Potentiation of pomegranate rind extract (PRE) bactericidal activity by $ZnSO_4$ combination

A thesis submitted for the degree of

Doctor of Philosophy (Ph.D)

By

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February 2020

ACKNOWLEDGEMENT

First and foremost, I would like to Thank Allah Almighty the Most Gracious Most Merciful for blessing me with the opportunity to complete my Phd surrounded by the utmost supportive educators in the most astounding university.

I would like to recognize and thank all the people whose assistance played a major role in the success of my journey. First and for most I would like to thank my supervisor Dr. Charles Heard, who I am greatly indebted to for his constant support and advice.

I must also thank Dr. Ryan Moseley and Dr Rae Moses for facilitating the work with cell culture and confocal microscopy at his laboratory at dentistry school. Thanks go to all my lab colleagues and the entire academic team that crossed my path during my educational experience. I would like to recognize the invaluable assistance provided by Princess Noura University which granted me the chance to achieve my educational aspirations.

I wish to acknowledge the endless support and great love of my husband Nader who stood by me every step of the way. My daughters Dayala, Sadeem, and Rawan for being the greatest daughters one could ask for. Your patience and love helped me overcome all obstacles that I came across. I would like to dedicate this thesis to my father soul, my mother who kept me in her prayers and thoughts throughout the years. My deepest gratitude to my friend, Maria for her presence, assistance and kindness that I never failed to find.

I also like to place on record my genuine appreciation to anyone and everyone who directly and indirectly have lent me their time, wise words and encouragement in this adventure.

ABBREVIATIONS

24 WP	24 well plate
96 WP	96 well plate
AMR	Antimicrobial resistance
ANOVA	One way analysis of variance
CFU	Colony-forming units
COX	Cyclooxygenase
CRBC	chicken red blood cells
DI	Deionised
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
ECM	Extracellular matrix
EGF	Epidermal growth factor epidermal
EtOH	Ethanol
FACS	Fluorescence-activated cell sorting
FBS	Foetal bovine serum
FDA	Fluorescein diacetate
FDC	Franz diffusion cell
FeSO4	Ferrous sulphate
FGF	Fibroblast growth factor
HaCat	Immortalized human skin keratinocytes
FIC	fractional inhibitory concentration index
HPLC	High performance liquid chromatography
HSE	Heat separated epidermis
HSV	*Herpes simplex* virus
IC50	Inhibitory concentration 50
ICP MS	Inductively Coupled Plasma Mass Spectrometry
IL-1	Interleukin-1
LC-MS	Liquid chromatography–mass spectrometry

LDH	Lactate dehydrogenase
MBC	Minimum bactericidal concentration
MDCK	Madin-Darby canine kidney
MHA	Mueller Hinton agar
MHB	Mueller Hinton broth
MHB	Mueller Hinton broth
MIC	Minimum inhibitory concentration
MMP	Mixed metalloproteinase
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSSA	Methicillin-sensitive *Staphylococcus aureus*
MTT	Thiazolyl blue tetrazolium bromide
MTT	3-(4, 5-dimethylthiazol-2yl)-2,5-diphenyl tetra bromide
NADH	Nicotinamide adenine dinucleotide
NCTC	National Collection of Type Cultures
Pb	Phthalate buffer
PBS	Phosphate buffered saline
PDGF	Platelet-derived growth factor
PI	Propidium iodide
PRE	Pomegranate rind extract
PRE/Zn	Combination of PRE and Zn(II), as zinc sulphate ($ZnSO_4$)
ROS	Reactive oxygen species
SD	Standard deviation
SSTI	Skin and soft-tissue infections
TGF-α	Transforming growth factor-alfa
TGF-β	Transforming growth factor-beta
TSA	Tryptone soya agar
TSB	Tryptone soya broth
VEGF	Vascular endothelial growth factor
Zn(II)	Zinc in oxidation state II (synonymous with Zn^{2+})
ZOI	Zone of inhibition

SUMMARY

The addition of Zn(II) to PRE was previously found to increase virucidal activity of PRE by seven log reduction in plaque forming units against *Herpes simplex* virus. This thesis tested the same system for antibacterial and bactericidal activity against bacteria that are often associated with skin and wound infections. Antibacterial synergistic activity of PRE + Zn(II) was tested using a checkerboard test and showed synergy with MRSA and *S. epidermidis* with FIC index ≤0.5 and MIC level of PRE in the combination at (62.5, 31.25 μg/mL) and Zn(II) at (1600, 400 μM). In addition, suspension time-kill testing showed synergistic bactericidal activity with PRE 1 mg/mL + $ZnSO_4$ (0.125 and 0.25M). The cytotoxicity of PRE ± Zn(II) was investigated using MTT and FACS analyses, short term exposure showed that PRE (5-50 μg/mL) was tolerated and no significant toxicity on HaCaT cell line was observed. *In vitro* scratch wound model was investigated and showed that PRE at lower levels mediates HaCaT cell migration compared to untreated group. Significant cell migration mediation was observed with PRE 10 μg/mL and Zn(II) (25-100 μM), as did the combination that contained PRE (5, 10 μg/mL) with $ZnSO_4$ 50 μM. Optimization of topical hydrogel formulations resulted in three formulations that showed promising permeation and penetration profiles using *in vitro* porcine epidermis. Formulations applied to inoculated porcine in simulated in-use conditions again demonstrated syndergistic bactericidal activity of PRE + Zn(II) with >5 log reduction in MRSA inoculated porcine kin after 20 minutes contact time compared to ~1 log reduction for the control.

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Chapter 1: General introduction

1.1 Overview

Due in part to the increase of bacterial resistance to conventional antibiotics, new sources of antimicrobial agent are urgently needed. Wounds and particularly chronic wounds are a big burden to the NHS and new treatments are needed. Natural sources have been extensively studied because of antimicrobial potency and generally good safety profile in the human body. This thesis investigated the possibility of the development of a new therapeutic system of antimicrobials with its foundations in the fruit rind extracts of *Punica granatum*, or pomegranate, which is referred to as PRE. Specifically, the research built upon prior observations that potent activities of an antiviral nature occurred with the combination of PRE with zinc ions, herein referred to as Zn(II), as a synergising or potentiating agent. Although the benefits in terms of antimicrobial and health, of the pomegranate have been familiar for hundreds of years, it had been discovered more recently that adding zinc sulphate ($ZnSO_4$) significantly enhanced virucidal activity of PRE against *Herpes simplex* virus.

The aim of this research was to explore the potential of combining PRE and $ZnSO_4$ as a new system for treating skin infections and wounds. A number of multi-disciplinary areas of science were explored, including antibacterial activity cytotoxicity and wound healing. This chapter includes the background information about health benefits of pomegranate, skin and wound physiology during the process of healing that is relevant and the impact of bacteria on the process of wound healing.

1.2 Pomegranate uses in traditional medicine

The pomegranate (*Punica granatum L. Punicaceae*) is a type of seeded or granular apple. The name pomegranate originates from the Latin term ponusandgranatus. It is an ancient fruit that has altered little in its form during human history. The pomegranate

fruit plays an important role in Jewish, Christian and Muslim customs. It is indigenous to Iran, Afghanistan, China and the Indian sub-continent. In ancient times the pomegranate had already been cultivated for thousands of years in Iran to Pakistan, eastern India, and China (Celik et al. 2009; Lansky and Newman, 2007). The pomegranate is consumed throughout the world today, with the major producers and exporters being India, China, Turkey, Iran, South Africa, the US, Peru, Chile, Argentina and Spain. Approximately 2 million tons of pomegranates are grown globally and the main importer of the fruit is the European Union (EU). Kalaycıoğlu and Erim, (2017) noted that 67,000 tons were imported to Europe in 2013, in addition to production within the continent.

Different societies have identified that pomegranate skin has medicinal potential. The fruit is utilised to treat conditions in numerous forms of medical practice. The Egyptians have used pomegranate peel extract as a remedy for a number of widespread conditions including inflammation, diarrhoea, intestinal worms, coughs and infertility (Lansky, and Newman, 2007), with pomegranate peel extract acting as an astringent in the treatment of diarrhoea and dysentery (Prashanth et al. 2001). Jurenka (2008) indicates that Ayurvedic medicine uses the pomegranate to combat parasites, as a blood tonic, and to treat diarrhoea and aphthae and other ulcers, while the Unani medical practice of the Middle East and India utilises the fruit to treat diabetes.

Al-Saeedi et al. (2003) published a study from Saudi Arabia, in which structured interviews were conducted with a sample of 1039 diabetic patients regarding traditional cures, including the pomegranate. The relationship between belief and socio-demographic and diabetes-related factors was evaluated via chi-square tests. It was found that 15.6% of respondents thought that traditional medicines were safe and effective and 25.8% that they could be helpful, 328 interviewees (31.6 %) believed in the therapeutic application of pomegranate and its health benefits. A third of the respondents utilised traditional cures. A statistically significant association was

demonstrated between belief in traditional medicine and variables including female gender, family history of diabetes, duration of this illness and dietary factors.

1.3 General health benefits of pomegranates

Various studies have demonstrated the therapeutic effects of pomegranate on a number of conditions; for example cancer prevention and therapy (Turrini et al. 2015) and prostate cancer more specifically (Wang and Martins-Green 2014), chronic inflammatory conditions (Viladomiu et al. 2013), cardiovascular diseases (Vlachojannis et al. 2015), spoilage and pathogenic microorganisms (Tanveer et al. 2015), obesity (Al-Muammar and Khan 2012) and as an anti-inflammatory agent (Houston et al. 2017a).

Miguel et al. (2010) referred to a number of ongoing clinical trials that investigated the therapeutic benefits of pomegranate extract. It was noted that a number of applications of pomegranate, among other species, had been patented recently, demonstrating their usefulness: skin treatments (Gross and Gross 2009) and, more specifically, to enhance skin appearance (Jacobs 2010), to maintain and enhance skin moisture levels (Vilinsky 2010), to prevent skin dryness and aging (Cho et al. 2010); to inhibit the impact of mental stress on the hair (Briese et al. 2010) and to combat alopecia via external application (Jo et al. 2009); to tone the waist and abdomen (Khan and Zaidi 2009); to prevent dental conditions such as plaque (Babu et al. 2009); and to treat sickle cell disease (Desai and Desai 2009; Desai 2010). There are also patents relating to aspects of the health benefits of pomegranate.

1.4 Phytochemical properties of pomegranates

Pomegranates are consumed in various forms: as fresh fruit, as other food products (including jam and jellies), as beverages (such as juices and wines), and as an extract

utilised as a botanical component of herbal medicines and dietary supplements. Different part of pomegranate that has been extracted is illustrated in (Figure 1-1). The use of such extracts has recently increased (Afaq et al. 2005; Negi et al. 2003; Seeram, 2006; Zahin et al. 2010). Approximately 80% of the overall weight of the pomegranate fruit is edible, of which 80% is juice and 20% is seed. There are numerous phytochemicals that have been found in different parts of the pomegranate tree, and in pomegranate fruits and seeds; although most are concentrated in the pericarp (rind).

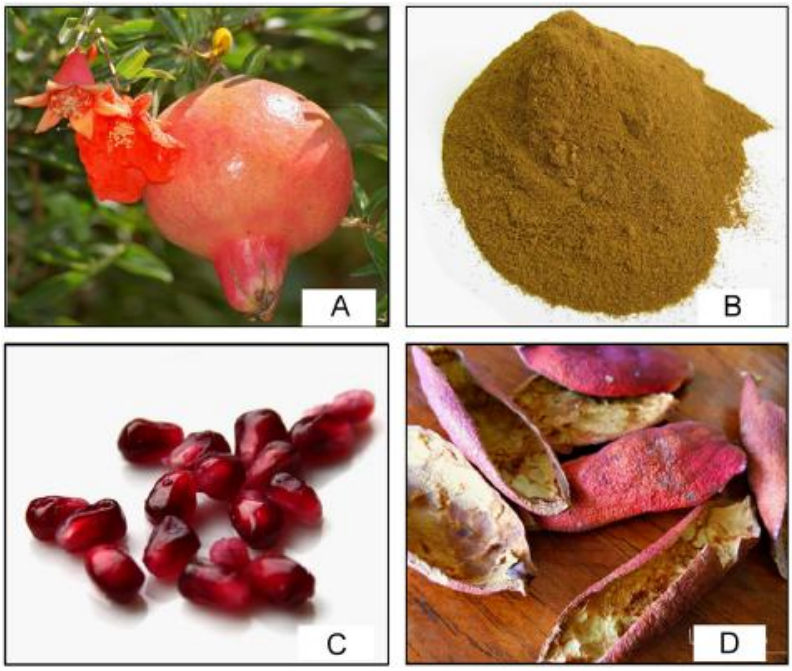


Figure 1-1 Pomegranate fruit (A) and its anatomical components, pomegranate peel powder (B) pomegranate arils (C) and sun-dried pomegranate peel or rind (D) (Ismail et al. 2012).

The main group of phytochemicals found in pomegranates are polyphenols, which contain phenolic ring multiple hydroxyl groups. Pomegranates are recognised for containing a number of particularly useful water soluble tannins, including hydrolysable ellagitannins (punicalagins and punicalins) (Gil et al. 2000), condensed tannins (proanthocyanidins) (Poyrazoglu et al. 2002), anthocyanins (Hernández et al. 1999), phenolic acids (gallic acid and ellagic acid) (Mousavinejad et al. 2009), and organic acids (malic acid) (Poyrazoglu et al. 2002). All these substances exhibit high levels of antioxidant activity (García-Alonso et al. 2004). Polyphenols occur in all fruits and vegetables and significantly influence their colour, texture and flavour, along with antioxidant (Hernandez et al. 1999) and antibacterial activities (Negi and Jayaprakasha, 2003). Non-tannin pomegranate polyphenols include falavonoids (falvovols, falvanols and anthocyanins). Flavonoids include flavonols such as luteoline quercetin, and kaempferol occurring in the rind extract and anthocyanins present in the aril. Anthocyanins are water-soluble pigments (originating from delphinidin, cyanidin and pelargonidin), giving the pomegranate fruit and juice with its distinctive red (Hernández et al. 1999). Other phytochemicals present include organic and phenolic acids, sterols and triterpenoids, fatty acids, triglycerides, and alkaloids.

Hydrolyzable tannins (HT) occur in the membrane and pith of the pomegranate. They are the major polyphenols contained in pomegranate extract. HTs (punicalin, pedunculagin, punicalagin, gallagic and ellagic acid esters of glucose) contribute 92% of the antioxidant activity of the entire fruit. The main HT is punicalagin, which accounts for approximately 50% of antioxidant activity in pomegranate extract (Gil et al. 2000; Passamonti et al. 2003; García-Alonso et al. 2004). Punicalagin is a large polyphenolic substance with a molecular formula of $C_{48}H_{28}O_{30}$, a molecular mass of 1084.718 and 11 cyclic rings with two ester bridging groups (Figure 1-2). It is suggested that punicalagin and its degradation compounds are the major bioactive extracts occurring in pomegranate rind extract PRE (Seeram et al. 2005).

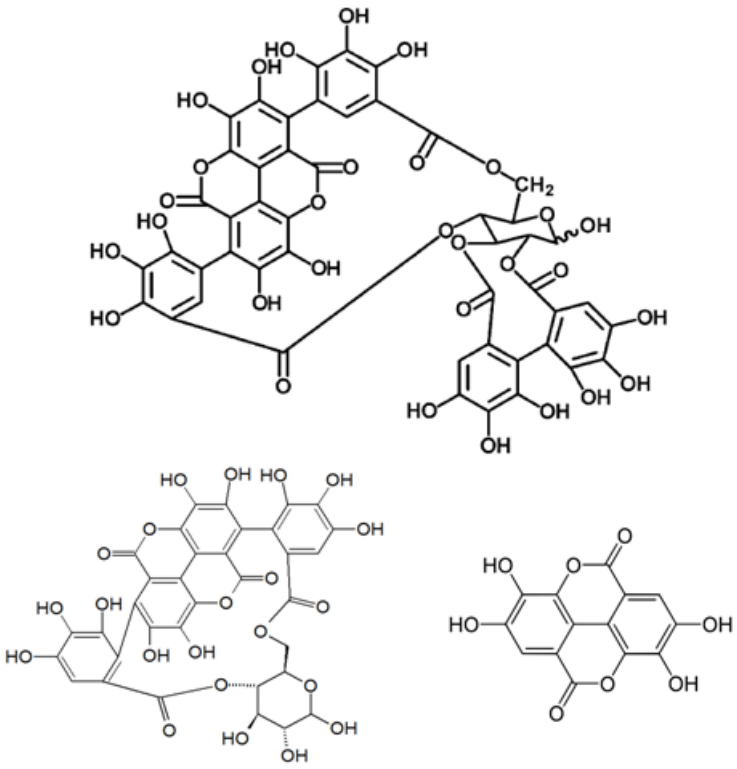


Figure 1-2 Chemical structures of punicalagin (top), the main polyphenol in pomegranate extract, and its constituent moieties punicalin (lower left) and ellagic acid (lower right) that resulted when degradation of punicalagin occur .

1.5 Antioxidant activity of pomegranates

The free radical reactive oxygen species (ROS) such as hydrogen peroxide has been linked to many diseases and their presence in high level will increase the oxidative stress on cell leading to its damage. Elevated levels of ROS and impaired mechanism of detoxification of the free radical ROS, has been found in non-healing wound site (Cano Sanchez et al. 2018). Antioxidant activity conferred by polyphenolic substances is known to provide health benefits via free radical scavenging activity, which leads to reduced oxidative stress and limit their damaging effect. These include hydrolysable tannins, which are mostly found in the peel and mesocarp of pomegranates. Polyphenols contribute significantly to the organoleptic properties of pomegranate arils and juices, as

they result in the attractive red colour and slight astringency that is typical of the fruit (Gil et al. 2000; Fischer et al. 2011). Elfalleh et al. (2009) identified antioxidant activity in pomegranates via independent testing. There was significant linear correlation between phenolic concentration and antioxidant activity. The coefficient for correlating the total amount of phenols and flavonoids with antioxidant activity in peel or juice was determined using the ABTS and DPPH methods.

1.6 Antimicrobial activity of pomegranates

The antibacterial properties of phenolic substances can alter enzymes (Furneri et al. 2002) and they can also attach to substrates such as minerals, vitamins and carbohydrates, rendering them inaccessible to microorganisms (Stern et al. 1996). Moreover, phenols can be absorbed into the cell wall, leading to perturbation of the membrane structure and function (Hugo and Bloomfield 1971). Tannins are phenolic compounds with a high molecular weight, being found in many plants, including pomegranate (*Punica granatum L.*) exocarp. Tannins are able to form complexes, mostly with proteins, but also with carbohydrates, resulting from the occurrence of numerous phenolic hydroxyl groups (Hassanpour et al. 2011a, 2011b). Duman et al. (2009) report on research indicating the significance of physicochemical properties of pomegranate in relation to antimicrobial activity.

1.6.1 E. coli

E. coli is a food-borne bacterium – more specifically a toxin-producing enteropathogen - that induces bloody diarrhoea, a haemorrhagic form of colitis, and haemolytic uremic syndrome (Bruins et al. 2006). Quantitative research has been conducted into the antimicrobial activity of pomegranate fruit and peel extracts against *E. coli* (Pagliarulo et al. 2016). Crude pomegranate juice extract exhibited inhibition of the development of *E. coli* isolate at 40 μg/μL. The minimum inhibitory concentration MIC values of pomegranate extracts determined in different studies vary considerably. Pomegranate

peel extracts revealed the following antimicrobial activity against *E. coli*: MIC 30 µg/µL and MBC 70 µg/µL. It may be concluded from the findings from microbiological testing that the antimicrobial capacity of crude peel extracts is higher than that of purified extracts and crude peel extracts are especially effective at opposing the development and survival of clinical isolates. The development, survival and resilience of the *E. coli* isolates were also studied. A concentration of 160 µg/µL in crude pomegranate juice extract had a complete bactericidal effect on *E. coli* and the bactericidal level was 70/µL in crude pomegranate peel extracts.

The antimicrobial properties of a several tropical plants from Puerto Rico were investigated utilising the disc diffusion method in relation to *E. coli* and *S. aureus* (Melendez and Capriles 2006). It was found that pomegranate extract generated inhibition zone sizes of 11 for *E. coli*. MIC levels have been determined in a number of studies, extending from 0.62 to 10 mg/mL antagonizing *E. coli* (Navarro et al. 1996).

1.6.2 Staphylococcus aureus and MRSA

A study by Biedenbach et al. (2004) into *Staphylococcus aureus* declared that it is one of the major sources of infections in hospitals, and it is the second most responsible microorganism causing bacteraemia, and is a major factor in many sepsis cases on a global scale. Over the past two decades, *S. aureus* and *MRSA* have emerged as two of the most resistant and dangerous bacteria strains. Both strains are particularly strong against a variety of antibiotics, few of which – including macrolides, fluoroquinolones, or β-lactams – can have an effect on these pathogens (Klugman and Koornhof 1989). Cosgrove et al. (2003) identified that MRSA infections are harder to combat than those caused by *S. aureus*, which is more susceptible to methicillin, and that the former tend to have a higher mortality rate. A Brazilian study investigated the impact of pomegranate extract on *Staphylococcus aureus* development and subsequent generation of enterotoxin (Braga et al. 2016). The interaction of a *P. granatum* methanolic extract with five antibiotics was observed in relation to 30 clinical isolates of Methicillin-Resistant

Staphylococcus aureus (MRSA) and methicillin-sensitive *S. aureus.* The relevant antibiotics were chloramphenicol, gentamicin, ampicillin, tetracycline and oxacillin. A synergistic effect of the pomegranate extract and all five antibiotics was identified against the *S. aureus* isolates, with the synergy with ampicillin being the most notable. This interaction extended the time before bacterial growth occurred by three hours (compared with that of ampicillin in isolation). It was also bactericidal, demonstrated by a 72.5 % decrease in methicillin-sensitive organisms and a 99.9% decrease in MRSA. The findings from this research and previous studies indicate that the ellagitannin, punicalagin, is the main component resulting in the reported antibacterial activity.

Quantitative research was conducted into the antimicrobial activity of pomegranate fruit and peel extracts opposing *S. aureus* (Pagliarulo et al. 2016). The development of *S. aureus* isolate was blocked by a concentration of 65 µg/µL of juice extract. Pomegranate peel extracts revealed the following antimicrobial activity in relation to *S. aureus*: MIC 20 µg/µl and MBC 50 µg/µL. The effects of crude pomegranate juice and rind extracts were determined on the development, survival and resilience of *S. aureus.* A complete bactericidal effect on *S. aureus* was exhibited by the juice extract at 160 µg/µL and the peel extract at 50 µg/µL. Melendez and Capriles (2006) studied the antimicrobial activity of various tropical plants from Puerto Rico that antagonising *E. coli* and *S. aureus*. The disc diffusion technique was utilised. It was established that pomegranate extract resulted in inhibition zone sizes of 20 mm in relation to *S. aureus*.

1.6.3 Other bacteria

Choi et al. (2011) report on a study of *in vitro* and *in vivo* antimicrobial activity of *P. granatum* peel ethanol extract (PGPE) opposing 16 varieties of *Salmonella enteritidis*. The disc diffusion method produced Zones of Inhibition ranging from 13.3 to 18.6 mm against tested bacteria. Each disc was loaded with 500 µg of PGPE per disc. The study also relates the increase in dose loading per disc had led to increase in zones of inhibition in dose-dependent manner. The minimum inhibitory levels MIC of PGPE

ranged from 62.5–1000 µg/mL. The *in vivo* antibacterial function of the PGPE extract was also evaluated in relation to *S. typhimurium*. Mice were infected with *S. typhimurium* and subsequently with PGPE. It was shown that PGPE had considerable impact on mortality and the amount of viable *S. typhimurium* extracted from faeces. Although clinical effects and histological damage were infrequently found in these mice, untreated controls exhibited signs of sluggishness and histological damage of the liver and spleen. The collated findings of the research suggest that PGPE has the capacity to deliver successful treatment for salmonellosis.

Another study was undertaken on the antimicrobial activity of extracts from pomegranate skins antagonising certain food-borne pathogens, deploying both *in vitro* (agar diffusion) and *in situ* (food) techniques (Al-Zoreky 2009). The MIC of 80% methanol extract (WME) in relation to *Salmonella enteritidis* was the highest, at 4 mg/mL. Phytochemical examination indicated the occurrence of active inhibitors in peels, such as phenolics and flavonoids.

While the prevalence of gastric cancer has systematically reduced in the last half-century, Watanabe et al. (1998) reiterate that it is still the second most common cancer globally and continues to pose a major threat to health. *Helicobacter pylori* was first identified by Warren and Marshall (1983), and its actions in the development of gastric carcinogenesis have been widely researched since. Watanabe et al. (1998) note that during 1994, at a Working Group of the World Health Organisation's International Agency for Research on Cancer, *H. pylori* was categorised as a group 1 carcinogen for humans. There is increasing prevalence of *Helicobacter pylori (H. pylori)*, which is resistant to many antibiotics and a primary cause of gastric cancer. *P. granatum* extracts were analysed opposing multiple antibiotic-resistant *Helicobacter pylori*, as reported by Voravuthikunchai and Mitchell (2008). MIC was established via the agar dilution technique in Petri dishes with a Millipore filter membrane. Minimal bactericidal concentrations (MBC) were identified with the extract that generated a significant MIC in relation to each variety of bacteria, by positioning the Millipore filter membrane on a

fresh agar plate. Ethanolic extracts of *P. granatum* inhibited the development of all types of *H. pylori*, with a MIC of 0.8 and a MBC of 3.1 mg/mL.

Pomegranate, *Punica granatum* L. is sometimes utilised to treat gastro-intestinal resistant organisms, oral bacteria, extremely pathogenic and antibiotic-resistant organisms (*Salmonellae* and *Pseudomonas aeruginosa*) and certain skin infecting pathogens, such as fungus and MRSA. Moorthy et al. (2013) describe research into the *in vitro* antimicrobial activity and phytochemistry of ethanolic pericarp extract of *P. granatum*, against 21 microorganisms (19 bacteria and 2 fungi). Disc diffusion and broth dilution techniques were used, along with a standard procedure to determine the phytoconstituents. Ethanolic extract of pericarp exhibited notable bactericidal function opposing *Yersinia enterocolitica* (25.8 mm), *Staphylococcus epidermidis* (24.6 mm), *Burkholderia cepacia* (19.4 mm), *S. aureus* (19.2 mm), *Salmonella enterica* (21.2 mm), *Salmonella paratyphi* A (18.8 mm), *Salmonella typhimurium* (18.6 mm), *Escherichia coli* (18.4 mm) and *P. aeruginosa* (18.2 mm). Utilising the broth dilution method, the lowest concentration of ethanolic pericarp extract at 512 μg/mL exhibited a high level of activity blocking *S. epidermidis* and *Y. enterocolitica* and a MIC of 1,024 μg/mL was established opposing *S. aureus*, *Streptococcus mutans*, *S. paratyphi* A, *S. typhimurium*, *Salmonella brunei*, *P. aeruginosa* and *B. cepacia*. The ethanolic extract of *P. granatum* pericarp revealed considerable inhibitory function compared to other organisms. The greatest minimum inhibitory function was noted in relation to bacteria rather than fungi. This function may result from the occurrence of certain secondary metabolites, such as phenolic compounds, flavonoids, terpenoids, phytosterols, glycosides and tannins detected in the ethanolic pericarp extract of *P. granatum.*

Punica granatum (pomegranate) extract inhibits dental plaque. A hydroalcoholic extract of *Punica granatum* fruit (HAEP) was examined for antibacterial activity opposing dental plaque microorganisms (Menezes et al. 2006). A cohort of 60 healthy patients who had fixed orthodontic appliances were allocated randomly to three groups of 20: (1) a control group who rinsed with 15 mL distilled water, (2) a group who rinsed with 15

mL chlorhexidine, a standard antiplaque mouth wash; and (3) a group who rinsed with a 15 mL HAEP solution. Rinsing lasted for one minute and dental plaque was gathered from each patient before and afterwards. Samples were diluted and placed on Meuller-Hinton agar and incubated at 37 °C for 48 hours. HAEP reduced the amount of colony forming units (CFU) of dental plaque bacteria by 84%, similar to chlorhexidine (79 % reduction) but considerably outperforming the control rinse which was associated with a decrease of 11 %. Both HAEP and chlorhexidine successfully inhibited *Staphylococcus, Streptococcus, Klebsiella*, and *Proteus* species, as well as *E. coli.* The ellagitannin, punicalagin, is considered to be the component driving the pomegranate's antibacterial activity.

Ahmad and Beg (2001) report on research involving ethanolic extracts of 45 plants from India that were traditionally utilised in medicine. The antimicrobial functions of these extracts were analysed in relation to certain drug-resistant bacteria. It was observed that alcohol extracts of pomegranate fruits exhibited wide ranging antibacterial function opposing *S. aureus, E. coli* and *Shigella dysentriae.*

1.6.4 Antiviral activity of pomegranate

Pomegranate has been a common remedy in traditional medicine for a broad range of conditions such as upper respiratory tract infections and influenza (Nonaka et al. 1990). Haidari et al. (2009) reported a study into how pomegranate purified polyphenol extract (PPE) suppressed the influenza virus and also exhibited a synergistic effect with oseltamivir. The research showed that PPE constrained the agglutination of chicken red blood cells (CRBC) by the influenza virus and that it was virucidal. Single-cycle growth conditions revealed that, separate from this virucidal effect, PPE inhibited viral RNA replication as well. PPE did not modify virus ribonucleoprotein (RNP) entry into nucleus or translocation of virus RNP from nucleus to cytoplasm in Madin-Darby canine kidney (MDCK) cells. Four main polyphenols in PPE were investigated: ellagic acid, caffeic acid, luteolin, and punicalagin. It was found that punicalagin is the effective, anti-

influenza constituent of PPE. Punicalagin prevented replication of the virus RNA, blocked agglutination of CRBCs by the virus and was virucidal. It was also demonstrated how PPE worked in combination with oseltamivir. This was beneficial since it can result in a potentially higher potency, improved clinical efficiency and reduced dosage, reduced toxicity and side effects, and increased cost-effectiveness.

Su et al. (2010) observe that polyphenols in pomegranate juice have also effectively combated food borne viral infections. In the absence of culturable human noroviruses, feline calicivirus (FCV/F9), murine norovirus (MNV/1), and MS2 positive-sense single-stranded RNA (ssRNA) bacteriophage were utilised as food borne viral substitutes. Different concentration of pomegranate juice (8, 16, and 32 mg/mL of polyphenols) were added to two types of viruses in high titers ($\sim$7 $\log_{10}$ PFU/mL) and ($\sim$5 $\log_{10}$ PFU/mL). This combination was incubated for 1 hour at room temperature. The low titer of FCV-F9, MNV-1, and MS2 were decreased by 2.56, 1.32, and 0.32 $\log_{10}$ PFU/mL, respectively, and 1.20, 0.06, and 0.63 $\log_{10}$ PFU/mL, respectively, for high titers. The study concluded that pomegranate juice was able to reduce the food borne viral substitutes FCV-F9, MNV-1 and MS2 (particularly for low titers). It was suggested that these samples had good potential as natural means of suppressing or mitigating human norovirus infections.

It has recently been shown that the innate antimicrobial activity of PRE is considerably enhanced by the co-application of zinc sulphate, or Zn(II) ions. Up to 7-log reduction potency was identified in relation to the *Herpes simplex* virus (HSV), which is the microorganism responsible for anogenital herpes and oral cold sores (Houston, 2017b).

1.6.5 Antifungal activity of pomegranate

Both Beck-Sagué and Jarvis (1993) and Nguyen et al. (1996) referred to the greater prevalence of opportunistic fungal infections, as well as the problems raised by drug-resistant strands, especially those of the *Candida* strain, in the last few decades. Santos

et al. (2009) refer to punicalagin isolated from pomegranate skin being effective in combatting *Candida albicans* and *Candida parapsilosis* and also interacting favourably with fluconazole. Transmission electron microscopy revealed that cells treated with punicalagin exhibited a thickened wall, altered space between the wall and the plasma membrane, vacuoles, and decreased cytoplasmic content. Denture stomatitis is often connected to *C. albicans* although other *Candida* species have been extracted from lesions. Ethnobotanical research carried out in Brazil has proven the application of pomegranate in oral health (Santos et al. 2009).

Tayel and El-Tras (2009) established that methanol, ethanol and water extracts of pomegranate rinds successfully reduced *C. albicans* growth. Additionally, it was found that aerosol extracted from pomegranate rind effectively and comprehensively sanitised semi-closed sites from *C. albicans* growth. Therefore, it could play a role in preventing *C. albicans* contamination and growth in susceptible locations.

1.7 PRE and metal ion combination

When PRE and Cu^{2+} or Cu(II) ions interact, they show increased antimicrobial activity opposing *E. coli*, *Ps. aeruginosa* and *P. mirabilis* and moderate activity opposing *S. aureus,* compared to the individual activity of PRE and of Cu(II) ions. Adding large volumes of vitamin C significantly increased the function of both PRE/Fe(II) and PRE/Cu(II) combinations opposing *S. aureus*. Further research was being undertaken into the effect of PRE/ Cu(II) amalgamations on clinical isolates of *Ps. Aeruginosa* and *S. aureus* (McCarrell et al. 2008).

Yehia et al. (2011) analysed antibacterial activity opposing Gram positive bacteria *Staphylococcus s*pp., *B. subtilis, Bacillus indicus* and Gram negative bacteria *E. coli, Enterobacter aerogenes*, *Serratia marcescens* and *Brucella spp*. Mixtures of PRE with metal and PRE with vitamin c and metals were investigated using Zone of Inhibition test ZOI. The three metals that had been used in this study were $CuSO_4$, $MnSO_4$ and $ZnSO_4$.

Tested mixture prepared by adding 330 µL of PRE was added to 700 µL of each metal salts used ($CuSO_4$, $MnSO_4$ and $ZnSO_4$) and each salt was prepared in concentration of 4.8 mM. Among these mixtures tow mixtures of PRE: vitamin C and PRE: $ZnSO_4$were with highest antibacterial activity than the rest of mixtures. A combination of PRE and $ZnSO_4$ showed increased antimicrobial activity opposing *B. subtilis, Staphylococcus spp.* and *Brucella spp.*, each at 15 mm with 15, 15 and 15 mm zones of inhibition respectively. A combination of PRE and vitamin C (1:1) had greater inhibitory effect on *E. coli* and *B. indicus* than the three metal mixtures that reached 20 and 18 mm zones of inhibition respectively. Additional research was being conducted to determine how this functioned, as well as the process of enhancement by metal salts and vitamin C. This study showed also that the mixture of PRE and $MnSO_4$ only inhibited the yeast *Saccharomyces cerevisiae* at10 mm. Moderate antimicrobial activity was observed when combining PRE, vitamin C and metal salts against the various microorganisms. It was determined that an combination of PRE, vitamin C and $MnSO_4$, whereas a mixture of PRE/vitamin C/ $CuSO_4$ suppressed *Brucella* spp. at 10 mm. A PRE - vitamin C - $ZnSO_4$ combination did not impact on any of the tested types of bacteria. The PRE in isolation in this study did not show antimicrobial function opposing any of the tested isolates (Yehia et al. 2011). It is noticeable that the extraction method in this study was done by blending 50 g of the seed with 100 ml of distilled water for 10 min. The final extract underwent filtration through muslin Whatman No. 1 filter paper and then autoclaved for 15 minutes at 121 °C before storage at – 20 °C.

A patent disclosed by Stewart et al. (1995), showed that PRE and iron sulphate produces a potent phagicidal activity. They showed that addition of $FeSO_4$ to pomegranate rind extract gave 11 log reduction of plaque forming within 3 minutes with no effect on the host cell. However, the stability of ferrous sulphate and PRE combination was low (30 min) due to rapid oxidation to ferric sulphate to a blackened material – this also coincided with a loss of antimicrobial activity.

Recent work done by Houston (2011) at Cardiff University, showed that addition of ferrous sulphate to PRE was a potent virucidal against *Herpes simplex* virus, although the stability was an issue with ferrous salt because of the short window of activity and black by product, reflecting the low stability of the combination as observed by Stewart et al. (1998). However, it was noticed that the formation of black by product did not occur when ferrous sulphate was replaced with zinc sulphate – Zn(II) is far more stable than Fe(II). His work showed that that a potentiation of antimicrobial and microbicidal activity of pomegranate extract can be established by the addition of zinc sulphate ($ZnSO_4$). The activities increased by 7log reduction against *Herpes simplex* virus. It was also stated that Zn^{2+} ions (hereafter referred to as Zn(II)) remained stable after addition of $ZnSO_4$ to pomegranate rind extract (PRE). No change in colour or redox change upon the addition of $ZnSO_4$ to PRE and no new compound yielded by the combination because of the zinc ion stability. Preliminary work also carried out in CSPPS noted the same combination also shows potentiated activity against a number of bacteria including *E. coli*, MRSA and *Ps aeruginosa.* Thus the combination is stable in terms of microbial activity, and both PRE and $ZnSO_4$ have good safety profiles for the human body. The precise mechanism of potentiated activity, which has a clear optimum ratio of PRE/Zn, has not yet been clearly established; but the high tannin content of pomegranate extract is believed to be associated with destabilization of microbial membrane, allowing the entry of toxic levels of zinc ions.

Considering the previous work on the antimicrobial activity of PRE in relation to potentiation of virucidal and bactericidal activity by Zn(II) ions, it is proposed that PRE could demonstrate similar levels of bactericidal activity which could be exploited as novel topical broadspectrum antimicrobial therapeutic systems. Consequently, this project set out to investigate such a possibility in relation to skin and wound infections.

1.8 The Skin

The human skin is the body's biggest organ and is representative of its first defence line with against external foreign subject. Alongside protection, there are two other key functions of the skin: sensation and regulation. Figure 1-3 shows cross sectional of human skin shows the three main layers: epidermis, dermis and hypodermia (Zhang and Michniak-Kohn 2012).

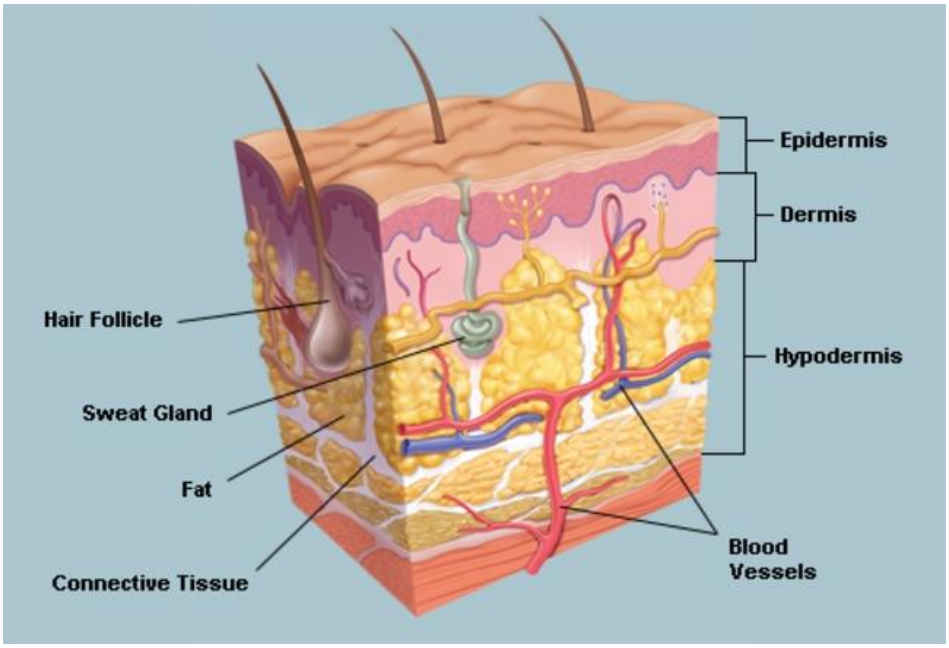


Figure 1-3 DiaGram shows human skin's three layers: epidermis, dermis and hypodermis. (www.webmd.com/skin-problems-and-treatments, 2020) .

1.8.1 Skin and soft tissue infections (SSTIs)

Skin and soft tissue infections (SSTIs) are condition that mainly caused by bacterial infection that involve skin's upper layer of or it can exacerbate to involve underlying tissue. *Staphylococcus aureus* has been found to be the primary pathogen that is responsible of SSTI and foremost methicillin-resistant Staphylococcus aureus (MRSA)

related skin infection can be life threatening infections. Other bacterial pathogens that can cause SSTI include Gram positive staphylococcus epidermidis or Gram negative *Pseudomonas aeruginosa*. Most of the time combinations of more than one microorganism will happen in SSTIs such as S. aureus and/or Streptococcus pyogenes infection in impetigo (Figure 1-4).

The severity of such infection varies from mild superficial infection e.g. pyoderma to complicated severe skin and soft tissue infection (cSSTI) e.g necrotising fasciitis. Clinical sign and symptoms of SSTI vary according to the severity of infection that includes: erythema, edema, pain and warmth in minor infections. While systemic sign and symptoms can occur in sever SSTIs including: increase in body temperature, increase in blood pressure, increase heart rate more than 100 beats/min altered mental status and extreme pain (Vincent and Coleman 2008). Many risk factors can increase the progression of Mild skin infection to sever one e.g. (diabetes mellitus, immune compromised condition, burns, skin trauma, contaminated wounds, venous insufficiency disease).

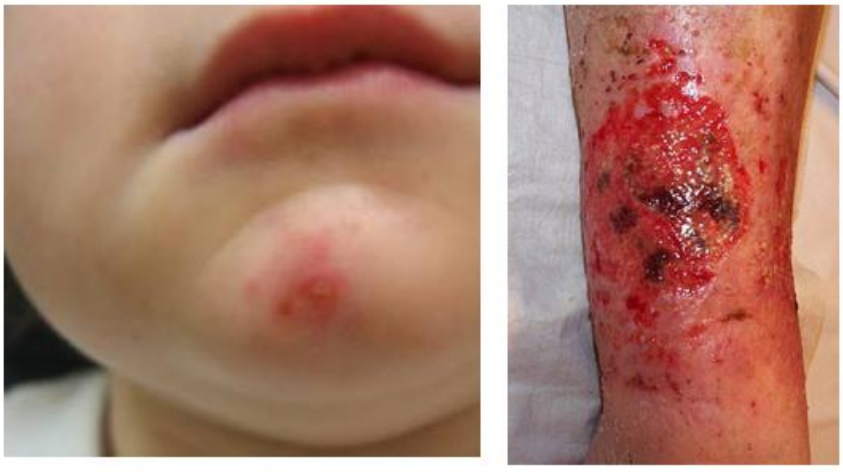


Figure 1-4Impetigo skin infection clinical presentations. (Left) Typical crusting lesions of nonbullous impetigo. (Right) Blistering lesions characteristic of non-bullous impetigo (Williamson et al. 2017)

Scarlet fever is a bacterial SSTI occurring as a result of a group A streptococcus infection, *Streptococcus pyogenes*. It most commonly affects children between 5-15 years of age, and has a bright red rash which feels like sandpaper and the tongue may be red and bumpy (figure 1-5)

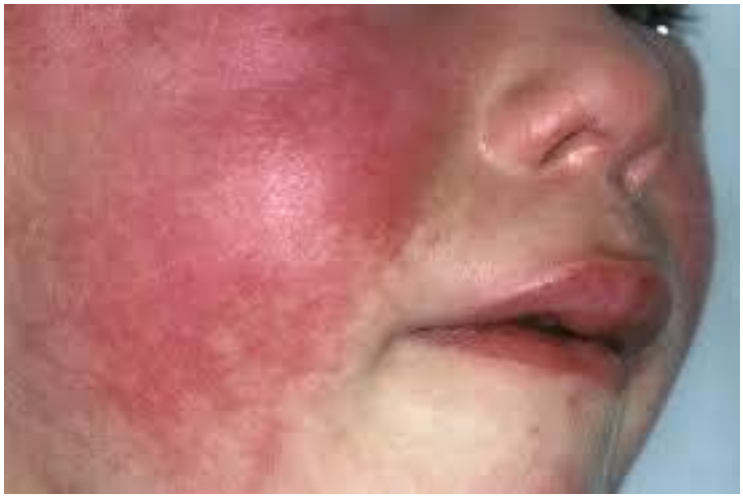


Figure 1-5 clinical presentation of Scarlet fever

Cold sores are a common viral infection, caused by the *Herpes simplex* virus (HSV-1) – closely related to the one that causes genital herpes (HSV-2. They are start as, fluid-filled blisters on and around the lips. These blisters are often grouped together in patches. After the blisters break, a crust forms over the resulting sore. Cold sores usually heal in two to four weeks without leaving a scar. Cold sores spread from person to person by close contact. Both of these viruses can affect your mouth or genitals and can be spread by oral sex. Cold sores are contagious even if invisible. There's no cure for HSV infection, and the blisters may return. Antiviral medications can help cold sores heal more quickly and may reduce how often they return. There are several distinct stages as shown in Figure 1-6.

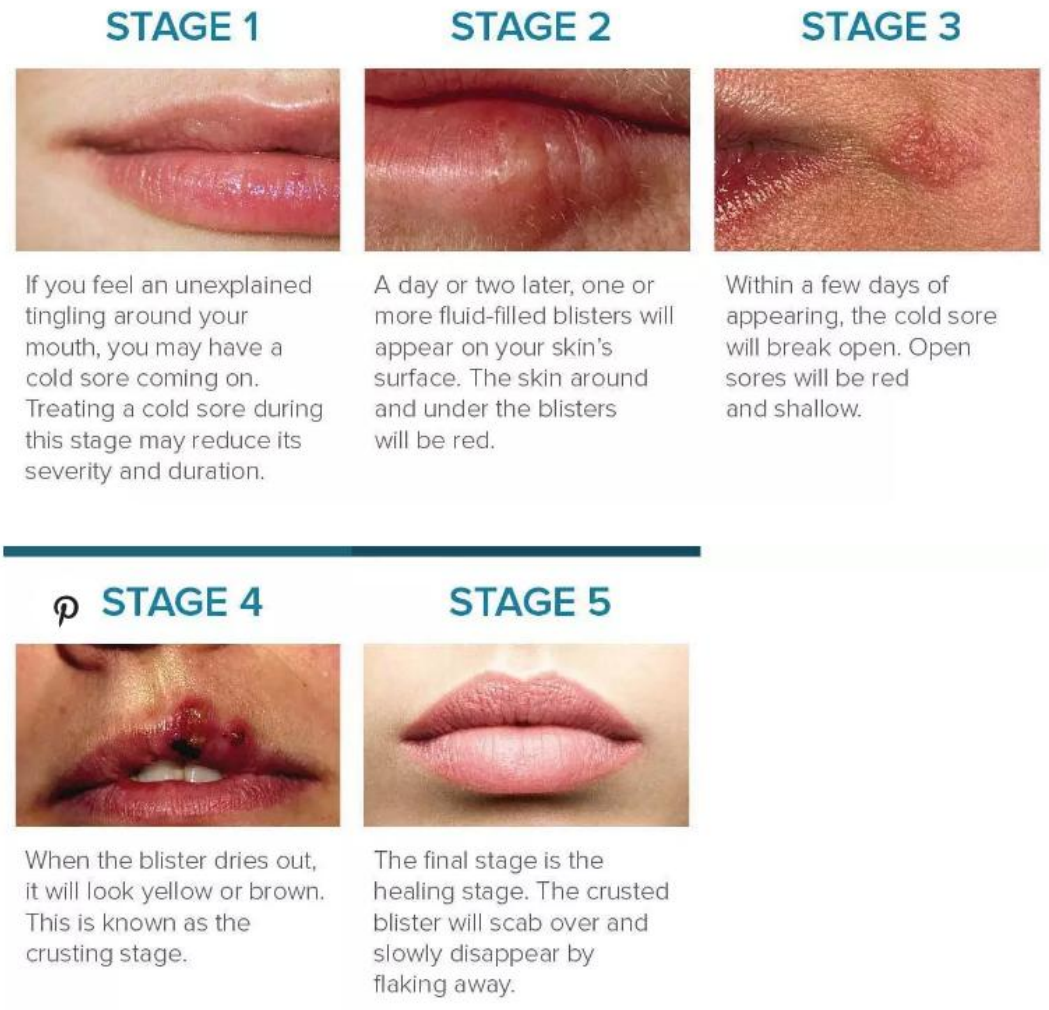


Figure 1-6 Stages of peri-oral cold-sore development from tingling throughcrusting and finally healing, at whcih stage the virus returns to its latenst stage within the body

Another type of skin infection that is potentially relevant to this work is cellulitis. Cellulitis occurs when bacteria enter a break in the skin and spread laterally and often extensively. The result is infection, which may cause swelling, redness, pain, or warmth (Vinken et al. 2001). Cellulitis is a serious condition as there is high risk of progression to systemic infection and sepsis (figure 1-7). It is typically treated with systemic antibiotics although topical therapy is may be appropriate in the early stages.

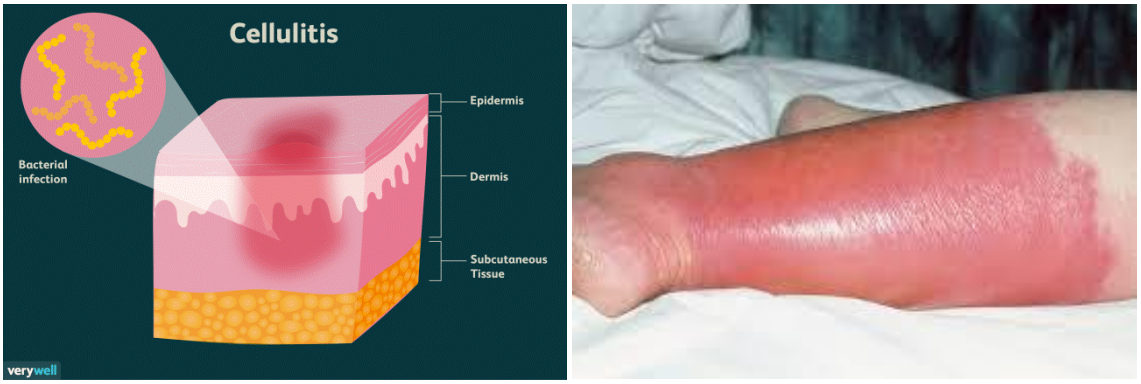


Figure 1-7 Left: depiction of cellulitis. Right: Cellulitis case present in patient's lower leg whereby an MRSA infection has spread laterally under the skin (photo from https://emedicine.medscape.com/)

1.8.1.1 **Current Treatment for SSTI**

Treatment options in SSTI patients can be received and administered as outpatient if the infection is minor or as inpatient and hospitalization will be required in sever SSTI. Minor skin infection usually involves prescription of topical broadspectrum antibiotic such as fusidic acid and mupricin and in this case patient can use and apply at home. Hospitalization or recurrent treatment of SSTI at clinics, will be required in patient who can potentially develop complicated sever SSTI including: diabetes mellitus, MRSA related infections, burn patient, heavily contaminated wounds or immune compromised condition. When severe complicated SSTIs and MRSA related infection occurs, oral or systemic antibiotic will be used for treatment, such as: vancomycin and inezolid (Lambert 2011).

Additionally, prophylactic therapy is considered highly important to prevent skin and wound related infections especially after surgery to reduce surgical site infections SSIs and usually includes treatment with oral antibiotic such as clindamycin and application of topical antibacterial or antiseptic agents such as chlorhexidine, povidine iodie (Lambert 2011; Williamson et al. 2017).

Topical and oral antibacterial drug therapy is a growing approach. A combined regimen of both systemic and topical antibiotics can provide an additive, more potent effect that can exceed the benefit of using one drug alone (Scher and Peoples 1991).

The combination of topical and oral antibiotics have been found to be a successful approach to eradicate the resistance type of bacteria methicillin-resistant *Staphylococcus aureus* (MRSA) colonisation in hospitalized patients. Combined topical and oral antimicrobial therapy for the eradication of MRSA was investigated in a retrospective chart study that included 207 adults in patient with MRSA colonisation, at Sunnybrook Health Sciences Centre hospital affiliated with the University of Toronto, Canada. The study was set for MRSA eradication treatment using topical 4% chlorhexidine soap for bathing and washing, 2% mupirocin ointment topically applied to interior nostril three times per day and oral antibacterial rifampin (300 mg twice daily) and either trimethoprim/ sulfamethoxazole (160 mg/800 mg twice daily) or doxycycline combination treatment for seven days. The study found that combination of topical and systemic antibacterial agent was effective to eradicate MRSA from selected patient 98% and 94% and of treated patient with combined therapy showed culture negative MRSA after completion of therapy and 90 % of those after 3, and 6 month follow up showed successful decolonisation (Fung and Simor 2002).

1.8.2 Wounds

The definition of a wound is that it is the disruption of the continuity of a tissue in both anatomic and cellular terms and may happen due to trauma, either with thermal, chemical, physical microbial or immunological tissue. Wounds can compromise the self-image of a patient as well as well-being, independence and working capacity. According to Maver et al. (2015) and Leach (2004) wound management that is effective is thus necessary on both the individual and the community level.

1.8.3 Wound-healing process

At the insult time, multiple pathways both cellular and extracellular are activated, in a stringently coordinated and regulated manner, with the target to restore the integrity of the tissue. Orthodox wound-healing processes comprise four specific phases: haemostasis, inflammation, proliferation, and tissue remodelling (Harper et al. 2014).

1.8.3.1 **Haemostasis**

Homeostasis phase, which involves the constriction of blood vessels to prevent flow of blood. The decreased blood flow to that tissue results in hypoxia and acidosis which trigger nitric oxide production and other vasodilator. Then after that, platelet aggregation happened on blood vessels wall and finally formation of blood clot at the site which will prevent extra bleeding (Harper et al. 2014). At early stage, when the skin is injured, there is disruption to the epidermal barrier and a release by keratinocytes of prestored interleukin-1 (IL-1). IL-1 is the initial signal alerting the cells that are surrounding of barrier damage. Additionally, components of blood are released into the site of the wound which activates the clotting flow. The subsequent clot provokes haemostasis, providing a matrix of inflammatory cells to invade the area (Barrientos et al. 2008). There is a degranulation of platelets that release alpha granules and these secrete factors of growth such as platelet-derived growth factor (PDGF), epidermal growth factor

(EGF) and transforming growth factor-beta (TGF-β). Together with pro-inflammatory cytokines such as IL-1, PDGFs are significant in the attraction of neutrophils to the site of the wound in order to remove contaminating bacteria (Harper, Young, and McNaught, 2014).

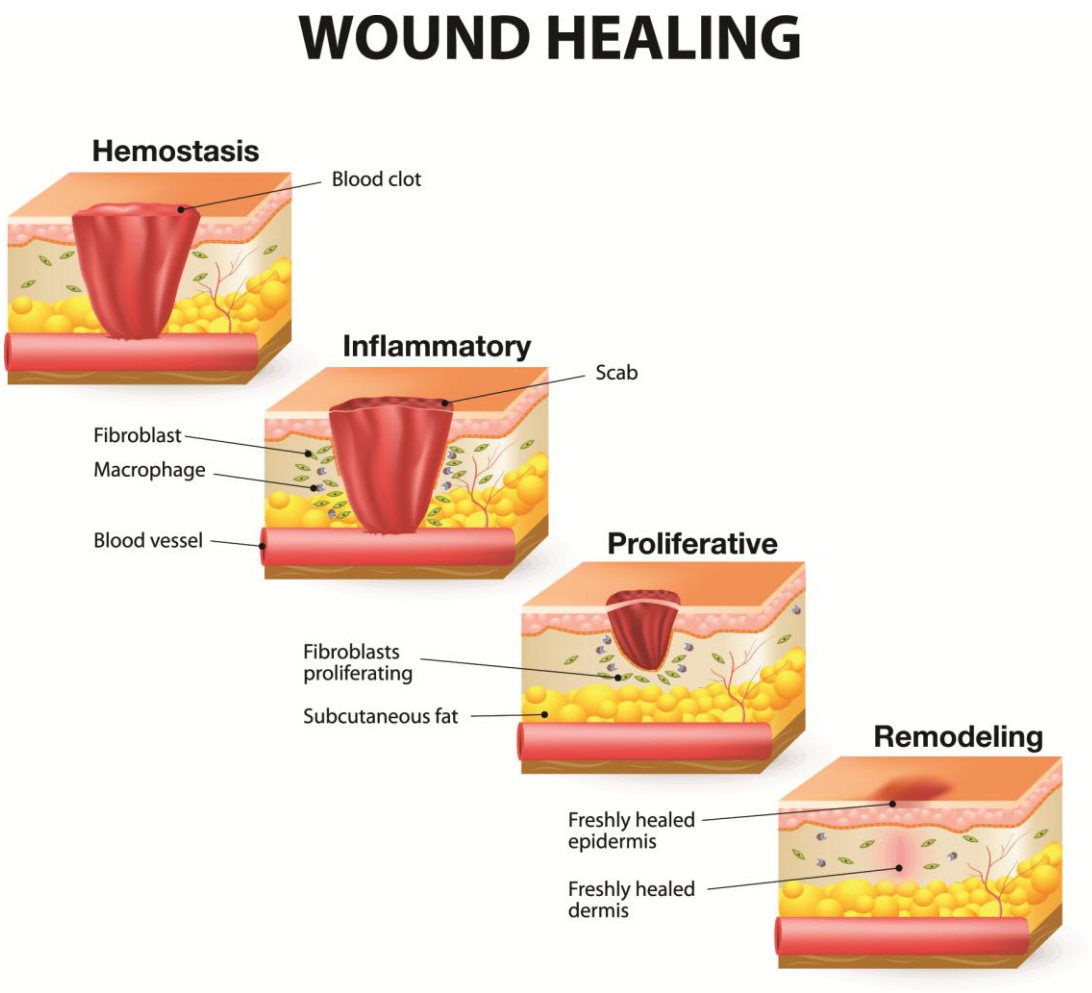


Figure 1-8 DiaGram showing the 4 different stages of wound healing process.

1.8.3.2 **Inflammation**

The second stage is the inflammation phase which is characterized by infiltration of neutrophils, macrophage and T-lymphocyte in the wound site. Neutrophils act as a cleaning machine against bacteria and debris in the wound area. These ‘first responders’

are known as neutrophils and are highly ambulatory cells. According to George et al. (2006), within an hour of the insult, these cells infiltrate the wound, migrating in constant levels for the initial 48 hours. This is arbitrated through different signalling methods that are chemically based, which includes the complement cascade, interleukin activation and the signalling of transforming growth factor-beta (TGF-b), leading to neutrophils travelling down a chemical slope towards the wound. This process is known as chemotaxis. Neutrophils clean wound debris by phagocytosis process or release of oxygen free radicals, reactive oxygen species (ROS), or by release of toxins and proteases that destroy bacteria such as chromatin protease, lactoferrin and cathepsin. Neutrophils after that go under apoptosis or phagosytosis by macrophage cells. The core aim of this phase in wound healing is the prevention of infection at the injury site. No matter the wound's aetiology, the mechanical obstruction which was at the forefront against the invasion of microorganisms is broken. Macrophages usually reach the sustained concentrations at wound area at 48 to 72 hours after injury time. With TGF-b's assistance, monocytes are transformed into macrophages which play a crucial role in the augmentation of the response of inflammation and the debridement of tissue (Barrientos et al. 2008). There is a large amount of growth factors harboured in macrophages, such as EGF and TGF-b, which are significant in the regulation of inflammatory response. In addition, they help with the stimulation of angiogenesis and the augmentation of granulation tissue formation (George et al. 2006; Harper et al. 2014). Macrophages implement the tissue granulation development through the release of a number of pro-inflammatory cytokines (IL-1 and IL-6) and growth factors (EGF, fibroblast growth factor [FGF], PDGF and TGF-b) (Barrientos et al. 2008).

1.8.3.3 **Proliferation**

This complicated procedure incorporates angiogenesis, the deposition of collagen, granulation tissue formation and re-epithelialisation which happens at the same time (Harper et al. 2014).

1.8.3.3.1 Angiogenesis

The triggering of angiogenesis happens at the point of the formation of the haemostatic plug, with platelets releasing PDGF, TGF-b and FGF. Vascular endothelial growth factor (VEGF) is released in reply to hypoxia. Together with the other cytokines, this process induces endothelial cells to activate neovascularisation and repair blood vessels that have been damaged. Mixed metalloproteinase (MMP) are a set of enzymes triggered by the invasion in the hypoxic tissue of neutrophils. Through liberating VEGF, they promote angiogenesis and extracellular matrix (ECM) remodelling (Harper et al. 2014; Siefert and Sarkar 2012).

According to Barrientos et al. (2008), with the help of FGF and platelet-released VEGF, there is a proliferation of endothelial cells and ensuing angiogenesis, an essential process for the deposition, synthesis and organisation of a new ECM.

1.8.3.3.2 Fibroblast migration

There is a stimulation of fibroblasts after the wound insult to reproduce by growth factors which the haemostatic clot release predominantly by PDGF and TGF-b and then it migrates to the wound. By day three, fibroblasts enrich the wound which lay down ECM proteins (hyaluronan, proteoglycans and fibronectins) and subsequently fibronectin and collagen are produced. Harper et al. (2014) explain that the result is a vascular pink, fibrous tissue called granulation tissue, formed as a replacement for the clot at the wound site. PDGF and TGF-b also cause phenotypic alterations in these cells through the conversion into myofibroblasts of fibroblasts which arrange themselves as a generated constructive power along the ECM's borders, which facilitates the closure of the wound (Barrientos et al. 2008; Hantash et al. 2008).

1.8.3.3.3 Epithelialisation/ re-epithelialisation

Epithelialisation is initiated within hours of the wound and EGF, FGF and TGF-a are released which act as a stimulant for the migration and proliferation of the epithelial cells. This procedure starts with the dissolution of the contacts of cell-substratum and

cell-cell with the migration and polarisation of keratinocytes following over the ECM that is provisional. With the achievement of the closure of the wound (100% epithelialisation), the differentiation and stratification of keratinocytes occur for the restoration of the barrier. Removing the granulation tissue via revascularisation is required for the formation of the matrix. Elastin and collagen fibres form a framework as a replacement for the granulation tissue, which according to Barrientos et al. (2008) is then saturated with glycoproteins and proteoglycans.

1.8.3.4 **Remodelling**

It can take up to 24 months for the final stage of healing for a wound, culminating in the scar tissue's normal maturation developing (Harper et al. 2014). This entails new collagen synthesis mediated by TGF-b, and old collagen breakdown by PDGF. The end product of this procedure is scar tissue.

1.8.4 Acute and chronic wounds

Disregarding the wound's aetiology, the processes of repair are similar. The result of a wound is tissue damage and this stimulates a physiological response that is coordinated for the provision of haemostasis and the initiation of the proliferation of the inflammation and remodelling processes. Acute wounds generally pass through these stages comparatively quickly. Wounds that display healing that is delayed three months after the first insult are referred to as 'chronic wounds (Harper et al. 2014; George et al. 2006), frequently as a result of pathological inflammation that is prolonged and maturation phase delayed or not attained.

According to Gottrup (2004), it is estimated that, during their lifetime between 1% and 2% of the population within countries that are developing will experience a chronic wounds. Chronic wounds are a significant cause of morbidity, with over 1% of the population of the UK affected at a cost in terms of treatment of at least £1 billion annually (Thomas and Harding, 2002). The chronicity of wounds has a tendency to

happen to individuals with a heightened risk of bacterial incursion or due to local factors that are poor such as venous hypertension, arterial insufficiency and trauma, or because of systemic disease including rheumatoid arthritis or diabetes mellitus (Falanga, 1993).

1.8.5 Effect of bacteria on wound healing

The healing of wounds is complex with a number of possible factors delaying the healing process. There is a growing interest in the impacts of bacteria on different wound healing phase. Bacteria colonises all wounds, with low bacterial levels being of benefit to the process of wound healing. Injury infection is damaging to wound healing, however, according to Edwards and Harding (2004), the management and diagnosis infection to a wound is controversial and varies among clinicians.

Bacteria from the skin surrounding contaminate all wounds as well as bacteria from the regional environment and endogenous sources of patients. For patients that are hospitalised, the local surroundings are particularly relevant. Wound's simple contamination and colonisation can be serious when the colonisation exacerbates to be critical colonisation until it reaches infection stage (Frank et al. 2005). The definition of colonisation is that of the existence of bacteria that proliferate without a perceptible host response. Wound colonisation may augment or impede the healing of a wound, depending upon the bacterial encumbrance. Loads of bacteria up to 10^5 organisms/g of tissue is considered equivalent to normal flora load and in case of injuries, it can help in wound healing process, although as Kingsley (2003) points out, this threshold may change through the immune system of the host's status and the number and sorts of species of bacteria present. An intermediate phase in the continuum of wound infection is increasingly being recognised and defined as 'critical colonisation' or local infection. This makes reference to a transitional stage between the wound simple colonisation and invasive infection as shown in Figure 1-7 (Edwards and Harding, 2004).

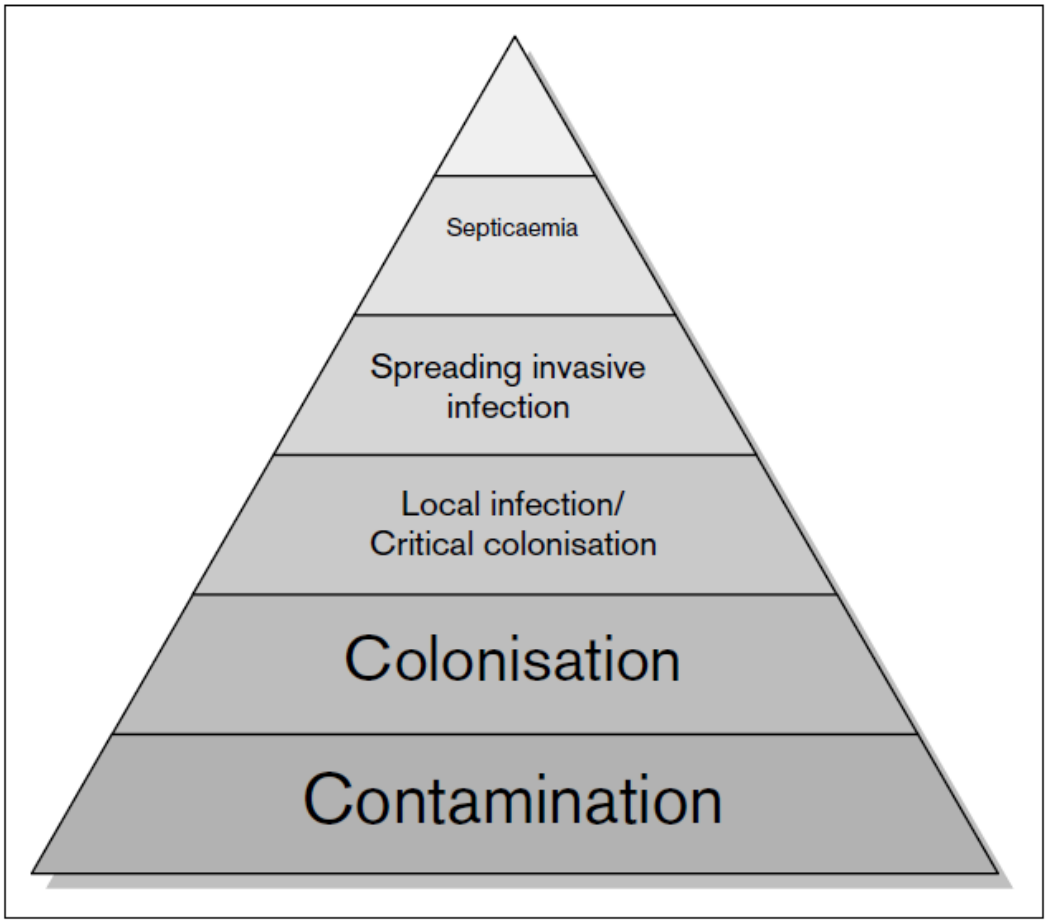


Figure 1-7 The clinical spectrum of bacterial affect on wound from colonisation to infection (Edwards and Harding, 2004).

According to Kingsley (2003) the characterisation of critical colonisation is a burden of bacteria that is increased or an infection that is covert, and at this stage, the wound may enter a chronic, non-healing, inflammatory condition. Frank et al. (2005) and Siddiqui and Bernstein (2010) describe the signs of critical colonisation as being deterioration or atrophy of granulation tissue, grey or deep red discolouration of granulation tissue, increased friability of the wound and increased drainage.

Infection transition happens when the proliferation of bacteria overcomes the immune response of the host and there is the occurrence of the host injury (Salcido, 2007). There

are several factors determining the transition to infection from colonisation: according to Wysocki (2002), these are the bioburden itself, the bacterial virulence, differing species' synergistic action, and the host's capability to mount an immune response. Malnutrition, diabetes, obesity, steroid use over the long-term and advanced age changes the immune system's efficacy and thereby increases the risk of infection progression. There are a number of other factors potentially preventing the body from preparing an efficient immune response such as foreign bodies, poor perfusion, necrosis, tunneling and undermining (Bowler 2001; Siddiqui and Bernstein 2010).

1.8.5.1 **Impact of wound infection on different stage of wound healing**

1.8.5.1.1 Impact of wound infection on inflammation phase:

The general impact of infection is heightened complement of protein consumption, which according to Robson et al. (1990) results in a reduction of chemotaxis as well as platelet depletion. Cellular response, in particular activity that is chemotactic and bactericidal, neutrophils, enhanced accumulation of white cell and macrophages. The function of white cells is impaired when infection is present, which includes short chain products of fatty acid by bacteria that is anaerobic (Wall et al. 2002). According to Edwards and Harding (2004), tissue damage is increased through heightened cytotoxic enzyme production and free oxygen radicals. Thrombosis that is localised and the release of metabolites that are vasoconstrictive increase the hypoxia of tissue and promote the proliferation of bacteria and the destruction of tissue (Robson et al. 1990).

1.8.5.1.2 Impact of wound infection on the formation of granulation tissue and angiogenesis:

The turnover increases more, due in part to bacterial enzymes' production, which results in haemorrhagic, oedematous, friable granulation bed and the formation of scarring that is excessive. Interleukin production is stimulated by endotoxins in wounds as well as TNF, which induces MMP production (Konturek et al. 2001; Power et al. 2001). There

is an imbalance between MMPs and their inhibitors of tissue and a decline in the production of growth factor results.

1.8.5.1.3 Impact of wound infection on epithelialisation

This will decline if the load of the bacteria is over 10^5 because bacterial metabolites restrain epithelium migration and digest proteins that are dermal as well as polysaccharides (Edwards and Harding, 2004; Lawrence 1983; Lawrence 1987). Orgill and Demling (1988) state that at state of infection, increased production neutrophil's proteases enzymes of damages epithelium that is vulnerable.

1.8.5.1.4 Impact of wound infection on the production of collagen:

This results in fewer numbers and fibroblast proliferation, with the production of collagen being disorganised. Both the production increase and the collagen breakdown have the overall impact of decreasing the strength of the wound (Robson, Stenberg and Heggers, 1990). Metzger et al. (2002) stated that cross linking and deposition of collagen is decreased by endotoxins and is connected to infection resulted from surgical dehiscence. Moreover, wound contraction can be significantly delayed by wound infection (Robson et al. 1990).

1.8.6 Current wound treatment

1.8.7 Antibacterial and antiseptic agents

As in skin infection wound care and antibacterial treatment is highly dependent on the severity of the infection and underlying patient's risk factors that could deteriorate the wound healing process. Bacterial colonisation in wound can be managed by healthy immune system and that depend on many factors including the bacterial load itself, type

of bacteria and many other factors that compromise human immune system to opportunistic infection. If colonisation has developed to cause local infection in wound site the therapy of an initial nature is normally empiric and should be assessed by the infection's severity and on any data of a microbiological nature that is available, such as types of bacteria that causing the infection. Agents that are of a broad spectrum should start therapy for infections that are severe as well as moderately chronic, more extensive infections. These observed antibiotics should be active against cocci that is Gram-positive (which includes MRSA in locations where it is common to find this pathogen), as well as obligate anaerobic and Gram-negative organisms. Topical antibacterial pharmaceutical product are intended to work on the wound site and have the advantage of eliminating or decreasing systemic toxicity when used in clinical dosage.

The majority of mild infections and a number of moderate ones can be treated through the use of agents which have a comparatively narrow spectrum, such as those applicable only to aerobic cocci that are Gram-positive point out Although organisms that are anaerobic are isolated from a number of serious infections, they are not very frequent infections that are mild to moderate (Lipsky et al. 1990).

Topical antiseptic agents have high antibacterial or bactericidal effect on microorganism that will act on the external surface of the skin**.** Antiseptics work against resident and transient type of flora on the surface of the skin. Most of antiseptic agents have non-selective mechanism of action which makes them broadly active against several types of bacteria, viruses and fungi. These high activities and no selectivity make them work even on wound healing skin cells that could counter act normal healing process. Most commonly types of antiseptics that have been used on wound managements including: chlorhexidine, alcohol, povidone-iodine acetate (Figure 1-8), sodium hypochlorite, hydrogen peroxide (H_2O_2), silver nitrate and silver sulfadiazine (Drosou et al. 2003; Atiyeh et al. 2009; Edlich et al. 2010).

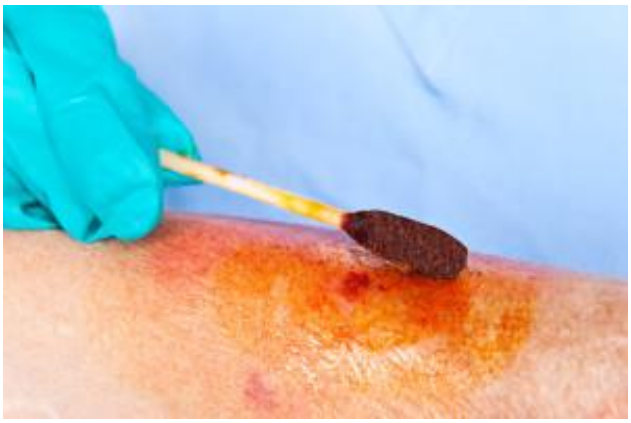


Figure 1-8 Povidone iodine treatment on infected skin.

Silver-containing topical products are commonly used for prevention and treatment in acute and chronic wound infection. The mechanism of antibacterial effect from silver containing product is attributed to the highly reactive silver molecule that will bind to the bacterial negatively charged particle such as proteins, DNA, RNA, and chloride ions which may lead cell membrane permeability destruction, interfere with electron transport and inhibit DNA replication. It has broadspectrum activity against bacteria, fungi, mould and even resistance type of bacteria such as MRSA.

Silver sulfadiazine (Flammazine) is one of the most common silver-containing product that was introduced in 1968. It consists of two products, silver and sulfadiazine antibiotic. Resistance type of salmonella to silver is very serious condition that can cause death if septicaemia occurs. Salmonella resistance silver has been reported with a death of three burns patient at Massachusetts General, which was the main reason that lead to closure of the burn unit at the time (Gupta et al. 1999; Murphy and Evans 2012). The silver concentration level that can cause the antibacterial activity has damaging effects on human healthy cell at the wound site. Delayed reepithelialization and inhibition of keratinocyte growth has been reported with silver nano particles (Innes et al. 2001; Lam et al. 2004)

1.8.7.1 **Wound cleaning, irrigation and disinfection**

As previously mentioned, all wounds acute and chronic will be colonised with bacteria either from the surrounding skin normal flora or from external environment such as nosocomial infection. In chronic wounds, cleaning and disinfection plays very important role in preventing any exacerbation of bacterial colonisation towards critical or local infection level. In cleaning procedure, the risk benefit balance to the wound should be considered ahead. The bacterial load at infected wound can be significantly decreased by debridement and can be defined as the removal of dead tissue and plaque from the wound site.

Wound cleaning solution can be described as the use of fluid to take off any inflammatory contaminant that present on the surface of wound and it can be with or without antiseptic activity (Rodeheaver and Ratliff 1997). The topical Antiseptic agent can slow or stop the growth of microorganism on external surface of the skin. In previous study, wound cleanser of with and without antiseptic activity were compared in terms of toxicity index to show that the combined antiseptic with wound cleanser showed higher level of toxicity indices than the cleanser that don't have any antiseptic activity (Hellewell et al. 1997).

Wound irrigation is a constant flow of cleaning solution on open wound and pressure applied by the hydraulic forces that removes debris from wound site. The pressure force from continuous flow of fluid should be more than the adhesion force that holds the debris to the wound site in order to be logically effective (Rodeheaver and Ratliff 1997). The pressure applied in irrigation has been reported to enhance the removal of bacterial at wound site. Higher pressure up to 25 pounds per square inch (psi) showed more efficient than the low-pressure irrigation produced by e.g. bulb syringe (Rodeheaver et al. 1975; Rodeheaver and Ratliff 1997). Most commonly and practical irrigation method is perform is using syringe filled with wound cleanser and flush the wound with continuous pressure and to attaches the syringe with needle or catheter to increase the pressure of irrigation. According to Longmire et al. (1987) the pressurised irrigation

using a 35-mL syringe attached to 19-gauge needle showed a significantly enhancement of bacterial removal from wound site and a subsequent decrease in incidence of wound infection than the irrigation with low pressure using a bulb syringe. Several methods and techniques of irrigation vary from complex pulsatile lavage methods to simple use of 1 L bag of sterile normal saline. The flow rate and time needed for irrigation can vary from 0.05 L/min when use 60 mL syringe with 19-gauge needle up to 2 L/min irrigation method described by Karuppasamy et al. (2004) with using of one liter normal saline bag and full cutting of the outer port to produce a nozzle.

1.8.8 Antimicrobial resistance

Antimicrobial resistance (AMR) happens when microorganisms (such as bacteria, fungi, viruses, and parasites) change when they are exposed to antimicrobial drugs (such as antibiotics, antifungals, antivirals, antimalarials and antihelminthics). Microorganisms that develop antimicrobial resistance are sometimes referred to as "superbugs". Antimicrobial resistance (AMR) threatens the effective prevention and treatment of an ever-increasing range of infections caused by bacteria, parasites, viruses and fungi. As a result, the medicines become increasing less effective and infections persist, increasing the risk of spread to others; and in increasing cases to the point the infection becomes untreatable. One of the most aggressive types of bacteria is MRSA - it can cause topical infection and if untreated or treated unsuccessfully it can progress to cause systemic life-threading infection. Hospital acquired or nosocomial bacterial infection in wound is commonly caused by MRSA (Köck et al. 2010). The high risk of developing local or generalized systemic MRSA bacterial infection caused by surgical wound or pressure ulcer was addressed in a cohort study that involved 479 hospital patients who were initially colonized with MRSA. This study took 2 years to complete and showed that almost 53 % of the patients in the cohort actually developed MRSA infection in surgical wounds and pressure ulcer. The epithelial discontinuity in a wound can make the access of MRSA to the underlying tissue far more likely than intact skin (Coello et al. 1997).

The lack of new antibiotics threatens global efforts to contain drug-resistant infections. Therefore, there is an urgent need for the development of new treatment products to treat infections. In his previous work, Houston (2011) showed that PRE/Zn have synergistic or potentiated antiviral activity against HSV and acyclovir-resistant HSV-1 and HSV-2.

1.9 Research question and aims

Given the need for new products to treat infected wounds, along with previous observations of the synergised antimicrobial activity of PRE combined with Zn(II), the research question in this thesis is "Does PRE and Zn(II) have potential as a novel therapeutic system to treat skin infections and wounds?"

1. To determine the antibacterial and bactericidal activity of PRE alone and in combination with Zn(II) against a panel of bacteria associated with skin and wound infections.

2. To determine the stability PRE alone and in combination with Zn(II) under different pH and temperature conditions

3. To determine the *in vitro* cytotoxicity and anti-proliferative effects of PRE and Zn(II) on the HaCaT keratinocyte cell line.

4. To determine the effects of PRE and Zn(II) on the scratch-test wound closure model

5. The preparation of topical formulation contains PRE and Zn(II)

6. To determine the in vitro skin permeation and penetration of punicalagin and Zn(II) into skin

7. To determine activity of topical formulation contains PRE and Zn on inoculated ex vivo skin model.

Chapter 2: Materials and general methods

2.1 Materials

24 well plates (24 wp)	Thermo Fisher Scientific. (Loughborough, UK)
50 mL sterile blue cap	(Greiner Bio-One Ltd. (Stonehouse, UK)
96 well plates (96 wp)	Thermo Fisher Scientific (Loughborough, UK)
Antibiotic (100 µg/ mL streptomycin sulphate, 0.25 µg/mL amphotericin B and 100 U/mL penicillin G sodium)	Sigma-Aldrich Company Ltd. (Poole, UK)
Carbopol 974 PNF	Thermo Fisher Scientific (Loughborough, UK)
Carrageenan, iota type	Thermo Fisher Scientific (Loughborough, UK)
Chlorhexidine 4% Hibiscrub	Boots pharmacy (Cardiff, UK)
Crystal violet	Sigma-Aldrich Company Ltd. (Poole, UK)
Dimethyl sulfoxide (DMSO)	Thermo Fisher Scientific (Loughborough, UK)
Dulbecco's Modified Eagle Medium (Gibco DMEM 1X) contains 4.5 g/Lglucose and 0.11 g/L sodium pyruvate stored at 4°C,	Sigma-Aldrich Company Ltd. (Poole, UK)
Eppendorf tubes (1.8 mL)	Sigma-Aldrich Company Ltd. (Poole, UK)
Ethanol (HPLC grade)	Thermo Fisher Scientific (Loughborough, UK)
Ethylene diamine	Sigma-Aldrich Company Ltd. (Poole, UK)
Foetal calf serum (FCS) fetal bovine serum (FBS)	Thermo Fisher Scientific (Loughborough, UK)
Fluorescein diacetate (FDA)	Sigma-Aldrich Company Ltd (Poole, UK).
Iodine, Gram Stain Solution	Thermo Fisher Scientific (Loughborough, UK)
Glycerine	Thermo Fisher Scientific (Loughborough, UK)
Glycerol	Sigma-Aldrich Company Ltd. (Poole, UK)
Fluorescein Diacetate (FDA)	Sigma-Aldrich Company Ltd. (Poole, UK)
Fluorescence-activated cell sorting	Sarstedt (Sarstedt Ltd., UK)

(FACS) tubes.	
High vacuum grease	Dow Corning (Barry, UK)
Hydrochloric acid (HCl)	Sigma-Aldrich Company Ltd. (Poole, UK)
L-glutamine	Sigma-Aldrich Company Ltd. (Poole, UK)
Hydroxymethylcellulose	Sigma-Aldrich Company Ltd. (Poole, UK)
Methanol (HPLC grade)	Thermo Fisher Scientific (Loughborough, UK)
Methocel 856N	Thermo Fisher Scientific (Loughborough, UK)
Micro stirrer bars (2x5 mm)	Thermo Fisher Scientific (Loughborough, UK)
Microscope slides	Thermo Fisher Scientific (Loughborough, UK
Millex®HA syringe-driven filter unit	Millipore (Watford, UK)
Mueller Hinton Agar (MHA)	Sigma-Aldrich Company Ltd. (Poole, UK)
Mueller Hinton Broth (MHB)	Sigma-Aldrich Company Ltd. (Poole, UK)
Nitrocellulose transfer membrane (Protran® BA85) 0.45μm pore size	Schleicher and Schuell (Dassel, Germany)
Pasteur pipettes	Sigma-Aldrich Company Ltd. (Poole, UK)
Phenylmethyl sulphonyl fluoride (PMSF)	Sigma-Aldrich Company Ltd. (Poole, UK)
Phosphoric acid	Thermo Fisher Scientific (Loughborough, UK)
PBS tablets phosphate buffered saline tablets	Thermo Fisher Scientific (Loughborough, UK)
Polyethylene glycol 400 (PEG 400)	Sigma-Aldrich Company Ltd. (Poole, UK)
Polyethyleneglycol (100) stearate	Thermo Fisher Scientific (Loughborough, UK)
Polyoxyethylene-sorbitan monolaurate (Tween 20)	Sigma-Aldrich Company Ltd. (Poole, UK)
Pomegranate	Asda (Cardiff) Pomegranate (original source from Spain)
Ponceau S solution (0.1%, w/v in 5% acetic acid)	Sigma-Aldrich Company Ltd. (Poole, UK)

Potassium hydrogen phthalate	Thermo Fisher Scientific (Loughborough, UK)
Potassium hydroxide	Thermo Fisher Scientific (Loughborough, UK)
Punicalagin	Phytolab (Vestenbergsgreuth, Germany)
Propidium Iodide (PI)	Sigma-Aldrich Company Ltd. (Poole, UK)
Safranin stain solution, 1%	Thermo Fisher Scientific (Loughborough, UK)
Sellotape	Asda (Cardiff)
Sodium bicarbonate	Sigma-Aldrich Company Ltd. (Poole, UK)
Sodium chloride (NaCl)	Sigma-Aldrich Company Ltd. (Poole, UK)
Sodium hydroxide	Sigma-Aldrich Company Ltd. (Poole, UK)
Spectrophotometer microcuvettes	Bio-Rad Laboratories Ltd. (Hertfordshire, UK
Sterile pipettes 10 mL and 25 mL	Greiner Bio-One Ltd. (Stonehouse, UK)
Super glue	Asda (Cardiff)
Syringe (5 mL, Sterile)	Sigma-Aldrich Company Ltd. (Poole, UK).
Syringe needles	Sherwood-Davies and Geck (Gosport, UK)
T 75, tissue culture flasks	Sarstedt (Sarstedt Ltd., UK),
Thiazolyl blue tetrazolium bromide (MTT)	Sigma-Aldrich Company Ltd. (Poole, UK)
Triflouroacetic acid (TFA)	Sigma-Aldrich Company Ltd. (Poole, UK).
Trypan blue stain (0.4%),	Sigma-Aldrich Company Ltd. (Poole, UK)
Trypsin-EDTA 0.05% (1X) (Gibco)	Sigma-Aldrich Company Ltd. (Poole, UK)
TSA Tryptone Soya agar	Thermo Fisher Scientific (Loughborough, UK)
TSB Tryptone Soya Broth	Thermo Fisher Scientific (Loughborough, UK)
Whatman filter paper	Thermo Fisher Scientific (Loughborough, UK)
Water, HPLC grade	Thermo Fisher Scientific (Loughborough, UK)
Zinc sulphate ($ZnSO_4$) heptahydrate	Thermo Fisher Scientific (Loughborough, UK)

2.2 General methods

2.2.1 Solution preparation

2.2.1.1 **Pomegranate rind extract (PRE)**

Pomegranate rind extract was prepared using the method of Stewart *et al.,* 1998. Four pomegranates were bought from the local market, and the source was from Spain. Pomegranate rind was cut into thin long strips and then blended with deionized water 25% w/v. Then, the solution was boiled for 10 minutes in order to extract tannins from the rind. Centrifuging of the solution was done for 40 minutes (6 x) using Heraeus™ Multifuge 3 S-R centrifuge at 5980 g. After that, the solution was vacuum filtered through 0.45 µm nylon membrane filter. The total volume of the filtrate was 289.5 mL. Part of the solution was freeze-dried using a Scanivac™ freeze drier and the rest was stored at -20˚C. The extraction steps are summarised in Figure (2-1).

2.2.1.2 **Reconstitution of freeze-dried PRE**

When needed, freeze dried PRE was reconstituted with phthalate buffer (pH 4.5) to a known concentration. Several concentrations of PRE were used during this project, for example to prepare 2 mg/mL concentration of PRE, a 20 mg of freeze dried PRE was added to 10 mL phthalate buffer (pH 4.5). In the same manner, if 5 mg/mL stock solution of PRE was needed, a 25 mg of freeze dried PRE was added to 5 mL phthalate buffer (pH 4.5). After preparation, sonication for 10 min at 50-60 Hz of solution then filtered through 0.45 µm Millex-FG syringe driven filter unit. Finally, the solution was protected from light. In the stability study water were used as solvent for PRE samples with pH 7.

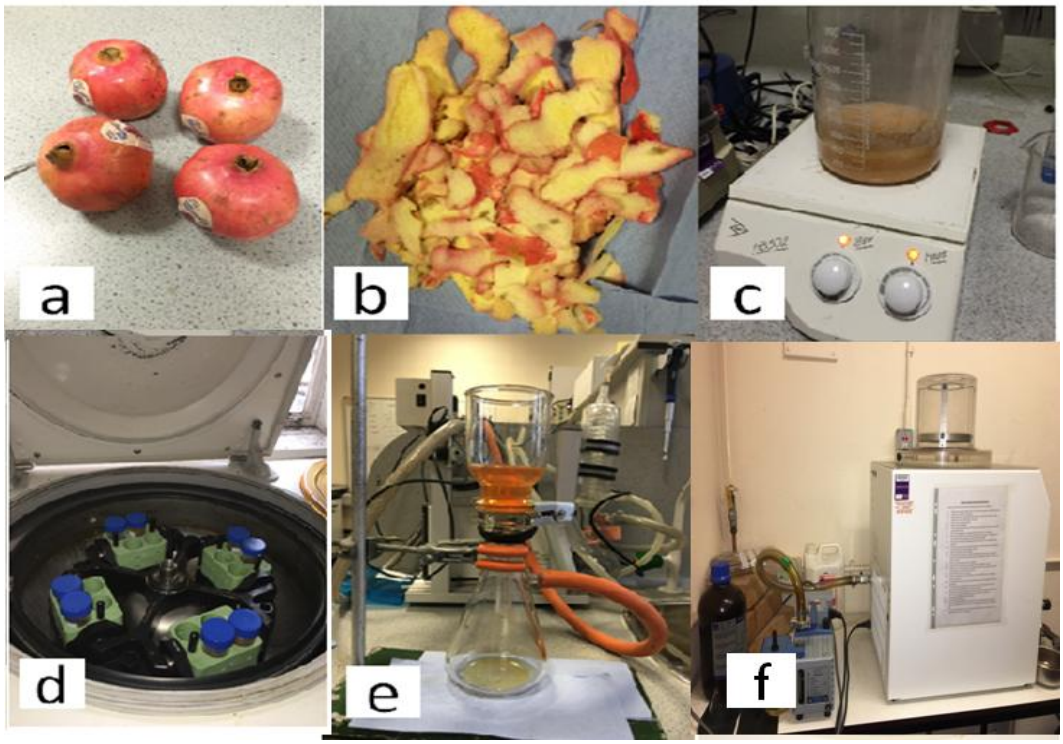


Figure 2-1 Summary of the steps involved in pomegranate rind extraction, where (a) pomegranates as purchased from local store, (b) excised pomegranate rind, (c) boiling for 10 minutes, (d) centrifugation at5980 g (e) vacuum filtration though 0.45 µm nylon filter (f) freeze drying.

2.2.1.3 **Phthalate buffer pH4.5**

To prepare phthalate buffer with pH value of 4.5, potassium hydrogen phthalate and sodium hydroxide were prepared separately (0.1 M) in deionised water. In a 1L volumetric flask, 250 mL of potassium hydrogen phthalate was added to 87 mL of sodium hydroxide and the volume was completed with deionised water. Phosphoric acid was used to adjust the final pH to 4.5, and the buffer was stored at 2-4 ˚C until further use (Houston, 2011).

2.2.1.4 **Zinc sulphate ($ZnSO_4$)**

In 10 mL volumetric flask (2.875 g) of $ZnSO_4$ heptahydrate was added to 10 mL phthalate buffer (pH4.5) to prepare a stock solution of zinc sulphate (1 M). The solution underwent sonication for 10 minutes at 50-60 Hz. Once fully dissolved the solution was filtered through 0.2 µm Minisart® syringe driven filter unit. The solution was stored in 2-4 °C (refrigerator) until further use. Further dilution of $ZnSO_4$ was prepared when needed. In the stability study experiment water were used as solvent in $ZnSO_4$ solution with (pH 7).

2.2.1.5 **Preparation of PRE and $ZnSO_4$ combined solutions**

A stock concentration of PRE and $ZnSO_4$ were prepared in phthalate buffer (pH4.5) and when needed further dilutions were made to give final concentration desired in combination. In the stability study experiment water was used as solvent in combination stated (pH 7).

2.2.1.6 **Phosphorous buffered saline (PBS)**

A pre-prepared tablet of PBS was dissolved with deionized water 100 mL, once fully dissolved after stirring using magnetic stirrer; the solution was autoclaved at 121 °C for 15 min.

2.2.1.7 **Punicalagin solution**

Punicalagin solution was made by adding punicalagin (2 mg) to deionized water (2 mL.) The resultant solution was sonicated at 50-60 Hz for 10min. After complete dissolution of punicalagin, further (serial) dilutions were made using phthalate buffer (pH 4.5).

2.2.2 Analytical methods

2.2.2.1 **Quantitative analysis of PRE and punicalagin by reverse-phase HPLC**

An Agilent series 1100 HPLC system was used for analysis. The column used for separation was Phenomenex (Kinetex®, USA) 5µm C18 100A 4.6 mm x 150 mm). A gradient elution involved two mobile phases A: methanol with 0.1% trifluoroacetic acid (TFA) and B: deionised water with 0.1% TFA. Detection was performed at 258 nm and the analysis was performed at ambient temperature. Injection volume was 20 µL and the column was conditioned with mobile phase for 10 min beforehand. Flow rate was 1.5 mL/min and mobile phase run time was 32 min. The gradient elution timetable is shown below in Table 2-1.

Time (min)	**A. % MeOH + 0.1% TFA**	**B. % H_2O + 0.1% TFA**
0	5	95
7	10	90
15	20	80
20	40	60
25	60	40
30	5	95
32	5	95

Table 2-1 Gradient elution timetable used for punicalagin and pomegranate rind extract.

The PRE chromatogram shown in Figure 2-2 highlights punicalagin which exists as two structural isomers or anomers: α with retention time of 7.38 minute and anomer β-punicalagin with retention time 11.74 minutes. Detection of punicalagin anomers was done using external standard method. Spiking with standard punicalagin gave rise to the both α and β punicalagin peaks (Houston, 2011).

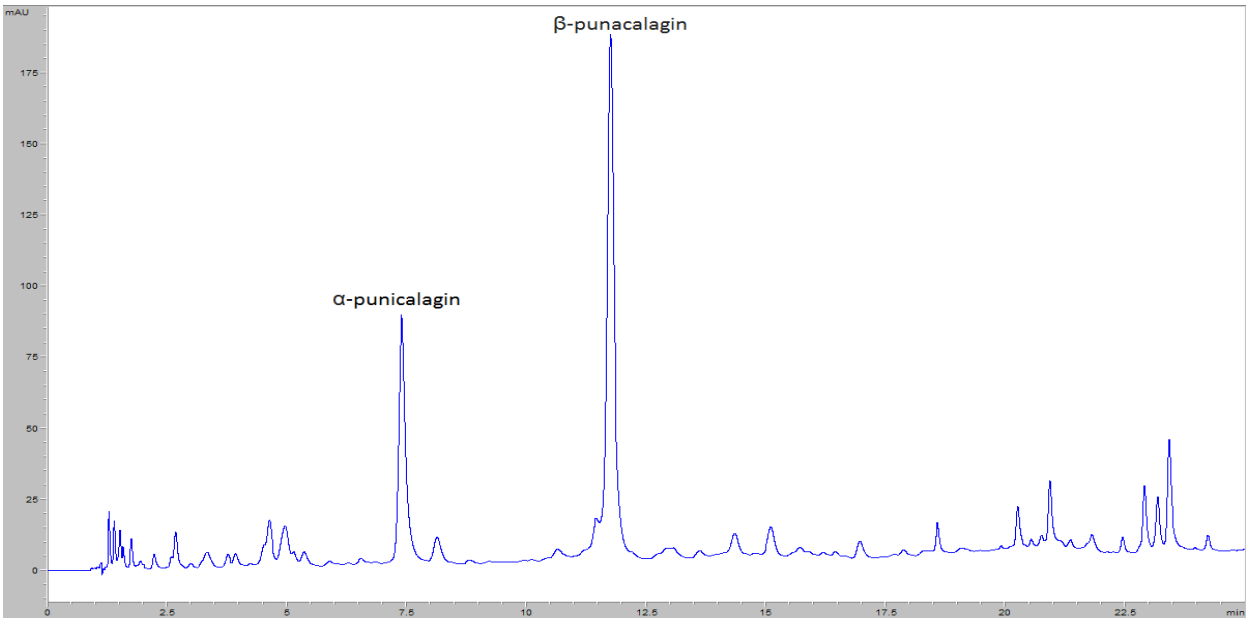


Figure 2-2 HPLC chromatogram of PRE showing α and β punicalagin in typical 1:2 ratio

2.2.2.1.1 Standard punicalagin and construction of calibration curve

Standard punicalagin was used to construct the calibration curve of punicalagin. Serial diluted solutions of standard punicalagin were prepared with deionized water, to yield a calibration plot of area under curve against serials of punicalagin concentration as shown in Figure 2-3. The peak areas were plotted against the corresponding standard concentrations using Microsoft Excel, and corresponding regression equation and R^2 determined. The entire process was carried on three separate occasions to obtain the mean calibration coefficient. The relationship between concentration and mean peak area was linear (R^2 >0.99) and therefore the both α and β-punicalagin concentration could be estimated using the derived equation. The α punicalagin ratio to β was 1:2 as shown by area under curve values and this is in accordance to previous noted by Houston (2011).

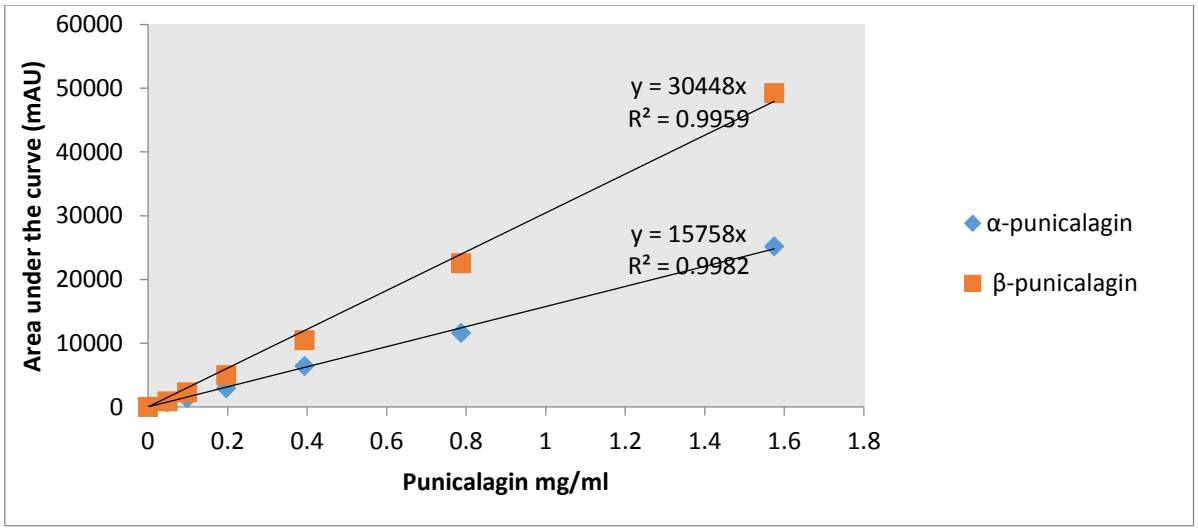


Figure 2-3 Calibration curves of standard α and β punicalagin showing relationship between punicalagin concentration and area under curve produced by HPLC analysis.

In order to determine the quantity of punicalagin present in PRE, a serial dilution of freeze dried PRE was prepared and the mean concentration was calculated of β punicalagin using the calibration curve produced by standard β-punicalagin (Figure 2-3). Consequently, a plot was constructed to show the concentration of punicalagin in PRE as shown in Figure 2-4. The relationship between PRE concentration and punicalagin concentration is shown in Figure 2-4. Using the equation y=4.61x, where y is PRE concentration and x is β-punicalagin concentration, the punicalagin present as 1 mg in each 4.61 mg of PRE.

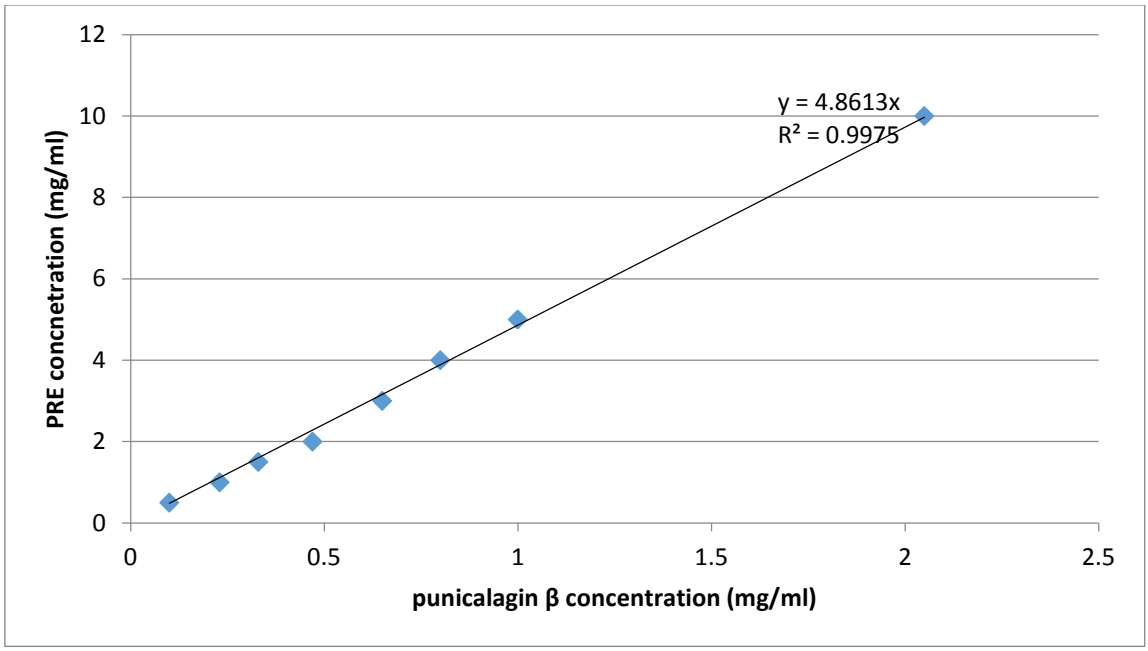


Figure 2-4 The relationship between total mass of PRE extracted and β-punicalagin within the freeze-dried PRE.

2.2.2.2 **Zinc detection using Inductively Coupled Plasma Mass Spectrometry (ICP-MS)**

Zinc permeation through full thickness porcine skin was detected using Thermo Elemental X Series 2 ICP-MS system that has been fitted with a Plasma Screen. All samples were analysed in the School of Chemistry, Cardiff University. ^{66}Zn was used as analytical mass for each analysis. Calibration was performed using standard single element solutions prepared from synthetic standards. The accuracy was checked out by analysis of a solution of the international rock standard JB1a as an unknown. Samples were firstly digested using HF/HNO_3 and then HNO_3 using methods described previously (Parkinson and Pearce 1998; Li et al. 2012).

2.2.3 Skin preparation methods

2.2.3.1 **Porcine ear skin preparation**

Porcine ear skin was provided by a local slaughterhouse. The ears were cut immediately after slaughter and washed and transferred in cooled container to the laboratory. At the lab the skin was cleaned using running tap water at cool temperature and any excess hair was removed using electric clippers. Then the full thickness skin was carefully separated from the dorsal cartilage site by blunt dissection using a sterile scalpel using and excess subcutaneous fat layer was removed. Then the skin was divided into small sections area with (2 x 2 cm) and of 0.5–0.8 mm thickness used.

2.2.3.2 **Heat-Separated Epidermis (HSE)**

Some of the previously prepared porcine skin in Section 2.2.3.1 underwent a further step to isolate the epidermis layer when needed. According to the published method by Kligman and Christophers (1963), epidermis layer can be isolated from the dermis by soaking the extracted skin in deionized water at high temperature (55 °C) for 1-2 minutes. The epidermis layer was removed by hand using gloves and carefully taking the outer layer from the underlying dermis. Any abnormality or holes were observed in skin samples were excluded. The epidermis samples to be used were cut into (2cm x 2cm) squares.

2.2.4 Microbiological methods

2.2.4.1 **Aseptic technique**

Aseptic technique was employed at all stages in microbiology methods using microbiology safety cabinets and routinely cleaning with 70% ethanol. To maintain sterility, all solutions and some plastics that have been used as phosphate buffered saline (PBS), water and pH 4.5 phthalate buffer were autoclaved at 123 °C, 15 lb/m^2 for 15

minutes. Water baths, incubator and cabinets were routinely cleaned using water and 70 % ethanol.

2.2.4.2 **Preparation of Mueller Hinton Agar (MHA) and Tryptone Soy Agar (TSA)**

Mueller Hinton Agar (MHA) was prepared as instructed on the bottle (Sigma-Aldrich Company Ltd, Poole, UK) by adding 38 g to 1 litre of deionised water. After mixing and completely dissolved autoclave at 121 °C for 15 min and stored at 60 °C before pouring into sterilized plates. Tryptone Soy Agar (TSA) was prepared by adding 40 g of TSA (CM0131) to 1 L deionised water. The mixture was placed on hot plate until boiling and fully dissolved. Then mixture then underwent sterilization by autoclave at 121 °C for 15 min and stored at 60 °C until pouring to sterilized plates.

2.2.4.3 **Preparation of Mueller Hinton Broth (MHB) and Tryptone Soy Broth (TSB)**

MHB prepared as instructed on the bottle (Sigma-Aldrich Company Ltd, Poole, UK) by placing 21.0 g in 1 L of deionized water then mix to dissolve completely. After that, the mixture was autoclaved at 121 °C for 15 min and stored at 60 °C before pouring into sterilized plates. Tryptone Soy Broth (TSB) was prepared as instructed on the bottle. 30 g TSB (CM0129) was added to one litre deionised water. After mixing well, the broth was sterilized by autoclave at 121 °C, then stored at 2-4 °C until further use. Fresh nutrient broth was prepared every 2 weeks.

2.2.4.4 **Preparation of bacterial cultures**

Five bacterial strains were used for investigation used in this study:

1. Methicillin resistant *Staphylococcus aureus* (MRSA) (NCTC 12493)
2. *Escherichia coli* (*E. coli*) (NCTC 12923)
3. *Pseudomonas aeruginosa* (*P. aeruginosa)* (NCTC 6750)
4. *Staphylococcus epidermidis* (NCTC 11047)
5. Methicillin sensitive *Staphylococcus aureus* (MSSA) (NCTC 10788)

All bacterial strains were provided by CSPPS, Cardiff University. The bacterial stock was stored in Microbank™ cryo-protective beads (Pro-Lab Diagnostics Ltd., UK) at -80° C until further use. Culture preparation of each bacterium followed the working culture preparation method. When needed fresh bacterial culture was prepared every 2 weeks and stored at 2-4 ˚C until further use.

2.2.4.5 **Preparation of fresh bacterial culture**

To prepare fresh bacterial cultures, fresh inoculations of tested bacteria were made onto agar plates using a 10 µL inoculation loop. The streak plate method was used for inoculation in order to spread the concentrated bacteria as solution on agar plate, as shown in Figure 2-2. Incubation was at 37 °C for 24 h and stored at 2-4 °C until needed. Fresh bacteria were prepared every 2 weeks.

2.2.4.6 **Preparation of working culture**

Inoculate bacteria from freshly prepared bacterial culture stock onto agar plate using 10 µL loop and then incubate at 37 ˚C for 24 h. A single colony of bacterial growth was transferred into sterile universal tubes containing 10 mL of broth and incubated at 37 ˚C

for 24 h in a shaking incubator set at 100 revolutions per minute (Thermo Fisher Scientific, Loughborough, UK).

2.2.4.7 **Preparation of 0.5 McFarland (10^8 CFU/mL) bacterial culture**

Overnight bacterial cultures of tested bacteria were centrifuged using a Beckman Avanti J-20XP Centrifuge at 35000 g for 15 min. After bacterial pellet was formed, the supernatant broth was discarded and phosphate buffer solution (PBS) was used to re-suspend the bacteria and further dilute it to the 0.5 McFarland standard ($1x10^8$ bacteria cell density). The optical density of the diluted bacteria was measured using a spectrophotometer (Jenway 6305, Bibby Scientific Ltd, UK) at wavelength 625 nm, where 0.5 McFarland should have an absorbance between 0.08-0.13 mAU.

2.2.4.8 **Broth dilution method to determine MIC**

Broth dilution method using 96 well microtitre plate was performed to calculate the minimum inhibitory concentration (MIC) of PRE and $ZnSO_4$ of the investigated bacteria. At first, bacterial culture with 0.5 McFarland standard previously prepared by the method in Section 2.2.4.7 was diluted 1:100 using MH broth. Then 100 µL of bacterial culture was added to 9.9 mL of MHB to prepare 10^6 CFU/mL of bacterial suspension. In sterile Eppendorf tubes, 1 mL of test solution was added in the first tube and 12 other tubes were filled with 500 µL of MHB. An aliquot of 500 µL of the first Eppendorf tube that contained drug was pipetted into a 2nd vial to give a 50% of the concentration. These were then mixed using a vortex mixer; and then 500 µL taken of that and transferred to the next Eppendorf. Each time doing this will be dilute the tested concentration by 50%. Add 100 µL of the diluted tested antimicrobial decreasing in concentration across the 96 well plates. Add a 100 µL volume of the diluted microbial suspension on top to make a 200 µl total volume. Negative control (MH broth alone) and positive control (bacterial suspension alone) was added. Then, the 96 well plate was incubated at 37 °C for 24 h. After incubation the MIC was determined visually, if no growth. According to CLSI guidelines for antimicrobial susceptibility testing, (2014) the

MIC point is recorded by visual unaided eye. A micro plate reader was employed as confirmation step (clear well) compared to that of the positive (microbial) control. The MIC was obtained in accordance to the guidelines of CLSI twenty-fourth informational supplement: Performance standards for antimicrobial susceptibility testing (2014).

2.2.4.9 **Universal quenching agent (UQA)**

In order to prepare 1 L of UQA, 1 g sodium thiosulphate, 1 g peptone, 0.7 g lecithin and 5 g Tween-80 was transferred to 1 L volumetric flak and completes the volume with deionised water and adjusted to pH7. Once fully dissolved the mixture was autoclaved at 121°C for 15 min (Johnston et al. 2002)

2.2.4.10 **Streak plate method**

To determine the culture purity a streak plate of tested bacteria was performed routinely. Form overnight bacterial culture, a 10 µL was transferred using sterile inoculation loop and plate out on agar plate. The streaking method was done by inoculating parallel lines near to one edge of the agar plate, then from each end a perpendicular lines are streaks and same for the third time as shown in Figure 2-5.

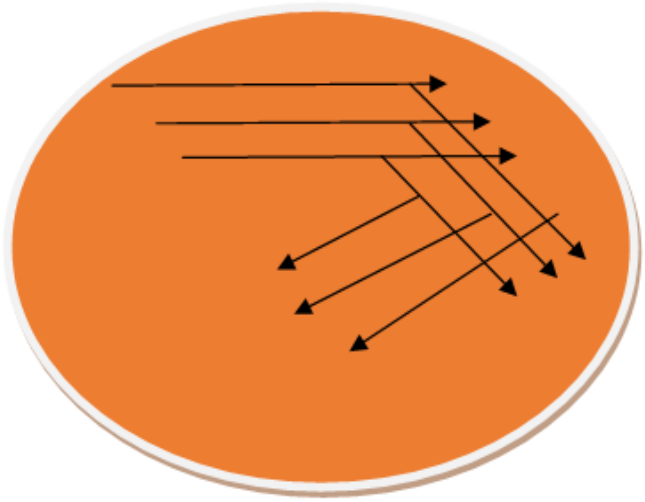


Figure 2-5 Schematic figure shows streak plate method to isolate bacterial colonies.

2.2.4.11 **Gram staining of tested bacteria**

A selected single colony of bacteria from streak agar plate were transferred using a sterile inoculation loop with 10 µL on slide of microscope and film is created with sterile de ionized water. After slide had dried, heat fixation of the bacteria was done by rapidly pass the slide on flame. Crystal violet reagent was added to stain the bacteria and left for 1 minute then Grams iodine added and left for 1 minute. The following step is rinsing of the slide with 70 % ethanol for 15 sec. The last step was to apply the red counter stain safranin for 1 minute. After that oil immersed slide is viewed light microscope magnification (CK2 Inverted Microscope, Olympus UK Ltd., Middlesex, UK)

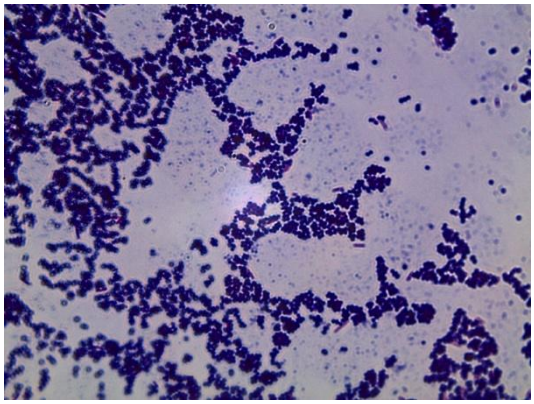


Figure 2-6 Gram stain of *S. epidermidis* (NCTC 11047)

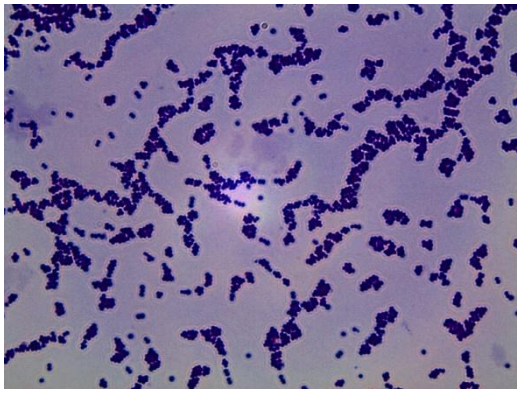


Figure 2-7 Gram stain shows *Staphylococcus aureus* (NCTC 10788)

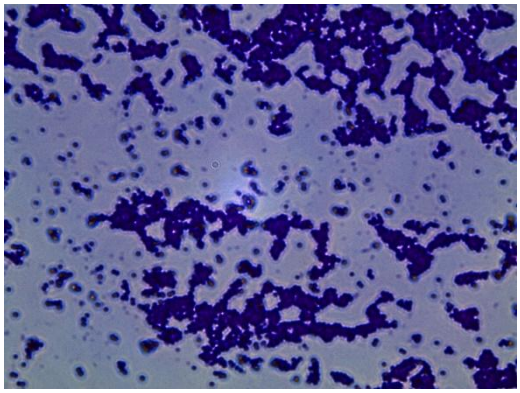


Figure 2-8 Gram stain shows methicillin resistant *Staphylococcus aureus* MRSA (NCTC 12493)

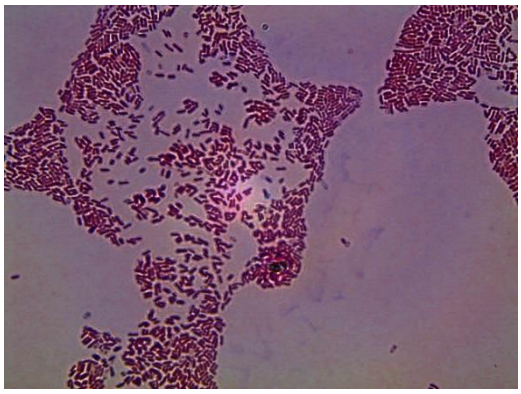


Figure 2-9 Gram stain shows of *Escherichia coli* (NCTC 12923)

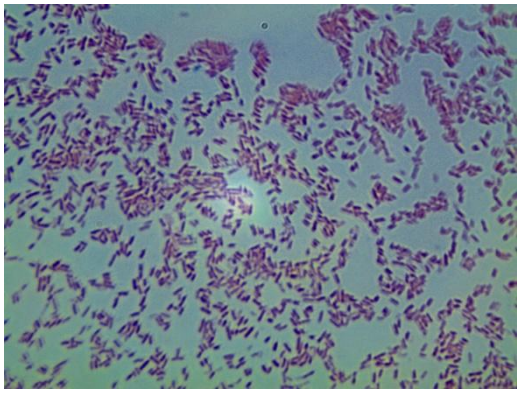


Figure 2-10 Gram stain showing *Pseudomonas aeruginosa* (NCTC 6750)

2.2.5 Cell culture general methods

2.2.5.1 **Aseptic technique**

Aseptic technique was employed at all stages of tissue culture using mammalian cell culture safety cabinet. Routine cleaning and sterilization of material using autoclave has been previously mentioned in Section 2.2.5.1.

2.2.5.2 **Human adult, spontaneously immortalized, epidermal keratinocytes (HaCaT cells)**

Human adult, spontaneously immortalized, epidermal keratinocytes (HaCaT cells) were provided by the School of Dentistry, Cardiff University, UK. The cells were cultured in 10 % serum containing media (SCM). The seeding density was $1.5x10^5$ in 75 cm tissue culture flask. Incubation of HaCaTs cultures were in 37 °C, 5 % CO_2/ 95 % air, with media replaced every 24-48h. Cell splitting and sub-cultured was carried out when the HaCaT cells reached 70-80% confluence. The cells were re suspended in SCM and seeding density was counted using a haemocytometer.

2.2.5.3 **Culture media preparation**

Foetal calf serum (FCS) was thawed overnight from frozen state to liquid in 2-4 °C then heat inactivation was performed. The FCS was placed in water bath with temperature of 56 °C for 30 minutes then aliquots of 50 mL each was prepared. The inactivated FCS was stored at -20 °C for further use, and in 2-4 °C for regular use up to 2 weeks. The 10% serum culture media (SCM) composed of Dulbecco's Modified Eagle's Medium (DMEM) + 10% foetal calf serum media + 1% antibiotic/ antimycotic (100 U/mL penicillin G sodium, 100 µg/mL streptomycin sulphate and 0.25 µg/mL amphotericin B) and 1% L-glutamine).

2.2.5.4 **Trypan blue cell staining**

One of the earliest techniques is the live /dead cell staining by Trypan blue dye, in which same concept of cell counting previously detailed in chapter two. Briefly, the Trypan blue technique will appear under the microscope taken up by dead cells that don't have intact cell membrane, but expelled out by healthy live cell to show dead cell in dark blue and healthy on in transparent colour. The methods simply count the cells at the beginning of the assay and then after the exposure to treatment media and then calculate the percentage cell survivor (Kun and Abood. 1949).

2.2.5.5 **Cell sub culturing and counting**

To maintain an appropriate cell density for working with HaCaTs, the cells were subcultured and counted when they reached 80% confluency. First, the old media was removed from tissue culture flask and washed with PBS then three millilitre of trypsin was added to facilitate detachment of cells from the flask. Then, the flask was returned to the incubator at 37 °C, 5 % CO_2/ 95 % air for 5 minutes. After observing that all cells were detached by light microscope at x100 magnification (CK2 Inverted Microscope, Olympus UK Ltd., Middlesex, UK), the trypsin was neutralized with 6 mL of 10% SCM previously detailed in section 2.2.5.3, the centrifuged at 1500 rpm for 5 minutes. The supernatant was removed and the pellet was re-suspended with 10 mL of SCM. To carry out cell counting, an equal volume of cell suspension and trypan blue (15µL + 15µL) was mixed. Then the haemocytometer was loaded with the cell mixture and counted under a light microscope at x100 magnification (Figure 2-11). The number of viable cells in the outer four squares of the haemocytometer were counted, if too many per square the cell suspension was diluted with SCM, the counted cells number were from twenty to seventy cell in each square. The cells that entrapped in the outer four squares were counted and the one that present outside the square were not. The Haemocytometer consist of 9 gridded square each has 1 x 1 mm (1 mm^2). Total number of cells from 80-300 were counted and then the following equation was applied to obtain the number of cells per 1 mL (cell density):

Number of cells in 4 corners/4 = B

B x 2 (dilution factor by trypan blue) x 10^4 = cells/mL

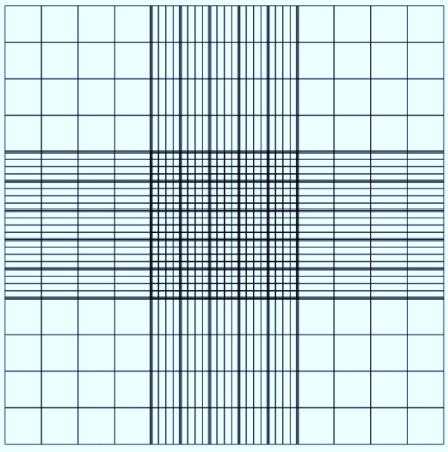


Figure 2-11 Schematic figure showing the 9 gridded squares of haemocytometer

Trypan blue will be taken in by all cells, live and dead. The live cells will pump the dye out and appear with clear cytoplasm, bright in the middle, while dead ones will retain the dye in and appear in dark blue cytoplasm with irregular shape. Viable cells arecounted to obtain density. Following cell counting, the cells were subcultured in 10 mL K-SCM at density of $1.5x10^5/75$ cm^2 tissue culture flask.

2.2.5.6 **Cell Freezing and cell thawing**

After sub-culturing the cells from the tissue culture flask, the pellets produced were re suspended in 1mL of freezing mix (90% foetal calf serum and FCS +10% DMSO). The desired cell density to be stored is from 1 - 2 $x10^6$ cells/mL in pre-cooled cryovials. The cryovial that contained the cell mixture was then placed in afreezing pot (Mr. FROSTY, Sigma-Aldrich Company) and stored at -80 ˚C for 24 hours. The Mr. FROSTY freezing container contains isopropanol that is designed to control the rate of cooling of cells by -1 °C per minute, which is the optimal condition that is desired for preserving cells. After overnight storing at -80 °C, the cryovials were transferred to liquid nitrogen foir storage at -196°C.

When needed, the cells were retrieved from liquid nitrogen store by rapidly placing the cryovials in 37 °C water bath. Then, 9 mL of 10% SCM previously detailed in section 2.2.5.3, was added to the cells and then mixed and centrifuged at 1500 rpm for 5 minutes. The supernatant was removed and the pellets were mixed thoroughly with 10 mL of SCM and transferred to 75 cm^2 tissue culture flasks to be incubated.

Chapter 3: Antibacterial and bactericidal activity of PRE alone and in combination with Zn(II)

3.1 Introduction

It is of increasing interest to treat a range of conditions using >1 drug in combination. It is possible that combined use allows drugs to treat a single disease or target more efficaciously by taking advantage of the most applicable functions of the different drugs. There are four main motivations for using drugs in combination. First, such methodology may make the therapeutic effect more impactful. Second, it allows for a reduced dosage of each drug without compromising efficacy of treatment, this mitigates against toxicity. Third, drug combinations diminish the likelihood of the evolution of drug resistance. Finally, it allows for selective synergism, whether against the host (toxicity antagonism) or the target (efficacy synergism) (Chou, 2006).

To understand drug combination outcomes, it is useful to examine some examples. One example of potentiation or enhancement is if Drug A (which has a therapeutic effect) is combined with Drug B (which has little or no therapeutic effect), and produces a greater impact than if Drug A is used in isolation. This potentiation or enhancement is described in terms of –fold of potentiation or percent enhancement. Should Drug A and Drug B both have a treatment effect, their combination may result in an effect described as additive, synergistic or antagonistic. An additive effect is somewhat less impactful than synergism, and antagonistic effect is even lesser still (Chou 2006; Williamson 2001).

For antibacterial (inhibiting growth or replication) and bactericidal (killing) synergy testing, several methods have been established to evaluate the synergy activity for in vitro experiments. Reviewing the literature, two major methods for the synergy testing were the checkerboard test and the bacterial suspension test or time kill assy. Many researchers have utilized the checkerboard test because it can give an accurate prediction of any synergy in inhibition of bacterial growth. Historically the dilution methods used to be in macro level in test tubes but in checkerboard test it is done on 96 well plates and the dilution goes in micro level, which saves any un necessary loss of the tested antibacterial agent. Mathematical representation of the results is done by calculation the

Fractional Inhibitory Concentration (FIC). The accurate definition of FIC is the minimum inhibitory concentration of the drug in the combination divided by the minimum inhibitory concentration of the drug when used alone. Then the sum of FIC is calculated using the following formula:

$\sum FIC = FIC\ A + FIC\ B$

FIC A is the MIC of drug A in the combination divided by the MIC of drug A alone.

FIC B is the MIC of drug B in the combination divided by MIC of drug B alone.

Then after calculation the combination is considered to be synergistic, antagonistic, additive or indifferent according to the sum of FIC ∑FIC. (White *et al.* 1996; Bonapace *et al.* 2002).

∑ FIC Value	Interpretation
≤0.5	Synergistic
>0.5 and < 4	Additive
≥4	Antagonistic

Table 3-1FIC index interpretation (Bonapace et al. 2002)

The second most well known method to assess synergism activity is by bactericidal suspension test or time kill assay (White *et al.*1996). The bactericidal suspension test has been used in many investigational studies in different contact time depending on the use of tested antibacterial agent. In our study different contact time from 5 min to 1 h was chosen and to check the antibacterial activity PRE and Zn(II) combination.

3.1.1 Pomegranate use in wound treatment

Skin is the largest organ in human body and it plays very important rule as first line defence mechanism that protect human body. Major and minor injuries to the skin can cause different kind of acute or chronic wounds. Natural products have been used from ancient years as treatment for wounds that usually resulted from injuries or burns. Still a day, natural product is used as primary treatment in 80 % of the time worldwide (Maver et al. 2015). Pomegranate extract has been extensively studies in wound treatment. In the past ten years several studies have investigated *in vivo* and invitro effect of pomegranate extract for its potential benefits in wound injuries and wound healing activity (Schmidt et al. 2009; Adiga et al. 2010; Hayouni et al.2011; Huan et al. 2013; Mo et al.2014; Ma et al. 2015; Fleck et al. 2016; Nasiri et al. 2017; Asadi et al. 2018). Wound infection is very serious complication that happened in chronic wound and diabetic wound ulcers. *Staphylococcus aureus* alongside with *Pseudomonas aeruginosa* is considered to be the two most common pathogens that has been involved in wound infections (Serra et al. 2015).

Hospital acquired or nosocomial bacterial infection in wound is commonly caused by MRSA (Köck et al. 2010). The high risk of developing local or generalized systemic MRSA bacterial infection caused by surgical wound or pressure ulcer was addressed in cohort study that involved 479 hospital patients who were initially colonized with MRSA. This study took 2 years in duration and showed that almost 53 % of the patients in the cohort actually developed MRSA infection in surgical wounds and pressure ulcer. The actual opening of the skin in wound can make the access of MRSA to the underlying tissue more easily than intact skin (Coello et al. 1997). Another bacterium that has been reported to have major role in nosocomial wound infection is Gram negative *Escherichia coli (E. coli)* (Bereke et al. 2012).

3.1.2 Bacteria resistance in biofilm

A very important factor that can decrease the antibacterial activity is location of these bacteria inside a protective biofilm, which is known to represent a serious complication of chronic wound infection. Biofilm and its related infections account for ~80% of total nosocomial infection in particular involving *S. aureus* chronic biofilm infections (Reffuveille et al. 2017) and are present in cases of impetigo and furuncle (Akiyama et al. 1997) and are also believed to be involved in other skin disorders such as acne or psoriasis (Gannesen et al. 2018). Biofilm is a complicated environment that consists of the attachment of bacterial cells to either biological or non-biological surfaces. Inside the biofilm interface the bacteria forms microcolonies implanted in 'slimy' liquid extracellular matrix (ECM) of hydrated extrapolymeric substances (EPS). Water channels are dispersed inside EPS that act as circulatory system providing nutrition needed and removal of metabolic waste products. Biofilm provides an excellent environment for bacterial growth because of the physical protection by EPS that prevent and inhibit permeation of antibacterial agents and for bacteria microcolonies to communicate to each other which result in more virulence and propensity to cause infections (Davis et al. 2008; Siddiqui and Bernstein 2010). A recent review indicated that bacterial presence inside a multi-structured biofilm is considered 10 to 1000 times more resistant to traditional treatment by antibacterial agents than free un-attached floating bacteria or 'planktonic' bacteria (Reffuveille et al. 2017).

Considering the previous work on the antimicrobial activity of PRE in relation to potentiation of virucidal and bactericidal activity by Zn(II), here we tested the hypothesis that PRE/Zn(II) might also demonstrate bactericidal activity, which could be exploited as novel broadspectrum antimicrobial therapeutic systems for wound infection.

In this chapter, antibacterial and bactericidal activity is investigated based on a panel of bacteria that are commonly involved in skin and wound infections.

3.2 Objective and aims

To investigate the antibacterial activity of PRE and $ZnSO_4$ against a range of Gram positive and Gram negative bacteria.

- To determine antibacterial and bactericidal activity of PRE with and without Zn(II) against 5 relevant bacterial strains: methicillin sensitive *Staphylococcus aureus* MSSA, methicillin resistant *Staphylococcus aureus* MRSA, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa* and *Escherichia coli* .
- To investigate the synergistic effects between different combination.
- Measure the fractional inhibitory concentration index of several combinations against tested bacteria.
- To determine the activity of PRE and Zn(II) containing combination against *S. aureus* embedded in a single-specie biofilm.

3.3 Materials and methods

3.3.1 Materials

All materials used in this chapter previously detailed in Chapter 2, Section 2.1.

3.3.2 Solution preparation

Preparation of PRE and $ZnSO_4$ was carried out according to the previously detailed solution preparation detailed in Chapter 2, Section 2.2.1 all prepared samples used phthalate buffer pH4.5.

3.3.3 Aseptic technique

Aseptic technique was employed at all stages in microbiology studies using microbiology safety cabinets and aseptic technique previously described in Chapter 2, Section 2.2.4.1.

3.3.4 Microbiology culture preparation

Five bacterial were used for investigation used in this study, methicillin resistant *S. aureus* MRSA (NCTC 12493), *E. coli* (NCTC 12923), *P. aeruginosa* (NCTC 6750), *S. epidermidis* ATCC 14990 (NCTC 11047) methicillin sensitive *S. aureus* (NCTC 10788), all bacteria strains were provided by CSPPS, Cardiff University. Standards and storing conditions were previously detailed in Chapter 2, Section 2.2.5.

3.3.5 Determination of minimum inhibitory concentration (MIC)

Broth dilution method was use in accordance to the method detailed in Chapter 2, Section 2.2.4.8 for broth dilution method to obtain PRE and $ZnSO_4$ MIC values against the tested microorganism. Individual drug MICs were obtained in accordance to the guidelines of CLSI twenty-fourth informational supplement: 'Performance standards for antimicrobial susceptibility testing (2014)'. At higher concentrations PRE showed problems of turbidity at first we tried to solve it by dissolving PRE at 2.5% DMSO, but still the turbidity remained. Another approach to eliminate turbidity was to decrease the amount of broth that has been used in each well. Finally, 50 µL of double strength MHB

was used and water was used the final volume was 100 µL of tested substance and 50µL of tested bacteria.

3.3.6 Checkerboard test

To determine antibacterial synergy, a checkerboard assay was performed using different concentrations of two antibacterials in a 96 well plate. One drug (drug A) is diluted against the abscissa and the other (drug B) is diluted along the ordinates. The starting concentration for each one should be at least double the MIC value as recommended by CLSI guidelines. MIC values were tested for PRE and $ZnSO_4$ in separate against five bacterial strains. In the checkerboard test each concentration of PRE and $ZnSO_4$ was made initial 4 times the concentration to be tested. For example, if PRE concentration to be tested is 1000 µg/mL then the initial concentration will be 4000 µg/mL. Using 2mL Eppendorf vials, PRE was serilly diluted to 9 times to give the concentration range of (15.625-4000 µg/mL). At first 50 µL was added from each eppindorf to its representative concentration starting from column no. 10 for the highest concentration of PRE and serially diluted along the abscissa to column no. 2 with lowest PRE concentration. Columns 1, 11 and 12 were kept as PRE-free wells. The same for the $ZnSO_4$ if the wanted concentration was 6400 µM so, 25600 µM was made in 2 mL Eppendorf and serial dilution was made up to 8 times from each Eppendorf, 50 µL was added to its representative concentration of $ZnSO_4$ serial dilution along the ordinate in the 96 well plate, starting from A1-A10 for the higher concentration going down to G1-G10 for the lower concentration and leaving H1-H10 and column 11,12 zinc-free wells. Column11 represented positive control drug-free wells and column 12 was negative control for sterility test. A 50 µL aliquot of bacterial suspension was added to all wells except column no.12. MHB at 50 µL was added to the following wells H2-10, column 1 and column 12. Finally, 50 µL of double strength MHB was added to all wells. Separate duplicate a 96 well plate with same serial dilution except its bacteria free media which represent negative control to be used in calculation as negative control. The turbidity

was measured at 600 nm Tecan Sunrise™ microplate reader. Samples combination is illustrated in Table 3-2.

Position in 96 well plate	**PRE content (µg/ml)**	**$ZnSO_4$ content (µM)**
A1	-	6400
A2	15.625	6400
A3	31.25	6400
A4	62.5	6400
A5	125	6400
A6	250	6400
A7	500	6400
A8	1000	6400
A9	20000	6400
A10	40000	6400
B1	-	3200
B2	15.625	3200
B3	31.25	3200
B4	62.5	3200
B5	125	3200
B6	250	3200
B7	500	3200
B8	1000	3200
B9	20000	3200
B10	40000	3200
C1	-	1600
C2	15.625	1600
C3	31.25	1600

C4	62.5	1600
C5	125	1600
C6	250	1600
C7	500	1600
C8	1000	1600
C9	20000	1600
C10	40000	1600
D1	-	800
D2	15.625	800
D3	31.25	800
D4	62.5	800
D5	125	800
D6	250	800
D7	500	800
D8	1000	800
D9	20000	800
D10	40000	800
E1	-	400
E2	15.625	400
E3	31.25	400
E4	62.5	400
E5	125	400
E6	250	400
E7	500	400
E8	1000	400
E9	20000	400
E10	40000	400
F1	-	200
F2	15.625	200
F3	31.25	200

F4	62.5	200
F5	125	200
F6	250	200
F7	500	200
F8	1000	200
F9	20000	200
F10	40000	200
G1	-	200
G2	15.625	200
G3	31.25	200
G4	62.5	200
G5	125	200
G6	250	200
G7	500	200
G8	1000	200
G9	20000	200
G10	40000	200
H2	15.625	-
H3	31.25	-
H4	62.5	-
H5	125	-
H6	250	-
H7	500	-
H8	1000	-
H9	20000	-
H10	40000	-

Table 3-2 Concentration range of PRE and $ZnSO_4$ tested using the checkerboard test

3.3.7 Fractional inhibitory concentration index

The fractional inhibitory concentration index (FICI) of PRE and $ZnSO_4$ from checkerboard test were calculated according to the following formula:

$\sum FIC = FIC\ A + FIC\ B$

FIC A is the MIC of drug A (PRE) in the combination/MIC of drug A (PRE) alone.

FIC B is the MIC of drug B ($ZnSO_4$) in the combination/MIC of drug B ($ZnSO_4$) alone.

Then after calculation the combination is considered to be synergistic, antagonistic, additive according to the sum of FIC according to Table 3-1.

3.3.8 Time kill (log reduction) assay

Bactericidal activity of PRE and $ZnSO_4$ alone and in combination was tested using time kill assay.The tested substance in volume of 990 µL was added to a 10 µL aliquot of 0.5 McFarland standard of tested bacteria in a 2 mL Eppendorf vial and allowed to incubate for different time points: 5, 10, 20, 60 minutes (for *S. aureus* MSSA an extended period of up to 6 hours). After each timepoint, 100 µL was transferred to 900 µL of neutralizing agent (UQA) and allowed to set for 60 min. Next, serial dilution of the neutralized mixture was made up to 8 times. The Miles and Misra technique was used for the enumeration viable cell colonies; briefly, 3 drops in 10 µL volume of each dilution was transferred on dried Muller Hinton Agar and incubated under aerobic condition at 37 ˚C for 24 h. then, counted colony and calculate the value results were expressed as colony forming unit (CFU) per 1 mL (CFU/mL) (Miles et al. 1938).

3.3.9 Calculation of log reduction value

After incubation for 24 hours at 37 ˚C under aerobic condition, the number of surviving colonies were counted then the following formula was used to calculate the colony forming unites per one millilitre (CFU/mL):

$$\frac{\boldsymbol{no\ of\ colonies \times dilution\ factor}}{\boldsymbol{volume\ of\ culture\ media}}$$

The Log_{10} reduction in colony forming units per 1 mL was calculated against control (phthalate buffer pH) using the following formula:

$\boldsymbol{log_{10}}$ **(A) -** $\boldsymbol{log_{10}}$ **(B)**

Where A is CFU/mL of the control (phthalate buffer) and B is CFU/mL of test sample.

3.3.10 Effects of PRE and Zn(II), alone and in combination, against pre-formed biofilm-embedded *S. aureus*

A major proposed application for the combination of PRE and Zn(II) is for use on chronic wounds, samples of PRE that contained (1 and 2.5 mg/mL) ± $ZnSO_4$ at 0.25 M was eval;uated on preformed biofilm-embedded *S. aureus*. The work in this section was performed in the laboratory of Dr. Wing Man Lau (School of Pharmacy, Faculty of Medical Sciences, Newcastle University, UK.). *S. aureus* (NU118) was obtained and isolated in Newcastle University, UK. The effects of the PRE on pre-formed biofilms were evaluated in 96 well plates - briefly, *S. aureus* was cultured in tryptic soy broth (TSB, Formedium, England) supplemented with 2% glucose at 1x 10^8 CFU/mL with optical density at 600 nm (OD 600nm) of 0.1, 200 µL per well. The culture was

incubated at 37 °C for 48 h to allow biofilm formation. After 48 h, the planktonic phase was removed, and the biofilm was washed once with 200 µL PBS. TSB (90 µL) was added in each of the well with 10 µL of the formulation added on top. *S. aureus* cultured in TSB alone was served as positive control, TSB alone without bacterial was served as negative control to ensure the experiment was not contaminated and the extraction formulation without PRE as background control to minimise the effects of the solvent used. The plates were then incubated for 20 h at 37 °C. After 20h, the supernatants were removed and the plate was washed once with 100 µL PBS. 100 mL of 0.1 % crystal violet (CV) solution was added to all wells and incubated for 15 min at room temp. After 15 min, the excess CV was removed by washing three times with 100 µL PBS. 100 µL of 7% acetic acid was added to dissolve the CV stain. The solution was then transferred to a new 96-microtiter plate for absorbance detection at 570 nm (BioTek Synergy HT) after dilution of 1:20 (Stepanović, et al, 2000).

3.3.11 Statistical analysis

The results were determined using Microsoft Excel® proGram of each sample number of log reduction in CFU/mL in different contact time. In each test, data are expressed as means ± SD, otherwise stated. Statistical analysis of log reduction value was carried out using One way analysis of variance (ANOVA) with Tukey‘s post test to compare statistical significances between groups. PRE and $ZnSO_4$ combinations were compared to control and individual PRE and $ZnSO_4$ at the same concentration used in that combination. Results were expressed as significant and very significant with $p < 0.05$ and $p < 0.01$ respectively. The analysis was carried out using InStat for Macintosh, version 3.0 (GraphPad Software Inc, San Diego, CA).

3.4 Results

3.4.1 Determination of minimum inhibitory concentration (MIC)

The MIC is defined as the lowest concentration of drug that can inhibit bacterial growth under standardized conditions. In the broth dilution method MIC was recorded when no turbidity was observed in the well (i.e. clear well). According to CLSI guidelines MIC point is recorded by visual unaided eye. A microplate reader was employed as confirmation step. The lowest MIC level was observed in *S. epidermidis* (NCTC 11047) for PRE at 125 µg/mL, and $ZnSO_4$ at 1600 µM. Table 3-3 shows the MIC concentrations for each microorganism for PRE and $ZnSO_4$ when applied individually.

Bacteria strain	**PRE MIC (µg/mL)**	**$ZnSO_4$ MIC (µM)**
MRSA (NCTC 12493)	1000	6400
***P. aeruginosa* (NCTC 6750)**	2000	1600
S. epidermidis (NCTC 11047)	125	1600
MSSA (NCTC 10788)	2000	3200
***E. coli* (NCTC 12923)**	NA	1600

Table 3-3 MIC values of PRE and $ZnSO_4$ against five bacterial strains: MRSA (NCTC 12493), *E. coli* (NCTC 12923), *P. aeruginosa* (NCTC 6750), *S. epidermidis* (NCTC 11047) and *S. aureus* (NCTC 10788) (n=3).

3.4.2 Checkerboard test

The results of the checkerboard analysis are shown in Figure 3-1 representing the entire 96 well plate. In this representation, an opaque well means growth, whereas a white well means no growth (clear).

To fully understand and explain if synergistic activity had occurred or not, the FIC index was calculated and explanation is mentioned in the Table 3-1. Looking at the Table 3- 4, it is apparent that synergism effect of PRE/Zn(II) happened in against two of the tested bacteria: MRSA and *S. epidermidis* - no antagonistic effect were observed at MSSA *S. aureus* and *P. aeruginosa*.

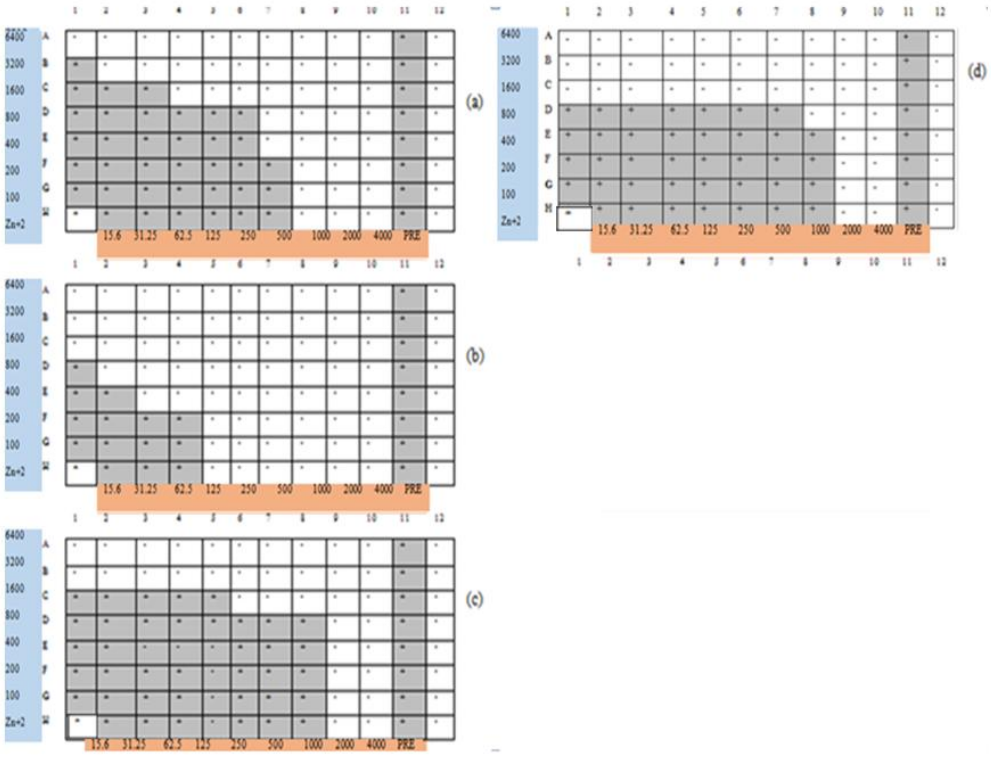


Figure 3-1 Checkerboard representation of 96 well plate where an opaque well means bacterial growth and clear one represents no growth for: (a) MRSA (NCTC 12493), (b) S. epidermidis (NCTC 11047), (c) MSSA (NCTC 10788) and (d) P. aeruginosa (NCTC 6750) (n=3).

Interestingly, the resistant *S. aureus* MRSA showed synergism while the methicillin sensitive *S. aureus* MSSA showed only additive effect. In the same manner *P. aeruginosa* FIC index value is 0.75 which is interpreted as additive or indifferent effect. For *E. coli* no inhibition could have been recorded because of previous mentioned

problem of turbidity of PRE at high concentrations, although $ZnSO_4$ alone showed inhibition at 1600 μM (Section 3.2.3.3).

Bacteria strain	**PRE (μg/mL) MIC**	**$ZnSO_4$ (μM) MIC**	**PRE (μg/mL) MIC in combination**	**$ZnSO_4$ (μM) MIC in combination**	**FIC index**	**Interpretation**
MRSA NCTC 12493	1000	6400	62.5	1600	0.3125	Synergism
***S. epidermidis* NCTC 11047**	125	1600	31.25	400	0.5	Synergism
MSSA *S. aureus* NCTC 10788	2000	3200	250	1600	0.625	Additive
***P. eruginosa* NCTC 6750**	2000	1600	1000	800	0.75	Additive
***E. coli* NCTC 12923**	NA	1600	NA	NA	NA	NA

Table 3-4 FIC index results from checkerboard test, with MIC level of PRE and $ZnSO_4$ at: MRSA (NCTC 12493), *E. coli* (NCTC 12923), *P. aeruginosa* (NCTC 6750), *S. epidermidis* (NCTC 11047) and *S. aureus* (NCTC 10788).

3.4.3 Bactericidal time kill test

3.4.3.1 **Methicillin resistant *Staphylococcus aureus* MRSA (NCTC 12493),**

Based on the previous MIC results and checkerboard assay, MRSA was chosen to optimize the concentration range for testing using time kill assay. The major advantage of this method is that it shows the extent of kill, i.e. log reduction value in bacterial viability at different contact times. The contact time was set at 5, 10, and 20 minutes and

up to 1 hour. PRE concentration used was the minimum inhibitory concentration against MRSA which was at concentration of 1 mg/mL. At first, a lower concentration of $ZnSO_4$ was tested with and without PRE at 0.0256, 0.064, 0.125, 0.25 M with PRE 1mg/ml $ZnSO_4$. The concentration range of $ZnSO_4$ was 4xMIC, 10xMIC and 20xMIC of $ZnSO_4$ with addition of higher concentrations 0.5 and 1M of $ZnSO_4$.

As shown in Figure 3-2, $ZnSO_4$ at the highest two concentrations (0.5, 1M) showed a 5.6 log reduction at 20 minutes and 1 hour contact time and consequently the same effect was seen when combined with PRE 1 mg/mL (figure 3-3)

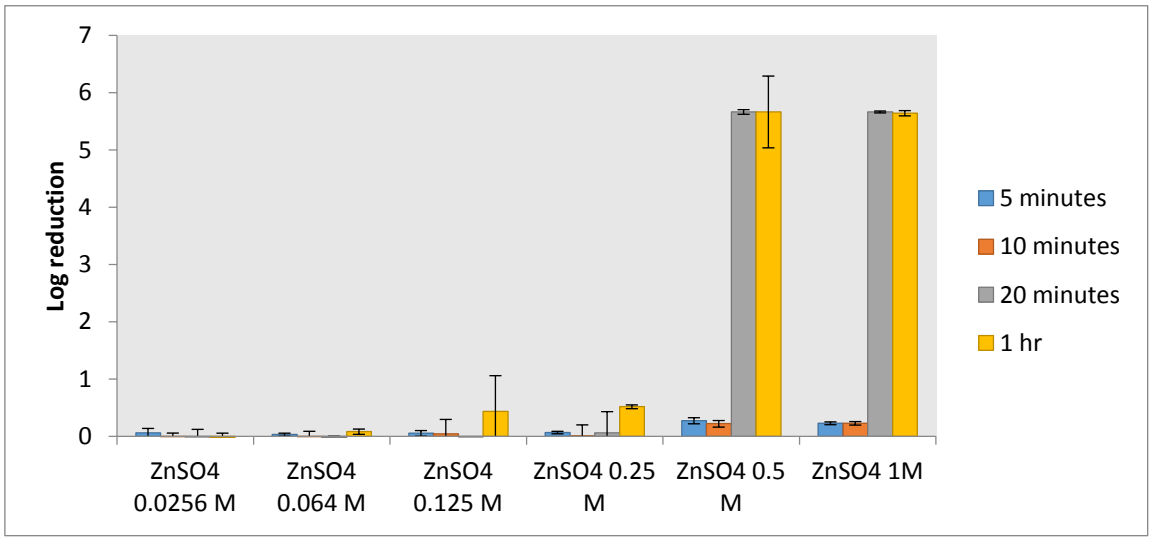


Figure 3-2 Bar chart showing log reduction in the colony forming unit (CFU) of MRSA (12493) by ZnSO4 (M) alone in comparison to control (phthalate buffer pH 4.5) (n=3 ± SD).

Very interesting activity happened with $ZnSO_4$ at 0.25M. PRE at 1 mg/mL alone showed the least bactericidal activity at all contact times and $ZnSO_4$ at 0.25M individual sample showed less than 0.5 log reduction value at all time up to one hour. Figures 3-2 and 3-3 present the log reduction values that have been obtained by this method against MRSA.

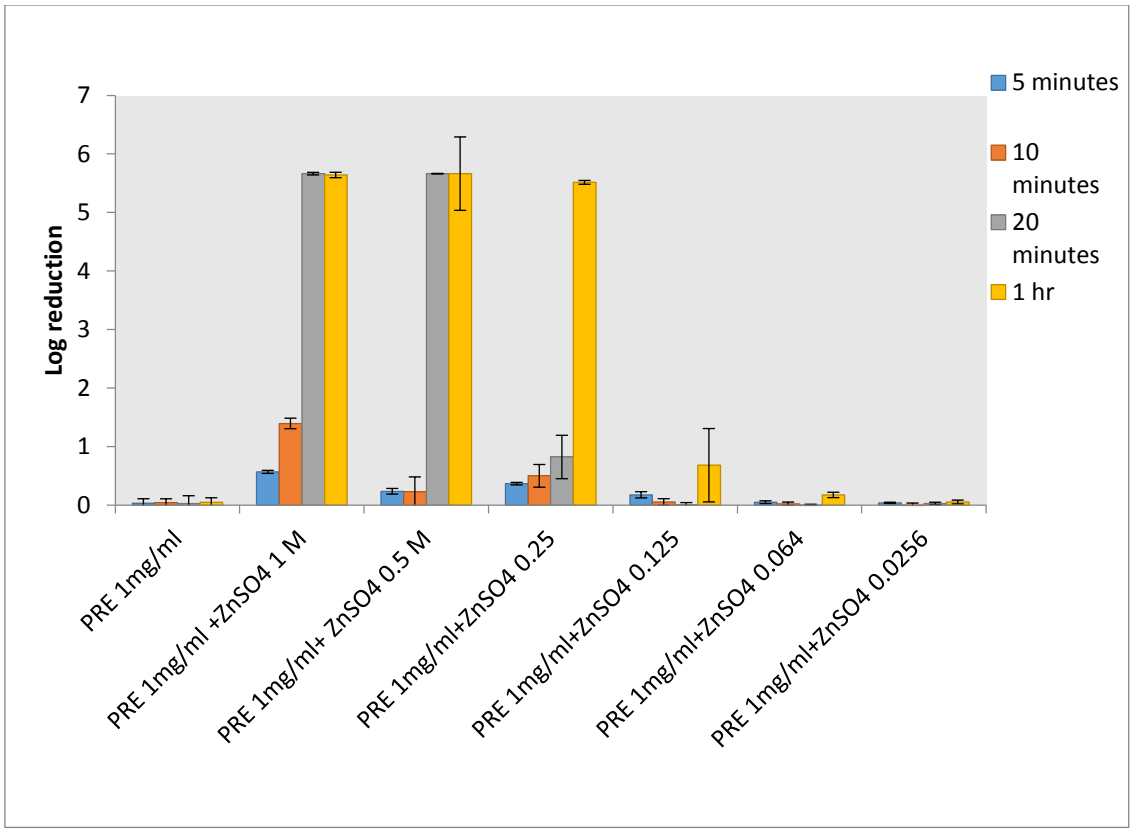


Figure 3-3 Bar chart showing log reduction in the colony forming unit (CFU) of MRSA by PRE alone and in a combination with $ZnSO_4$ in comparison to control (phthalate buffer pH 4.5) (n=3 ± SD).

The top half of the Table 3-5, it shows the log reduction values of the combined PRE and different $ZnSO_4$ concentrations and the lower half shows the $ZnSO_4$ activity by its own. At lower concentration of $ZnSO_4$ (0.0256, 0.064 0.125 M), the combination with PRE showed no significant difference in log reduction value when compared to $ZnSO_4$ or PRE individual samples.

Looking at the results at 10 minutes $ZnSO_4$ at 1M, a significant difference was detected ($p < 0.01$) since the $ZnSO_4$ alone shows 0.23 log reduction while adding the PRE at the same contact time shows a much greater 1.39 log reduction. The results showed a very significant increase in the bactericidal activity at (PRE 1 mg/mL with $ZnSO_4$ at 0.25 M) at all contact times when compared to individual $ZnSO_4$ at 0.25 M at those time points

($p < 0.05$). For the longest contact time at 1 hour, $ZnSO_4$ at 0.5 and 1 M showed very good bactericidal activity alone (5.6 log reduction), which remained the same when adding 1 mg/mL PRE. On the other hand, PRE alone and $ZnSO_4$ alone 0.25 M showed 0.05 and 0.51 log reduction after treatment for 1 hour, while the combination of both showed very significant increase in the bactericidal activity ($p < 0.01$). Since the higher concentration $ZnSO_4$ at 0.5 and 1 M already showed more than 5 log reduction value on its own, the combination (PRE 1 mg/mL with $ZnSO_4$ at 0.5 M, PRE 1 mg/mL with $ZnSO_4$ at 0.5 M) similar activity was kept, when compared to the individual respective $ZnSO_4$ group. It important to note that there was no evidence of antagonistic effect was observed when adding PRE to $ZnSO_4$ at all time points.

Log reduction	5 min	10 min	20 min	1 h
PRE 1mg/mL	**0.03**	**0.04**	**0.03**	**0.05**
PRE 1mg/mL + $ZnSO_4$ 1 M	**0.57**	**1.39**	**5.66**	**5.64**
PRE 1mg/mL + $ZnSO_4$ 0.5 M	**0.24**	**0.23**	**5.66**	**5.66**
PRE 1mg/mL + $ZnSO_4$ 0.25M	**0.37**	**0.50**	**0.82**	**5.51**
PRE 1mg/mL + $ZnSO_4$ 0.125M	**0.17**	**0.05**	**0.00**	**0.68**
PRE 1mg/mL + $ZnSO_4$ 0.064M	**0.05**	**0.02**	**0.00**	**0.17**
PRE 1mg/mL+$ZnSO_4$ 0.0256M	**0.04**	**0.01**	**0.03**	**0.05**
$ZnSO_4$ 0.0256 M	**0.06**	**0.01**	**0.01**	**0.02**
$ZnSO_4$ 0.064 M	**0.03**	**0.00**	**0.02**	**0.08**
$ZnSO_4$ 0.125 M	**0.05**	**0.04**	**0.01**	**0.43**
$ZnSO_4$ 0.25 M	**0.07**	**0.01**	**0.06**	**0.51**
$ZnSO_4$ 0.5 M	**0.27**	**0.22**	**5.66**	**5.66**
$ZnSO_4$ 1M	**0.23**	**0.23**	**5.66**	**5.64**

Table 3-5 Log reduction value in the colony forming unit (CFU) of MRSA by PRE alone, $ZnSO_4$ alone and combination in comparison to control (phthalate buffer pH 4.5) (n=3).

3.4.3.2 **Pseudomonas aeruginosa (P. aeruginosa) (NCTC 6750)**

In a similar manner to MRSA, PRE at a concentration of 1 mg/mL showed minimal bactericidal activity and less than 1 log reduction at all contact time up to 1 hour when compared to the control group as shown in Figure 3-4. Figure 3-4 also shows that there was a marked increase in the log reduction value at one hour contact time at all $ZnSO_4$ containing samples showed very significant difference ($p < 0.01$), compared to the control and gave >5 log reduction value. At the same time, no significant change between $ZnSO_4$ individual samples when compared to the respective combination ($p > 0.05$) at the same concentration and all gave good bactericidal activity (>5 log reduction). Another important result was seen with the combination (PRE 1 mg/mL with $ZnSO_4$ at 0.25 M and 0.5 M) at 10 min contact time - the combination in both concentrations showed very significant increase in the log reduction value ($p < 0.01$) to reach 5.47 log reduction, while individual log reduction value was 1.07 and 1.55 at $ZnSO_4$ at 0.25 M and at 0.5 M respectively. No significant difference was observed for the combination that contains $ZnSO_4$ at 1 M at all time when compared to $ZnSO_4$ individual samples since at 10 minutes all samples showed more than five log reduction value whether alone or in combined with PRE 1mg/ml. At 10 minutes contact time, a significant increase ($p < 0.05$) in the bactericidal activity (2.10 log reduction) was observed in the combination (PRE 1 mg/mL with $ZnSO_4$ at 0.125M) when compared to the individual samples. At 20 min contact time, for the low concentration 0.125 M of $ZnSO_4$ there was 0.83 log reduction of *P. aeruginosa* and PRE 1 mg/mL showed < 0.5 log reduction, while the combination of two showed very significant potentiation of the bactericidal activity ($p < 0.01$) and gave 5.47 log reduction (Figure 3-4). Log reduction values of PRE ± $ZnSO_4$ at all contact time are summarised in Table 3-6.

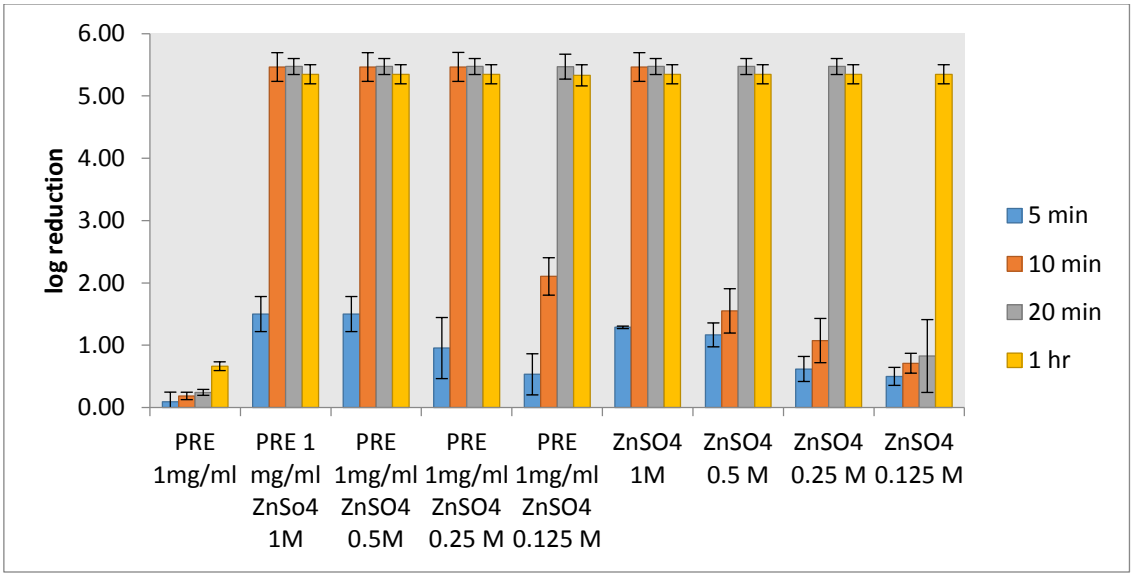


Figure 3-4 Bar chart showing log reduction in the number of CFU of *P. aeruginosa* by PRE 1 mg/mL alone, $ZnSO_4$ alone and in combination in comparison to control (phthalate buffer pH 4.5) (n=3 ± SD).

Sample	5 min	10 min	20 min	1 h
PRE 1 mg/mL	0.09	0.19	0.24	0.66
PRE 1 mg/mL $ZnSO_4$1M	1.50	5.46	5.47	5.35
PRE 1mg/mL $ZnSO_4$ 0.5M	1.50	5.46	5.47	5.35
PRE 1 mg/mL $ZnSO_4$ 0.25 M	0.95	5.47	5.47	5.35
PRE 1 mg/mL $ZnSO_4$ 0.125 M	0.53	2.10	5.47	5.33
$ZnSO_4$ 1M	1.29	5.46	5.47	5.35
$ZnSO_4$ 0.5 M	1.17	1.55	5.47	5.35
$ZnSO_4$ 0.25 M	0.62	1.07	5.47	5.35
$ZnSO_4$ 0.125 M	0.50	0.71	0.83	5.35

Table 3-6 Log reduction in the number of CFU of *P. aeruginosa* by PRE 1 mg/mL alone, $ZnSO_4$ alone and in a combination in comparison to control (phthalate buffer pH 4.5) (n=3).

3.4.3.3 **Staphylococcus epidermidis (S. epidermidis) (NCTC 11047)**

S. epidermidis was one of the three *Staphylococcus* bacteria that were investigated by bacterial suspension test. The PRE alone sample has very low activity against *S. epidermidis* and the maximum log reduction value been 0.1 log reduction at one hour. Closer analysis of the data presented at the Figure 3-5 and Table 3-7 show a significant effect ($p < 0.01$) at the following combinations when compared to the $ZnSO_4$ alone samples (PRE 1mg/mL with $ZnSO_4$ at 1 M) at 5, 10 and 20 minutes, (PRE 1mg/mL with $ZnSO_4$ at 1 M) at 10 and 20 minutes (PRE 1 mg/mL with $ZnSO_4$ at 0.125 M and 0.25 M) at 1 hour contact time. The highlight of these results is that $ZnSO_4$ at lower concentration (0.125 M and 0.25 M) showed only 0.79 and 1.57 log reduction respectively, while the combination in each with PRE gave much higher 5.57 log reduction values.

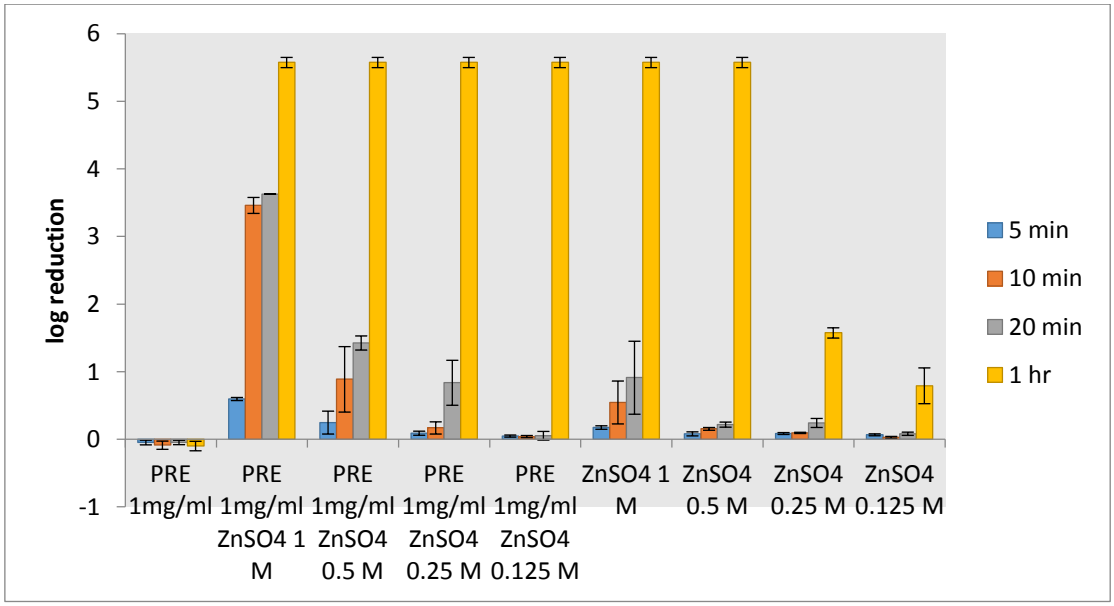


Figure 3-5 Bar chart showing log reduction in the number of CFU of *S. epidermidis* by PRE 1 mg/mL alone, $ZnSO_4$ alone and in a combination in comparison to a control (phthalate buffer pH4.5) (n=3 ± SD).

Sample	5 min	10 min	20 min	1 h
PRE 1mg/mL	0.05	0.09	0.05	0.10
PRE 1mg/mL + $ZnSO_4$ 1 M	0.60	3.46	3.63	5.57
PRE 1mg/mL + $ZnSO_4$ 0.5 M	0.25	0.89	1.43	5.57
PRE 1mg/mL + $ZnSO_4$ 0.25 M	0.09	0.17	0.84	5.57
PRE 1mg/mL + $ZnSO_4$ 0.125 M	0.05	0.04	0.05	5.57
$ZnSO_4$ 1 M	0.17	0.55	0.91	5.57
$ZnSO_4$ 0.5 M	0.08	0.15	0.22	5.57
$ZnSO_4$ 0.25 M	0.09	0.10	0.24	1.57
$ZnSO_4$ 0.125 M	0.07	0.03	0.08	0.79

Table 3-7 Log reduction in the number of CFU of *S. epidermidis* by PRE 1mg/mL alone, $ZnSO_4$ alone and in a combination in comparison to control (phthalate buffer pH 4.5) (n=3).

3.4.3.4 **Methicillin sensitive *Staphylococcus aureus* MSSA (NCTC 10788)**

PRE at 1mg/mL and $ZnSO_4$ in concentration 1, 0.5, 0.25 and 0.125 M were employed to test bactericidal activity against *S. aureus*. At first, the same contact times that had been used on MRSA were used to test bactericidal activity against *S. aureus* which was 5, 10, 20 min and up to 1 hour. Minimal activity in all tested samples was observed within a contact time of up to 1 hour. The only significant difference ($p < 0.01$) was observed in the combination that contained the tow highest concentration of $ZnSO_4$ at 0.5 and 1M to reach 0.82 and 0.84 respectively. The increase was small but still counts as significant when compared to the individual $ZnSO_4$ at 0.5 and 1M ($p < 0.05$). Consequently, contact time was increased to include 6 h to determine if it will increase the bactericidal activity.

At 6 h all $ZnSO_4$ samples showed low bactericidal activity except $ZnSO_4$ at concentration of 1 M as shown in Figure 3-6. Comparing the individual PRE 1 mg/mL

and $ZnSO_4$ at 0.125, 0.25 and 0.5 M to the combination of PRE with $ZnSO_4$ at those concentrations, the results showed statistically significant increase ($p < 0.01$) in the bactericidal activity at 6 h contact time to reach 5.75 log reduction while it was 0.41, 1.52 and 0.86 log reduction in individual $ZnSO_4$ samples respectively. The log reduction value of PRE ± $ZnSO_4$ at all contact times is summarised in Table 3-8.

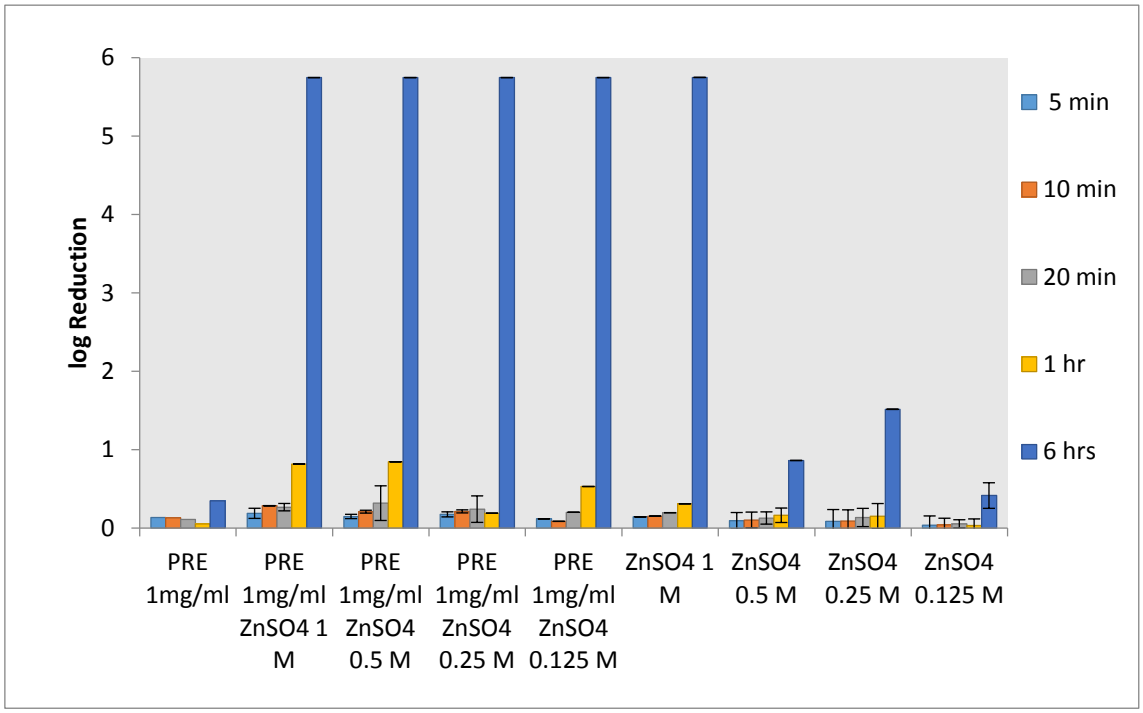


Figure 3-6Bar chart showing log reduction in the number of colony forming units (CFU) of S. aureus by PRE 1 mg/mL alone, $ZnSO_4$ alone and in combination in comparison to control (phthalate buffer pH 4.5) (n=3 ± SD).

Sample	5 min	10 min	20 min	1 h	6 h
PRE 1mg/mL	**0.14**	**0.13**	**0.11**	**0.06**	**0.35**
PRE 1mg/mL $ZnSO_4$ 1 M	**0.19**	**0.28**	**0.27**	**0.82**	**5.75**
PRE 1mg/mL $ZnSO_4$ 0.5 M	**0.15**	**0.21**	**0.32**	**0.84**	**5.75**
PRE 1mg/mL+$ZnSO_4$0.25 M	**0.18**	**0.21**	**0.24**	**0.19**	**5.75**
PRE 1mg/mL+$ZnSO_4$0.125 M	**0.12**	**0.08**	**0.20**	**0.53**	**5.75**
$ZnSO_4$ 1 M	**0.14**	**0.15**	**0.19**	**0.31**	**5.75**
$ZnSO_4$ 0.5 M	**0.09**	**0.10**	**0.13**	**0.16**	**0.86**
$ZnSO_4$0.25 M	**0.08**	**0.09**	**0.13**	**0.16**	**1.52**
$ZnSO_4$ 0.125 M	**0.04**	**0.04**	**0.05**	**0.03**	**0.41**

Table 3-8 Log reduction in the number of colony forming units (CFU) of *S. aureus* by PRE 1 mg/mL alone, $ZnSO_4$ alone and in combination in comparison to control (phthalate buffer pH 4.5).

An additional concentration of PRE (5 mg/mL) was tested against *S. aureus* in order to determine if this would increase the effect the bactercidal activity at shorter contact time. As shown in Figure 3-7 the the higher concentration of PRE alone at 5mg/mL did not increase bactericidal activity at 5, 10, 20 min and no significant increase ($p < 0.05$) in the bactercidal activity was observed comparing the combination with its respective individual samples .only one concentration of $ZnSO_4$ (1M) at one hour that showed significant increase when addded to the PRE 5 mg/mL to show 0.81 log reduction. At 6 hours contact time, no significant difference was seen when PRE 5 mg/mL was added to the $ZnSO_4$, since PRE individual and combinations showed high bactericidal activity (5.75 log reduction value). Table 3-9 summarises the results obtained *S. aureus* (MSSA).

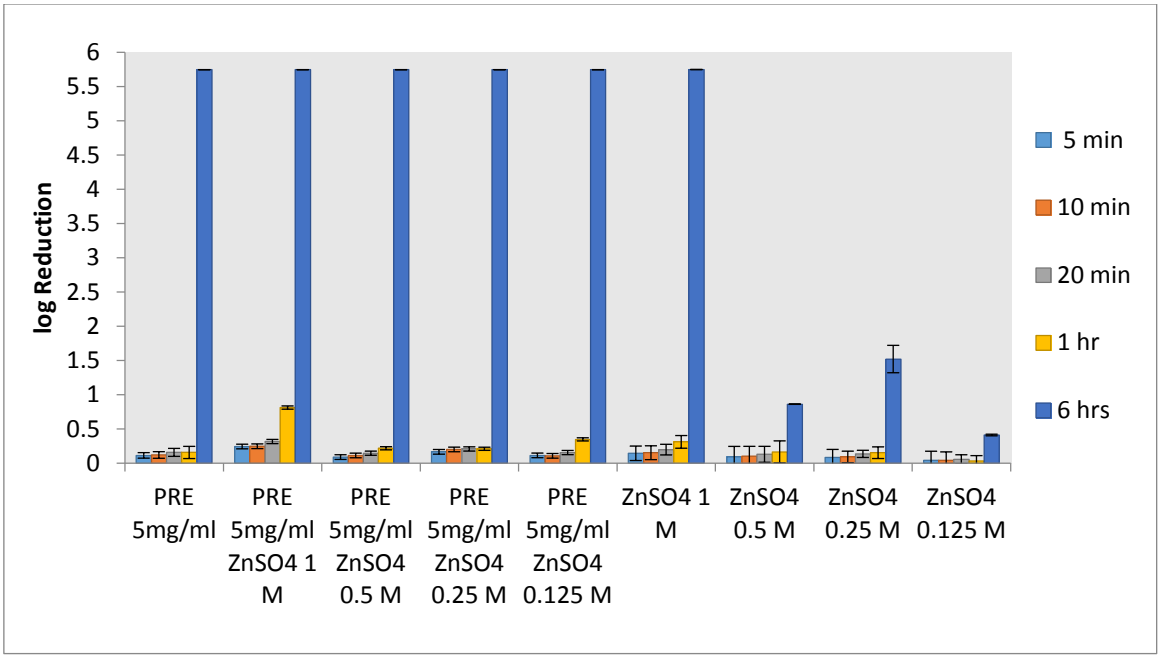


Figure 3-7 Bar chart showing log reduction in the number of colony forming units (CFU) of *S. aureus* MSSA by PRE 5 mg/mL alone, $ZnSO_4$ alone and in combination in comparison to control (phthalate buffer pH4.5) (n=3 ± SD).

Sample	5 min	10 min	20 min	1 h	6 h
PRE 5mg/mL	**0.11**	**0.12**	**0.15**	**0.16**	**5.75**
PRE 5mg/mL+$ZnSO_4$ 1 M	**0.24**	**0.24**	**0.32**	**0.81**	**5. 75**
PRE 5mg/mL+$ZnSO_4$ 0.5 M	**0.09**	**0.11**	**0.14**	**0.21**	**5. 75**
PRE 5mg/mL+$ZnSO_4$ 0.25 M	**0.16**	**0.19**	**0.21**	**0.20**	**5. 75**
PRE5mg/mL+$ZnSO_4$0.125 M	**0.11**	**0.11**	**0.15**	**0.35**	**5. 75**
$ZnSO_4$ 1 M	**0.14**	**0.15**	**0.19**	**0.31**	**5.75**
$ZnSO_4$ 0.5 M	**0.09**	**0.10**	**0.13**	**0.16**	**0.86**
$ZnSO_4$ 0.25 M	**0.08**	**0.09**	**0.13**	**0.16**	**1.52**
$ZnSO_4$ 0.125 M	**0.04**	**0.04**	**0.05**	**0.03**	**0.41**

Table 3-9 Log reduction in the number of colony forming units (CFU) of *S. aureus* MSSA by PRE 5 mg/mL alone, $ZnSO_4$ alone and in combination in comparison to control (phthalate buffer pH 4.5).

3.4.3.5 **Escherichia coli (E. coli) (NCTC 12923)**

PRE at a concentration of 1mg/mL was tested in time kill assay alone and in combination with $ZnSO_4$ at 1, 0.5, 0.25 and 0.125 M against Gram negative bacteria *E. coli*. PRE at all contact time showed no more than one log reduction value, compared to the control at 5 minutes contact time almost doubled the log reduction value was observed and significant increase ($p < 0.01$) in the following combination when compared to the PRE and $ZnSO_4$ individual samples (PRE with $ZnSO_4$ at 1 M, 0,25M and 0.125 M) to reach 4.41, 2.1 and 1.89 log reduction. At 10 minutes contact time, the combination of PRE in the same combinations (PRE with $ZnSO_4$ at 1 M, 0,25 and 0.125 M) showed significant ($p < 0.01$) increase in the log reduction value when compared to individual $ZnSO_4$ and PRE samples as shown in Table 3-10. All combinations at 1 h showed high bactericidal activity (6.5 log reduction) as shown in Figure 3-8. Comparing the combination to its respective $ZnSO_4$ individually, only one combination that contained $ZnSO_4$ at 0.125 M showed significant increase ($p < 0.01$) in the log reduction value when compared to the individual PRE, $ZnSO_4$ to reach 6.5 log reductions. There was more than 6 log reduction value at all the other combinations and individual $ZnSO_4$, resulting in no significant difference being observed since all gave good/maximal bactericidal activity. At 20 minutes contact time, $ZnSO_4$ in concentration of 0.125 and 0.25 M did not show more than 1.35 log reduction, while the combination of containing PRE at those concentrations showed significant increase in bactericidal activity ($p < 0.05$) of PRE to reach 2.53 and 2.78 in individual $ZnSO_4$ respectively. Table 3-10 and Figure 3-8 summarises the log reduction and bactericidal activity against *E. coli*.

Sample	5 min	10 min	20 min	1 h
PRE 1mg/mL	**1.03**	**1.04**	**0.99**	**1.04**
PRE 1mg/mL + $ZnSO_4$ 0.125 M	**1.89**	**1.89**	**2.53**	**6.53**
PRE 1mg/mL + $ZnSO_4$ 0.25 M	**2.10**	**2.34**	**2.78**	**6.53**
PRE 1mg/mL + $ZnSO_4$ 0.5 M	**2.56**	**2.59**	**4.54**	**6.53**
PRE 1mg/mL + $ZnSO_4$ 1M	**4.41**	**6.59**	**6.54**	**6.53**
$ZnSO_4$ 1M	**2.43**	**6.59**	**6.54**	**6.53**
$ZnSO_4$ 0.5 M	**2.47**	**2.18**	**4.54**	**6.53**
$ZnSO_4$ 0.25M	**1.00**	**1.49**	**1.30**	**6.53**
$ZnSO_4$ 0.125M	**0.62**	**0.95**	**1.35**	**1.52**

Table 3-10 Log reduction in the number of colony forming units (CFU) of *E. coli* by PRE 5 mg/mL alone, $ZnSO_4$ alone and in combination in comparison to control (phthalate buffer pH 4.5) (n=3).

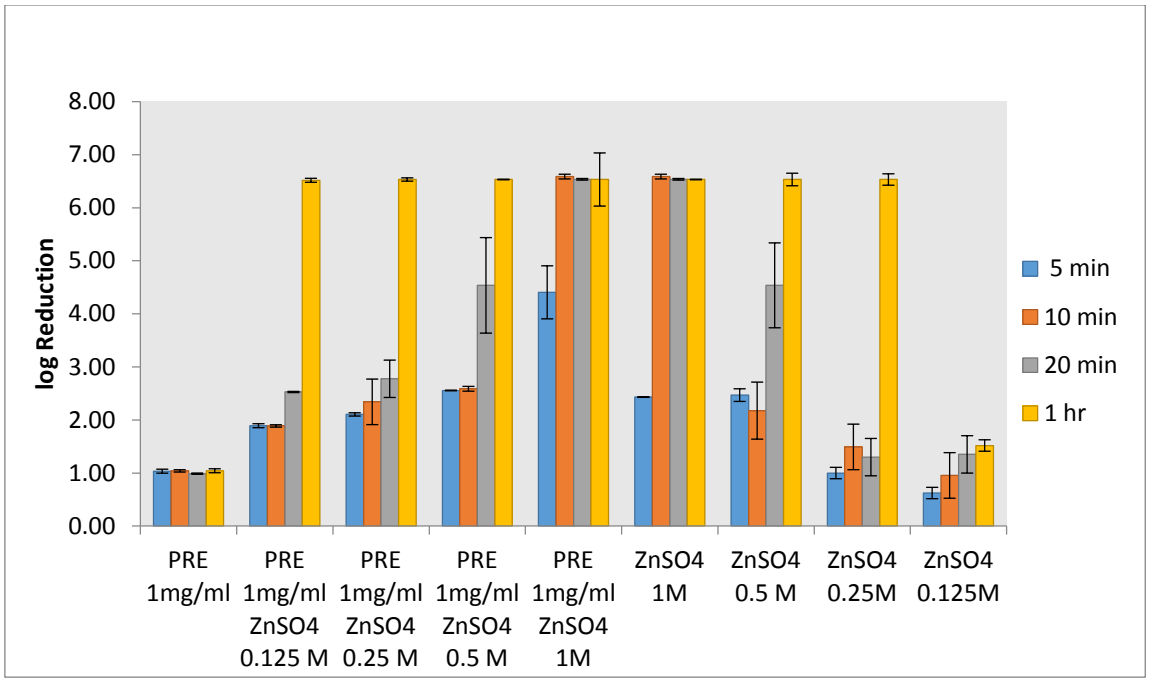


Figure 3-8 Bar chart showing log reduction in the number of CFU of *E. coli* by PRE 1 mg/mL alone, $ZnSO_4$ alone and in combination in comparison to a control (phthalate buffer pH 4.5) (n=3 ± SD).

3.4.4 Effects of the PRE and Zn(II) alone and in combination against pre-formed biofilms embedded *S. aureus*

PRE and $ZnSO_4$ at different concentrations, alone and in combination, showed no significant antibiofilm activity against the *S. aureus* preformed biofilm, as shown in Figure 3-9.

The survival rate of *S. aureus* ≥ 100 % in all tested groups and significant growth in *S. aureus* was observed in tested samples that contained PRE at 2.5 mg/mL ($p < 0.05$).

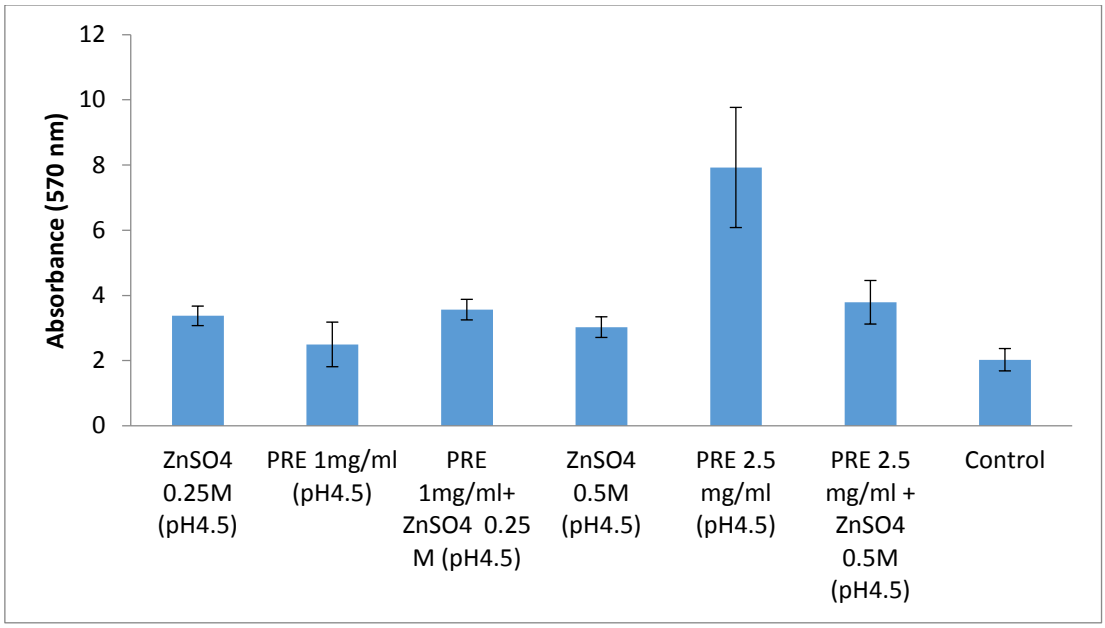


Figure 3-9 Effects of the PRE ± Zn(II) against pre-formed biofilm-embedded *S. aureus* after 24 hours incubation in the presence and absence of compounds. Results are shown as mean absorbance of bacteria (indicates growth) ± SD at 570 nm from single replicate from 6 experimental replicates.

3.5 Discussion

Due to the increase of bacterial resistance to the conventional antibiotics, new sources of antimicrobial agent are needed. Natural sources have been excessively studied because of its antimicrobial activity and relatively good safety profile to human body. The pomegranate in particular has been used since ancient cultures to treat a wide range of conditions and in modern civilized culture the fruit has been studied scientifically for its benefits to human health. Several studies examined the antibacterial activity (Choi. et al. 2011; Menezes et al. 2006; Meléndez and Capriles 2006; Pagliarulo et al. 2016; Panichayupakaranant et al. 2010; Prashanth et al. 2001; Tanveer et al. 2015), antiviral activity (Haidari et al. 2009; Su et al. 2010) and antifungal of different forms of

pomegranate extract (Santos et al. 2009; Tayel. and El-Tras 2009). In our research we examined the combined effect of pomegranate alone and with another agent, Zn(II). The recent discovery of potentiated antiviral activity of PRE by Zn(II) in this School was the driving influence for our ongoing research for the antibacterial activity of the combination (Houston, 2011).

In our study, the effect of adding on antibacterial activity of PRE was investigated by means of checkerboard test and time kill assay against MRSA, *E. coli*, *P. aeruginosa*, *S. epidermidis* and MSSA.

There is considerable variation between studies regarding the antibacterial activity of PRE against *S. aureus* (sensitive and resistant) as reviewed in the literature. The MIC levels of pomegranate extracts as established by different studies, ranging from 0.62 μg/μL and up to 10 μg/μL against *S. aureus* (McCarrell et al. 2008; Prashanth et al. 2001). Machado et al. (2003) reported that the total methanolic extract and water extract of *Punica grantum* showed MIC levels > 250 mg/L against *Staphylococcus aureus:* methicillin sensitive MSSA and methicillin resistant MRSA and our results showed that water extract of pomegranate rind showed MIC level of 1 mg/mL and 2 mg/mL against MRSA and MSSA respectively. Those MIC levels went down when combined with $ZnSO_4$ to reach 0.0625 mg/mL against MRSA and 0.5 mg/mL against MSSA as shown in the checkerboard test. Our findings showed the lowest MIC level of PRE alone against *S. epidermidis* at 0.125 mg/mL. The published MIC of pomegranate extract ranged from 9 to 25 μg/μL against *E. coli* and our results showed that *E. coli* need higher concentration than the tested. *P. aeruginosa* showed the same level of MIC as MSSA (2 mg/mL) as in previous reports (Navarro et al. 1996; Prashanth et al. 2001; Machado et al. 2003; Melendez and Capriles 2006; Pagliarulo et al. 2016). Such variation in the level of antibacterial activity may be explained by various factors such as the means of extraction, tannin (in particular punicalagin) content, seasonality, the cultivation process where the pomegranate is grown in different geographical areas (Zografou et al. 2013; McCarrell et al. 2008).

As shown by the checkerboard test, the FIC index calculation indicated that MRSA surprisingly was more sensitive than MSSA strain and synergistic effect was seen in MRSA and *S. epidermidis.* Interestingly, this was the reflected in time kill log reduction assay. This result was very encouraging to show the activity against MRSA bacteria because of its bad reputation in aggressive wound infection. MRSA is known for its resistance to beta lactam antibiotics and other antibiotics (Hartmann et al. 1997). Hospital acquired MRSA wound infection can lead to life threatening generalized MRSA infection (Coello et al. 1997).

In our study PRE alone at high concentration (5 mg/mL), without Zn(II), gave a high bactericidal effect against MSSA at 6 hours contact time to reach 5.75 log reduction values. While in low concentration of PRE (1mg/mL) alone showed minimal bactericidal effect up to 6 h contact time (0.35 log reduction), it showed a significantly higher ($p < 0.01$) bactericidal effect (>5 log reduction) against *S. aureus* (MSSA) when $ZnSO_4$ was added at 0.125, 0.25 and 0.5 M, but needed 6 hours to give the action. Furthermore, bactericidal activity against MRSA showed that PRE 1 mg/mL potency was increased by $ZnSO_4$ 0.25 M at all contact times but significant increase was recorded at one hour contact ($p < 0.01$) a high bactericidal (5.5 log reduction), while this activity wasn't seen in the individual samples. The results for *S. epidermidis* showed that $ZnSO_4$ at lower concentration (0.125 M and 0.25 M) gave only 0.79 and 1.57 log reduction respectively while the combination in each with PRE 1 mg/mL gave 5.57 log reduction values and significant potentiation was observed.

Our results showed that the activity of PRE (1 mg/mL) was increased against Gram negative bacteria *E. Coli* and *P. aeruginosa* when $ZnSO_4$ was introduced. The addition of $ZnSO_4$ potentiated the bactericidal activity of PRE against *E. coli*. The results showed that there was a potentiation and significant increase ($p < 0.01$) in the bactericidal effect against *E. coli* was observed with combination of PRE 1 mg/mL and $ZnSO_4$ at 0.125 and 0.25 M at 5, 10 and 20 minutes contact time when compared to individual of PRE 1 mg/mL and $ZnSO_4$. The other Gram negative bacteria *P. aeruginosa* showed similar

potentiation at $ZnSO_4$ in concentration of ($ZnSO_4$ 0.125 M at 20 min), (0.25 and 0.5 M at 10 min). In the *P. aeruginosa* experiment the pomegranate rind extract at 1 mg/mL alone showed less than one log reduction at all contact time up to 1 h. $ZnSO_4$ alone in all concentration gave a high bactericidal activity (> 5 log reduction) at 1h but the potentiation was seen at 20 min contact time with PRE/0.125 M and at 10 min contact time with PRE/0.25 M $ZnSO_4$ and PRE/0.5 M $ZnSO_4$ in contrast to each one individually.

The antibacterial activity of $ZnSO_4$ was reported in a previous study that showed the MIC level of different bacterial strain isolated from rat and human wound. The study showed MIC levels of Zn(II) in the range (2-4 mmol/L) against MSSA *S. aureus* and MIC level of *E.coli* at (4-8 mmol/L). These findings are in accordance with our results that showed MIC level of Zn(II) at 3200 μM against MSSA *S. aureus* while higher MIC was obtained with MRSA (6400 μM) and lower MIC level was recorded by *E.coli* (1600 μM). There are several mechanisms proposed in which Zn(II) could act as an antibacterial agent. Zn(II) ion binding to the bacterial surface and or generation of hydrogen peroxide which may lead to increase the cell membrane permeability, intracellular outflow and eventually cell growth inhibition and cell death (Sirelkhatim et al. 2015).

Most of the pathogens involved in chronic wounds, for instance *Pseudomonas* spp and methacillin resistant *Staphylococcus aureus* (MRSA), are typical producers of biofilm. These bacteria can develop very high resistance levels to conventional treatment because of lack of drug permeability through the biofilm itself provided by extrapolymeric substances (EPS) (Donlan et al. 2001; 2002). Another defense mechanism of bacteria that is present in biofilm is that this type of bacteria grows less quickly, and this lack of growth may cause a decrease in antibacterial agent uptake, as well as other changes of a physiologic nature that could impact on the effectiveness of the drug (Mandell et al. 2005; Siddiqui and Bernstein 2010; Wolcott et al. 2010).

The results in this chapter are very encouraging involving *planktonic* bacteria. However, chronic wounds and skin infections are typically associated with biofilms and our results, showed a resistance of the preformed *S. aureus* biofilm to the PRE/Zn solutions that contained PRE (1 mg and 2.5 mg) ± $ZnSO_4$ (0.25M). Activity could be limited by low permeation through the ECM, such that insufficient reaches the bacterial load to elicit an effect.

Although the results are considered empiric because of low replicate number, it is still an important observation, revealing data that merits inclusion in this chapter. Additionally higher PRE concentration showed higher growth and survival of *S. aureus* biofilm and that could be result of higher glucose levels in crude extract of PRE (2.5 mg/mL) (Waldrop, et al. 2014). It is not possible to make further deductions without further work being done and higher replicates performed. Biofilm bacteria according to Donlan et al (2001; 2002) have been reported as being 500 times more resistant to antibiotics than those that are freely living or unattached (planktonic). This results broadly agree with previous findings in which concluded that planktonic *S. aureus* study showed more susceptibility to the antibiotic treatment at first 48h then a tolerance will be developed after mature biofilm is formed on the wound (Wolcott et al. 2010). The study has demonstrated a new insight in relation to the resistance of biofilm with young or mature type biofilm and biofilm susceptibility testing and clinical biofilm maturity experiments, showed that 1 day planktonic *S. aureus* is more susceptible to the antibacterial agent gentamicin and tolerance was produce at mature biofilm after 48h. However, a positive effect of debridement procedure (i.e. biofilm removal from wound surface) on mature biofilm was explained, that it will remove the mature biofilm and resulted bacteria in to planktonic or young biofilm type will be more susceptible to the antibacterial agent. The study recommended an application time window of antibacterial is first 48-hour after debridement in order for that treatment to be effective.

In this chapter, we showed that a synergistic and or potentiation effect can be achieved with PRE/Zn(II) combination. Our findings are in line with other studies that show a

synergistic effect could result from combination of metal and pomegranate extract (McCarrell et al. 2008), although this is the first to show such activity with Zn(II). Synergistic effects against viruses have been reported by Houston et al (2017b). In his work, Houston (2011) showed that the addition of $ZnSO_4$ increased microbicidal activity of PRE from ~1 to 7log reduction against *Herpes simplex* virus (Houston *et al*, 2017b). In another study, McCarrell et al. (2008) indicate that synergistic effect was seen when Cu(II) was added to PRE, it show increased in antimicrobial activity opposing *E. coli*, *Ps. aeruginosa* and *P. mirabilis* and opposing *S. aureus,* compared to the individual activity of both. Although McCarrell et al. (2008), reported the potentiation of PRE bactericidal activity upon addition of Cu(II), the study failed to find the same kind of synergistic effect with Zn(II) ion that showed minimal increase in the antibacterial activity of PRE against tested microorganisms (*Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa* and *Proteus mirabilis*).

In contrast to McCarrell et al. (2008), our study showed a potentiation of antimicrobial activity of PRE upon addition Zn(II) obtained by checkerboard synergy test against methicillin resistant *Staphylococcus aureus* and *Staphylococcus epidermidis* and an additive effect was seen in *Staphylococcus aureus and Pseudomonas aeruginosa*. There are several factors to be considered when analyzing the opposing results of the both studies regarding the antibacterial activity of PRE and zinc, or Zn(II) ion, one of them is that different salts of zinc were employed in each test while McCarrell et al. (2008) used zinc oxide (ZnO) and in our study we used $ZnSO_4$. Due to its tight bonding, ZnO is highly insoluble in aqueous media as noted by Houston *et al* (2017b) and may not liberate sufficient free Zn(II) to potentiate PRE adequately. Our results showed a high bactericidal effect on MSSA *S. aureus* at 5mg/mL concentration of pomegranate peel extract, while Pagliarulo (2016) recently reported that PRE should give complete bactericidal effect at 50 µg/µL.

The mechanism by which pomegranate can induce antibacterial activity is not fully understood, but a proposed mechanism is that active phenolic/tannin substances can alter

enzymes or it can be absorbed into the cell membrane peptidoglycan, of Gram positive and Gram negative bacteria leading to perturbation of the membrane structure and function (Hugo and Bloomfield 1971; Furneri et al. 2002). Another possible mechanism is because polyphenols can precipitate microbial cell membrane protein and or inhibit glycosyltransferase enzyme at the same time leading to bacterial cell wall damage and breakdown (Stern et al. 1996; Karimi et al. 2017). Figure 3-10 shows the possible mechanisms of action polyphenol could act on Gram positive and Gram negative bacteria.

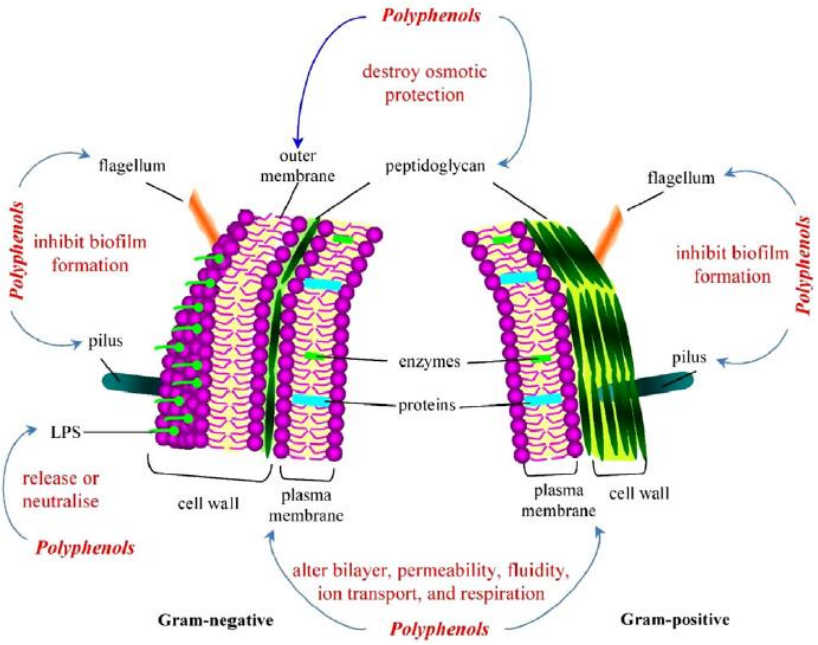


Figure 3-10 Hypothesized meachanism of antibacterial action of polyphenol and interactions cell wall components and plasma membrane of Gram-negative and Gram-positive bacteria (Papuc et al. 2017)

However, the above mechanisms do not account for the synergy observed with Zn(II). Mechanisms which the PRE/Zn(II) combination induces the potentiated activity, remains to be established. Houston (2011) studied the virucidal effects of different salts of Zn(II) in addition to PRE and he found that it is indeed the Zn(II) that triggered the potentiation and not the SO_4^{2-} ion. (Houston 2011). Possible antibacterial mechanism of

action of $ZnSO_4$ could be attributed to the Zn(II) ion and this theory could be confirmed by testing different zinc salts against investigated bacteria. Both PRE and Zn(II) possess low levels of antibacterial/bactericidal activity but the combination of two gave more activity as it was revealed in this chapter.

It is feasible that the multiple phenol groups of the tannins (i.e. punicalagin) attach to the bacterial cell peptidoglycan first and compromise its function and structure of cell wall enabling toxic levels of Zn(II) to be absorbed causing cell death. Another possible mechanism is that Zn(II) will act first on the cell wall and compromise its permeability and function, leading to polyphenol substance to be absorbed or interact with bacterial resulting in protein denaturation and or destabilization of microbial cell wall leading to its damage.

3.6 Conclusion

Findings in this chapter demonstrate that there is a synergistic effect of PRE microbicidal activity upon the addition of Zn(II).

- From the checkerboard test the potentiation was seen at PRE/Zn (II) concentration (62.5 μg/mL + 1600 μM) and (31.25 μg/mL + 400μM) against MRSA and *S. epidermidis* respectively. Whereas MSSA and *P. aeruginosa* demonstrated an additive effect only. No antagonistic effects were observed at any time.
- From the time kill assay, the concentration level of Zn(II) that has the capability of working with PRE 1 mg/mL as broadspectrum bactericidal system were $ZnSO_4$ at 0.125 and 0.25 M. The combination of PRE and Zn(II) are capable *in vitro* of effectively killing Gram-positive bacteria, MSSA *S. aureus*, MRSA and *S. epidermidis* and the development of Gram-negative bacteria, *E. coli* and *P. aeruginosa.*

- On the other hand, based on the limited study we were able to carry out, the biofilm embedded *S. aureus* (NU118) results didn't show susceptibility to PRE/Zn. However, the formulation may have potential use following biofilm debridement.

As the antibacterial/bactericidal activity of PRE/Zn system has revealed very encouraging results, the cytotoxicity of such combination is very important as a following step. In the next Chapter, the cytotoxicity of PRE ± Zn(II) will be investigated *in vitro* using an immortalised epithelium cell line.

Chapter 4: *In vitro* cytotoxicity and anti-proliferative effects of PRE and Zn(II)

4.1 Introduction

The aim of this project was to explore the potential of PRE/Zn as novel treatment for bacterial skin infections – these can involve intact epidermis or compromised epidermis. For example, impetigo is a common and contagious skin infection caused when bacteria such as *Staphylococcus aureus*, MRSA or *Streptococcus pyogenes* infect the epidermis; either intact skin (primary impetigo) and also in broken skin (secondary impetigo). When infections involve broken skin where the epidermis has been compromised and there is thus a wound healing aspect that must be considered in the development of any new topical treatment. Whereas the primary function of a potential new treatment based upon PRE and Zn(II) is to address the microbial load resident within affected areas of skin, it is also important that the formulation does not have adverse affects at the cellular level, which could prevent cell proliferation and re-epithelialisation across the wound bed in a timely manner or even cause the cells to die.

4.1.1 Cell viability

Determination of cell viability and proliferation activity (cytotoxicity) in cell culture experiments is thus considered to be the most important assay in the development of novel pharmaceutical products. In cytotoxicity assays measuring what is defined as healthy viable cell is the key purpose in these kinds of experiments (Stoddart 2011). Typically such data involves cell proliferation portrayed relative to an untreated control which is set at 100% - a test substance that produces a result less than 100% shows reduced cell proliferation and is deemed cytotoxic, and the lower the percentage the greater the cytotoxicity. On some occasions, cell proliferation is found to be greater than control (>100%) indicating the test substance may have stimulated or accelerated cell proliferation relative to control.

There are several methods used to probe cell viability and cell proliferative activity in mammalian cell cultures. Measuring cell viability by using of metabolic dye that is present in healthy cells became more sophisticated and complex with time.

4.1.1.1 **Trypan blue**

One of the earliest techniques is the live /dead cell staining by Trypan blue dye, in which same concept of cell counting previously detailed in Chapter 2, Section 2.2.5.4 was employed.

4.1.1.2 **MTT assay**

Another method very commonly used in cell cytotoxicity assays is the use of metabolic days and one of the early discover dye is MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). MTT assay is colorimetric in type in which depends on the presence of live cell metabolic activity that will transform the MTT yellow dye into purple color when added to the live cell culture. Around the early 1940s, small-scale research and case studies began to use tetrazolam salts as detective measure of live cell metabolic activity (Kun and Abood 1949). The mechanism of action of MTT assay involves quantification of the presence of live cells enzyme called nicotinamide adenine dinucleotide phosphate NADPH dependent cellular oxidoreductase enzyme that will reduce the yellow coloured MTT dye to the purple coloured insoluble product, formazan. The cells are typically exposed to the MTT reagent for 4 hours at least (Kun and Abood. 1949; Kupcsik 2011).

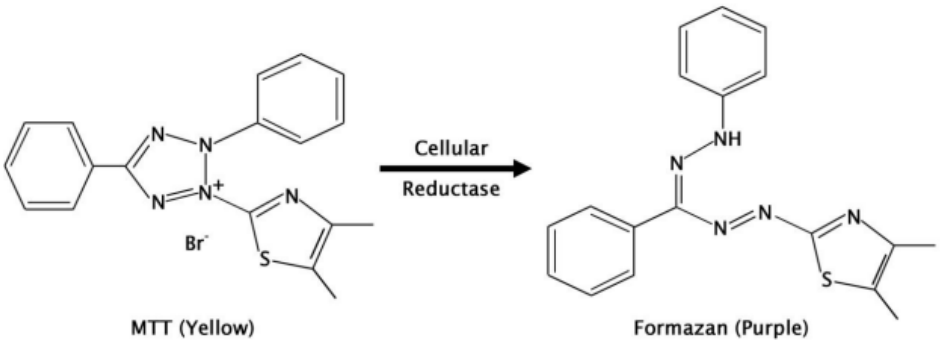


Figure 4-1 Schematic of cellular reduction of MTT to formazan

After formation of insoluble formazan crystals, dimethyl sulfoxide (DMSO) is added to the media to dissolve these crystals and reading of the absorbance is the final step in this assay, typically by plate reader at 540-570 nm. An advantage of this assay that it can be performed using multiwell plates and exposure to the treatment media can be over a prolonged timescale. Numerous considerations should be taken into account when exposure time is prolong and one of the main factors is that seeding density should be considered when long exposure time is applied, since overgrowth of the cells can lead to lower metabolic rate and false estimation and readings if the seeding density was too high from the beginning. Another important factor is the refreshment of the media every other day, since exhausted media lack in glucose level and decreased glycolysis in the cell that will lead to lower MTT production (Berridge and Tan 1993; Berridge et al.1996; Kupcsik 2011).

4.1.1.3 **MTS**

In the same concept as MTT, others have detected viable cells through metabolic activity such as MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium), XTT (sodium3-[1-(phenylaminocarbonyl)-3,4-tetrazolium)-bis-(4-methoxy-6-nitro) benzene sulphonic acid hydrate) and WST derivatives. This method have increased sensitivity because they both are used in the

presence of an intermediate electron acceptor PMS (phenazine methosulphate) which make the assay highly stable (Cory et al. 1991; Stoddart 2011).

4.1.1.4 **LDH**

Measuring cell death in the culture is a more direct technique for detecting cell viability in response to the presence of a test substance. When death happens to normal cells, the mechanisms of cell death can be divided into two types: apoptosis and necrosis. Apoptosis can be defined as proGrammed cell death that can occur inside the healthy cell to get rid of unwanted cells during normal development of cell growth cycle. It can also happen when permanent damage beyond the repair of the cell happens due to infection period or cell undergo stressed conditions. On the other hand, necrosis is non-proGrammed cell death due to trauma or toxin presence that trigger premature cell death. When necrosis happens to the cell, a certain enzyme called lactate dehydrogenase (LDH) is released from the damaged cell membrane into the extracellular medium. Colorimetric detection of LDH enzyme is widely used as a measure of cytotoxicity and a number of LDH kits are commercially available. The reading of the end-point coloured product spectophotometrically can be used to detect and quantify the LDH present. The LDH enzyme is considered to be stable up to 48h after cell death. The increased LDH release in the cell culture supernatant is directly proportional to the number of lysed cells in the culture (Wong et al. 1982; Hipler et al. 2007; Gerets et al. 2011; Stoddart 2011).

4.1.1.5 **FACS**

Another assay for cell viability is the Fluorescence Activated Cell Sorting system (FACS). The FACS analysis most routinely used is the ‘dual dye’ method. It depends on staining the cell culture after treatment with two fluorescent reagents: propidium iodide (PI) and fluorescein diacetate (FDA). FDA can penetrate the cellular membrane of the viable cell and the dye transform to the fluorescence green colour. The esterase enzyme in the cell membrane is responsible for the production of this green fluorescence colour and higher fluorescence green is a mark of high proliferative cell presence when

analyzed under flow cytometry. On the other hand, PI is excluded from viable cells because of the presence of intact cellular membrane that is not permeable to the PI, rather it is a DNA binding dye that can bind to dead cells nucleic acid without cell membrane and turn into red fluorescence state. Cellular fluorescence is measure by flow cytometer measurement after staining with PI and FDA at the end to estimate the cell cytotoxicity (Ross et al. 1989; Stoddart 2011).

4.1.2 HaCaT cell line

HaCaT keratinocytes were chosen as the appropriate cell model in this work. HaCaT cells are a spontaneously immortalised human keratinocyte cell line originally obtained from histologically normal skin distal to an excised melanoma. The name HaCaT refers to **H**uman **A**dult skin keratinocyte origin and because cells were propagated in low **Calcium** medium and at an elevated **Temperature** (Boukamp et al. 1988). Spontaneous immortalisation of human cells is quite rare but early in the immortalisation process; HaCaT cells differentiated and stratified under high calcium conditions, but proliferated as a monolayer under low calcium. Further passages reduced the effects of low calcium levels and cells that survived increased temperature were able to grow autonomously in culture. Marker chromosomes resulted which were able to show that the cell line was monoclonal by karyotyping, showing that HaCaT cells were aneuploid but many cells in late passages were polyploid. Like normal keratinocytes HaCaT cells develop into a well-structured epidermis, having the same keratin expression profile, exhibiting normal differentiation at higher passages. A commonly used technique for cells immortalisation involves virus infection (e.g. Simian Virus-40), which tends to produce irregular growth, keratinisation and differentiation (Boukamp et al. 1988).

Since their discovery, HaCaT cells have been widely utilized as a model for determining cytotoxic effects due to their high ability to differentiate and proliferate in vitro. As a human keratinocyte model, the cell line is reproducible and overcomes issues such as short culture lifespan and variations between cell lines. Chu et al. (2017) used HaCaT

cells to characterise cytotoxcity of tea tannin, epigallocatechin gallate. Ellagic acid (a component of PRE) was found to have a protective effect to HaCaT cells against UV induced oxidative stress and apoptosis (Hseu et al. 2012), and pomegranate phenolics were found to ameliorate H_2O_2–induced oxidative stress and cytotoxicity in HaCaT cells (Liu et al. 2019).

4.2 Objective and Aims

The objective of this chapter was to determine the cytotoxic (cell viability and proliferation) effects of PRE ± Zn(II) on a standard keratinocyte cell line, HaCaT, using:

- MTT colorimetric assay
- FACS analysis using propidium iodide (PI) and fluorescein diacetate (FDA).

4.3 Material and methods

4.3.1 Materials

The materials used in this chapter are detailed in Section 2.1

4.3.2 Sample preparation

PRE ± $ZnSO_4$ solutions at pH4.5 were prepared using previously detailed methods in Chapter 2, Section 2.2.1.

4.3.3 Aseptic techniques

Aseptic measures and methods were employed at all stages in cytotoxicity investigation on HaCaT cells using cell culture safety cabinets and methods previously detailed in Chapter 2, Section 2.2.5.1.

4.3.4 Cell culture general methods

Human adult, spontaneously immortalized, epidermal keratinocytes (HaCaT cells) were used in the investigation of cytotoxicity studies. Details of the cell culture techniques may be found in Chapter 2, Section 2.2.5.

4.3.5 Culture media preparation

The culture media composed of (Dulbecco's Modified Eagle's Medium (DMEM) + 10% foetal calf serum media + 1% antibiotic/ antimycotic (100U/mL penicillin G sodium, 100µg/mL streptomycin sulphate and 0.25µg/mL amphotericin B) and 1% L-glutamine). All prepared according to previously detailed method in Chapter 2, Section 2.2.5.3.

4.3.6 Cell sub culturing and counting

To maintain appropriate cell density whilst working with HaCaTs, the cells were subcultured and counted when they reached 80% confluency. Counting and subculturing methods are detailed in Chapter 2, Section 2.2.5.5.

4.3.7 Cell Freezing and cell thawing

For cell storing and cell retrieval, a previous method is detailed in Chapter 2, Section 2.2.5.6.

4.3.8 Cell growth study

HaCaT growth behaviour and pattern was studied under the same laboratory conditions that were used in the all cell culture assays. Resulted growth curve will demonstrate the different stages of cell growth in accordance to the time. The experiment was carried out in duplicate, n=2. An overnight culture of HaCaT cells was detached from the flask using trypsin (trypsinisation) and a cell suspension with density of to 2×10^4 cells mL^{-1} was prepared using haemocytometer counting method. Cells were seeded in three 24-well plates by adding 1 mL of cell suspension in each well. The plates were incubated in mammalian culture incubator at 37°C, 5% CO_2/95% air for 6 dyes. Cell counting was firstly performed at 24 h, then twice daily counting was done for the following days. Cells media within the wells was first removes and washed twice with phosphate buffer saline (PBS). Trypsinization of the cells was performed by adding 100 µL of trypsin and incubated for 10 minutes. After that, in order to neutralize the trypsin, fresh culture media was added (1 mL) to each well and mixed well by pipetting up and down for several times. At each time point samples were transferred from the 24-well plates to labelled Eppendorfs and the 24-well plate was returned to the incubator for the following time points. Cell medium was changed with fresh medium every other day.

4.3.9 MTT assay

In the current work, cell viability test was evaluated by MTT assay. Overall each assay took ten days long, three day for preparation and 7 day exposure time. Firstly, four 96 well plates was seeded with 5×10^4 HaCaT cells/mL in each well and 100 µL total volume in culture media. Culture media used contains Dulbecco's Modified Eagle's Medium (DMEM), 1% antibiotic, 1% L-glutamine and 10% FCS. The four plates were labelled for day one, day three, day five and day seven of contact time. The cells were suspended in F-SCM and placed in a incubator at 37°C and 5% CO_2/95% air for 24 hours. The next day, the medium was replaced with serum-free medium and returned in the incubator for 24h and this step of serum starvation is done to synchronize the cells to

the same cell cycle. The following day the medium was replaced with test compounds accompanied with Dulbecco's Modified Eagle's Medium (DMEM), 1% antibiotic, 1% L-glutamine and 1% FCS. This medium was used for the rest of the experiment.

The MTT assay was performed on both PRE and $ZnSO_4$ alone and in combination. PRE concentrations ranged from 5-100 µg/mL, $ZnSO_4$ from 12.5-100 µM/mL. The total test samples examined by MTT assay in this work are listed in Table 1. After 24 hours of cell exposure to the test compound media, 25 µL of thiozoly blue tetrazolium bromide (MTT) reagent was added to each well of the 96 well plate labelled day one and returned to the incubator for four hours. After that time, the media and MTT dye was removed and 100 µL of dimethyl sulfoxide DMSO was added to each well in order to solubilise the formazan crystals, wrapped with clingfilm and incubated for 30 minutes at 37°C and 5% CO_2 /95% air. Next, the absorbance of each well was measured by plate reader at a wavelength of 570 nm (Sunrise^{TM}, Tecan Trading, Switzerland).

The cell's exposure time was set to 7 days. The MTT assay was performed on day one, three, five and seven of exposure time. The treatment media was replaced with fresh one every other day. Each test was done in triplicate. Percentage cell viability was calculated using the following formula were control well is refereed to well treat with buffer only:

$$\%\ \text{Cell viability} = \frac{\text{Mean absorbance of tretment media wells}}{\text{Mean absorbance in control wells}} \text{X } 100$$

PRE samples
5 µg/mL
10 µg/mL
20 µg/mL
50 µg/mL
100 µg/mL
PRE 5 µg/mL
$ZnSO_4$ samples
12.5 µM
25 µM
50 µM
100 µM
Combination samples
PRE 5µg/mL + $ZnSO_4$ 12.5µM
PRE 10µg/mL + $ZnSO_4$ 25µM
PRE 20µg/mL+ $ZnSO_4$ 25µM
PRE 50µg/mL + $ZnSO_4$ 50 µM
PRE 100µg/mL + $ZnSO_4$ 50 µM
PRE 100µg/mL+ $ZnSO_4$ 100 µM

Table 4-1Samples tested using the MTT assay.

Day 1	Seed the cells in 96 well plates (5 x 10^4 cells/mL), incubate at 37°C and 5% CO_2/95% air for 24 hours (medium 10% serum, 1% antibiotic/ antimycotic, 1% L-glutamine.
Day 2	Replace the medium with serum-free media +1% antibiotic /antimycotic, 1% L-glutamine and incubate for 24 hours.
Day 3	Replace the medium with medium containing treatment compounds +1% antibiotic /antimycotic, 1% L-glutamine and incubate for 24 hours.
Day 4	Add 25µL of MTT to day 1 plate and incubate for 4 hours then remove the media and MTT, add 100µL DMSO, wrap with cling film and incubate for 30 minutes. Read absorbance at 570nm.
Day 5	Replace the treatment medium with fresh one.
Day6	Add 25µL of MTT to day 3 plate and incubate for 4 hours then remove the media and MTT, add 100 µL DMSO wrap with cling film and incubate for 30 minutes. Read absorbance at 570 nm.
Day 7	Replace the treatment medium with fresh one.
Day 8	Add 25µL of MTT to day 5 plate and incubate for 4 hours then remove the media and MTT, add 100 µL DMSO wrap with cling film and incubate for 30 minutes. Read absorbance at 570nm.
Day 9	Replace the treatment medium with fresh one.
Day 10	Add 25 µL of MTT to day 7 plate and incubate for 4 hours then remove the medium and MTT, add 100µL DMSO, wrap with cling film and incubate for 30 minutes. Read absorbance at 570nm.

Table 4-2 Summary of MTT steps used with HaCaT cell line.

4.3.10 FACS analysis using (PI) propidium iodide and (FDA) fluorescein diacetate

The cytotoxicity of PRE ± $ZnSO_4$ was evaluated using PI/FDA double staining FACS method. The method used two dyes in which dual staining was applied: propidium iodide and fluorescein diacetate. At first 1 mL of HaCaT cell suspension were seeded

using 24 well plate with cell density of 5×10^4 cell per well. Culture media contained Dulbecco's Modified Eagle's Medium (DMEM), 1% Antibiotic, 1% L-glutamine and 10% FCS. Then 24 well plate was incubated over night in mammalian culture incubator at 37°C, 5% CO_2/95% air. After 24 h the culture media was replace with culture media containing tested groups of PRE ± $ZnSO_4$. There were nine groups to be investigated in this experiment, 6 groups of them are individual in range of concentrations and two combination samples. Number of control groups used in this method was four including: control group with non treated unstained cells for FACS calibration, control group with non treated cell but stain with FDA to calibrate the cell viability, non treated cell in which fixed with 200 µL of ethanol at first for a duration of ten minutes to cause cell death and then non-viable cell stained with PI for PI calibration and finally non-treated cells stained with both PI/FDA to calibrate both staining. An additional control group was added after calibration as one of the treatment media and it was a control group with phthalate buffer to determine the vehicle effect, stained with both dyes.

After 24 hours of treatment media exposure the supernatant of each well were collected in eppendorfs and centrifuged at 500 g for five minute and then supernatant were discarded. Each well was washed with PBS twice and then 200 µL trypsin/ EDTA was added for 10 min. Then 800 µL of culture media was added to each well to stop the trypsinaization process. Each well was collected in the corresponding Eppendorfs and then centrifugation at 500 g for another 5 minutes. After pellet formation, the supernatants were discarded then further wash of the cells with 1 mL of PBS and centrifuge at 500g for 5 minutes. Gentle shaking of each Eppendorf tube with hand was employed to loosen the formed pellet then addition of PI and FDA to each sample tube. The dual florescent dyes were as the following; 300 µL of 10 µg/mL FDA in 00.2% DMSO/PBS and 300 µL of 5 µg/mL PI of in PBS. Gentle mixing was applied and then the tubes were placed on ice prior to analysis by flow cytometry (FACSCalibur^{TM}, Becton Dickinson, Heidelberg, Germany) using the CELLQUEST^{TM} proGramme. Analysis took place within one hour after mixing with the dyes with an excitation

wavelength of 490 nm and an emission wavelength of 526 nm for FDA. PI excitation wave length at 535 nm and an emission at 617 nm for PI.

Calibration of the system was done using the four control previously detailed for calibration purposes. Separate channels were used for the examination of each dye: fluorescence on with FDA on FL1 channel and PI on FL3 channel; and data were collected in log mode. Linear mode collection was employed using Forward scatter FCS and side scatter SSD. To confirm that cell will be detected in the proper location, adjustment of the following: Voltage, "FACS threshold", was done in the machine. The number of events was set to be 20000. Each treatment media and controls were done in duplicate (n=2). The cell stained percentage with FDA/PI for tested PRE $\pm ZnSO_4$ was calculated using the following equation:

$$\text{FDA/PI stained cells \%} = \frac{100 \text{ x (FDA/PI Geo Mean in treatment wells)}}{\text{(FDA/PI Geo Mean in control wells)}}$$

4.3.11 Data analysis

In each test, data are expressed as means ± s.d., otherwise stated. Statistical analysis of HaCaT cytotoxicity was carried out using One way analysis of variance (ANOVA) with Tukey's post test to compare statistical significances between groups and or One way analysis of variance (ANOVA) with post-test Dunnett analysis to compare groups against the control. Results were expressed as significant and very significant with $p < 0.05$ and $p < 0.01$ respectively. The analysis was carried out using InStat for windows, version 7.0 (GraphPad Software Inc, San Diego, CA).

4.4 Results

4.4.1 Cell growth study

The cell growth cycle consists of four stages or phases: the first one is the lag phase where the cells normally grow in size rather than in the number. The second phase is the log or exponential phase, where cell division actually takes place and cells grow in number until a certain confluence is reached - normally from 80 to 100 % confluence. This phase is the typical phase for passaging cells (cell splitting) where cells are highly proliferative and in a viable state. Cell culture experiments typically take place in the log phase were 80% of cell confluence is reached. The log phase time period is highly dependent on the cell culture type and cell seeding density. After the log phase, the plateau or stationary phase starts and it starts with slowing down of the proliferation rate because of the overcrowded cell density natural progress of the cell cycle leads to the final stage which is cell death, where decline number of viable cells happened.

In the cell growth study, Figure 4-2 shows the curve of the log number of cell against the time in hours. The three stages of cell growth cycle were identified and labelled. The data obtained from the log phase were plotted in linear regression curve as shown in Figure 4-3. The lag phase was calculated by the linear regression equation and extrapolating of the linear regression line to reach the seeding density at the y-axis. The lag phase took approximately 25 hours and the log phase lasts nearly four days.

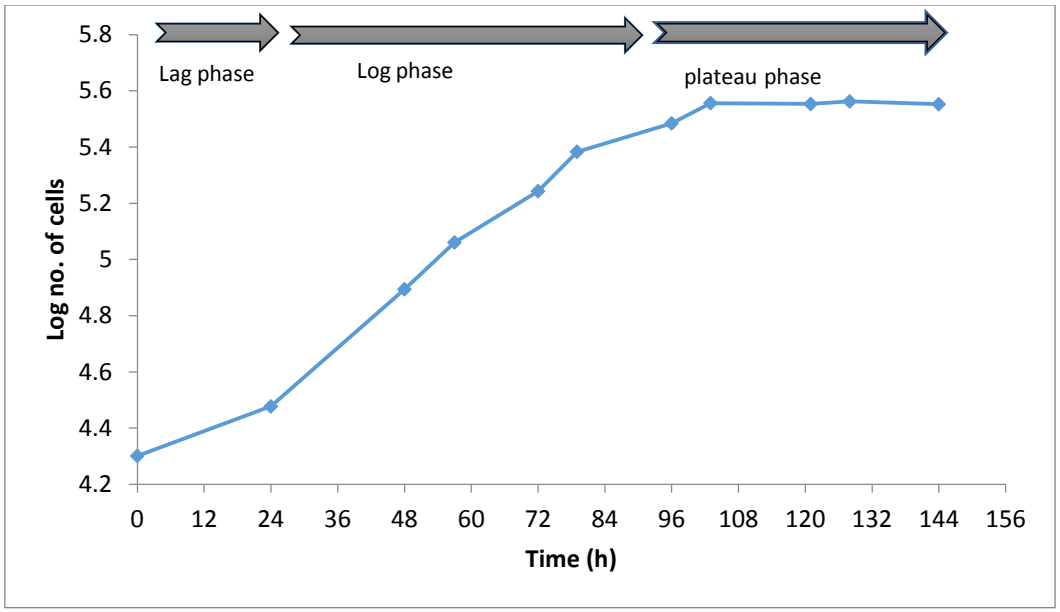


Figure 4-2 Cell growth curve of HaCaT cells against time in hours with lag, log and plateau stage presented. Representative of 2 determinations.

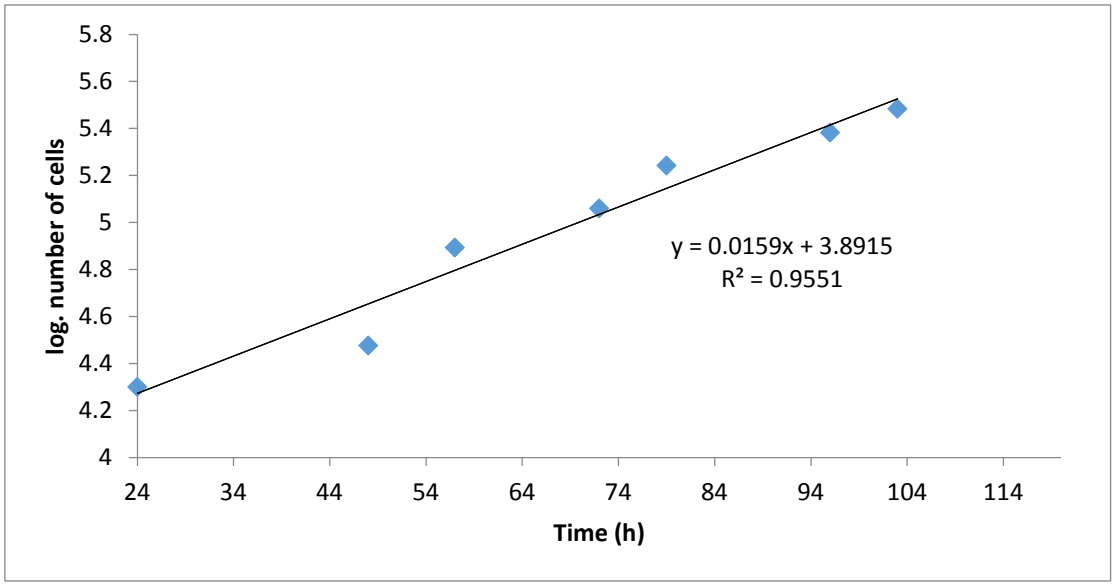


Figure 4-3 Linear regression of the log stage of HaCaT cell growth cycle.

4.4.2 MTT assay

Cytotoxicity analysis of HaCaT cell line was investigated for PRE at concentration range from 5 to 100 μg/mL and $ZnSO_4$ in concentration from 12.5 to 100 μM in individual and in combination group for 168 hours (7 days). The vehicle only treatment that contained phthalate buffer at pH4.5 was included to serve as control.

At first, a short preliminary assay for 2 days (data not shown) showed less than 50% inhibition in the HaCaT cell proliferation. Based on the observation of these results, a longer cytotoxicity study was designed to show the effect of PRE over 7 days (168 h). After short exposure of HaCaT to PRE to 24 hours, two concentration of PRE (5 μg/mL and 10 μg/mL), showed significant increases of 12% and 9% in cell viability compared to the control group ($p < 0.05$). On the other hand, no positive or negative effect was observed in PRE at 20, 50 and 100 μg/mL when compared to the control group ($p > 0.05$) at the first 24 h.

Longer exposure time to PRE treatment showed no cytotoxic effect in HaCaTs proliferation and cell viability at 72 h in PRE at 5 and 10 μg/mL compared to control with cell viability of 103 % and 102 % respectively. Contrarily, higher PRE concentrations of 20, 50 and 100 μg/mL showed observed inhibition in cell viability to give 82.44 %, 67.98% and 57.36 % respectively. PRE showed dose dependent anti-proliferative activity on HaCaT cell as shown in Figure 4-4 after 24hrs contact time.

The IC_{50} value (concentration of PRE required to inhibit 50 % of HaCaT cell growth against control) couldn't be calculated at the first 24 hr since all PRE concentration gave more than 90% cell viability. The IC_{50} value determined (Table 4-3) calculated using best fit line, plotting the % cell viability against log concentration of PRE at 72, 124 and 168 hours, the calculation of IC_{50} at all represented graphs was with linear regression $R^2 > 0.9$. For better comparison PRE % cell viability compared to control untreated group are summarised over the tested time point in Figure 4-5.

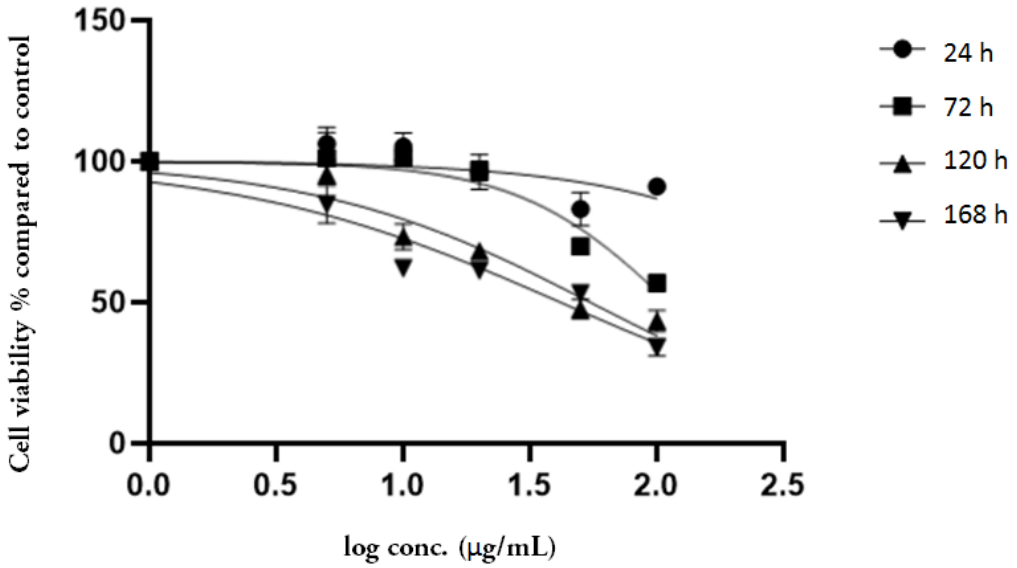


Figure 4-4 Dose dependent cytotoxicity curve of PRE in concentrations (5,10,20,50,100 mg/mL) over 24h, 72h, 124h, 168h. Data are expressed as three separated replicates with 6 experimental replicates each time (n=3±SD)

	72 h	**120 h**	**168 h**
PRE IC50 (µg/mL)	111.9 µg/mL	54.99 µg/mL	41.92 µg/mL
95% confidance interval	96.54 to 136.8	45.43 to 68.56	33.45 to 54.18
R^2 value	0.9118	0.9234	0.9039

Table 4-3 The IC_{50} of PRE obtained from the MTT assay after 72, 120 and 168 h treatments. The regression coefficient and the 95% confident interval also showed.

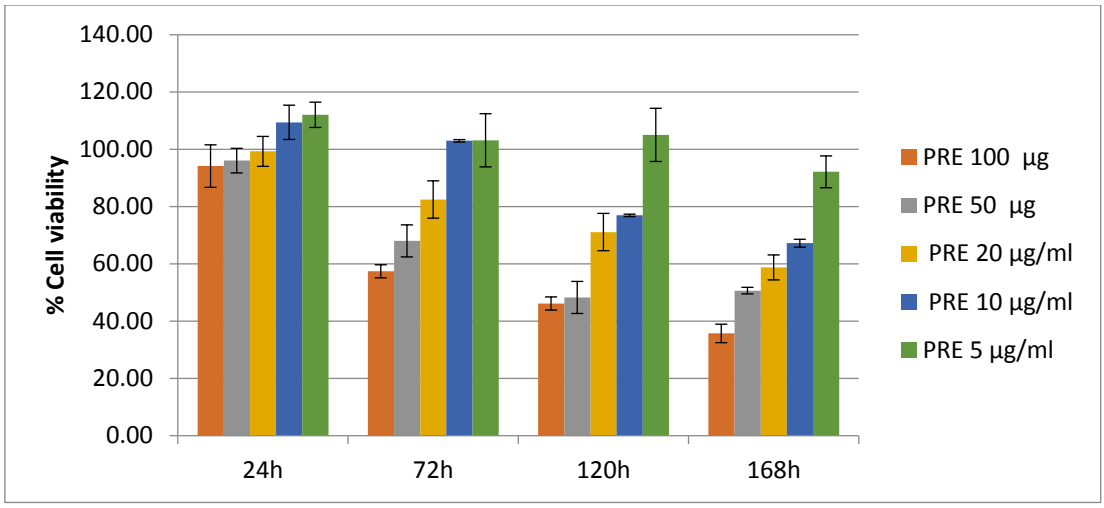


Figure 4-5 MTT assay results shows percentage of HaCaT cell viability compared to control after treatment with different PRE concentration (5 µg/mL, 10 µg/mL, 20µg/mL, 50µg/mL and 100 µg/mL) over 168. Data are expressed as mean of three separate replicates with 6 experimental replicates each time (n=3±SD).

Next all samples contained $ZnSO_4$ in concentration range 12.5 -100 µM didn't show any significant change to the cell proliferation at the first 24 hour when compared to the control. Longer exposure to $ZnSO_4$ showed enhanced cell proliferation (>12%) in HaCaT cells compared to the control group ($p < 0.05$) at 120 h with the lowest two concentrations of $ZnSO_4$ at 25 and at12.5 µM.

The concentration range of $ZnSO_4$ that have been investigated were very well tolerated by HaCaT cell line and up to 7 days of treatment, no antiproliferative activity was observed over the concentration range 12.5 -100 µM as shown in Figure 4-6. The IC_{50} $ZnSO_4$ was more than 100 µM (the maximum concentration tested using MTT).

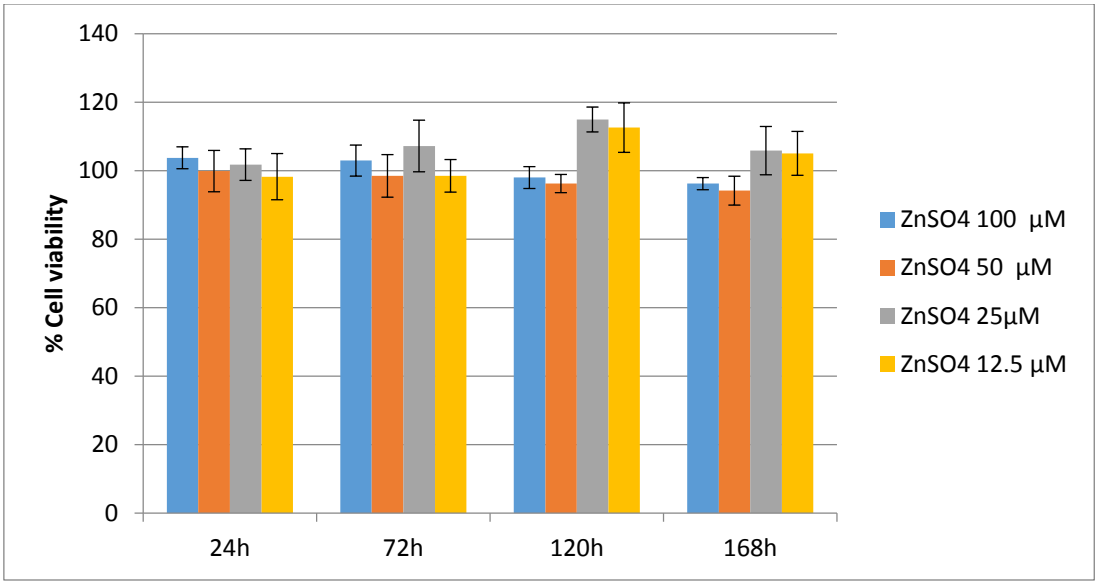


Figure 4-6 MTT assay results showing percentage of HaCaT cell viability compared to control after treatment with different $ZnSO_4$ concentration (12.5µM, 25µM, 50 µM, 100µM) over 168. Data are expressed as mean of three separate replicates with 6 experimental replicates each time (n=3±SD).

Turning now to the results for the combination samples listed in Table 4-1 which, three combinations that contained (PRE 100 µg/mL + $ZnSO_4$ 100 µM, PRE 100 µg/mL + ZnSO4 50µM, PRE 50 µg/mL + $ZnSO_4$ 50µM) showed significant inhibition in the proliferation at the first 24 h with cell viability of 77.55%, 86.73 and 82.81 % respectively. While the combinations that contained PRE in the range (10, 20, 5µg/mL) with $ZnSO_4$ 25 µM, 12.5 µM) showed more than 90 % survival rate of HaCats cell at the first 24 hours with.

For longer duration of exposure, up to 168h, analysis showed only one combination that contained PRE 5 µg/mL and $ZnSO_4$ 12.5µM showed no cytotoxicity effect on HaCaT cell line proliferation up to 7 days. Intended application would be related to the first 24 cytotoxicity levels of the PRE and combination results.

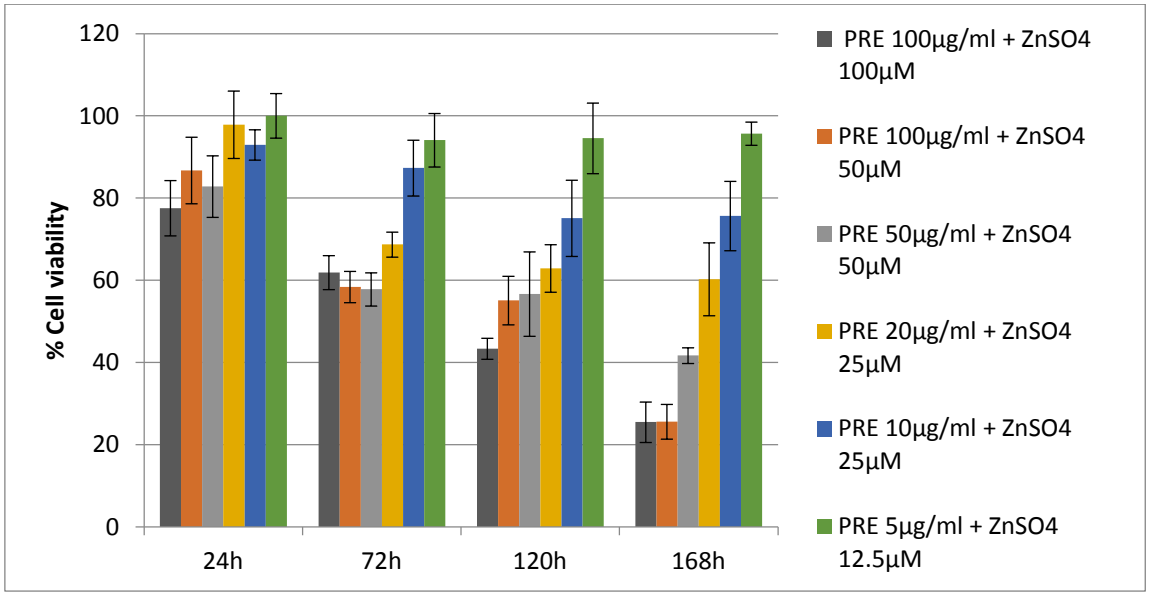


Figure 4-7 MTT assay results shows percentage of HaCaT cell viability compared to control after treatment with PRE+$ZnSO_4$ combination at different concentrations. Data are expressed as mean of three separate replicates with 6 experimental replicates each time (n=3±SD).

4.4.3 FACS analysis

For further investigation of the cytotoxic effects of PRE ±$ZnSO_4$ dual staining using FACS analysis was employed. In order to select desired population of HaCaTs cells. At first, 20000 cells from the non-treated non-stained control were aspirated to flow cytometer. Then plotting of forward scatter (FSC) versus side scatter (SSC) and make surrounding gate (P1) (Figure 4-8) to select most desired HaCaT cell population. Then the selected population from P1 gate will be used for further gating of PI and FDA stained cells. Then, plotting of PI-h against FDA-h was carried out, where cells stained with PI were positioned on the upper left (UL) of the plot (Figure 4-9) and the stained cells with FDA were positioned on the lower right (LR) right of the plot.

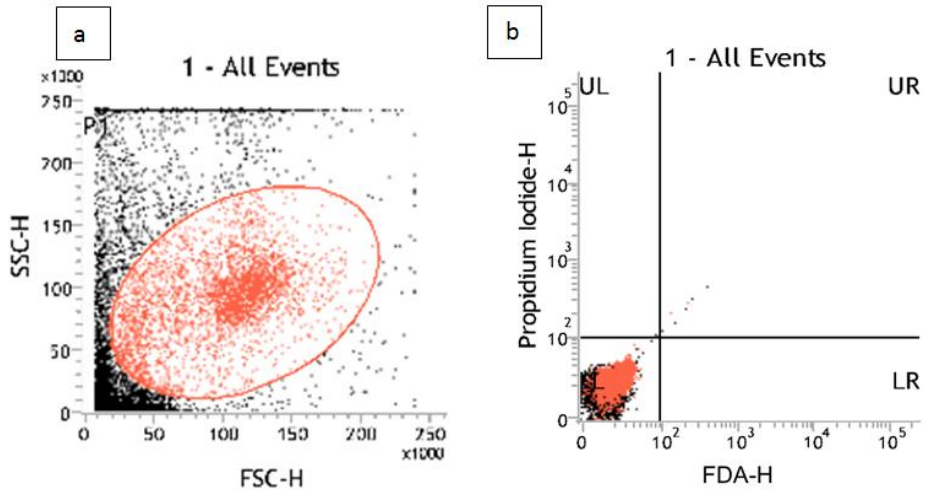


Figure 4-8 cells of intrest selection and gate setting in dot plot, using non treated and non stained cell control which appear as red dot population (P1) as shown in Figure a) dot plot of FSC vs. SSC, b) PI-H dot plot position when plotted against FDA-H in which non stained cells shows at the corner left of the plot.

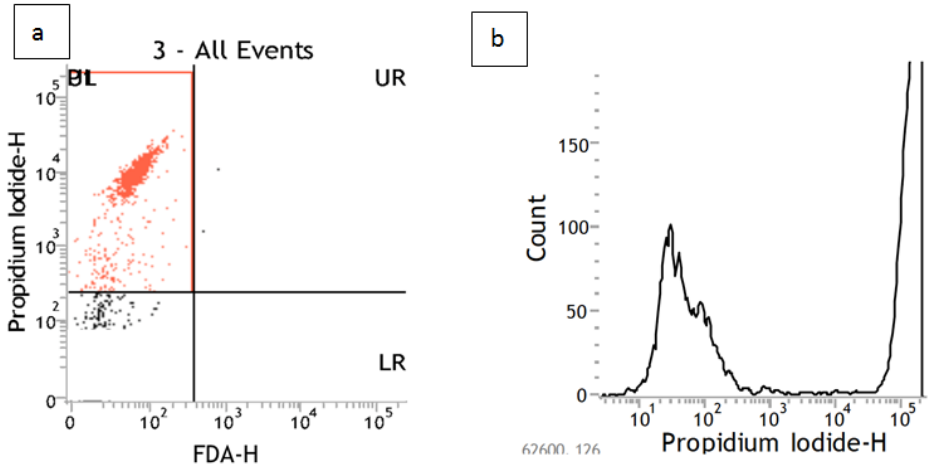


Figure 4-9 FACS analysis setting the region of PI stained cells. The PI dye will be taken up by dead cells and will accumulate with show of red florescence colour as shown in a) the dead cell florescent in the upper left corner b) PI-H histoGram.

Controls that composed of stained non-treated cells was applied to differentiate between PI stained red cells on the UL of the plot form FDA stained cell which appears on the bottom LR of the plot.

The cell cytotoxicity was analysed by collecting dead and viable cell from the culture medium , where dead will be floating in the supernatant and viable will be attached to the well . Then the tested samples will be stained with PI, and the data are presented as the percentage of PI stained cells (dead cells) in each sample against non-treated control stained with PI was calculated as % of PI stained cells compared to control.

At the flow cytometer the PI-H plot against FDA-H will show red fluorescent which represent PI stained dead HaCaTs cells on UL because the PI dye will be taken up by nonviable cell and accumulate in the nuclei. While green fluorescence will be showing on the LR of the plot in which represent FDH stained that has been taken by viable HaCaT cells.

PRE IC50 was calculated at 72 h using MTT assay to be (96.5 - 136.8 μg/mL) and when PRE 100 μg/mL was investigated using FCAS analysis, it showed similar cytotoxicity to HaCaTs cell using dual staining technique.

Figure 4-10 showed an increase in the % PI stained cell at PRE 100 μg/mL in compare to control ($p < 0.05$). In the same manner, as MTT results showed no toxicity of $ZnSO_4$ at 50 and 100 μM, FACS results didn't show significant effect on HaCaT proliferation with same concentration of $ZnSO_4$. Also two combinations were investigated using non-toxic concentrations that has been revealed by MTT (PRE 5 μg/mL and 10 μg/mL) and $ZnSO_4$ 50 μM and 100 μM) and same results were established by FACS, i.e. that no toxicity was observed at these two combinations. Altogether, combining non-toxic concentrations of PRE and $ZnSO_4$, results show the same non-toxic effects on HaCaT cell proliferation.

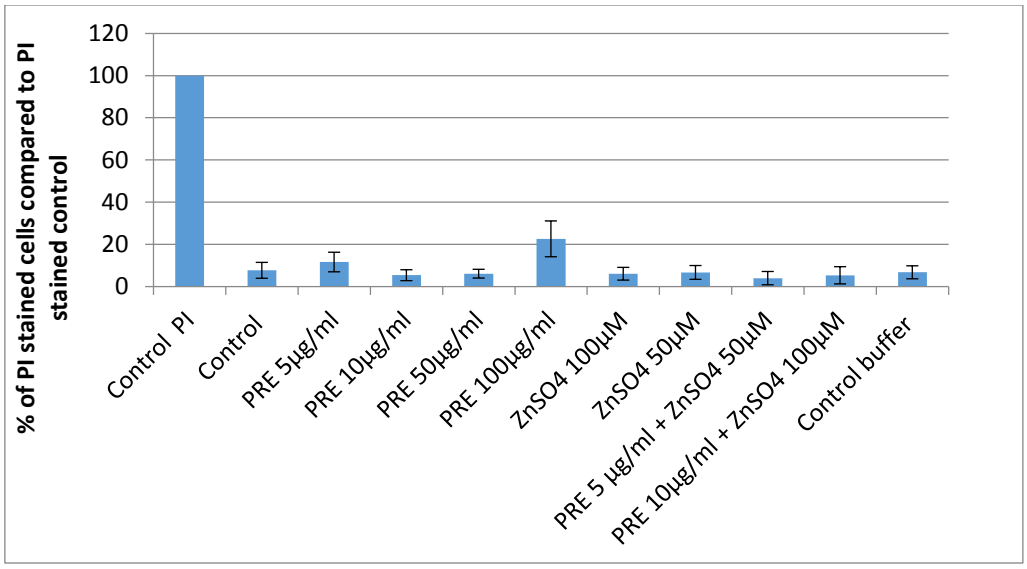


Figure 4-10 FACS analysis results using dual staining of HaCaT cells with PI and FDA after 24h incubation with PRE ± $ZnSO_4$. Data are expressed as percentage of PI stained cells to PI stained control of three separate replicates with two experimental replicates (n=2).

4.5 Discussion

In this chapter, the application of the treatment media meant to be as antiseptic for the wound, the short-term exposure to treatment media is the main interest in this Section. However, the long-term exposure was conducted to investigate other application possibilities of the treatment media using PRE and $ZnSO_4$. The PRE range from 5 -100 µg/mL showed little or no toxcicity effect on HaCaT cell line up to 24 h >94% as revealed by MTT test. At lower concentrations (5 µg/mL and 10 µg/mL), PRE showed significant enhanced in proliferation of HaCaT cell line. Since short exposure showed low toxicity we needed to increase the exposure time up to 7 days exposure. Longer term of exposure to the treatment media showed dose dependent cytotoxicity effect of PRE. On the other hand, PRE at higher concentration (≥100 µg/mL), decreased cell

viability by as shown by FACS analysis while no toxicity was observed at 50 μg/mL and lower concentration at 24 hours.

Several studies have investigated the cytotoxicity effect of pomegranate polyphenols obtained from either juice, leaves, seeds or other extracts. Different pomegranate extracts showed differences in the cytotoxicity range, depending on cell line tested and method used. Our results showed that PRE ≥100 μg/mL was cytotoxic to HaCaT cells and this results broadly supports the work of previous study in this area linking In vitro antiproliferative effect of pomegranate juice on human oral, and prostate tumor cell line (Seeram et al. 2005). This study recorded inhibition of cell viability by 30 to 100% when treated with 'pomegranate juice' at concentration range from 12.5 μg/mL to 100 μg/mL on cancer cell line for 48 h. The same study highlighted that the potent effect of total pomegranate juice showed antiproliferative and antioxidants in higher manner than separated polyphenols obtained from pomegranate when tested alone such as isolated punicalagin, ellagic acid and total polyphenol tannins TPT. The study concluded that such activity could be attributed to the synergistic and or additive effect of multiple bioactive compounds acting together with total pomegranate extract (Seeram et al. 2005). Pomegranate has been reported with selective toxicity to give >50% inhibition in proliferation of cancer colon cell at PRE 250 μg/mL with preserved >55% of normal colon cell viability using the same concentration of PREand exposure time of 72 hours (Sukri et al. 2019).

Houston (2017) studied the cytotoxicity effect of $ZnSO_4$ and PRE alone and on combination on Vero cell line (green monkey kidney epithelial), and he found that PRE from 100 μg /mL didn't show any toxicity, up to 72h incubation time. Also this study showed no toxic effect on Vero cell line when treated with $ZnSO_4$ up to 0.1 M concentration. The short time cytotoxicity results of our study up to 24 hours are consistent with Houston work; however the long term exposure for the higher PRE concentration showed significant cytotoxicity on HaCaT cell line. The different types of cell line could be the reason why keratinocytes showed more cytotoxic effect. On the

same manner, recent study showed dose dependant cytotoxicity effect of pomegranate leaves extract (PLE), concentration < 50 μg/mL was not toxic up to 24 hours contact time. In that study, PLE was investigated in concentration range from 6.25 and up to 50 μg/mL. After 24 hours, PLE treated samples showed no or little toxicity on three different cell line: African monkey kidney cell line (Vero), human embryonic cell line (HEK 293) and human normal liver cell line (LO2) (Li et al. 2016). The higher PLE in concentration ≥ 100 μg/mL, showed cell death and cytoxicity effect was documented. The mechanism of action at which high dose of pomegranate rind extract induced cytotoxicity has not been fully revealed yet. Li et al. (2016) found that pomegranate leave extract decreased mitochondrial membrane potential and thus induce apoptosis in cell via mitochondria- mediated apoptic pathway and cell cycle arrest.

In our results, the cytotoxicity study revealed that HaCaT cells tolerated $ZnSO_4$ at various concentrations (12.5, 20, 50 and 100 μM) and no toxic effect was observed during 7 days exposure time. $ZnSO_4$ at 25 and 12.5 μM showed significant increase ($p < 0.05$) in proliferation of HaCaT viability by more than 12% when compared to control group at 120 h. When comparing the individual results of our study, $ZnSO_4$ individual samples are in line with a previous study that showed $ZnSO_4$ 14 μM showed better proliferation of HaCaT cells when compared to untreated keratinocytes cells (Deter et al. 2003) which was an *in-vitro* study to investigate two different salts of zinc and its effect on the proliferative activity and physiological function on human keratinocytes. The concentration of zinc was set to be 14 μM of zinc histidine and $ZnSO_4$ which was selected according to the average serum zinc concentration. Proliferation activity of HaCaT cells increased in the zinc-containing groups in comparison to the control group after nine days of incubation. Further investigation, reported no cytotoxic effect was observed in zinc containing groups when tested by MTT and LDH assay. A previous study reported by Ågren et al. (1991) revealed that concentrations of $ZnSO_4$ up to 100μM showed no negative effect on the cell proliferation compared to control.

For the PRE/Zn combination, our results showed that using combination of non-toxic level of PRE with non-toxic level of $ZnSO_4$ resulted in non-toxic combined effect on HaCaT cell line. Enhanced proliferation observed with PRE 5 μg/mL and $ZnSO_4$ 12.5 μM in individual samples was maintained within the first 24 hours.

As shown in Chapter 3, antibacterial synergy of PRE/Zn with MIC levels (62.5 μg/mL + 1600 μM) against MRSA and (31.25 μg/mL + 400 μM) against *S. epidermidis.* At these levels, no significant toxicity was observed up to 24h. No toxic effect was recorded of Zn(II) up to 100μM over 7 days exposure time. Previous data was reported by Houston (2011) also showed that $ZnSO_4$ up to 0.1 M didn't show significant toxicity on Vero epithelial cell viability up to 72h.

4.6 Conclusion

PRE with and without $ZnSO_4$ was investigated for cytotoxicity on HaCaT cell line using 2 methods. The results showed:

- MTT testing showed that PRE at 5 and up to 100 μg/mL showed no toxic effect and enhanced cell growth was observed with PRE 5 and 10 μg/mL at 24h exposure time.

- Longer exposure, up to 7 days, showed concentration-dependant cytotoxicity at PRE > 10 μg/mL.

- FACS analysis showed that PRE at 50 μg/mL and lower concentrations, showed no significant inhibition in cell viability ($p < 0.05$), while PRE ≥100 μg/mL did show some cytotoxic effects.

- Zn(II) in the concentration range 12.5-100 μM showed no cytotoxcity by MTT and FACS analysis up to 24 h. Longer exposure to $ZnSO_4$ showed enhanced cell

proliferation (>12%) in HaCaT cells compared to the control group ($p < 0.05$) at 120 h with the $ZnSO_4$ at 25 and 12.5 µM.

- When PRE in the range (10, 20 and 5 µg/mL) was added to $ZnSO_4$ (25µM, 12.5 µM), the survival level was > 90% over the first 24 hours.

These results are highly encouraging and support the development of a formulation of PRE and Zn(II) to be used as antiseptic application for chronic or acute wounds. In the next Chapter, *in vitro* effects of PRE/Zn are investigated in the scratch wound model.

Chapter 5: Effect of PRE and Zn(II) on cell migration and wound closure in HaCaT cell cultures

5.1 Introduction

Skin infections can involve unbroken skin (eg cellulitis), but are more typically associated with disruption or trauma to the epidermis and dermis (e.g. impetigo and MRSA infections) – i.e. a wound. For any new product intended for application to an open wound, it is important to understand how the formulation may modulate the skin healing process. In general a retarding effect would be considered a negative result, whereas an accelerative could be viewed as beneficial to the development of a topical product. In this chapter we explored the potential for PRE and Zn(II) formulations to modulate the rate of wound healing using cultured HaCaT epidermal cells.

5.1.1 Wound healing processes

After skin injuries or postoperative skin incisions the healing process is a progression of four overlapping phases: homeostasis (pre-inflammation), inflammation, proliferation and remodelling (maturation) (Figure 5-1) as detailed in Chapter 1, Section 1.9.1.

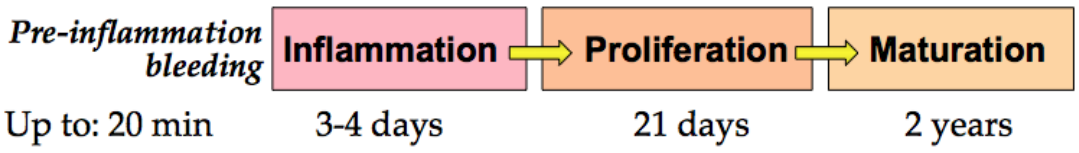


Figure 5-1 Normal progression and timescales of wound healing

Wound healing is a very complex series of processes and requires the involvement of many growth factors and cytokines at each stage. In this chapter keratinocyte cells were used as an *in vitro* model for scratch wound test and cell migration and epithelialization was also investigated. Epithelialization, or re-epithelialization, is a term referring to the process of epithelial cell covering the damaged surface or wounded area. The success of regulating epithelialization process is important for complete and timely wound healing. Epithelialization is a process that is initiated at the early stage of wound injury and is

highly dependent on complicated signal integration that synchronises the cellular processes, regulated by chemokines, cytokines, growth factors integrins, keratins, matrix metalloproteinases (MMPs) (Pastar et al. 2014). First cell migration of epidermis keratinocyte cell in which the cells at the edges of wound will migrate to the inner side via freshly formed matrix. First, keratinocyte migration process is stimulated and regulated by epidermal growth factors HB-EGF, EFG and TGF-α that act as direct stimulants for the migration process. The movement of keratinocytes to the newly formed extracellular matrix ECM is also promoted by metalloproteinases MMPs enzyme. Regulation between the MMPs enzymes and tissue inhibitor metalloproteinases (TIMPs) ratio must be achieved through epithelialization process since imbalance between those enzymes could lead to chronic wound formation. The second important cellular activity involved in epithelialization is keratinocyte proliferation. The proliferation of keratinocytes is directly stimulated by same growth factors mentioned earlier HB-EGF, EGF and TGF-α in addition to other insulin-like growth factors (IGF)-1 and other factors including components of ECM and integrins. MMPs enzymes that present in the ECM can release the growth factor from wound site matrix by the proteolytic activity. In addition MMPs can convert inactive forms of growth factors to the active form such as (IGF-1). The growth factor receptor pathway can be activated by the action of ECM and integrins together to give more functional activity of these growth factors. The stimulation of keratinocyte proliferation by combined effect of MMPs, ECM, integrins and growth factors will continue till fully epithelial layer is formed and the activated proliferation state stimulant will be decrease and the pathway will return to the normal differentiation state (Leigh 1995; Pastar et al. 2014; Barrientos et al, 2008; Harper et al. 2014; Frykberg and Banks, 2015).

5.1.2 Wounds and chronic wounds

Chronic wounds can be defined as those that fail to progress from the proliferation phase to the remodelling phase in a timely manner. Overall, acute or chronic wounds can be a huge economical burden on that affects the health system since it has been estimated that

wound treatment is responsible for 4% of the overall health system costs (Wilhelm et al. 2017). Wound infection is one of the important factors that can lead to the impaired and non-healing wounds alongside with external factors, such as sustained pressure, temperature, and moisture, also play important roles in determining rate of wound healing (Han and Ceilley 2017). Chronic wound can be classified by the processes that give rise to them, including diabetic ulcers, pressure ulcer and venous ulcers. The venous ulcers usually happened in legs where lower blood circulation is involved especially in elderly people. Pressure ulcers usually occur in bedridden patients where he wound site is affected by the physical pressure in which overcomes the circulation pressure. Diabetic ulcers happened to patient with diabetes mellitus, where can be caused from injuries or even small scratch can lead to chronic wound at the site. The chronic wounds in general share many of the following characteristics including, extended inflammation, frequent infection and bacterial biofilm formation on service that is resistance to the ordinal antibiotics (Frykberg and Banks 2015).

Figure 5-2 Chronic wounds can be considered as stuck in-between the inflammatory and proliferative stages of healing.

5.1.3 Infected chronic wounds

Many wounds have population of microorganisms – often these reside harmlessly as colonies within the wound bed and this referred as colonisation stage. Usually the colonisation burden of bacterial is $< 10^5$ CFU/mL. However, if the microorganisms invade the surrounding tissue, in addition to other factors, such as decreased vascular supply, intrinsic bacterial virulence (including *Staphylococcus aureus*), and host immune factors, a critical colonisation can appear and topical infection occurs (Siddiqui and Bernstein 2010). Clinical spectrum of bacterially affect on wounds, from colonisation to

infection, was previously detailed in chapter 1, Section 1.9.3. Infection of wounds is known to retard the healing process.

Generally, there has been the suggestion that wound chronicity starts with a persistent bacterial level in tissue, in particular the production of endotoxins and competing with normal healing cells on the nutrition and oxygen resourced. According to Konturek et al. (2001) and Power et al. (2001) this results in the protracted elevation of cytokines that are pro-inflammatory such as tumour necrosis growth factor-α and interleukin-1). Robson et al. (1990) assert that this condition subsequently leads to an augmented amount of matrix MMPs, together with a decreased amount of their inhibitors of tissue TIMPS, and a reduction in the growth factor production (Tarnuzzer and Schultz 1996). A normal element of the process of wound healing is inflammation; however the process of repair is prolonged if there is an excessive inflammatory response (Ladwig et al. 2002; Bucknall, 1980). Wounds infected with antibiotic resistant bacteria pose a growing threat, in particular ciprofloxacin-resistant *Pseudomonas aeruginosa* and Methicillin-resistant *Staphylococcus aureus* MRSA.

5.1.4 Modulation of wound healing time

Many factors are involved in determining the time it takes for a wound to heal. Adverse effects and delayed wound healing can happen if the patient suffers from diabetes, renal failure, immunodeficiency, malnutrition and infection. During the wound healing, regulation of growth factors, cytokines and MMPs and other cellular components are essential for successful wound to heal. Wound healing time could be severely altered when one or more of these components is disrupted (Thiruvoth 2015). Impact of bacterial infection on different stages of wound healing process including; inflammation, formation of tissue granulation, production of collagen and epithelialization process has been previously detailed in Section 1.9.3.1.

5.1.5 Wound healing models

Wound healing models can be classified into *in silico, in vitro, ex-vivo* and *in vivo*. The in *silico* model can be used as a screening tool to predict the effect of a drug on a cell and help in planning of your experiment. The lack of biophysical characteristic is the main disadvantage of this model although when used with other models can give a better experimental prediction of wound healing (Ud-Din and Bayat, 2017; Sami et al. 2019). Secondly in vitro models can be either single layer cell culture or co-cultured cell culture. Monolayer cell culture involve seeding of human epidermal keratinocytes on culture plates and mechanical scratch is introduced to mimic injury of wound and then study cell migration and wound retraction in the presence of treatment substances (Liang et al. 2007).

On the other hand more sophisticated *in vitro* model uses both human epidermal keratinocytes and fibroblasts in a system called a 'Boyden chamber'. In this system two compartment of the chamber are connected by filter membrane with known pore size. In cylindrical chamber the cells are seeded on the top part in serum free media and impeded for a certain period of time in a cell culture plates on the other side that contain serum and tested solution. Cell migration count or cell invasion towards the bottom compartment can be measured by removal of the filter membrane and consequent cell staining and counting method (Sami et al. 2019).

Ex vivo model is well-established method where full thickness skin membrane is used to study wound healing and inflammation process in presence of treatment substances. It has the advantages of providing 3D structure that is lack in the *in vitro* models. This model provides more of an understanding of inter- and intracellular interaction between cells and other biochemical and physical environment where wound healing actually takes place (Ud-Din and Bayat 2017).

Finally *in vivo* models can be either animal or human. Several types of animal wounds have been studied e.g. incisional, excisional, burn and full thickness wound model. In human wounds persistence chronic wound or in volunteered different types of acute wound can be studied e.g. blister model, tape stripping, abrasive model and full thickness wound model. Human model is commonly comes subsequently after in vitro models studies. Human models has the importance of its direct relevant when studying of new investigational treatment (Ud-Din and Bayat 2017; Wilhelm et al. 2017).

5.1.6 Scratch testing model of wound healing

The basic steps involve culturing a monolayer of cells appropriate to the systems being modelled for example for skin wounds, epithelial cells and fibroblasts. A wound is then created in the monolayer using a "scratch" in which the two sides of the wound are completely separated. Images are then captured at the beginning and at regular intervals during cell migration until the scratch is no longer visible, or “healed”, in the presence and absence (control) of test compounds which are introduced at the start of the migration phase (Liang et al. 2007). The images and then used to quantify and visualise the migration rate of the cells (Grada et al. 2017). The technique has been used to investigate the effect on rate of wound healing of a wide range of test substances, where an accelerative effect is noted by a reduced rate relative to control, whereas a deleterious and potentially toxic effected is noted by a reduced rate of closure (Hardwicke et al. 2010; Moses et al. 2014; D’Agostino et al. 2015)

In this work we used HaCaT keratinocytes – this is a spontaneously transformed aneuploid immortal keratinocyte cell line derived from human skin. It is widely used in scientific research because of their high capacity to differentiate and proliferate in vitro, compared to primary cells and thus have a long culture lifespan (Boukamp et al. 1988).

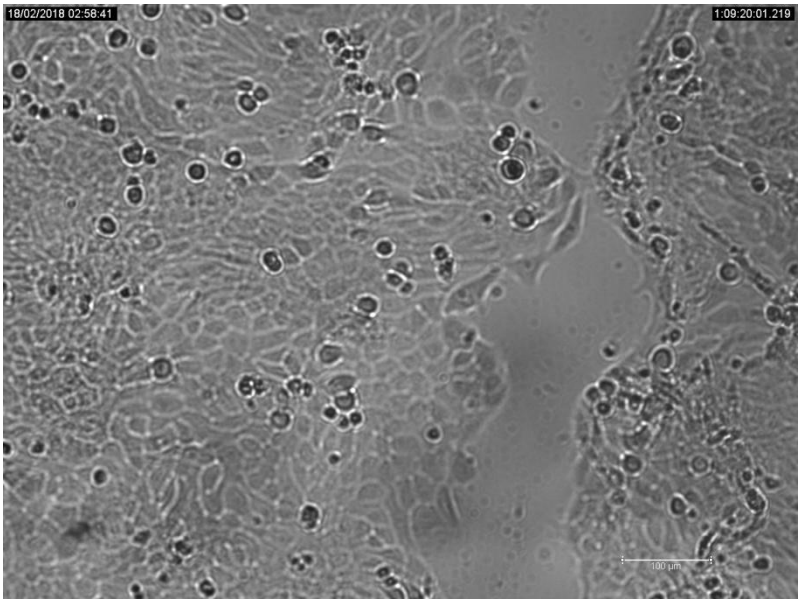


Figure 5-3 HaCaT cell line taken by Leica TCS SP5 Confocal Microscope.

5.2 Objective and aims

In this chapter, the objective was to investigate the potential effects of administering PRE/Zn to a wound, using the scratch test wound closure model. The aims were:

- To study the effect of PRE on wound healing activity of HaCaT cells.
- Study the effect of $ZnSO_4$ on wound healing activity of HaCaT cells.
- Study the effect of PRE/$ZnSO_4$ combination on wound healing activity of HaCaT cells.

5.3 Materials and methods

5.3.1 Materials

All materials used in this chapter are detailed in Chapter 2, Section 2.1.

5.3.2 Aseptic technique

Aseptic technique was employed at all stages in cell culture methods using safety cabinets using aseptic methods for routinely cleaning and autoclaving of material previously detailed in Chapter 2, Section 2.2.5.1.

5.3.3 Human adult, spontaneously immortalized, epidermal keratinocytes (HaCaT cells)

Human adult, spontaneously immortalized, epidermal keratinocytes (HaCaT cells) were provided by Dr. Ryan Moseley and Rachael Moses (Wound Biology Group, Tissue Engineering & Reparative Dentistry, School of Dentistry, Cardiff University). Details of the cell culture techniques may be found in Chapter 2, Section 2.2.5.

5.3.4 Culture media preparation

The culture media composed of (Dulbecco's Modified Eagle's Medium (DMEM) + 10% foetal calf serum media + 1% antibiotic/ antimycotic (100U/mL penicillin G sodium, 100 µg/mL streptomycin sulphate and 0.25 µg/mL amphotericin B) and 1% L-glutamine) was prepared according to the previously detailed method in Chapter 2, Section 2.2.5.3.

5.3.5 Cell sub culturing and counting

To maintain appropriate cell density through working with HaCaT, the cells were subculture and counted when they reached 80% confluence. Counting and subculturing method is detailed in Section 2.2.5.5.

5.3.6 Cell Freezing and cell thawing

For cell storing and cell retrieval a pervious detailed methods in Section 2.2.5.6.

5.3.7 Cell Migration Assay

In v*itro* scratch wound model was performed using HaCaT cells which were kindly donated by Dr. Ryan Moseley (School of Dentistry, Cardiff University). HaCaT cells monolayer was detached from the flask using trypsin (trypsinisation) and a cell suspension with density of to 7.5×10^4 cells mL^{-1} was prepared in cultured DMEM medium containing 10% FBS. One millilitre of cell suspension was transferred to each well of a 24 well tissue culture plate and incubated overnight at 37 ℃ and 5% CO_2 /95% air for 48 hours. At the 3rd day of experiment, cell confluence in each well was inspected under light microscope at x100 magnification. The media in each well were changed to serum free media and returned to the incubator for 24 hours. The following day, the serum free medium was removed from the 24 well plate and an artificial gap, or scratch, was made using a sterile 200 µL plastic pipette tip to produce a vertical straight line in the middle of each well. The tip was changed for each well. In order to remove any remaining debris, the cells were washed twice with PBS after wound was induced. Then, one millilitre of 1% serum cultured media that contained different concentration of PRE and $ZnSO_4$ were respectively added to each well of the 24 well plate firstly individually then in different combinations as presented in Table 5-1. For the control 1% FCM with phthalate buffer (pH 4.5) was used to represent untreated cells.

The next step was to incubate the 24 well plate under Time Lapse Microscopy, using the Leica TCS SP5 Confocal Microscope (Leica Microsystems (UK) Ltd., Buckinghamshire, UK). Cell migration was monitored and digital images were taking every 20 minutes for 48h time period. The cell migration in each well was monitored in 3 position and images were converted into video using LAS AF Lite (Leica Software, Version 4.0.11706, Leica Microsystems (UK) Ltd). Analysis of wound healing was performed using ImageJ Software (NIH Software, Version 1.49) and "MRI wound healing" tool for ImageJ was used to calculate the area of scratch that contains no cells at 0h, 24 and 48h l. Percentage closure of wound was calculated by comparing the images at 24 and 48 hours to the original image at 0h.

Sample name	**PRE conc.**	**$ZnSO_4$ conc.**
PRE 2.5 µg/mL	2.5 µg/mL	-
PRE 5 µg/mL	5 µg/mL	-
PRE 10 µg/mL	10 µg/mL	-
PRE 20 µg/mL	20 µg/mL	-
PRE 50 µg/mL	50 µg/mL	-
PRE 100 µg/mL	100 µg/mL	-
$ZnSO_4$ 25 µm	-	25 µm
$ZnSO_4$ 50 µM	-	50 µM
$ZnSO_4$ 100 µM	-	100 µM
PRE 5 µg + $ZnSO_4$ 25 µM	5 µg/mL	25 µM
PRE 5 µg + $ZnSO_4$ 50 µM	5 µg/mL	50 µM
PRE 5 µg + $ZnSO_4$ 100 µM	5 µg/mL	100 µM
PRE 10 µg + $ZnSO_4$ 25 µM	10 µg/mL	25 µM
PRE 10 µg + $ZnSO_4$ 50µM	10 µg/mL	50 µM
PRE 10 µg + $ZnSO_4$ 100 µM	10 µg/mL	100 µM

Table 5-1 Concentration range of PRE ± $ZnSO_4$ used in cell migration assay.

5.3.8 Data analysis

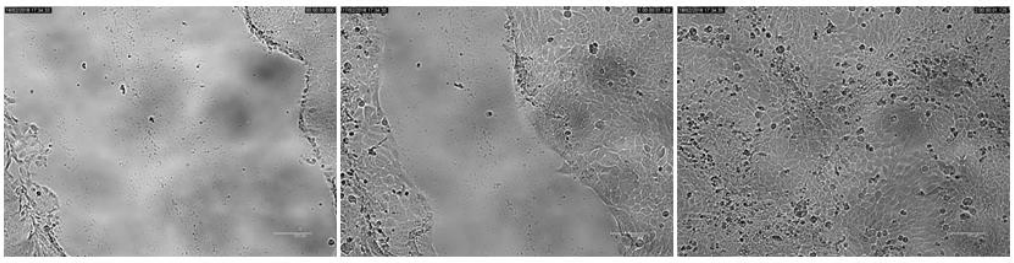
In each test, data are expressed as means ± SD, otherwise stated. Statistical analysis of HaCaT wound repopulation was carried out using One way analysis of variance (ANOVA) with Tukey‘s post test to compare statistical significances between groups and or One way analysis of variance (ANOVA) with post-test Dunnett analysis to compare groups against the control. Results were expressed as significant and very significant with $p < 0.05$ and $p < 0.01$ respectively. The analysis was carried out using InStat for windows, version 3.0 (GraphPad Software Inc, San Diego, CA).

5.4 Results

5.4.1 Cell migration assay

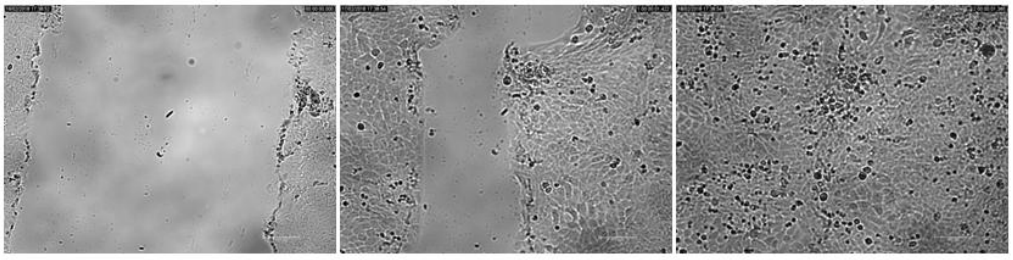
Wound closure is represented as percentage area closure at two time point 24 h and 48 h compared to the original wound area at time 0h. At first, PRE at the lower concentrations (2.5 μg/mL, 5 μg/mL) promoted cell migration in a similar rate as the control untreated cell at the first 24 hours with 49.6% and 44.1% respectively as shown in Figures 5-5 and 5-6 in comparison to the control in Figure 5-4, which showed 38.3% wound closure.

At 48h time period results showed complete wound closure with the both PRE 2.5 μg/mL and 5 μg/mL and control. The higher concentration of PRE at 10 μg/mL significantly promote the cell migration and percentage of healed area ($p < 0.05$) when compared to untreated control cells and showed 61.54 % healing at the first 24 h. At the 48 hours time point, PRE 10 μg/mL showed 98.5% wound closure with 10 μg/mL samples as shown in Figure 5-7. Statistical analysis of the results of cell migration at 48 h showed no significant difference ($p > 0.05$) in wound healing in comparison to the control with PRE at 2.5, 5 and 10 μg/mL since all gave complete wound closure at 48h.



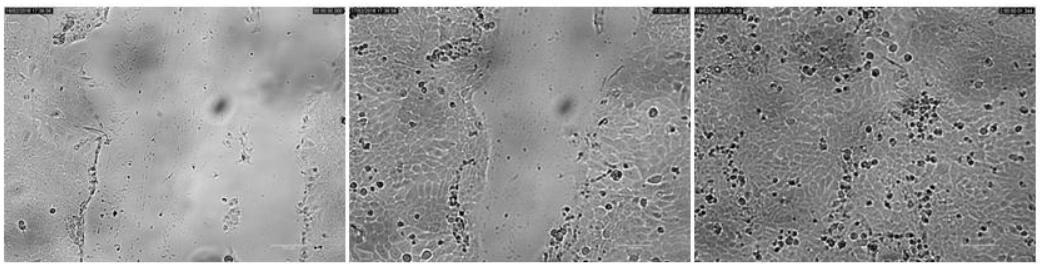
(a) (b) (c)

Figure 5-4 HaCaT Cell migration under Leica TCS SP5 Confocal Microscope in presence of 1% FCS media containing control buffer at (a) 0h, (b) 24h and (c) at 48h time point.



(a) (b) (c)

Figure 5-5 HaCaT Cell migration under Leica TCS SP5 Confocal Microscope in presence of 1% FCS media containing PRE 2.5 µg/mL at (a) 0h, (b) 24h and (c) at 48h time point.



(a) (b) (c)

Figure 5-6 HaCaT Cell migration under Leica TCS SP5 Confocal Microscope in presence of 1% FCS media containing PRE 5 µg/mL at (a) 0h, (b) 24h and (c) at 48h time point.

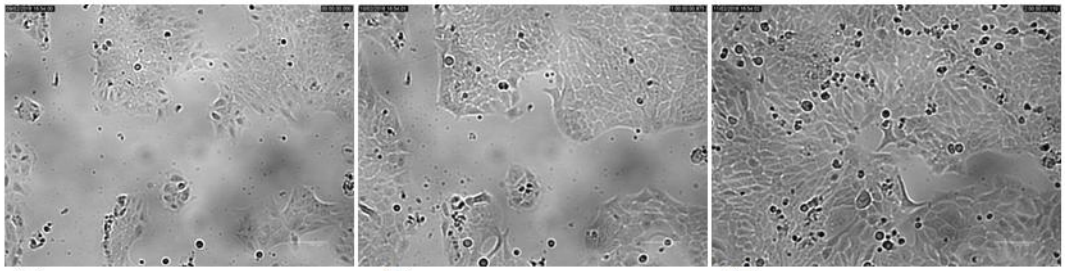


Figure 5-7 HaCaT Cell migration under Leica TCS SP5 Confocal Microscope in presence of 1% FCS media containing PRE 10 µg/mL at (a) 0h, (b) 24h and (c) at 48h time point.

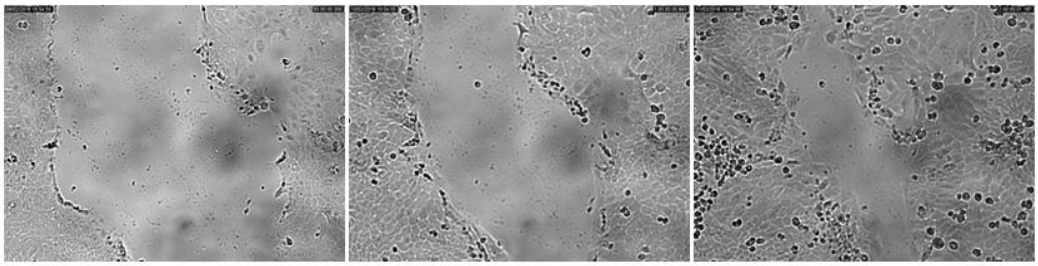
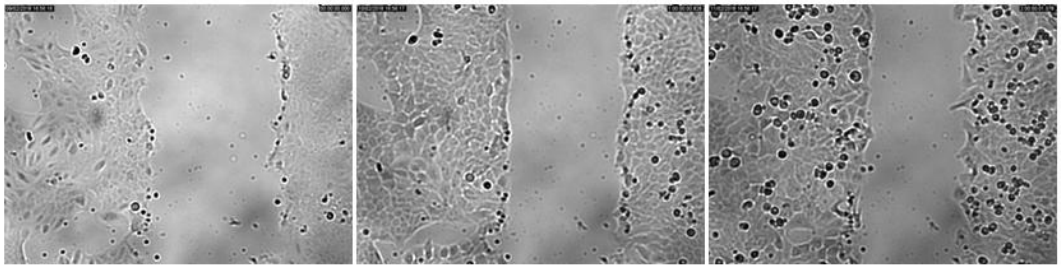


Figure 5-8 HaCaT Cell migration under Leica TCS SP5 Confocal Microscope in presence of 1% FCS media containing PRE 20 µg/mL at (a) 0h, (b) 24h and (c) at 48h time point.

The next set of samples that contained PRE at 20 µg/mL didn't show significant differences to the control at 24 h with 31.85% ($p > 0.05$) and adversely delayed the cell migration at 48 as shown in Figure 5-7 to give 70% closure as shown in Figure 5-8.

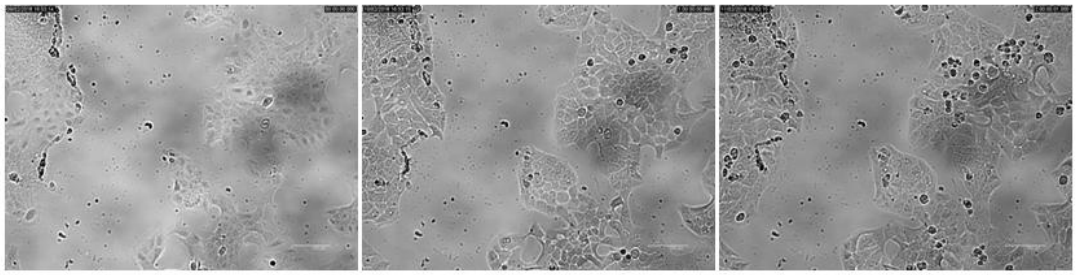
PRE in higher concentration (50 & 100 µg/mL) showed lower process of cell migration in contrast to the control and a significant decrease ($p < 0.01$) in the percentage of the scratch wound healing, with 23% of wound closure at 50 µg/mL and less than 5%

wound closure was seen at 100 µg/mL as shown at 48 hours, as shown in Figures 5-9 and 5-10.



(a) (b) (c)

Figure 5-9 HaCaT Cell migration under Leica TCS SP5 Confocal Microscope in presence of 1% FCS media containing PRE 50 µg/mL at (a) 0h, (b) 24h and (c) at 48h time point.



(a) (b) (c)

Figure 5-10HaCaT Cell migration under Leica TCS SP5 Confocal Microscope in presence of 1% FCS media containing PRE 100 µg/mL at (a) 0h, (b) 24h and (c) at 48h time point.

Figure 5-11 shows a summary of the concentration range of PRE that was used individually and percentage wound closure using *in vitro* scratch wound model. The % closure was calculated at 24 h and 48h time points compared to the original scratch at 0 hours.

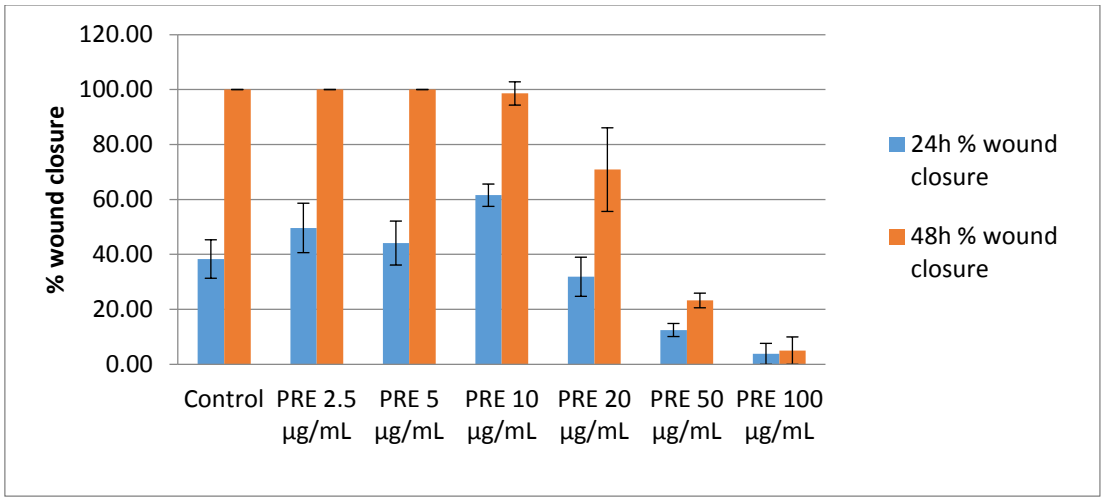


Figure 5-11 Effect of pomegranate rind extract on wound closure percentage in scratch test at 24 and 48 h (37 °C; 5% CO_2) in DMEM medium containing 1% FBS: (1) Control buffer, (2) PRE 2.5 µg/mL, (3) PRE 5 µg/mL, (4) PRE 10 µg/mL, (5) PRE 20 µg/mL, (6) PRE 50 µg/mL, (7) PRE 100 µg/mL. Data are expressed as percent wound area closure in comparison to the original scratch area at 0h (n=3±SD).

Next, the results of $ZnSO_4$ samples at lower concentration (25, 50, 100 µM), were found to promote cell migration and showed complete wound closure at higher rate than the control over the first 24 hours. The cell migration in samples treated with $ZnSO_{4\ at}$ 25, 50 and 100µM showed very significant increase in wound closure over the first 24h when compared to the control ($p < 0.001$) to give 91.13%, 74.19% and 96.34% respectively as shown in Figures 5-12, 5-13 and 5-14.

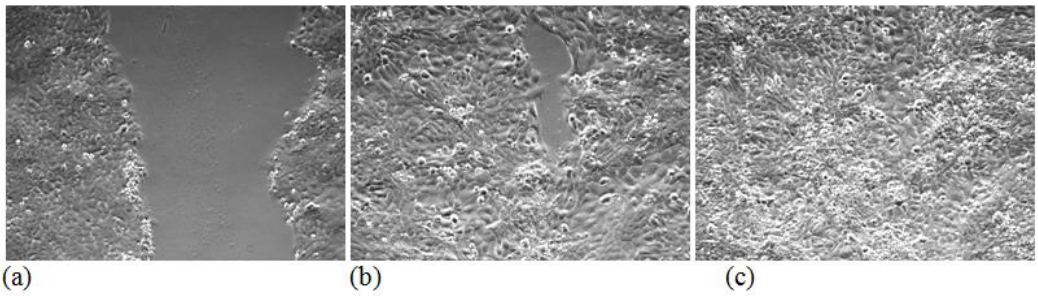


Figure 5-12 Cell migration under Leica TCS SP5 Confocal Microscope in presence of 1% FCS media containing $ZnSO_4$ 25 µM at (a) 0h, (b) 24h and (c) at 48h time point.

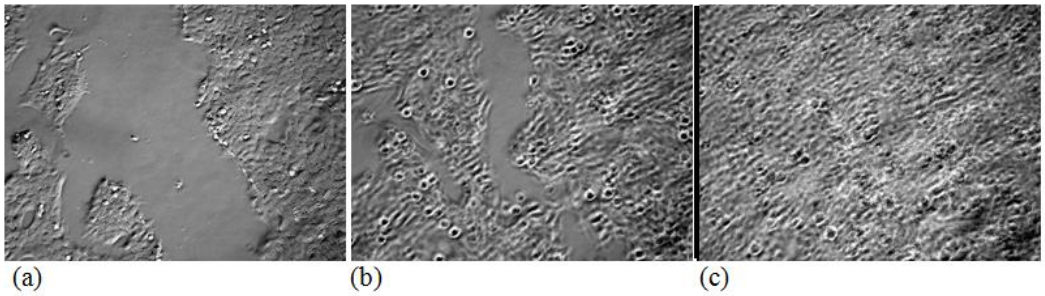


Figure 5-13 Cell migration under Leica TCS SP5 Confocal Microscope in presence of 1% FCS media containing $ZnSO_4$ 50 µM at (a) 0h, (b) 24h and (c) at 48h time point.

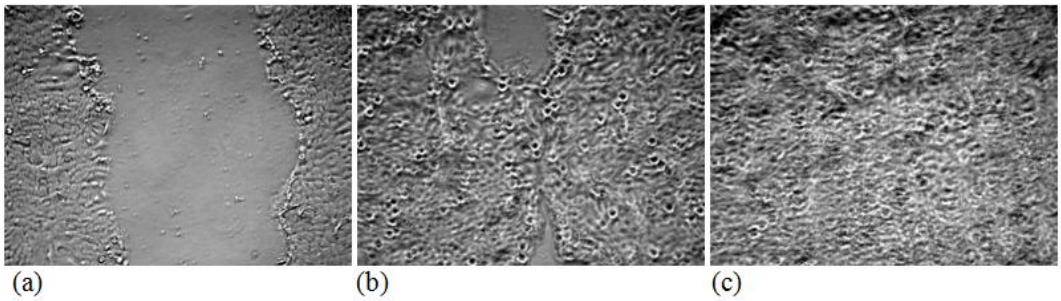


Figure 5-14 Cell migration under Leica TCS SP5 Confocal Microscope in presence of 1% FCS media containing $ZnSO_4$ 100 µM at (a) 0h, (b) 24h and (c) at 48h time point.

Figure 5-15 shows a summary of $ZnSO_4$ concentration range used and their effect on wound closure using cell migration assay compared to control untreated cells at 24 and 48 hours post scratch introduction.

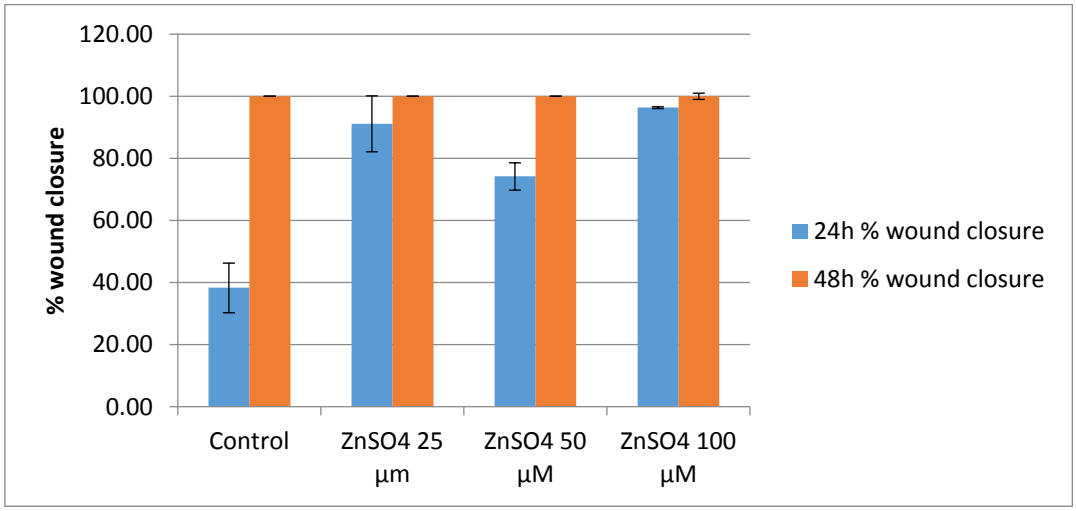


Figure 5-15 Effect of $ZnSO_4$ on the wound closure percentage in scratch test at 24 and 48 h (37 °C; 5% CO_2) in DMEM medium containing 1% FBS: (1) $ZnSO_4$, (2) 25 µM $ZnSO_4$ (3) 50 µM, (4) $ZnSO_4$ 100µM. Data are expressed as percent wound area closure in comparison to the original area at 0h (n=3±SD).

The next set of samples, which were all PRE/Zn combinations were first compared to its relevant individual results. The first three combination that contained PRE 5 µg/mL (PRE 5 µg/mL+ 25 $ZnSO_4$ µM; PRE 5 µg/mL $ZnSO_4$ 50 µM and PRE 5 µg/mL $ZnSO_4$ 100 µM) showed significant increase ($p < 0.01$) in wound healing over the first 24 hours, to give 86.88%, 93.26 and 92.97% closure respectively, compared to the PRE 5 µg/mL samples, which gave 44.13% wound closure over this timescale. High cell migration covered the wounded area after first 24 h at these combinations as shown in Figure 5-16, 5-17 and 5-18.

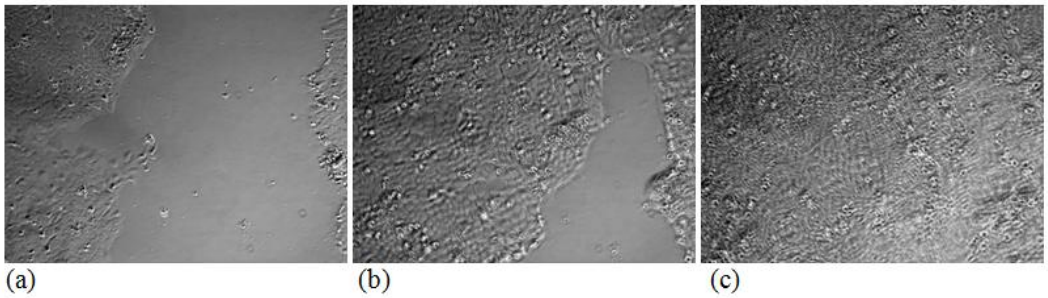


Figure 5-16 Cell migration under Leica TCS SP5 Confocal Microscope in presence of 1% FCS media containing combination 1 (PRE5 µg/mL+$ZnSO_4$ 25 µM) at (a) 0h, (b) 24h and (c) at 48h time point.

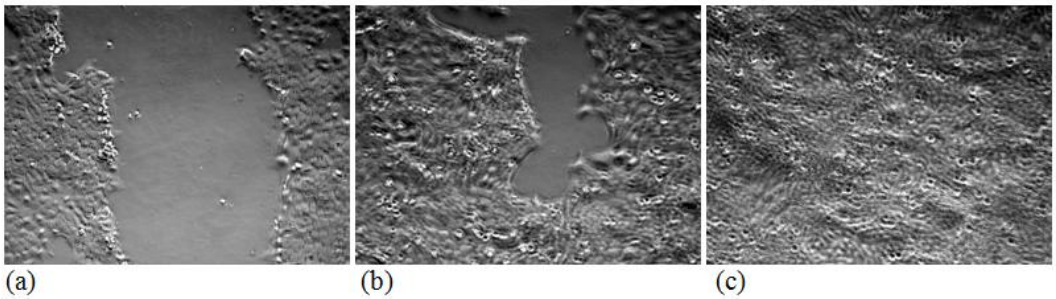


Figure 5-17 Cell migration under Leica TCS SP5 Confocal Microscope in presence of 1% FCS media containing combination 1 (PRE5 µg/mL+$ZnSO_4$ 50 µM) at (a) 0h, (b) 24h and (c) at 48h time point.

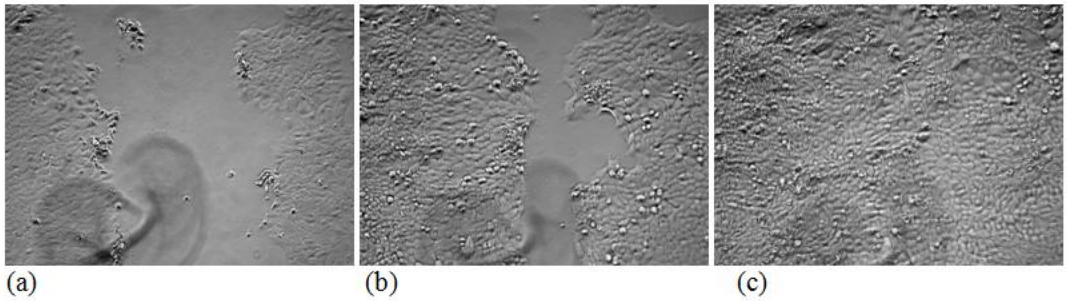


Figure 5-18 Cell migration under Leica TCS SP5 Confocal Microscope in presence of 1% FCS media containing combination 1 (PRE5 µg/mL+ZnSO4 100 µM) at (a) 0h, (b) 24h and (c) at 48h time point.

The other thee combinations that contained PRE 10 µg/mL (PRE 10 µg/mL+ 25 ZnSO4µM; PRE 10 µg/mL $ZnSO_4$ 50 µM and PRE 5 µg/mL $ZnSO_4$ 100 µM) showed

significant increase ($p < 0.05$) in wound healing at the first 24 h to give 89.17%, 94.54 and 100% closure respectively when compared to the PRE 10 µg/mL samples which gave 61.54% wound closure at the first 24h. HaCaT cell migration results of these combinations are illustrated in Figures 5-19, 5-20 and 5-21.

Taking together all 6 combinations mentioned above, that contained PRE ranging concentration from 5-10 µg/mL and $ZnSO_4$ concentration range 25-100 µM, they all showed very significant increase in the percentage of wound closure at the first 24 hours when compared to the control ($p < 0.01$) and showed complete wound closure at 48 h.

Next, when comparing the last 6 combinations that showed the positive effect on wound healing to its respective $ZnSO_4$ level, two combinations (PRE 5 µg/mL $ZnSO_4$ 50 µM and PRE 10 µg/mL $ZnSO_4$ 50 µM), showed significant increase ($p < 0.05$) in wound healing percentage to give 93.26% and 94.54% respectively when compared to the individual $ZnSO_4$ 50 µM which gave 74.19% in wound closure over 24 hours. On the other hand, no significant differences ($p > 0.05$) were observed when comparing the rest of the combinations to their relative $ZnSO_4$ concentrations at 25 and 100 µM, since all gave good healing results but in the same range relative to $ZnSO_4$ alone at 24 h.

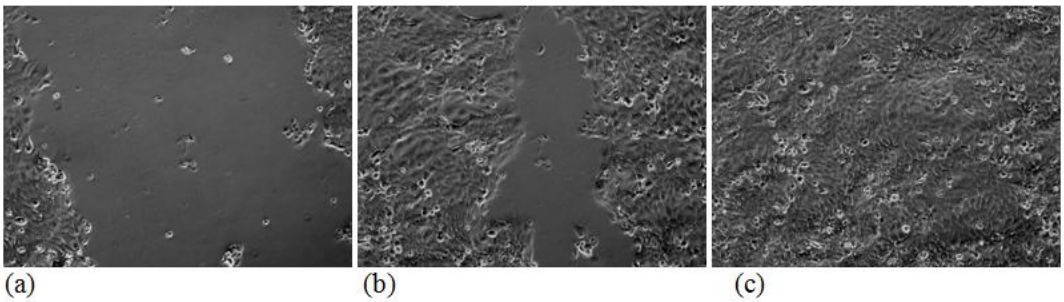


Figure 5-19 Cell migration under Leica TCS SP5 Confocal Microscope in presence of 1% FCS media containing combination 1 (PRE 10 µg/mL+$ZnSO_4$ 25 µM) at (a) 0h, (b) 24h d (c) at 48h time point.

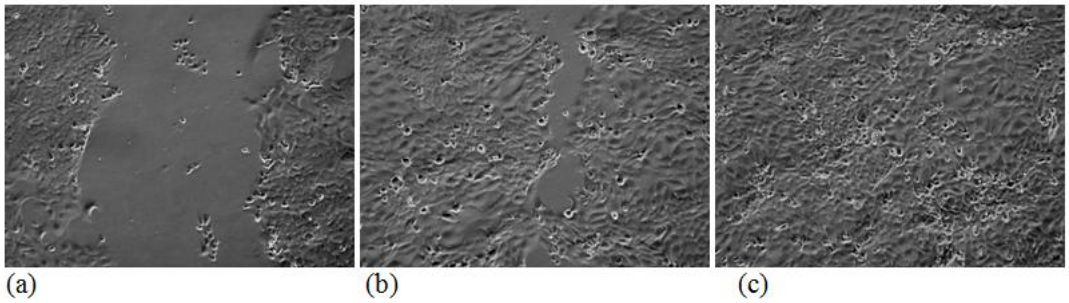


Figure 5-20 Cell migration under Leica TCS SP5 Confocal Microscope in presence of 1% FCS media containing combination 1 (PRE 10 µg/mL+$ZnSO_4$ 50 µM) at (a) 0h, (b) 24h and (c) at 48h time point.

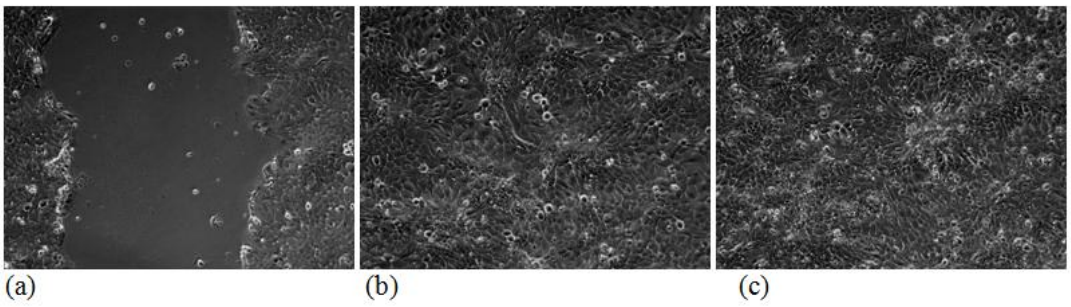


Figure 5-21 Cell migration under Leica TCS SP5 Confocal Microscope in presence of 1% FCS media containing combination 1 (PRE 10 µg/mL + $ZnSO_4$ 100 µM) at (a) 0h, (b) 24h and (c) at 48h time point.

Figure 5-22 shows a summary of wound closure percentage at 24h and 48 h time point for 6 combinations that contained PRE (5 µg/mL, 10 µg/mL) + $ZnSO_4$ (25 µM, $ZnSO_4$ 50 µM, $ZnSO_4$ 100 µM) and gave positive effect using cell migration assay.

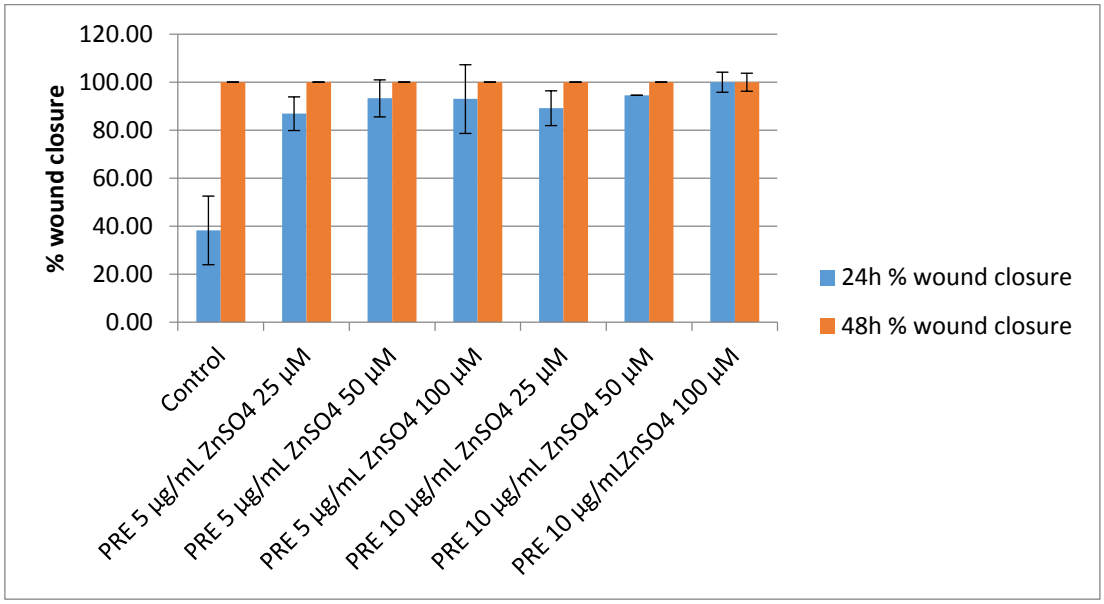


Figure 5-22 Effect of 6 different PRE +$ZnSO_4$ combination on the wound closure percentage at 24 and 48 h using scratch test performed on of HaCaT keratinocyte monolayer (37 °C; 5% CO_2) DMEM medium containing 1% FBS compared to control untreated cells. Data are expressed as percent wound area closure in comparison to the original area at 0h (n=3±SD).

% wound closure at 24 h	$ZnSO_4$ 0 µM	$ZnSO_4$ 25 µM	$ZnSO_4$ 50 µM	$ZnSO_4$ 100 µM
PRE 0 µg/mL		91.13	74.19	96.34
PRE 5 µg/mL	44.13	86.88	93.26	92.97
PRE 10 µg/mL	61.54	89.17	94.54	100

Table 5-2 Wound closure percentage of tested combinations contained PRE (5µg/mL, 10 µg/mL) and in combination with $ZnSO_4$ (25 mM, 50 µM, 100 µM) at 24 using scratch test performed on HaCaT monolayer (37 °C, 5% CO_2) in DMEM medium containing 1% FBS. Data are expressed as percent wound area closure in comparison to the original area at 0 hours (n=3).

5.5 Discussion

The effect of PRE ± Zn(II) was studied on the cell migration of immortalised HaCaT keratinocyte monolayer *in vitro* using a scratch wound model – to the best of our knowledge, this is a novel approach which has not been studied previously. However, a variety of pomegranate plant extracts have been investigated for its potential in wound healing properties using other models, typically involving *in vivo* rodent models. Huan et al. (2013) studied the wound healing properties of 'pomegranate polyphenol peel, PPP' gel in Alloxan-induced diabetic rats as a model for complicated chronic wounds. The results showed that wound healing time was significantly shorter with PPP gel treated rats compared to control. Mo et al. (2014) used a standardized pomegranate rind extracts (SPRE) at 1%, 2.5 and 5% to treat two types of dermal rat wounds: excision and burn wound and they showed significant facilitation in wound healing against control group which has been treated with saline only. The wound healing acceleration was in dose-dependent manner with SPRE 5% (the highest concentration evaluated) being the most effective. They concluded that the benefits of SPRE in wound healing may be

attributed to various factors such as enhancement of collagen synthesis, antibacterial activity and anti inflammatory activities. Nasiri et al. (2017) used pomegranate flower extract to investigate in vivo healing potential against burn-induced wounds in rats. The pomegranate flower extract at 1%, 5% and 10 % was compared to negative normal saline treatment and positive silver sulfadiazine treatment. The study showed that at day 15, the average size of wounds that were treated with pomegranate flower extract were significantly smaller than silver sulfadiazine comparator group, 2.8 cm^2 against 8.4 cm^2 respectively. The wound healing was observed in 10 days earlier with pomegranate flower extract group than in the other treated groups.

Hayouni et al. (2011) studied the in vivo effect of a 5% methanolic extract of pomegranate peel extract ointment in Guinea pigs excision wounds compared to a cetrimide 2% cream and a placebo ointment. The study showed that faster wound healing and contraction was achieved with both pomegranate peel ointment and cetrimide group compared to the placebo control group. After complete wound healing a tensile strength test was carried out in involving the force needed to reopen the wound by mechanical measured force. Although the mechanism at which 5% pomegranate peel extract ointment promoted healing and cell proliferation has not been established by his study (Hayouni et al. 2011).

In our study, higher concentrations of PRE at 50 and 100 µg/mL showed significantly lower percentage closure compared to control, while lower concentrations of PRE showed promising results. PRE at concentrations from 2.5 µg/mL - 20µg/mL promoted wound healing of scratch test on HaCaT cells samples and even higher percentage of wound closure was observed at the first 24 hours when PRE 5 and 10 µg/mL was combined with $ZnSO_4$ at 25, 50 and 100 µM concentration range compared to untreated cell.

Although the mechanism of action of PRE (and indeed PRE/Zn) in wound healing promotion has not been fully elucidated, Huan et al. (2013) found increased expression

of TGF-β1, VEGF and EGF in a diabetic rat wound area treated with gel contained 'pomegranate polyphenol peel'. The mechanism and important role of these growth factors, as outlined in Section 1.9.1, is important in collagen regeneration, fibroblast infiltration, vascularisation and epithelialisation stage at wound healing process. Vascular endothelial growth factor (VEGF) has an essential role in promotion of angiogenesis stage of wound healing and induces endothelial cells to activate neovascularisation and repair blood vessels that have been damaged (Harper et al. 2014). Epidermal growth factor (EGF) and transforming growth factor-beta (TGF-β) Together with pro-inflammatory cytokines such as IL-1, PDGFs are significant in the attraction of neutrophils to the site and activate inflammatory response. In addition, EGF and TGF β are significant stimulants for the migration and proliferation of the epithelial cells and responsible for mediation of collagen synthesis and breakdown (Harper et al. 2014; George et al. 2006).

In a similar manner a previous report has shown the effect of pomegranate rind and seed extracts on MMP-1 enzymes, which is the main enzymes responsible of collagen destruction (Aslam et al. 2006). The study showed that pomegranate peel extract stimulated the proliferation dermal fibroblast and induced type I procollagen synthesis dermal fibroblasts concomitantly with inhibition of MMP-1 enzymes was observed in cell treated with pomegranate peel extract. This could explain promotion of skin repair by inhibiting collagen destruction and synthesis of procollagen. Pitz et al. (2016) reported the effect cytoprotective properties of jaboticaba fruit peels in wound healing processes. The study showed the strong cytoprotective of jaboticaba fruit peels extract treated cells after oxidative stress induced by hydrogen peroxide exposure for 3 h in murine fibroblasts cell line. The antioxidant activity was attributed to the phenolic content (anthocyanins) of the fruit in which same group are found in abundance in pomegranate rind extract PRE.

Zinc formulations have been investigated previously for wound care and many reports showed that topical zinc therapy can reduce the wound debris and promote

epithelialisation process. Zinc oxide topical has been established to treat wound in acute or chronic wounds including leg ulcers, diabetic ulcers and pressure ulcers (Ågren et al. 2006: Strömberg and Ågren 1984). In our results, cell migration study revealed that HaCaT cells treated with $ZnSO_4$ at concentration (20, 50 and 100 µM), showed significant increase with more than 35% wound closure at the first 24h compared to control untreated cells. It has been reported that zinc oxide topical treatment showed promotion by more than 30% in wound healing of domestic pigs and stimulate the epithelialisation phase in wound healing process (Ågren et al. 1991). Although the same study showed that $ZnSO_4$ at 0.03% and 0.3% concentration showed no positive or negative effect to the wound healing compared to untreated wounds. Several mechanisms of action have been proposed to explain the role of Zn(II) in wound healing, including as being essential for the metalloenzyme synthesis and signalling that is needed for cell proliferation and division pathway. The mitotic activity of cell involved in wound healing at the wound site is maximal at the first 24-48 hours and this can explain the higher zinc demand as its essential for cell replication enzymes including DNA and RNA polymerase and others which can lead to the increase mitosis and epithelialisation process (Iwata 1999; Lansdown et al. 2007). Another important action that it promotes healing in necrotic ulcers which has been reported from *in vitro* study that showed zinc oxide promoting the collagen destruction from wound debris by activate MMPs (Ågren et al. 1993).

While a majority of previous studies showed the effect of either PRE alone or Zn(II) alone containing formulation on wound healing, our results show effect of combined PRE ± $ZnSO_4$ on the cell migration of HaCaT cell line using *in vitro* scratch wound. Pomegranate extract has been investigated in combined formulation and used in one case study of chronic wound in form of non-healing leg ulcer. A leg ulcer for 76 years old woman had failed to heal for over a year, then a pomegranate peel extract 2% hydrogel was applied once daily with zinc oxide ointment to the wound. It took six weeks for the ulcer to reduce to one quarter of its original size (6.8 x 4.3 cm) and complete healing occurred after 90 days responded positively to pomegranate peel

ethanolic extract. This case study supports the effect of pomegranate peel extract accompanied by the metal zinc in form of zinc oxide ointment (Fleck *et al.*, 2016); however, the Zn(II) as the sulphate salt is a free Zn^{2+} ion in solution, whereas zinc oxide is an *insoluble salt* so the zinc cannot behave in the same way.

The combination of both PRE and Zn (II) showed promotion of cell migration in 6 combinations. The combination that contained $ZnSO_4$ 50 µM (PRE 5µg/mL+ $ZnSO_4$ 50 µM) and (PRE 10µg/mL+ $ZnSO_4$ 50 µM) showed significant increase in wound healing % at the first 24 h when compared to un treated control group, PRE individual groups (PRE 5 µg/mL or 10 µg/mL) or $ZnSO_4$ 50 µM individual group ($p < 0.05$) with more than 93% wound closure at the first 24 hr. The rest of combinations that contained PRE in (5, 10 µg/mL) or $ZnSO_4$ in (25, 50,100 µM) showed high wound closure percent at the first 24h > 86 % when compared to than the control which gave 38.31 % at the same time.

5.6 Conclusion

This chapter has demonstrated the effect of PRE and $ZnSO_4$ activity upon wound healing using an *in vitro* wound model in form of scratch test performed on HaCaT cells. The results showed that:

- PRE at 10 µg/mL and $ZnSO_4$ at (25, 50,100 µM) showed a significant increase in the cell migration and % wound healing in comparison to the control group ($p > 0.05$) over 24 hours.

- PRE at 20-50 µg/mL showed cell migration and proliferation but in a delayed matter compared to the control untreated group.

- PRE ≥100 µg/mL showed significant delay in cell migration and that was expected from the Chapter 4 cytotoxicity work of PRE at this concentration.

- PRE and $ZnSO_4$ at the tested concentrations showed accelerated healing properties compared to the blank control, and that property was maintained when combinations of the two were used.

- The combination (PRE 5μg/mL+ $ZnSO_4$ 50 μM) and (PRE 10 μg/mL+ $ZnSO_4$ 50 μM) showed significant increase wound closure (> 93 %) over the first 24 hours when compared to untreated control group, PRE individual groups (PRE 5 μg/mL or 10 μg/mL) or $ZnSO_4$ 50 μM individual group

- The rest of combinations that contained PRE (5, 10 μg/mL) or $ZnSO_4$ in (25, 50,100 μM) showed > 86 % wound closure over the first 24 hours, possible due to mediation action of Zn (II) for those combinations.

The antimicrobial results properties of PRE/Zn along with wound healing properties in this chapter show that the combination has potential as a product for use in wound repair and those which have bacterial infection. The next Chapter will investigate stability and the possible effect of different pH and temperature conditions on individual PRE and PRE/Zn solutions.

Chapter 6: Stability of PRE solution with and without $ZnSO_4$ under different pH and temperature conditions

6.1 Introduction

The patent by Stewart et al. (1995) disclosed that the combination of PRE and iron sulphate produces a potent phagicidal activity, that arises due to a Fenton reaction whereby Fe(II) oxidises to Fe(III) in the presence of PRE. However, the activity is short-lived, lasting for up to 30 minutes, and is accompanied by a blackening of the solution. The work of Houston (2011), found that when Zn(II) was used instead of Fe(II) the solution did not suffer from solution blackening, and activity that was not limited to 30 minutes. However, the exact timescale for enhanced activity was not determined. In the previous Chapter of this thesis, the enhanced activity of PRE was found against bacteria, when combined with Zn(II). Again, no discolouration or blackening of the solution was observed and time kill assay data indicated stability of microbiological action. This microbiological stability infers there is also chemical stability when PRE is in solution along with Zn(II) – this is extremely important, should a product based on this system be proposed for commercialization.

The purpose of this chapter was therefore to conduct a chemical stability study for PRE solutions, alone and in the combination with $ZnSO_4$. Thermal stability of pomegranate either juice or rind extract has been previously studied in the literature. The pH level in which pomegranate is extracted at or stored, has a major effect on the total phenolic content of the extract. Low pH level from 5-3.5 and temperature <30˚ C, have been reported to be best condition in relation to the stability of punicalagin isomer in pomegranate extract (Houston 2011; Lu et al. 2010; Panichayupakaranant et al. 2010; Qu et al. 2012; Qu et al. 2014). Very few studies have yet explored the affect of metal ion on the stability of PRE. Combination of PRE with Fe(III) showed a rapid decline in the punicalagin's absorbance although a new compound was not apparent in HPLC chromatograms (Houston 2011; Lu et al. 2010)- this could be due to low solubility of the product. Here, we aimed to assess how the pH level and storage temperatures impact on the stability of punicalagin in PRE solution with and without co-formulated Zn(II).

Determination of stability is of key importance in the development of new pharmaceutical products. The guidelines implemented by the International Council for Harmonisation (ICH) or other relevant regulatory agencies must be followed during stability study analysis. Despite typically concerning the capacity to bear chemical losses throughout decomposition, "stability" in pharmaceutical terms refers to the length of time for which a drug can be stored in its dosage form before it starts to degrade and/or becomes inefficacious or dangerous for use. An expiration date of a medical product must thus be established (Waterman and Adami 2005) – this is the date before which the quality of the product can be guaranteed. Typically, approaches such as the direct monitoring of drug loss or observing the appearance of degradants in the formulation to determine the product's shelf life. Such tests may be carried out at the intended storage conditions (real time) or at higher temperatures (stress condition), after which extrapolation to the intended storage temperature must be performed (Darrington and Jiao 2004).

It is through accelerated stability analysis that data pertaining to the physicochemical stability of drugs can be initially assessed, and such data can allow for the product's likely decomposition path to be established (Sengupta et al. 2018). Conventionally, accelerated stability studies subject the drug to elevated temperatures to make reactions occur more quickly. To estimate ambient stability of drugs, it is important that the reaction rate is determined at different temperatures in advance, and that the chemicals are subsequently extrapolated to the intended temperature. Here, it is possible to establish the reaction rate at a given temperature, as well as the extrapolation process. Research exploring rates of change for various physicochemical features may be used to predict the stability and design of drug formulations.

Usually, chemical stability is represented in the form of a rate constant, *k*, which signifies either drug degradation or product formation. Arrhenius kinetics typically determines reaction rates for most products created through the drug degradation process. Such kinetics are linearly reliant on a reaction rate logarithm against the

reciprocal of the absolute temperature *T* (*R* represents gas constant and *A* the entropy of process (Waterman and Adami 2005).

$$ln\ k\ =\ lnA - \frac{Ea}{RT}$$

kT in this equation refers to the degradation rate at temperature *T* in kelvins, whilst *k* represents the degradation rate at temperature *T* (typically the temperature required to make the prediction). The rate constant can be calculated as the amount of product created in the unit time, if low conversions are presumed. Most frequently, rate constants acquired through experimental data at a given temperature are determined by employing linear least squares techniques to fit the data. This is particularly common for liquid dosage forms (Waterman and Adami 2005; Bajaj et al. 2012).

The results of such experiments can indicate the storage conditions under which a product must be kept over its shelf-life, for example: room temperature, refrigeration at 2-4 ˚C, freezing at -20 ˚C or lyophilization. A solution that may be maintained at room temperature is ideal as it may be used straight away by a patient. Storage at 2-4 ˚C or -20 ˚C are unfavoured, but acceptable although formulation would need to be brought to room temperature prior to use, and more likely to be single-use, as degradation may occur once opened. Lyophilised or freeze-dried formulations must be reconstituted prior to use with an appropriate buffer, which does not lend itself to OTC availability and more likely to require the involvement of a healthcare professional.

Furthermore, data acquired through stability analysis is crucial in ensuring regulatory approval for a new formulation or drug (Singh et al. 2000). The shelf-life of a new drug can be defined as the time required for are pharmacological activity to drop to the lowest acceptable amount, and ultimately determined in real-time. Although this definition is by no means universal, an acceptable level of decomposition is determined to be approximately 10 %, except in cases where product decomposition can be toxic. The following decomposition routes are the most common ones identified in

pharmaceuticals: oxidation, hydrolysis, racemization, photochemical degradations, thermal decomposition, microbial degradation and chemical interactions (Bajaj et al. 2012). However, in a natural product extract such as PRE, the situation is far more complex as the hydrolysable ellagitannins could potentially undergo acid- or base-catalysed ester cleavage. On the other hand, the innate anti-oxidant properties of tannins and smaller polyphenols, which are also present in PRE may confer protection against oxidation reactions.

Combining PRE and metal ions in a formulation presents further potential complication in that metals have the potential to interact with the components of PRE. This can potentially include: 1) homogeneous catalysis, a catalytic reaction where the catalyst is in the same phase as the reactants. 2) complex formation. The zinc ion (Zn^{2+}) or Zn(II) possesses a filled *d* orbital (d^{10}) and therefore does not participate in redox reactions as with Fe(II), but could function as a Lewis acid in accepting a pair of electrons (Williams 1987). Such processes have the potential to impact on stability and render the product inefficacious.

6.2 Objective and aims

To determine the stability of PRE (ie punicalagin) in the absence and presence of Zn(II) over a range of temperature and pH conditions.

1. To seek evidence about the effect of pH 4.5 and pH 7 on the stability of PRE solution with and without $ZnSO_4$

2. Study the effect of temperature on PRE solution with and without $ZnSO_4$.

3. Seek evidence about the effect of long-term addition of $ZnSO_4$ to PRE and effect on overall stability compared to PRE individual samples.

4. Compare α and β punicalagin anomers during the degradation process.

5. Estimate a shelf life based on the accelerated stability study.

6.3 Materials and methods

6.3.1 Materials

Materials used in this project are detailed in Chapter 2, section 2.1

6.3.2 Sample preparation

PRE individual solution was prepared according to previous method of freeze-dried PRE Reconstitution in Chapter 2, Section 2.2.1.2. $ZnSO_4$ individual samples were prepared according to Chapter 2, Section 2.2.1.4. The preparation of combined solution of PRE and $ZnSO_4$ was prepared according to the detailed method in Chapter 2, Section 2.2.1.5. The two pH values investigated were: phthalate buffer (pH 4.5) as the optimum determined for virucidal action (Houston 2011) and neutral water (pH 7).

6.3.3 Real time stability study

In order to demonstrate different storage temperature effect on stability of PRE solution alone and in combination with $ZnSO_4$, three different temperatures were employed: -20˚C, 2-4˚C, 20 ˚C representing freezer, fridge and room temperature storage respectively. In addition, two different pH levels were tested (in phthalate buffer pH 4.5 and water at pH 7) – in total, 12 test solutions were examined as summarised in Table 6.1. Aliquots of each sample were stored in glass vials occluded from light. Data collection was performed on days 0, 30, 60, 90, 150 and 180, by analyzing for punicalagin content by HPLC method previously detailed in Chapter 2, Section (2.2.2), The testing frequency was set to be no more than four weeks apart. Three replicates of each sample were tested at each time point to give a total analysis count of 36 samples every occasion.

Sample name	PRE conc.	$ZnSO_4$ conc.	Storage temperature	pH value
PRE pH4.5 (-20˚)	3mg/mL	0	-20˚C	4.5 (phthalate buffer)
PRE/Zn pH 4.5 (-20˚)	3mg/mL	0.25M	-20˚C	4.5 (phthalate buffer)
PRE pH4.5 (2-4˚)	3mg/mL	0	2-4 ˚C	4.5 (phthalate buffer)
PRE/Zn pH 4.5 (2-4˚)	3mg/mL	0.25M	2-4˚C	4.5 (phthalate buffer)
PRE pH4.5 (20˚)	3mg/ml	0	20˚C	4.5 (phthalate buffer)
PRE/Zn pH 4.5 (20˚)	3mg/mL	0.25M	20˚C	4.5 (phthalate buffer)
PRE pH 7 (-20˚)	3mg/mL	0	-20˚C	7 (water)
PRE/Zn pH 7 (-20˚)	3mg/mL	0.25M	-20˚C	7 (water)
PRE pH 7 (2-4˚)	3mg/mL	0	2-4 ˚C	7 (water)
PRE/Zn pH 7 (2-4˚)	3mg/mL	0.25M	2-4˚C	7 (water)
PRE pH 7 (20˚)	3mg/mL	0	20˚C	7 (water)
PRE/Zn pH 7 (20˚)	3mg/mL	0.25M	20˚C	7 (water)

Table 6-1 Summary of test solutions used in stabilty study.

6.3.4 Accelerated storage stability testing

The purpose of carrying out the accelerated storage testing was to determine sample stability at 4 and -20 °C, as these samples showed low degradation relative to room temp. PRE samples with and without $ZnSO_4$ were stored in sealed glass vials occluded from light at and the accelerated stability testing was conducted at three two stress temperature 40 ˚C and 50 ˚C. The samples were kept at incubator and at time for analysis the samples were collected and the vial was return to the incubator immediately. The sample collection was carried out on a weekly basis for a period five weeks. Using HPLC method previously detailed in Chapter 2, Section (2.2.2.1.), PRE and PRE/Zn samples were tested for punicalagin α and β content and converted area under the curve showed in HPLC chromatogram to concentration using calibration curve of punicalagin. Stability testing was conducted according to International Conference on Harmonisation (ICH) guidelines for stability testing of new drug substances and product ICH Topic Q 1 A (R2) (2003). Construction of Arrhenius plot was made using natural logarithm of

reaction rate constant k of 40 °C and 50 °C against 1/T (absolute temperature in kelvin). The prediction of the degradation rate at lower temperature was made by extrapolating of Arrhenius plot to give ln k at 20 °C and 4 °C which represent room temperature and fridge storing temperature.

6.3.5 Data Processing

After HPLC analysis of punicalagin α and β anomers in the samples, the concentration was calculated in accordance to the calibration curve of Punicalagin previously constructed in Chapter 2, Section 2.2.2.1.1. Punicalagin anomer (α and β) concentrations were calculated separately and in total and percentage punicalagin content was calculated in relation to the initial concentration at time zero. In each test, data were expressed as mean ± SD, otherwise stated. Statistical analysis was carried out using One way analysis of variance (ANOVA) with Tukey's post test to compare statistical significances between groups. PRE and $ZnSO_4$ combinations were compared to control and individual PRE. Results were expressed as significant with $p < 0.05$. The analysis was carried out using InStat for Macintosh, version 3.0 (GraphPad Software Inc, San Diego, CA).

6.4 Results

6.4.1 Real time stability determination

The data in the real time stability test revealed that both temperature and pH had a significant effect on the stability of punicalagin in both PRE and in PRE combined with $ZnSO_4$ after being stored for 180 days. Table 6-3 shows a summary of α punicalagin concentration of at different pH and temperature. At first, data analysis at the lower pH value (pH 4.5) showed that as the temperature increased, the loss in α punicalagin concentration increased. At the end of the testing period, it was apparent that samples at the higher temperature (20˚C) showed significantly decrease with 16-18 % loss in α

punicalagin concentration in both PRE with $ZnSO_4$ and PRE individual samples ($p < 0.05$). After 180 days storage time of both PRE and PRE/Zn sample at 20 ˚C and pH 4.5, the percentage recovery of α punicalagin were 81.8 % and 83.3 % respectively. On the contrary, no loss in α punicalagin concentration was observed at the lowest tested temperature -20 °C ($p > 0.05$) in both PRE samples and PRE with $ZnSO_4$ as shown in Table 6-3. However, the refrigerated samples which were stored at temperature 2-4 °C showed reduction of <8% of the original α punicalagin concentration in both PRE and PRE/Zn samples after stored for 180 days.

Moreover, the higher pH 7 (water) was found to negatively impact on α punicalagin concentration at both temperatures 2-4 °C and 20 °C to give α punicalagin percent of 84.9 % and 84.8 % at 2-4 ˚C and 74 % and 72.8 % at 20 ˚C in PRE and PRE/Zn respectively. Comparing the PRE and PRE/Zn samples at same temperature (20 °C), the pH level significantly affect ($p < 0.05$) the stability and percentage loss of initial α punicalagin with pH 4.5 positively added to the stability of both PRE and PRE/Zn. Data showed that the addition of $ZnSO_4$ to PRE didn't affect the of punicalagin and that remained true for all samples tested in this project regardless of the pH or temperature level ($p >0.05$).

α Punicalagin concentration (mg/mL) in PRE with and without $ZnSO_4$ in phthalate buffer <u>pH 4.5</u> at three different storage temperature (-20, 2-4˚C, 20˚C) in time period of 180 days.						
Days	PRE pH 4.5 (-20˚)	PRE/Zn pH 4.5 (-20˚)	PRE pH 4.5 (2-4˚)	PRE/Zn pH 4.5 (2-4˚)	PRE pH 4.5 (20˚)	PRE/Zn pH 4.5 (20˚)
0	0.39	0.39	0.39	0.39	0.39	0.39
30	0.38	0.38	0.38	0.38	0.36	0.35
60	0.39	0.39	0.38	0.38	0.35	0.34
90	0.39	0.39	0.38	0.37	0.34	0.35
120	0.39	0.38	0.38	0.38	0.34	0.34
150	0.39	0.39	0.36	0.37	0.33	0.33
180	**0.39**	0.40	0.36	0.36	0.32	0.32
α punicalagin concentration (mg/mL) in PRE with and without $ZnSO_4$ in water <u>pH 7</u> at three different storage temperature (-20, 2-4˚C, 20˚C) in time period of 180 days.						
Days	PRE pH7 (-20˚C)	PRE/Zn pH 7 (-20˚C)	PRE pH 7 (2-4˚C)	PRE/Zn pH 7 (2-4˚C)	PRE pH 7 (20˚C)	PRE/Zn pH 7 (20˚)
0	0.40	0.40	0.40	0.40	0.40	0.40
30	0.39	0.40	0.37	0.36	0.36	0.36
60	0.40	0.39	0.35	0.36	0.35	0.35
90	0.40	0.40	0.36	0.36	0.35	0.35
120	0.40	0.40	0.37	0.37	0.34	0.35
150	0.41	0.40	0.35	0.35	0.33	0.32
180	0.39	0.41	0.34	0.34	0.29	0.29

Table 6-2 α punicalagin concentration (mg/mL) in PRE with and without $ZnSO_4$ in pH 4.5 and pH 7 at three different storage temperature (-20 °C, 2-4 °C, 20 °C) in time period of 180 days.

A similar pattern was observed for levels of β punicalagin. From Table 6-3, the first set of samples at pH 4.5 and stored at room temperature (20 °C), showed the minimum punicalagin % recovery among all samples at the same pH level. The Table below shows that at 180 days, β punicalagin concentration at PRE and PRE/Zn at 20 °C and pH 4.5 was 82.4 % and 84.8 % of initial concentration respectively. The next set of samples which were stored at 2-4 ˚C and being with the same pH level (4.5), the PRE and PRE with Zn(II) showed less than 6 % loss of punicalagin concentration at after 180 days. Almost unchanged, ($p > 0.05$), the concentration of β punicalagin in PRE and PRE/Zn that had been stored at -20˚C was more than 99.6 % of initial concentration at pH 4.5 at the end of 180 days.

Similar to α punicalagin, higher pH had a negative effect on β punicalagin stability and greater loss of initial concentration was seen at high pH (pH 7) with high temperature (20˚C). On the second set of data presented in Table 6-3, PRE and PRE/Zn samples prepared at pH 7 and stored at 20 °C, showed significant loss ($p < 0.05$) of β punicalagin after 180 days with recovery of 68 % and 66.1 % of initial concentration respectively. At 2-4 °C, the β punicalagin recovery after 180 days was 84.5 % and 85.6 % in PRE and PRE/Zn accordingly. The same positive effect of lower temperature (-20 °C) under pH 4.5 conditions, the β punicalagin content at pH 7 was reserved and more than 99.6% of initial concentration was recovered after 180 days. Again as previously shown with α punicalagin, $ZnSO_4$ addition to PRE samples didn't show any significant affect and same pattern of β punicalagin loss in PRE samples was seen in PRE/Zn samples regardless pH or temperature level.

β punicalagin concentration (mg/mL) in PRE with and without $ZnSO_4$ in phthalate buffer <u>pH 4.5</u> at three different storage temperature (-20, 2-4˚C, 20˚C) in time period of 180 days.						
Days	PRE pH4.5 (-20˚C)	PRE/Zn pH 4.5 (-20˚C)	PRE pH4.5 (2-4˚C)	PRE/Zn pH 4.5 (2-4˚C)	PRE pH4.5 (20˚C)	PRE/Zn pH 4.5 (20˚C)
0	0.41	0.42	0.41	0.42	0.41	0.42
30	0.42	0.43	0.42	0.42	0.43	0.43
60	0.42	0.42	0.41	0.42	0.41	0.40
90	0.43	0.43	0.42	0.42	0.37	0.37
120	0.43	0.43	0.41	0.42	0.35	0.35
150	0.41	0.42	0.41	0.41	0.33	0.35
180	0.41	0.42	0.40	0.40	0.34	0.36
β punicalagin concentration (mg/mL) in PRE with and without $ZnSO_4$ in water <u>pH 7</u> at three different storage temperature (-20, 2-4˚C, 20˚C) in time period of 180 days.						
Days	PRE pH7 (-20˚C)	PRE/Zn pH 7 (-20˚C)	PRE pH 7 (2-4˚C)	PRE/Zn pH 7 (2-4˚C)	PRE pH 7 (20˚C)	PRE/Zn pH 7 (20˚C)
0	0.44	0.45	0.44	0.45	0.44	0.45
30	0.45	0.45	0.44	0.44	0.40	0.41
60	0.44	0.45	0.43	0.42	0.40	0.39
90	0.45	0.45	0.44	0.43	0.35	0.35
120	0.46	0.46	0.43	0.44	0.35	0.34
150	0.45	0.45	0.41	0.41	0.34	0.33
180	0.45	0.46	0.37	0.38	0.30	0.30

Table 6-3 β punicalagin concentration (mg/mL) in PRE with and without $ZnSO_4$ in pH 4.5 and pH 7 at three different storage temperature (-20 °C, 2-4 °C, 20 °C) in time period of 180 days.

Figures 6-1 to 6-9 shows the total punicalagin α and β concentration and chromatogram report after storage for 180 days at pH 4.5, pH 7 and three different temperatures. As shown in Figure 6-1 no statistically significant difference was observed in the concentration of total punicalagin isomer after 180 days storage at temperature -20 °C in both PRE samples and PRE with $ZnSO_4$ ($p > 0.05$). This was expected as observed earlier, each isomer in separate didn't show any significant loss at this low temperature. The pH difference showed no affect at this low temperature and all samples remain stable for 180 days.

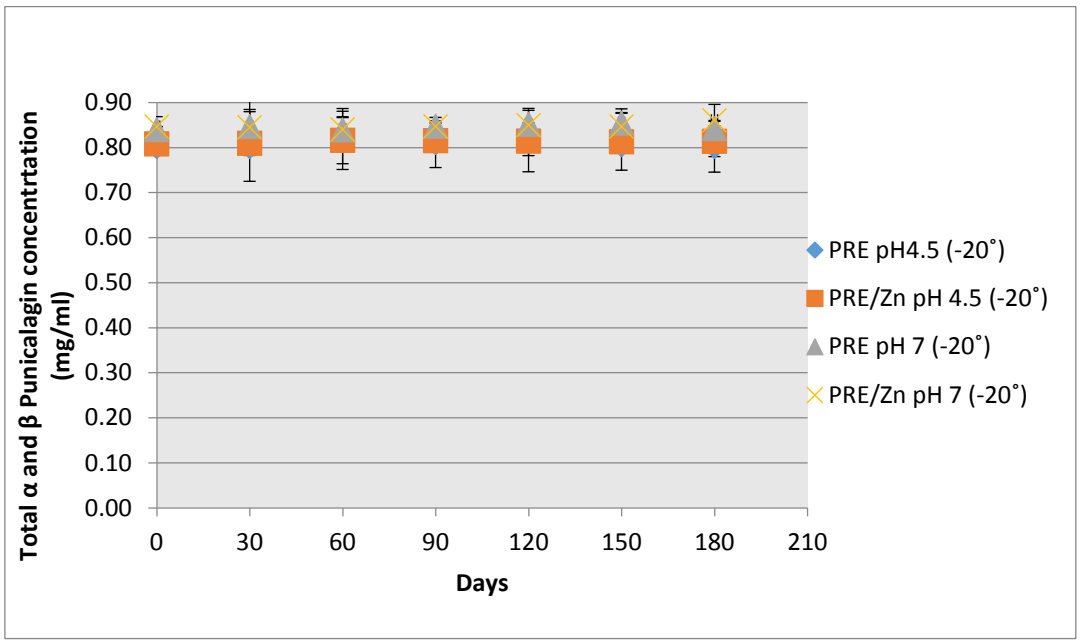


Figure 6-1 Total α and β punicalagin concentration (mg/mL) in PRE with and without $ZnSO_4$ in pH4.5 and pH 7 at (-20˚C) after 180 days storage time (n=3 ± SD). This shows stable concentration of punicalagin recovered over 6 months (-20 °C).

The chromatogram report showed no change in punicalagin α and β peak was observed in PRE individual samples or combined with Zn(II) when storage condition was at low temperature (-20 °C). Figure 6-2 illustrates chromatogram of punicalagin content in PRE ± Zn(II) at pH 4.5 and Figure 6-3 shows same samples at pH 7. From both chromatograms Figures, no inhibition in punicalagin α or β peaks was observed and no

new peaks were introduced, showing original chromatogram at zero time in blue line and overlaid chromatogram (in red line) after 6 month storage time at -20 ˚C.

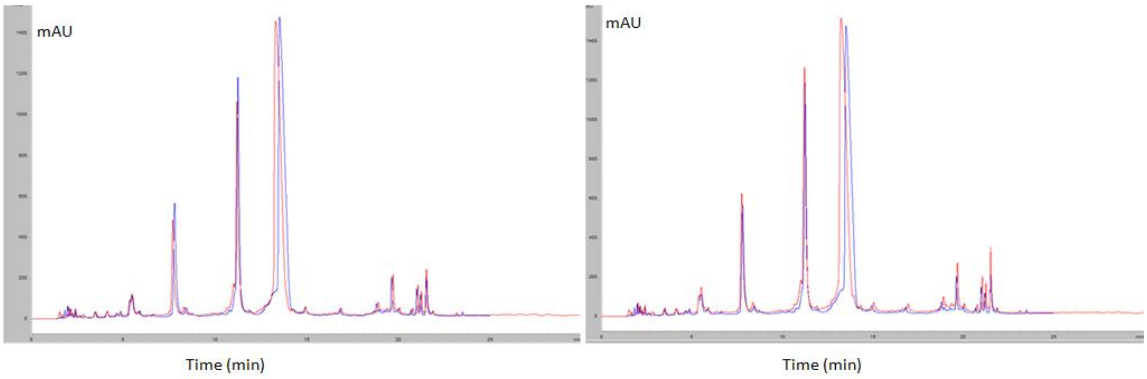


Figure 6-2 HPLC chromatogram showing α and β punicalagin peaks in PRE individual sample (right chromatogram) and PRE+$ZnSO_4$ (left chromatogram). Blue line shows peaks at initial concentration at time zero, red line shows peaks after 6 months storage in pH4.5 at -20 °C. Punicalagin α and β peaks show same areas under peak after 6 months storage).

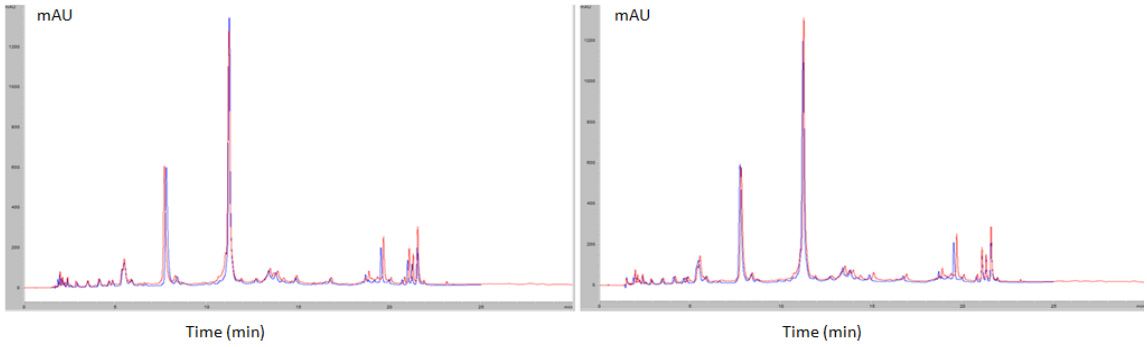


Figure 6-3 HPLC chromatogram showing α and β punicalagin peaks in PRE individual sample (right chromatogram) and PRE+$ZnSO_4$ (left chromatogram). Blue line shows peaks at initial concentration at time zero, red line shows peaks after 6 month storage in pH7 at -20 °C. Punicalagin α and β peaks show same area under peak after 6 months storage.

The following set of results, Figure 6-4, shows the total punicalagin under 2-4 °C storing condition with different pH value at PRE individual samples and combined with Zn(II). While the pH difference didn't affect punicalagin stability at temperature of -20 ˚C in both PRE and PRE/Zn samples, it showed a significant effect on samples stored at 2-4 °C when compared to each other ($p < 0.05$). The higher pH at 7 gave total punicalagin of 84.7 % and 85.2 % of initial concentration while lower pH 4.5 showed 94 % and 93.2 % in PRE and PRE/Zn respectively after stored for 180 days.

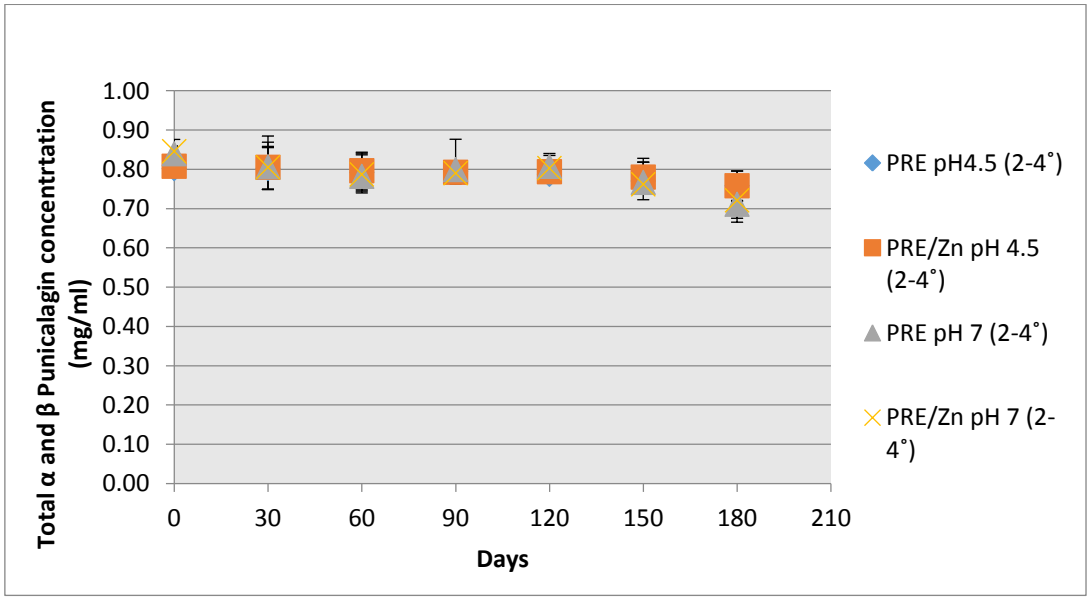


Figure 6-4 Total α and β punicalagin concentration (mg/mL) in PRE with and without $ZnSO_4$ in pH 4.5 and pH 7 at (2-4 °C) (n=3 ± SD) the plot shows >93% of punicalagin was kept at pH 4.5 and > 84% at pH 7 after 180 days storage time.

The chromatogram report of PRE ± Zn(II) indicated reduction of both α and β punicalagin peaks as shown in Figure 6-5 at pH 4.5 and Figure 6-6 at pH 7. It is shown in chromatogram of punicalagin, no significant effect Zn(II) addition to the PRE samples were observed since similar inhibition was observed in PRE with and without Zn(II) and no new peaks were introduced after Zn(II) combination.

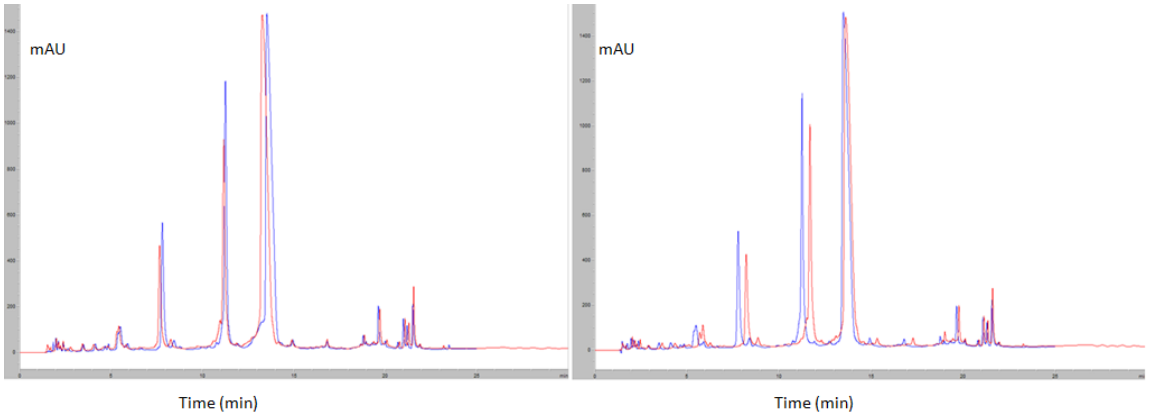


Figure 6-5 HPLC chromatograms showing α and β Punicalagin peaks in PRE individual sample (right chromatogram) and PRE+$ZnSO_4$ (left chromatogram). Blue line shows peaks at initial concentration at time zero, red line shows peaks after 6 month storage in pH 4.5 at 2-4 ˚C. The plot shows that punicalagin α and β peaks gave 94 % (left) and 93.2 % (right) of original area under peak after 6 months storage.

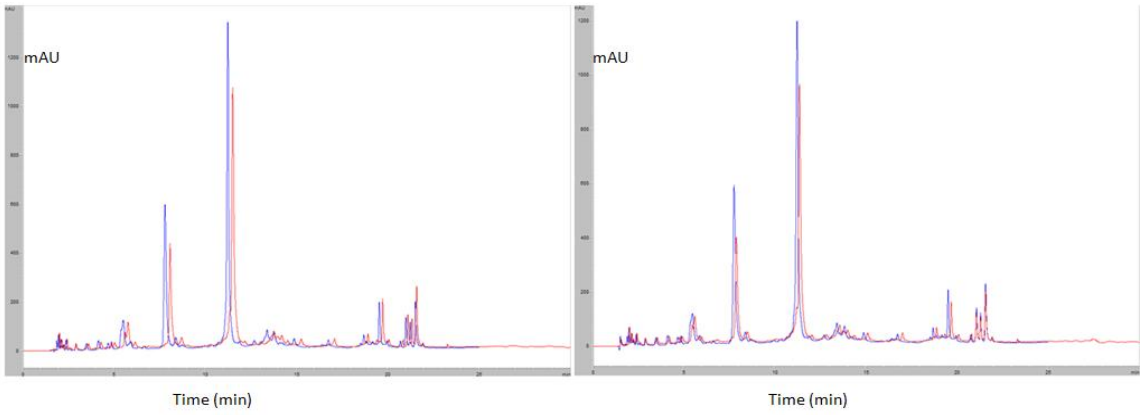


Figure 6-6 HPLC chromatograms showing α and β Punicalagin peaks in PRE individual sample (right chromatogram) and PRE+$ZnSO_4$ (left chromatogram). Blue line shows peaks at initial concentration at time zero, red line shows peaks after 6 month storage in pH 7 at 2-4 °C. The plot shows that punicalagin α and β peaks gave 85.2 % (left) and 84.7 % (right) of original area under peak after 6 months storage.

The lowest concentrations of punicalagin were found in samples with higher pH (7.0) and temperature 20 °C, and this was true for both PRE and PRE/Zn samples. As shown in Figure 6-6 the loss in punicalagin isomer was significant ($p > 0.05$) in all samples after being stored for 180 days at 20 °C. At 20 °C, the pH difference showed punicalagin content kept higher at pH 4.5 with punicalagin percent of 75.2 % and 75.9 % than pH 7 with 70.8 % and 69.3% of punicalagin recovered from PRE and PRE/Zn samples accordingly after stored for 180 days at the same temperature.

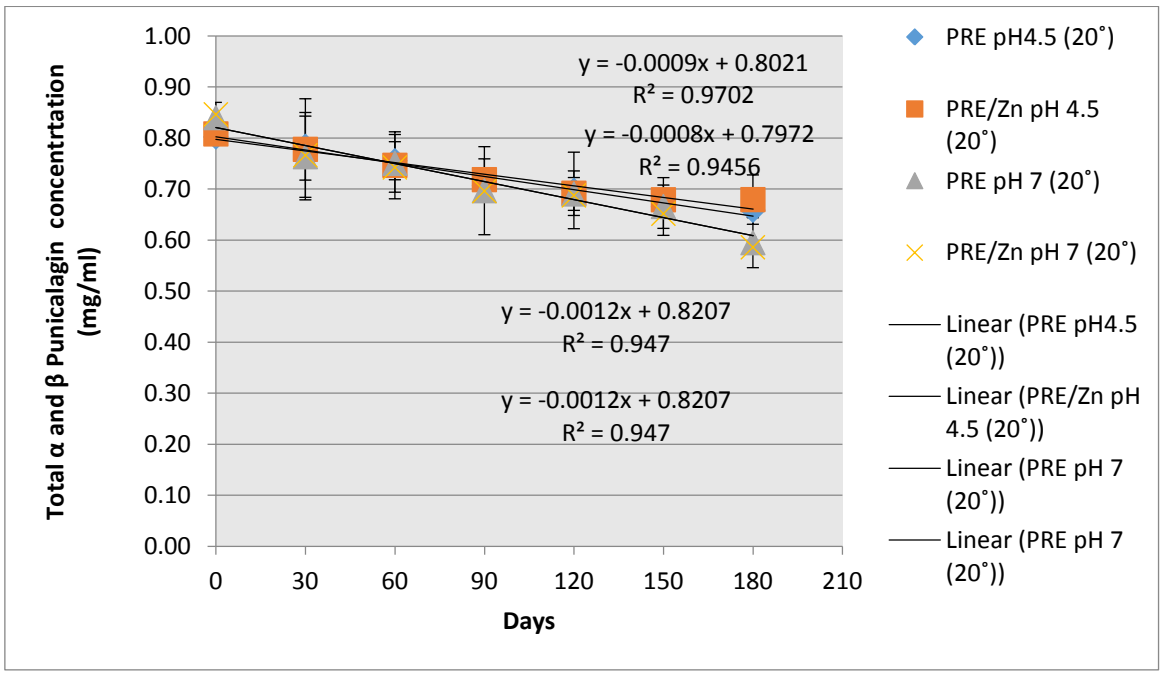


Figure 6-7 Total α and β punicalagin concentration (mg/mL) in PRE with and without $ZnSO_4$ in pH 4.5 and pH 7 at (20 ˚C) after 180 days storage time (n=3 ± SD). Plot shows significant loss in punicalagin content at both pH 4.5 and pH7.

PRE with and without Zn(II) chromatogram reports showed a significant inhibition of area under peak of punicalagin α and β when temperature was 20 °C and pH 4.5 as shown in Figure 6-8. And even lower inhibition in punicalagin area under peak was observed at same pH 7 and 20 °C as shown in Figure 6-9. At this temperature the punicalagin peak decreased, and an increase in another peak was observed at 22 minutes

and that was later identified as ellagic acid, which is one of the known degradants of the punicalagin molecule.

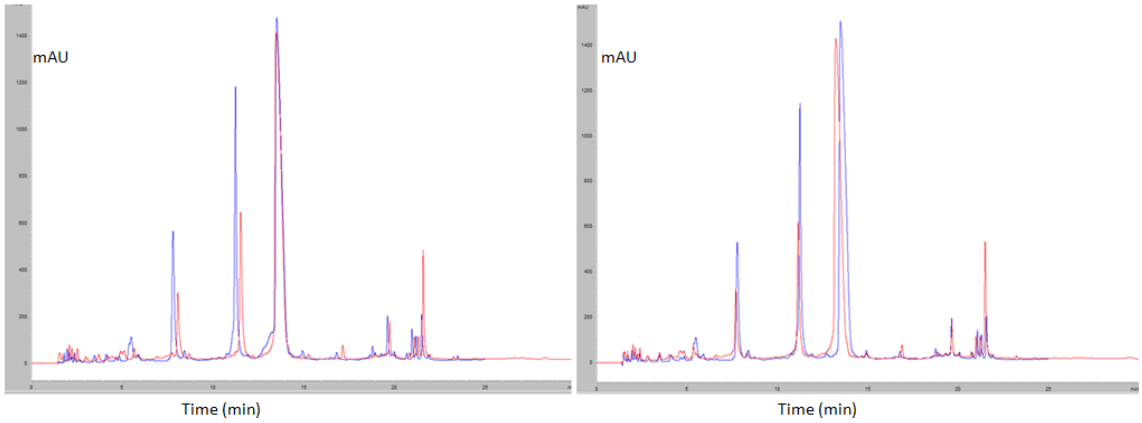


Figure 6-8 HPLC chromatogram showing α and β punicalagin peaks in PRE individual sample (right chromatogram) and PRE+$ZnSO_4$ (left chromatogram). Blue line shows peaks at initial concentration at time zero, red line shows peaks after 6 months storage in pH 4.5 at 20 °C. The chromatograms show that punicalagin α and β peaks gave 75.2 % (left) and 75.9 % (right) of original area under peak and increase in ellagic acid peak at 22 minutes after 6 months storage.

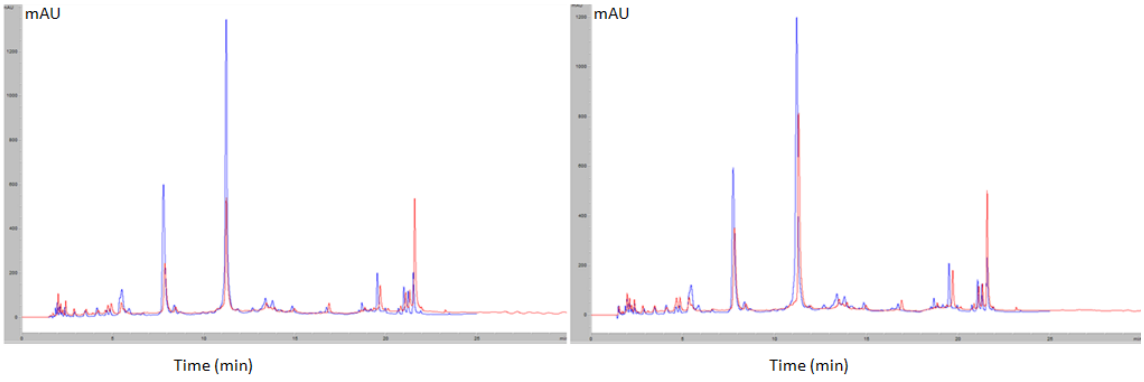


Figure 6-9 HPLC chromatogram showing α and β punicalagin peaks in PRE individual sample (right chromatogram) and PRE+$ZnSO_4$ (left chromatogram). Blue line shows peaks at initial concentration at time zero, red line shows peaks

after 6 months storage in pH 7 at 20 °C. The chromatograms show punicalagin α and β peaks gave 70.8 % and (left) and 69.3% (right) of original area under peak and increase in ellagic acid peak at 22 minutes after 6 months storage.

These results are in accordance with previous studies that showed a lower pH (3.5) maintained the stability of phenolic content in pomegranate peel extract during extraction (Qu et al. 2012; Qu et al. 2014). The study showed that both high temperature and pH value had a negative impact on the soluble phenolic content of pomegranate peel extract including both α and β punicalagin. Qu et al. (2012) suggests that the lower pH value (pH 5.0) used for pomegranate extraction, the higher concentration of a particular phenolic compound will be maintained in the sample. The study concluded that even lower pH level of around 3.5 should be used if the process is intended to keep punicalagins isomer stable. Another study advised the storage of punicalagin-containing sample at temperature 30 °C or lower (Lu et al. 2010). In a more general context, they also reported that punicalagin extract from pomegranate peel was relatively stable when kept at pH between 4 to 7, and when the pH <3.0 or pH >9.0 conditions were applied; there was a rapid decline in punicalagin level. However, in the later study statements were based on the stability of purified punicalagin concentration from pomegranate husk.

6.4.2 Accelerated stability testing

Figure 6-10 shows the percentage of total of α and β punicalgin in PRE samples with and without Zn (II) at 40 °C and 50 °C over a period of four weeks. The punicalagin isomer degradation was very extensive ($p < 0.05$) at 50 °C in which reached more than 94.77 % and 95.06 % loss in punicalagin content in both PRE and PRE/Zn samples respectively. In the same manner, samples stored at 40 °C showed significant loss in punicalagin content after four weeks, to give only 35.58 % and 35.22 % recovery of punicalagin in accordance to the initial concentrations of punicalagin present in PRE and PRE/Zn. These results are in accordance with previous research that showed extreme

temperature had a negative impact on punicalagin isomer. Research by Lu et al. (2010) indicated that some 8.6 % punicalagin had decomposed when heated for just sixty minutes at 80 °C.

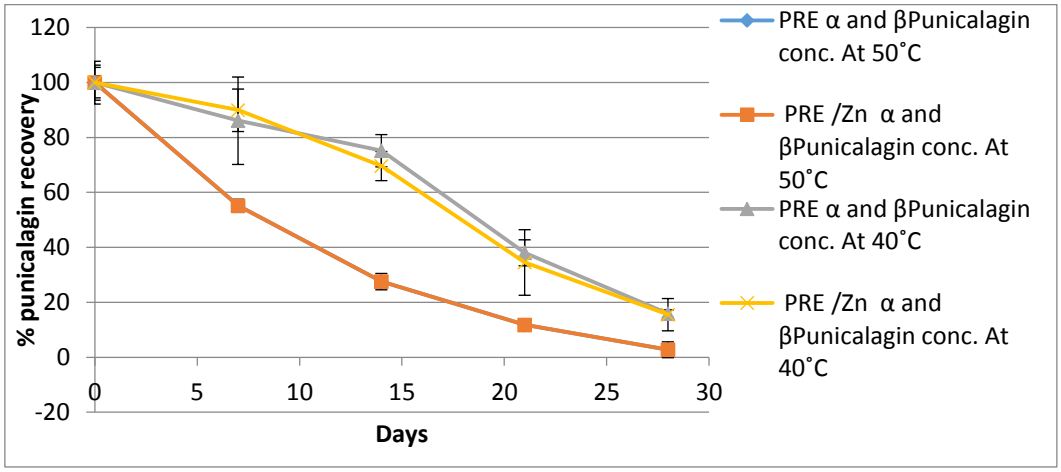


Figure 6-10 Accelerated stability profiles showing total α and β-punicalagin percentage in PRE samples in the absence and presence of Zn(II), pH 4.5 phthalate buffer at three temperatures: 40 °C and 50 °C over a 4 week period

From the previous accelerated stability results, a 0-order graph of punicalagin natural log conc. (mg/mL) versus the time (days) was made and degradation rate constant for PRE with and without $ZnSO_4$ at temperature 40 °C and 50 °C was determined.

In order to construct Arrhenius plot, the natural logarithmic values of the rate constants were plot against 1/T (absolute temperature, Kelvin). Table 6-2 shows the data needed for Arrhenius plot construction, including the predicted degradation rate of punicalagin at 20 ˚C and 4 ˚C. Ideally more than three stress temperatures are needed to construct Arrhenius plot, but here only two temperatures were used. The shelf life which is the time of the drug to be stable and have 90% of its active ingredient depend on order of the reaction and is calculated using following equation (Pugh 2002).

$$t_{90} = \frac{0.105}{k}$$

On this basis, a shelf life for storage at fridge was estimated at 1.31 and 1.34 years for PRE solution and PRE Zn(II) respectively in phthalate buffer solution pH 4.5. For storage at -20 °C the estimated shelf life for PRE±Zn at pH 4.5 was much greater, at approximately 71.9 years.

PRE				
Temperature in Celsius	**ln k value**	**Temperature in Kelvin**	**Inverse ln k**	**1/T**
50 °C	-2.48	323.15	0.084	0.003095
40 °C	-3.62	313.15	0.0267	0.003193
20 °C	Predicted -6.15	293.15	Predicted 0.002133	0.00341
4 °C	Predicted -8.43	277.15	Predicted 0.000218	0.00361
-20 °C	predicted -12.4	253.15	Predicted 0.000004	0.00395
PRE+Zn(II)				
	ln k value	**Temperature in Kelvin**	**k**	**1/T**
50 °C	-2.47	323.15	0.0846	0.003095
40 °C	-3.62	313.15	0.0268	0.003193
20 °C	Predicted -6.15	293.15	Predicted 0.002133	0.00341
4 °C	Predicted -8.45	277.15	Predicted 0.000214	0.00361
-20 °C	predicted -12.42	253.15	Predicted 0.000004	0.00395

Table 6-4 Accelerated stability data used to construct Arrhenius plot.

As shown in Figure 6-2 and 6-3, Arrhenius plot was made using the K_{50} and K_{40} values obtained from accelerated temperature study for both PRE and PRE/Zn. The degradation rate prediction of punicalagin at 20 °C was the same for both PRE and PRE/Zn 0.00213 mg/mL day^{-1}. On the other hand, the estimation of PRE and PRE/ Zn punicalagin degradation rate at 4 °C were equal, at 0.00218 mg/mL day^{-1} and 0.00214 mg/mL day^{-1} accordingly.

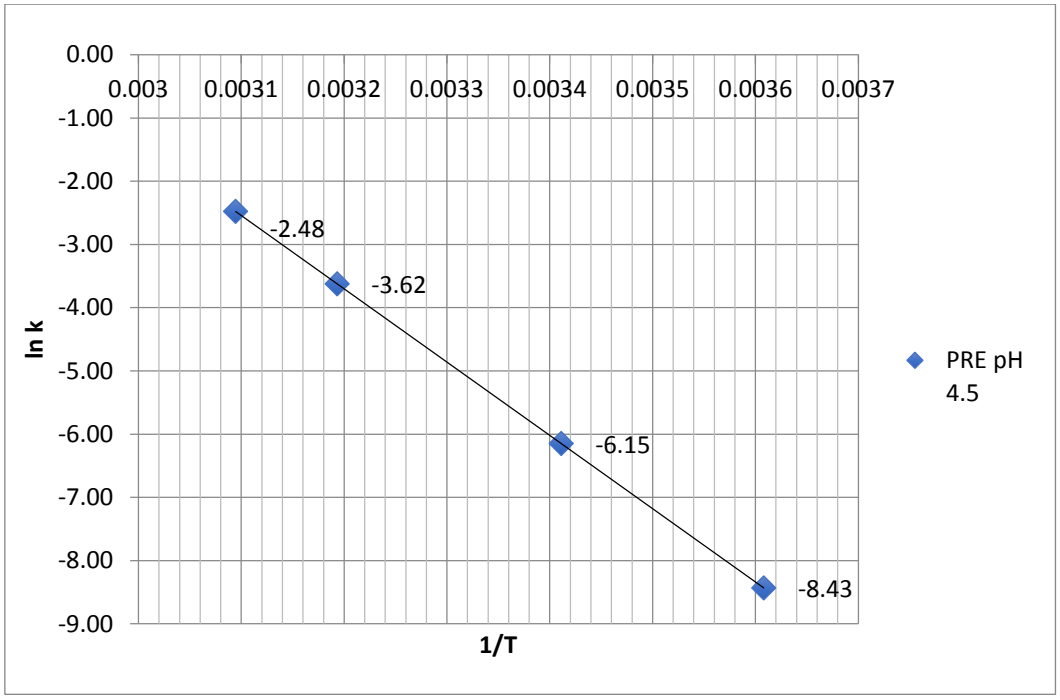


Figure 6-11 Arrhenius plot for PRE solution in pH4.5 (n=2, ± SD). ln k value of 50 °C, 40 °C and extrapolation was made to predict the k value at 20 °C and 4 °C plot against 1/T (Kelvin).

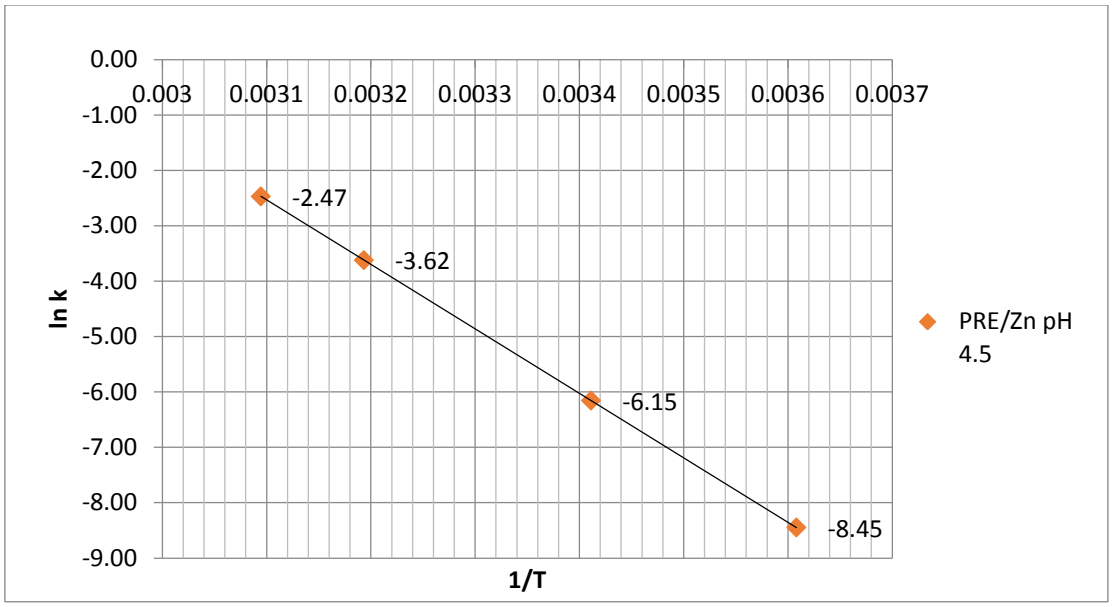


Figure 6-12 Arrhenius plot for PRE/$ZnSO_4$ solution in pH 4.5 (n=3, ± SD). ln k value of 50 °C, 40 °C and extrapolation was made to predict the k value at 20 °C and 4 °C plot against 1/T (kelvin).

Comparing the estimated degradation rate of punicalagin from the Arrhenius plot, the predicted k_{20} were 0.002133 mg/mL day^{-1}, while the real time degradation rate k_{20} as shown in Figure 6-6 was much lower at 0.0009 mg/mL day^{-1} and 0.0008 mg/mL day^{-1} for PRE and PRE/Zn at pH 4.5 accordingly. The difference can be explained that Arrhenius plot shows estimation but not the real degradation rate, and as pointed by Bajaj et al. (2012) the more stress temperatures tested, the more accurate the prediction of degradation rate.

6.5 Discussion

In the present study, the effect of temperature and pH value on PRE individual samples or combined with $ZnSO_4$ were under investigation throughout real time stability study for 6 month period time. Both variables at high, pH 7 and temperature 20 ˚C, showed a significant impact on the stability of punicalagin α and β isomer after stored for 180 days. At temperature of -20 °C the effect of pH was diminished since all samples showed >99% of punicalagin initial concentration after stored for 180 days. The largest loss in punicalagin content was seen at pH 7 and 20 °C to give 29.2 % and 30.7 % after 180 day, compared to 17.9 % and 16 % loss resulting from punicalagin at pH 4.5 and 20 °C. Comparing the last results together, the pH difference at 20 °C, showed a significant effect ($p < 0.05$) and the % loss of punicalagin after 6 months, was almost half with lower pH 4.5. In accordance with the present results, previous studies have demonstrated the stability of liquid phenolic extracts produced from pomegranate peel over a period of 180 days and under three storage temperatures (4, 20 and 37 °C). The storage at higher temperature was found to have a negative impact throughout the whole time of storage. Storing extracts at low temperatures is thus advised to uphold an acceptable level of quality and stability. In that study, products stored at 4 °C for up to 180 days, retained 67 % of the initial total soluble phenolic content (Qu et al. 2014).

Moreover, other research also investigated the stability of purified punicalagin under a variety of conditions. Findings indicated that over 8.6 % punicalagin had decomposed when they were heated for just one hour at 80 °C. However, almost all of the punicalagin was stable at a constant temperature of 30 °C or lower. During the punicalagin extraction process, it is thus important to keep temperatures below 30 °C. This component was found to possess the most elevated absorbance at pH 3.0. Furthermore, it was relatively stable when stored at pH 4-7, in that a slowly decreased absorbance was identified. When the pH <3.0 or pH >9.0 conditions were applied however, there was a significant decrease in its absorbance at 260nm. No reactions were found between other metal ions and punicalagin, with punicalagin samples possessing

the same levels of absorbance when mixed with other metal ions as that of the punicalagin solution alone. Houston (2011) studied the stability of a prototype hydrogel which included the PRE and $ZnSO_4$ as active ingredient and he found that total punicalagin concentration showed no loss after being stored for 12 months occluded from light and air stored at room temperature and pH 4.5. Furthermore, the positive effect of lower pH value obtained in our results broadly agreed with the previous reported study by Akbarpour et al. (2009), that PRE phenolic stability is greater at lower pH value.

Next, the results are considered regarding to the stability of punicalagin in presence of metal combination Zn(II). In our results, individual PRE punicalagin content were compared the combined PRE with $ZnSO_4$ and no negative or positive affect were seen ($p > 0.05$) at PRE after addition of $ZnSO_4$ and that remain true for all tested pH and temperature condition. This was pointed out by Houston (2011), when comparing PRE combinations with either $FeSO_4$ or $ZnSO_4$. Analysis of punicalagin through HPLC, showed that combining $FeSO_4$ with PRE results in new unidentified peak and as the $FeSO_4$ concentration increased, the punicalagin peak decreased and the new peak increased. Redox reaction between the Fe^{2+} + punicalagin → Fe^{3+}, and black by product resulted from the combination. It was speculated that the new peak must be due to the formation of a complex between PRE (ie punicalagin) and Fe(III).

From our results, the addition of $ZnSO_4$ to PRE showed no additional new peak in the HPLC chromatogram of punicalagin and no peak was identified and that could be attributed to the stability of Zn(II) ion to resist reaction. Another paper investigated the effect of multiple metal combinations on the punicalagin chromatogram and eight different metal ion solutions was investigated, including Na^+ (NaCl), K^+(KCl), Ca^{2+} ($CaCl_2$), Cu^{2+}($CuCl_2$), Zn^{2+}($ZnCl_2$), Mg^{2+}($MgCl_2$), Fe^{3+} ($FeCl_3$), and Al^{3+}($AlCl_3$). Only two metal ions, Cu^{2+} and Fe^{3+}, showed a rapid decline in the punicalagin's absorbance and through alterations to its molecular structure while no such alteration was made with Zn^{2+}. The authors (Lu et al., 2010) concluded that it is important not to use iron or

copper vessels during the process of extracting and storage of punicalagin samples. However, from our perspective, it can be concluded that Zn(II) does not form a significant stable complex with punicalagin, supporting the chemical stability of such admixtures and indicating a much different mechanism of microbicidal action of PRE/Zn compared to PRE/Fe.

Since the real time stability showed relatively lower degradation of solution at pH 4.5 with PRE ±Zn solution at 4 and -20 °C compared to room temperature, accelerated stability was performed to predict the possible shelf life (10% active drug loss) and the shelf life estimated for PRE and PRE/Zn at pH 4.5 was up to 1.3 years at fridge and 71.9 years at freezer storage condition. These are useful timescales for a marketed product.

Degradation mechanism

As mentioned in the Introduction, the punicalagin molecule is a large molecule comprised of 3 major sub-units, or moieties: gallagic acid, glucose and ellagic acid. Seeram, et al. 2005, reported that punicalagin degradation involves liberation of ellagic acid, and punicalin (gallagic acid + glucose) as shown in Figure 1-2. As shown by accelerated stability testing the higher temperature at 50 °C gave significant increase to the degradation rate value with 0.084 mg/mL day^{-1} and 0.0846 mg/mL day^{-1} when compared to the k_{40} with 0.0267 mg/mL day^{-1} and 0.0268 mg/mL day^{-1} at PRE and PRE+ Zn(II) ($p < 0.05$). This indicates that higher temperature catalyses the kinetics of punicalagin isomerisation in PRE ± Zn(II). Examination of the HPLC chromatograms obtained at 20 °C revealed that when punicalagin levels decreased, there was a simultaneous increase another peak at the chromatogram – that was identified as ellagic acid as shown in the chromatogram at Figures 6-8 and 6-9.

From the data revealed in real time stability, high temperature level at 20 °C catalyses the degradation of punicalagin as it was observed at the accelerated stability testing. Figure 6-9 showed that combined high pH at 7 and high temperature at 20 ˚C catalysed the degradation even more to give highest decrease in punicalagin area under peak and

increased liberation of ellagic acid. Critically, the degradation rate was not affected by presence of Zn(II), and that was shown by chromatogram after addition of Zn(II). PRE chromatogram of same concentration shows same punicalagin isomer peaks when compared to PRE/Zn combination as shown in the Figure 6-13.

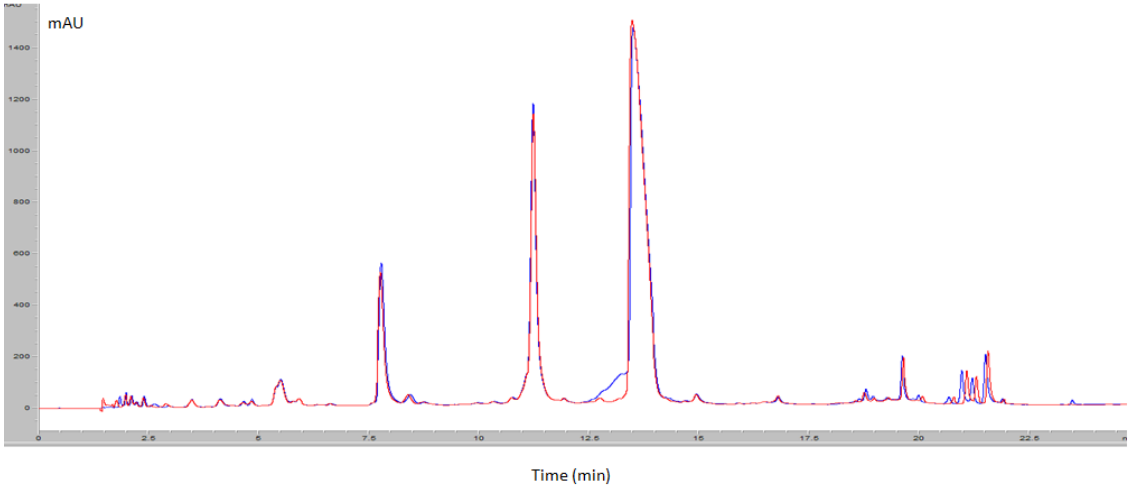


Figure 6-13 HPLC chromatogram showing α and β punicalagin of PRE alone (blue line) and PRE with ZnSO4 (red line). The chromatogram shows no new peak introduction and no alteration of the original peaks of PRE chromatogram after adding Zn(II).

6.6 Conclusion

Pomegranate rind extract solution with and without $ZnSO_4$ was examined for stability as determined by means of punicalagin loss after 6 month storage at two pH value (pH 4.5, pH 7) and three storage temperature (-20 °C, 2-4 °C, 20 °C). The main conclusions from this chapter are:

1. Both higher temperature and pH value had a significant impact on the degradation of punicalagin (α and β) content after 180 days storage time period.

2. At temperature ≤ 4 °C , punicalagin was recovered at extent >90 % of initial concentration at pH 4.5 after 180 days storage time period.

3. Overall PRE and PRE/Zn solution showed acceptable stability and recovery percent of initial punicalagin when stored at pH 4.5 and stored at fridge or cooler temperature.

4. The absence of additional peaks in HPLC chromatograms suggest no complexation between PRE and Zn at both pH 4.5 and pH 7

5. Shelf life estimation (90% of active drug retained) of PRE and PRE/Zn solution at pH 4.5 was 1.3 and 1.34 years when stored in the fridge (2-4 °C) and more than 71.9 years when stored at freezer (-20 °C). These are useful timescales for a commercial product.

Chapter 7: Preparation of topical hydrogel formulations containing PRE and $ZnSO_4$

7.1 Introduction

Wound infection is common problem that occur in wound healing process. If the wound healing process is prolonged > 12 weeks then it can be defined as chronic wound (Harper, Young and McNaught, 2014). Treatment of chronic wounds is a process that consists of multistep. These steps can be known by the TIME acronym. Initially the damaged non-viable tissues (T) and debris are taken away by scissor or surgical debridement. Then, infection (I) at the wound site should be treated with antimicrobial and anti-inflammatory agents. Next, moisturizing (M) the area of the wound can be done by use of a suitable dressing. Final step is addition of selected therapy such as growth factors in order to promote the formation of granulation tissue and epithelialization (E) process (Demidova-Rice et al. 2012).

Skin and wound antiseptics and irrigation treatments are very important to the healing process itself and mostly importance in non healed or infected wound to sanitize and remove superficial debris or excessive amount of exudates or any substances and leftover from previous dressing (Main 2008). The first modern use of wound antiseptic use was in 1865 by Joseph Lister, when reported the use of carbolic acid on wound involved in compound fracture (Badoe 1994; Salami et al. 2006). For more than a century, numerous antiseptics have been introduced for wound cleansing and irrigation purposes. Recently a considerable literature has grown up around the importance role of using antiseptic in wounds and some has defined it as a detrimental to the healing process (Drosou et al. 2003).

For chronic wound, antiseptic and cleansing agents should be used in routinely basis to decrease the risk of persistence wound infection. Certain properties should be considered in those agents. First and most important is to decrease the infection risk by decreasing microbial count at the infection site. Additionally antiseptic agent should be non-toxic to human and don't cause any sensitivity reaction. Finally those agent should be in reasonable coast which will make it easy available an accessible to use (Main 2008). A

variety of antiseptic classes has been introduced such as: alcohols, biguanides, bisphenols, iodine compounds, quaternary ammonium compounds, silver compounds, chlorine compounds, and peroxygens (Drosou et al. 2003). One of the widely used antiseptic agents in wound is chlorhexidine from the biguanides class and it is commercially available in two concentrations 0.05 and 4%.

Among practicing nurses, anecdotal evidence reported by Main (2008) that chlorhexidine 4% is most often used because of its availability and widely used as hand sanitizer. Although the main indication of the 0.05% is to irrigate the wound and 4% is clinically indicated as hand sanitizer and not to be applied on body cavities. Chlorhexidine use as antiseptic in wounds has been controversial despite its strong activity as an antimicrobial agent with broad spectrum to kill Gram positive and Gram negative bacteria, fungi and viruses (Sibbald et al. 2000), the cytotoxicity has been in question in many of the literature. Chlorhexidine was one in several antiseptics that has been studies by Brennan et al. (1985) using rabbit ear champers model for wound healing. The study investigating the effects of several antiseptic agents, including chlorhexidine 0.05%, on granulation tissue used a rabbit ear chamber which is a technique for in vivo microscopic studies of cells and tissues including revascularisation process that appear in wound healing process. Change and toxic effect was seen by laser Doppler in capillary circulation at all contact time with chlorhexidine from five minute and up to 60 minutes. The author has come to emphasise that the balance between antibacterial activity and high toxicity of chlorhexidine is in fine line. Additionally, wound-healing properties has been an issue with chlorhexidine since the wound healing was delayed in reported study that compared three antiseptic and irrigation agents effect on wound healing process (Salami et al. 2006). Wound healing time was longer when chlorhexidine dressing was used in comparison to tap water and Normal saline dressings. The *in vivo* study involved the evaluation of 2cm x 2cm wound healing on Wister rats. The study showed a significant decrease in wound healing time with chlorhexidine group with almost 27 days for complete healing while with tap water and normal saline it was 17 and 16 days respectively.

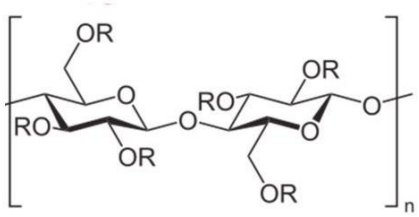
Figure 7-1 Commercially available chlorhexidine-based sanitisers at concentrations of 0.05% (Baxter antiseptic solution) and 4% (Hibiscrub skin cleanser).

In this research, preparation of a prototype of topical formulation to be used as wound antiseptic was carried out. The aim of this chapter was to develop a simple liquid formulation and test for physical appearance, rheological profile, wettability and drug content stability. The main criterion is a fluid formulation that resists excessive run-off, once applied to the infection or wound surface, by inclusion of a thickening agent.

7.1.1 Thickening agents

Thickening agents, or viscosity modifiers, can be described as substances which increase its viscosity of a liquid formulation without substantially modifying its other properties. Thickening agents are (with the exception of fumed silica) long chain polymers that increase viscosity in proportion to the amount added. This can provide 'body', improve suspending action, as well as provide enhanced adhesion properties and reducing dose 'run-off'. Three thickening agents were tested in this work.

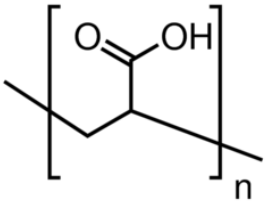
HPMC 856 is a hydroxypropyl methylcellulose, and the abbreviated name hypromellose is often used. Chemically it is semi-synthetic soluble methylcellulose ethers. HPMC is used as a thickening agent, binder, film former, and hydrophilic matrix material and is available in various viscosity grades ranging from 4000–100,000 mPa s.



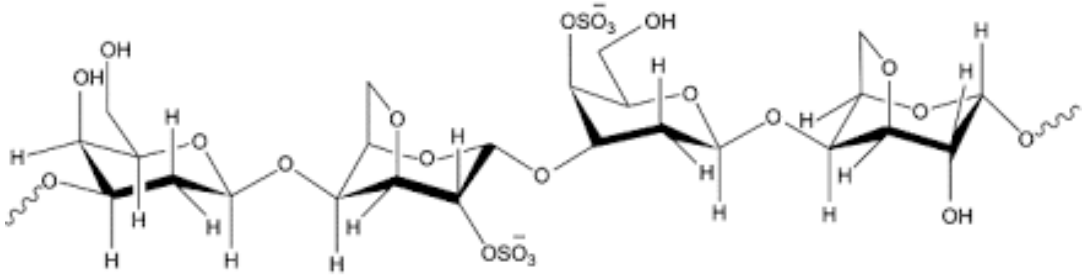
R = –H, –CH_3, –CH_2–CHOH–CH_3

HPMC

Carbopol 974 All Carbopol® polymers are high molecular weight, crosslinked polyacrylic acid polymers. Carbopol 974 is a homopolyer of medium crosslink density, which provides medium-high aqueous gel viscosity. Polyacrylic acids, such as Carbopol® polymers must be neutralized in order to achieve maximum viscosity. Once a neutralizer such as NaOH is added to the dispersion, thickening gradually occurs, with a maximum seen at a pH 6.0 - 7. 0. The concentration of Carbopol® can be increased in order to obtain high viscosity at pH values below 5 and above 9 (R Varges et al 2019; Lubrizol 2020).



Carrageenan Carrageenan is a natural product that is generally extracted from marine red algae. Carrageenan is composed of sulfated galactans that are mostly 1,3-linked β-d-galactose and 1,4-linked α-d-galactose.



They are widely used in many cosmetic and topical formulations because of their thickening and gelling properties (Stiger et al. 2016)

7.1.2 Rheology

Evaluation of flow properties and particle behaviour in pharmaceutical formulation can be studied by rheology test (Mastropietro et al. 2013). Viscosity is essential when analyzing any flow behaviour of materials of fluids and semisolid preparations. Early on in (1687) Isaac Newton described the relationship in his Newton's viscosity law's states: "the resistance which arises from the lack of slipperiness of the parts of the liquid, other things being equal, is proportional to the velocity with which the parts of the liquid are separated from one another" (Tanner and Walters 1998). When shear (γ) rate (γ) is plotted against shear stress (σ) in fluids that obey Newtonian law, the relationship will show a linear line originated from zero as shown in Figure 7-2. Water is an ideal example of Newtonian flow, it shows a linear relationship between shear rate with the applied applied shear stress. The gradient coefficient in newton law is the viscosity coefficient (η) and its steady in this flow behaviour.

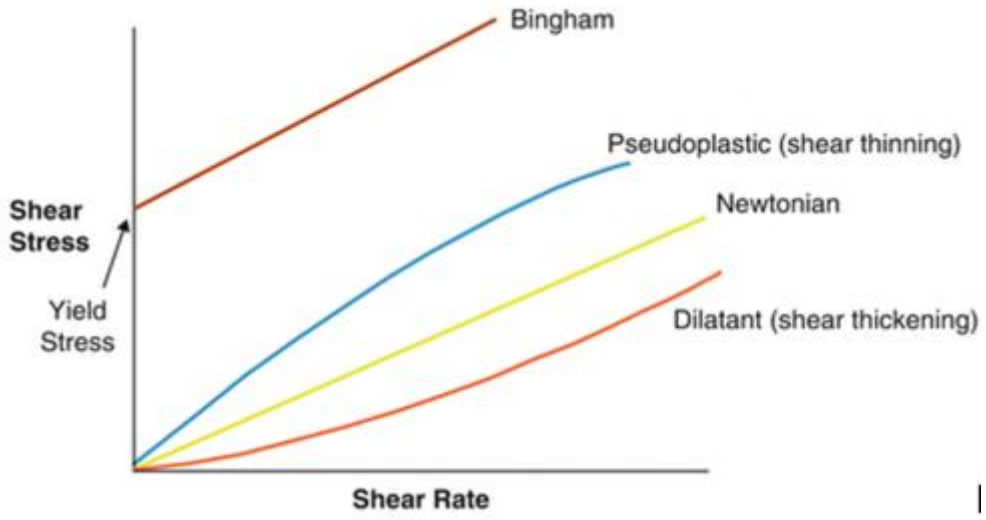


Figure 7-2 Shear stress as function of shear rate in different flow profile (George and Qureshi 2013)

Non-Newtonian flow generally does not include simple solution, but has complex mixtures such as gel, cream, pastes, or polymeric solutions. The material behaviour in

non-Newtonian flow, can be classified according to the flow behaviour into mainly four different categories. First, Bingham plastic materials show a non-Newtonian flow that has a linear relationship between shear stress and shear rate yet it requires a yield stress (τ_y) at first to flow. Once the flow begins, the Bingham plastic behaves as Newtonian flow as shown on the curve Figure 7-2. Secondly when increasing the shear stress the resistance to flow can increase or decrease when the resistance of the material to flow with the increase they are called shear thickening or *dilatant* material. As shown on the curve, shear thickening flow is originated from the zero and doesn't require a yield stress (τ_y). On the contrary, another non-Newtonian flow that is also start from the origin, is the shear thinning or *pseudoplastic* flow that showed decreased resistance to flow once the shear stress start with increasing the shear rate. Apparent viscosity in both the shear thinning and shear thickening is always recorded and the change increase or decrease is also must be quoted with increased shear rate but the viscosity still to be independent at all shear time. Finally, the time-dependent with continuous shear type. In this type, when the applied shear stress end, the material continues to shear either by increased or decreased shear rate and doesn't return to its primary rheological state. A distinctive phenomena happen with these type of flow behaviour, is the hysteresis loop in which indicate that breakdown in the structure took a place. The breakdown extent is proportional to the hysteresis loop area. This also be known by thixotropy in which a temperature dependent material can loos its consistency under shearing stress and gradual recovery happened when the stress is removed.

7.1.2.1 **Cone and plate**

This viscometer uses the cone and plate technique in which the tested subject is applied on the centre of fixed horizontal plate on the lower part of the apparatus while the cone with very on the upper part of the machine will rotate in spinning from the above in direction to the plate and shear stress will be measured by torque sensor. The cone movement either in constant speed and shear stress requires will be recorded, or the cone

movement will be in constant stress and shear rate can be developed (Barnes, Hutton and Walters 1989).

In a small-angle cone-and-plate rheometer Viscosity in (Pa s) units could be calculated by the following equation:

$$\eta = \frac{3\mathrm{W}\ \mathrm{T}}{2\pi\ \mathrm{r}^3\ \alpha}$$

Where T is the torque, Ꞷ is the angular velocity of the plate, r is the cone radius and α is refer to the angle between plate and cone.

7.1.3 Viscosity

Viscosity is a property of a fluid (materials that flow) and may be defined as: resistance to deformation at a given rate. For liquid and in particular liquid formulations, it is generally conceptualised in terms of ‘thickness. As an example, syrup has a higher viscosity than water or syrup is thicker than water. Viscosity can be conceptualized as quantifying the frictional force that arises between adjacent layers of fluid that are in relative motion. For instance, when a fluid is forced through a tube, it flows more quickly near the tube's axis than near its walls. In such a case, experiments show that some stress (such as a pressure difference between the two ends of the tube) is needed to sustain the flow through the tube. This is because a force is required to overcome the friction between the layers of the fluid which are in relative motion: the strength of this force is proportional to the viscosity. A fluid that has no resistance to shear stress is known as an ideal or inviscid fluid. Zero viscosity is observed only at very low temperatures in superfluids. Otherwise, the second law of thermodynamics requires all

fluids to have positive viscosity such fluids are technically said to be viscous or viscid. A fluid with a high viscosity, such as pitch, may appear to be a solid.

7.1.4.1 **Vibrational viscometer**

There are numerous methods to determine viscosity. Kinematic methods include falling-ball and Ostwald viscosity tubes, and dynamic viscosity is typically determined with a rotational (Brookfield) viscometer. A relatively new approach is the sine wave vibrational viscometer, and here a A&D SV10 Vibro Viscometer was used, which works by sending uniform frequency vibrations out from two sensor plates which sit submerged in the test fluid. The thicker (more viscous) the substance, the more driving current is required to maintain the vibration frequency, the viscosity is then worked out by the positive correlation between the driving electric current and the viscosity, in SI units of Pascal second (Pa s).

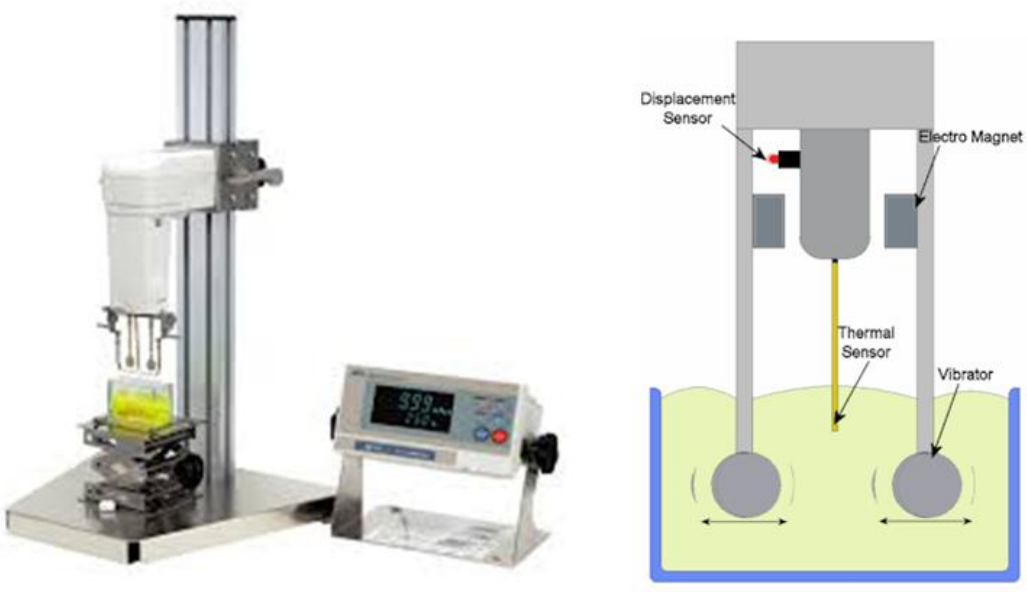


Figure 7-3 Left: A&D SV10 Vibro Viscometer; Right: schematic of the vibrational viscometer sensor.

7.1.5 Contact angle measurement and topical drug delivery

Contact angle is a quantitative measure of how a droplet of a liquid (formulation) wets a contact surface (skin or wound). Contact angle is a geometric parameter, being the angle formed by a liquid at the three-phase boundary where a liquid, gas and solid intersect. The Young equation describes the balance at the three-phase contact of solid-liquid and gas.

$\gamma sv = \gamma sl + \gamma lv \cos \theta Y$

where γ_{sv}, γ_{lv}, γ_{sl} is the surface tension of solid surface, liquid surface, and solid–liquid interface, respectively and θ is the inherent contact angle on the solid surface.

The interfacial tensions, γsv, γsl and γlv, form the equilibrium contact angle of wetting or Young contact angle. Figure 7-4 shows that a low contact angle indicates the liquid spreads onto the surface, or high wettability. On the other hand, a high contact angle indicates poor spreading and poor wettability. A contact angle of < 90° shows the liquid wets the surface, 0° contact angle represents complete wetting. If contact angle is > 90°, the surface is said to be non-wetting with that liquid and therefore its surface hydrophilicity or lipophilicity, ascribed to free fatty acids (Alkhyat et al. 2014).

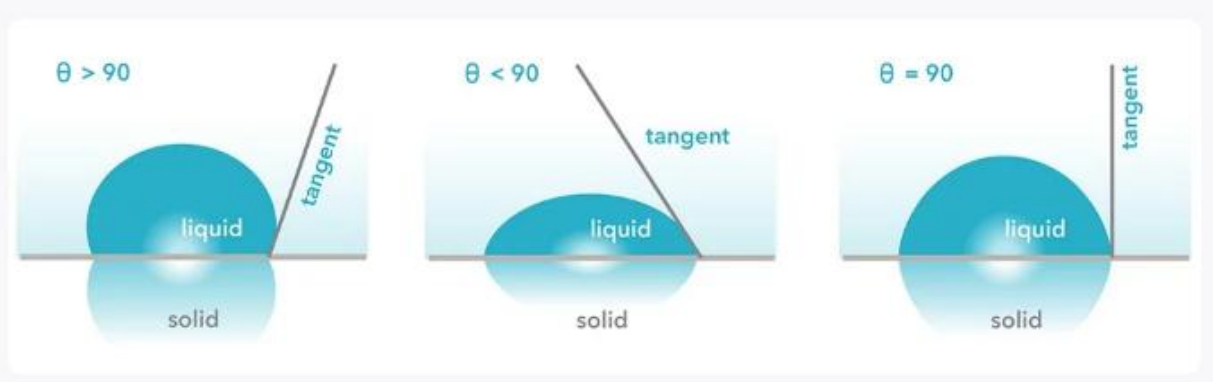


Figure 7-4 contact angle measurement of different material with different contact angles.

Contact angles can be divided into static and dynamic angles. Static contact angles are measured when droplet is standing on the surface and the three-phase boundary is not moving. Static contact angles are utilized in quality control and in research and product development. When the three-phase boundary is moving, dynamic contact angles can be measured, and are referred as advancing and receding angles. Contact angle hysteresis is the difference between the advancing and receding contact angles. Contact angle hysteresis arises from the chemical and topographical heterogeneity of the surface, solution impurities absorbing on the surface, or swelling, rearrangement or alteration of the surface by the solvent. Advancing and receding contact angles give the maximum and minimum values the static contact angle can have on the surface.

McGinn et al. (1968) performed contact angle analysis for droplets of water on viable human skin coated with a range of treatments. Clean and degreased skin gave a high contact angle with water (> 100 °) whereas paraffin, stearic acid, a lanolin derivative, a quaternary ammonium salt, and hydrophilic and hydrophobic silicone treated skin reduced the water droplet contact angle when applied to the skin.

Hagens et al. (2007) used contact angle analysis to determine how the substantivity of sunscreen formulations is affected by the wash-out rate of ultraviolet-absorber and -reflector compounds in water. It was found that formulations that give CAs above 30 degrees may be categorised as waterproof.

Static and dynamic in vitro contact angle measurements were used to determine the ability of a polymeric spray formulation sunscreen film to prevent outside water from reaching the skin-like substrate surface. It was found that acrylates/octylacrylamide copolymer enhanced the occlusive properties of continuous ethanol-based sunscreen (EBS) sprays, which was demonstrated via the in vitro static and dynamic contact angle measurements and confirmed by the *in vivo* TEWL studies (Dueva-Koganov et al. 2012).

Skin tribology (the study of friction) can also be used to monitor changes in skin, induced by application of exogenous formulations such as moisturisers, in terms of eg friction coefficient (Alkhyat et al. 2014), and the effect of skin surface roughness on contact angle has also been studied (Bhushan et al. 2012).

7.1.5.1 **Contact angle measurement and topical drug delivery**

The higher the wettability of a formulation, the greater the contact area and thus greater the possibility of a drug being released in order to exert its topical effect (Singh et al. 1968). Azarbayjani et al. (2010) studied the effect of surface tension and contact angle on the permeation of haloperidol across human skin using cyclodextrin derivatives. Contact angle measurements showed that vehicles with higher skin wettability decreased contact angle therefore increased the contact of the drug with the skin surface and therefore resulted in higher drug permeation across human epidermis.

7.2 Objective and Aims

To prepare and characterise several topical formulations that contain PRE and $ZnSO_4$.

- Formulations containing the following viscosity enhancers: carrageenan, HPMC and Carbopol.
- Determine physical appearance of formulations
- Determine viscosity properties at room temperature and 32 °C
- Determination of formulation/skin wettability by contact angle measurement and adhesion by measuring run-off.
- Determine drug content and stability profile of selected formulation

- Confirm the bactericidal activity of the formulations containing PRE and Zn(II) against planktonic *S. aureus*.

7.3 Materials and methods

7.3.1 Materials

Materials used in this chapter previously detailed in Chapter 2, Section 2.1.

7.3.2 Preparation of Zinc and PRE solutions

Preparation of PRE and $ZnSO_4$ was carried out according to the previously detailed solution preparation detailed in Chapter 2, Section 2.2.1 all prepared samples used phthalate buffer pH4.5.

7.3.3 Hydrogel formulation using Methocel 856

A hot-cold method was used to prepare this hydrogel. 2.5 g of Methocel 856 N hydroxypropyl methylcellulose (HPMC) was added to 40 mL of hot water at 80 °C with stirring under magnetic stirrer at 350 rpm until all particles were completely dissolved. Then, 2 g of polyethylene glycol PEG was added with continuous stirring until dissolved. Next, 60 mL of cold or iced water was added to the mixture with continuous stirring. The formulation was then cooled over night at 2-4 °C. The hydrogel was prepared with PRE and $ZnSO_4$ in combination and in individual formulations. PRE tested concentrations were as follows: 0.1, 0.5, 1 and 20 mg/mL while $ZnSO_4$ concentrations were: 0.1, 0.25 and 1M.

7.3.4 Hydrogel formulation using Carbopol 974

Stock solution of polymer gel was prepared in double concentration to the final desired one (Dodov et al. 2005). One g of Carbopol 974 was added to 100 mL of deionised water and mix well under magnetic stirrer at 350 rpm for a 4 hour time period until clear gel and completely dissolved particles was achieved. The pH of the formulation was adjusted using 0.1 NaOH.

To prepare the formulations a stock solution of PRE and $ZnSO_4$ prepared in advance in phthalate buffer solution pH4.5. Then, for example to prepare the polymer $ZnSO_4$ combination, 10 mL of $ZnSO_4$ stock solution was added slowly to 10 mL of the stock Carbopol 974 gel with continuous stirring at 350 rpm. Each formulation was prepared in separate by adding 10 mL of stock polymer to 10 mL of PRE and $ZnSO_4$ in different concentrations and give final Carbopol 974 of 0.5 % (w/w) concentration. PRE tested concentrations were: 0.1, 0.5, 1 and 5 mg/mL while $ZnSO_4$ concentration: 0.1, 0.25 and 1M. The formulations were stored at 2-4 °C.

7.3.5 Topical gel formulation using carrageenan

Carrageenan gel was prepared by dissolving 1g of carrageenan in 100 mL of deionized water at temperature of 75 °C under continuous stirring at 350 rpm until complete dissolution was observed. Next the hot plate was removed and desired amount of zinc sulphate alone or in combination with PRE was added to the clear gel mixture. The formulation mixture was cooled to room temperature then stored at 2-4 °C.

7.3.6 Viscosity

Viscosity testing was performed using an A+D Vibro viscometer at St Mary's Pharmaceutical Unit, Cardiff. Approximately 35 mL of formulation was placed into the

sample well and the equipment switched to the on setting. Viscosity was tested at 32 °C and room temperature.

7.3.7 Determination of formulation/skin wettability by contact angle measurement

The instrument of choice to measure contact angles and dynamic contact angles is an optical tensiometer, however rudimentary measurements can also be made by eye using a protractor. Full thickness porcine ear skin was liberated from the underlying cartilage by blunt dissection using a scalpel. The hairs were then trimmed with electric clippers then shaved gently under running water using a Bic double bladed razor. Some of this skin was then subjected to heat treatment (55 °C for 1 min) in order to isolate epidermal sheets and epidermis-free dermis (Kligman and Christophers 1963). On a ceramic plate, the skin was cut into sections of 1 x 1 or 1 x 4 cm in order to receive 50 µL aliquots of the 4 test formulations previously detailed in Table 7-5; the skin was dry to the touch. Formulations were stored at room temperature or 32 °C prior to dosing onto the skin using a Gilson pipette. Images were then captured using a camera as per the set up in Figure 7-5, which were then imported into and measured on a computer monitor with a protractor in order to establish a contact angle as measured by protractor, n=3.

Figure 7-5 Contact angle camera set up for capturing side-on images of 50 μL quots of formulation on porcine skin.

7.3.8 Formulation stability

A stability study investigated the percentage punicalagin content of each formulation over a six months period. The PRE and $ZnSO_4$ final selected formulations were stored in sealed light-resistant containers stored at 2-4˚C. Drug content of punicalagin was quantified using HPLC analytical method described in Chapter 2, Section 2.2.2.1. As formulations are more viscous in nature than the solution, a dilution step was done at first and kept the same for all testing samples. Percentage drug punicalagin content was calculated according to the initial punicalagin concentration calculated using calibration curve of standard punicalagin Section 2.2.2.1.1 at real time data of 0, 3, and 6 month storage time. Experiments were conducted in triplicate (n = 3).

7.3.9 Confirming the bactericidal activity of formulations and solution containing PRE and Zn(II) against planktonic *S. aureus*

The encouraging microbiological data determined in Chapter 3 was obtained using PRE and Zn(II) solutions. Here we aimed to establish that synergistic bactericidal activity would be retained in the presence of the thickening agents, whilst also obtaining independent confirmation of activity of the solution. This part of the work was performed using samples sent to the laboratory of Dr. Wing Man Lau (School of Pharmacy, Faculty of Medical Sciences, Newcastle University, UK.). *S. aureus* (NU118) were obtained and isolated in Newcastle University, UK.

The effect of the PRE ± Zn(II) in phthalate buffer and three formulation on planktonic bacteria was studied against *S. aureus*. First on 96 well-plates, each well contains 100 µL of 1x 10^6 CFU/mL of *S. aureus* in brain heart infusion broth (BHI). 100 µL of the tested formulation was added in the well. The plate was then incubated at 37 °C for 48 h. BHI was added as positive control and no bacteria well was used as negative control. After 48 h, the supernatants were removed; the plate was washed once with 100 µL of PBS. 100 mL of 0.1 % crystal violet (CV) solution was added to all wells and incubated

for 15min at room temp. After 15 min, the excess CV was removed by washing three times with 100 μL PBS. 100μL of 7% acetic acid was added to dissolve the CV stain. The solution was then transferred to a new 96-microtiter plate for the absorbance detection at 570 nm (BioTek Synergy HT) with 8 times dilution. The three formulations were investigated by this method were: HPMC 856, Carbopol 974 and carrageenan, in addition to solutions containing different concentrations of Zn and PRE, as summarised in Table 7-1.

Test solution	**PRE conc (mg/mL)**	**$ZnSO_4$ conc. (M)**
$ZnSO_4$ 0.25 M	**0**	**0.25**
PRE 1mg/mL in phthalate buffer (pH4.5)	1	0
$ZnSO_4$ 0.5M in phthalate buffer (pH4.5)	0	0.5
PRE 2.5 mg/mL in phthalate buffer (pH4.5)	2.5	0
PRE 2.5 mg/mL +$ZnSO_4$ 0.5M in phthalate buffer (pH4.5)	2.5	0.5
PRE 1mg/mL+ $ZnSO_4$ 0.25 M in phthalate buffer (pH4.5)	1	0.25
HPMC 856 formulation	1	0.25
Carbopol 974 formulation	1	0.25
Carrageenan formulation	1	0.25

Table 7-1 Summary of test formulations and solutions used for the investigation of antibacterial activity against planktonic *S. aureus* (NU118).

7.4 Results

7.4.1 Hydrogel formulation using Methocel 856N

Clear gel was formed with HPMC 856N polymer alone formulations and that is in accordance with previous report (Dodov et al. 2005). Upon addition of $ZnSO_4$ up to the 0.25%, no change in the gel formulation was observed (Table 7-2). With high concentration of Zn(II) at 1M, the formulation showed complete separation between gel and liquid phase and white gel floating matter was formed. On the other side all tested PRE alone concentration up to 20 mg/mL showed thick gel formulation with slight yellow colour. The table below shows the different concentrations of $ZnSO_4$ and PRE that were incorporated using this method.

Formulation name	**HPMC 856 (w/w)**	**PEG (w/w)**	**$ZnSO_4$ (M)**	**PRE mg/mL**	**Physical appearance**
MA	2.5%	2%	0	0	Gel
MB	2.5 %	2%	0.1	0	Gel
MC	2.5 %	2%	0.25	0	Gel
MD	2.5 %	2%	1	0	Phase separation
ME	2.5 %	2%	0	0.1	Gel
MF	2.5 %	2%	0	0.5	Gel
MG	2.5 %	2%	0	1	Gel
MH	2.5 %	2%	0	20	Gel
MI	2.5 %	2%	0.25	1	Gel
MJ	2.5%	2%	0.1	0.1	Gel

Table 7-2 Physical appearance of different HPMC 856N formulations that include PRE and $ZnSO_4$ in combination or individually.

7.4.2 Hydrogel formulation using Carbopol 974

Carbopol alone polymer gave a clear gel with all $ZnSO_4$ tested concentrations as shown in Table 7-3. These results broadly agree with previous work reported by Keegan et al. (2007) in their assessment of zinc/Carbopol complex. When pH adjustment was made using NaOH 0.1M. At higher pH 5.5, adding PRE to the $ZnSO_4$ /Carbopol transformed the gel to a liquid state with precipitation due to an interaction.

Formulation name	Carbopol 974 (w/w)	$ZnSO_4$ (M)	PRE mg/mL	Physical appearance
CA	0.5%	0	0	Gel
CB	0.5%	0.1	0	Gel
CC	0.5%	0.25	0	Gel
CD	0.5%	1	0	Gel
CE	0.5%	0	0.1	Phase separation, white precipitate
CF	0.5%	0	0.5	Phase separation, white precipitate
CG	0.5%	0	1	Phase separation, white precipitate
CH	0.5%	0	5	Phase separation, white precipitate
CI	0.5%	0.25	1	Phase separation, white precipitate
CJ	0.5%	0.1	0.1	Phase separation, white precipitate

Table 7-3 Physical appearance of different Carbopol 974 formulations that include PRE and $ZnSO_4$ in combination or individually.

When the same method was repeated with lower pH (3.5) and adding 2 % PEG to the mixture, the result was viscous homogenous mixture. Formulation with Carbopol 974 contained PRE 1 mg/mL and $ZnSO_4$ was selected for further investigation.

7.4.3 Topical gel formulation using carrageenan

The carrageenan alone showed a clear gel formation and with added $ZnSO_4$ up to 0.25 M no change in the gel uniformity or the colour was observed (Table 7-4). This is in accordance with previous studies that have investigated use of carrageenan with $ZnSO_4$ as a gel based system (Chen et al. 2016; Fernández-Romero et al. 2012). In tested concentration addition of PRE up to 1 mg showed slight yellow colour formation with gel texture intact.

Formulation name	Carrageenan	$ZnSO_4$ (M)	PRE mg/mL	Physical appearance
Cg A	1%	0	0	Gel
Cg B	1%	0.1	0	Gel
Cg C	1%	0.25	0	Gel
Cg E	1%	0	0.1	Gel
Cg F	1%	0	0.5	Gel
Cg G	1%	0	1	Gel
Cg H	1%	0.25	1	Gel

Table 7-4 Physical appearance of different carrageenan formulations that include PRE and $ZnSO_4$ in combination or individually.

Finally, three selected formulations were chosen for further investigation alongside the simple phthalate buffer solution combination that contained PRE and $ZnSO_4$. Table 7-5 shows the concentration PRE and $ZnSO_4$ at each formulation and polymer percentage used.

Formulation	$ZnSO_4$ (M)	PRE mg/mL	PEG (w/w)	HPMC 856 (w/w)	Carrageenan	Carbopol 974	PRE (w/v)	$ZnSO_4$ (w/v)
HPMC 856	0.25	1	2%	2.5%	0	0	0.1%	7.19%
Carbopol 974	0.25	1	2%	0	0	0.5%	0.1%	7.19%
Carrgeenan	0.25	1	0	0	1%	0	0.1%	7.19%
Phosphate buffer pH4.5 solution	0.25	1	0	0	0	0	0.1%	7.19%

Table 7-5 Formulations selected for further investigation.

7.4.4 Viscometry

The viscosity measurements made on the 3 formulations and blank, are detailed in Table7-6. As expected, the unthickened blank gave the lowest reading and HPMC the highest; the general progression was: blank < Carbopol < carrageenan < HPMC. This trend was for both Room temperature and skin temperature (32 °C). In all cases, viscosity was clearly lower for 32 °C relative to room temperature, even though the temperature interval was quite small (i.e. 25 – 32 °C).

Temperature	Blank	Carbopol	Carrageenan	HPMC
25 °C (room temp.)	1.53 mPa s	2.09 mPa s	178 mPa s	1060 mPa s
32 °C (skin surface)	1.30 mPa s	1.81 mPa s	130 - 147 mPa s	4.5-9.8 mPa s *

Table 7-6 Viscosity in mPa s of Carbopol, carrageenan and HPMC formulation, and unthickened blank using an A&D SV10 Vibro Viscometer (performed by Kay Pothecary, Senior QC analyst, St Mary's Pharmaceutical Unit, Cardiff). *The HPMC gel was very watery with lots of clumps at 32 ˚C and it was difficult to get a stable reading.

Figure 7-6HPMC formulation showing at room temperature (left) and at 32 °C inside the viscometer sample well (right).

7.4.5 Contact angle on skin

Contact angle as measured for each formulation is summarised in Table 7-7. All formulations except carrageenan formulation showed contact angle < 90 °C which may indicate high wettability action. The side-on images captured of the skin are shown in Figure 7-7.

Contact angle θ, °	**PRE/Zn in phthalate buffer**	**PRE/Zn in Carbopol**	**PRE/Zn in Carrageenan**	**PRE/Zn in HPMC**
Full thickness/stratum corneum, RT	58 ± 3	88 ± 4	109 ± 2	86 ± 1
Full thickness/stratum corneum, 32 °C	60 ± 2	60 ± 1	95 ± 1	73 ± 2
Dermis, RT	44 ± 4*	72 ± 2	102 ± 2	83 ± 3
Dermis, 32 °C	29 ± 6*	50 ± 2	93 ± 3	75 + 2

Table 7-7 Contact angles, in degrees, for 50 μL aliquots of each formulation on porcine skin, (n=3 ± SD) * estimation, as formulation absorbed into tissue.

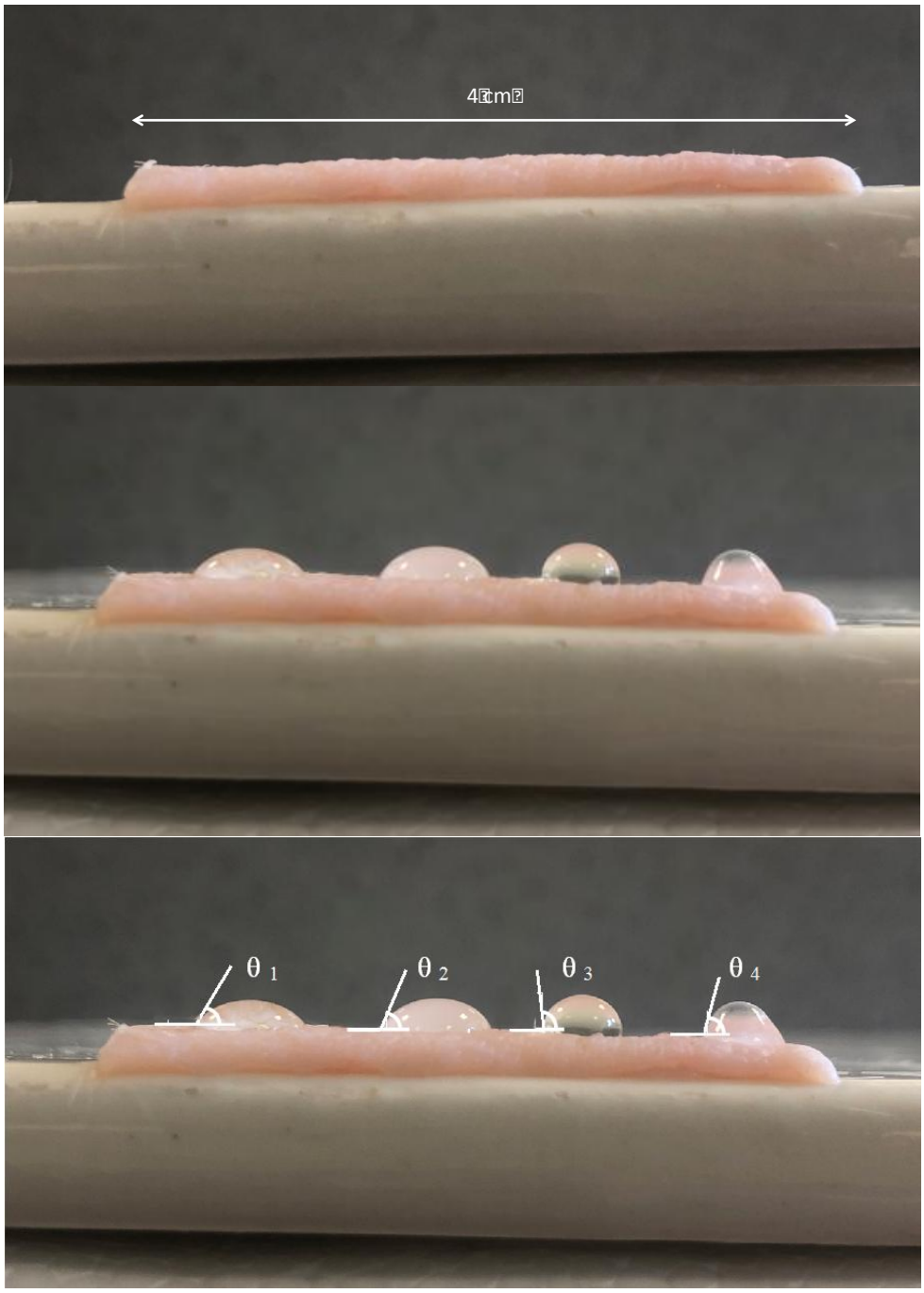


Figure 7-7 Side-on images of porcine full thickness skin. Upper: untreated. Middle: added 50 μL of PRE/Zn formulations (L-R) aqueous (θ_1), Carbopol (θ_2), carrageenan (θ_3) and HPMC (θ_4) at 32 °C. Lower: Formulation/ skin contact angles, θ for each formulation.

7.4.6 Stability and percentage drug content

The punicalagin drug content was used as a biomarker for the stability of the selected formulation (HPMC formulation, carrageenan formulation and Carbopol 974 formulation) over six-month storage time. Stability data obtained in Chapter 6 showed stability of the PRE + $ZnSO_4$ at a storage temperature of <4 °C. The formulations were stored at fridge for entire stability study. Punicalagin content in each formulation was calculated as % punicalagin content in relation to the initial punicalagin concentration at time = 0.

Figure 7-9 shows punicalagin content in all three formulations and stability in term of punicalagin content was kept > 90% over 6-months for all three formulations stored at 2-4˚C. The stability showed more than 95% of punicalagin was retrieved from two formulations (HPMC formulation and Carbopol 974 formulation), while carrageenan showed relatively lower punicalagin content of 93 % of after stored for 6-months.

This is in accordance with our stability results that has been revealed in Chapter 6, that stability of punicalagin in PRE/Zn in solution form will remain > 90% over six months storage at 2-4 ˚C.

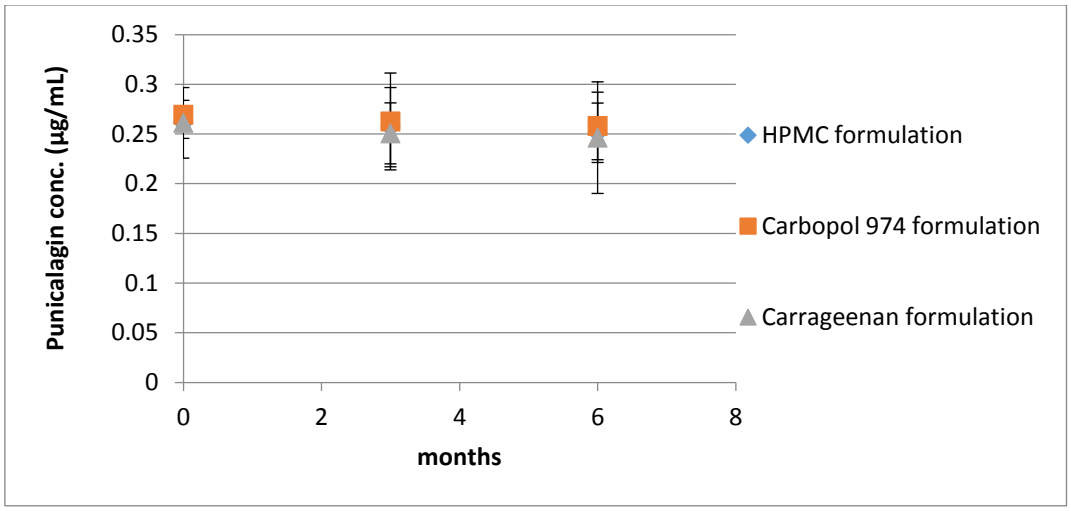


Figure 7-8 stability data showing punicalagin content in (µg/mL), the three formulations all contain (PRE 1 mg/mL + $ZnSO_4$ 0.25M): Carbopol 974, carrageenan and HPMC over six month storage at 2-4 °C. Data are expressed as mean punicalagin concentration (n=3±SD).

7.4.7 Conformation of bactericidal activity of formulations and solution containing PRE and Zn(II) against planktonic *S. aureus*

Indirect quantification of microbe death using crystal violet staining was used to perform planktonic results against *S. aureus* (NU118). Adding PRE 1 mg/mL to $ZnSO_4$ (0.25 M) showed significant decrease in *S. aureus* survival percent with 18.5 % while each one at individual didn't show this activity with higher cell survival at 82 % and 66 % ($p < 0.05$). On the other hand, higher uptake of crystal violet was seen in the PRE 2.5 mg/mL ($p < 0.05$) and no significant change was seen with $ZnSO_4$ (0.5 M). In the same manner, addition of the PRE 2.5 to $ZnSO_4$, showed a significant decrease in the *S. aureus* survival rate to give 27.76 % while PRE and Zn(II) at the individual showed higher bacterial survival > 90%. These results validate the synergistic activity that has been established in Chapter 3 regarding the potentiating effect using combined PRE 1mg/mL and Zn(II) 0.25M, and it shows the activity of three formulations that contain the same concentration of PRE/Zn. All formulations showed significant decrease in the bacterial

survival percent to give 55.6 % and 40.10 % and 37.7 % bacterial survival ($p < 0.05$) in the carrageenan formulation, Carbopol 974 formulation and HPMC 856 formulation respectively (Figure 7-10).

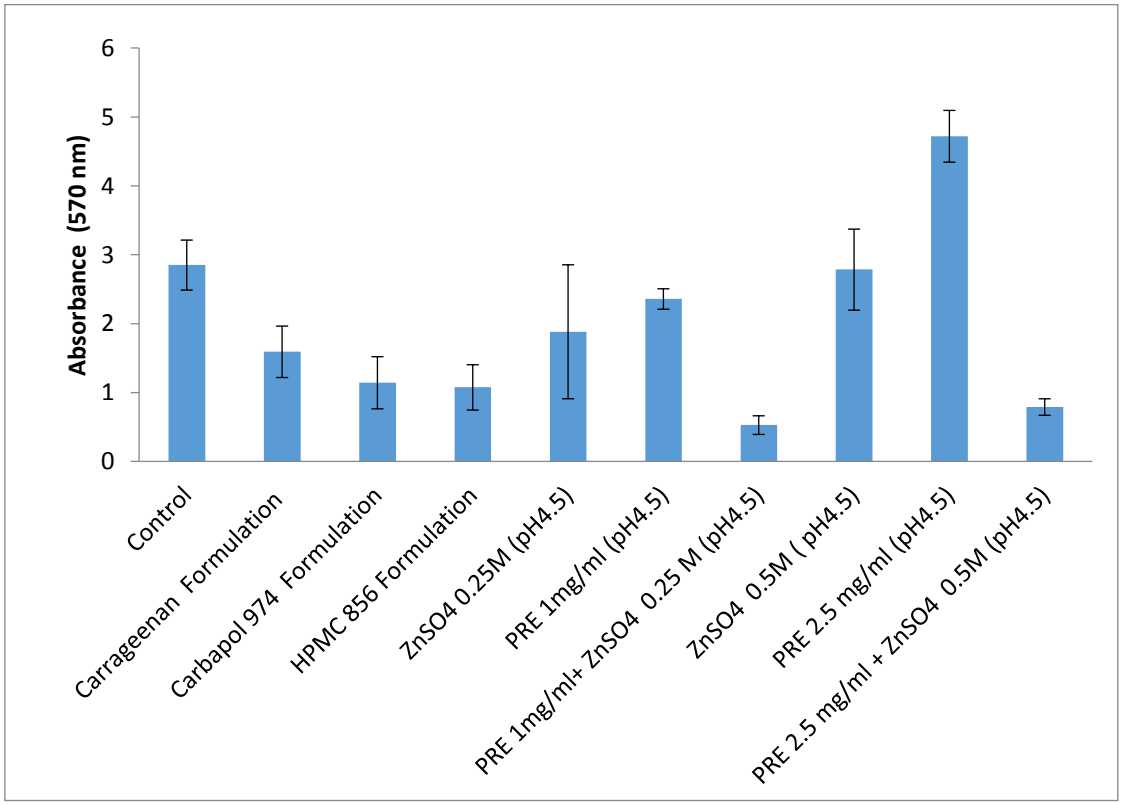


Figure 7-9 Effect of the PRE ± Zn(II) in solution form and formulations against planktonic *S. aureus* after 24 hours incubation in the presence and absence of tested compounds. Results are shown as mean absorbance of bacteria (indicate growth) ±SD from a (single replicate and 6 experimental replicates).

7.5 Discussion

Because of the combination system between the PRE and metal ion it was challenging to take in consideration the interaction that can occurs between polymers and PRE or polymer and Zn(II) or even both. Metal ion such Zn(II) presence in a formulation, even

in trace amount, can be a challenge because of the high reactive nature (Chen et al. 2016). Ionic polymers viscosity is highly affected and can be lost in presence of other metal ions (Kawakami et al. 2006). With the Carbopol 974P, the higher pH and $ZnSO_4$ to instantly break down the gelling formation and gave a white precipitate. Another approach was made to prepare the polymer without pH adjustment, which gave a homogenous viscous mixture with active ingredient PRE and $ZnSO_4$.

Gel formation was observed with all studied polymers in this project without the active ingredient incorporation. Among investigated polymers, HPMC856N and Carrageenan showed optimal gelling effect with the PRE and $ZnSO_4$. This is in accordance with Chen et al. (2016) when $ZnSO_4$, as a highly reactive metal ion, showed desired gelling activity with these tow polymers. In the reported method the carrageenan polymer was used with $ZnSO_4$ at 2% concentration and formed homogenous gel. Carrageenan as topical gel thickening agent has a good safety profile for use in human and it has been investigated to incorporate several metal ion for topical formulation including zinc acetate and zinc sulphate (Chen et al. 2016; Fernández-Romero et al. 2012). With HPMC polymer, the loss of gelling characteristic was observed with higher $ZnSO_4$ concentration at 1M formulation MD. On the contrary, no effect was seen with higher PRE up to 20 mg/mL loaded formulation MH. This result is in accordance to the previous work described by Houston *et al.* (2017) with slight modification and added polymer to the formulation. A bioadhesive polymer such as Carbopol has been earlier studies and showed and proved to make moderate gel formulation with PRE (Fan and Liu 2017) and $ZnSO_4$ (Keegan et al. 2007). With Carbopol 974 the formulation showed light viscous formulation at lower pH (3.5) while at higher pH (5.5) when adding the PRE, a phase separation appeared. The viscosity value shows higher viscosity value with HPMC 856 formulation with 1060 mPa s, while viscosity of Carbopol 974 was the lowest 1.78 mPa s. The lower viscosity of Carbopol because in our study Carbopol preparation was used without pH adjustment and pH was modified by the phthalate buffer vehicle at pH (4.5) which gave viscous formulation. Carbopol has been reported in earlier study to be used without need for neutralization and found to form viscous gel (Proniuk and Blanchard 2002). The final formulation selected to be further investigated from these preparations, were with

the desired ratio of PRE and $ZnSO_4$ (1 mg/ 0.25M) that showed the good antibacterial activity in Chapter 3.

The contact angle established between a droplet and a surface is governed by a number of factors, including relative lipophilicities of the two, surface asperity, temperature and viscosity - all of which contribute to the phenomenon of wettability. However, there was no obvious correlation between contact angle and viscosity in this work. The stratum corneum possesses high asperity due in part to the irregular packing of the corneocytes which are in early or late stages of desquamation (Figure 7-5). The surface has further irregularities in terms of folds/wrinkles and the appendages, hair/ hair follicles and sweat pores. The surface of the stratum corneum is largely lipophilic due to the intercellular lipids and sebum exudate. As shown from the contact angle in Table 7-7 the contact angle established between the formulation and dermis can be considered a model for a superficial wound or abrasion.

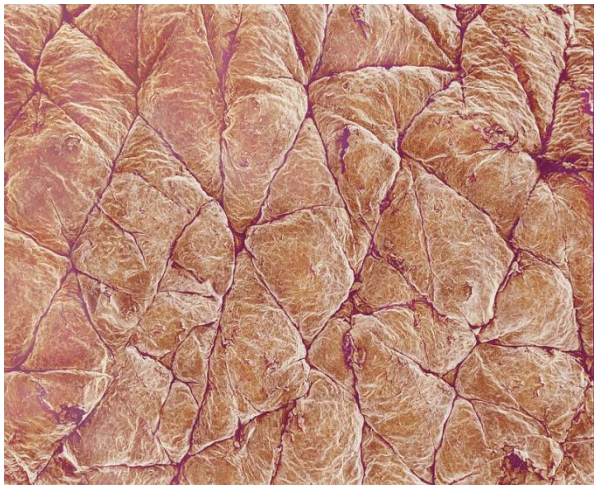


Figure 7-10Scanning electronic microscope SEM image of human skin stratum corneum (Sciencephoto.com)

The stability study in Chapter 6 showed that the optimal temperature to store PRE and $ZnSO_4$ solution was at temperature < 4˚C, ultimately all formulations were stored in the

fridge and stability study performed over 6-month storage time. In all 3 tested formulations, the stability of punicalagin > 93 % over this period.

For optimization of the formulation all three formulation were tested for antibacterial activity against planktonic *S. aureus*. The results showed that a combination of PRE 1 mg/mL and Zn(II) 0.25 M that has been used in tested formulation (carrageenan formulation, HPMC 856 formulation and Carbopol 974) or in simple solution (of PRE 1 mg/mL + $ZnSO_4$ 0.25 M at pH4.5; PRE 2.5 mg/mL + $ZnSO_4$ 0.5 M at pH4.5), significantly decrease in *S. aureus* (NU118) viability when planktonic test was performed in comparison to control group ($p < 0.05$). The results in this Section validate the potentiating activity achieved by PRE 1mg/mL and Zn(II) 0.25 M earlier revealed in Chapter 3.

7.6 Conclusion

Three different polymers were used to prepare PRE/Zn formulations with different viscosity properties. The conclusions that may be drawn from this chapter are:

1. Three formulations with the same PRE/Zn loadings were selected that showed acceptable homogeneity, wettability. The gels had varying viscosities.

2. Stability of the active constituent, punicalagin, was demonstrated over 6-month storage time at 2-4 °C and -20 °C.

3. Retention of microbiological activity of the gels and the solution was established independently against *S. aureus* in an external collaborator's laboratory.

As it is intended for the formulation to be used as topical formulation to infected skin and wounds, the skin permeability and penetration will be investigated in the following Chapter.

Chapter 8: *In vitro* skin penetration and permeation of punicalagin and Zn(II) from solution and hydrogel formulations containing PRE and $ZnSO_4$

8.1 Introduction

Although the topical application of an antimicrobial system is generally intended to provide its activity on the surface, and ideally the actives would remain at the point of application, this is rarely achieved as some will pass through the upper layers of the skin. In this chapter, the skin penetration and permeation of punicalagin (from PRE) and Zn(II) (from $ZnSO_4$) were investigated from a topically-applied solution and several topical formulations. *Skin penetration* can be defined as an amount of chemical compound entering and resident within a membrane, i.e. skin; whereas *skin permeation* can be defined as the amount and/or rate of a compound passing out of tissue (skin) following the penetration stage.

While the PRE/Zn system will ideally be active on the surface of the skin for chronic and acute wounds as an antiseptic, both skin penetration and permeation are investigated here. A skin penetration study can indicate the mass (or moles) of actives that have been absorbed into the skin, and show any reservoir formation within the outer layers. The formation of a reservoir can be useful in providing a *substantive* effect, i.e. the antimicrobial activity is sustained even after the initial colonies have been killed, thereby inhibiting recolonisation. As the epidermis is constantly being formed and removed by desquamation, actives located in lower layers migrate towards the surface where they will provide sustained colonisation inhibition (Li et al. 2018). The substantivity of the antibacterial activity of chlorhexidine antiseptic has been reported to be superior when compared to isopropyl alcohol in an in vivo study comparing the antimicrobial effect on the resident microflora of hands (Aly and Maibach 1979). Mertz et al. (1984) compared the substantivity of the antibacterial effect of two antiseptic agents on inoculated partial thickness wound *in vivo.* The study revealed that the antibacterial activity treatment of 10% povidone-iodine was prolonged post application at 24h, compared to the short acting antiseptic 70% isopropyl alcohol.

The initial purpose of the permeation study was to demonstrate the level of applied dose that may reach systemic circulation. However, if it can be demonstrated that PRE/Zn permeates at sufficient levels through intact, unbroken skin, it might be a potential new treatment for sub-surface skin infections known as *cellulitis*.

8.1.1 *In vitro* porcine skin model

In topical drug development and optimisation processes, animal skin is generally used as the model of research in the preclinical trials stage of drug development (Semlin et al. 2011; Flaten et al. 2015). *In vitro* or *ex vivo* pig/porcine ear skin is the most frequently used model for studying drug permeation and penetration. Pig skin has an advantage over other animals such as rat and monkeys, due to its physiological and anatomical similarity to human skin. It has also been established to be a good model for human skin in terms of epidermal thickness, blood vessel density, and dermal to epidermal thickness (Dick and Scott 1992; Godin and Touitou 2007). Several methods have been used to prepare skin, including: full or split thickness skin, heat separated epidermis HSE, and trypsin isolated stratum corneum. Porcine skin and ears are readily accessible and easy to obtain from local slaughterhouses, and are generally considered as animal waste.

It is important to take into consideration and exclude any scarred or inflamed areas, since the integrity of such skin could jeopardise the results obtained (Henning et al. 2009; Meyer et al. 2006). Another important consideration is whether the skin has been frozen before use, and for how long, as this this is known to affect results. In this chapter only fresh/unfrozen porcine skin was used, in conjunction with Franz diffusion cells (FDCs).

8.1.2 Franz Diffusion Cell (FDC)

To study skin membrane permeation and penetration, one of the most common techniques is to use FDCs, which come in a diversity of shapes and sizes. According to the guidelines, FDCs should be made of inert non-adsorbing material, and in our study all-glass FDCs (Corbo et al. 1993) are used, with a nominal diffusional area of 0.88 cm^2 and a receptor phase volume of 3 mL. The FDCs consist of two parts: the donor and receptor phase, with a skin sample mounted between the two on greased flanges. The donor compartment should be in contact with the upper part of the tested skin, with the stratum corneum facing upwards. The receptor phase fluid should not interfere or limit the penetration of tested formulation through the skin. As the active molecules in this work were polar and hydrophilic in nature, the receptor phases were filled with phosphate-buffered saline (PBS) solution, pre-warmed to 37 ˚C. The fluid dynamics in the receptor phase should be in continuous movement and in order be in line with these criteria, a micro-stirrer bar was added to the receptor phase and the receptor fluid was under continuous stirring on multi-stirrer plates the whole time of experiment. The two parts were put together using a clamp, with vacuum grease applied to the edges of each side, with a skin membrane in between.

The application of dose or formulation to the donor phase can be either a precise, 'in-use' dose (*finite*) that mimics the actual reported therapeutic dose, or an *infinite* dose can be applied to determine the maximal skin permeation and penetration of investigated substances, as it is assumed the concentration and hence driving force will not change with time. The FDC should be maintained under controlled temperature (30-32 ˚C) to represent general skin surface temperature, by placing the FDC in a temperature-controlled environment such as an incubator or water bath, or by using specific water jackets for the FDC (Corbo et al. 1993; Courtellemont et al. 1999; Sartorelli et al. 2008; Baccarin and Lemos-Senna 2017; Houston et al. 2017). The experiment commences once the drug dose has been applied. For permeation data, the receptor phases are removed at specific time points and retained for analysis, then replaced with a fresh

receptor phase (Figure 8-1). The data is processed to allow the construction of permeation profiles, drug/cm^2 versus time. Linear plots or linear parts of plots are generally 0-order and can yield steady state flux data, e.g. drug/cm^2/h.

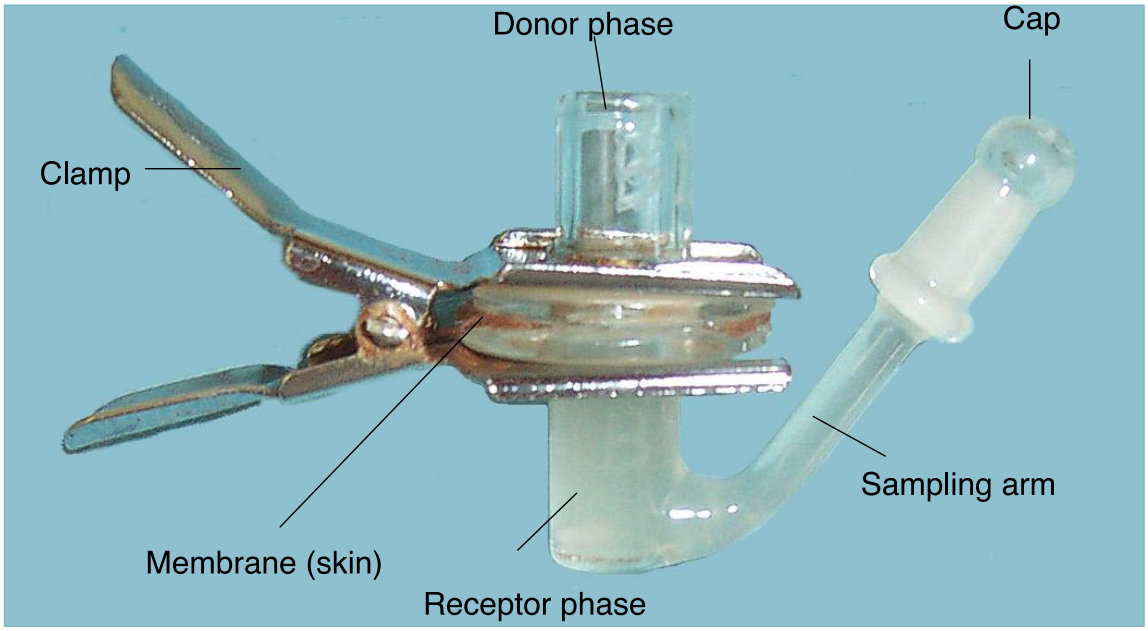


Figure 8-1 All glass Franz diffusion cell (FDC).

8.1.3 Skin Tape stripping

Tape stripping is one of the main methods that has been used to investigate drug penetration and localisation within the stratum corneum and epidermis. At first, the topical drug is applied for a certain time to the skin, then stripped via the application and removal of adhesive tape on the outer layer of the skin. The stripping is done in a repeated manner to the same area of skin using a new piece of tape each time; each piece of tape contains tissue from deeper within the skin. The drug on each strip is then desorbed and quantified (Escobar-Chávez, et al. 2008; Lademann et al. 2010; Raber et al. 2014). Figure 8-2 shows the general steps of the tape stripping technique used here.

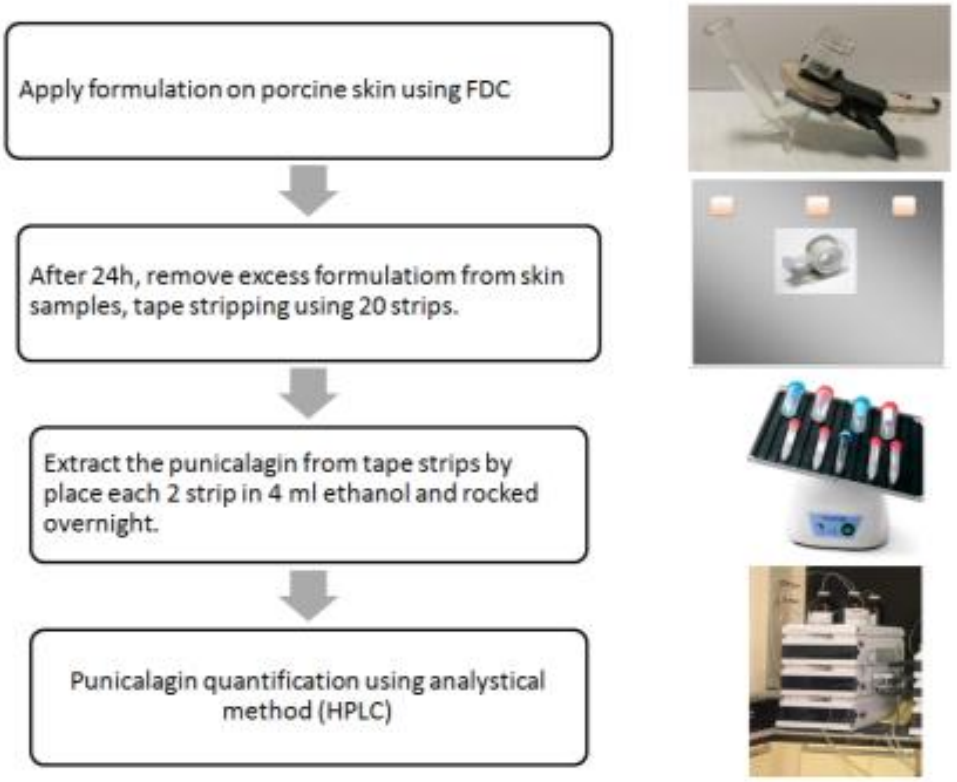


Figure 8-2 General steps shows the tape stripping method using porcine skin.

8.2 Objectives and aims

- To use the tape stripping technique to determine the *in vitro* skin penetration profile of three different formulations containing PRE 1mg/mL and $ZnSO_4$ + 0.25M and phthalate buffer solution loaded with PRE 1mg/mL and $ZnSO_4$ + 0.25M using HSE.

- To determine the *in vitro* skin permeation profiles of punicalagin and zinc from three different formulations containing PRE 1mg/mL and $ZnSO_4$ 0.25M and phthalate buffer solution loaded with PRE 1mg/mL and $ZnSO_4$ 0.25M using a heat-separated epidermis HSE model.

- To relate delivery data to wound healing and antibacterial data to estimate potential effects.

8.3 Materials and methods

8.3.1 Materials

All materials used in this chapter were previously detailed in Chapter 2, Section 2.1.

8.3.2 Preparation of formulation and solution contain PRE and ZnSO4

A solution of PRE 1 mg/mL + $ZnSO_4$ 0.25M in pH 4.5 phthalate buffer was prepared according to the method described in Chapter 2, section 2.2.1. The three semi-solid formulations, based on the thickened HPMC 856 formulation, Carbopol 974 formulation, and carrageenan, selected for further investigation are detailed in Chapter 7. A blank control was prepared using the same method for each formulation without the incorporation of PRE and $ZnSO_4$.

8.3.3 Full thickness porcine ear skin preparation

Porcine ear skin was obtained from a local slaughterhouse and used without prior freezing. The skin cleaning and preparation method is described in detail in Chapter 2, Section 2.2.3.1.

8.3.4 *In vitro* depth profile using tape stripping technique and skin remaining

Tape stripping method previously described in Chapter 8 (Section 1.1) was used to determine the skin penetration through the HSE prepared by method in detailed in Chapter 2, section 2.2.3.2. The skin that was used to study the permeation of punicalagin and zinc through heat-separated epidermis (HSE) was removed from the FDC and placed on a porcelain tile, after which the excess formulation was removed using a cotton bud with constant and uniform pressure applied each time. The skin was placed on a porcelain tile with the stratum corneum facing upwards, in order to perform the tape stripping. Twenty strips of regular adhesive tape were used for each porcine skin sample. Several considerations were taken in mind when performing the tape stripping. The pressure applied and removal velocity was kept the same each time. For each sample of twenty strips, two strips were put together in one vial and added to 4 mL of ethanol, and rocked overnight using a laboratory rocker. After that, the ethanol was evaporated and the residue reconstituted in 1 mL of deionised water. Each vial was centrifuged at 1500 rpm for 15 minutes, then the supernatant was retained. Thereafter, the remaining skin samples were cut and divided into small pieces in order to transfer them to a vial containing 2 mL of ethanol. The chopped skin pieces were then homogenised using a Stuart® SHM1 homogeniser (supplied by Fisher Scientific, Loughborough, UK). After each use, the homogeniser was cleaned and the probe changed. After complete homogenisation the samples were centrifuged at 1500 rpm for 30 minutes using a Thermofisher Scientific Heraeus Megafuge 8R (supplied by Fisher Scientific, Loughborough, UK). Clear supernatant was transferred to a vial and placed in a water bath at 37 ˚C overnight until all ethanol had evaporated, then 1 mL of deionised water was added to each sample and rocked until all solids had dissolved (Escobar-Chávez et al. 2008; Ruela et al. 2016). The samples were then tested for punicalagin content via the previously-described reverse phase HPLC method (Chapter 2, section 2.2.2.1) (Weerheim and Ponec 2001), and tested for zinc using the ICP-MS method described in Chapter 2, Section 2.2.2.2 *(note: here we refer to zinc rather than Zn(II), as the instrument analyses total element rather than specific chemical form).*

8.3.5 Permeation of punicalagin and zinc through Heat Separated Epidermis (HSE)

Using the Franz diffusion cell (FDC) method detailed in Chapter 8 and an HSE membrane prepared according to the method detailed in Chapter 2, Section 2.2.3.2, the selected formulation and solution of PRE/$ZnSO_4$ with infinite dose was applied to the phase of the porcine HSE with 1 g of formulation or solution being applied. All formulations contained the same concentrations of PRE/$ZnSO_4$ (PRE 1 mg/mL + $ZnSO_4$ 0.25M), and infinite dose conditions were selected to predict the *maximal* permeability of the epidermis membrane. The solution in the receptor phase was taken after 24 hours, and punicalagin content was analysed using the HPLC method and zinc determined by ICP-MS, as previously described in Chapter 2, sections 2.2.2.1 and 2.2.2.2.

For the cumulative permeation study, the solutions in the receptor phases were collected and replaced with fresh ones at 1, 3, 6, 12, and 24 hour time intervals. For each time interval, the total fluid in the receptor phase was taken using a sterile pipette and replaced with the same volume of fresh PBS solution. The samples were stored in a light-protective glass container for further use. Analysis of punicalagin and zinc content used HPLC and ICP-MS, respectively, with both methods having been previously described in Chapter 2, sections 2.2.2.1 and 2.2.2.2. The permeation flux per unit of skin surface area was determined from the linear regression of the initial linear part of the curve.

8.3.6 Data analysis

In each test, data are expressed as means ± SD, unless otherwise stated. Statistical analysis was carried out using One way analysis of variance (ANOVA) with Tukey‘s post test to compare statistical significances between groups and or One way analysis of variance (ANOVA) with post-test Dunnett analysis to compare groups against the control. Results were expressed as significant and very significant with $p < 0.05$ and $p <$

0.01 respectively. The analysis was carried out using InStat for windows, version 3.0 (GraphPad Software Inc, San Diego, CA).

8.4 Results

8.4.1 *In vitro* depth profile using Tape stripping technique and skin remaining

Tape stripping was done for four samples, including one solution and three semi-solid formulations. As shown in Figure 8-3, all formulations that contained the same amount of PRE (1 mg/mL) penetration depth profile for punicalagin through the epidermis were similar to all formulations with a punicalagin concentration of $\leq$ 0.3 nM/cm^2 in each layer. There was no punicalagin recovery in the control group with no PRE and Zn included in the formulation, since no peak was identified in the HPLC analysis which make the detectable amount of punicalagin from the formulation significant ($p < 0.05$).

When the tape stripping results were pooled for each formulation, both HPMC 856 formulation and carrageenan formulation showed a slightly higher – 1.79 and 1.8 nM/cm^2 ($p > 0.05$) – but statistically insignificant penetration through the epidermis than the Carbopol and phthalate buffer solution with 1.34 and 1.55 nM/cm^2 (Figure 8-4). The depth profiles showed that all formulations achieved an equal level of punicalagin at each layer, indicating that the equilibrium of punicalagin took place and a reservoir effect could be happening here.

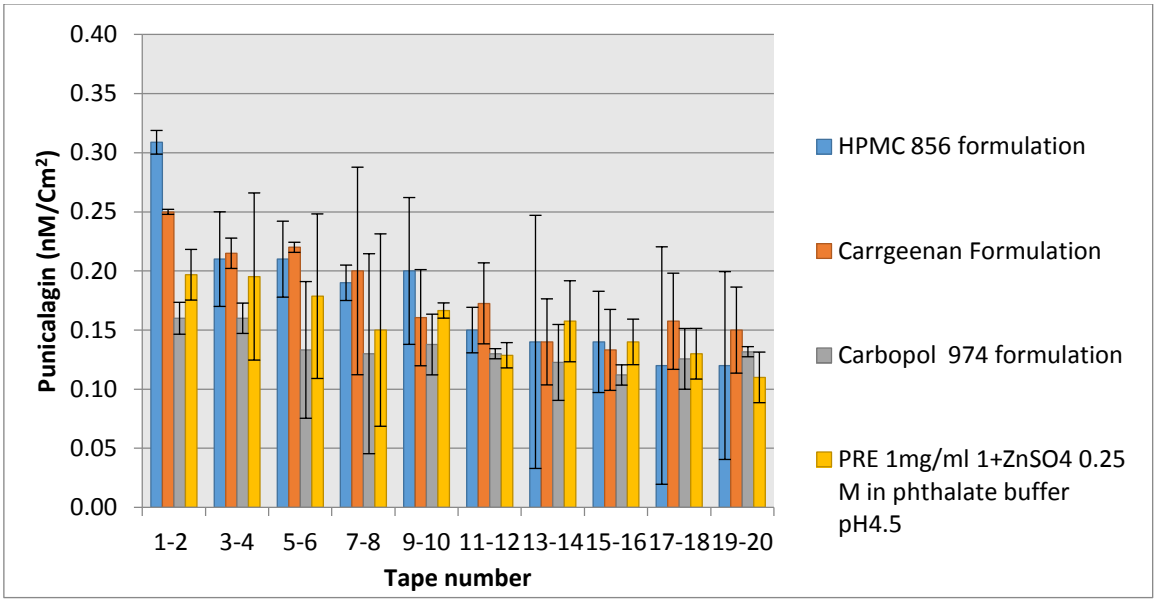


Figure 8-3 Punicalagin penetration profile through HSE recovered from tape stripping after application of 1g of three topical formulations at 24h: blue, HPMC 856 formulation; orange, carrageenan formulation; grey, Carbopol formulation and yellow, solution. *All formulations contained same concentration of PRE/$ZnSO_4$ (PRE 1mg/mL+$ZnSO_4$ 0.25M)* (n=3±SD).

Data obtained from the remaining skin after tape stripping is referred to as remaining skin absorption, which is defined as epidermal skin excluding the stratum corneum (Corbo et al. 1993). As shown in Figure 8-4, a greater amount of punicalagin was obtained from the HPMC 856 formulation compared to other groups, with 3.21 nM/cm^2 ($p < 0.05$). The Carbopol formulation showed the lowest dermal penetration, even lower than the phthalate buffer PRE/Zn solution, although the decrease is not significant ($p > 0.05$).

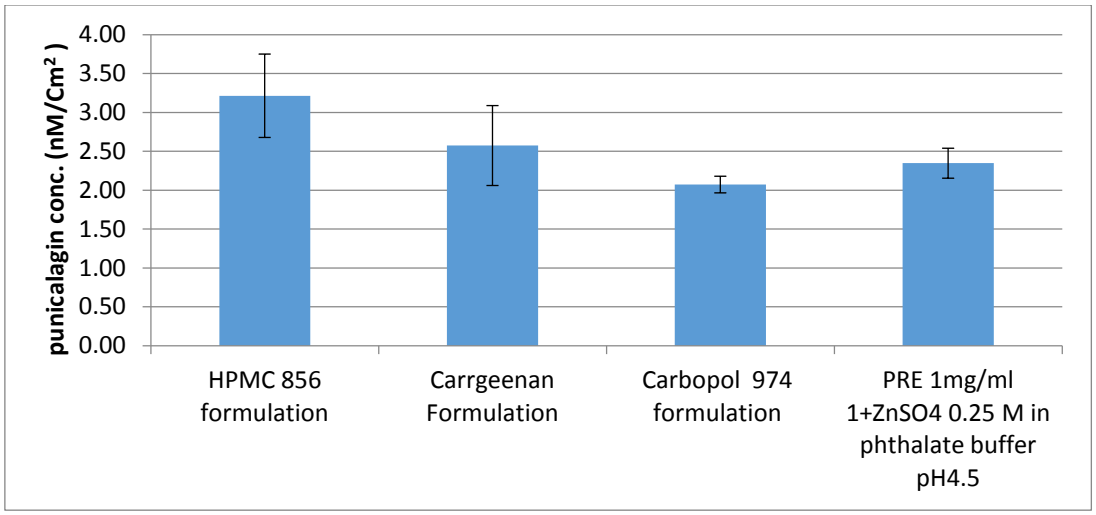


Figure 8-4 Bar chart showing average mass of punicalagin recovered from remaining skin after tape stripping method, after application of 1 g of three formulations and solution that contained same concentration of PRE/$ZnSO_4$ (PRE 1mg/mL+$ZnSO_4$ 0.25M) compared to control (vehicle only no PRE or $ZnSO_4$) at 24 hours (n=3 ±SD).

Comparing both amounts of punicalagin recovered from the tape strips and remaining skin, punicalagin recovery was largest from the remaining skin (epidermis remaining after tape stripping of HSE) rather than the amount of tape stripes recovered, as shown in Figure 8-5.

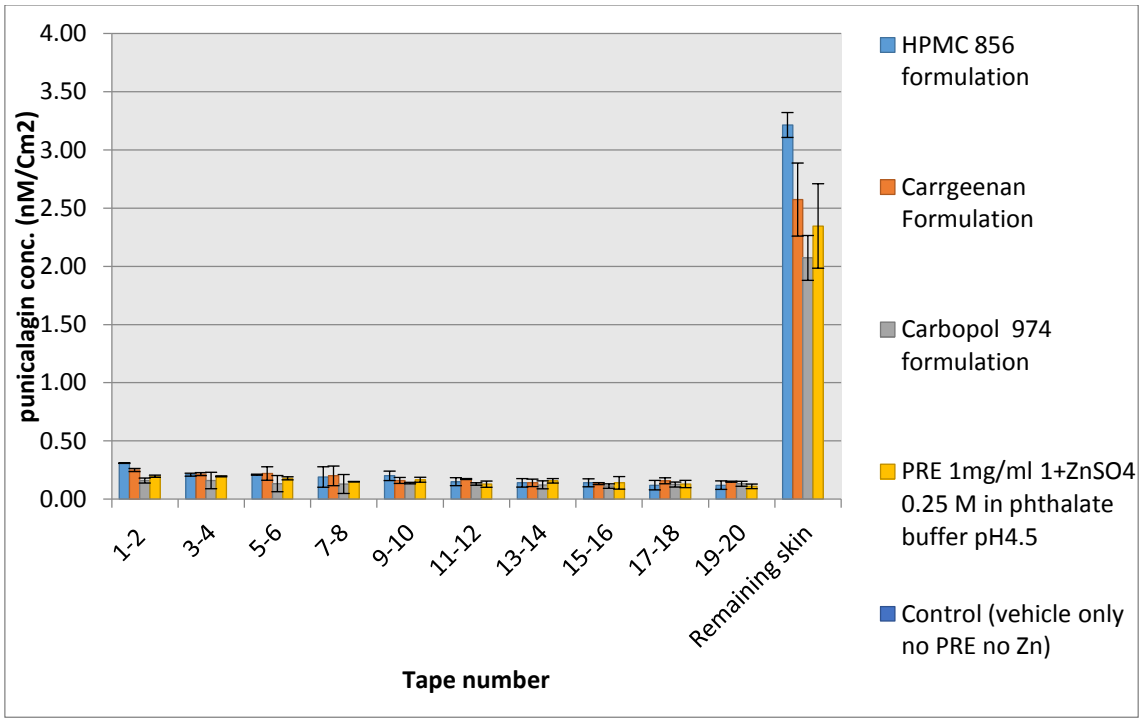


Figure 8-5 Bar chart showing the depth permeation profile of punicalagin through HSE recovered from tape stripping and skin remaining of after application of 1g of three formulation and solution that contained same concentration of PRE/$ZnSO_4$ (PRE 1mg/mL + $ZnSO_4$ 0.25M) compared to control (vehicle only no PRE or zinc) at 24 hours, (n=3±SD).

Figure 8-6 shows the average mass of zinc recovered after tape stripping of HSE porcine skin membrane compared to the control, which contained only vehicle with no PRE or Zn (II). As shown, the amount of zinc recovered from the epidermis was slightly higher in the carrageenan formulation, but not significantly different when compared to the control. Similarly, all formulations and control zinc from the dermis (skin remaining) layer showed no significant difference to the control ($p > 0.05$). Consistent with the literature, the average mass of zinc that was epidermic in the control group was 76.7 nM/cm^2 ± 3.9, which is considered to be within the previous reported levels of zinc

recovered from the untreated group, and could be explained as the presence of endogenous zinc in the porcine skin (Gamer et al. 2006; Houston 2011).

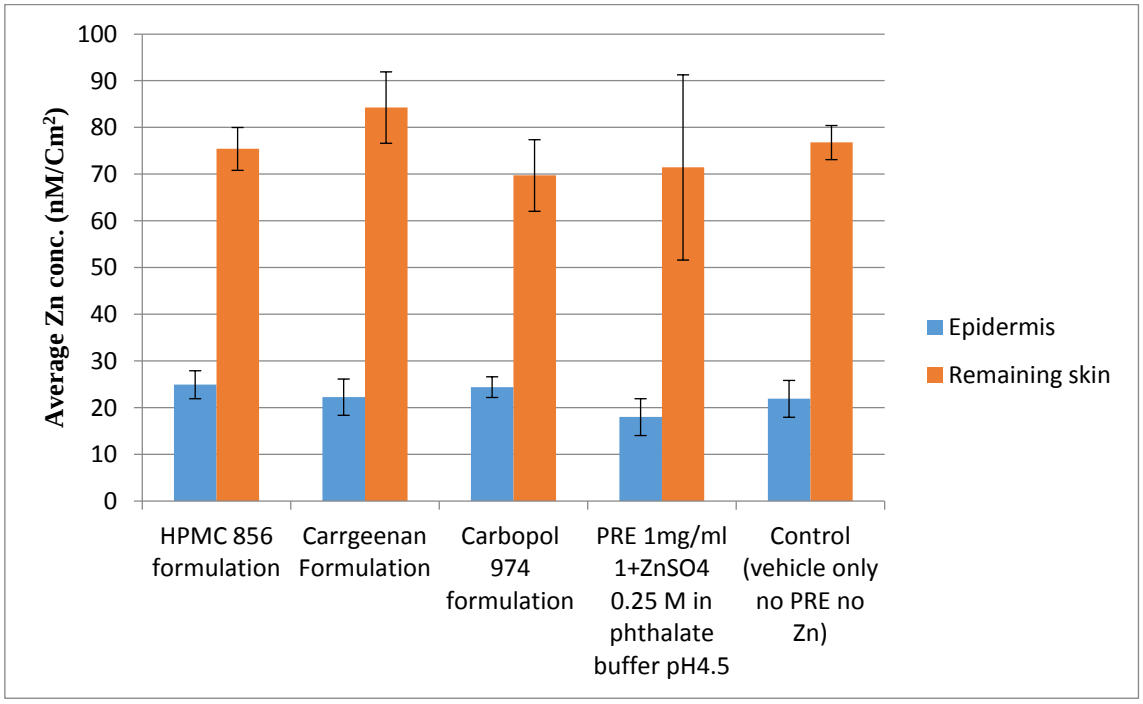


Figure 8-6 Average mass of zinc recovered from tape stripping of HSE membrane, showing epidermis and (skin remaining) after application of 1g of three formulation and solution that contained same concentration of PRE/zinc (PRE 1mg/mL + $ZnSO_4$ 0.25M), compared to control (vehicle only no PRE or zinc) at 24 hours (n=3 ±SD).

8.4.2 Permeation release of punicalagin and zinc through HSE

8.4.2.1 **Punicalagin**

Figure 8-7 shows the cumulative release of punicalagin through heat-separated epidermis (HSE) over 24 hours. From the permeation curve, HPMC 856 formulation showed the highest punicalagin amount at 24 hours, with 6.9 nM/cm^2, which is statistically significant when compared to the control and the phthalate buffer solution of PRE 1mg/mL+$ZnSO_4$ 0.25M. In the same manner, carrageenan formulation showed a high amount of punicalagin at 24 h with 5.2 nM/cm^2, which is statistically significant when compared to control group or the phthalate buffer solution with PRE + Zn(II) ($p<0.05$). Since PEG was added as a permeation enhancer to the original HPMC 856 formulation developed earlier by Houston (2011), the HPMC 856 formulation with PEG showed a higher permeation through the epidermis at 24 hours, but this difference was statistically insignificant compared to the permeation data reported earlier from HPMC 856 without PEG with 6.9 and 7 nM/cm^2, respectively. Permeation across HSE at 24 h showed that the HPMC 856 formulation delivered almost double the amount of punicalagin permeating through the epidermis than the phthalate buffer solution, with 3.2 and 6.9 nM/cm^2, respectively. Unfortunately, the carbopol data was not included.

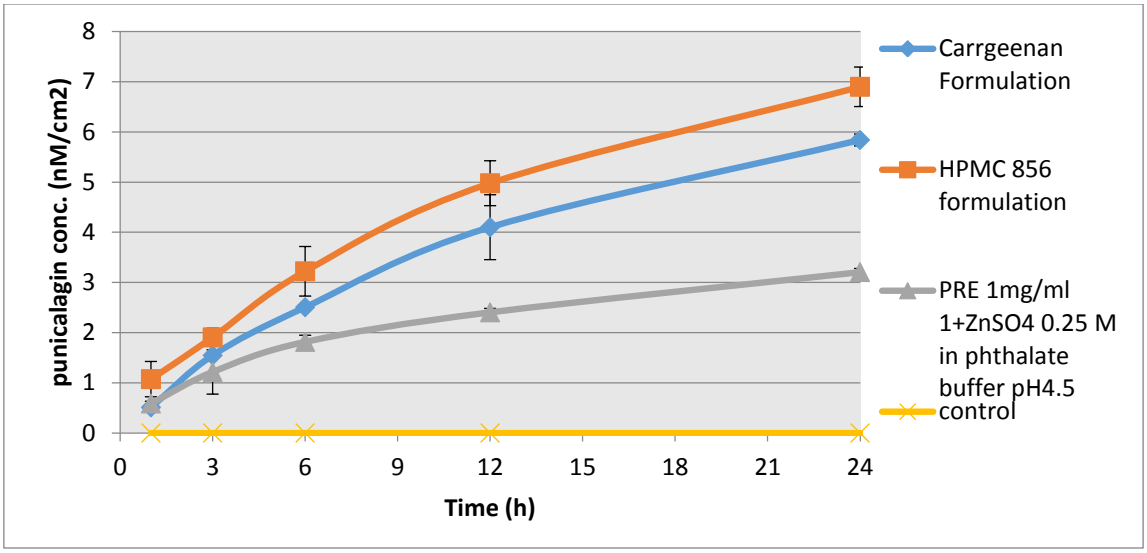


Figure 8-7 Cumulative permeation of punicalagin through Heat Separated Epidermis HSE (receptor phase content), over 24 hours after application of 1g of three formulation and solution that contained same concentration of PRE/$ZnSO_4$ (PRE 1mg/mL + $ZnSO_4$ 0.25M), HPMC 856 formulation (orange line), carrageenan formulation (blue line), PRE 1mg/mL + $ZnSO_4$ 0.25 M in phthalate buffer pH4.5 (gray line) and blank control (vehicle only, no PRE or $ZnSO_4$) (yellow line) (n=3±SD).

The permeation curve analysis of punicalagin showed the highest flux as being from the HPMC 856 formulation with 0.34 nM/cm^2/h, and the lowest being from the phthalate buffer solution with PRE + Zn with 0.13 nM/Cm^2/h. Carrageenan formulation showed flux that was in the middle between the two previous value with 0.28 nM/cm^2/h. Comparing the two HPMC 856 formulations and carrageenan formulation, the difference between them is minimal and statistically insignificant ($p > 0.05$). In the control group, no peak of punicalagin was recorded at any time of the experiment testing time intervals. Consistent with the previous assumption of reservoir effects, the cumulative release showed a plateau curve after 12 hours of administration of 1 g of each formulation that contained PRE 1mg/mL and $ZnSO_4$.

8.4.2.2 **Zinc**

Zinc analysis results were pooled and analysed using ICP MS. The data obtained for permeation through HSE is shown in Figure 8-8. Significantly more zinc permeated the HSE in a higher concentration from the HPMC 856 formulation and carrageenan formulations ($p < 0.05$). The detectable amount of zinc in control group was expected because of the endogenous cellular zinc leaching from the porcine skin.

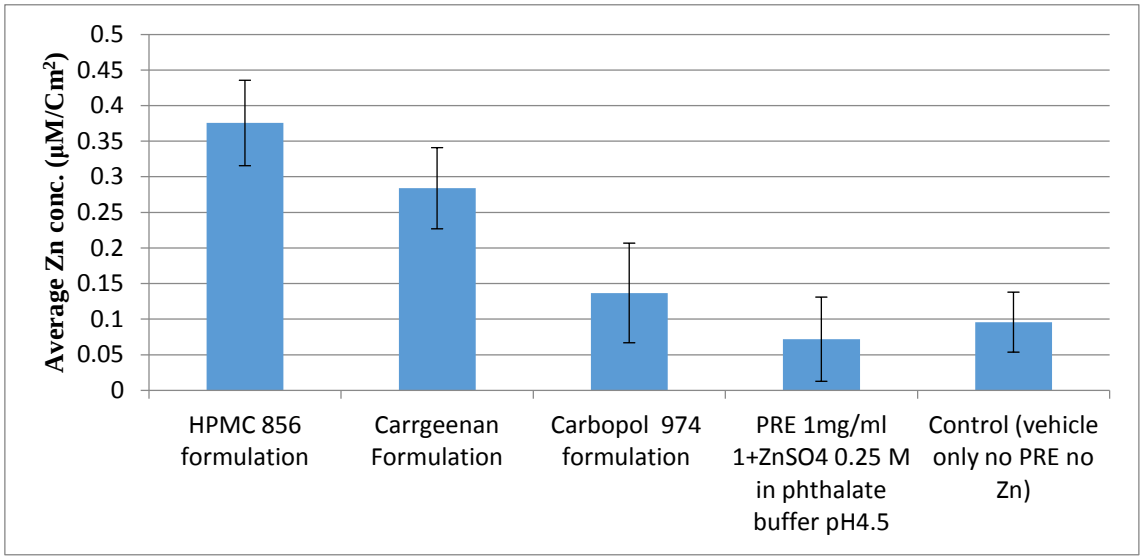


Figure 8-8 Zinc permeation through Heat Separated Epidermis (HSE) (receptor phase content) at 24 hours after application of 1g of three formulation and solution that contained same concentration of PRE/$ZnSO_4$ (PRE 1mg/mL + $ZnSO_4$ 0.25M) compared to control (vehicle only no PRE or zinc) at 24h, (n=3 ±SD (n=2±SD).

8.5 Discussion

Punicalagin is the primary tannin present in pomegranate rind extract, and its count as the main polyphone that is responsible for the bioactivity and

antimicrobial activity (Seeram et al. 2005). In vitro permeability and penetration of different PRE/Zn formulations has been tested in this project using porcine ear skin, and samples were tested for presence of zinc and punicalagin. Punicalagin is by far the most abundant tannin in the PRE, although it is possible that other PRE components will have penetrated and permeated the skin; however, it was beyond the scope of this thesis to pursue their analysis further.

The amount of punicalagin that penetrated the epidermis and remaining skin was highest from HPMC 856 and carrageenan formulation, with 5.27 and 4.18 nM/cm^2, respectively, compared to Carbopol 974 and phthalate buffer solution containing the same amount of PRE/Zn ($p < 0.05$). This result is in accordance with our finding from cumulative release of punicalagin across HSE epidermis, since the same formulations showed better permeation than phthalate buffer solution containing the same concentration of PRE, at 1 mg/mL and $ZnSO_4$ at 0.25M. Overall, the amount of punicalagin recovered from tape stripping and skin remaining produced results which corroborate the findings of a great deal of the previous work on punicalagin permeation across porcine skin membranes (Houston, 2017).

Comparing the punicalagin permeation through HSE to punicalagin permeation through full thickness membrane (data not shown), punicalagin was mainly retained in the upper skin layer (epidermis) since no punicalagin was found in the FDC receptor phase when full thickness skin was used. Our results are in accordance with previous findings when in vitro skin penetration of nanoemulsion contained pomegranate peel tannins showed that punicalagin was retained mainly in the upper skin layer at a similar level (6 $\mu g/cm^2$) (Baccarin and Lemos-Senna. 2017). A possible explanation for punicalagin retention in upper skin layers may be because in fact punicalagin is a large molecule which is lipophilic in nature, with a high molecular weight of 1084.7 g/mol, while the upper limit for drugs to penetrate skin is assumed to be 500 g/mol (Bos and Meinardi 2000).

As illustrated in the tape stripping of HSE, the recovery of zinc was observed in high level form the epidermis layer from all formulations, but no significant difference was observed compared to the control. Consistent with the literature, the average mass zinc that was recovered from remaining skin and epidermis in the control group was 76.7 ± 3.9 nM/Cm^2. This is in accordance with the previous findings of Houston (2011). In his study, when comparing between two phthalate solutions, one containing only PRE and the other PRE/Zn, he found that the permeation of zinc was not affected by the addition of PRE to the solution, a slightly higher level was recovered from the tape strips and dermis layer in the zinc individual sample, but yet statistically insignificant ($p > 0.05$) to the combination of PRE and zinc. Additionally, a higher level of zinc was recorded with HPMC 856 and carrageenan formulation using the HSE permeation model in the formulation than the control group or phthalate buffer ($p < 0.05$).

The formulations were prepared taking into consideration PRE-to-zinc ratio, which showed the potentiation of the bactericidal activity previously detailed in Chapter 3, with optimum potentiation activity at a PRE concentration of 1 mg/mL with $ZnSO_4$ at (0.125 M and 0.25M), which is equal to (71.9 mg/mL and 35.95 mg/mL), and the ratio of Zn/PRE that is needed for potentiation at 0.0139 and 0.027, respectively.

When the same amount of PRE+$ZnSO_4$ containing formulation was investigated for permeation through the epidermis (HSE), two formulations showed a better permeation profile of PRE/Zn (HPMC 856 and carrageenan formulation when compared to phthalate buffer solution and Carbopol formulation containing PRE 1 mg/mL +$ZnSO_4$. The zinc-to-punicalagin ratio which permeated the HSE layer is within levels that can show the potentiation of the antibacterial activity for both analytes – which was 0.018432 and 0.020579 in HPMC 856 and carrageenan, respectively – with large Zn(II) excess. This is in accordance with Houston's (2011) work, and the same observation showed that Zn(II) permeated an excess amount through the epidermis membrane compared to punicalagin.

Additionally, in Chapter 5, it was found that PRE also gave enhanced wound migration and proliferation of HaCaT skin cell line with *in vitro* at 24 h with a concentration of PRE at 5 and 2.5 μg/mL, although a statistically significant increase was seen at 10 μg/mL. The equivalent concentration of punicalagin present in these PRE concentrations was calculated using the relationship between total mass of PRE extracted and β-punicalagin within the freeze-dried PRE previously established in Chapter 2, Section 2.2.2.1.1. The equivalent level of punicalagin to enhance cell migration was found to be 0.23 nM.

The results of the tape stripping show that a large amount of punicalagin was found to be retained in the epidermis layer. Potential reservoir effect was hypothesised because of the equal amount of punicalagin concentration found in each layer of the upper skin using tape stripping, which was in the range of $\leq$ 0.3 nM/cm^2, and the total amount added with the remaining skin to give 5.27 and 4.18 nM/cm^2 of HPMC 856 and carrageenan formulation. This level of punicalagin would be expected to promote cell healing and migration. Lower levels were recorded with Carbopol and phthalate solution, with punicalagin at 3.22 and 2.94 nM/cm^2, but these values are still within the level of punicalagin that can enhance cell migration.

The surface of the stratum corneum continually desquamates and is continually replaced by the layers underneath. As the tape-stripping data has shown that punicalagin and zinc are present in these lower layers, it can be inferred that they will remain present as the lower layers emerge onto the surface and still be able to provide antibacterial activity and therefore substantivity in antibacterial activity (Long, et al, 1985). Ideally, this would need to be confirmed in follow up experiments.

From the depth penetration and epidermis permeation profile of PRE/Zn revealed in this chapter, the system could potentially be used for other skin conditions that need an active component to be available at the epidermis layer, such as cellulitis.

8.6 Conclusion

In this chapter the permeation profile of three different formulations and one solution contained the same active component of PRE 1mg/mL + $ZnSO_4$ 0.25M has been illustrated through *in vitro* porcine skin membrane.

- Two formulations showed the optimum penetration and permeation profile through in vitro epidermis model (HPMC 856 and carrageenan formulation) in order to potentiate the antibacterial activity. Punicalagin and zinc penetrated the epidermis in levels that should produce potentiated antibacterial activity.

- Our intended formulations were made to provide bactericidal potentiation concentration at the surface of the skin, but with this depth profile of skin penetration and HSE permeation, such system could also be beneficial to treat other sub-surface skin infection, such as cellulitis.

In the next Chapter the antiseptic activity of such formulations are investigated using an inoculated porcine *in vitro* skin model.

Chapter 9: *In vitro* 'in-use' skin sanitation potential of PRE and Zn(II) solution and formulations

9.1 Introduction

The bacterial suspension test data, as detailed in Chapter 3, gave promising results in regard to the enhanced antibacterial activity of PRE and Zn(II) in combination. The activity associated with the combination was one of a synergistic effect when FIC was calculated relative to the MICs of PRE and Zn(II) against MRSA. However, it is important to be able to demonstrate that the microbiological data determined under such laboratory conditions can be reflected in a simulated 'in-use' scenario. Obtaining similar effects on an infected biological surface would support the further development of a topical sanitation product based on the PRE and Zn(II) combination.

Although the mechanism of synergy between PRE and Zn(II) remains to be elucidated, it is likely to involve interplay between the zinc and PRE (punicalagin) (Houston 2011). Many factors could potentially interfere with and diminish this interplay and antibacterial enhanced activity, including drying, the presence of materials such as irregular surfaces of organic tissue, and phospholipid interactions.

In this chapter, the bacterial recovery from inoculated *in vitro* porcine skin following the application of PRE formulations and solution is investigated. The *s. aureus* was used as a test microorganism, as it is one of the most common bacteria to have been associated with severe skin and wound infections, including surgical site infection (Wang et al. 2018). Furthermore, the PRE and Zn(II) combination has shown promising synergised activity against MRSA, as previously detailed in Chapter 3. A control group will be treated with skin antiseptic formulation (Hibiscrub) with 4% of the active ingredient chlorhexidine. Chlorhexidine was chosen as the control in this chapter due to its superior antiseptic activity compared to isopropyl alcohol, as has been reported in a previous *in vivo* study comparing the antimicrobial effect on the resident microflora of the hand (Aly and Maibach 1979).

9.2 Objective and aims

To evaluate the potential of PRE and Zn(II) solution and formulations to disinfect *in vitro* porcine skin in a simulated in-use scenario.

- Confirm synergised bactericidal activity of formulation containing PRE and Zn(II) after application onto porcine skin inoculated with MRSA

9.3 Materials and methods

9.3.1 Materials

All materials used in this chapter were previously detailed in Chapter 2, Section 2.1.

9.3.2 Preparation of PRE and $ZnSO_4$ solutions

Solutions containing individual PRE in phthalate buffer were prepared using methods previously detailed in chapter 2, Section 2.2.1.2. Zinc sulphate individual samples in phthalate buffer pH4.5 were prepared according to the method in Section, 2.2.1.4. Test solution in combinations of PRE and $ZnSO_4$ were prepared using the method previously described for preparing combined solution in Section 2.2.1.5 in phthalate buffer pH 4.5.

9.3.3 Aseptic technique

An aseptic technique was maintained through all experiments in this chapter using routine microbiological safety cabinets previously detailed in Chapter 2, Section 2.2.4.1.

9.3.4 Preparation of microbiology agar media and broth

Mueller Hinton agar and broth were prepared according to the methods previously detailed in Chapter 2, Sections 2.2.4.2 and 2.2.4.3.

9.3.5 Preparation of bacterial culture

An overnight bacterial culture of methicillin-resistant *Staphylococcus aureus* (MRSA) (NCTC 12493) was prepared according to the routine working bacterial culture preparation previously described in Section 2.2.4.6. Then, preparation of 0.5 McFarland (1.5×10^8 bacteria cell density) followed the methods detailed in Chapter 2, Section 2.2.4.7

9.3.6 Preparation of formulations

The selected three formulations were HPMC 856, Carbopol 974, and carrageenan, and followed the method of preparation previously detailed in Chapter 7, sections 7.3.3, 7.3.4, and 7.3.5. Additional blank formulations without any PRE or $ZnSO_4$ were prepared using the same method of preparation, but excluding the active ingredients (PRE, $ZnSO_4$). An additional positive control group of skin samples were challenged with a commercially available chlorhexidine 4% skin sanitiser product (Hibiscrub 4%).

9.3.7 Full thickness porcine ear skin preparation and skin sterilization prior to the experiment

Porcine ear skin was provided by a local slaughterhouse. The skin cleaning and preparation methods are described in Chapter 2, Section 2.2.3.1. Avoiding imperfections, prepared porcine skin was cut with sterile scissors into similar squares with a 2 cm^2 surface area. Each skin sample was placed in a sterile petri dish and kept in

a laminar air flow safety cabinet. To ensure that inoculated bacteria were the only bacteria on the skin sample, the ears were sterilised beforehand.

Two methods were evaluated in a preliminary experiment: 1) UV lamp: a cycle of two 10 minute pulses of UV lamp (Figure 9-1) was employed to each side to clean and sterilise the skin from any microflora as much as possible; 2) the second method for sterilisation involved spraying with 70 % ethanol: all surfaces were thoroughly wetted for 10 min before ethanol being allowed to evaporate in a laminar air flow cabinet. The tested sterilised skin was then swabbed with a sterile cotton bud swab presoaked with MHB, then spread on a dried Mueller Hinton agar plate for inoculation, and incubated at 37 ˚C for 24 hours.

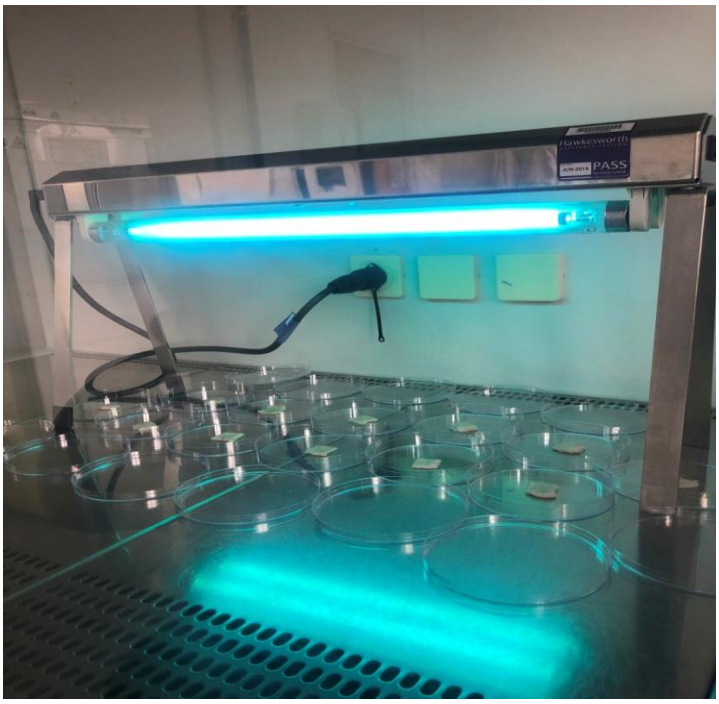


Figure 9-1 Sterilisation of porcine skin samples by treatment with UV lamp to ensure skin microbes had been eradicated prior to the inoculating with bacteria.

9.3.8 Bacterial recovery from inoculated porcine skin following application of PRE formulations and solution

The method used in this section is an adaptation of previously reported protocol that investigated the bacterial survival on skin and their sensitivity to antibacterial agent by Messager et al. (2004).Skin samples pretreated with a UV lamp were placed in a laminar air flow cabinet for the entire duration of the experiment. An overnight culture was used to inoculate skin samples by adding 10 µL of methicillin resistant *Staphylococcus aureus* MRSA (NCTC 12493) with 1.5 $x10^8$ CFU/mL in the centre of every 2 cm sampling area. Each area was then dosed with 100 µL of test formulation challenge or control, and then massaged using a sterile rod using the same protocol each time: 10 rotations with light pressure applied. After 20 minutes exposure time, skin areas were swabbed with a sterile cotton bud pre-soaked in 5 mL MHB for 10 seconds. The swabbing method was done in an identical manner for all samples: five times moving the bud in a horizontal line up and down, and five times for a vertical line in the same sampling area. The tip of each cotton bud was cut off using sterilised scissors, and then transferred to 5 mL of MHB and vortex mixed for 20 seconds. Then 100 µL was transferred to 900 mL of neutralising agent previously detailed in Chapter 2, Section 2.2.4.9, and allowed to sit for 60 minutes. Six serial dilutions were then prepared. After that, the Miles and Misra (1938) drop count technique was employed for each dilution using 3x10 µL drop onto agar plates then incubated for 24 hours at 37 °C. Colony forming units per millilitre (CFU/mL) of the bacteria and bactericidal activity were calculated for each group as a log reduction value, as previously described in Chapter 3, sections 3.3.8 and 3.3.9.

To investigate bacterial survival on such a skin model, initially two samples of skin were each inoculated with the same concentration of MRSA: 10 µL of (1.5 $x10^8$ CFU/mL). The first skin sample was swabbed and the drop count method was applied on an agar plate to calculate the bacterial concentration at 0 hours. After incubation of the tested

skin for 3 hours at 37 °C, the second skin sample was swabbed and counted for bacterial concentration. Both agar plates were incubated for 24 hours at 37˚C. This step was initially made to determine the dryness effect and bacterial survival of MRSA after inoculation on an *in vitro* skin model.

9.3.9 Statistical analysis

In each test, data are expressed as means ± SD, otherwise stated. Statistical analysis of was carried out using One way analysis of variance (ANOVA) with Tukey‘s post test to compare statistical significances between groups and or One way analysis of variance (ANOVA) with post-test Dunnett analysis to compare groups against the control. Results were expressed as significant and very significant with $p < 0.05$ and $p < 0.01$ respectively. The analysis was carried out using InStat for windows, version 3.0 (GraphPad Software Inc, San Diego, CA).

9.4 Results

9.4.1 Full thickness porcine ear skin preparation and skin sterilisation prior to the experiment

As shown in Figure 9-2, the UV lamp swab sterilisation method showed better results as illustrated after a skin swab growth test on MH agar, while the 70% ethanol still showed some microbial growth. Based on these results, all further skin samples were treated with a UV lamp prior to experimentation.

Figure 9-2 Muller Hinton agar plate showing microorganism growth from swab taking from porcine skin after 70% ethanol sterilization (left) and sterilization with UV for 10 minutes pulses was employed to each side (right).

9.4.2 Bacterial recovery from inoculated porcine skin following application of PRE formulations and solution

The *in vitro* method to test the antibacterial activity on skin was based on that reported previously (Messager et al. 2004). Figure 9-3 shows the initial concentration of MRSA at time zero, which was 7.58 ± 0.23 $\log_{10}$ CFU/mL, and that original concentration significantly increased after 3 hours incubation to reach 8.15 ± 0.13 $\log_{10}$ CFU/mL ($p < 0.05$), which is an indication of bacterial survival over the skin model.

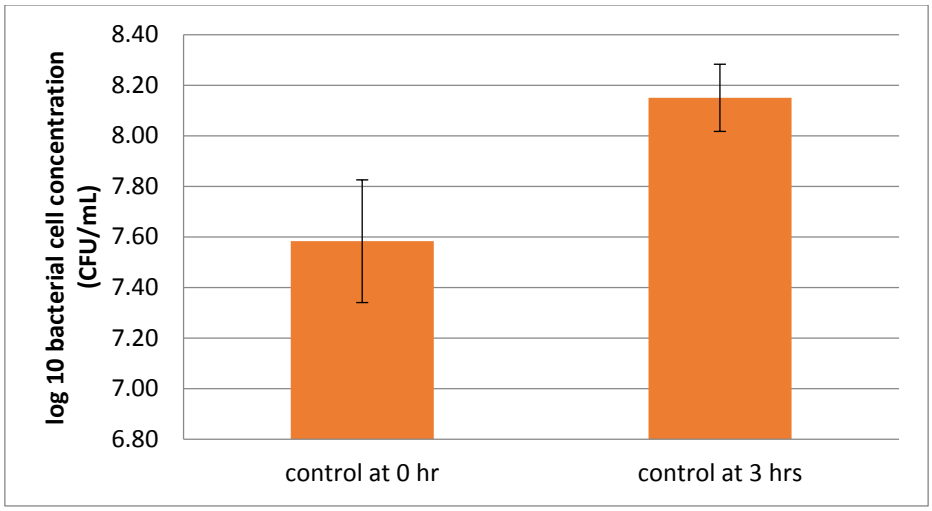


Figure 9-3 Viable cell number (log10 CFU/mL) of MRSA recovered from skin after incubation for 0 and 3 hours (n=2±SD).

All tested samples were applied to inoculated skin and incubated for 20 minutes. In order to determine the bactericidal activity, the log reduction value was calculated against the control group, as shown in Figure 9-4.

The chlorhexidine 4%, carrageenan formulation, HPMC 856 formulation and Carbopol 974 formulations achieved a log reduction value of more than 4, at 5.83, 5.75, 4.63, and 4.53, respectively. All three tested formulations and chlorhexidine 4% showed a significantly higher log reduction value than the simple (non-thickened) solution of PRE 1mg/mL + $ZnSO_4$ 0.25M ($p < 0.05$).

Insignificant bactericidal effects were observed using the blank formulation of carrageenan formulation, HPMC 856 formulation and Carbopol 974 where no PRE or $ZnSO_4$ was present. This was an overall <1 log reduction in bacterial growth, and when compared each blank formulation to its relative active formulation, it showed a statistically significantly lower log reduction value than the active formulation loaded with PRE 1 mg/mL and $ZnSO_4$ 0.25M ($p < 0.05$), as shown in Figure 9-5.

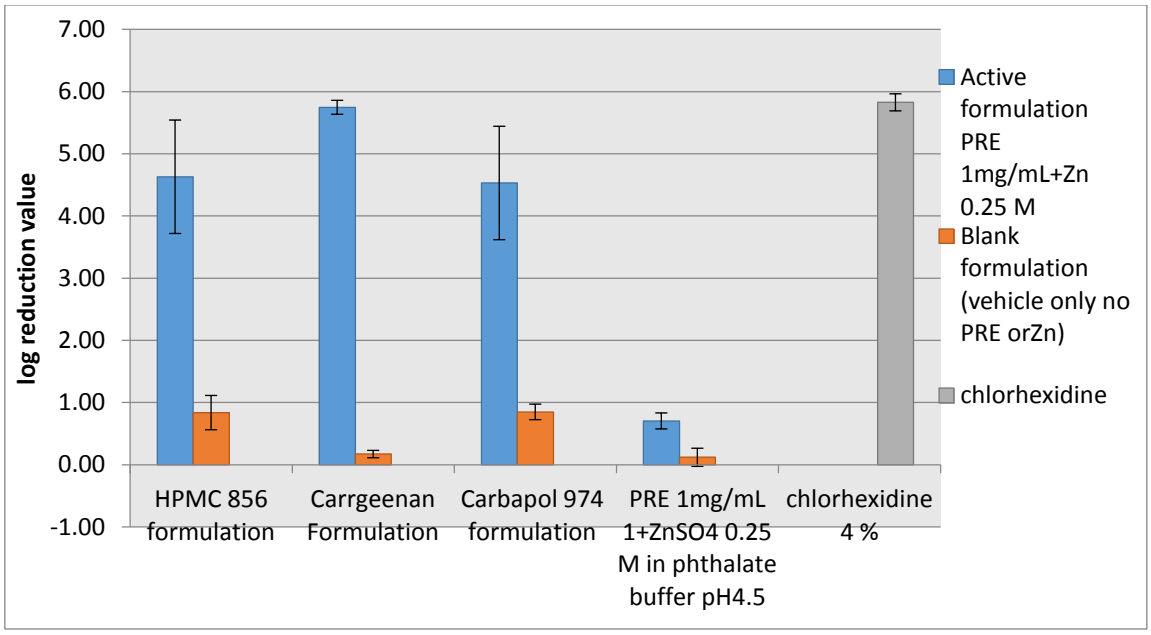


Figure 9-4 log reduction value of MRSA inoculated *in vitro* skin after 20 minutes exposure timeto (100 µL) of test formulations: HPMC856, carrageenan and Carbopol 974 PRE 1mg/mL + $ZnSO_4$ 0.25 M (pH4.5) and chlorhexidine 4% after application . Results are shown as mean log redcution value compared to control untreated group (n=2±SD).

9.5 Discussion

Initially, our inoculated bacterial (MRSA) showed survival and increased in growth after 3 hours incubated using an *in vitro* skin model. This was a very important step to measure at first, because simple dryness could kill bacteria if inoculated on the skin model at a lower concentration (Messager et al. 2004).

Although the same concentration of PRE/Zn(II) was used in the preparation of the three formulations and phthalt buffer solution, all containing (PRE 1 mg/$ZnSO_4$), the formulations showed significant high log reduction value at 20 minutes contact time ($p<0.05$) compared to the phthalate solution containing PRE /Zn. The results show that with a 20 minute application time of each formulation (carrageenan formulation, HPMC

856 formulation, and Carbopol 974), a >4.5 log reduction value was achieved against MRSA. The resulting bactericidal activity was compared to a blank formulation that contained the same excipient but not the active ingredient (PRE 1 mg/mL + $ZnSO_4$ 0.25 M). All blank combinations showed a <l log reduction and significantly lower activity when compared to the comparator formulation containing actives/s ($p < 0.05$). The observed bactericidal activity with blank formulations could be attributed to the lower pH value (pH4.5) or sensitivity to some of excipient presence in the formulation. This is in accordance with previous report showed that MRSA susceptibility could be restored when acidic media is in use (Lemaire, et al. 2007). This demonstrates evidence of PRE/Zn activity under simulated in-use conditions.

The 3 thickened formulations all performed similarly, and it was not possible to select the most active. This might be possible by diluting the formulations appropriately and re-running the experiment, although such a dilution would disrupt the integrity and viscosity properties of the gels.

On the other hand, the non-thickened solution of PRE 1mg/mL + $ZnSO_4$ 0.25 M at phthalate buffer pH4.5 gave a <1 log reduction value, and this was expected from previous findings in Chapter 3, which showed that the lowest contact time for bactericidal activity to appear was 1 hour. The additive effects of active drug to be incorporated in formulation rather than solution forms, was further explained by Nicoletti et al. (1993). In his study, chlorhexidine 4% in formulation product showed more rapidly antiseptic activity than when compared to the laboratory-prepared solution of the same concentration of chlorhexidine against *S. aureus.* Another explanation for the higher bactericidal activity achieved in the formulation but not the solution is the substantivity effect, conferred by the thickening agents used. This effect can be defined of the persistence of the antimicrobial activity of a substance even after rinsing and drying (Nishioka, et al. 2018). In Chapter 8, the permeation through HSE and epidermis layer achieved for both Zn(II) and punicalagin in higher amounts with HPMC 856 and carrageenan formulation, and our results in this chapter show that highest bactericidal

activity was observed in the latest formulation with 5.75 log reduction value, which further adds to the reservoir theory. In a previous study, the excellent long-term activity of chlorhexidine was greatly attributed to its substantivity. After the application of chlorhexidine, the compound bound to the skin stratum corneum will act as a reservoir of the antiseptic activity, and even after rinsing or drying resulted in extended bactericidal effect. The study reveals that only chlorhexidine showed substantivity activity among the three tested antiseptics, including povidone-iodine and sodium hypochlorite (Macias et al. 2013).

In our study, chlorhexidine 4% (Hibiscrub) showed high bactericidal activity (>5 log reduction value) after application to *in vitro* full thickness porcine skin inoculated with MRSA. This is in line with a previous report showing that chlorhexidine 4% gave a ≥ 4.6 log reduction value against clinically isolated MRSA at 30 minutes exposure time (Nicoletti et al. 1993). In another study, chlorhexidine 2% gave a log reduction value of 5 when the activity was investigated using bacterial suspension tests against MRSA, which broadly confirms our results achieved with chlorhexidine 4% (Maillard et al. 1998). Although the same study reported lower bactericidal activity of chlorhexidine recorded when bacteria was inoculated in an *in vitro* skin model. The study highlighted that chlorhexidine 2 % bactericidal activity decreased significantly when using the inoculated skin model to reach a log reduction value of almost 1 in comparison to the bacterial suspension test (5 log reduction value) at the same contact time ($p < 0.05$). While our results show high bactericidal activity of chlorhexidine 4% using the *in vitro* inoculated skin model, this could be explained by the fact that we used a higher concentration of chlorhexidine (at 4%) and a longer contact time (20 minute) in contrast to 2% and 10 minutes contact time in Maillard et al.'s (1998) study.

Overall, all three formulations which contained (PRE 1 mg/Zn 0.25M) showed bactericidal activity against MRSA that is comparable to chlorhexidine 4% (Hibiscrub) ($p<0.05$) after application on the inoculated *in vitro* skin model for 20 minutes contact time.

9.6 Conclusion

Our data from this simulated in-use skin sanitation work shows that:

1. All three formulations (carrageenan formulation, HPMC 856 formulation, and Carbopol 974) that contained the same concentration of active ingredient (PRE 1 mg/mL + ZnO_4 0.25 M) achieved a >4 log reduction value against MRSA (NCTC 12493). This was statistically greater than the unthickened formulation.

2. The enhanced activity of the formulations supports the development of a topically applied bactericidal product.

Chapter 10: General discussion

10.1 General Discussion

The antimicrobial activity of pomegranate has long been recognised and extensively studied in the literature, and has shown promising antimicrobial activity against a wide range of microorganisms (Melendez and Capriles, 2006; Biedenbach et al. 2004; Choi et al. 2011; Moorthy et al. 2013; Pagliarulo et al. 2016). While the activity of pomegranate has been investigated by means of fruit extracts from either the juice, pulp, seeds, flower, or rind, interest in the pomegranate peel extract has recently increased because of the higher antimicrobial activity of the rind compared to extracts from various other parts of the fruit (Fawole et al. 2012; Baccarin 2015; Ma et al. 2015; Nasiri et al. 2017; Asadi et al. 2018).

In this project we set out to investigate the potential use of the natural products pomegranate rind extract (PRE) and Zn(II) as a potential topical product to treat skin infections, both superficial and wounds. The premise for the work was the significant findings of Dr David Houston, who discovered that the co-application of PRE and $ZnSO_4$, in particular ratios, created synergistic and potent virucidal activity (the term 'potentiated' was used at the time, although it is more likely to be synergistic, as both possess some degree of antimicrobial action).

Viral skin infections are important, including the *Herpes simplex* virus, which commonly manifests as peri-oral coldsores and anogenital herpes, and the work of Houston (2011) indicated that a new topical product was feasible to treat these conditions. Additionally, as PRE/Zn is cidal in nature it has potential action in the later stages of a cold sore, whereas antiviral therapy only works in the earlier replication stages.

However, many other skin infections are caused by bacteria, and these are potentially far more life-threatening, especially if the infection enters the system. A particularly problematic topical infection is MRSA, and other bacteria which are becoming

increasingly resistant to first-line antibiotics, such as ciprofloxacin. Therefore, one of the first tasks in this work was to determine if the PRE/Zn had any effect against a panel of bacteria associated with wound infections.

The first major achievement of this work was to establish that PRE/Zn was indeed active against MRSA. In Chapter 3, the investigation of antibacterial activity of PRE/ Zn(II) showed that a synergistic effect was observed against MRSA and *S. epidermidis* when FIC index value of PRE/Zn was ≤ 0.5 in both microorganisms. An additive effect was also seen in MSSA *S. aureus*, *E.coli*, and *P. aeruginosa*. The activity varied depending on the species being challenged, but overall each was sensitive to PRE/Zn. Importantly, the activity was verified independently in an external laboratory setting (Dr Wing Man Lau, Newcastle University).

It is rare that a compound or co-administered compounds are active against both viruses and bacteria, because they are such different organisms. PRE/Zn exerts both antimicrobial and microbicidal activities. Unpublished work has determined that new ligand complexes are <u>not</u> formed in solution (Heard, personal communication). The mechanism of action, although not the focus of this work, probably lies in an interplay between the components of PRE (i.e. the tannin punicalagin, as shown by Houston) and Zn(II). An important point, then, is that the PRE/Zn system can only work when applied together topically to challenge the microorganism cooperatively – if administered intravenously for example such cooperativity is unlikely to occur due to dilution in the bloodstream.

Most drugs for treating microorganism infections are anti-microbial in nature – i.e. they interfere with the life cycle in some manner – the ratio of Zn(II) to PRE (punicalagin) is clearly of importance to achieve the best effect. Microbicides are rarer as pharmaceuticals, and one reason is that they not only kill microbes, they also have adverse effects on human tissues; i.e. they are overly cytotoxic.

Therefore, in the the next part of this project, the cytotoxicity of PRE with and without Zn(II) was investigated using the HaCaT keratinocyte cell line as a standard model of epidermal cells. Using the standard MTT cytotoxicity test, no cytotoxicity effect was observed on HaCat cell line when treated with PRE from 2.5 μg/mL and up to 100 μg/mL, at 24 hours. While FACS analysis narrowed the toxicity level to PRE ≥100 μg/mL, all investigated Zn(II) concentration (12.5-100 μM) showed no cytotoxic effect during 7 days exposure time. The previous study by Houston (2011) showed that $ZnSO_4$ showed no cytotoxic effect on the epithelial cell line at 0.1 M up to 72 hours. The antibacterial potentiation concentration that is needed for PRE to work against MRSA and *S. epidermidis* is (62.5 and 31.25 μgmL) and ZnSO (1600, 400 μM), respectively. These findings reveal that there was little or no cytotoxic effect observed in PRE/Zn in the concentration range that is needed for the potentiating of antibacterial activity up to 24 contact times. So, if PRE/Zn is generally safe to use at the cellular level, how does it affect the rate of wound closure?

Several studies have shown the potential of pomegranate extract application in wound healing, especially against aggressive types of non-healing wounds such as diabetic foot ulcers (Fleck et al. 2016). With this in mind, an *in vitro* scratch wound model was used to investigate PRE/Zn on cultured HaCaT cell migration following the introduction of an artificial scratch. The cell migration of the scratched wound model was followed using confocal microscopy over a period of 48 hours. The cell migration process showed that PRE at a lower concentration (2.5 and 5 μg/mL) appeared to show promotion within the first 24 hours, whereas PRE 10 μg/mL significantly enhanced the migration and proliferation at within this period to give 61% wound closure compared to the control (untreated group) with 38%. The same positive effect on wound healing was observed in $ZnSO_4$ in concentrations of 25, 50, and 100μM, with 91, 74, and 96% closure within the first 24 hours. All treatments in addition to the control showed complete wound closure by the 48h mark. The combination of PRE (5 or 10 μg/mL) was made with $ZnSO_4$ 25, 50 and 100 μM) and all combinations at all times gave >86% closure in the first 24 hours. Observed increase in wound healing at (PRE 10 μg/mL $ZnSO_4$ 50 μM) and (PRE 5

µg/mL + $ZnSO_4$ 50 µM) which gave 94 and 93% wound closure at the first 24 hours, respectively, when compared to the PRE and Zn(II) individual results. Overall, these positive effects seen at the cellular level suggest that PRE/Zn could indeed be beneficial by accelerating wound healing *in vivo*.

To have any chance of reaching the marketplace, PRE/Zn must demonstrate stability over a reasonable timescale. A stability study was thus performed over six months, and three main effects were investigated: the effect of Zn(II) application on PRE, the effect of three different temperatures (-20, 2-4, 20 °C), and the effect of pH value (pH 4.5 in phthalate buffer and 7 in water). Using punicalagin content as a marker, both higher temperatures and high pH value had a significant effect on the stability of PRE/Zn formulations. At six months storage time, a significant loss of punicalagin was observed with high pH (7) and high temperature (20 °C), with an almost 30 % loss in punicalagin content. No negative or positive effect of Zn(II) addition to PRE was observed at any condition. From this study, we conclude that the optimum stability of such a combination will be at pH 4.5 with a temperature storage condition of ≤ 4 °C. This could be considered an unwelcome finding, as it is far more preferable for a product to be stored and be stable at room temperature.

The work carried out thus far involved PRE/Zn as a solution; however, this is of limited value in terms of translating the positive effects observed into tangible pharmaceutical products. Therefore, we next wanted to prepare a formulation that could be used for topical application of the PRE/Zn combination. Three formulations were selected, which used three types of thickening agent: carrageenan, HPMC856, and Carbopol 974. Optimisation of the preparations was made by means of: physical appearance, stability investigation, viscosity, wettability, and finally validation of the formulation's synergistic antibacterial activity against planktonic *S. aureus.* The three selected formulations all contained the same active ingredients: PRE 1 mg/mL + $ZnSO_4$ 0.25 M.

Next, the formulations were investigated for their ability to deliver PRE (punicalagin) and Zn(II) into and across the skin. The rationale for carrying this out was that a

reservoir might be created for these actives, which could facilitate a longer term or more substantive effect on the skin surface. Also, initially mainly for completeness, we wanted to determine the extent to which the actives permeated skin, using freshly excised porcine ear skin. Consequently, depth profiling (by tape stripping) and permeation was investigated *in vitro* using heat-separated epidermis membranes. The potentiation of bactericidal activity was observed at two combinations: PRE 1mg/mL and $ZnSO_4$ 0.125M and PRE 1mg/mL and $ZnSO_4$ 0.25M. Two formulations (HPMC 856 and carrageenan) showed the permeation ratio of both analytes through the HSE is at levels which can show potentiation of the antibacterial activity for both analytes. It became apparent that this finding was more significant than first realised, as it supported the application of these formulations for the treatment of sub-surface skin infections, such as cellulitis. From the tape stripping of the HSE, the punicalagin was found in levels that could increase the reservoir effect of PRE. The punicalagin level detected at the outer layer would be sufficient for PRE to promote cell proliferation and cell healing.

Finally, we wanted to end the thesis by establishing that the formulations provide bactericidal activity in an in-use scenario. An *in vitro* skin sanitation study was therefore performed, again using freshly excised porcine as a substrate and MRSA as test bacterium. The skin was firstly sterilised used UV irradiation for 10 minutes, then inoculated with MRSA. The areas inoculated were then dosed with the PRE/Zn formulations and, after certain timepoints, bacterial recovery was determined. The results show that the unthickened solution gave a < 1 log reduction after 20 minutes of contact time. However, a >5 log reduction value was achieved against MRSA with all three formulations (HPMC 856, Carbopol 974, and carrageenan), which was the same as the positive control chlorhexidine 4% (Hibiscrub).

To summarise the main findings of this thesis:

1. PRE/Zn shows synergised activity against a panel of bacteria, as well as viruses such as HSV as known from previous works

2. Little evidence of cytotoxicity using HaCaT cells

3. Positive effects on cell migration and wound closure

4. Good stability was demonstrated at temperatures of ≤ 4 ˚C

5. Three hydrogels were prepared and characterised, which are demonstrated bactericidal activity

6. Of the tested formulations, PRE/Zn penetrates the skin to an extent that could provide a reservoir effect.

7. Of the tested formulations, PRE/Zn permeates skin at levels that could be used to treat sub-surface infections

8. Applied topically to skin, the formulations provide good bacterial sanitation, to a similar level of the chlorhexidine control

Further work

This thesis has covered a lot of ground in determining the feasibility of developing a PRE/Zn product for use as a skin and wound sanitiser. However, we are still some way away from a refined marketable system.

Given more time, we would have liked to investigate activity across even further bacterial biofilms, since it would make for an extremely interesting system across a wide range of sanitising applications. Adding further bacterial species and clinical isolates would also help to establish the broad spectrum activity of PRE/Zn.

As the current system is based on natural product (pomegranate rind extract), development of a standardised method to decrease the extraction batch variations, i.e. varying polyphenol content levels, would be advantageous.

In preliminary studies, we used a preparative HPLC system to isolate punicalagin from PRE, and it showed a high purity level of about 89% (data not shown). It is worth reinvestigating the punicalagin isolation method, and studying the stability and antibacterial activity of this compound in isolation.

Pomegranate polyphenols antibacterial activity can be studied in separate such as punicalagin and ellagic acid. More data antibacterial activity would give more insight into the antibacterial activity part; specifically, whether it is advantageous to use isolated polyphenol or the whole PRE in combination with Zn (II).

Mechanism of action can be further studied using several Zn (II) salts to confirm that the antibacterial activity comes from the Zn(II) rather than its salt (i.e SO_4^{2-}). In addition, a binding interaction between PRE and Zn(II) can be investigated using isothermal titration calorimetry (ITC), in order to investigate if the combination made a new substance or simple complexation took place.

Finally, given the high correlation of the porcine skin model to human skin, we showed the encouraging antiseptic and permeation profile activity of PRE/Zn, although it is important to note that all work was performed under "*ex vivo*" conditions. Ultimately, an *in vivo* study on an animal model or in humans would need to be conducted, especially when proposing the treatment of clinical conditions such as impetigo and cellulitis.

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