donor human skin, which were predominantly normal skin commensals. Anaerobic *Propionibacterium spp.*, the prevalent skin microorganism, were not recovered from excised donor skins in this study, which may be due to storage and processing conditions of the skin samples prior to incubation in anaerobic conditions. All the isolates were sensitive to CHG; however the selected skin isolates showed increased resistance to common antibiotics such as penicillins. In addition, only a negligible amount of CHG was demonstrated within the donor skin samples assessed. This is advantageous in one instance as these skin samples may be used within skin models to evaluate CHG penetration without interference from residual CHG. More importantly, the lack of CHG in the donor skin samples indicates a need for enhanced delivery of CHG into the deeper layers of skin including hair follicles where microorganisms reside often in a biofilm-mode of growth. The subsequent chapters of this thesis will therefore concentrate upon the antimicrobial activity of CHG against microorganisms in a planktonic and a biofilm modes of growth; the development of a skin model to assess the penetration of CHG within the skin, and methods to enhance the delivery of CHG within the skin.

3. CHAPTER: ANTIMICROBIAL ACTIVITY OF CHLORHEXIDINE DIGLUCONATE AND ITS PERMEATION INTO EXCISED HUMAN SKIN

3.1. Introduction

Effective skin antisepsis is essential in preventing infections associated with invasive procedures, such as intravascular catheter insertion or surgery. A range of skin antiseptic agents are available in the clinical setting, including alcoholic and aqueous solutions of povidone-iodine and chlorhexidine (CHG) compounds at various concentrations. However, 2% (w/v) CHG solution is currently the recommended antiseptic agent to be used prior to invasive procedures by the EPIC (Evidence-based Practice in Infection Control) and CDC (Centres for Disease Control and Prevention) guidelines (O'Grady *et al.*, 2002, Pratt *et al.*, 2001). Chlorhexidine is a well established skin antiseptic, which has a broad spectrum of antimicrobial activity against Gram-positive and negative bacteria, fungi and some viruses (McDonnell and Russell, 1999), however, its efficacy is reduced in the presence of organic compounds and at low pH (McDonnell and Russell, 1999). In addition, the antimicrobial activity of CHG is reduced against microbial biofilms which are often associated with foreign bodies including intravascular catheters (Donlan and Costerton, 2002, Gristina *et al.*, 1989). Microorganisms residing as microcolonies on the skin surface and within the skin tissue are also less susceptible to antimicrobial agents (Edwards and Harding, 2004).

Currently there is very little known about the kinetics of CHG permeation within human skin (Lafforgue *et al.*, 1997, Wang *et al.*, 1990). Effective permeation of CHG within the skin tissue is essential as microorganisms not only colonise the skin surface but are also found to inhabit hair

follicles and lower skin depths (Hendley and Ashe, 1991). Many antimicrobial agents exhibit restricted permeation of the skin (Hendley and Ashe, 1991) and fail to reach the deeper layers, including hair follicles (Brown *et al.*, 1989, Hendley and Ashe, 2003, Hendley and Ashe, 1991, Leeming *et al.*, 1984, Malcolm, 1980). These resident microorganisms may therefore persist within the skin following antisepsis (Elliott *et al.*, 1997, Leeming *et al.*, 1984) and cause infection post surgery (Langgartner *et al.*, 2004, Safdar and Maki, 2004, Worthington and Elliott, 2005). Effective and rapid permeation of the antiseptic agent into the deeper layers of skin is therefore essential in preventing infections associated with invasive procedures.

3.2. Aims of this study

The aim of this part of the study was firstly to evaluate the antimicrobial efficacy of CHG *in vitro*; minimum inhibitory (MIC) and minimum bactericidal (MBC) or fungicidal concentrations (MFC) of aqueous CHG were assessed against a panel of microorganisms, in both a planktonic and biofilm modes of growth, which are commonly associated with catheter-related infections (*S. epidermidis, S. aureus,* MRSA, *P. acnes, Acinetobacter spp., Ps. aeruginosa* and *C. albicans*). The second part of this study was to develop a skin model based on a Franz cell system in order to determine the permeation of 2% (w/v) CHG within excised human skin.

3.3. Materials

3.3.1. Chemicals

Horse serum was purchased from Southern Group Laboratory ltd (Northamptonshire, UK). Sodium heptane sulphonate, diethylamine (both HPLC grade), PBS, aqueous 20 % (w/v) CHG and Tween 80 were purchased from Sigma-Aldrich (Dorset, UK). Methanol, glacial acetic acid (both HPLC grade) and glucose were purchased from Fisher Scientific (Leicestershire, UK). ViaLight MDA Detection Kit was purchased from Cambrex (USA).

Mueller-Hinton agar (MHA), Mueller-Hinton broth (MHB), Sabouraud-dextrose agar (SAB agar), Sabouraud-dextrose broth (SAB), brain heart infusion (BHI) agar and tryptone soya broth (TSB) (Oxoid, UK) were prepared and sterilised in line with the manufacturers' recommendations. Congo red agar was prepared as described in 2.3.1. Alcian blue stain was prepared by adding 1 g of Alcian blue 8GX (Sigma-Aldrich, Dorset, UK) with 3 mL acetic acid (Fisher Scientific) and 97 mL distilled water.

White walled, clear bottom, tissue culture treated 96-well microtitre plates were obtained from Corning Incorporated (NY, USA). Clear, round bottom 96-well microtitre plates were purchased from Barloworld Scientific (Staffordshire, UK) and clear, flat bottom 96-well microtitre plates from Thermo Labsystems (Franklin, USA).

3.3.2. Equipment

High performance liquid chromatography (HPLC) equipment used in this study was as stated in 2.3.2. Cryotome was purchased from Bright Instruments (Cambs, UK). Fluorescence were measured with a microtitre plate reader (Lucy 1, type 16 850, Rosys Anthos Labtech Instruments).

3.3.3. Microbial cultures

Type culture microorganisms used in antimicrobial susceptibility assays were *S. epidermidis* RP62A, NCTC 11047 and NCTC 9865, MSSA NCTC 6538, MRSA EMRSA15, *S. hominis* ATCC 35982, *Ps. aeruginosa* ATCC 15442 and *P. acnes* NCTC 737.

Clinical isolates of *S. epidermidis* (TK1, TK2 and TK3) and *C. albicans* (n= 5) (both recovered from blood cultures of infected patients) were kindly donated by Dr A. Casey (UHB NHSFT) and the clinical isolates of MSSA (n= 4), MRSA (n= 4), *Ps. aeruginosa* (n= 4) and *Acinetobacter spp.* (n= 5) were kindly donated by Professor P. Lambert (Aston University, Birmingham, UK). Clinical isolates of *P. acnes* (n= 4) were kindly donated by Dr T. Worthington (Aston University). Microorganisms were stored on MicroBank beads (Pro-Lab Diagnostics, Cheshire, UK) at -70°C until required.

3.3.4. Skin samples

Full thickness human skin samples used in this study were as stated in section 2.3.4.

3.4. Methods

3.4.1. Evaluation of the antimicrobial activity of CHG against microorganisms in a planktonic mode of growth

3.4.1.1. Preparation of the microbial inocula

Staphylococcus spp., *Ps. aeruginosa* and *Acinetobacter spp.* were recovered from beads stored at -70°C and inoculated onto MHA. Cultures were incubated at 37°C in air for 24 h. *Propionibacterium acnes* was inoculated onto BHI agar and incubated at 37°C in anaerobic conditions for 72 h, and *C. albicans* was inoculated onto SAB agar and incubated at 30°C in air for 48 h. All cultures were checked for purity. Microbial suspensions for the antimicrobial assays were prepared by inoculating 10 identical colonies from fresh microbial cultures into sterile PBS. The concentration of microorganisms was adjusted to 1 x 10^8 cfu/ mL by diluting the culture with sterile PBS and measuring the optical density at 570 nm, with the exception of *C. albicans* which was measured at 420 nm (standard curves cfu versus absorbance were performed for each microorganism prior to the study, see Appendix Figures 9.1.- 9.6). The suspensions were further diluted to 1 x 10^6 cfu/ mL with MHB, with the exception of *C. albicans* which was diluted with SAB broth.

3.4.1.2. Determination of MIC, MBC and MFC of CHG against microorganisms in a planktonic mode of growth

Minimum inhibitory concentration, MBC and MFC of aqueous CHG was determined using a broth microdilution assay in line with NCCLS guidelines (NCCLS, 2000) as described in 2.4.3.2. The final CHG concentration ranged between 0.125 µg/ mL to 128 µg/ mL. Microtitre plates containing 1 x 10^5 cfu/ well of *Staphylococcus spp., Ps. aeruginosa* and *Acinetobacter spp.* were incubated in air at 37°C for 24 h, *P. acnes* in anaerobic conditions at 37°C for 72 h and *C. albicans* in air at 30°C for 48 h. The suspension assays were repeated in the presence of 10% (v/v) horse serum.

3.4.2. Evaluation of the antimicrobial activity of CHG against *S. epidermidis* in a biofilm mode of growth

3.4.2.1. Establishing S. epidermidis biofilms

Antimicrobial activity of CHG was evaluated against *S. epidermidis* in a biofilm mode of growth. The ability of five clinical isolates of *S. epidermidis* and two reference strains (RP62A and NCTC 11047) to produce slime was evaluated by culturing the bacteria on Congo red agar as described in 2.4.2.3. The ability of the isolates to produce biofilm was confirmed by the Alcian blue method (Adams, 2006). The number of microorganisms in the biofilm were evaluated by the scrape and wash method (Adams, 2006).

3.4.2.1.1. Alcian blue staining to confirm the presence of bacterial biofilm

Suspensions of *S. epidermidis* were prepared in MHB and TSB with or without 2% (w/v) glucose and 10% (v/v) horse serum as described in 3.4.1.1, to obtain a final concentration of 1 x 10^6 cfu/ mL. Two hundred microlitres of the microbial suspensions were placed into the wells of 96-well flat bottomed microtitre plate in quadruplicate and incubated in air at 37°C for up to 72 h. Four wells were left blank to serve as controls. During the incubation period the culture broth was removed from the wells by aspiration with a pipette after 24 h and replaced with fresh culture medium to remove any unbound bacteria and encourage growth of the bound microorganisms.

Following 72 h of incubation, the wells were carefully washed with PBS to remove any unbound microorganisms and the wells were stained with Alcian blue by placing 250 µL of Alcian blue stain into all wells on the microtitre plate and incubating the plates at room temperature for 10 min. The stain was removed and the wells washed once with PBS. Two hundred and fifty microlitres of ethanol was placed into all of the wells before reading the absorbance at 570 nm.

3.4.2.1.2. Determination of the number of microorganisms within the biofilm: the scrape and wash method

After establishing the optimum growth conditions for biofilm production, the number of microorganisms contained within the biofilm was determined by the scrape and wash method (Adams, 2006); in brief, the culture broth was removed from the wells and washed once with sterile PBS. The microbial biofilms were removed from the wells by placing 200 µL of PBS into the wells and scraping the wells with a sterile pipette tip 10 times in each direction vertically, horizontally, diagonally both ways, clockwise and anticlockwise. The suspensions were removed

and placed into sterile eppendorf tubes. The procedure was repeated four more times to yield a final total volume of 1 mL. Serial dilutions (10^{-3} to 10^{-6}) of the final suspension were performed in PBS after mixing the suspensions by vortexing for 30 s. One hundred microlitres of each dilution was placed onto sterile Petri dishes in duplicate and mixed with molten MHA cooled down to 45°C. The set culture plates were incubated at 37°C in air for 24 h. The colony forming units were counted and the cfu per mm^2 of biofilm culture calculated.

3.4.2.2. Preparing S. epidermidis biofilms

Suspensions of *S. epidermidis* were prepared using culture conditions producing optimum biofilm (section 3.4.2.1.1.). The microbial biofilms were prepared by placing 200 µL of the microbial suspensions containing 1 x 10^6 cfu/ mL into the wells of white walled, clear bottom, tissue culture treated 96-well microtitre plates. Four wells in the last column of each plate were left blank to serve as bioluminescence negative control. Microtitre plates containing *S. epidermidis* cultures were incubated in air at 37°C for 48 h.

3.4.2.3. Determination of MIC and MBC of CHG against S. epidermidis biofilms

Microtitre plates containing *S. epidermidis* biofilms were washed once with sterile PBS to remove any unbound bacteria. Serial double dilutions of CHG were performed with MHB to obtain CHG concentrations ranging from 128 µg/ mL to 0.25 µg/ mL. Two hundred and fifty microlitres of CHG was added to the wells in diminishing concentrations from column 1 to column 11. The wells on the last column served as controls containing the biofilm and saline alone and MHB alone without bacterial biofilm.

Following incubation in air at 37°C for 24 h the wells were washed once with sterile PBS and the microbial viability determined using an ATP bioluminescence assay (ViaLight MDA Bioassay kit); one hundred microliters of Bactolyse was added with 100 μL of saline into each well and the plates sonicated using a 50 Hz water bath sonicator (Kerry ultrasonics, UK) for 30 min to release and lyse the cells of the microbial biofilm. Fifty microlitres of ATP-monitoring reagent was added to each well and luminescence measured (Lucy 1, type 16 850 fluorescence measurer, Rosys Anthos Labtech Instruments). The minimum inhibitory concentration was defined as the minimum concentration of antimicrobial agent that inhibited further growth of the initial biofilm (control well containing biofilm treated with saline), and the concentration which produced below, or equal, to the background level of luminescence (empty well) as MBC. The assay was performed in duplicate microtitre plates.

3.4.3. Permeation of 2 % (w/v) CHG into excised human skin

Skin permeation studies were performed with vertical Franz diffusion cells (Figure 3.1.). The receptor compartment was filled with 29 mL of PBS and maintained at 37° C by a circulating water jacket and agitated by stirring with a magnetic bar. Skin samples were thawed in PBS at room temperature, dried with an absorbent towel and mounted onto Franz diffusion cells with the stratum corneum (SC) uppermost facing the donor compartment. The surface area exposed to the test compound was 3.14 cm^2 (2 cm in diameter). All entrapped air was removed between the skin and receptor fluid and the skin was allowed to equilibrate for 30 min to reach the skin surface temperature of 32°C.

Twenty percent (w/v) aqueous CHG was diluted with distilled water and 0.1% (v/v) Tween 80 to obtain the final test solution 2% (w/v) CHG. One millilitre of test solution was spread over the skin surface in the donor compartment and the compartment was sealed with a moisture resistant film (Parafilm M®, Alcan packaging, USA) to prevent evaporation. One millilitre of receptor fluid was removed every 30 min for 2 h, every hour between 2 to 6 h and at 8 h, 12 h and 24 h. Fluid removed from the receptor compartment was immediately replaced with an equal volume of fresh PBS solution. All samples were filtered through a 0.45 μm nylon filter (Kinesis, UK) and analysed by HPLC (the method for CHG analysis by HPLC was evaluated prior to the study as described in 2.4.4.1.). The assay was performed in triplicate and on two different donor skin samples.

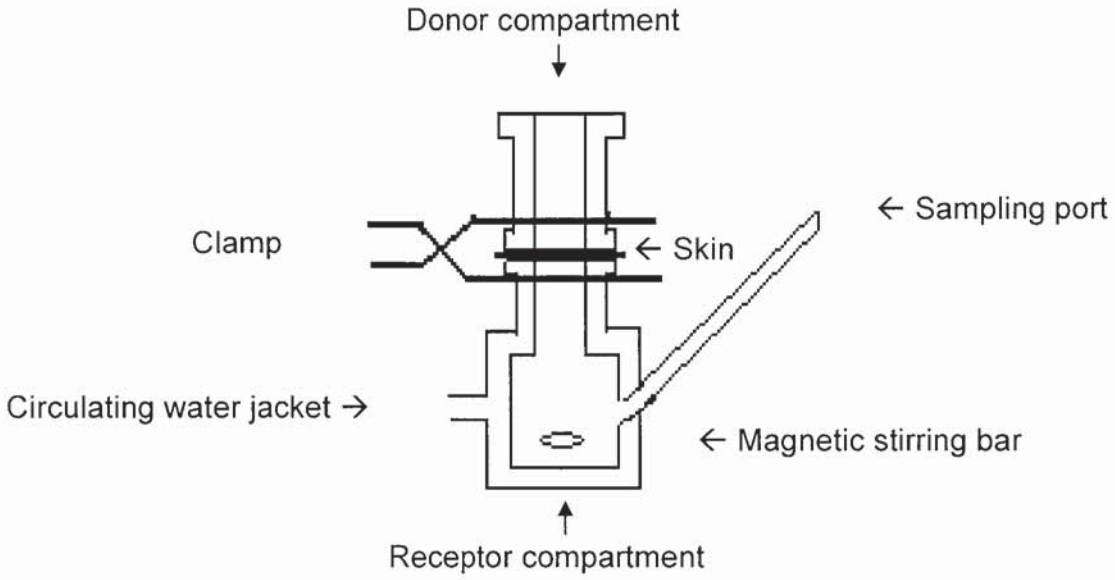

Figure 3.1. Diagram of Franz diffusion cell. Receptor compartment was filled with PBS, which was kept at 37°C by circulating water jacket. The skin was mounted between the receptor and donor compartment and clamped. The test drug was placed onto the donor compartment. The drug diffused through the skin was sampled by drawing up receptor fluid via sampling port.

3.4.4. CHG skin penetration profile studies

Excised full thickness human skin samples were mounted onto the Franz diffusion cells as described above, and exposed to 2% (w/v) CHG for 2 min, 30 min and 24 h. Following exposure, the skin samples were removed, washed with sterile PBS and dried with a sterile absorbent towel. The skin samples were immediately sprayed with a cryospray (Bright Instruments, Cambs, UK), frozen at -20°C and sectioned as described in 2.4.4.3. Chlorhexidine was extracted from the skin as described in 2.4.4.2. and the amount of CHG in the samples was analysed by HPLC. Control skin (skin without treatment) was analysed parallel to the test samples. The assay was performed in triplicate for each donor skin.

3.4.5. Statistical analysis

The data obtained from skin penetration studies were analysed with student t-test using INSTAT3 GraphPad (San Diego, CA, USA)

3.5. Results

3.5.1. Antimicrobial activity of CHG against microorganisms in a planktonic growth mode

Minimum inhibitory concentrations, MBCs and MFCs for microorganisms in a planktonic growth mode are given in table 3.1. Gram-positive *Staphylococcus spp.* and *P. acnes* were more susceptible to CHG (median MIC of 3 μg/ mL and MBC of 4 μg/ mL) compared to fungi (median MIC of 32 μg/ mL and MFC of 64 μg/ mL) and Gram-negative bacteria (median MIC of 48 μg/mL and MBC of 64 μg/mL). The presence of 10% (v/v) serum reduced efficacy of CHG against Gram-positive bacteria *S. epidermidis*, with a 4-fold increase in MIC and MBC, which was not seen in fungi or Gram-negative bacteria (Table 3.1.).

Table 3.1. Median MIC, MBC and MFC (and range in μg/ mL) of aqueous CHG against Gram-positive and Gram-negative bacteria and fungi in a suspension with or without 10% (v/v) horse serum. The susceptibility assay was performed by the broth microdilution method in triplicate (NT= not tested).

Microorganism	No serum (n=5)		With 10 % (v/v) serum (n=3)	
	MIC	**MBC or MFC**	**MIC**	**MBC or MFC**
S. epidermidis	2 (2-4)	4 (2-4)	8 (8-16)	16 (8-16)
MSSA	2 (2-16)	4 (4-32)	NT	NT
MRSA	4 (4)	4 (4-8)	NT	NT
P. acnes	2 (0.5-16)	2 (1-16)	NT	NT
Acinetobacter spp.	64 (64)	64 (64)	NT	NT
Ps. aeruginosa	32 (8-32)	32 (32-64)	32 (32)	64 (32-128)
C. albicans	32 (32-64)	64 (64)	32 (32-64)	64 (64-128)

3.5.2. Antimicrobial activity of CHG against *S. epidermidis* in a biofilm mode of growth

3.5.2.1. Establishing S. epidermidis biofilms

Congo red agar and Alcian blue assays confirmed *S. epidermidis* RP62A, NCTC 11047 and two clinical isolates (TK1 and TK2) as slime and biofilm producing isolates. Furthermore, Alcian blue assay on *S. epidermidis* RP62A and NCTC 11047 showed MHB with 2% (w/v) glucose, incubated for 48 h, produced a confluent *S. epidermidis* biofilm with approximately 3.6×10^4 cfu per mm^2 (Figure 3.2.).

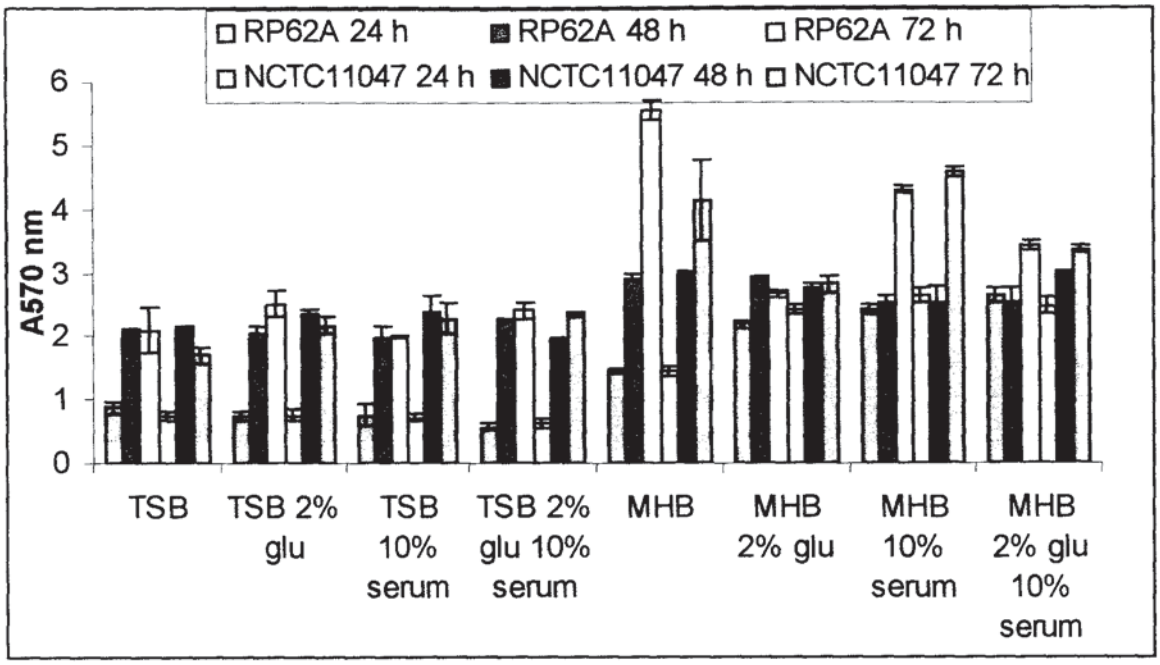

Figure 3.2. Alcian blue assay to evaluate the effect of various growth media and time of incubation on the biofilm production of *S. epidermidis* RP62A and NCTC 11047: tryptic soya broth (TSB) and Mueller-Hinton broth (MHB) ± 2% (w/v) glucose and with or without 10% (v/v) serum. The results are presented as the mean absorbance at 570 nm ± SEM (n= 4).

3.5.2.2. MIC and MBC of CHG against S. epidermidis biofilms

Chlorhexidine median MIC and MBC (and range) against *S. epidermidis* RP62A, TK1 and TK2 biofilms were 8 µg/ mL (8) and 16 µg/ mL (16) respectively.

3.5.3. Permeation of CHG in excised human skin using a Franz cell model

No CHG was detected in the receptor compartment during the 24 h exposure of excised full thickness human skin to 2 % (w/v) aqueous CHG suggesting that CHG does not permeate through the skin but is retained within the tissue.

3.5.4. Skin penetration of CHG in excised human skin using a Franz cell model

After 2 min, 30 min and 24 h, concentrations of chlorhexidine within the skin were highest in the surface 100 μm sections, and lower below depths of 300 μm (Figures 3.3. and 3.4.). The concentrations of CHG within the top 100 μm sections of skin were 0.157 (± 0.047) μg/ mg tissue and 0.077 (± 0.015) μg/ mg tissue after 2 min and 30 min exposure to 2% (w/v) CHG respectively (figure 3.3.). The concentration of CHG within the deeper layers (below 300 μm) was less than 0.002 μg/ mg tissue following both 2 min and 30 min exposure. The difference between the amount of chlorhexidine within the top layers between 2 min and 30 min exposure was not significant ($p > 0.05$). The concentration of CHG was significantly higher ($p < 0.05$) within all skin sections following 24 h exposure to CHG compared to exposure times of 2 min and 30 min. The concentration of CHG was 7.88 (± 1.37) μg/ mg tissue within the upper 100 μm sections, and less than 1 μg/ mg of tissue at depths of 300 μm and below.

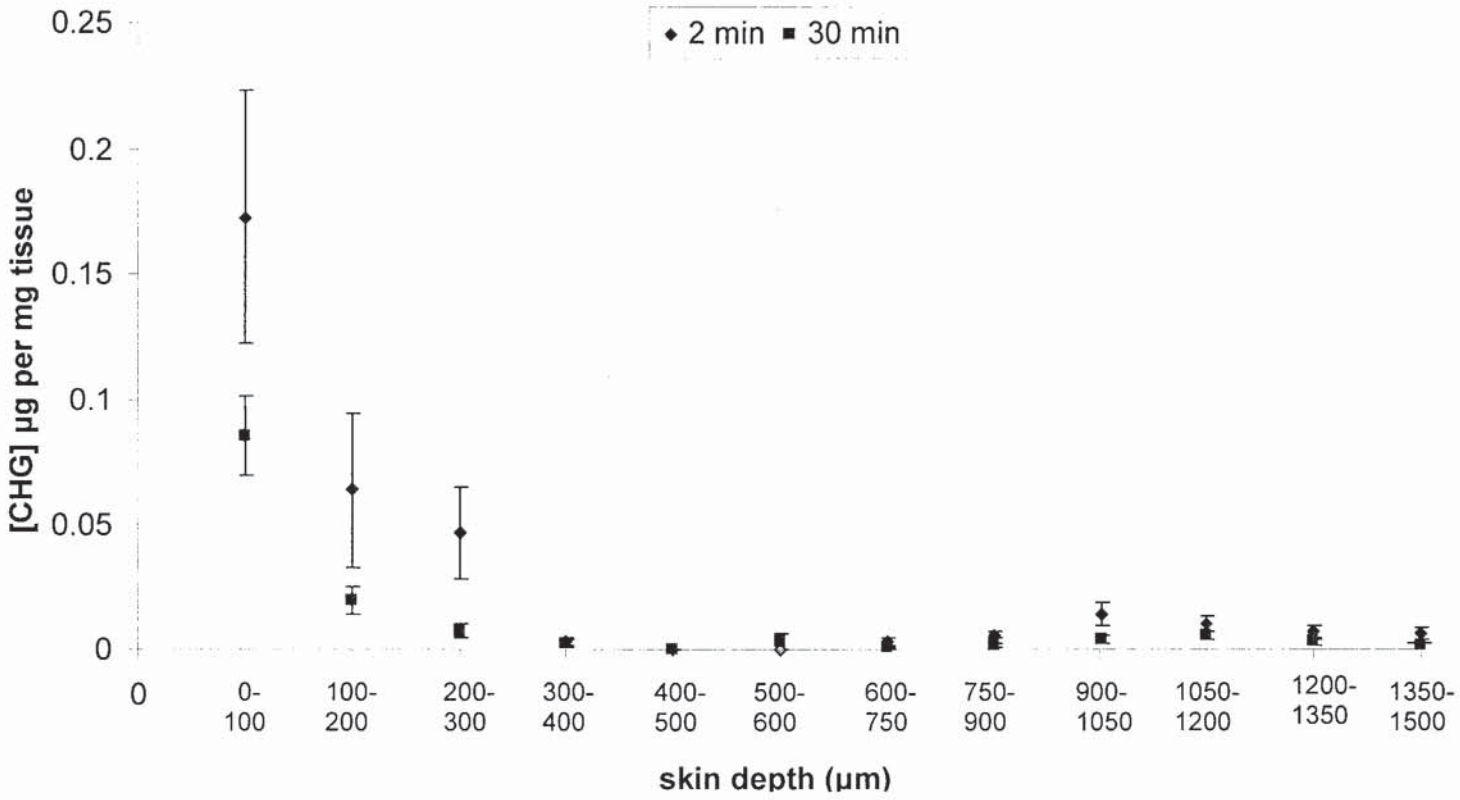

Figure 3.3. Penetration profile showing the location and concentration of CHG (µg/ mg tissue) in excised human skin after 2 min and 30 min exposure to aqueous 2% (w/v) CHG (mean ± SEM, n= 15).

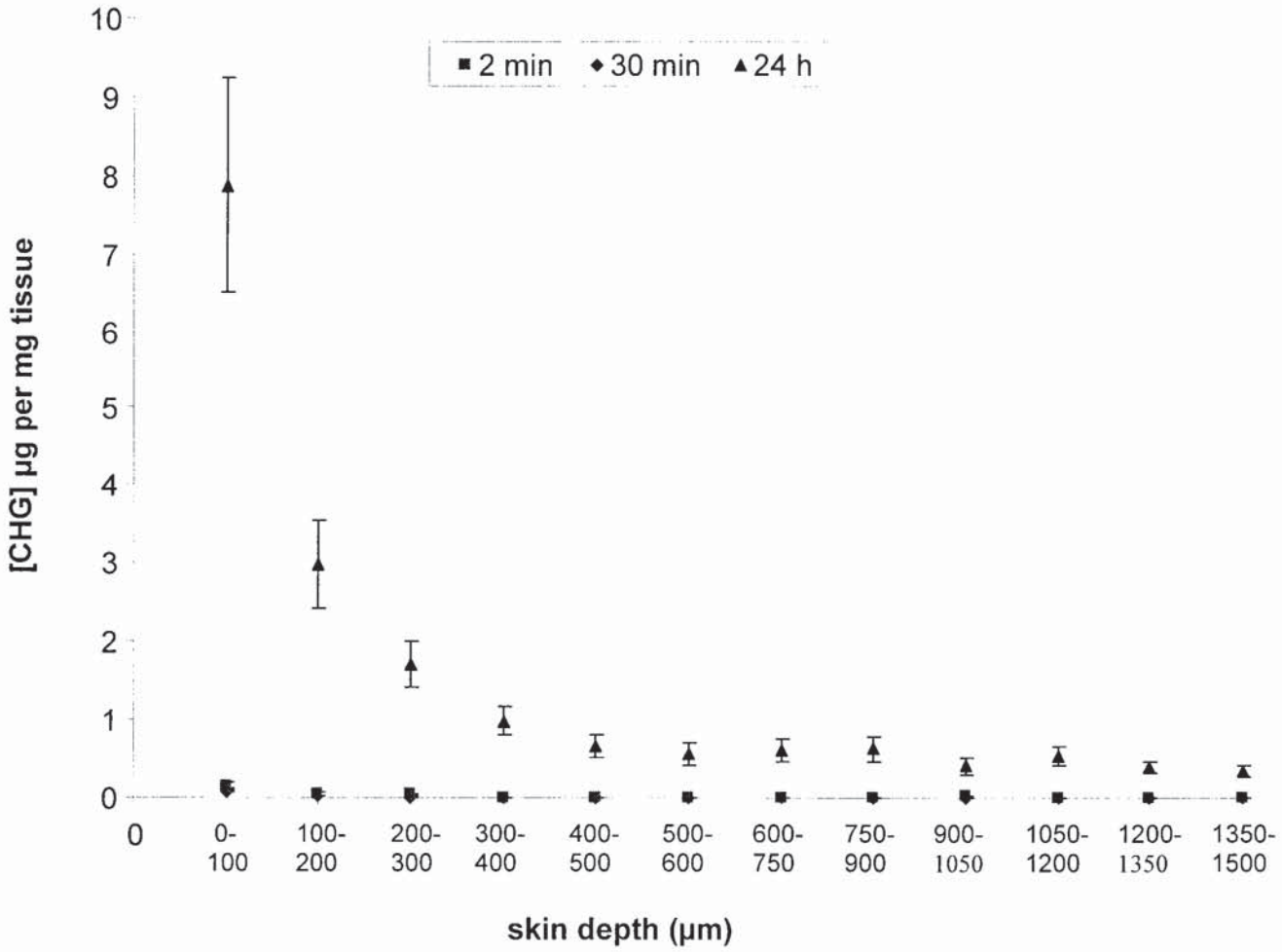

Figure 3.4. Penetration profile showing the location and concentration of CHG (µg/ mg tissue) in excised human skin after 2 min and 30 min (n= 15) and 24 hours (n= 30) exposure to aqueous 2% (w/v) CHG (mean ± SEM).

3.6. Discussion

In this study, the antimicrobial efficacy of aqueous CHG and penetration into excised human skin was evaluated *in vitro*. The antimicrobial activity of CHG was assessed against common skin microorganisms often associated with CRI in both planktonic and biofilm modes of growth. The results of this study confirmed the efficacy of CHG against Gram-positive and Gram-negative bacteria and *Candida*; furthermore Gram-positive bacteria were more susceptible to CHG compared to Gram-negative bacteria and Candida, which is in line with previous studies (Koljalg *et al.*, 2002, McDonnell and Russell, 1999, Lambert, 2004). These results are not surprising as the cationic compound, CHG, interacts with negatively charged microbial cells and the cell walls of Gram-positive bacteria are more negatively charged than those of Gram-negative bacteria (as discussed in section 1.2.1). The thick cell wall of *Candida spp.* is mainly composed of glucans and mannans, but also contains chitin, proteins and lipids (Olsen, 1990), which may affect CHG uptake into the yeast cell (McDonnell and Russell, 1999). More importantly, the ionic interaction of CHG with the cytoplasmic membrane phospholipids is hindered within Gram-negative bacteria due to the lipopolysaccharide (LPS) outer layer, which is not present in Gram-positive bacteria or yeast (Castillo *et al.*, 2006, Denyer and Stewart, 1998, Sheppard *et al.*, 1997). Diffusion of CHG to the inner cell membranes in Gram-negative bacteria may be compromised due to CHG binding to the negatively charged LPS layer (Papo and Shai, 2005). Furthermore, the LPS layer in *Ps. aeruginosa* is more tightly linked than other Gram-negative bacteria due to high concentration of Mg^{2+} ions (McDonnell and Russell, 1999). The relatively large molecular size of CHG (898 Da) is also likely to restrict its passage through the Gram-negative bacterial cell wall porins (Denyer and Stewart, 1998).

In addition to the differences in CHG efficacy against Gram-positive and Gram-negative bacteria, this study also found a 2-fold increase in the median MIC of CHG against MRSA compared to MSSA. Meticillin-resistant *S. aureus* has been shown in previous studies to be less sensitive to CHG compared to MSSA with a 1.5 to 3- fold higher MIC (Brill *et al.*, 2006, Suller and Russell, 1999); however, the correlation between reduced biocide susceptibility and antibiotic resistance has not yet been proven (Lambert, 2004, Suller and Russell, 1999).

The presence of interfering organic compounds has been reported to reduce the antimicrobial efficacy of CHG, which is partly due to interference with the ionic interactions of the cationic compound and the negatively charged microbial cell wall. A study comparing various growth conditions on the antimicrobial efficacy of the cationic compounds demonstrated that activity of CHG against Gram-positive bacteria is more affected by the interfering compounds than Gram-negative bacteria (Brill *et al.*, 2006), which may be due to a greater influence on the negative charge of the Gram-positive bacteria. The results from this study support previous findings, as the activity of CHG against *S. epidermidis* was more affected by the presence of 10% (v/v) serum in the growth media compared to *Ps. aeruginosa* and *C. albicans*.

The antimicrobial efficacy of CHG against *S. epidermidis* in a biofilm mode of growth was also assessed in this study. Bacterial biofilms are a major contributor to HCAI, commonly associated with intravascular catheters and other implants. Biofilms are generally regarded as less susceptible to antimicrobial agents compared to microorganisms in a planktonic phase of growth (Saginur *et al.*, 2006, Donlan and Costerton, 2002). This is believed to be related to reduced diffusion of the antimicrobial agents through the biofilm, sessile or slow growing microbial cells,

and physiological changes in the microenvironment within the microbial biofilm (Donlan and Costerton, 2002). This study showed a 4-fold higher median MIC against *S. epidermidis* in a biofilm mode of growth compared to planktonic cells. The diffusion of cationic CHG in the biofilm is thought to be hindered by the negatively charged extracellular matrix, changing the physicochemical properties of the extracellular matrix and its tertiary structure (Hope and Wilson, 2004). Furthermore, CHG efficacy against Gram-positive bacteria has been shown to be more affected by the growth environment, such as low pH and reduced levels of oxygen (Brill *et al.*, 2006).

In addition to the antimicrobial efficacy of CHG, its penetration into excised human skin was also evaluated in this study. The results from this investigation demonstrate that 2 % (w/v) chlorhexidine, the antiseptic agent recommended within EPIC and CDC guidelines for skin antisepsis prior to central venous catheter (CVC) insertion, permeates poorly into deeper layers of skin after 2 min and 30 min exposure to the antiseptic. The concentration of CHG within the upper 100 μm sections of skin was 0.157 (± 0.047) μg/ mg tissue and 0.077 (± 0.015) μg/ mg tissue after 2 min and 30 min respectively. If 1 g of tissue is estimated to equal 1 mL, these levels are higher than the concentration required to kill many common skin microorganisms such as *S. epidermidis in vitro* (see section 2.5.3.1). Below 300 μm, the CHG concentration remained less than 0.002 μg/ mg tissue, which may not be effective at eradicating many microorganisms within the skin (Messager *et al.*, 2001), especially microorganisms residing deep in the hair follicles. Furthermore, CHG activity is reduced in the presence of organic compounds, such as fatty acids and at lower pH and therefore may reduce the efficacy of skin antisepsis with CHG (McDonnell and Russell, 1999). Previous *in vivo* studies assessing the CHG concentration on skin following

CHG application demonstrated higher levels of CHG on the surface of skin, however higher concentration and repeated applications were used; following a single application of 5% (w/v) CHG the level of CHG recovered after 5 min was 50.4 μg/ cm^2, and CHG persisted on the skin over 72 h (the mean residual CHG on the skin 7.8, 2.7 and 1.4 μg/ cm^2 at 24, 48, and 72 h respectively) (Carret *et al.*, 1997). Another study using a semi-quantitative method to evaluate the CHG concentration on skin after washing the skin of healthy volunteers with either 4% (w/v) CHG soap, followed by rinsing with water, or 2% (w/v) CHG non-rinse cloth at the evening before, morning or both; they demonstrated higher levels of CHG on the skin after using the 2% (w/v) non-rinse cloth (361.5 μg/ mL to 2031.3 μg/ mL) compared to 4% (w/v) CHG soap with rinsing (17.2 μg/ mL to 1494 μg/ mL) (Edmiston *et al.*, 2008). However, they demonstrated CHG levels below detection on few of the study volunteers.

In this study, an exposure time of 2 min was used to reflect antiseptic application times used in clinical practice prior to surgery (Elliott *et al.*, 1997). Although the 2 min study appeared to have a higher concentration of bound CHG compared with the 30 min exposure study, there was variability in concentrations measured in the top layers and the difference in CHG concentrations following the 2 and 30 min exposure time was not significant ($p > 0.05$). These results were expected following the shorter exposure period of 2 min (Wagner *et al.*, 2000). It is likely that a steady state has not been reached following the 2 min exposure to the antiseptic. A similar phenomenon was reported by Wagner *et al.* (2002). Skin was also exposed to 2% (w/v) CHG for 24 h and the concentration of CHG in the deeper sections, i.e., beyond 300 μm, was less than 1 μg/ mg tissue. Whilst this concentration of CHG exceeds the MBCs for many skin commensals (section 2.5.3.1), this level was only obtained after a prolonged skin/ antiseptic contact time of 24

h. In this study no detectable amounts of CHG were recovered from the receptor compartment suggesting that aqueous CHG does not permeate through full thickness of excised skin and is retained within the tissue. These results support previous research on another CHG-based compound, chlorhexidine phosphanilate, which was also shown not to permeate through full thickness skin samples (Wang *et al.*, 1990).

Skin permeation studies are commonly performed *in vitro* with vertical or horizontal diffusion cells using skin or artificial membranes. This study was performed using vertical diffusion cells (Franz type diffusion cells), to evaluate the delivery of CHG through excised full thickness human skin. Such conditions mimic the *in vivo* environment by maintaining the physiological receptor fluid at body temperature and the skin surface temperature of 32°C (Franz, 1975, Wagner *et al.*, 2002). Skin permeation studies generally evaluate drug delivery through skin by measuring drug diffusion into the receptor fluid through the SC or epidermis, which are the main barriers for skin permeation. However, the use of stripped skin layers, such as isolated SC or epidermal layers, for drug permeation studies may influence the results as the potential retention of the drug in the dermal layers of skin is not considered. Full thickness skin was used in this study to determine the location of CHG throughout the skin samples, rather than studying flux of the drug through the barrier layers. Following exposure to CHG, the full thickness human skin was sectioned to a depth of 1500 μm by sequential sectioning with a microtome producing a total of 60 sections per skin sample. Skin sectioning has been used in many previous studies (Touitou *et al.*, 1998), however the SC is often removed by tape stripping prior to sectioning of skin. In this study full thickness skin samples were sectioned throughout the sample without prior removal of the surface layers.

This study demonstrates that CHG permeation through full thickness skin is not linear, which was expected due to the variation in structure at various layers. The top 100 μm layer of skin, which contains SC (average 10- 20 μm thick) and other epidermal layers (50 – 100 μm thick), contained the highest amount of CHG following exposure to 2% (w/v) CHG over all time points studied. Previous research has shown that the main permeation barrier for skin absorption is the SC (Cal *et al.*, 2001, Lafforgue *et al.*, 1997, Wang *et al.*, 1990), which is thought to be due to its high lipid matrix and packed layers of keratinised epithelial cells. Furthermore, this study found that below 300 μm, at the dermal layer, the level of CHG remained consistently low. Depending on the body site, dermis contains hair follicles and other appendages, including sebaceous glands and sudoriferous glands (sweat producing glands), which are of interest in skin antisepsis as they may be niches for microbial colonisation of skin following antisepsis (Hendley and Ashe, 1991, Hendley and Ashe, 2003). It is generally recognised that skin antisepsis does not sterilize the skin; our study demonstrates that it may be due to poor permeation of CHG into the deeper layers of skin.

3.7. Conclusion

Chlorhexidine has greater efficacy against Gram-positive bacteria than Gram-negative bacteria and *Candida spp*, and its efficacy against Gram-positive *S. epidermidis* is reduced in the presence of serum and in a biofilm mode of growth. Two percent CHG poorly penetrates into the deeper layers of skin, and therefore its efficacy in eradicating microorganisms residing beneath the surface of skin is limited. Furthermore, the model presented in this study is a valuable tool for determining a skin penetration profile for CHG (and potentially other antiseptics) through full thickness skin *in vitro*. This study lays the foundation for further research within this area with a view to potentially adopting alternative strategies for enhanced skin antisepsis in clinical practice.

4. CHAPTER: ANTIMICROBIAL ACTIVITY OF ESSENTIAL OILS ALONE AND IN COMBINATION WITH CHLORHEXIDINE

4.1. Introduction

Chlorhexidine (CHG) is one of the most widely used antimicrobials within clinical practice for skin antisepsis and is currently recommended within the EPIC and Healthcare Infection Control Practices Advisory Committee (HICPAC) guidelines (O'Grady *et al.*, 2002, Pratt *et al.*, 2007). Unfortunately, healthcare associated infections (HCAI) do arise following incision of skin (HPA, 2007, NAO, 2000) and are likely to be associated with increased prevalence of microbial resistance to antibiotics and antiseptics and inadequate skin antisepsis which encompasses both the contact time between skin and antiseptic prior to incision and permeation of the antiseptic within the skin (Elliott *et al.*, 1997, Hendley and Ashe, 2003, Hendley and Ashe, 1991, Koljalg *et al.*, 2002, Langgartner *et al.*, 2004). Therefore additional strategies for skin antisepsis or improvement of existing methods need to be considered.

The antimicrobial efficacy of essential oils has been known for several years and many studies have demonstrated broad spectrum activity against bacteria, fungi and viruses (Cowan, 1999). More recently, in the light of increased antimicrobial resistance within the clinical setting, the potential of essential oils for the prevention and treatment of infection has been researched in several studies (Al-Shuneigat *et al.*, 2005, Caelli *et al.*, 2000, Dryden *et al.*, 2004, Messager *et al.*, 2005, Warnke *et al.*, 2006). Indeed, tea tree oil (TTO) has recently been shown to be more effective than CHG at treating superficial skin sites and lesions colonised with MRSA (Dryden *et al.*, 2004). Many essential oils contain the lipophilic component, terpene, which is thought to

exhibit its antimicrobial activity through the disruption of microbial cell membranes (Cowan, 1999). However, essential oils are mixtures of many heterogeneous compounds and the main components responsible for their antimicrobial activity and the mode of their activity is not well understood (Cowan, 1999). Furthermore, essential oils have been shown to act as effective penetration enhancers, increasing permeation and improving retention of drugs within the skin (Biruss *et al.*, 2007, Fang *et al.*, 2004).

4.2. Aims of this study

The aim of this study was to investigate the antimicrobial efficacy of six essential oils (citronella, EO, juniper oil, lavender oil, TTO, and thymol) against a panel of microorganisms commonly associated with skin and infections related to intravascular devices. The antimicrobial efficacy of the oils was assessed against the microorganisms in both planktonic and biofilm modes of growth. Furthermore, the antimicrobial efficacy of combinations of EO, TTO and thymol with aqueous CHG against planktonic and biofilm cultures of *S. epidermidis* was investigated.

4.3. Materials

4.3.1. Chemicals

Congo red agar and Alcian blue stain were prepared as described in 2.3.1. and 3.3.1. respectively. Agar and broth culture media were prepared as described in 3.3.1. Tea tree oil (40.2% terpin-4-ol and 3.5% cineole), eucalyptus oil (EO) (82.9% cineole), thymol (>99.5%), citronella (85% geraniol/ 35% citronellal), lavender oil and dimethylsulphoxide (DMSO) were purchased from Sigma-Aldrich, UK. Juniper oil was purchased from Holland and Barrett (Nuneaton, UK) and ViaLight MDA Detection Kit was purchased from Cambrex (USA).

Tween 80, DMSO, Triton X-100, sodium thioglycolate and L-cysteine were purchased from Sigma-Aldrich. Letheen broth was purchased from Difco (BD, Sparks, MD, USA). Sodium metabisulphite were purchased form FSA Laboratory Supplies (Loughborough, UK), sodium thiosulphate pentahydrate from BDH Ltd (Poole, UK) and lecithin were purchased from Fisher Scientific (Loughborough, UK). Other chemicals and microtitre plates were as described in 3.3.1.

4.3.2. Microbial cultures

Microorganisms used in this study are described in section 3.3.3. The microorganisms were stored on MicroBank beads (Pro-Lab Diagnostics) at -70°C until required.

4.4. Methods

4.4.1. Preparation of the microbial suspensions

The microbial suspensions were prepared as described in 3.4.1.1. The final concentrations were adjusted with liquid culture media to 1 x 10^7 cfu/ mL (for the time-kill assays) 1 x 10^6 cfu/ mL (for the suspension assays) and 1 x 10^5 cfu/ mL (for the agar diffusion assay and biofilm preparation).

4.4.2. Preparation of the antimicrobial stock solutions

Aqueous CHG was diluted with MHB to obtain a stock solution of 512 µg/ mL. Citronella, EO, juniper oil, lavender oil, TTO and thymol were diluted with MHB to obtain the stock suspensions of 512 mg/ mL. Five percent (v/v) DMSO was added to the essential oil stock suspensions to enhance the solubility of the oils.

4.4.3. Preliminary evaluation of the antimicrobial activity of essential oils by the agar diffusion method

Citronella, EO, juniper oil, lavender oil, TTO and thymol, were assessed in a preliminary assay for antimicrobial activity by an agar diffusion assay. Mueller-Hinton agar was used for *Staphylococcus spp., Ps. aeruginosa* and *Acinetobacter spp.*, BHI agar was used for *P. acnes* and SAB agar for *C. albicans*. To obtain confluent growth, 500 µl of each microbial suspension

adjusted to 1 x 10^5 cfu/ mL, was spread over the surface of the agar plates using a sterile cotton swab. Wells (0.5 mm in diameter) were cut into the agar plates inoculated with microorganisms using a flame sterilized Pasteur pipette and 75 µL of 25% (v/v) of each essential oil (16.3 mg to 17.25 mg), except thymol 5% (w/v) (3.75 mg), was placed into the wells in duplicate. The plates were left for 30 min at 4°C to allow any diffusion of the oils.

Plates inoculated with *Staphylococcus spp.*, *Acinetobacter spp.* and *Ps. aeruginosa* were then incubated in air at 37°C for 24 h, *P. acnes* in anaerobic conditions at 37°C for 72 h and *C. albicans* in air at 30°C for 48 h. Following incubation the zone of growth inhibition around the wells were measured.

4.4.4. Determination of MIC, MBC and MFC of essential oils against microorganisms in a planktonic and a biofilm modes of growth

4.4.4.1. Antimicrobial activity of essential oils against Staphylococcus spp., P. acnes, Ps. aeruginosa, Acinetobacter spp. and C. albicans in a planktonic mode of growth

Minimum inhibitory concentrations of essential oils were determined using a broth microdilution assay in line with NCCLS (now CLSI) guidelines (NCCLS, 2000) as described in 2.4.3.2. Serial double dilutions of citronella, EO, juniper oil, lavender oil, TTO and thymol ranging between 512 mg/ mL to 0.5 mg/ mL were performed in liquid culture media in a 96-well round bottom microtitre plate. The antimicrobial activity of DMSO [ranging from 5% (v/v) to 0.015% (v/v)] was also determined on a separate microtitre plate. The assay was performed in triplicate and repeated in the presence of an organic load comprising 10% (v/v) serum

4.4.4.2. Antimicrobial activity against S. epidermidis in a biofilm mode of growth

Biofilms of *S. epidermidis* RP62A, TK1 and TK2 were prepared as described previously in section 3.4.2.2. Minimum inhibitory concentrations and MBC of EO, TTO and thymol (which demonstrated the greatest antimicrobial efficacy in the suspension assay) were determined against *S. epidermidis* in a biofilm mode of growth as described in 3.4.2.3. Serial double dilutions of the essential oils were performed with liquid culture media to obtain the final concentrations ranging from 256 mg/ mL to 0.25 mg/ mL. The assay was performed in duplicate microtitre plates and the antimicrobial activity of 5% (v/v) DMSO was assayed alongside the study.

4.4.5. Evaluation of the antimicrobial activity of TTO, EO and thymol in a time-kill assay

4.4.5.1. Evaluation of the neutraliser efficacy against TTO, EO and thymol

The efficacy of a non-toxic neutraliser to quench the antimicrobial activity of EO, TTO and thymol was determined prior to the time-kill assay. The neutralisers are given in table 4.1. The neutralising agent efficacy was assessed by placing 890 µL of a neutralising agent and 100 µL of the essential oils (512 mg/ mL) in to a bijou bottle and the suspensions were incubated at room temperature for 30 min after which time, 10 µL of a suspension containing 1 x 10^7 cfu/ mL of *S. epidermidis* was added; the suspensions were further incubated in air at 37°C for 20 h, and then observed for microbial growth (turbidity). The agents demonstrating neutralising efficacy towards EO, TTO and thymol were then evaluated quantitatively. The quantitative assay was performed by repeating the method described above over a 4 h period; the microbial growth was evaluated by removing 100 µL of the suspension, serially diluting in PBS (10^{-1} to 10^{-3}) and

placing the dilutions into sterile Petri dishes in duplicate and mixing with molten MHA cooled to 45°C. The set culture plates were incubated in air at 37°C for 20 h and the number of cfu/ mL determined. The control suspensions of MHB with inocula, neutraliser with inocula and essential oils with inocula were assayed alongside the study. The assay was performed in triplicate.

Table 4.1. The composition of neutralising solutions tested against TTO, EO and thymol and *S. epidermidis*.

Neutraliser number	Content	Reference
1	Letheen broth [beef extract (5g/ L), Proteose Peptone No 3 (10 g/ L), Polysorbate 80 (5 g/ L), Lecithin (0.7 g/ L) and Sodium Chloride (5 g/ L)] with an additional 2% (v/v) of Tween (Polysorbate) 80.	(Russell, 1999)
2	Letheen broth, 2% (v/v) Tween 80, 0.1% (v/v) Triton X-100, 0.5% (w/v) Sodium Thiosulphate.	(Adams, 2006)
3	12 mL (129 g) Tween 80, 2.5 mL of 40% (w/v) sodium metabisulphite, 1.569 g of sodium thiosulphate pentahydrate, 1 g sodium thioglycolate, 0.3 g L-cysteine, 0.4 g Lecithin. Adjusted with distilled water to 100 mL and pH 7 (double strength).	(Espigares *et al.*, 2003)
4	12 mL (129 g) Tween 80, 2.5 mL of 40% (w/v) sodium metabisulphite, 1.569 g of sodium thiosulphate pentahydrate. Adjusted with distilled water to 100 mL and pH 7 (double strength).	(Espigares *et al.*, 2003)
6	12 mL (129 g) Tween 80, 2.5 mL of 40% (w/v) sodium metabisulphite, 1.569 g of sodium thiosulphate pentahydrate, 0.4 g Lecithin. Adjusted with distilled water to 100 mL and pH 7 (double strength).	(Espigares *et al.*, 2003)
7	30 g/ L Tween 80, 3 g/ L lecithin, 1 g/ L histidine, 5 g/ L sodium thiosulphate, 34 g/ L potassium dihydrogen phosphate in TSB.	(Messager *et al.*, 2005)

4.4.5.2. Time-kill assay of EO, TTO and thymol against S. epidermidis in a planktonic mode of growth

The antimicrobial efficacies of EO, TTO and thymol were further assessed by evaluating the kill rate of *S. epidermidis* RP62A, TK1 and TK2 in a time-kill assay. One millilitre of each essential oil was diluted with MHB to obtain final concentration of 2 x MBC. The suspensions were inoculated with 10 µL of the suspension containing 1 x 10^5 cfu and vortexed for 15 s. The suspensions were incubated in a rotary shaker (150 rpm) at 37°C and 100 µL samples were withdrawn at 10 min, 30 min, 1 h, 2 h, 3 h, 4 h and 6 h and serial dilutions (10^{-1} to 10^{-3}) in a suitable neutraliser were performed. Each dilution was placed into sterile Petri dishes in duplicate and mixed with molten MHA cooled to 45°C. The set culture plates were incubated in air at 37°C for 20 h and the number of cfu/ mL determined. The assay was performed in triplicate.

4.4.5.3. Time-kill assay of EO, TTO, thymol and CHG against S. epidermidis in a biofilm mode of growth

The antimicrobial efficacies of EO, TTO and thymol were further assessed by evaluating the kill rate of *S. epidermidis* RP62A, TK1 and TK2 in a biofilm mode of growth, in a time-kill assay. Chlorhexidine were tested alongside the essential oils for comparison. Confluent biofilms were prepared as described in 3.4.2.2. Eucalyptus oil, TTO, thymol and CHG were diluted with MHB to obtain final concentrations of 2 x MBC. Two hundred and fifty microlitres of the antimicrobial agents were placed, in quadruplicate, into wells of microtitre plates containing biofilms of *S. epidermidis*. Plates were then incubated for 30 min, 1 h, 2 h, 4 h, 6 h, 8 h and 12 h. The microbial viability was evaluated as described in 3.4.2.3.

4.4.6. Checkerboard assay to assess the antimicrobial activity of CHG in combination with EO, TTO and thymol against *S.epidermidis*

4.4.6.1. Checkerboard assay against S. epidermidis in a planktonic mode of growth

The antimicrobial activity of aqueous CHG in combination with EO, TTO and thymol was assessed against *S. epidermidis* RP62A and TK1 by a checkerboard method (Shin and Lim, 2004). In brief, serial double-dilutions of the antimicrobial compounds were prepared (256 mg/ mL to 1 mg/ mL for natural compounds and 64 µg/ mL to 0.5 µg/ mL for CHG). Fifty microlitres of each CHG solution was added to the rows of a 96-well microtitre plate in diminishing concentrations and 50 µL of each essential oil to the columns in diminishing concentrations. The wells were then inoculated with 100 µL of the microbial suspension containing 1 x 10^5 cfu. Columns 10, 11 and 12 served as controls containing MHB and inoculum alone, and antimicrobial compounds separately with the inoculum. The microtitre plates were incubated in air at 37°C for 24 h and MIC for all antimicrobial compounds in combination was determined. To assess synergistic or antagonistic activity of antimicrobial combinations, the fractional inhibitory concentration (FIC) and FIC index (FICI) were determined using the following formulae:

$$\text{FIC} = \frac{\text{MIC of CHG or natural compound in combination}}{\text{MIC of CHG or natural compound alone}}$$

$$\text{FICI} = \text{FIC of natural compound} + \text{FIC of CHG}$$

FICI $\leq$ 0.5 were regarded as exhibiting a synergistic effect, values between > 0.5 to 4.0 as indifferent and over 4.0 as displaying antagonistic activity. The assay was performed in duplicate microtitre plates.

4.4.6.2. Checkerboard assay against S. epidermidis in a biofilm mode of growth

Microtitre plates containing *S. epidermidis* RP62A and TK1 biofilms were washed once with sterile PBS to remove any unbound bacteria. Thymol, TTO, EO and CHG were diluted with MHB as described previously and 125 µL of each antimicrobial dilution placed into each well in decreasing concentrations. Columns 10 and 11 contained biofilm and antimicrobial compounds alone, and column 12 served as a control with biofilm with saline and clear wells with saline. The plates were incubated in air at 37°C for 24 h after which the microbial viability was evaluated as described in 3.4.2.3. and FIC and FICI values determined. The assay was performed in duplicate microtitre plates.

4.5. Results

4.5.1. Preliminary evaluation of the antimicrobial activity of essential oils by the agar diffusion method

All of the essential oils (thymol, lavender oil, TTO, EO, citronella and juniper oil) demonstrated antimicrobial activity against the test microorganisms (Table 4.2. and Figure 4.1). Thymol showed the greatest efficacy against all the test microorganisms and the yeast *C. albicans* demonstrated the highest sensitivity to all of the test compounds.

Table 4.2. The antimicrobial efficacy of essential oils [25% (v/v) in MHB, except thymol at 5% (w/v) solutions] against Gram-positive and negative-bacteria and yeast in the agar diffusion assay. The results are expressed as the mean size of zone of growth inhibition (radius in mm). The tests were performed in duplicate.

	S.epidermidis (n= 5)	*S.aureus* (n= 5)	MRSA (n= 5)	*P.acnes* (n= 5)	*P.aeruginosa* (n= 5)	*Acinetobacter spp.*(n= 4)	*C.albicans* (n= 4)
Thymol	4	9	12.2	16	6.3	11.9	20
Lavender oil	1	1	3.5	2.8	< 1	2.6	6.9
Tea tree oil	2	2	10.7	6	1.5	9	20
Eucalyptus oil	6.5	2	6.2	5.5	3.3	7.5	6
Citronella oil	1	1	7.5	8	< 1	3.4	20
Juniper oil	1	2	5.7	8	< 1	4	4.5

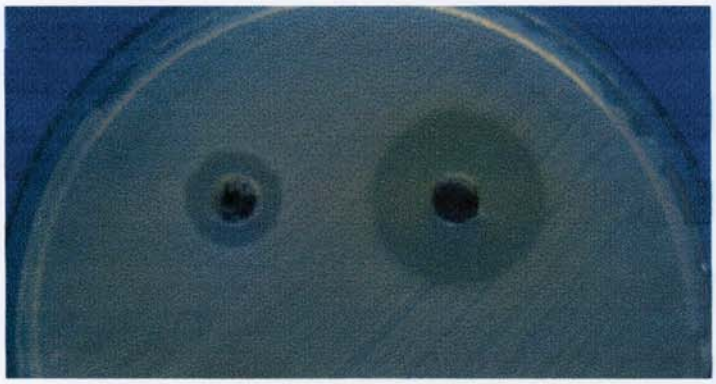

Figure 4.1. The zone of inhibition of citronella (left) [25% (v/v)] and thymol [5% (w/v)] in the agar diffusion assay against MRSA (incubated in air for 24 h at 37°C).

4.5.2. Determination of MIC, MBC and MFC of citronella, EO, juniper oil, lavender oil, TTO and thymol

4.5.2.1. Antimicrobial activity citronella, EO, juniper oil, lavender oil, TTO and thymol against microorganisms in a planktonic mode of growth

Thymol, TTO and EO demonstrated the greatest antimicrobial efficacy against the panel of test microorganisms, demonstrating median MIC and MBC or MFC levels of $\leq$ 128 mg/ mL (Figures 4.2. and 4.3.). Citronella demonstrated low efficacy against *Ps. aeruginosa* (MIC $\geq$ 256 mg/ mL). In addition, juniper oil demonstrated poor antimicrobial activity against *Staphylococcus spp.*, *Ps. aeruginosa* and *C. albicans* with MIC and MBC or MFC concentrations of $\geq$ 256 mg/ mL. The efficacy of TTO, EO and thymol against Gram-positive and Gram-negative bacteria and yeast was not affected by the presence of 10% (v/v) serum (Figure 4.4.).

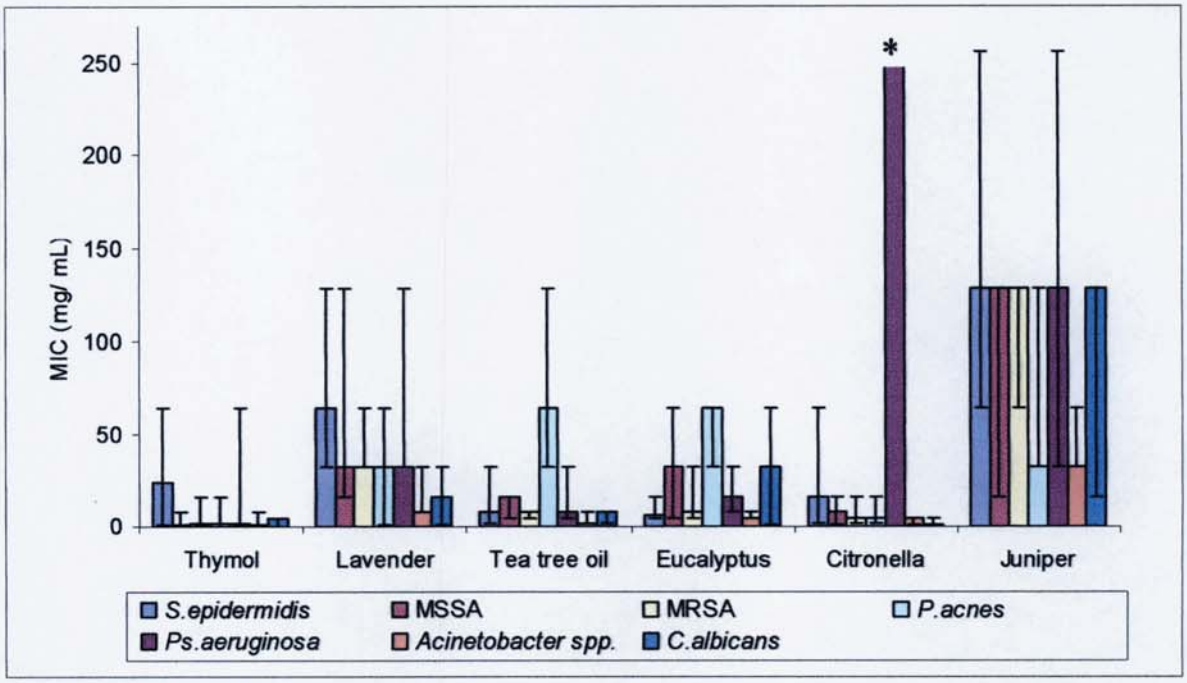

Figure 4.2. Median MIC (and range) of essential oils against Gram-positive and Gram-negative bacteria and fungi (n= 5). The assay was performed by the broth microdilution assay in triplicate (*MIC ≥ 256 mg/ mL).

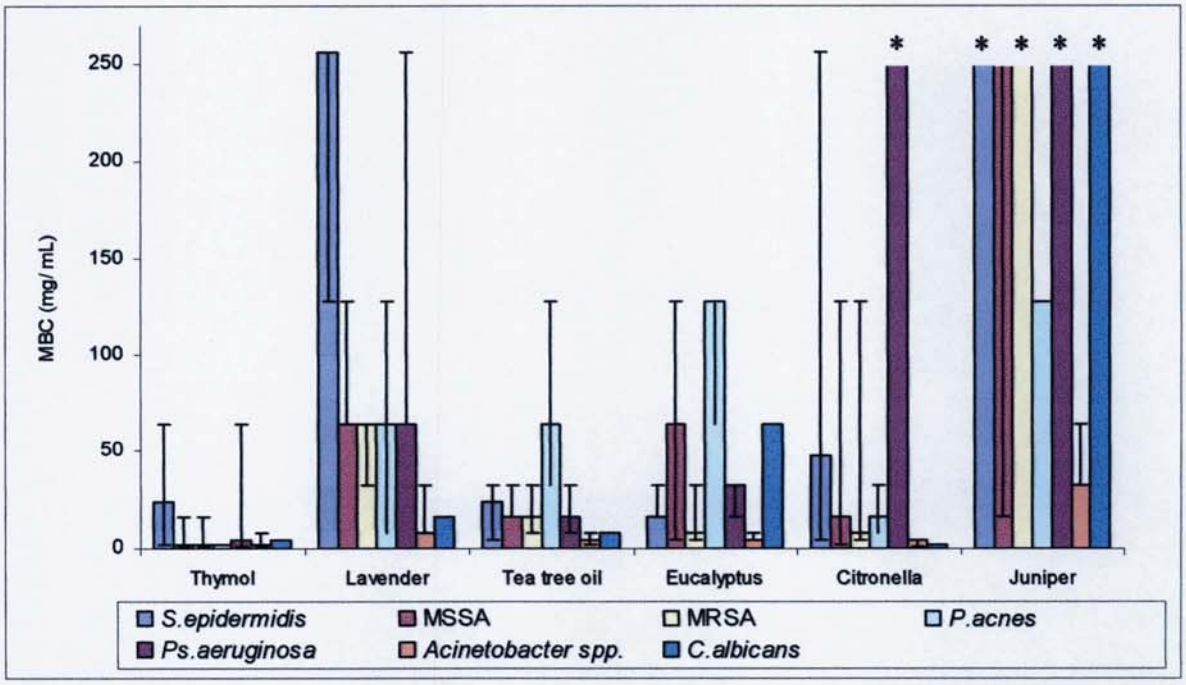

Figure 4.3. Median MBC or MFC (and range) of essential oils against Gram-positive and Gram-negative bacteria and fungi (n= 5). The assay was performed by the broth microdilution assay in triplicate (* MBC or MFC ≥ 256 mg/ mL).

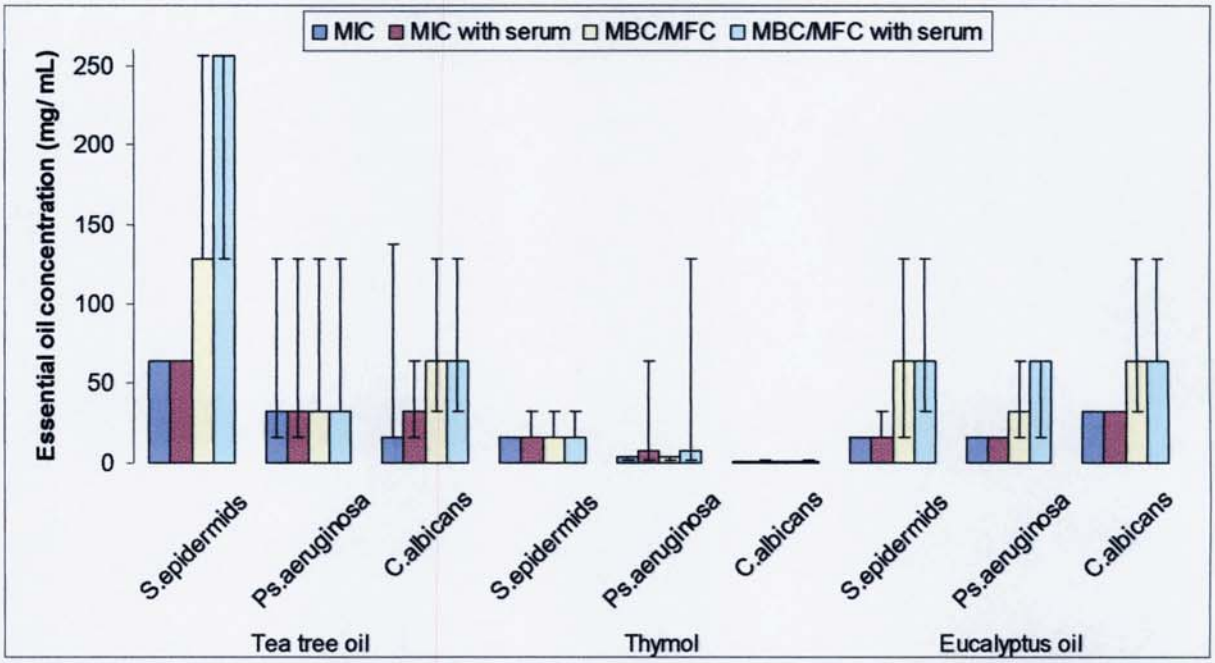

Figure 4.4. Median MIC, MBC or MFC (and range) of TTO, thymol and EO against *S. epidermidis*, *Ps. aeruginosa* and *C. albicans* with and without 10% (v/v) horse serum (n= 3). The assay was performed by the broth microdilution assay in triplicate.

4.5.2.2. Antimicrobial activity of TTO, EO and thymol against S. epidermidis in a biofilm mode of growth

Eucalyptus oil, TTO and thymol demonstrated bactericidal activity against *S. epidermidis* in a biofilm, reducing the cell viability to a negligible level as detected by the ATP bioluminescence method, with concentrations of < 256 mg/ mL (Table 4.3). Of the three essential oils tested, thymol demonstrated the greatest efficacy against *S. epidermidis* in a biofilm with MBC 2-8 mg/ mL. Tea tree oil and EO MBCs ranged between 32- 64 mg/ mL and 256 mg/ mL respectively. Five percent (v/v) DMSO, which was used as a co-solvent in the oil suspensions, did not demonstrate antimicrobial activity against *S. epidermidis* in either biofilm or planktonic modes of growth.

Table 4.3. MIC and MBC of aqueous CHG, EO, TTO and thymol against clinical isolate TK1 and RP62A of *S. epidermidis* in a suspension and in a biofilm mode of growth. The suspension assay was performed by the broth microdilution method and the biofilm assay was performed on pre-formed (48 h) bacterial biofilms (the ATP bioluminescence method was used to determine the cell viability). The assay was performed in triplicate.

Isolate	Antimicrobial agent	MIC (mg/ mL for essential oils, µg/ mL for CHG)		MBC (mg/ mL for essential oils, µg/ mL for CHG)	
		Suspension	Biofilm	Suspension	Biofilm
RP62A	Eucalyptus	4	32	64	256
	Tea tree oil	2	16	4	64
	Thymol	4	0.5	16	2
	Chlorhexidine	2	8	4	16
TK1	Eucalyptus	8	64	32	256
	Tea tree oil	16	16	64	32
	Thymol	0.5	0.5	4	8
	Chlorhexidine	2	8	4	16

4.5.3. Evaluation of the antimicrobial activity of TTO, EO and thymol in a time-kill assay

4.5.3.1. Evaluation of the neutraliser efficacy against TTO, EO and thymol

None of the neutralisers tested quenched the antimicrobial activity of TTO, EO or thymol, and therefore a dilution (1:100) technique was used in the time-kill assays as used in previous studies (Brady *et al.*, 2006, Denyer and Stewart, 1998, Mermel *et al.*, 2001). The neutralisers were not toxic to *S. epidermidis*.

4.5.3.2. Time-kill assay of EO, TTO and thymol against S. epidermidis in a planktonic mode of growth

Thymol demonstrated the best antimicrobial activity against *S. epidermidis* RP62A, TK1 and TK2 in a time-kill assay with bactericidal activity within 10 min (Figure 4.5). Tea tree oil and EO eradicated *S. epidermidis* in a planktonic mode of growth in 3 h and 2 h respectively. The assay was performed in triplicate on three isolates.

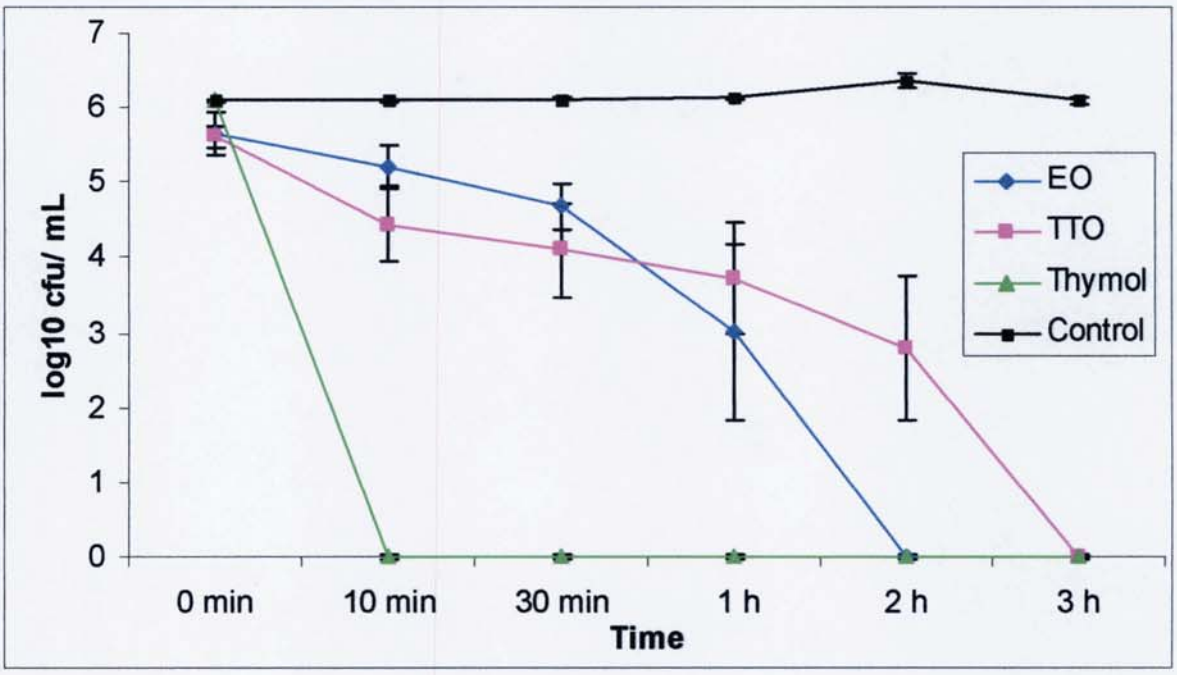

Figure 4.5. Time-kill assay of EO, TTO and thymol against *S. epidermidis* RP62A, TK1 and TK2 in a planktonic mode of growth (the results are expressed as mean $\log_{10}$ cfu/ mL ± SEM). The assay was performed in triplicate (the cultures were incubated in air at 37°C in rotary incubator with 150 rpm).

4.4.5.3. Time-kill assay of EO, TTO, thymol and CHG against S. epidermidis in a biofilm mode of growth

Thymol demonstrated the greatest antimicrobial efficacy of the three essential oils (i.e. thymol, TTO and EO) against *S. epidermidis* RP62A, TK1 and TK2 in a biofilm, reducing the ATP luminescence from the bacterial biofilms to below negligible levels within 30 min (Table 4.4.). For CHG, this was achieved within 8 h.

Table 4.4. Time-kill assay of EO, TTO, thymol and CHG against *S. epidermidis* RP62A, TK1 and TK2 in a biofilm mode of growth. The assay was performed in duplicate on pre-formed (48 h) bacterial biofilms. The bacterial viability was evaluated using the ATP bioluminescence method.

Isolate	**EO**	**TTO**	**Thymol**	**CHG**
RP62A	12 h	12	30 min	8 h
TK1	>12 h	> 12 h	30 min	8 h
TK2	12	> 12 h	30 min	8 h

4.5.4. Checkerboard assay to assess the antimicrobial activity of CHG in combination with EO, TTO and thymol against *S. epidermidis*

In the initial experiments, TTO, EO and thymol were the most efficacious essential oils against the Gram-positive and Gram-negative bacteria and *Candida*, and were therefore further investigated for their antimicrobial activity in combination with CHG. Combinations of CHG with TTO, EO and thymol demonstrated indifferent activity (i.e. no synergy or antagonism) against *S. epidermidis* RP62A and TK1 when grown in a suspension (Table 4.5). In a biofilm,

CHG in combination with EO demonstrated synergistic activity against both isolates of *S. epidermidis*, with FICI of 0.156 and 0.188 for *S. epidermidis* RP62A and TK1 respectively (Table 4.6.).

Table 4.5. The antimicrobial activity of aqueous CHG in combination with EO, TTO or thymol against clinical isolate TK1 and RP62A of *S. epidermidis* grown in a planktonic mode of growth. The assay was performed in duplicate by the checkerboard method. The results are expressed as Fractional Inhibitory Concentration (FIC) and FIC Index (FICI), with FICI ≤ 0.5 regarded as synergistic activity, FICI > 0.5 to 4.0 as indifferent activity and FICI > 4.0 as antagonistic activity.

Isolate	*Antimicrobial combination*	*MIC of essential oil (mg/ mL) in combination/ alone*	*FIC of essential oil*	*MIC of CHG (µg/ mL) in combination/ alone*	*FIC of CHG*	*FICI*	*Result*
RP62A	CHG + Eucalyptus	4 / 4	1	2/ 2	1	2	Indifference
	CHG + Tea tree oil	2 / 2	1	2/ 2	1	2	Indifference
	CHG + Thymol	1/ 4	0.25	2/ 2	1	1.25	Indifference
TK1	CHG + Eucalyptus	8 / 8	1	2 / 2	1	2	Indifference
	CHG + Tea tree oil	16 / 16	1	2 / 2	1	2	Indifference
	CHG + Thymol	0.25 / 0.5	0.50	2 / 2	1	1.50	Indifference

Table 4.6. The antimicrobial activity of aqueous CHG in combination with EO, TTO and thymol against clinical isolate TK1 and RP62A of *S. epidermidis* grown in a biofilm mode of growth. The assay was performed in duplicate with pre-formed (48 h) bacterial biofilms. The synergism was evaluated by the checkerboard method. The results are expressed as Fractional Inhibitory Concentration (FIC) and FIC Index (FICI), with FICI ≤ 0.5 regarded as synergistic activity, FICI > 0.5 to 4.0 as indifferent activity and FICI > 4.0 as antagonistic activity.

Isolate	Antimicrobial combination	MIC of essential oil (mg/mL) in combination/ alone	FIC of essential oil	MIC of CHG (µg/mL) in combination/ alone	FIC of CHG	FICI	Result
RP62A	CHG + Eucalyptus	4 / 32	0.125	0.25/ 8	0.031	0.156	Synergy
	CHG + Tea tree oil	4 / 16	0.25	4/ 8	0.5	0.75	Indifference
	CHG + Thymol	0.5 / 0.5	1	8/ 8	1	2	Indifference
TK1	CHG + Eucalyptus	4 / 64	0.063	1 / 8	0.125	0.188	Synergy
	CHG + Tea tree oil	16 / 16	1	8 / 8	1	2	Indifference
	CHG + Thymol	0.25 / 0.5	0.5	4 / 8	0.5	1	Indifference

4.6. Discussion

Essential oils have been widely studied for their antimicrobial activity and they have been used in many hygiene products (Cowan, 1999). They may also have an important role in preventing infections in healthcare environment, such as TTO in eradicating MRSA skin colonisation (Carson *et al.*, 1995). Therefore, in this study six essential oils (TTO, EO, thymol, juniper oil, citronella and lavender oil) were evaluated against a panel of skin microorganisms often associated with intravascular catheter related infections (CRI). Their activity was assessed alone and in combination with CHG, the agent currently recommended for skin antisepsis prior to intravascular catheter insertion, against microorganisms in a planktonic and a biofilm mode of growth.

This study demonstrates that TTO, EO, thymol, juniper oil, citronella and lavender oil have antimicrobial activity against *Staphylococcus spp.*, *P. acnes*, *Acinetobacter spp.*, *Ps. aeruginosa* and *C. albicans*. Of these six essential oils EO, TTO and thymol were the most efficacious demonstrating antimicrobial activity against Gram-positive and Gram-negative bacteria and *Candida* in planktonic cultures, and against *S. epidermidis* in a biofilm, which is in line with other studies (Brady *et al.*, 2006, Hammer *et al.*, 1996, Hammer *et al.*, 1999, Loughlin *et al.*, 2008). However, the concentration of essential oils required to achieve the same level of growth inhibition as CHG is several orders of magnitude higher (mg/ mL for essential oils compared to µg/ mL for CHG). Of the all essential oils assessed, thymol was the most efficacious. Interestingly, a higher concentration of thymol was required to eliminate *S. epidermidis* growing in a planktonic culture compared to the biofilm. This is an unusual finding, as biofilms are

considered more resistant to antimicrobial agents compared to microorganisms in a planktonic mode of growth (Saginur *et al.*, 2006). Partitioning of the oil, especially pure thymol, in the oil suspension as well as in the extracellular matrix of bacterial biofilm, may alter thymol activity which may account for this unusual finding. Indeed, in a previous study by Nostro and colleagues (2007) only small differences in susceptibility to thymol between biofilm and planktonic cultures of *Staphylococcus spp.* was demonstrated. Furthermore, in the study by Al-Shuneigat *et al.* (2005) *Staphylococcus spp.* in a biofilm mode of growth demonstrated increased susceptibility to an essential oil based formulation compared to cells in a planktonic phase of growth which concurs with our findings in relation to thymol. Thymol is a phenolic compound that has both hydrophilic and hydrophobic properties, which may enhance diffusion of this compound in a biofilm and allowing its access to bacterial cells where it alters permeability of plasma membranes (Nostro *et al.*, 2007). Furthermore, thymol demonstrated rapid antimicrobial activity against *S. epidermidis* in both a suspension and in a biofilm (< 10 min and 30 min respectively); CHG reduced the level of viable cells in the biofilms of *S. epidermidis* to below detection only after 8 h.

Combining CHG with TTO, EO and thymol did not improve its antimicrobial activity against *S. epidermidis* TK1 and RP62A isolates in a planktonic phase of growth; however, reductions in CHG and EO concentrations required to inhibit growth of both *S. epidermidis* isolates in biofilm were observed. Of the three essential oils used in this investigation, EO demonstrated the best potential for combination with CHG. Synergistic activity between EO and CHG was demonstrated against biofilms of both isolates of *S. epidermidis* (FICI 0.188 and 0.156 for TK1

and RP62A respectively). To our knowledge, this is the first report of synergism between EO and CHG.

Previous research that has investigated the synergistic activity of an essential oil and antimicrobial agent has suggested the synergism may be due to their action on both different (Fyfe *et al.*, 1997) or similar targets on the bacterial cells (ie. cell membranes) (Filoche *et al.*, 2005). Eucalyptus oil, and its main component 1,8-cineole, are thought to act on the plasma membrane of microorganisms, the same target as CHG. However, TTO (and its main antimicrobial component terpinen-4-ol) and thymol also have lipophilic properties and target cellular membranes, without showing synergy in combination with CHG. Therefore, the interaction of EO with CHG requires further research to fully establish the mode of action of the potential synergism. It is possible that not only one component is involved in the synergistic interaction between EO and CHG, but a mixture of several components. Moreover, it has been suggested that cationic CHG diffusion in the biofilm is hindered by the negatively charged extracellular matrix, changing the physicochemical properties of the extracellular matrix and its tertiary structure (Hope and Wilson, 2004). Chlorhexidine is likely to remain in the aqueous phase in the oil suspension [LogP of CHG 0.037 (Farkas *et al.*, 2007)]; both EO, which consists of several heterogeneous compounds, and CHG have hydrophilic and hydrophobic properties, and it may be possible that they alter ionic interactions in the extracellular matrix of the biofilm, as well as acting on the same target on the bacterial cell. However, further studies are needed to establish the mode of action of EO and CHG in combination.

The use of essential oils for the prevention and treatment of infection has been gaining popularity within the research field over the past decade (Sherry *et al.*, 2001b, Sherry *et al.*, 2004, Sherry *et al.*, 2003, Warnke *et al.*, 2006). Furthermore, the antimicrobial activity of TTO (Caelli *et al.*, 2000, Dryden *et al.*, 2004), thymol (Nostro *et al.*, 2007) and EO (Cimanga *et al.*, 2002) has been reported against several important pathogens. However, there has been little research to assess the efficacy of essential oils in combination with CHG against *S. epidermidis,* which is the major microorganism associated with skin related HAI.

Chlorhexidine is widely used as a skin antiseptic within the clinical setting and is the recommended antimicrobial within the EPIC and HICPAC guidelines (O'Grady *et al.*, 2002, Pratt *et al.*, 2007). However, infection rates associated with surgical incision of the skin remain high (HPA, 2007). The current strategies adopted for skin antisepsis therefore need to be considered with a view for improvement. The antimicrobial activity of CHG alone *in vivo* is reported as being bacteriostatic (Beighton *et al.*, 1991) and may be one factor which contributes to the survival of *S. epidermidis* within the skin following antisepsis and its association with subsequent infection. Furthermore, sub-inhibitory concentrations of chlorhexidine may increase a biofilm-mode of growth of *Staphylococcus spp.* (Houari and Di Martino, 2007), which may reduce the efficacy of skin antisepsis if inappropriate levels of antiseptic are used. The synergistic action of CHG in combination with EO may therefore be one way forward for enhancing both skin antisepsis and potentially disinfection of hard surfaces. The environment in the healthcare setting contributes to the spread of pathogens and transfer of microorganisms between patients and healthcare workers (Boyce, 2007). Microorganisms may reside on surfaces in aggregates embedded in a biofilm rendering them less susceptible to cleaning and disinfection. Furthermore,

many medical devices such as central venous catheter hubs and needleless connectors also become colonised with microorganisms capable of producing a biofilm (Casey *et al.*, 2003). At present chlorhexidine-based compounds or isopropyl alcohol (IPA) are commonly used for disinfecting these medical devices prior to use. The synergistic activity between CHG and EO in combination may therefore be of benefit in the clinical setting, for example, in improved skin antisepsis and the elimination of *S. epidermidis* existing as microcolonies which are likely to exhibit increased resistance to CHG alone, and also potentially hard surface disinfection.

4.7. Conclusion

Tea tree oil, EO and thymol are effective antimicrobial agents against microorganisms associated with CRI. Furthermore, EO demonstrates synergistic antimicrobial activity in combination with CHG against *S. epidermidis* biofilms, which may be beneficial in the clinical environment eradicating microbial biofilms in medical devices and hard surfaces and in improved skin antisepsis and the elimination of *S. epidermidis* existing as microcolonies on and within the skin. However, whilst much of the research data advocates the potential use of essential oils in the clinical setting for preventing and treating infection there is little information regarding safety in relation to their use, which needs to be taken into consideration. Therefore further studies are warranted.

5. CHAPTER: PERMEATION OF AQUEOUS AND ALCOHOLIC CHLORHEXIDINE INTO EXCISED HUMAN SKIN ALONE AND IN COMBINATION WITH EUCALYPTUS OIL

5.1. Introduction

The previous research in this thesis has shown that whilst chlorhexidine has a broad spectrum of antimicrobial activity, its penetration into excised human skin is poor. Furthermore, in the previous chapter, it was demonstrated that EO exhibits synergistic antimicrobial activity with chlorhexidine against biofilms of *S. epidermidis*. Many essential oils, including EO, contain terpenes, which have skin penetration enhancing properties (Aqil *et al.*, 2007). Indeed, EO has previously been shown to be an effective penetration enhancer (Biruss *et al.*, 2007) and a constituent terpene, 1,8-cineole, has been shown to enhance skin penetration of both lipophilic and hydrophilic compounds (Narishetty and Panchagnula, 2005, Yamane *et al.*, 1995, Femenia-Font *et al.*, 2005). With its broad spectrum of antimicrobial activity and synergy in combination with CHG this naturally occurring essential oil may serve as a potential candidate for improving the delivery of CHG within the skin and increasing the concentration of antiseptic achieved within the deeper layers.

5.2. Aims of this study

The aim of this current study was to evaluate the skin permeation of CHG and retention of CHG at the increasing depths of excised human skin following exposure to 2% (w/v) CHG in 70% (v/v) IPA and in combination with varying concentrations of EO.

5.3. Materials

5.3.1. Chemicals

Isopropyl alcohol was purchased from Sigma-Aldrich, UK, and the other chemicals used in this study are described in sections 2.3.1. and 4.3.1.

5.3.2. Equipment

The high performance liquid chromatography equipment and the cryotome are described in 2.3.2.

5.3.3. Skin samples

The human skin samples and the ethical committee approval are as described in 2.3.4. The full thickness human skin was frozen on the day of excision and stored at -70˚C until required.

5.4. Methods

5.4.1. Skin permeation of 2% (w/v) CHG with 5%, 10%, 20% and 50% (v/v) EO and 70% (v/v) IPA

Skin permeation studies were performed as described in 3.4.3. Twenty percent (w/v) aqueous CHG was diluted with distilled water, IPA and EO to obtain the final concentrations of 2% (w/v) CHG in 70% (v/v) IPA and 2% (w/v) CHG with 5%, 10%, 20% and 50% (v/v) EO. Tween 80 [0.1% (v/v)] was added to the test solutions to enhance EO solubility in the vehicle. One millilitre of test solution was spread over the skin surface in the donor compartment and the receptor fluid was sampled every 30 min for 2 h, every hour between 2 to 6 h and at 8 h, 12 h and 24 h. The samples were analysed for CHG by HPLC (as described in 2.4.4.1.). The assay was performed in triplicate.

5.4.2. CHG penetration profile studies with 2% (w/v) CHG with 5%, 10%, 20% and 50% (v/v) EO and 70% (v/v) IPA

Chlorhexidine penetration into a full thickness human skin was evaluated as described in 3.4.4. The excised full thickness human skin samples mounted onto the Franz diffusion cells were exposed to 2% (w/v) CHG in 70% (v/v) IPA and 2% (w/v) CHG with 50% (v/v) EO (both with 0.1% (v/v) Tween 80) for 2 min, 30 min and 24 h. Two percent (w/v) CHG with 20%, 10% and 5% (v/v) EO with 0.1% (v/v) Tween 80 were evaluated for CHG penetration following 24 h

exposure. Skin samples were serially sectioned with a cryomicrotome and the CHG was extracted from the skin sections as described in 2.4.4.2. The concentration of CHG (μg/ mg of skin) in the skin sections was determined by HPLC as described previously in section 2.4.4.3. Control skin (skin without treatment) was analysed parallel to the test samples. The assay was performed in triplicate.

5.4.3. Statistical analysis

The data obtained were analysed by a student t-test using INSTAT3 software (Graph pad software version 3.06) with a $p < 0.05$ level of significance.

5.5. Results

5.5.1. Skin permeation of 2% (w/v) CHG with 5%, 10%, 20% and 50% (v/v) EO and 70% (v/v) IPA

Chlorhexidine was not detected in the receptor compartment (LOD 0.0157 μg/ mL) during the 24 h permeation study. Negligible levels of CHG (< 0.0016 % of the applied dose) were detected in the receptor compartment during the permeation studies in one out of the three donor skin following 24 h exposure to 2 % (w/v) CHG in 70% (v/v) IPA and 2% (w/v) CHG with 50 % (v/v) EO.

5.5.2. CHG skin penetration profile studies with 2% (w/v) CHG with 5%, 10%, 20% and 50% (v/v) EO and 70% (v/v) IPA

5.5.2.1. CHG skin penetration following 2 min and 30 min exposure

The concentration of CHG which penetrated and was retained within the skin was significantly higher following treatment with CHG in combination with 50% (w/v) EO compared to CHG in aqueous solution (section 3.5.4.) or CHG with 70% (v/v) IPA; after 2 min exposure there was a significant difference in mean CHG concentrations at the deeper layers of the skin when exposed to CHG with 50% (v/v) EO compared to aqueous CHG (300 to 1500 μm depths; $p< 0.05$; Figure 5.1.) with a mean concentration of CHG (and SEM) within the tissues at this depth of 0.0270 μg/ mg (± 0.0021 μg/ mg) tissue following combined antisepsis with 2% (w/v) CHG/ 50% (v/v) EO

and 0.0048 µg/ mg (± 0.0008 µg/ mg) tissue following antisepsis with 2% (w/v) CHG alone. The difference between 2% (w/v) CHG/ 50% (v/v) EO and 2% (w/v) CHG in 70% (v/v) IPA were significant at all skin depths with a mean CHG concentration (and SEM) in the top 100 µm 0.1167 (± 0.0313) and 0.0226 (± 0.007) for 2% (w/v) CHG/ 50% (v/v) EO and 2% (w/v) CHG/ 70% (v/v) IPA respectively (Figure 5.1).

At 30 min the concentrations of CHG at the all skin depths were significantly higher ($p<0.05$) with 2% (w/v) CHG/ 50% (v/v) EO compared to 2% (w/v) CHG alone or 2% (w/v) CHG/ 70% (v/v) IPA; at the depths of 300 µm to 1500 µm CHG concentration increased >9.5-fold between 2% (w/v) CHG/ 50% (v/v) EO and 2% (w/v) CHG or 2% (w/v) CHG/ 70% (v/v) IPA, with mean CHG concentration (and SEM) of 0.0190 (± 0.0015) µg/mg tissue, 0.0021 (± 0.0004) µg/mg tissue and 0.0022 µg/mg (± 0.0018) tissue for 2% (w/v) CHG/ 50% (v/v) EO, 2% (w/v) CHG and 2% (w/v) CHG/ 70% (v/v) IPA respectively (Figure 5.2.). Within the top layers of skin (0-300 µm) the mean CHG concentrations were 4.8 to 6.4-fold higher with 2% (w/v) CHG/ 50% (v/v) EO compared to 2% (w/v) CHG alone and 2.7 to 20- fold higher with 2% (w/v) CHG/ 50% (v/v) EO compared to 2% (w/v) CHG/ 70% (v/v) IPA.

5.5.2.2. CHG skin penetration following 24 h exposure

The concentration of CHG extracted from all layers of the skin (0–1500 µm) was significantly higher in the presence of 50% (v/v) EO compared to CHG alone after 24 h permeation ($p< 0.05$; Figure 5.3.); the top 100 µm layer had a >2-fold increase (7.880 µg to 16.841 µg per mg tissue for CHG and CHG/ EO treatments respectively) in the CHG concentration and the difference increased >5-fold within the deeper layers of the skin at 300 to 1500 µm depths [0.581 (± 0.0466)

to 3.123 (± 0.16470) µg per mg tissue for CHG and CHG/EO respectively]. Data from this retention study were achieved by pooling together the data from five consecutive 20-µm and 30-µm skin sections (i.e. 100-µm sections from top to 600 µm depth and 150-µm sections from 600 to 1500 µm depth).

5.5.2.3. CHG skin penetration following 24 h exposure to 2% (w/v) CHG with 5%, 10%, 20% and 50% (v/v) EO and 70% (v/v) IPA

The optimum concentration of EO which enhances CHG penetration into the skin was evaluated (Figure 5.4.). Five percent (v/v) EO enabled significantly greater CHG skin penetration at the deeper layers of the skin (below 300 µm $p < 0.05$) and 10% (v/v) EO significantly enhanced CHG skin penetration ($p < 0.05$) within the top 900 µm compared to CHG alone. There were no significant differences ($p > 0.05$) in the skin penetration of CHG from aqueous 2% (w/v) CHG and 2% (w/v) CHG with 70% (v/v) IPA.

The optimum EO concentration, which enhanced CHG penetration into the full thickness human skin, was further evaluated in combination with 2% (w/v) CHG in 70% (v/v) IPA. Ten percent (v/v) EO in combination with 2% (w/v) CHG in 70% (v/v) IPA demonstrated enhanced CHG skin penetration after 2 min and 30 min exposure compared to 2% (w/v) CHG/ 70% (v/v) IPA alone ($p < 0.05$; Figures 5.5. and 5.6.).

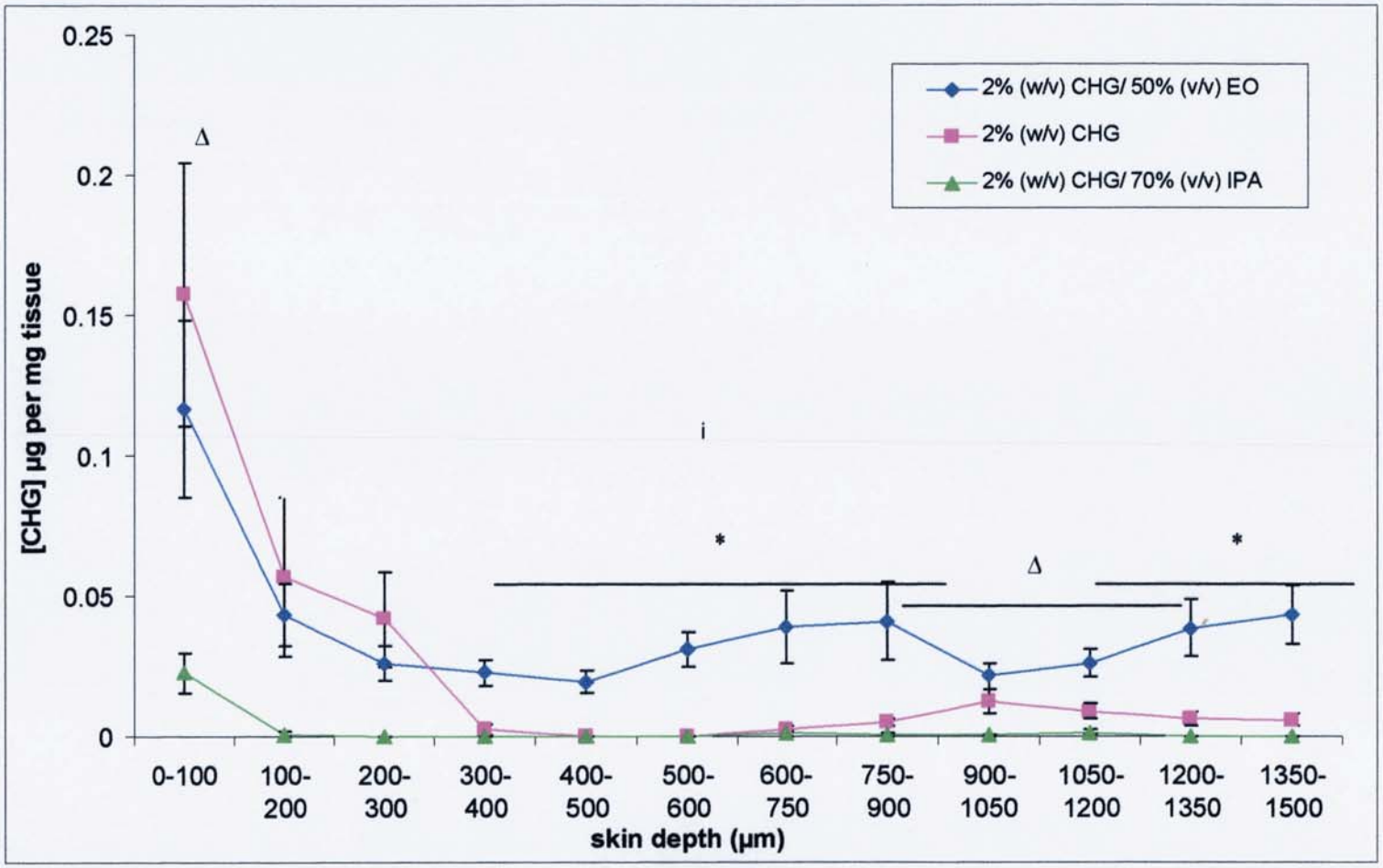

Figure 5.1. Penetration profile showing the location and concentration of CHG (µg/ mg tissue) in excised human skin after 2 min exposure to 2% (w/v) CHG with 50% (v/v) EO and 70% (v/v) IPA (mean ± SEM, n= 15) ($p< 0.05$ at all depths between CHG/EO and CHG/IPA; * $p< 0.05$ between CHG/EO and CHG; Δ $p< 0.05$ between CHG and CHG/IPA).

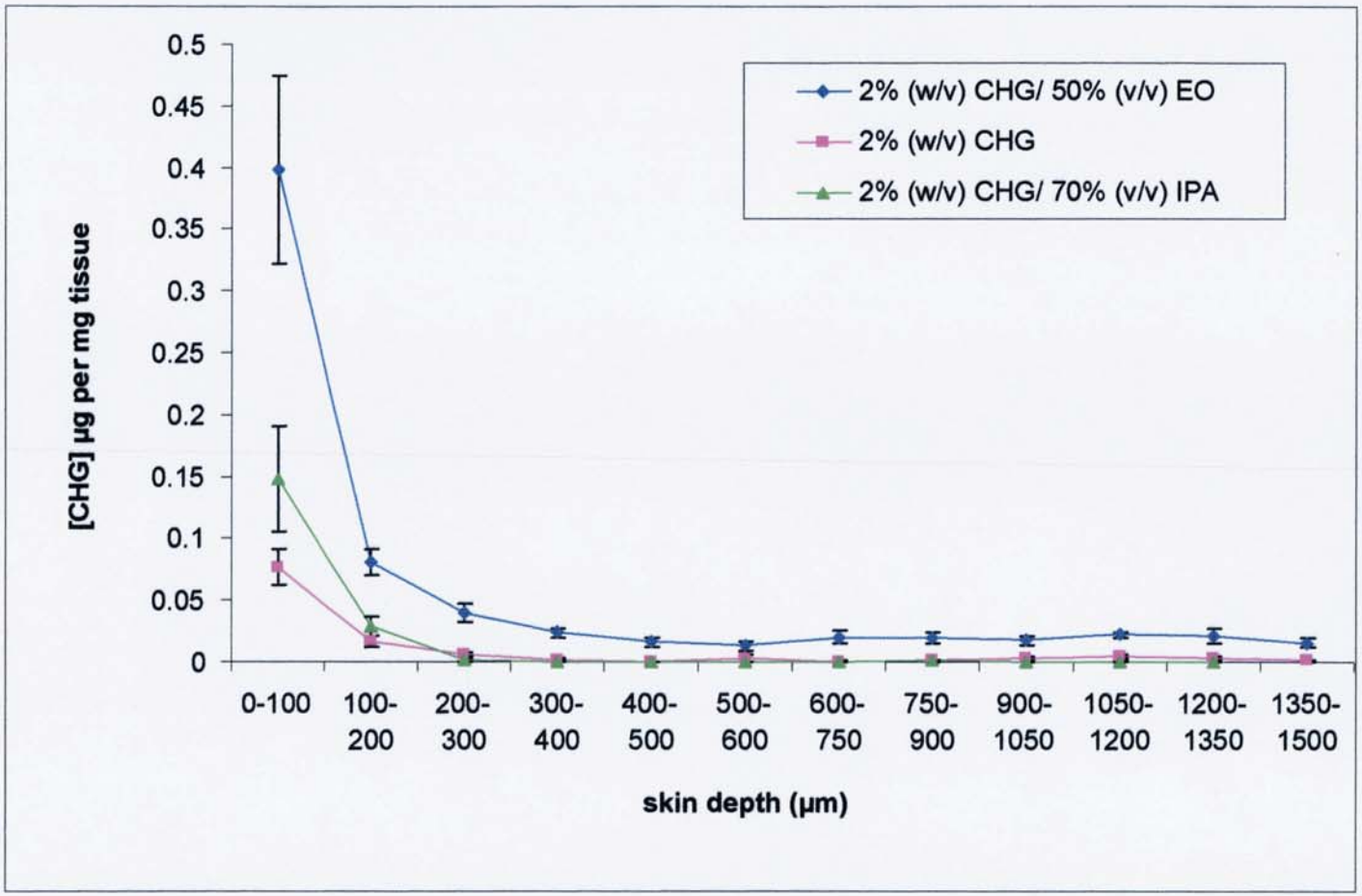

Figure 5.2. Penetration profile showing the location and concentration of CHG (µg/ mg tissue) in excised human skin after 30 min exposure to 2% (w/v/) CHG with 50% (v/v) EO and 70% (v/v) IPA (mean ± SEM, n= 15) ($p < 0.05$ at all skin depths between CHG/EO and CHG or CHG/IPA).

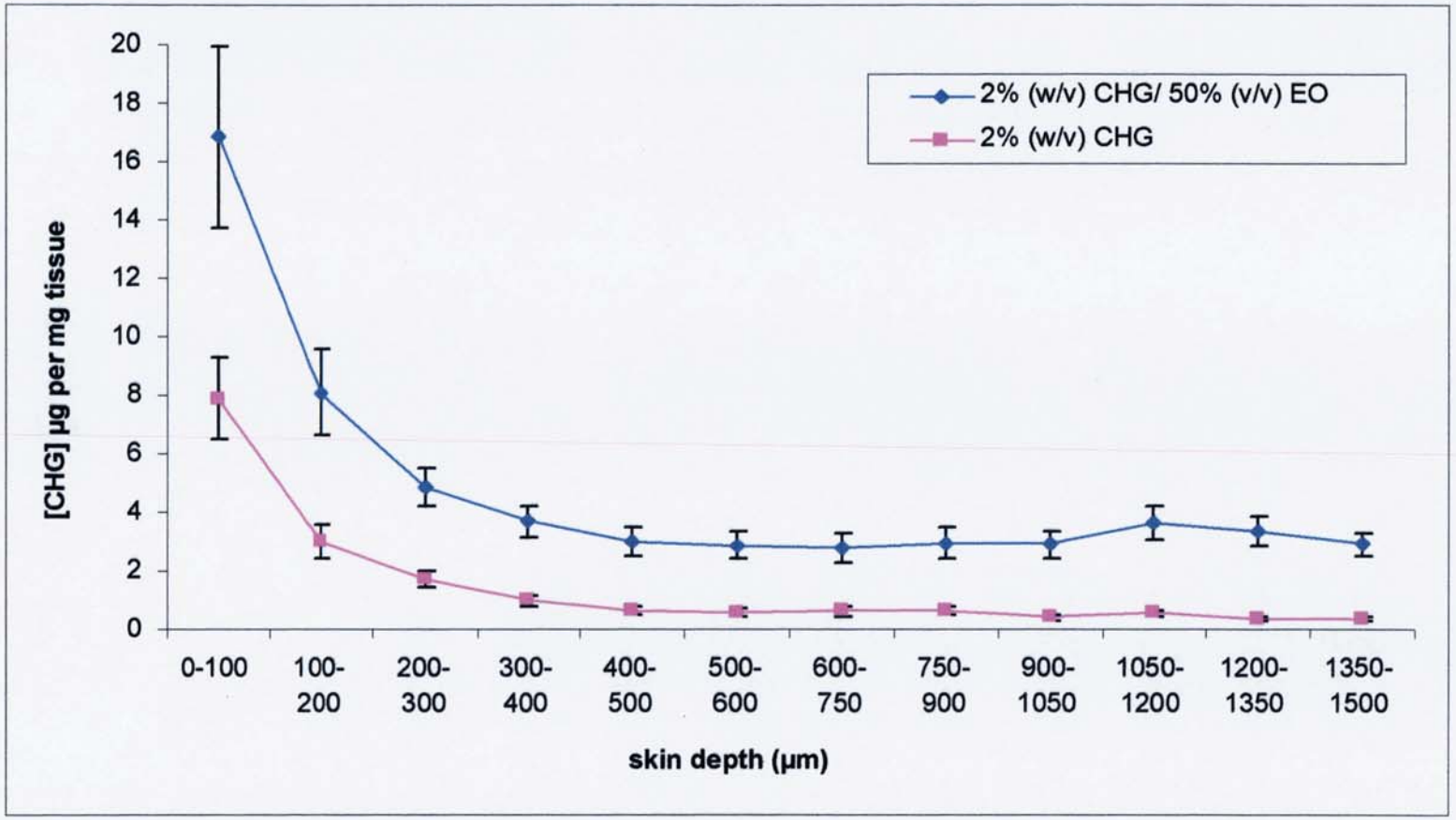

Figure 5.3. Penetration profile showing the location and concentration of CHG (µg/ mg tissue) in excised human skin after 24 h exposure to 2% (w/v) CHG with and without 50% (v/v) eucalyptus oil (mean ± SEM, n= 30) ($p < 0.05$ at all skin depths).

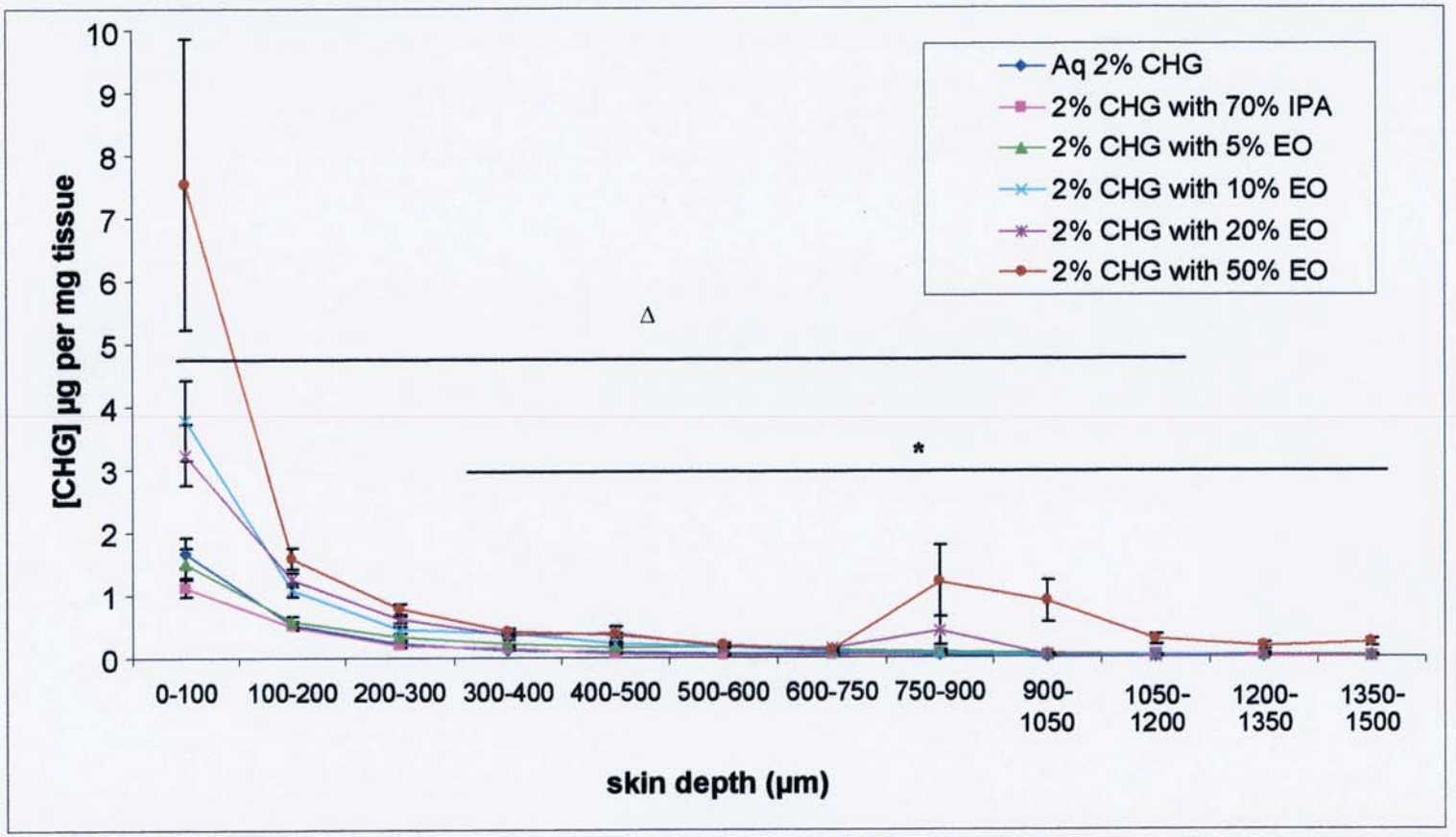

Figure 5.4. Penetration profile showing the location and concentration of CHG (µg/ mg tissue) in excised human skin after 24 hr exposure to 2% (w/v) aqueous CHG, 2% (w/v) CHG with 70% (v/v) IPA, and 2% (w/v) with various concentrations of EO (mean ± SEM, n= 15) [*p< 0.05 between 5% (v/v) CHG/EO and CHG; Δ p< 0.05 between 10% (v/v) CHG/EO and CHG].

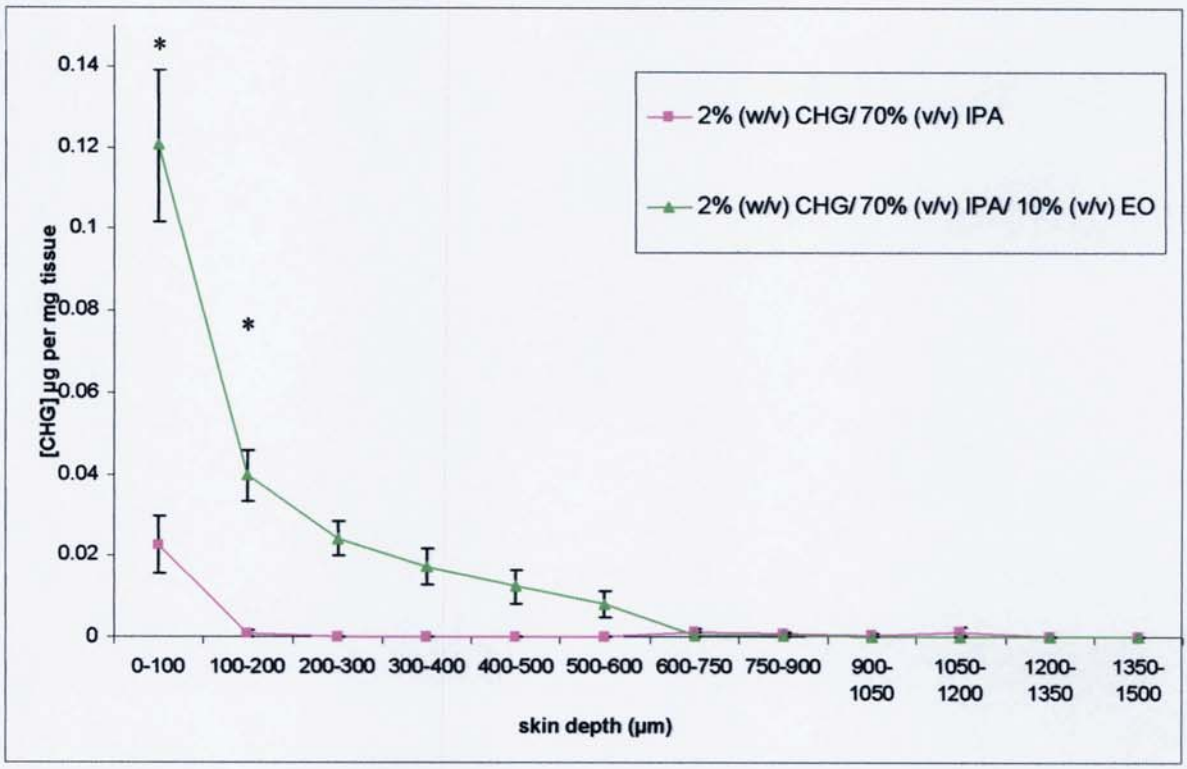

Figure 5.5. Penetration profile showing the location and concentration of CHG (µg/ mg tissue) in excised human skin after 2 min exposure to 2% (w/v) CHG/ 70% (v/v) IPA (n= 15) and 2% (w/v) CHG/70% (v/v) IPA with 10% (v/v) EO (n= 10 ; mean ± SEM)(* p< 0.0001).

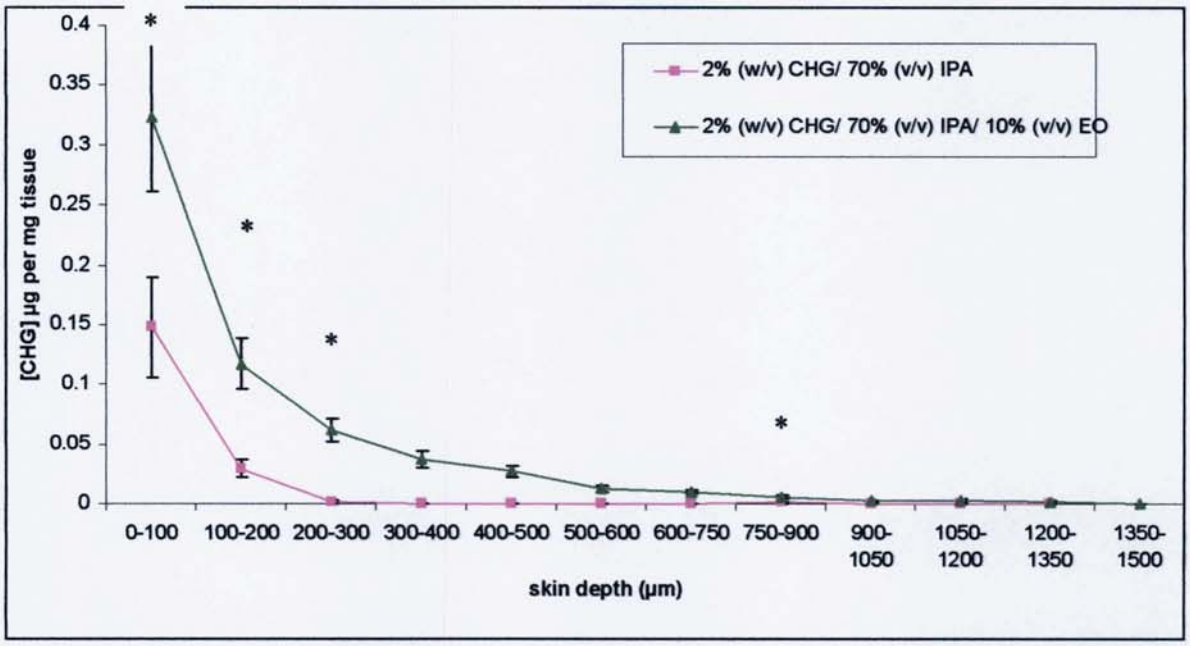

Figure 5.6. Penetration profile showing the location and concentration of CHG (µg/ mg tissue) in excised human skin after 30 min exposure to 2% (w/v) CHG/ 70% (v/v) IPA and 2% (w/v) CHG/ 70% (v/v) IPA with 10% (v/v) EO (n= 15; mean ± SEM) (* $p < 0.05$).

5.6. Discussion

In this current study the permeation of CHG within an excised human skin model was clearly enhanced when applied in combination with EO. Indeed, after a 2 min application of CHG with EO, the concentration of CHG was significantly higher at skin depths of 300 μm to 1500 μm when compared to CHG alone and at all depths compared to CHG in alcoholic solution. Furthermore, following 30 min and 24 h exposure to CHG with EO, the levels of CHG were significantly higher at all skin layers compared to aqueous CHG alone or in IPA. The results of this investigation demonstrate that EO enhances the permeation of CHG within the skin which suggests that combination antisepsis with CHG and EO may be one way forward in improving skin antisepsis in clinical practice. In addition, negligible levels of CHG were detected in the receptor compartment of the Franz diffusion cell suggesting that CHG does not permeate through the full thickness of excised skin and is retained within the tissue. These results concur with previous research on another CHG based compound, chlorhexidine phosphanilate, which was shown not to permeate through the full thickness of skin (Wang *et al.*, 1990).

The minimum concentration of EO required to enhance CHG skin penetration was also investigated in this part of the study; five percent (v/v) EO yielded greater CHG skin penetration at the deeper layers of the skin (below 300 μm $p< 0.05$) and 10% (v/v) EO significantly enhanced CHG skin penetration ($p< 0.05$) within the top 900 μm compared to CHG alone. There was no significant difference in CHG permeation in the skin using 20% (v/v) EO with 2% (w/v) CHG compared to 10% (v/v) EO with 2% (w/v) CHG.

It is tempting to speculate that the main skin permeation enhancing compound in the crude EO is the terpene 1,8-cineole, which has been studied for its activity on skin permeation. 1,8-cineole has been estimated to account for 44.3% to 80% of the crude EO extracted from *E. globulus* leaves (Cimanga *et al.*, 2002, De Vincenzi *et al.*, 2002, Sacchetti *et al.*, 2005). This compound is a monoterpene cyclic ether (Aqil *et al.*, 2007, De Vincenzi *et al.*, 2002) that has been previously been shown to enhance skin penetration of sumatriptan succinate (Femenia-Font *et al.*, 2005), zidovudine (Narishetty and Panchagnula, 2005) and 5-fluorouracil (Yamane *et al.*, 1995).

Terpenes, including 1,8-cineole, have been shown to bind in large quantities to SC (Cornwell *et al.*, 1996, Cal *et al.*, 2001). They are thought to enhance lipophilic drug penetration by increasing the partition coefficient (partitioning of drug between vehicle and SC), and hydrophilic drug penetration through increasing the diffusion coefficient (Cal *et al.*, 2001). Terpene 1,8-cineole has been found to increase skin permeation by disrupting intercellular lipids in SC (Cornwell *et al.*, 1996), which has been demonstrated by differential scanning calorimetry (Yamane *et al.*, 1995, Narishetty and Panchagnula, 2005), attenuated total reflection Fourier Transform Infrared spectroscopy (Narishetty and Panchagnula, 2005) and Raman spectroscopy (Williams *et al.*, 2006). The terpene 1,8-cineole has been shown to change SC membrane fluidity at the concentrations as low as 1% (Anjos *et al.*, 2007) to 5% (Gao and Singh, 1997). Yamane *et al.* (1995) showed that effect of lipid disruption was reversible, i.e. 1,8-cineole did not extract lipids from the SC. The study by Williams *et al.* (2006) also showed that the 1,8-cineole partitioning in the skin lipids is heterogeneous causing ordered and disordered areas in SC lipids. Furthermore, it has been shown that cineole does not permeate through the skin *in vitro* assays, but is retained in the skin (Cal *et al.*, 2006).

The skin penetration of CHG in 70% (v/v) IPA, the recommended agent to be used prior to CVC insertion and during the care of intravascular catheters in clinical guidelines (Pellowe et al., 2005), and CHG in 70% (v/v) IPA in combination with 10% (v/v) EO were also evaluated. Alcoholic solutions of CHG have been shown to have superior antimicrobial activity compared to 2% (w/v) aqueous CHG (Adams *et al.*, 2005, Hibbard *et al.*, 2002) however their efficacy in reducing CRI and intravascular catheter colonisation are comparable (Valles *et al.*, 2008). This study demonstrates that CHG from 70% (v/v) alcohol solution has poor permeation within the skin, and is comparable to 2% (w/v) CHG in aqueous solution. Alcohol, at high concentrations, has a rapid and broad spectrum of antimicrobial activity (Adams, 2006), but it has been shown to extract SC lipids (Van der Merwe and Riviere, 2005, dos Anjos *et al.*, 2007) and dehydration of SC proteins, therefore increasing the SC penetration barrier (Amini, 2001). Combining 2% (w/v) CHG with 70% (v/v) IPA and 10% (v/v) EO showed significantly higher CHG skin penetration compared to CHG/IPA alone. Previous studies on terpenes in combination with ethanol have shown increased skin permeation (Heard *et al.*, 2006), however at the high concentrations of alcohol the permeation was decreased (Obata *et al.*, 1991).

A two minute contact of the skin to the antiseptic agent is recommended prior to the invasive procedures, which is assumed to significantly decrease the level of microbial colonisation and help in prevention of infections associated with invasive procedures. However, permeation of many antimicrobial agents is poor below the surface of the skin (Hendley and Ashe, 1991) and therefore microorganisms may persist at the site of incision following the skin antisepsis procedure. Furthermore, Hendley and Ashe (1991) found that skin becomes re-colonised within 18 hours following removal of microorganisms from the site, suggesting a microbial reservoir

may reside in the deeper layers of the skin and sebaceous glands. Rapid permeation of the antiseptic agent into the deeper layers of the skin is therefore essential in preventing infection. These results clearly indicate that the combination of EO and CHG at the skin surface increases the concentration of CHG within the deeper layers which may therefore aid in preventing infection and microbial re-colonisation of the skin in clinical practice following invasive procedures.

In this study the skin permeation of CHG was evaluated down to the depth of 1500 μm. As expected, the permeation pattern through the full thickness skin was not linear, due to variations in structure of the various layers of the skin. The top 100 μm layer of the skin, which contains SC and other epidermal layers (Waller and Maibach, 2005), contained the highest amount of CHG following both treatments and at all time points. Previous research has shown that the main permeation barrier for skin absorption is the SC (Cal *et al.*, 2001, Wang *et al.*, 1990), which is thought to be due to high lipid matrix and packed layers of keratinised epithelial cells.

Concentrations of EO between five to 50% (v/v) (45.5 mg/ mL to 454.5 mg/ mL) were assessed in this current investigation. Ten percent EO in this study was shown to enhance the skin penetration of CHG, with significantly higher concentration recovered from the skin from surface to 900 μm depth compared to aqueous CHG; combination of 5% (v/v) EO with CHG enhanced skin penetration of CHG at the deeper layers of the skin (300- 1500 μm depth) compared to CHG alone. Previous studies have shown the skin permeation enhancing activity of EO at 45% (v/v) (Biruss *et al.*, 2007), however the potential for skin irritation at this concentration was not measured. Warnke *et al.* (2006) used an EO based ointment, containing 7% (w/w) EO and 15%

(w/w) of other essential oils, on head and neck necrotic ulcers, without skin irritation and ten percent (v/v) TTO has been used in topical skin preparations to reduce MRSA colonisation; however further studies are required to establish the skin tolerance of EO at 10% (v/v) in formulations.

5.7. Conclusion

In conclusion, this study demonstrated that 2% (w/v) CHG permeation into the deeper layers of skin was significantly enhanced with EO compared to CHG in aqueous solution or in 70% (v/v) IPA. At 10% (v/v), EO increased the skin penetration of CHG significantly compared to CHG in aqueous or alcoholic solutions, and at 5% (v/v) the CHG penetration was enhanced below skin depths of 300 and 400 μm compared to CHG and CHG/IPA respectively. Furthermore, 2% (w/v) CHG in combination with 70% (v/v) IPA and 10% (v/v) EO significantly increased the amount of CHG in the skin within 2 min compared to CHG/IPA. These preliminary results lay the foundation for further research within this area with a view to potentially adopting alternative strategies for enhanced skin antisepsis in clinical practice. However, further studies need to be undertaken to determine the skin tolerance of EO at 10% (v/v) concentration and skin penetration profiles of CHG from various solutions and co-enhancers.

6. CHAPTER: ANTIMICROBIAL ACTIVITY OF BENZYLIDENECARBOXAMIDRAZONES AND A THIOSEMICARBAZONE COMPOUNDS

6.1. Introduction

Resistance of microorganisms to antimicrobial agents including antibiotics and biocides is a major concern in the healthcare environment (Reynolds *et al.*, 2004). Reduced susceptibility of microorganisms to chlorhexidine has also been reported (Cookson *et al.*, 1991, Irizarry *et al.*, 1996). Furthermore, there are concerns of cross-resistance between antimicrobial agents and biocides (Smith *et al.*, 2008, Chapman, 2003). Therefore the search for new antimicrobial agents is warranted.

A team at Aston University, UK, has developed a novel benzylidenecarboxamidrazone compound, which has shown good antimicrobial activity against Gram-positive bacteria, including MRSA, and *Mycobacterium tuberculosis* (Rathbone *et al.*, 2006). Other carboxamidrazone compounds have also been shown to possess anti-malarial activity and tumour-suppressing activities (Gokhale *et al.*, 2001, Gokhale *et al.*, 2003). In addition, another group of antimicrobial compounds, the thiosemicarbazones, have demonstrated antibacterial and anti-parasitic activity (Aguirre *et al.*, 2004). Both of these groups of antimicrobial compounds, the carboxamidrazones and the thiosemicarbazones, therefore have the potential for therapeutic use; either for prevention of infections as an antiseptic agent or treatment of established infections.

6.2. Aims of this study

The aim of this study was to evaluate the antimicrobial activity of eight novel benzylidenecarboxamidrazones and a thiosemicarbazone compound against a panel of microorganisms commonly associated with skin and infections of the skin.

6.3. Materials

6.3.2. Chemicals

The microbiological culture media used in this part of study are described in section 3.3.1. Tween 80, DMSO, Triton X-100, sodium thioglycolate, L-cysteine and magnesium chloride were purchased from Sigma-Aldrich. Letheen broth was purchased from Difco (BD, Sparks, MD, USA). Ammonium dihydrogen orthophosphate and sodium metabisulphite were purchased form FSA Laboratory Supplies (Loughborough, UK), sodium thiosulphate pentahydrate from BDH Ltd (Poole, UK) and lecithin were purchased from Fisher Scientific (Loughborough, UK).

The lead novel benzylidenecarboxamidrazone compound (compound 1; Figure 6.1.) was previously prepared by Dr D L Rathbone (Pharmaceutical Sciences, Aston University); the benzylidenecarboxamidrazone analogues (compounds 2 to 8; Figures 6.2- 6.8) and the thiosemicarbazone (compound 9; Figure 6.9) were prepared by Ms L. Wheeldon (Life and Health Sciences, Aston University).

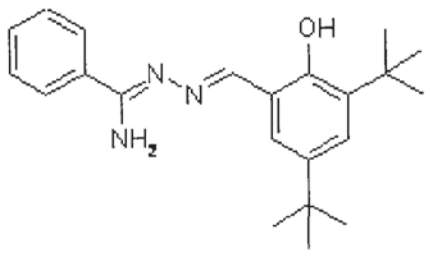

Figure 6.1. Chemical structure of compound 1, the lead benzylidenecarboxamidrazone compound, N^1-[3,5-di-(*tert*-butyl)-2-hydroxybenzylidene]-pyridine-2-carboxamidrazone

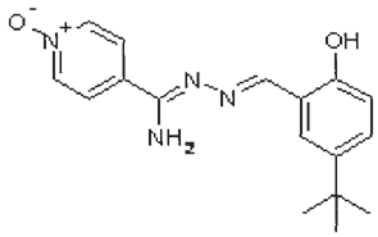

Figure 6.2. Chemical structure of compound 2, N^1-(2-Hydroxy-5-tert-butylbenzylidene)pyridine-4-carboxamidrazone-4-N-oxide.

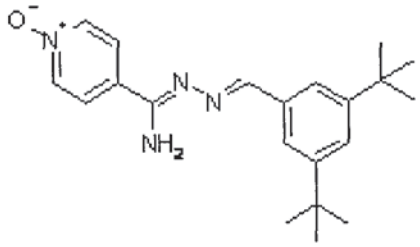

Figure 6.3. Chemical structure of compound 3, N^1-(3,5-Di-tert-butylbenzylidene)pyridine-4-carboxamidrazone-4-N-oxide.

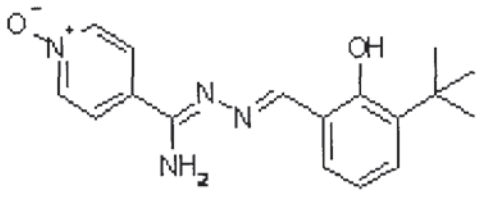

Figure 6.4. Chemical structure of compound 4, N^1-(2-Hydroxy-3-tert-butylbenzylidene)pyridine-4-carboxamidrazone-4-N-oxide.

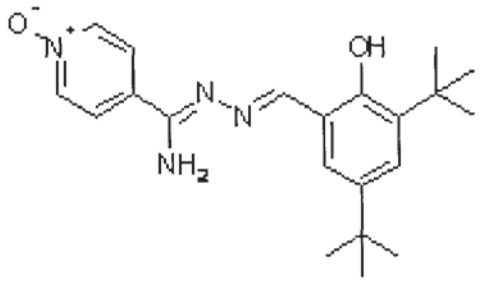

Figure 6.5. Chemical structure of compound 5, N^1-(3,5-Di-tert-butyl-2-hydroxybenzylidene)pyridine-4-carboxamidrazone-4-N-oxide.

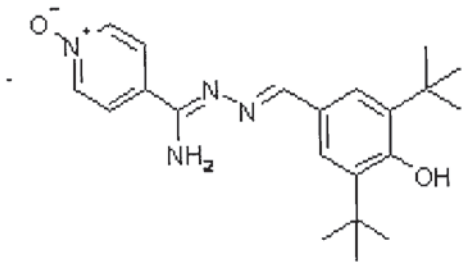

Figure 6.6. Chemical structure of compound 6, N^1-(3,5-Di-tert-butyl-4-hydroxybenzylidene)pyridine-4-carboxamidrazone-4-N-oxide.

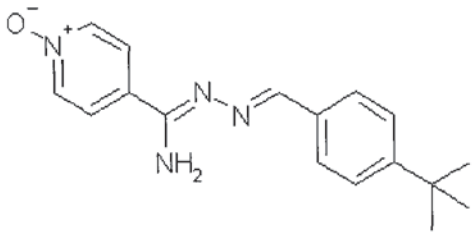

Figure 6.7. Chemical structure of compound 7, N^1-(4-tert-butylbenzaldehyde)pyridine-4-carboxamidrazone-4-N-oxide.

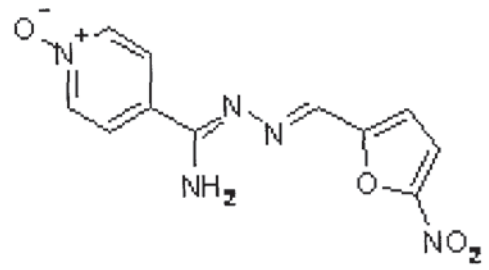

Figure 6.8. Chemical structure of compound 8, N^1-(5-Nitro-2-furylidene)pyridine-4-carboxamidrazone-4-N-oxide E/Z isomer mixture.

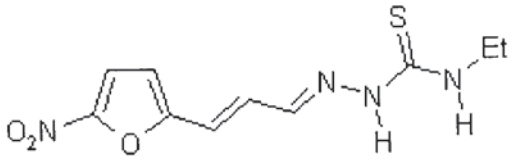

Figure 6.9. Chemical structure of compound 9, a thiosemicarbazone.

6.3.2. Microbial isolates

Microbial isolates used in this study are described in section 3.3.3. The microorganisms were stored on MicroBank beads (Pro-Lab Diagnostics, UK) at -70°C until required.

6.4. Methods

6.4.1. Preparation of the microbial suspensions

The microbial suspensions were prepared as described in 3.4.1.1. and the final concentrations were adjusted to 1 x 10^7 cfu/ mL (time-kill assays), 1 x 10^6 cfu/ mL (the suspension assays) and 1 x 10^5 cfu/ mL (the agar diffusion assay).

6.4.2. Preparation of the stock solutions of thiosemicarbazone and benzylidenecarboxamidrazone compounds

The thiosemicarbazone (compound 9) and benzylidenecarboxamidrazone compounds (1-8) were dissolved in DMSO to obtain stock solution of 5.12 mg/ mL, and were further diluted with a suitable culture broth to obtain the final test solution of 512 μg/ mL.

6.4.3. Preliminary evaluation of the antimicrobial activity of thiosemicarbazone and benzylidenecarboxamidrazone compounds by the agar diffusion assay

The thiosemicarbazone and benzylidenecarboxamidrazone compounds were screened for antimicrobial activity by an agar diffusion assay as described in section 4.4.3. The concentration of each of the test compounds inoculated into the agar wells was 512 μg/ mL and the assay was performed in duplicate on each test microorganism.

6.4.4. Determination of MIC, MBC and MFC of thiosemicarbazone and benzylidenecarboxamidrazone compounds

Minimum inhibitory concentration, MBC and MFC of thiosemicarbazone and benzylidenecarboxamidrazone compounds were determined using a broth microdilution assay in line with NCCLS guidelines (NCCLS, 2000) as described in 2.4.3.2. Serial double dilutions of the test compounds ranging between 512 µg/ mL to 0.5 µg/ mL were performed in liquid culture media on 96-well round bottom microtitre plate. The antimicrobial activity of DMSO [ranging from 5% (v/v) to 0.015% (v/v)] was also assayed on a separate microtitre plate alongside the assay. The assay was performed in triplicate.

6.4.5. Evaluation of the antimicrobial activity of thiosemicarbazone and benzylidenecarboxamidrazone compounds in a time-kill assay

6.4.5.1. Evaluation of the neutraliser efficacy against thiosemicarbazone and benzylidenecarboxamidrazone compounds

The neutralising solutions were prepared as described in Table 6.1. and sterilised. The efficacy of a neutraliser to quench the antimicrobial activity of the test compounds was evaluated prior to the time-kill assay as described in 4.4.5.1. The neutraliser efficacy was assessed against the test compounds at the concentration of 512 µg/ mL.

Table 6.1. The composition of neutralising solutions tested against compound 8 and compound 9 and *S. epidermidis*.

Neutraliser number	Content	Reference
1	Letheen broth, 2% (v/v) Tween 80.	(Russell, 1999)
2	Letheen broth, 2% (v/v) Tween 80, 0.1% (v/v) Triton X-100, 0.5% (w/v) sodium thiosulphate.	(Adams, 2006)
3	12 mL (129 g) Tween 80, 2.5 mL of 40% (w/v) sodium metabisulphite, 1.569 g of sodium thiosulphate pentahydrate, 1 g sodium thioglycolate, 0.3 g L-cysteine, 0.4 g lecithin. Adjusted with distilled water to 100 mL and pH 7 (double strength).	(Espigares *et al.*, 2003)
4	12 mL (129 g) Tween 80, 2.5 mL of 40% (w/v) sodium metabisulphite, 1.569 g of sodium thiosulphate pentahydrate. Adjusted with distilled water to 100 mL and pH 7 (double strength).	(Espigares *et al.*, 2003)
5	12 mL (129 g) Tween 80, 2.5 mL of 40% (w/v) sodium metabisulphite, 1.569 g of sodium thiosulphate pentahydrate, 0.3 g L-cysteine. Adjusted with distilled water to 100 mL and pH 7 (double strength).	(Modified from Espigares *et al.*, 2003)
6	12 mL (129 g) Tween 80, 2.5 mL of 40% (w/v) sodium metabisulphite, 1.569 g of sodium thiosulphate pentahydrate, 0.4 g Lecithin. Adjusted with distilled water to 100mls and pH 7 (double strength).	(Espigares *et al.*, 2003)
7	12 mL (129 g) Tween 80, 2.5 mL of 40% (w/v) sodium metabisulphite, 1.569 g of sodium thiosulphate pentahydrate, 0.3 g L-cysteine, 0.4 g lecithin. Adjusted with distilled water to 100 mL and pH 7 (double strength).	(Modified from Espigares *et al.*, 2003)
8	6 mL Tween 80, 0.2 g L-histidine, 0.6 g lecithin. Adjusted with distilled water to 100 mL and pH 7 (double strength).	(Espigares *et al.*, 2003)
9	6 mL Tween 80, 6 g Saponin, 0.2 g L-histidine, 0.2 g L-cysteine. Adjusted with distilled water to 100mls and pH 7 (double strength).	(Espigares *et al.*, 2003)
10	Skimmed milk.	
11	Soya milk (Alpro light, with 1.5% fat).	
12	Letheen broth, 2% (v/v) Tween 80, 20 mM Magnesium Chloride.	
13	Letheen broth, 2% (v/v) Tween 80, 20 mM Ammonium Dihydrogen orthophosphate.	

6.4.5.2. Time-kill assay in a suspension

Antimicrobial agents demonstrating efficacy in the suspension assay (6.4.4) were further investigated in a time-kill assay. Thiosemicarbazone and benzylidenecarboxamidrazone were evaluated against *S. epidermidis* as described in 4.4.5.2. The test compounds were assayed at the concentration of 10x MBC. The assay was performed in triplicate against three strains of *S. epidermidis*.

6.4.6. Checkerboard assay to assess the antimicrobial activity of thiosemicarbazone and benzylidenecarboxamidrazone compounds in combination with thymol, TTO and EO

The antimicrobial activity of compounds demonstrating good antimicrobial efficacy in the suspension assays were evaluated in combination with thymol, TTO and EO by the checkerboard method as described in 4.4.6.1. The highest concentrations of the compounds in the checkerboard assay were 2x MBC and the natural compounds were assayed at the concentrations ranging from 64 mg/ mL to 1 mg/ mL (thymol) and 256 mg/ mL to 2 mg/ mL (EO and TTO).

6.5. Results

6.5.1. Preliminary evaluation of the antimicrobial activity of thiosemicarbazone and benzylidenecarboxamidrazone compounds by the agar diffusion assay

The results of agar diffusion assays are given in table 6.2. Test compounds 9 (thiosemicarbazone), 1 and 8 (both benzylidenecarboxamidrazones) demonstrated antimicrobial activity against the Gram-positive bacteria *S. epidermidis*, *S. aureus* and *P. acnes*. No activity was shown against the Gram-negative bacterium *Ps. aeruginosa*; however compound 9 showed minimal inhibition against *Acinetobacter spp.* and the yeast *C. albicans* in agar diffusion assay. Compounds 2- 7 did not demonstrate inhibition of bacteria or fungi in the agar diffusion assay.

Table 6.2. The antimicrobial activity of benzylidenecarboxamidrazone compounds (1 and 8) and thiosemicarbazone compound 9 (75 µL of 512 µg/ mL) against Gram-positive and Gram-negative bacteria and fungi. The activity were measured by the agar diffusion method and results expressed as the mean size of zone of growth inhibition (radius in mm). The tests were performed in duplicate (n= number of isolates; X = no zone of inhibition).

Microorganism	Compound		
	1	8	9
S. epidermidis (n=5)	3.9	7.1 (5-9)	2.9 (2-4)
S. aureus (n=5)	2.6 (1-6)	4.7 (3-6)	< 5.0
MRSA (n=5)	2.5 (2-3)	4.0 (3-5)	2.3 (2-3)
P. acnes (n=5)	5.7	5.3	< 2.0
Ps. aeruginosa (n=5)	X	X	X
Acinetobacter spp. (n=4)	X	X	< 0.5
C. albicans (n=4)	X	X	< 0.5

6.5.2. Determination of MIC, MBC and MFC of thiosemicarbazone and benzylidenecarboxamidrazone compounds

Thiosemicarbazone compound 9 and benzylidenecarboxamidrazone 8 demonstrated the greatest efficacy against Gram-positive test microorganisms and compound 1 showed the least antimicrobial activity against all the test microorganisms as determined by MIC, MBC and MFC (Table 6.3.). Compound 9 also inhibited the growth of clinical isolates of *C. albicans* and *Acinetobacter spp.* Solvent DMSO were not bactericidal or fungicidal at the concentrations used at this study [< 5% (v/v)].

Table 6.3. Median MIC, MBC and MFC (and range in µg/ mL) of benzylidenecarboxamidrazone compounds 1 and 8 and thiosemicarbazone compound 9 against Gram-positive and Gram-negative bacteria and fungi. The assay was performed by the broth microdilution method in triplicate (n= number of isolates tested).

	n	1		8		9	
		MIC	MBC/ MFC	MIC	MBC/ MFC	MIC	MBC/ MFC
S. epidermidis	10	≥ 64	> 256	6 (4-16)	8 (4-32)	4 (2-8)	8 (4-16)
S. aureus	8	≥ 128	≥ 256	8 (8-32)	12 (8-128)	8 (4-64)	16 (4-128)
MRSA	8	128 (64-256)	> 256	16 (8-16)	16 (8-16)	8 (4-8)	12 (8-16)
P. acnes	8	≥ 4	≥ 128	16 (16-32)	64 (32-128)	12 (4-64)	48 (16- 256)
Ps. aeruginosa	5	N/A	N/A	N/A	N/A	N/A	N/A
Acinetobacter spp.	5	N/A	N/A	N/A	N/A	256	≥ 256
C. albicans	5	N/A	N/A	N/A	N/A	256	≥ 256

6.5.3. Evaluation of the antimicrobial activity of thiosemicarbazone and benzylidenecarboxamidrazone compounds in a time-kill assay

6.5.3.1. Evaluation of the neutraliser efficacy against thiosemicarbazone and benzylidenecarboxamidrazone compounds

The neutralisers 4 and 6 (Table 6.1) demonstrated antimicrobial quenching activity against thiosemicarbazone compound 9 (Figure 6.10.). Neutraliser 6 demonstrated statistically more efficient neutralizing activity against compound 9 and were not toxic against *S. epidermidis* ($p = 0.09$; one-way ANOVA). None of the neutralising agents was effective in quenching the antimicrobial activity of benzylidenecarboxamidrazone compound 8 against *S. epidermidis*; therefore dilution method was used for neutralisation in the time-kill studies.

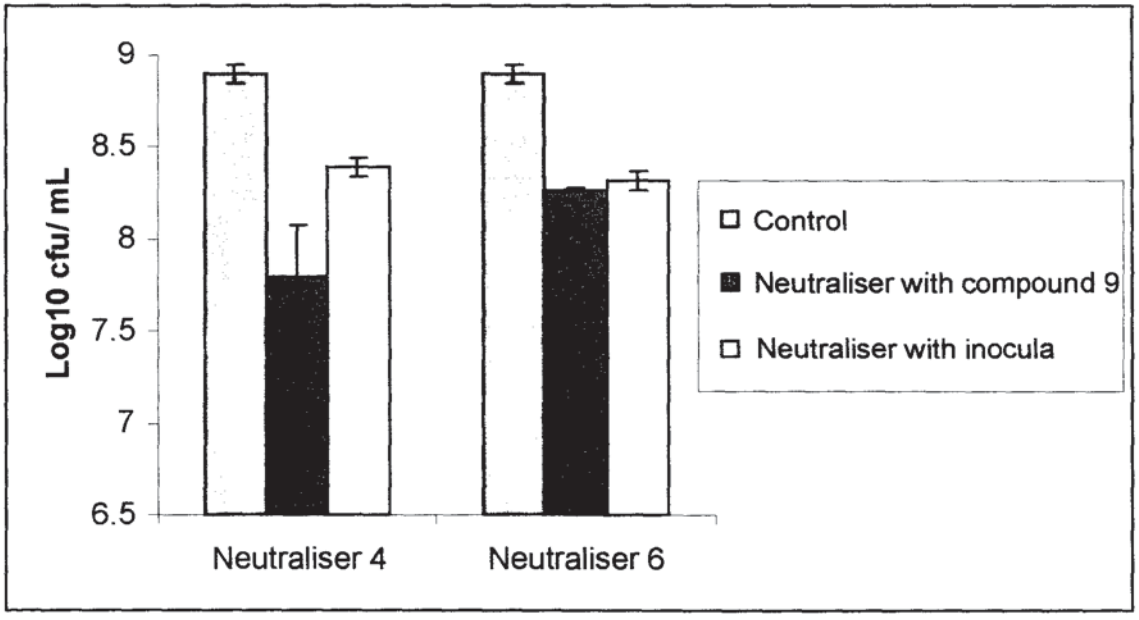

Figure 6.10. The neutraliser efficacy of two neutralising solutions against thiosemicarbazone compound 9 and *S. epidermidis* (n= 3; mean ± SEM).

6.5.3. Evaluation of the antimicrobial activity of thiosemicarbazone and benzylidenecarboxamidrazone compounds in a time-kill assay

6.5.3.1. Evaluation of the neutraliser efficacy against thiosemicarbazone and benzylidenecarboxamidrazone compounds

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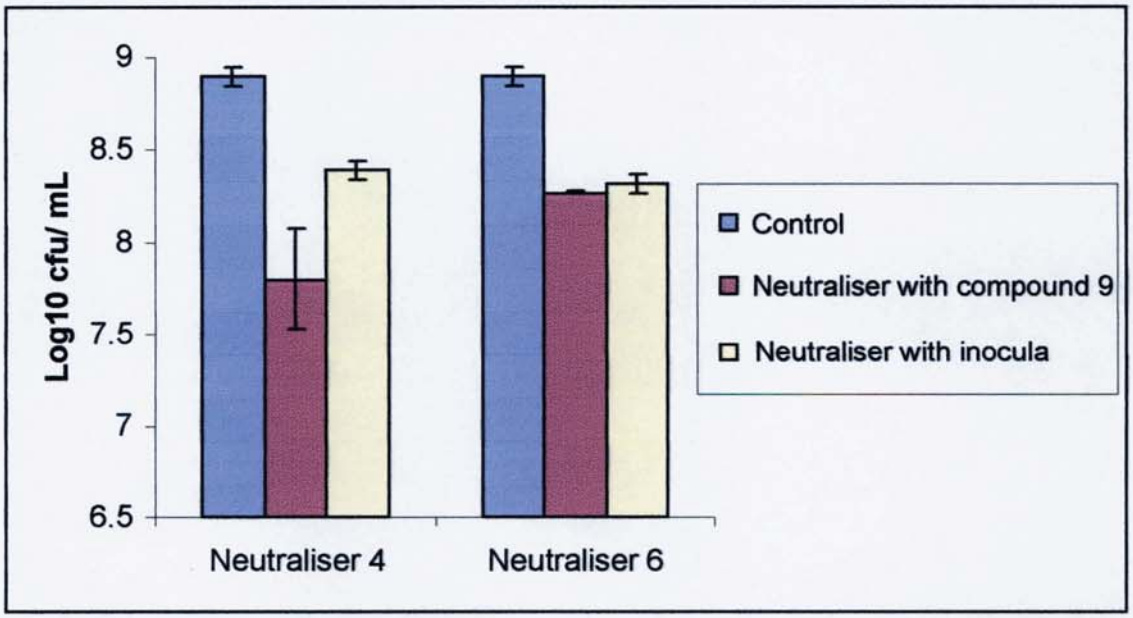

Figure 6.10. The neutraliser efficacy of two neutralising solutions against thiosemicarbazone compound 9 and *S. epidermidis* (n= 3; mean ± SEM).

6.5.3.2. Time-kill assay against S. epidermidis

The bactericidal activity of thiosemicarbazone compound 9 and benzylidenecarboxamidrazone 8 against *S. epidermidis* over 24 h is shown in Figure 6.11. Both compounds demonstrated antimicrobial activity with a log10 cfu reduction of 4.4 and 1.6 after 12 h for compounds 8 and 9 respectively.

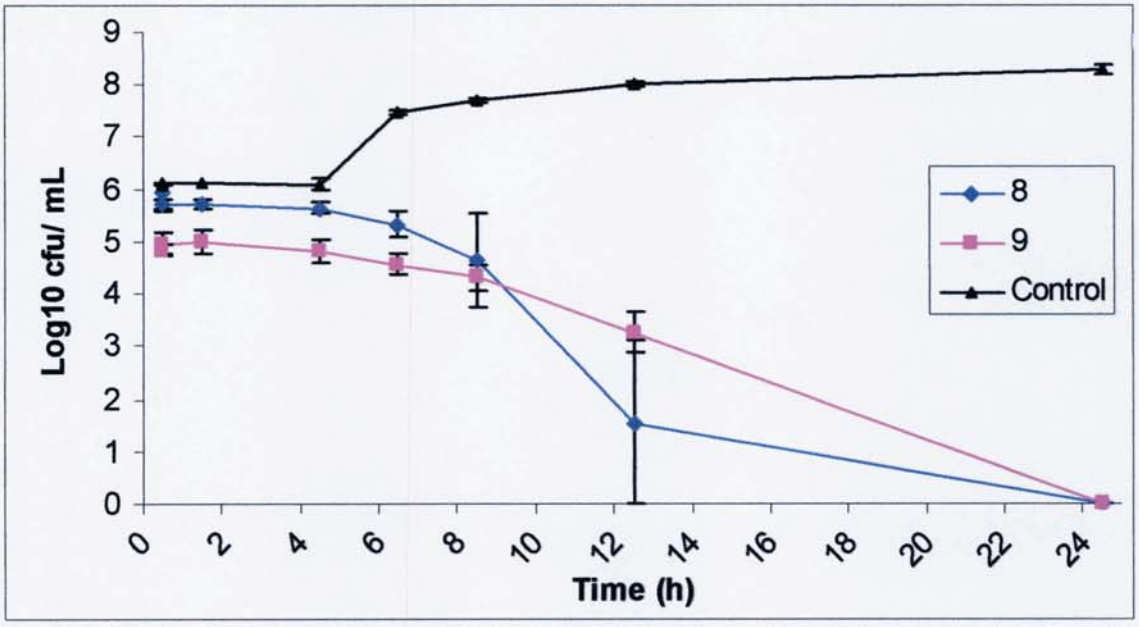

Figure 6.11. Benzylidenecarboxamidrazone compound 8 and thiosemicarbazone compound 9 activity against *S. epidermidis* (n=3) over 24 h (results are expressed as mean log_{10} cfu ± SEM). The assay was performed in triplicate.

6.5.4. Checkerboard assay to assess the antimicrobial activity of thiosemicarbazone and benzylidenecarboxamidrazone compounds in combination with thymol, TTO and EO

Antimicrobial activity of benzylidenecarboxamidrazone compound 8 and thiosemicarbazone compound 9, which demonstrated the greatest antimicrobial efficacy in the suspension assays, were further evaluated in combination with TTO, EO and thymol against *S. epidermidis* (Table 6.4). None of the combinations showed synergistic activity.

Table 6.4. The antimicrobial activity of benzylidenecarboxamidrazone compound 8 and thiosemicarbazone compound 9 in combinations with EO, TTO and thymol against *S. epidermidis* growing in a suspension. The assay was performed in duplicate by the checkerboard method. The results are expressed as Fractional Inhibitory Concentration (FIC) and FIC Index (FICI), with FICI ≤ 0.5 regarded as synergistic activity, FICI > 0.5 to 4.0 as indifferent activity and FICI > 4.0 as antagonistic activity.

Test compound	Natural compound	FIC of oil	FIC of test compound	FICI	Result
8	Thymol	0.5	0.25	0.75	Indifference
	EO	1.0	0.5	1.5	Indifference
	TTO	0.25	0.5	0.75	Indifference
9	Thymol	0.5	0.5	1.0	Indifference
	EO	1.0	1.0	2	Indifference
	TTO	0.25	0.5	0.75	Indifference

6.6. Discussion

Antimicrobial resistance is an increasing problem in the health care environment; therefore the search for new antimicrobial agents is warranted. This study evaluated the antimicrobial efficacy of novel benzylidenecarboxamidrazone compounds and a thiosemicarbazone against microorganisms commonly associated with the skin and infections of the skin. Benzylidenecarboxamidrazone compound 8 and thiosemicarbazone compound 9 demonstrated antimicrobial activity against Gram-positive bacteria, with a median MIC of 4- 12 µg/ mL and 6-16 µg/ mL for compound 8 and 9 respectively. Compound 8 did not show antimicrobial activity against Gram-negative bacteria or fungi and compound 9 only demonstrated antimicrobial activity at high concentrations (≥ 256 µg/ mL) against *Acinetobacter spp.* and *C. albicans.* Compound 1 showed limited antimicrobial activity against all test microorganisms. The remaining benzylidenecarboxamidrazone derivates did not demonstrate any antimicrobial activity in the preliminary investigations.

The mechanism of action of the two active benzylidenecarboxamidrazone compounds and the thiosemicarbazone is not yet understood (Billington *et al.*, 2000, Costello *et al.*, 2008, Rathbone *et al.*, 2006). The authors of previous studies on carboxamidrazone compounds (Billington *et al.*, 2000, Rathbone *et al.*, 2006) speculate that the phenolic hydroxyl moiety, the position of OH-group in the benzene ring, the lipophilic *tert*-butyl groups in the benzene ring and the 4-pyridyl moiety are important factors for the antimicrobial activity of carboxamidrazone compounds (Rathbone *et al.*, 2006). Furthermore, they suggest that the compound may act by chelating metal ions, however this was not confirmed. Compound 8, which is a benzylidenecarboxamidrazone,

shares structural similarities with the thiosemicarbazone, compound 9, as they both have a 5-nitrofuranyl moiety. Previous studies have shown that the 5-nitrofuranyl moiety plays an important role in the antimicrobial activity of these compounds (Costello *et al.*, 2008). However, these presumptions need further investigation to establish the site and the mode of antimicrobial activity.

Furthermore, the Gram-negative bacteria and yeast *Candida spp.* were not susceptible to benzylidenecarboxamidrazone compounds, which may be due to the differences in penetration of the compounds through the cell walls (Costello *et al.*, 2008); Gram-negative bacteria have an outer membrane layer containing LPS and lipoproteins, whereas the Gram-positive bacterial cells have a thicker but more open-structured cell wall comprising mainly of peptidoglycan. The yeast cell wall has a high proportion of glucan and mannoproteins not present in the bacterial cell wall (McDonnell and Russell, 1999). The thiosemicarbazone demonstrated limited activity against the yeast and Gram-negative bacteria. The carboxamidrazone compounds are highly lipophilic, with a calculated LogP of 5.1, compared to thiosemicarbazone which has LogP value around 2.8 (Costello *et al.*, 2008, Rathbone *et al.*, 2006), which may affect their diffusion through the microbial cell wall. However, further work is required to establish the site and the mode of action of these compounds.

In this study, agents to quench the antimicrobial activity of compound 8 were not found. This compound may act through ionic or physical interactions acting physically upon the membrane (as with ethanol and phenols) and may only be neutralised effectively by dilution (Denyer and Stewart, 1998). This would also concur with the hypothesis of Billington *et al.* However, the

antimicrobial activity of both the benzylidenecarboxamidrazone and the thiosemicarbazone compounds were delayed (over 12 h), which is considerably slower than many other antimicrobial compounds such as phenols. Further studies are required to establish the way these compounds interact with the microbial cells. The thiosemicarbazone compound was only neutralised by two solutions which contained Tween, sodium metabisulphite and thiosulphate pentahydrate; moreover, the solution with additional lecithin demonstrated good antimicrobial quenching activity. Tween and lecithin alone, which commonly neutralise ionic interactions of biocides such as biguanines and QAC's, did not neutralise the compound.

In this study the antimicrobial activity of thiosemicarbazone compound 9 and benzylidenecarboxamidrazone compound 8 were evaluated in combination with the natural compounds TTO, EO and thymol. Combination of two or several antimicrobial agents may be of benefit enabling lower concentrations to be used, therefore reducing the risk of toxicity or sensitisation. The benzylidenecarboxamidrazone lead compound (compound 1) has been shown to be toxic to human leucocytes (Coleman *et al.*, 2004, Rathbone *et al.*, 2006), and therefore synergy with another compounds would not only improve the efficacy of the compound but also potentially reduce the toxicity of the compound when lower concentrations are needed. However, the toxicity of the carboxamidrazone analogues such as compound 8, would require further studies. Other thiosemicarbazone compounds have not shown toxicity in *in vivo* studies (Aguirre *et al.*, 2004). Combinations of antimicrobial agents may also reduce the risk of development of resistance, if multiple targets on microbial cells are targeted simultaneously, and possibly increase their spectrum. However, in this study the combination of benzylidenecarboxamidrazone and thiosemicarbazone compounds with a natural compound demonstrating antimicrobial activity

did not indicate synergism. Further work is required to determine if these new antimicrobial agents have synergistic activity with other antiseptic agents such as CHG.

6.7. Conclusion

The novel benzylidenecarboxamidrazone compound 8 and the thiosemicarbazone compound 9 demonstrated antimicrobial activities against Gram-positive bacteria. The delayed antimicrobial activity and the narrow spectrum of activity demonstrated in this study may however limit their use as individual antiseptic agents. Further work in relation to these compounds should focus upon their activity in combination with other antimicrobial compounds and their mechanism of action.

7. CHAPTER: DISCUSSION

Effective skin antisepsis is imperative prior to incision of the skin, for example during surgery, insertion of intravascular devices and other invasive procedures. The current guidelines in prevention of CRI (EPIC in UK and CDC in USA), which are based on the research data available, recommend 2% (w/v) CHG [preferably in 70% (v/v) IPA] for skin antisepsis prior to insertion of intravascular catheters (O'Grady *et al.*, 2002, Pratt *et al.*, 2007). Two percent CHG has demonstrated superior antimicrobial activity compared to 0.5% (w/v) CHG, 70% (v/v) IPA or PVP-I (Balamongkhon and Thamlikitkul, 2007, Chaiyakunapruk *et al.*, 2002, Hibbard *et al.*, 2002). However, there are comparatively few investigations comparing aqueous CHG and alcoholic CHG (Hibbard *et al.*, 2002).

Microorganisms have been shown to persist within the skin following skin antisepsis, and the poor skin permeation of many antiseptic agents may contribute to this (Hendley and Ashe, 1991, Hendley and Ashe, 2003). This was corroborated in this study, where a large number and variety of microorganisms were recovered from full thickness human donor skin following skin antisepsis and excision, and negligible levels of CHG were detected within the donor skin (chapter 2). One of the main aims of this thesis was to develop a model for evaluating the concentration and the depth of skin permeation of CHG over time. The resulting Franz cell model clearly demonstrated the poor penetration of CHG into full thickness human skin using the *in vitro* human skin model, both from aqueous CHG and CHG in 70% (v/v) IPA (chapters 3 and 5). These findings concur with previous studies which demonstrated poor skin penetration of chlorhexidine compounds through intact skin (Lafforgue *et al.*, 1997, Wang *et al.*, 1990).

Following 2 min and 30 min exposure, the concentrations of CHG were low within the top layers (< 0.157 μm per mg tissue) and were negligible below the depth of 300 μm. Furthermore, following a 2 min application of aqueous CHG, higher concentrations of CHG were recovered in the top layers of skin (0- 100 μm depth) compared to CHG in alcoholic solution, but at the 30 min treatment skin penetration of CHG from aqueous and alcoholic solutions were comparable. Alcohol has been shown to have skin penetration enhancing properties, however at higher concentrations it has reduced skin permeation which is mainly due to skin dehydration (Amini, 2001, dos Anjos *et al.*, 2007, Van der Merwe and Riviere, 2005). However, further studies are required to establish the clinical significance of the poor CHG penetration in the presence of 70% (v/v) IPA, and to establish the optimum IPA concentration which supports both skin penetration enhancing properties and antimicrobial activity; previous studies have demonstrated IPA at concentrations above 50% (v/v) has a broad spectrum and rapid antimicrobial activity (Adams, 2006) and alcohol at the concentrations up to 63% (v/v) work as a skin penetration enhancer (Kurihara-Bergstrom *et al.*, 1990).

In addition to CHG, the activities of alternative antimicrobial agents for skin antisepsis were evaluated (chapters 4 and 6). Three out of six essential oils, EO, TTO and thymol, demonstrated a broad spectrum of antimicrobial activity *in vitro*, and were further evaluated in combination with CHG (chapter 4). The combination of CHG with EO demonstrated synergistic activity against *S. epidermidis* in a biofilm mode of growth, with significant reduction in both CHG and EO concentrations needed for inhibition of the biofilm mode of growth. This is the first reported finding of EO and CHG exhibiting synergistic activity against *Staphylococcus spp.* biofilms and is a concept that clearly requires further investigation and may have a potential clinical

application. For example, CHG in combination with EO has potential for use as a hard surface disinfectant which may be applied to medical devices that are often associated with staphylococcal biofilms in the clinical setting e.g. CVC hubs and needleless connectors (Casey *et al.*, 2007, Tebbs *et al.*, 1996, Worthington and Elliott, 2005). Microbial biofilms, such as *S. epidermidis*, are often associated with infections related with medical devices and often cause persistent infections unresponsive to antimicrobial treatment (Saginur *et al.*, 2006). Microorganisms in a biofilm mode of growth are generally regarded as more resistant to antimicrobial treatment than microorganisms in a planktonic mode of growth (Saginur *et al.*, 2006). Biofilms commonly occur on the surfaces of the foreign bodies as well as interfaces; microorganisms on the skin can also be present as aggregates or microcolonies which are similar to microbial biofilms, and are therefore less susceptible to skin antisepsis (Edwards and Harding, 2004). Furthermore, in addition to skin and other invasive medical devices, many surfaces in the hospital environment serve as a reservoir of opportunistic pathogens, some capable of biofilm formation; therefore effective cleaning of surfaces in the healthcare environment have an important role in preventing HCAI (Boyce, 2007).

Further studies are clearly warranted to evaluate the activity of CHG in combination with EO against biofilms on various inanimate surfaces, as well as the time required to eradicate microorganisms in a biofilm mode of growth. This study investigated the activity of CHG and EO against pre-formed *S. epidermidis* biofilms. Studies demonstrating increased biofilm expression following exposure to sub-lethal concentrations of antimicrobial compounds have been reported, therefore further studies investigating the effect of CHG and EO on formation of microbial biofilms at sub-MIC levels are needed, to establish their effectiveness at preventing

attachment and initiation of biofilm mode of growth. Furthermore, the toxicity and skin tolerance of EO, and EO in combination with aqueous or alcoholic CHG, are needed to establish the suitability of the compounds for clinical applications.

Essential oils, including EO, have been shown to enhance skin permeation of both lipophilic and hydrophilic compounds, which is thought to be due to the terpene content. Terpenes alone, such as the main terpene in EO, 1,8-cineole, have also demonstrated skin penetration enhancing properties (Aqil *et al.*, 2007, Narishetty and Panchagnula, 2005). In this study, EO, which demonstrated the greatest potential in the *in vitro* antimicrobial assays against bacterial biofilms, was evaluated for its skin permeation enhancing properties in the presence of CHG (chapter 5). This study demonstrated significantly enhanced skin penetration of CHG, with 5% (v/v) EO enhancing skin permeation at the deeper layers of the skin (300- 1500 μm depths) compared to CHG alone, and at 10% (v/v) EO the CHG concentration in the top layers (0- 900 μm) was significantly increased compared to CHG alone. Furthermore, CHG in 70% (v/v) IPA, which demonstrated poor skin permeation as a sole agent in this study, was evaluated in the presence of 10% (v/v) EO. The results demonstrated a significant increase in the CHG concentration and the depth of delivery following a 2 min and 30 min application of the compound with EO compared to CHG in IPA alone. Again, this is the first reported finding of EO enhancing the permeation of CHG within human skin. These results are encouraging, as antimicrobial agents have been shown not to totally eradicate the microorganisms in the skin, with recolonisation occurring after 18 h. This is thought to be due to poor permeation of the antimicrobial agent below the surface of the skin, hair follicles and sebaceous glands (Hendley and Ashe, 1991, Hendley and Ashe, 2003).

The results from this study clearly demonstrate the enhanced skin delivery of CHG with EO into the deeper layers of the skin and at higher concentrations, therefore enhancing skin antisepsis. These results clearly lay the foundation for future work. Clinical studies on skin penetration of CHG in combination with EO *in vivo* and the significance of the enhanced skin penetration of CHG on reducing microbial load on the skin *in vivo* are also warranted. However, the penetration of EO into the skin, its potential side effects and skin tolerability need to be investigated before *in vivo* studies are undertaken in the clinical setting. Potential future work may be the development of a skin wipe, biopatch or surgical bandage comprising a combination of CHG and EO which may be used in the pre- and post-surgical setting. 1,8-Cineole is the main component of the crude EO, and has been shown to enhance skin penetration of various drugs; however, further studies are warranted to determine the main component(s) of EO responsible for the skin penetration enhancing properties of CHG. Also, as discussed previously, alcohol has been used as a skin penetration enhancer, however as shown in this study, at a high concentration [70% (v/v)] it reduces skin penetration, therefore further studies are required to establish the optimum concentration of IPA in enhancing CHG skin penetration alone and in combination with EO.

In the healthcare environment the increased number of resistant microorganisms is a major concern. The wide use of many antimicrobial agents has contributed to the acquired resistance among many pathogens. Furthermore, the resistance genes can be transferred amongst the microorganisms through mobile genetic elements, such as plasmids, especially amongst the microorganisms in a biofilm mode of growth. The search for new antimicrobial agents is important for prevention and treatment of microorganisms carrying resistance to currently used antimicrobial agents, such as MRSA, VRE and *Acinetobacter spp*. This study evaluated the

antimicrobial activity of novel benzylidenecarboxamidrazone and a thiosemicarbazone compounds against a panel of microorganisms commonly associated with CRI (chapter 6). The results from this study demonstrated good antimicrobial activity of one carboxamidrazone and the thiosemicarbazone compound in an *in vitro* assay, however further studies are needed to establish their activity in combination with other antimicrobial compounds that have rapid antimicrobial activity as the onset of action of these agents alone is delayed. Furthermore, the mode of action of these two compounds as well as their toxicity requires additional work.

The main findings of this thesis: the enhanced antimicrobial activity of CHG in combination with EO against *S. epidermidis* in a biofilm mode of growth, and the enhanced skin delivery of CHG in combination with EO, are exciting and clearly lay the foundation for further work.

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9. APPENDICES

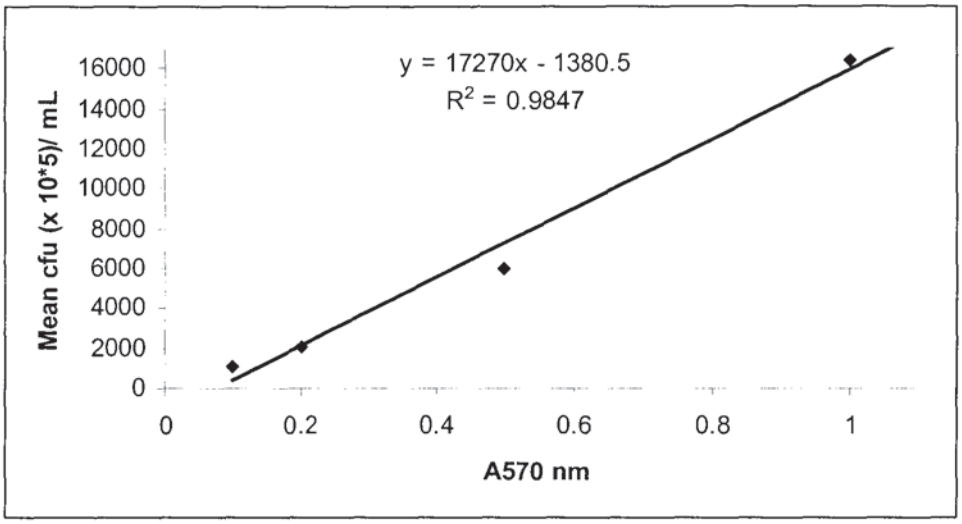

Figure 9.1. The number of colony forming units (cfu) of *Staphylococcus epidermidis* in a suspension versus turbidity of the suspension measured by absorbance at 570 nm (n= 5).

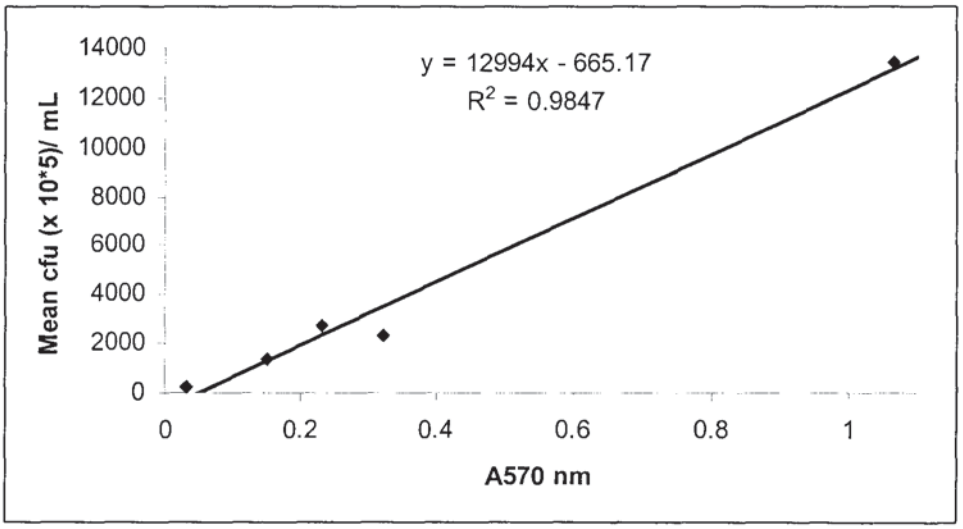

Figure 9.2. The number of colony forming units (cfu) of *Staphylococcus aureus* in a suspension versus turbidity of the suspension measured by absorbance at 570 nm (n= 5).

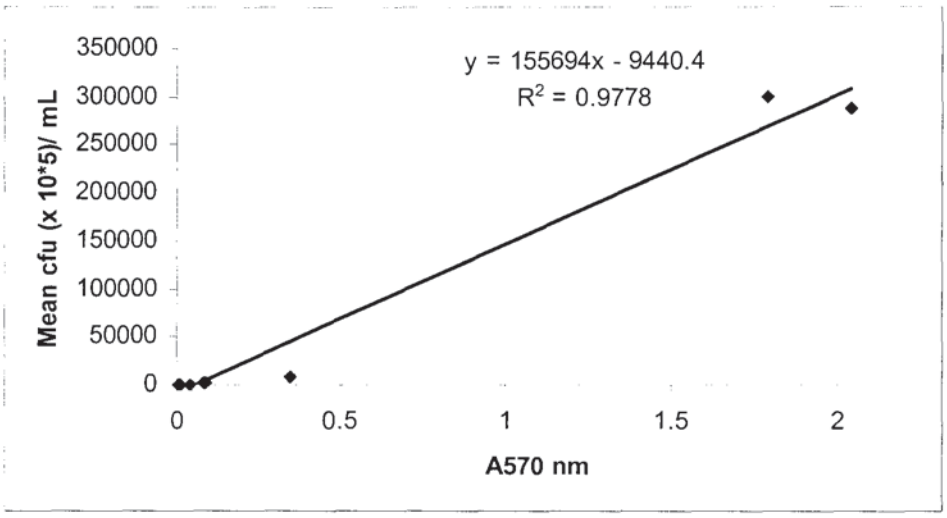

Figure 9.3. The number of colony forming units (cfu) of *Pseudomonas aeruginosa* in a suspension versus turbidity of the suspension measured by absorbance at 570 nm (n= 5).

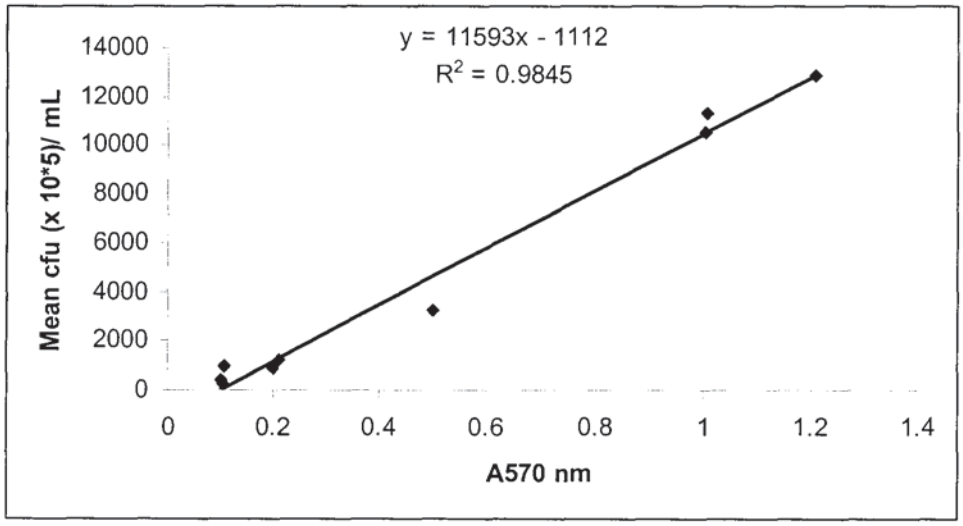

Figure 9.4. The number of colony forming units (cfu) of *Acinetobacter spp.* in a suspension versus turbidity of the suspension measured by absorbance at 570 nm (n= 5).

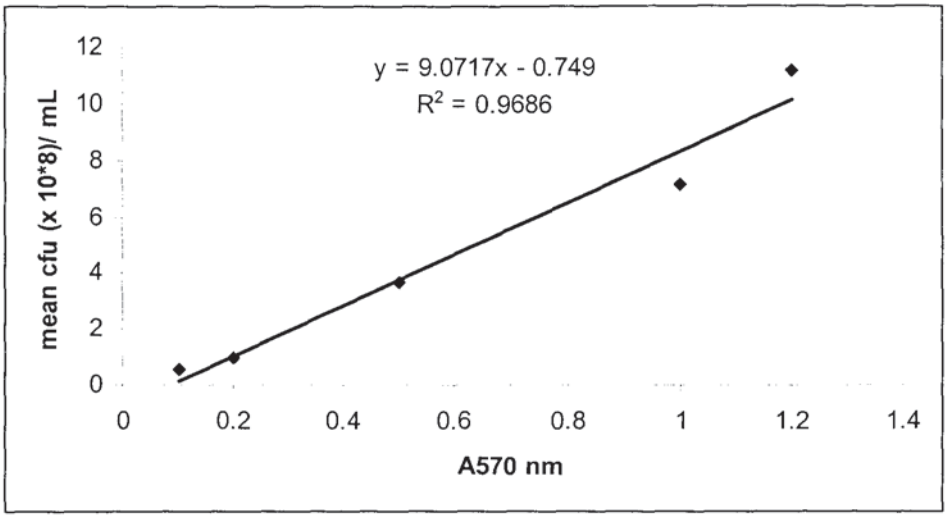

Figure 9.5. The number of colony forming units (cfu) of *Propionibacterium acnes* in a suspension versus turbidity of the suspension measured by absorbance at 570 nm (n= 5).

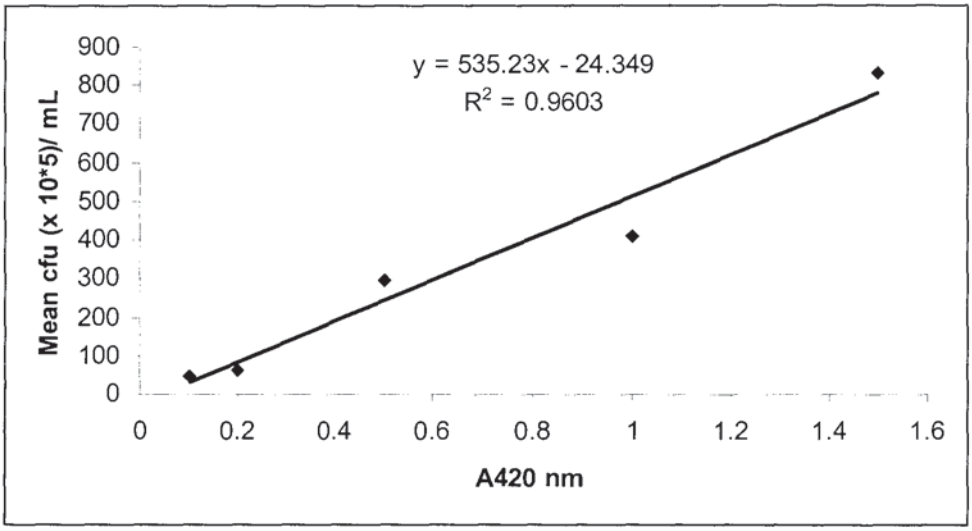

Figure 9.6. The number of colony forming units (cfu) of *Candida albicans* in a suspension versus turbidity of the suspension measured by absorbance at 420 nm (n= 5).

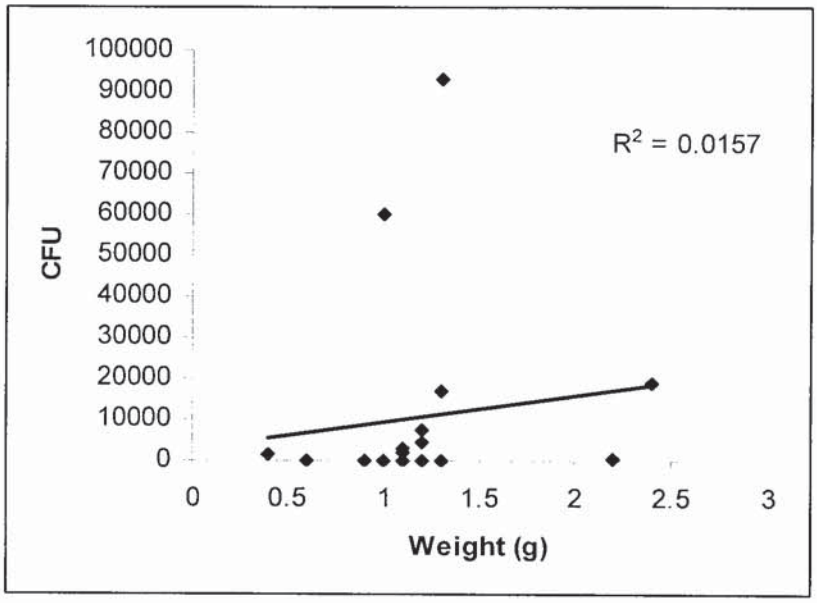

Figure 9.7. The number of microorganisms (expressed as colony forming units, cfu) recovered from ten different excised full thickness human skin samples in aerobic conditions versus the weight of donor skin sample. The frozen human skins were defrosted, homogenized in phosphate buffer saline and cultured on blood agar at 37°C (performed in duplicate).

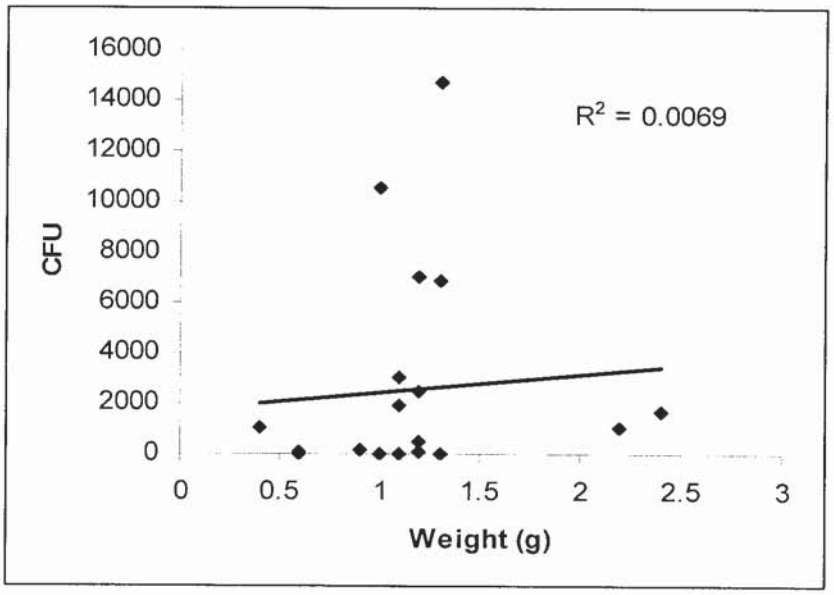

Figure 9.8. The number of microorganisms (expressed as colony forming units, cfu) recovered from ten different excised full thickness human skin samples in anaerobic conditions versus the weight of donor skin sample. The frozen human skins were defrosted, homogenized in phosphate buffer saline and cultured on blood agar at 37°C (performed in duplicate).

Table 9.1. Median minimum inhibitory concentration (MIC) and median minimum bactericidal concentration (MBC) of chlorhexidine digluconate (CHG) against the bacteria recovered from frozen excised full thickness human skins (n=10, from breast reduction surgery). The bacterial susceptibility assay was performed by the broth microdilution method in triplicate.

Type of microorganism	Number of isolates	Median MIC of CHG in µg/ mL (and range)	Median MBC of CHG in µg/ mL (and range)
S. epidermidis	26	1 (0.5- 1)	1 (0.5- 2)
Coagulase negative *Staphylococcus spp.* (other than *S. epidermidis*)	30	0.25 (0.06- 1)	0.25 (0.06- 8)
Acinetobacter spp.	2	0.25	0.25
Rzb. Rariobacter	1	1	1
Klebsiella spp.	1	0.5	4

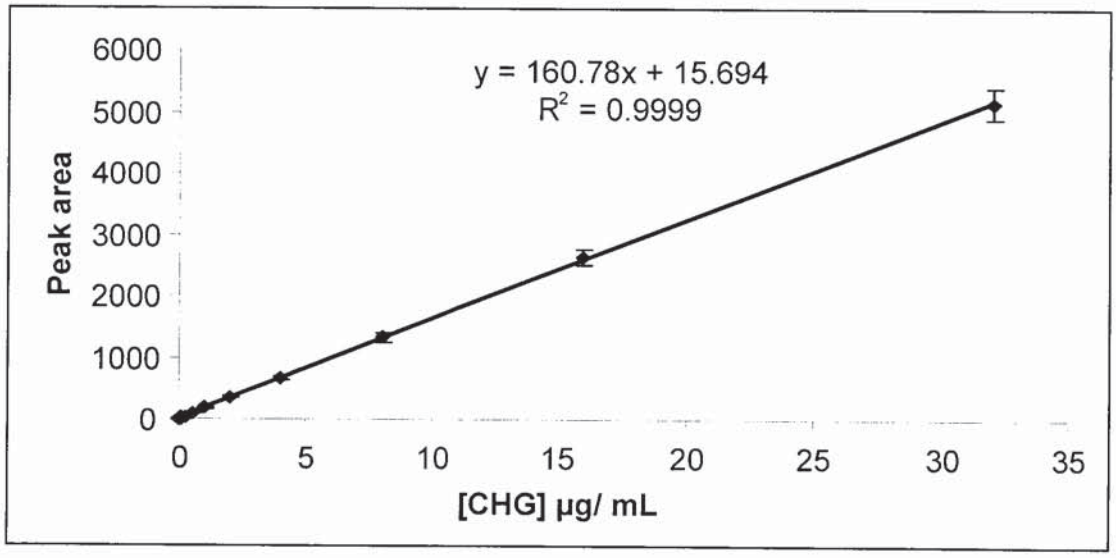

Figure 9.9. Standard curve for aqueous chlorhexidine digluconate (CHG) quantification by high performance liquid chromatography (HPLC; Agilent 1200 series, CPS2 Hypersil column, flow rate 1.2 mL/ min, UV detection 254 nm). Serial double dilutions of 20% (w/v) aqueous CHG were performed in PBS. The analysis of CHG was performed five times (mean ± SEM).

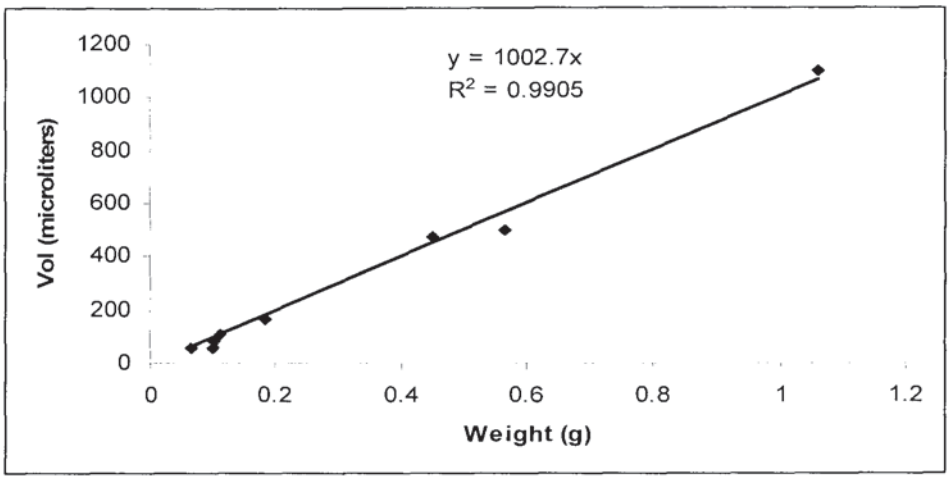

Figure 9.10. Correlation between the weight of full thickness human skin and the volume of skin (the assay was performed in duplicate from ten donor skins).

LIST OF PUBLICATIONS

Full papers

KARPANEN, T. J., WORTHINGTON, T., CONWAY, B. R., HILTON, A. C., ELLIOTT, T. S. J. & LAMBERT, P. A. (2008) Penetration of chlorhexidine into human skin. *Antimicrob Agents Chemother,* 52, 3633-3636.

KARPANEN, T. J., WORTHINGTON, T., HENDRY, E. R., CONWAY, B. R. & LAMBERT, P. A. (2008) Antimicrobial efficacy of chlorhexidine digluconate and combinations with eucalyptus oil, tea tree oil and thymol against planktonic and biofilm cultures of *Staphylococcus epidermidis*. *J Antimicrob Chemother,* 62, 1031- 1036.

Non-peer reviewed articles

KARPANEN, T. (2008). Central venous catheter related infections: an overview. *Microbiologist* 9 (1), 49-50.

Abstracts

KARPANEN, T.J., WORTHINGTON, T., RATHBONE, D. & LAMBERT, P.A. (2006) P6.18 Activity of Thiosemicarbazone and Carboxamidrazone Compounds and Essential Oils Against Microorganisms Associated with Intravascular Device Related Infections. *The Journal of Hospital Infection.* 6th International Conference of the Hospital Infection Society, Amsterdam, The Netherlands, 15-18th October.

KARPANEN, T.J., WORTHINGTON, T., CONWAY, B.R. & LAMBERT, P.A. (2007) P1117 Qualitative and quantitative evaluation of micro-organisms present in excised human skin used for *ex vivo* assessment of topical antimicrobials. *International Journal of Antimicrobial Agents* 29, S302. 17th European Congress of Clinical Microbiology and Infectious Diseases & 25th International Congress of Chemotherapy, Munich, Germany, 31st March – 3rd April.