School of Pharmacy

Anti-inflammatory and Antibacterial Effects of Kinos from Australian and Papua New Guinean Eucalyptus Species

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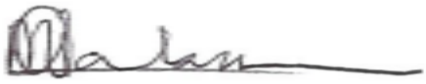
This thesis is presented for the Degree of
Doctor of Philosophy
of
Curtin University

June 2015

DECLARATION

To the best of my knowledge and belief this thesis contains no material previously published by any other person except where due acknowledgement has been made.

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.



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June 16, 2015

ACKNOWLEDGEMENTS

I would like to thank all the people who contributed to this Thesis in every way. First and foremost, I would like to thank my two supervisors, Dr Ricky Lareu and Dr Rima Caccetta for demanding a high quality of work in all my aspects of my PhD program and supporting my attendance at conferences. I would like to thank Dr Andrew S. McWilliam for his initial contributions towards my research. I would also like to thank Dr Simon Fox for his contributions for ensuring all reagents for my experiments were in stock at all times. Dr Connie Jackaman is thanked for her patience in training me on the use of flow cytometer. Ms Irma Larma-Cornwall assisted me with the analysis of the flow cytometry data for the phagocytosis assay. Dr Chi Ndi of University of South Australia is thanked for his assistance with the phytochemical screening of kinos.

I would like to thank my fellow PhD colleagues, Mr. Wolfgang Wimmer and Mr. Alex Richards for always working together as a team solving problems related to cell culture, immunology and molecular biology. Ms. Angie Samec and Mr. Giuseppe Luna assisted me with the Microbiology aspects of my research and sub-fractionation of kinos work, respectively, in this thesis. I would like to thank Ms. Gaewyn Ellison for going out of her way from her busy schedule for her experiments for her PhD program to proof-read different chapters of my thesis. Ms. Sangeetha Mathavan, a PhD colleague and a very close friend is thanked for always working together to solve problems for each other's research and morally supporting each other.

Without a sponsorship, I would not have studied at Curtin University and finally produce this thesis. I am very grateful to the Australian Government for giving me the Australian Leadership Award Scholarship that funded my tuition and living allowances during my 4 years PhD tenure. I would also like to thank the School of Pharmacy for giving me a fee waiver for 3 months when my 4 years sponsorship ended.

Finally, I would like to acknowledge family and friends who supported me during my study. First and foremost, I would like to thank my husband and my two daughters,

my mother, sisters, nephews and nieces for their constant love and support throughout this long academic journey. I would also like to thank Lydia Warubi, Jossie Bakri and Ericka Dawanincura for their friendship and support throughout my study period in Perth, Western Australia.

I would like to dedicate this thesis to my late father, my mother who lived up to see the final end-product of this thesis despite having arthritis and my two daughters, Grace Boker Mungkaje and Catherine Autamin Mungkaje.

LIST OF ABBREVIATIONS AND ACRONYMS

Abbreviation/ Acronym	Full name
APH	Acid phosphatase
BSA	Bovine serum albumin
CLSI	Clinical and Laboratory Standards Institute
DCM	Dichloromethane
E	*Escherichia or Eucalyptus*
E. coli	*Escherichia coli*
ELISA	Enzyme-linked immunosorbent assay
EOs	Essential oils
E. *calophylla*	*Eucalyptus calophylla*
E. *confertiflora*	*Eucalyptus confertiflora*
ERK	Extracellular-signal regulated kinase
FACS	Fluorescence-activated cell sorting
FBS	Foetal calf serum
AF1	Acidic fraction 1
BF2	Basic fraction 2
DCMF3	Dichloromethane fraction 3
h	Hour(s)
IκB	Inhibitory kappa beta
IL	Interleukin
JAK	Janus kinase
JNK	C-Jun N-terminal kinase
LPS	Lipopolysaccharide
LRP	Leukocyte common antigen-related phosphatase
MAPK	Mitogen-activated protein kinase
MBC	Minimum bactericidal concentration
MHA	Mueller-Hinton agar
MHB	Mueller-Hinton broth

LIST OF ABBREVIATIONS AND ACRONYMS CONTINUED

Abbreviation/ Acronym	Full name
MIC	Minimum inhibitory concentration
Min	Minute(s)
µg/mL	Microgram per millilitre
mg/mL	Milligram per millilitre
µL	Microlitre
mL	Millilitre
NCCLS	National Committee for Clinical and Laboratory Standards
NF-κB	Nuclear factor-kappa beta
NO	Nitric oxide
$ONOO^-$	Peroxynitrite
P. aeruginosa	*Pseudomonas aeruginosa*
pg/mL	Picograms per millilitre
PGN	Peptidoglycan
PNG	Papua New Guinea(n)
RA	Rheumatoid arthritis
RPMI	Roswell Park Memorial Institute
ROS	Reactive oxygen species
S. aureus	*Staphylococcus aureus*
TE	Total kinos extract
TLR	Toll-like receptors
TMB	3,5,3',5'-tetramethylbenzidine
TNF-α	Tumour necrosis factor alpha
USFDA	United States Food and Drug Administration
WA	Western Australia(n)

ABSTRACT

Kinos are astringent exudates from Eucalyptus species which are used by the first Australians to treat inflammatory conditions and bacterial infections. Tannins are the predominant chemical constituents in kinos as identified by the early investigations of kinos chemistry. The tannins and cathechins (constitutive of flavonoids), major groups of compounds are found in plants. Flavonoids are components of nutrition but have also been extensively investigated for their antimicrobial activities using *in vitro* and *in vivo* study models. However, limited antimicrobial studies have been conducted on kinos from *Eucalyptus* species and therefore, this study sets out to validate claims for their medicinal uses. The main goals were to investigate anti-inflammatory and antibacterial effects of kinos from Western Australian (WA) and Papua New Guinean (PNG) *Eucalyptus* species using *in vitro* study models.

Kinos from *Eucalyptus calophylla* (WA) and *Eucalyptus confertiflora* (PNG) were solubilized in a water-based buffer. Murine RAW264.7 macrophages were used for anti-inflammatory and phagocytosis studies. Gram negative and Gram positive bacteria were tested for their susceptibility to kinos. The level of cytokine secretion by the kinos treated LPS-stimulated macrophages were measured by ELISA, whilst nitric oxide (NO) levels were measured by the Griess method. Phagocytosis was evaluated by fluorescent flow cytometry and antibacterial effects were determined by agar diffusion and microdilution methods. Phytochemical analyses of kinos were conducted using thin layer chromatography and qualitative tests.

The RAW264.7 macrophage cells developed vacuoles after being exposed to kinos. The intensity of the vacuoles increased with increasing concentrations of kinos and incubation times. The vacuoles were more pronounced in cells exposed to WA kinos than to the cells exposed to the PNG kinos. The cells were 100% viable for the kinos concentrations lower than 250 µg/mL and they were 80% viable at this concentration. The cell viability decreased in a dose-dependent manner. Secretions of interleukin (IL)-6 and NO were significantly reduced by the kinos in a concentration dependent manner. In contrast, the secretion of tumour necrosis factor alpha was enhanced by the kinos whilst IL-10 was also enhanced by WA kinos but it was reduced by the PNG

kinos. The phagocytosis of Gram positive and Gram negative bacteria by macrophages was enhanced by the kinos. Gram positive bacteria were inhibited by the kinos with a minimum inhibitory concentration of 1 mg/mL and a minimum bactericidal concentration of 3 mg/mL. Gram negative bacterial growth was not affected by kinos.

Phenolics (flavonoids), tannin, and triterpenoid saponins are predominant in the kinos as evaluated by the phytochemical qualitative (flavonoid and triterpenoids saponin tests), quantitative (assays of total phenolics and total tannins) and spot tests (kinos spotted on thin layer chromatography plate). Biological activity was lost for WA kinos by fractionation whilst some fractions of PNG kinos retained activity. This suggests that the biological effects of kino on macrophage activity and potentially on growth and viability of Gram positive bacteria may be multifactorial and possibly synergistic in nature.

Through these studies we demonstrate that kinos predominantly induce an anti-inflammatory profile in mouse macrophages and possess antibacterial properties against Gram positive bacteria. Although limited to the *in vitro* context we provide evidence substantiating some of the claims by traditional medicine.

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CHAPTER 1

GENERAL INTRODUCTION

1.1. Introduction

Investigations into the therapeutic properties of natural extracts from plants, and more recently marine life, have been an area of intense research, particularly with the increasing resistance of microorganisms to current drugs world-wide. Properties under investigation include antibacterial, antifungal, antiviral, anti-inflammatory and antioxidant activities. The ineffectiveness of many drugs against various infections has contributed to numerous investigations into overcoming the resistance of pathogens to drugs. This type of research requires a multi-disciplinary approach to address the concern of resistance among pathogens around the world over the years to produce alternative drugs. Plants have been widely investigated for various bioactivities including effects on microorganisms for use as potential drugs. Chemical constituents of plants are isolated and tested for different biological properties *in vitro* before those chemicals that exhibit most efficacious activities are modified and/or synthesized and tested *in vivo* (Cowan, 1999; Dillard *et al.*, 2000). These lead drugs undergo extensive clinical tests before being approved by drug standards committees such as the US Food and Drug Administration (USFDA) and Australian Therapeutics Good Administration, for human use.

In the continuing search for drugs to replace those that have become ineffective against various pathogens, scientists have mainly targeted plants to obtain chemical compounds, known as secondary metabolites, to be purified, modified and possibly synthesized into drugs. A significant number of plant species have been investigated for their various chemical compounds and biological properties. However, there is a need for new investigations into such properties due to an increase in the development of resistance in pathogenic microorganisms to current drugs. This increase is at an alarming rate, as evidenced by high cases of morbidity and mortality in humans (Dias *et al*., 2012).

Many bacterial species have evolved mechanisms that enable them to quickly proliferate into large numbers on wound sites, resulting from mild through to severe adverse effects on the host. Inflammation is a standard response at the site of infection, and depending on the degree of infection and the site, the intensity of the inflammatory response will vary, which is initiated and propagated by the types and levels of cytokines released at the infection site. Therefore, a major aim of this thesis is to evaluate the anti-inflammatory and antibacterial properties of a natural plant product to alleviate persistent bacterial infections that elicit inflammation in the human host.

Various biological and chemical properties of leaves, barks and, to a lesser extent, various kinos have also been investigated (Grattendick *et al.*, 2008). The kinos are a term that refers to reddish-brown dried exudates from *Eucalyptus* species and several *Pterocapus* species (Grattendick *et al.*, 2008; Williams, 2011). There have been studies on the antibacterial and antifungal properties of the essential oils from the leaves of the *Eucalyptus* species in Australia and other countries (Ghalem & Mohamed, 2008; Gilles *et al.*, 2010; Sartorelli, *et al.*, 2007; Silva, *et al.*, 2003). However, their antiviral, anti-inflammatory and antioxidant properties have not been extensively studied. Similarly, a number of significant investigations on the chemistry of the leaves, bark and kinos of Australian *Eucalyptus* species have been conducted (Low *et al*., 1974; Maiden, 1890; 1892; Marzoug *et al*., 2010). Investigations of the chemistry of kinos were done using unsophisticated equipment in the late1800s (Maiden, 1890; 1892). In contrast, there have not been any studies on any of the biological and chemical properties of the *Eucalyptus* species in Papua New Guinea (PNG) where *Eucalyptus* plants are also abundant.

Differences in soil types and climatic conditions contribute to differences in the chemical composition and thus biological effects of plants. These may contribute to variations in antimicrobial effects of the plants due to the presence of secondary compounds, also known as natural products (Cowan, 1999).

1.2. Natural products

Natural products are chemical constituents that are produced by living organisms that inhabit terrestrial, fresh water and marine environments. They contain pharmacological substances for use in pharmaceutical drug discovery and drug design. Distribution and composition of chemical constituents of organisms vary from species to species and even among different parts of an individual organism (Cowan, 1999). For example, a study by Sultana *et al* (2009) demonstrated that flavonoids were found predominantly in the leaves of *Moringa oleifera* rather than in its bark. This study also showed differences in the composition of total phenols and flavonoids with different extraction methods. In a similar study, the essential oil, 1, 8-cineol, was more predominant in immature flowers than in the leaves, stem and fruits of the *Eucalyptus oleosa* (Marzoug *et al.* 2011).

Following the discovery by Alexander Flemming in 1928 that penicillin (*Penicillium notatum)* killed *Staphylococcus aureus*, investigations into microbial sources for the discovery of new drugs increased dramatically in the hope of identifying and

isolating more antibiotics from microorganisms (Butler, 2004; Dias *et al.*, 2012). Investigations into plant sources also increased in order to search for natural products that can be synthesized by drug companies for a variety of applications. Due to advances in equipment for undersea exploration, marine environments have also been investigated for organisms to be used for research into drug discovery from the late 1970s (Butler, 2004).

For each organism that was identified possessing potential therapeutic substances, investigations were undertaken to determine whether they contained any bioactive chemical compounds. These chemical compounds are known as natural products or secondary metabolites and they vary among individual organisms and also among parts of an organism (Cowan, 1999). For example, the chemical compounds that are found in the mature parts of a plant are different to those that are evident in young parts of this individual plant. The secondary metabolites are produced either to adapt to the surrounding environment or as defence mechanisms against predators (Cowan, 1999; Dewick, 2002). The different biosynthetic pathways such as photosynthesis, glycolysis and the Krebs cycle that take place in the different parts of the plants contribute to the production of various secondary metabolites (Cowan, 1999; Dewick, 2002).

The chemical diversity of plants is also evident by differences in their bioactivity. Many studies have shown one plant part can possess a vast array of bioactivities. Bioactivity results from individual interactions between molecules that may be antagonist, no effect, additive or synergistic. The synergistic effect refers to a very large increase in bioactivity which is much greater than that due to the sum of the individual components (Jia *et al*., 2009; Schro¨ der *et al*., 2011). The notion of chemical diversity is further supported by the isolation, characterization and identification of bioactive chemical compounds by advanced chemical analysis techniques.

Selection of plants and organisms for the isolation of pharmacologically active compounds have been mostly directed by their use in folklore medicine, in treating ailments including life-threatening diseases such as HIV-AIDS, cancer and diabetes. The use of natural medicine in most indigenous communities that rely partially or entirely on these natural remedies, are generally not well documented. However, they were verbally conceded from one generation to another prior to the 20th century (Pearn, 2005; Williams, 2010). For example, there were no written systematic

records of ethnobotany of people of the Khoikhoi and Western Cape and Karoo regions of South Africa, the oldest folk medicine system, see below (van Wyk 2008).

1.3. Link between folk medicine and natural products

Folk medicine has been in existence for as long as humans have lived on earth. There are several folk medicine systems documented that include: Africa, Asia, Ayurveda and Latin America (Bischoff, 2007; Mukherjee & Wahile, 2006; Patwardhan & Mashelkar, 2009; van Wyk 2008). The African traditional herbal medicine system is the oldest with numerous therapeutic effects compared to other herbal medicine systems. Five thousand species out of the 45,000 plant species are medicinally important in South Africa, due to tropical and sub-tropical climatic conditions for their growth and distribution (Mahomoodally, 2013). In contrast, the Ayurvedic medicinal system is the second oldest herbal, alternative and complimentary system but it is the oldest system for healing of psychotic disorders in the world ((Ranawat *et al*., 2015).

Plants were used for treating different ailments since 25 BC. Many philosophers from the BC era documented methods of plant extract preparations and their medicinal uses on engravings and drawings (Petrovska, 2012). For example, the gummy sap known as ‘manna’ from Alghagi maurorum Medik (commonly known as Camels thorn) contains melezoites, sucrose and inverted sugar (a mixture of glucose and fructose). The Ayurvedic people documented its use for the amelioration of anorexia, constipation, dermatosis, epistaxis, fever, leprosy and obesity as early as 25 BC (Butler, 2004). Plants used in folk medicine have been and still are the lead targets for the identification and isolation of natural products for further screening for bioactivity against various pathogens. For example, the Indian medicinal plants are known to be the major sources of natural products until now, used in the production of pharmaceuticals, agrochemicals, flavour and fragrance ingredients, food additives and pesticides (Patil & Gaikwad, 2011).

It has been argued that although investigations on drug discovery have been mostly based on antimicrobial effects of organisms and plants, it is also the chemistry aspect that complements such investigations. The bioassay-guided isolation and identification of chemical compounds from organisms and plants direct investigations into lead target compounds that were initially guided by their traditional uses (Butler, 2004). Investigators have adapted many chemical analyses

and screening methods but due to their costly nature, have been substantially scaled down in the early 2000s.

Many natural products isolated from plants and approved by the USFDA have been based on traditional medicinal remedies throughout the world. Acetylsalicylic acid and salicin (an alcoholic β-glucoside) are two natural anti-inflammatory agents isolated from the willow tree *Salix alba* L. (Locher & Currie, 2010; Patel *et al*., 2013). In the 1870s, investigation of the *Papaver somniferum* L., an opium poppy that was used medicinally by Sumerians and ancient Greeks, led to the isolation of alkaloids from which codeine was synthesized and is now used as an analgesic drug (Cragg & Newman, 2005). Digitoxin, isolated from *Digitalis purpurea* L, was seen to relieve cardiac conditions (Balunas & Kinghorn, 2005; Li *et al*., 2014). The antimalarial drug, quinine, isolated from the bark of *Cinchona succirubra* Pav. Ex Klotch, was the main antimalarial drug until the 1940's when chloroquine became used instead. In 1994, it was taken off the shelves by the FDA for treatment of leg cramps, but subsequently reapproved in 2004 as an antimalarial drug. It was re-approved for treating malaria because the newly introduced anti-malarial drug, chloroquinine, during that period became ineffective to the malarial parasites (Achan *et al*., 2011; Dias *et al*., 2012). This bark was commonly used for the relief of malaria throughout the world in the 1800s. Another plant-derived drug, pilocarpine, had formulations approved by the USFDA for chronic and acute glaucoma (Butler, 2004).

Drug discovery and development is a complex, technological and capital-intensive orientated process (Heinrich *et al*., 2009; Patwardhan & Mashelkar, 2009). Any investigations into drug discovery are also multi-disciplinary in nature and as such involve traditional herbalists, ethnopharmacologists, pharmacologists, phytochemists and antimicrobial scientists. An ethnobotanical approach into drug discovery investigation is suggested to be the best approach (McRae *et al.*, 2007). Such an approach enhances chances of isolating novel compounds from indigenous medicinal plants. There are several methods that are used in isolating novel compounds that could potentially treat persistent infections such as methicillin-resistant staphylococcal, vancomycin-resistant enterococcal and multidrug-resistant infections. Usually pre-screenings of extracts from traditional medicinal plants are employed which use cell-based assays to validate chemical isolation from a given extract for the chemical compound isolation after numerous tests. The chemical compounds that can undergo preclinical and clinical tests should be standardized, synergistic, safe and efficacious (Doughari *et al.*, 2009). Separation of compounds

from the plant extract mixture and the bioassays to test for their efficacies are difficult and costly. Therefore, academics and pharmaceutical companies have taken ownership of certain stages of any drug discovery investigations to minimize the costs and difficulties (Heinrich, 2009).

1.4. First Australian traditional medicine

Traditional medicine has been practised among first Australian communities. First Australians, which include the Aborigines and Torres Strait Islanders, have been utilising selected plants for alleviation of ailments for centuries prior to the arrival of the first Europeans in 1788. Debilitating conditions such as scurvy and diarrhoea encountered by the European settlers to Australia, due to long sea voyages, made it necessary for these settlers to search for plants onshore for remedies (Williams, 2010). These were the beginnings of the archival herbal medicine in Australia. Thus, the beginnings of documentations appear to have started with the arrival of the settlers. Herbal medicines that are commonly used in First Australian communities are considered sacred; they believe are controlled by the 'spirits' of their ancestors and geographical landmarks such as mountains and rivers. Traditional knowledge of herbal medicines in these ancient communities has been conceded from one generation to another and was not documented before 1788 (Pearn, 2005).

Plants are not the only source of traditional medicine known among the First Australian communities. A diverse group of insects are also highly regarded for their therapeutic uses besides being good dietary supplements (Williams, 2010). A link between plants and insects is evident in the type of infections they relieve. Some chemical compounds that are bioactive in the insects are also common in the plants that they associate with since insects such as butterflies and bees pollinate the flowers of plants. Hence, chemical compounds known as secondary metabolites are produced by specific biosynthetic pathways within pollinated parts of plants (Dewick, 2002).

There are over 27, 000 plant species in Australia, both native and introduced, many of which possess pharmacological properties. Well over 700 *Eucalyptus* species are native to Australia and most of them produce kinos. Different parts of *Eucalyptus* plants, including kinos, have been used traditionally to ameliorate various infections among the first Australians. Kinos are astringent exudates from many Australian *Eucalyptus* species and they have been used by the first Australians for the alleviation of wounds, burns, pains and diarrhoea (Lassak & McCarthy, 2001;

Maiden, 1892; Zhang *et al*., 2008). Kino is a dark, brownish liquid exudate which is produced from plants and hardens when exposed to air due to oxidation. Plant genera such as *Eucalyptus* and *Pterocarpus* are the most common kino-producing plants. The kinos are produced when live bark and wood cambium are damaged. Gum has been universally used to refer to these dried *Eucalyptus* exudates, and additionally, the gum denotes any *Eucalypts* in Australia (Zhang *et al.*, 2008). Essential oils from eucalypts have been used to develop lozenges, cough syrups, rubs and vapour baths which are commonly used as remedies for common colds throughout Australia, United States and Europe. Besides treating colds, there are ointments produced from essential oils of the *Eucalypts* that are used for relief from nasal and chest congestion. The essential oils are also available in insect sprays and in the form of body lotions and liquids to ameliorate arthritis and treat boils, sores and wounds (Zhang *et al.*, 2008). Having synthesized these products mostly from the leaves of *Eucalypts*, researchers can confidently perceive that some medicines can also be synthesized from kinos for the amelioration of infections. This can be achieved by isolating chemical compounds from the kinos and establishing their therapeutic potential through antimicrobial studies. Chemical structures of these compounds can be elucidated and modified to produce medicines with clinically established therapeutic effects.

Of more than 700 *Eucalyptus* species that are abundant in Australia, only eight *Eucalypts* are endemic to Western Australia (Williams, 2011). *The Eucalyptus* species are from the Order *Myrtales* and Family *Myrtaceae* and genus *Eucalyptus*. There are eight species that are only found in Western Australia (WA) and they grow in dry and sandy soil conditions. One of these species is *Eucalyptus calophylla,* R. Br and its common names are Marri and Port Gregory gum. Morphological and deoxyribonucleic acid (DNA) analyses of *E. calophylla* have shown it to possess a taxonomic relationship with the genus *Corymbia*. Therefore, *E. calophylla* is also classified as *Corymbia calophylla*. Two other WA *Corymbia* species, *Corymbia ficifolia* and *C. haematoxylon* have common taxonomic features with *C. calophylla* (Williams, 2011). The *E*. *calophylla* is one of the most commonly used *Eucalyptus* species for medicinal purposes by the first Australian communities in WA. The leaves, barks, fruits and kinos of *E*. *calophylla* have been used for treating inflammatory conditions, bacterial infections and toothache among these communities (Lassak & McCarthy, 2001; Williams, 2011).

Non-contemporary phytomedicines have been the basis for the development of some modern medicines. For instance, cinchona barks were used in Latin American communities for the amelioration of malaria and scientists subsequently synthesized quinine, guided by the Latin American folklore medicinal uses of these barks. Due to general knowledge of the treatment of malaria for centuries in Brazil with *Cinchona calisaya* and *C*. *succirubra*, three *Cinchona*-related plant species were explored for their antimalarial effects in mice (Andrade-Neto *et al.*, 2003). Only one of the three species showed antimalarial activity, by reducing the number of parasites in the *Plasmodium berghai* induced mice. This shows that plant physiology, their biosynthetic pathways, genetic composition and the environmental conditions can contribute to differences in a type of expected bioactivity. Quinine was common for the treatment of malaria in the 18thand 19th centuries (Olguín-Albuerne *et al.*, 2014) and many of its derivatives such as quinidine are being sold as prescription drugs for arrhythmia (Yang *et al.*, 2009). In their review article, Yang and co-workers reported previously discontinued quinidine to be efficacious when used in combination with verapamil which showed a synergistic effect in treating arrhythmia. Artemisinin is another example of a natural product that was synthesized from the leaves of *Artemisia annua* (qinghao), a plant used by the Chinese for treating malaria. Achan *et al* (2011) reported the bioactivity of *Artemisia* through screening processes for toxicity. Artemisinin and its derivatives have now become important therapeutic agents for treating malaria. In a recent review paper, Bilia *et al* (2014) documented other antimicrobial activities of essential oils of *Artemisia annua.* The essential oils were demonstrated to inhibit several pathogenic bacterial and fungal species using different methods as outlined in different studies reported in this review.

Similarly, in-depth aspects of the chemistry of kinos of Australian *Eucalyptus* species have been thoroughly reviewed by Locher and Curie (2010). The history, mechanisms of formation, medicinal uses and chemistry of kinos are covered in this review. Furthermore, a limited number of investigations into pharmacological aspects of kinos have been conducted and are also reviewed. With increasing improvements in facilities for pharmacological studies in this century, kinos like any other plant sources, will have to be investigated scientifically to establish claims for their use in various treatments for infections by the first Australians. Table 1.1 on page 10 outlines kinos from different *Eucalyptus* species and their respective medicinal uses in first Australian communities.

Table 1.1. Traditional medicinal uses of kinos from Australian *Eucalyptus* species.

Species	Type of infection treated	Reference(s)
E. acmenioides	Gastrointestinal infection	Lassak & McCarthy (2001); Low (1990)
E. calophylla	Gastrointestinal infection, Inflammation and wounds	(Powell, 1990)
E. camaldulensis	Gastrointestinal infection Wounds Sores, colds and fevers	Isaacs (2002) Lassak & McCarthy (2001) Low (1990)
E. crebra	Gastrointestinal infection	Lassak & McCarthy (2001)
E. gummifera	Venereal sores	Isaacs (2002); Lassak *&* McCarthy (2001); Low (1990)
E. haemastoma	Gastrointestinal infection and wounds	Lassak & McCarthy (2001)
E. maculata	Inflammation	Lassak & McCarthy (2001); Low (1990)
E. mycrocorys	Gastrointestinal infection and sores	Lassak & McCarthy (2001); Low (1990)
E. papuana	Bacterial infection of gums	Low (1990); Webb (1969)
E. polycarpa	Gastrointestinal infection, burns and bacterial infection of gums	Lassak & McCarthy (2001); Low (1990)
E. racemosa	Gastrointestinal infection, wounds and ulcers	Lassak & McCarthy (2001); Low (1990); Webb (1969)
E. rostrata	Gastrointestinal infection, colds and bacterial infection of gums	Low (1990); Maiden (1892)
E. tereticornis	Gastrointestinal infection	Lassak & McCarthy (2001); Low (1990)
E. terminalis	Gastrointestinal infection, inflammation, sores and wounds	Lassak & McCarthy (2001); Low (1990) Reid & Betts (1979)
E. tessellaris	Gastrointestinal infection and inflammation	Lassak & McCarthy (2001); Low (1990)
E. tetrodonta	Gastrointestinal infection and inflammation	Isaacs (2002); Lassak & McCarthy (2001)

E-Eucalyptus

In spite of well-known historic medicinal uses of kinos from different *Eucalyptus* species, only two antibacterial studies have been conducted on the kinos from Australian *Eucalyptus* species (Locher & Currie, 2010; von Martius, *et al*., 2012) whilst there have been no antibacterial studies of the kinos from any *Eucalyptus* species from PNG.

There are ten introduced *Eucalyptus* species in PNG. The PNG *Eucalypts* grow in abundance in dry savannah grasslands concentrated in the southern region of the country with a few species scattered in the other coastal regions. There is no documentation of their medicinal use, possibly because they are introduced, and thus more recent and less explored by the local people for possible medicinal values (Srivastava, 1996).

1.4.1. Chemistry of kinos and links between their chemical constituents and antimicrobial activity

According to reports by Maiden (1892), three main groups of kinos from the Australian *Eucalyptus* species were identified based on the texture of the gums after they have been submerged in water and spirit, as it was called during the era of Maiden. The three groups included: ruby, gummy and turbid groups. The ruby group of kinos was soluble either in cold water or cold spirit and was ruby in colour. The gummy group kinos were soluble in cold water but not in cold spirit. The members of the turbid group were soluble in hot water and hot spirit but precipitated upon cooling. The basic groupings of the kinos formed the basis for the earliest chemistry work on the kinos.

Tannic acids and flavonoids were the predominant chemical constituents that were first isolated and identified from the kinos from the *Eucalyptus* species (Maiden, 1890; 1892). The tannins and the flavonoids are polyphenolic compounds that are common chemical constituents that are predominantly found in plants (Cowan, 1999). However, these are not of the same chemical group of compounds. The tannins contain mostly hydroxyl and some carboxyl functional groups while the flavonoids contain a 15-carbon skeleton, which consists of two phenyl rings and a heterocyclic ring (Dai & Mumper, 2010).

Flavonoids are important dietary constituents present in fruits, vegetables and teas (Burbage *et al*., 2015; Sakakibara *et al*., 2003). Flavonoids are not only valued for their nutritional benefits, they have also been isolated from numerous plants and extensively investigated for their antimicrobial activities using *in vitro* and *in vivo*

study models (Sakakibara *et al*., 2003). One such study by Zhao *et al* (2011) isolated nine flavonoids from *Dalbergia odorifera* and found four of them exerted anti-*Ralstonia solanacaerum* activity. *Ralstonia solanacaerum*, an anaerobic, non-sporing, Gram negative plant pathogenic bacterium, was inhibited by these isolated flavonoid constituents. The reason for the different antimicrobial activities by similar compounds depends on the functional groups and their location on the basic chemical structure of plant chemical constituents such as flavonoids, alkaloids and anthraquinones, significantly influencing bioactivity (Zhao *et al.*, 2011). In another study, three isolated and identified flavonoids from a dichloromethane extract of *Euphatorium arnottianum* exhibited anti-inflammatory effects *in vitro* and *in vivo* (Clavin *et al.*, 2007). A study by Costa *et al* (2008), demonstrated that the same crude extracts of *Guatteriopsis* species varied in antibacterial activity against eleven bacterial species. This was due to differences in the cellular structures of Gram-positive and Gram-negative bacteria and these differences were also demonstrated in another study (Polančec *et al.*, 2012). Therefore, it can be strongly suggested that differences in a) the chemical constituents of extracts, b) solvents and c) methods used for extraction play an important role in the differences in bioactivity of phytochemicals (Demishtein *et al.*, 2015; Uribe *et al.*, 2013; Zhao *et al.*, 2011).

Tannins and flavonoids from plant sources have been investigated to be potent against inflammatory conditions, bacterial, fungal and viral infections (Dai & Mumper, 2010; Dias *et al*., 2012; Scalbert, 1991). One such investigation involved the proanthocynanidins (flavonoids) from the fruits of *Zizyptus jujube* var. *inermis* and *Zanthoxylum piperitum* which inhibited the methicillin-resistant *Staphylococcus aureus* (MRSA). In contrast, the tannins from the same fruits slightly suppressed the MRSA (Hatano *et al*., 2005).

Kim *et al* (2004) demonstrated that several flavonoids to modulate the pro-inflammatory gene expression of important biomolecules including cyclooxygenase-2, inducible nitric oxide synthase, and several pivotal cytokines. In addition, some flavonoids have been reported to have the inhibitory effects on the enzyme activities of various signalling pathways for inflammation.

Other studies also investigated potential antimicrobial activity of flavonoids in the essential oils of *Eucalyptus* species, which prominently exhibited antibacterial activities (von Martius *et al*., 2012; Ghalem & Mohamed, 2008; Marzoug *et al*., 2010). There were also antifungal (Sue *et al*., 2006; Musyimi & Ogur, 2008; Gilles *et al*., 2010; Marzoug *et al*., 2010) and anti-inflammatory (Silva *et al*., 2003) activities

found in essential oils. These previous studies strongly suggest that the *Eucalyptus* species possess different antimicrobial activities. The antimicrobial activities of tannins and flavonoids were not only detected *in vitro* but also *in vivo*.

1.5. Antimicrobial effects of *Eucalyptus* species

Numerous antimicrobial activities of *Eucalyptus* species have been investigated in Australia and many other countries where these plant species are abundant. Many of the *Eucalyptus* species have been used for traditional medicinal purposes in first Australian communities for centuries (Zhang *et al.*, 2008). In tropical and subtropical countries where these plant species are predominantly found, they have been the lead targets for the investigation of various antimicrobial activities (Bachir & Benali, 2012). Most of the investigations into antimicrobial properties, such as the antibacterial activity, of the *Eucalyptus* species have so far been mainly conducted on groups of compounds in selected extracts such as the essential oils, from leaves and barks, with limited antimicrobial studies on kinos. A study by von Martius *et al* (2012) demonstrated an intense antibacterial effect of hydrolysable and condensed tannins from fifteen Australian *Eucalyptus* species on Gram positive bacteria using the agar well diffusion method. However, they found no inhibitory effect on Gram negative bacteria and yeast. The minimum inhibitory concentrations (MICs) and the minimum bactericidal concentrations (MBCs) of the kinos were not demonstrated for the Gram negative bacteria in this study by von Martius *et al* (2012). The MICs and MBCs are two very important semi-quantitative variables in any antibacterial studies and are usually evaluated using agar and broth dilution methods (Fitting *et al.*, 2004; Zahin *et al.*, 2010).

Many of the essential oils of *Eucalyptus* species from both Australia and elsewhere have been investigated for their antibacterial effects against aerobic and anaerobic pathogenic bacteria using various methods approved by the then National Committee for Clinical Laboratory Standards (NCCLS), currently known as Clinical and Laboratory Standards Institute (CLSI). One of the earliest antibacterial studies on Australian *Eucalyptus* plants was the study by Atkinson and Brice (1955) which showed variability in the effects of the essential oils on *S. aureus, Salmonella typhi* and *Mycobacterium phlei* using Tween agar and broth methods. Similarly, Low *et al* (1974) showed antibacterial effects of chromatographically isolated fractions of essential oils of *Eucalyptus citriodora*, *E*. *dives*, *E*. *fruiticetorum*, *E*. *australiana* and three other non-*Eucalyptus* plant species. A synergistic antibacterial effect of

citrionellal and citrionellol from *E*. *citriodora* was exhibited on *S. aureus* and *Salmonella typhi* and it was enhanced four-fold when these two essential oils and cineol were mixed and tested for their antibacterial activity.

The chemistry of the antibacterial compounds of the *Eucalyptus* species was investigated prior to this date as reported in some of the pioneering chemistry work on various *Eucalyptus* species by Maiden and Smith 1895a; Maiden, 1895b; Maiden, 1889a; 1889b; 1889c cited in Locher and Currie, (2010). In comparison to the vast array of antibacterial studies, only a limited number of studies have investigated the antifungal and antiviral properties of the *Eucalyptus* species. There have been *in vivo* antifungal studies in India using *Eucalyptus* essential oils as fungicides (Batish *et al.*, 2008) while *in vitro* studies have been conducted in Taiwan (Su *et al.*, 2006), Kenya (Musyimi & Ogur, 2008), France (Marzoug *et al.*, 2010) and Jordon (Khalil & Dababneth, 2007). In addition, anti-inflammatory effects of chemical compounds apart from antifungal, antioxidant and anti-diabetic effects from plants have also been studied in Australia (Gilles *et al.*, 2010a).

Musyimi and Ogur (2008) conducted a comparative study on the antifungal properties of *Eucalyptus citriodora* and *E*. *globulus* in Kenya and the standard antifungal drugs clotrimazole, griseofulvin and nystatin using the agar well diffusion method. The extracts completely inhibited the growth of *C*. *albicans*, *Trychophyton mentagrophytes*, and *Microsporum gypsum*. This result agrees with other studies (Silva *et al.*, 2003; Su *et al.*, 2006) which evaluated antimicrobial properties of *E*. *citriodora* being effective against test microorganisms. It is likely that this is due to the presence of citrionellol, citronellal and 1, 8-cineole that have been reported to be the most active essential oils among the *Eucalyptus* species. It was suggested that the effectiveness of the extracts of *E*. *citriodora* on the test yeast and fungal species was due to synergy. The methanol extract of *E*. *globulus* was reported to be the least active and this may have been due to the chemical compounds not being able to diffuse efficiently though the agar, thus, their anti-infective effect was not exhibited.

Another study conducted in France by Marzoug *et al*. (2010) investigated antioxidant, antibacterial and antifungal properties of *Eucalyptus gracilis*, *E*. *oleosa*, *E*. *salubris* and *E*. *salmonophloia*. The 1, 1-diphenyl-2-picrylhydrazyl (DPPH) and 2'2-azinobis-3-ethylbenzothiazoline-6-sulfonate (ABTS) assays were employed to determine antioxidant activity and the agar diffusion assay was used to study antibacterial and antifungal activities. The antioxidant data demonstrated *E*. *gracilis*

possessed the highest antioxidant activity with an inhibitory concentration of 12 mg/mL compared to the other three *Eucalyptus* species. Essential oils of all four *Eucalyptus* species exhibited various intensities of efficacy on all test microorganisms except for *Staphylococcus aureus* and *Escherichia coli*. In comparison, *Eucalyptus oleosa* possessed the greatest antimicrobial potency among the *Eucalyptus* species investigated in this study. The effectiveness of essential oils on pathogens was consistent to the many investigations that have been conducted on different antimicrobial properties of many other *Eucalyptus* species.

In Taiwan, Su *et al* (2006) investigated antifungal properties of essential oils isolated from *Eucalyptus citriodora*, *E*. *grandis*, *E*. *camaldulensis* and *E*. *urophylla,* also using the agar well diffusion method. Several mildew strains and woody decay fungi were reported to be most susceptible to *E*. *citriodora* whilst *E*. *urophylla* had the least efficacy on the mildew strains. *Eucalyptus citriodora* was the most efficacious among the four *Eucalyptus* species. It is likely that the greater activity was due to the higher levels of 1, 8-cineole, citrionellal and citrionellol in its leaves as determined by the gas chromatography mass spectrometry. Silva and co-workers (2003) examined analgesic and anti-inflammatory effects of essential oils of *E. citriodora*, *E*. *tereticornis* and *E*. *globulus in vivo* using mice and rats as experimental models in Brazil. All three *Eucalyptus* species were reported to possess analgesic and anti-inflammatory effects against both animal models. These *Eucalyptus* species were demonstrated to possess anti-inflammatory effects with various intensities in rats. *Eucalyptus tereticornis* demonstrated the highest anti-inflammatory effect. It is likely that it was due to this *Eucalyptus* species possessing non-neutrophil-mediated components compared to the other two *Eucalyptus* species. The number of neutrophils was not reduced by the essential oils of the *E*. *tereticornis* as demonstrated by lowest level of swelling of the rat paws. In an earlier but similar *in vitro* study on the anti-inflammatory effects of eucalyptol (1, 8-cineole) in human blood monocytes, potent inhibition of inflammatory mediators (cytokines) were observed (Juergens *et al.*, 2004). Hence, these two studies point out that some essential oils possess non-neutrophil mechanisms that are effective against inflammatory infections.

The essential oils of *Eucalyptus olida*, *E*. *staigeriana* and *E*. *dives* were demonstrated in another study to possess different levels of antimicrobial activities. The essential oils were shown to be potent against Gram positive and Gram

negative bacteria, and a clinical fungal species at varying intensities. The highest antimicrobial activity was exhibited by *E*. *staigeriana,* which agrees with many other studies reporting 1, 8-cineole to be very effective against pathogens since this compound was found to be dominant in this *Eucalyptus* species (Gilles *et al.*, 2010).

A significant number of previous investigations have demonstrated the potential of eucalyptus species to be potent against non-multidrug-resistant pathogens. A study was undertaken to investigate the antibacterial activity of certain essential oils on multidrug-resistant bacterial pathogens (Mulyaningsih *et al.*, 2011). Methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant Enterococci strains and four multidrug-resistant Gram negative bacteria were tested for their susceptibility to different essential oils from *Eucalyptus* species using the microdilution method in 96-well microtiter plates. All the test multidrug resistant bacterial species were inhibited by the leaf and fruit extracts of the *Eucalyptus* species, except for the Gram negative bacteria which were completely uninhibited. The non-inhibition of Gram negative bacterial species by plant extracts and other pharmacological agents is attributed to multilayers of their cell envelopes which include the outer membrane, peptidoglycan cell wall and cytoplasmic membrane which could have prevented the permeability of chemical compounds into the target sites (Silhavy *et al*., 2010).

Serafino *et al* (2008), showed that essential oils stimulated the monocyte derived macrophages (MDMs) to a phagocytic response *in vitro* and *in vivo*. This study investigated phagocytosis as part of the innate immunity. The essential oils enhanced phagocytic capability of macrophages with toll-like receptors (TLR), especially the TLR-4 receptor. The authors analysed phagocytosis of fluorescent-labelled *E. coli* by confocal microscopy and flow cytometry. In the *in vivo* part of this study, the monocytes were obtained from the peripheral blood of immune-competent rats after oral administration of the essential oils and analysed using the Bio-Rad cytometry bead array human Th1/Th2 cytokine kit for the release of cytokines by the MDMs. The levels of the two proinflammatory cytokines (tumour necrosis factor alpha (TNF-α) and interleukin (IL)-6) and anti-inflammatory cytokines (IL-4 and IL-10) were reduced in MDMs treated with EOs. Nitric oxide (NO), a free radical oxygen species, was another mediator of inflammation that was investigated for *in vitro*-anti-inflammatory effect of *E*. *globulus* and *Thymus vulgaris* (Hillis, 1951). Production of NO is normally enhanced after endothelial cells have been exposed to an immunogenic substance, such as lipopolysaccharide (LPS) or trauma (Vigo *et al*, 2004). Nitric oxide is synthesized from L-arginine in endothelial cells of various

mammalian tissues and its production is enhanced by the endothelial and inducible NO synthases in addition to ATP (Lockwood *et al.*, 2014).

According to the literature regarding biological properties compiled in this section, it can be stated that the *Eucalypts* possess many different antimicrobial properties, which vary from one species to another. Furthermore, there are variations in the efficacy of essential oils of different *Eucalyptus* species. In addition, the different methods of antimicrobial tests using different medically important microorganisms with varying cellular structures contribute to differences in the bioactivity data. The antimicrobial methods have been modified to efficiently determine specific biological properties of the *Eucalyptus* species over the years. Table 1.2 summarizes the different biological properties that have been determined by different studies conducted around the world.

Table 1.2. Examples of bioactivities of leaves and barks of different *Eucalyptus* species in different countries of the world.

Species	Bioactivity	Country	Reference(s)
E. astrigen	Antibacterial	Australia	von Martius *et al* (2012)
E. botryoides	Antibacterial	Australia	von Martius *et al* (2012)
E. caesia	Antibacterial	Australia	von Martius *et al* (2012)
E. calophylla	Antibacterial	Australia	von Martius *et al* (2012)
E. camaldulensis	Antibacterial	Algeria Australia	Ghalem & Mohamed (2008) von Martius *et al* (2012)
E. citriodora	Antibacterial, Antifungal Anti-inflammatory	Australia Taiwan Kenya Brazil	von Martius *et al* (2012) Su *et al* (2006) Musyimi & Ogur (2008) Silva *et al* (2003).
E. comuta	Antibacterial	Australia	von Martius *et al* (2012)
E. densa	Antibacterial	Australia	von Martius *et al* (2012)
E. dives	Antibacterial, Antifungal	Australia	Giles *et al* (2010).
E. erythrocorys	Antibacterial	Australia	von Martius *et al* (2012)
E. ficifolia	Antibacterial	Australia	von Martius *et al* (2012)
E. flocktoniae	Antibacterial	Australia	von Martius *et al* (2012)
E. gracillis	Antibacterial, Antifungal	France	Marzoug *et al* (2010).
E. houseana	Antibacterial	Australia	von Martius *et al* (2012)
E. langiflorense	Antibacterial, Antifungal	Australia Iran Japan	von Martius *et al* (2012) (Safaei-Ghomi, 2010) Takahashi *et al* (2004)
E. maculata	Antibacterial, Antifungal	Australia	von Martius *et al* (2012)

E-Eucalyptus

Table 1.2 continued

Species	Bioactivity	Country	Reference(s)
E. olida	Antibacterial, Antifungal	Australia	Giles *et al* (2010).
E. platypus	Antibacterial	Australia	von Martius *et al* (2012)
E. robusta	Antibacterial, Antifungal	Brazil	Sartorelli *et al* (2007)
E. roycei	Antibacterial	Australia	von Martius *et al* (2012)
E. sagentii	Antibacterial	Australia	von Martius *et al* (2012)
E. saligna	Antibacterial, Antifungal	Brazil	Sartorelli *et al* (2007)
E. salmonophloia	Antibacterial, Antifungal	France	Marzoug *et al* (2010)
E. salubris	Antibacterial Antifungal	France	Marzoug *et al* (2010)
E.staigeriana	Antibacterial, Antifungal	Australia	Giles *et al* (2010).
E. stowardii	Antibacterial	Australia	von Martius *et al* (2012)
E. tereticornis	Antibacterial	Australia	von Martius *et al* (2012)

E-Eucalyptus

1.6. General methods used in drug discovery research

Many methods have been developed to investigate chemical compounds from plants, from simple screening assays to sophisticated high-throughput screening (HTS) assays (Cragg & Newman, 2013; Dias *et al*., 2012). Chemical analyses involving simple qualitative, quantitative and thin layer chromatography to high performance liquid chromatography, liquid chromatography-mass spectroscopy and nuclear magnetic resonance can also be techniques used in drug discovery investigations (Yuliana *et al.*, 2011). A combination of assays and chemical methods are used to investigate target compounds. Optimization of these methods is important to minimise costs of assays and the chemical tests and also to produce drugs with very minimal side effects to humans.

Simple screening assays involve testing of crude extracts and a limited number of chemical compounds isolated, for their bioactivities at a time. Promising compounds with high levels of bioactivities contribute to hundreds of compounds being investigated in the HTS assays (Yuliana *et al.*, 2011). The HTS assays involve testing of hundreds of synthesised chemical compounds from pharmaceutical companies for different bioactivities. The compounds that are bioactive are referred to as high-hits or targets and are further investigated in animal and clinical models. Cell-based assay products and molecular methods target specific cell molecules such as proteins in the HTS assays and they are more expensive to operate than the simple screening assays (Patwardhan & Mashelkar, 2009). The review article of Yulian *et al* (2011) describes metabolomics as an approach to take in drug discovery in order to reduce time for testing of natural products.

Some methods used in the drug discovery research are also based on methods that are used in preparations (whether they are infusions, decoctions, tinctures and macerations) of traditional remedies for the treatment of ailments in indigenous communities around the world (Cragg & Newman, 2013). Such methods used in the drug discovery research are modified and optimized to emulate those that are used in these communities and this is referred to as an ethnopharmacological approach. This approach for drug discovery research uses modified methods for testing the efficacy of a plant extract and is determined by the methods traditionally used for the preparation of plant remedies. In addition, these methods are also determined by specific traditional medicinal uses to alleviate infections and physio-pathological conditions. The ethnopharmacological approach in drug discovery research is also

known as a ‘reverse pharmacology’ since it involves non-conventional testing but instead clinical tests are undertaken prior to laboratory testing and confirmation of bioactivities of indigenous plant formulations (Patwardhan & Mashelkar, 2009). The different herbal formulations of the Ayurvedic and other Indian medicine systems have been used in the alleviation of various infections and medical conditions in Indian communities for thousands of years before being scientifically tested (Mukherjee & Wahile, 2006). Many of the Ayurveda traditional herbal formulations have been systematically and strategically utilised by pharmaceutical companies to produce target compounds for an array of investigations of various bioactivities *in vitro, in vivo* and clinical trials (Patwardhan & Mashelkar, 2009). Similar approaches for confirmation of traditional herbal medicines have been used for investigations for herbal formulations from other traditional herbal medicine systems such as the African, Asian, Chinese, and South American systems. Development of standardized, synergistic, safe and effective traditional herbal medicines with robust scientific evidences provide faster and economical alternative drugs for those that are no longer effective.

Hence, the research work in this thesis was conducted using an ethnopharmacological approach to test for the anti-inflammatory and antibacterial effect of kinos from *E. calophylla* (a Western Australian native *Eucalyptus* species) and *E. confertiflora* (a PNG and Northern Australian *Eucalyptus* species). There are claims by the first Australians that the kinos from *E. calophylla* alleviate bacterial infections and inflammatory conditions such as rheumatic arthritis and (Lassak & McCarthy, 2001; Williams, 2011) whilst there are no claims for medicinal use of kinos from the PNG eucalyptus species. In this study, the anti-inflammatory and antibacterial effects of the kinos from two different *Eucalyptus* sources were scientifically tested to assess claims by the first Australians of their medicinal efficacy.

In order to adequately interpret data for the anti-inflammatory and antibacterial effects of kinos in this study, aspects of immune systems, inflammation and its mediators, and the pathogenesis of bacteria are discussed in the following sections.

1.7. Innate and adaptive immune systems

There are two known immune response systems that occur in humans and animals that provide immunity when an infection or foreign particle is encountered. These systems are characterized into innate and adaptive immunity and feature specific immune cells, receptors and genetic characteristics (Akira, 2011; He, 2000).

1.7.1. Innate Immune system

The innate immune system provides a second line of defence in humans and animals once a physical barrier such as the skin has been damaged or breeched by pathogens. The body is protected against invading microbial pathogens such as bacteria, fungi and viruses penetrating into the inner body organs and tissues by the innate immune system (Awouafack *et al*., 2013). Importantly, the innate immune system is the first line of host defence against the pathogen and is mediated by phagocytes (for example, macrophages and dendritic cells), complement proteins and natural killer (NK) cells. Toll-like receptors which recognise pathogenic molecular patterns are found on the surfaces of both the innate and adaptive immune cells (Barros *et al.*, 2013).

There is a controversy over whether innate immunity is nonspecific or specific like the adaptive immunity. Despite this, it is still widely considered as being non-specific. Several studies (Davey & O'toole, 2000; Jaso-Friedmann *et al*, 2000) show the non-specific nature of the innate immune system. For example, after exposure to new environments, serum activated serum factor in stressed tilapia and other teleosts increased levels of the nonspecific cytotoxic cells (NCC) in the peripheral blood whilst the NCC response was low in tilapia that had anti-NCC monoclonal antibodies (Jaso-Friedmann, 2000). The view of innate immunity being nonspecific was further supported by a study that demonstrated *Francisella asiatica*, an intracellular bacterial species mutant (Δ*iglC*) prevented cultured fish from being infected by fish francisellosis with increasing treatments with the mutant (Hou *et al.*, 2014). However, several other reviews (Akira, 2011; Chatterjee *et al*., 2014; Huang *et al*., 2013; Laskin & Laskin, 2001) have argued that innate immunity is also specific and similar to adaptive immunity. Schulengburg *et al* (2007) showed that the specificity of the immune system of invertebrates is similar to that of the innate immune system. The similarity of the immune system is obvious in the genetic diversity, synergistic interactions of different receptors of host cells and the dosage effect. The severity of an infection and different types of infection occurring

simultaneously in hosts can trigger activation of multiple receptors on the innate immune cells to provide defence. It was demonstrated in another study that the C-lectin domain proteins of *Caenorhabtidis elegans*, a nematode, bound to the peptidoglycan of pathogenic Gram positive bacteria (Schulenburg *et al*., 2008). Thus, the C-lectin proteins like the receptors of an innate immune system recognize the Gram positive bacterial peptidoglycan and enhance engulfment and ingestion of the bacteria. Chatterjee *et al* (2014) also demonstrated that the innate immunity is specific as shown by expression of genes and proteins by hepatitis virus-free mice upon the onset and progression of infection by the hepatitis virus.

Innate immunity protection is concentrated on the epidermal tissues of body organs where specific immune cells provide initial protection when there is an invasion of pathogens. Innate immune cells, including antigen presenting cells (APCs), polymorphonuclear cells, macrophages, dendritic cells and NK cells are activated by TLRs (Akira, 2011; He, 2000). Pathogen recognition receptors (PRRs) such as TLRs which include TLR1, TLR2, TLR4, TLR5 and TLR6 recognize different microbial components. Akira *et al* (2006) reported TLRs to activate cascades of mitogen-activated protein kinases (MAPK) and extracellular signal-regulated kinases (ERK) that signal upon pathogen recognition. In this process, the specific TLRs, depending on the type of pathogens that have been recognized, trigger the cells to either upregulate or downregulate protein or gene expression. Recognition of pathogens by the TRLs of macrophage cells enhances phagocytosis and ingestion of pathogens which is reflective of innate immune system function (Tsang *et al.*, 2014). The receptor for β-glycan dectin-1, was expressed at low levels on macrophages and high levels on dendritic cells which used TLRs to recognize microbes in the mouse. The dendritic cell is not able to kill the pathogens after phagocytizing them but instead will act as APCs and migrate from the infected tissues to the lymph nodes where they present the antigens to the lymphocytes, thus activating the adaptive immune system (Akira, 2011). This process is detailed in Figure 1.1.

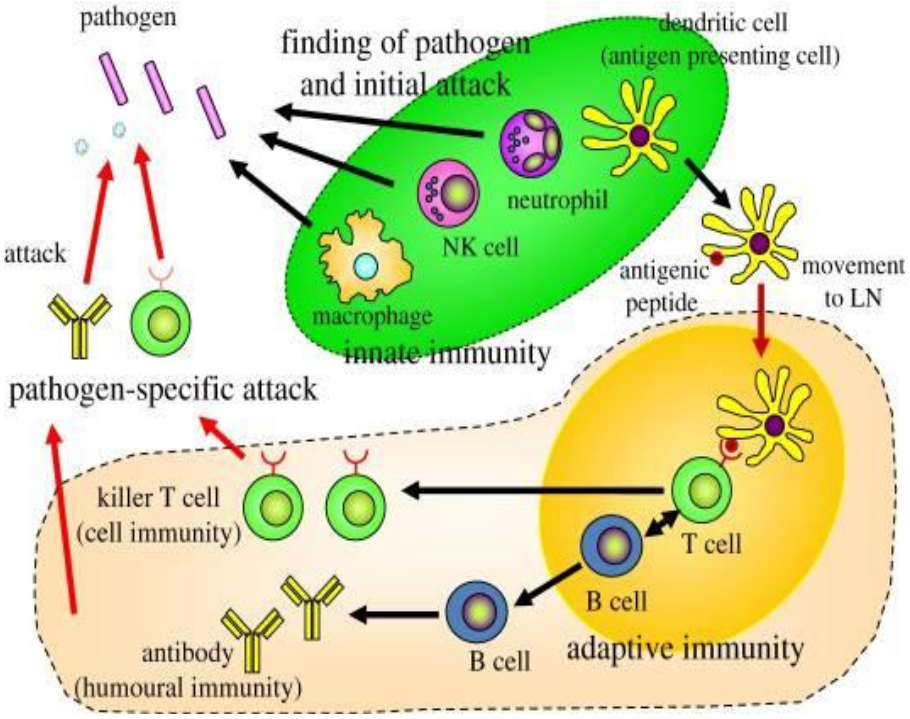


Figure 1.1. Interactions of innate and adaptive immunity

When pathogens invade the body, the innate immune system is activated first and participates in the initial attack against the pathogens. Among the cells involved in innate immunity, dendritic cells act as antigen-presenting cells and migrate from the infected tissue to the regional lymph nodes where they present the antigens to T cells. Subsequently, the adaptive immune system is activated, and antibody production and killer T cells are induced. The resulting antibodies and killer T cells specifically attack the pathogens. (Reproduced from Akira, 2011, with permission. Refer to Appendix A1 for the correspondence)

1.7.2. Adaptive Immune system

The adaptive immune system provides long-term protection in humans and other organisms when the innate system cannot further progress with its fight against pathogens. There are two components to the adaptive immune system: humoral and cellular. The humoral system involves B cell production of antibodies with the help of the T-cells to protect the body from the invading pathogens. In contrast, T-cells participate in cellular immunity of the body (Elbaz *et al*., 2012). Similar to the dendritic cells, macrophages and neutrophils of the innate immune system, cells of the adaptive immune system also have receptors on their surfaces. B-cells and T-cells also have receptors that recognize antigens such as bacteria and viruses in the body. In the process, the B-cells release the receptors as antibodies while the T-cell receptors remain on the surface of the cells to be activated by the specific antigens (Elbaz *et al*., 2012). The PRRs such as the TLRs trigger activation of the B-cells and the T-cell in the adaptive immune system (Schwandner *et al.*, 1999).

There are a number of major subsets of T-cells that are specific in their roles within cellular immunity of the body. These include the Helper T (Th)- cells (also called CD4+), cytotoxic T (Tc)-cells (also called CD8+) and the Regulatory T (Treg)-cells. The $CD4^+$ cells assist the $CD8^+$ cells in their cytotoxic activity against viruses-infected host cells (Elbaz *et al*., 2012). The antigen-specific CD4+ and CD8+ cells interact primarily with their respective specific major histocompatibility complex (MHC) antigens. The CD4+ cells interact with MHC class II in chronic viral infections (Fernando *et al.*, 2014) and tumours while the CD8+ cells are activated by the presence of MHC class I antigens in tumours to eliminate intracellular infections (Gopalakrishna *et al.*, 2014). Although the CD4+ cells are known for neutralizing viral infections in humans, it was reported in a review article that the human immunodeficiency-virus (HIV)-1 specific Th cells instead were impaired, resulting in low number of CD4+HIV-1 specific antigens (Licherfeld *et al*., 2004). This was due to the dynamics of CD4+ and CD8+ T cells being altered during HIV infection (Bacheler *et al.*, 2001; Catalfamo *et al.*, 2011). Catalfarno and colleagues (2011) demonstrated that memory CD4+ and CD8+ T-cells in HIV-positive patients possessed an increase in gene expressions associated with type I interferon and gamma chain cytokine signalling in CD4 T cell subsets but only the type I interferon in CD8 T-cell subset. This observation demonstrates homeostasis of the body due to differences in the gene expressions in the two subsets of the T-cells. It further shows that the CD4+ and CD8+ T-cells participate in specific signalling pathways.

However, some studies still maintained that high levels of antigen specific CD4+ cells eliminate viral infections and cancers usually with assistance from cytotoxic CD8+T-cells, making these cells non-specific (Gopalakrishna *et al*., 2014; Noguchi *et al*., 2005).

1.7.3. Inflammation and cytokines as the mediators

Inflammation is a pathophysiological response by immune cells such as macrophages, dendritic cells and mast cells in tissues to either destroy invading pathogens or to repair damaged tissues after an injury (Roy & Chatterjee, 2014b). There are two types of inflammation; acute and chronic inflammation. The acute inflammatory response is mostly part of the innate immune system and it can last for 24 to 48 hours and may persist for less than a week, in some cases (Roy & Chatterjee, 2014a). It is generated by neutrophils, eosinophils, basophils and mast cells, depending on the location of the injury in the body and the type of organism (Martin & Leibovich, 2005).

Macrophages are the key immune cells that participate in inflammation in both adaptive immunity, via antigen presentation (Akira, 2011) and IL-12 production (Wiegand *et al.*, 2008) and in innate immunity against bacterial, viral and fungal pathogens (Beta *et al.*, 2014). Small proteins known as cytokines such as TNF-α, IL-6 and IL-1β, and NO, a vaso-muscular molecule, participate in the protective role of inflammation. However, an overproduction of proinflammatory cytokines and NO causes adverse conditions in cells and tissues that accommodate these molecules.

There are specific mechanisms that activate inflammatory cells to produce high levels of cytokines and NO during inflammation. A previous study (Rahman *et al.*, 2002) reported NF-κB to be regulated by cellular signalling pathways such as MAPK. It is then released and translocated to the nucleus to activate the transcription of target genes and also those of the cytokines and inducible NO synthase during inflammation (Chatterjee *et al*., 2015; Roy & Chatterjee, 2014b). Lipopolysaccharide is one of the most potent endotoxins from Gram negative bacteria that plays a major role in inflammation by activating TLRs in macrophages and dendritic cells to produce cytokines such as TNF-α, IL-6 and IL-1β, and NO (Roy & Chatterjee, 2014b).

Tumour necrosis factor alpha is a plethoric pro-inflammatory cytokine that enhances the production of other proinflammatory cytokines including IL-6, IL-1β and prostaglandin E2. It occurs in acute and chronic inflammatory conditions such as

trauma, sepsis, infection and rheumatoid arthritis (Bhunia *et al.*, 2014; Matsuno *et al.*, 2002). TNF-α is a neutralizing cytokine for septic shock and parasitic infections such as malaria, as reported in a review article by Pfeffer (2003). Roles of TNF-α vary with the type of receptor on immune cells such as macrophages and monocytes in both acute and chronic inflammatory conditions. Each TNF-α either receptor 1 or 2 is very specific to accommodate the function of the different TNF cytokines: lymphotoxin (LT)-α, LT-β, TNF and LIGHT (Pfeffer, 2003). The TNF-α is regarded as the mediator of all other mediators (pro-inflammatory cytokines) of inflammation although they are not produced in high levels in some inflammatory conditions such as rheumatoid and psoriasis in the presence of TNF-α. Studies have, however, shown that TNF-α and IL-6 aggregate under these chronic inflammatory conditions, exhibiting a form of synergistic effect (Guilloteau *et al.*, 2010). Each of these cytokines is also multifunctional (Machiela *et al.*, 2015). Interleukin 17A, IL-22, oncostatin M, IL-1α and TNF-α have also been demonstrated to act in a synergistic manner to upregulate the expression of CXCL8 (IL-8) and β-defensin 2 (BD2). This shows that the synergism of different cytokines enhances the intensity of each cytokine.

Interleukin-6 is also a pleiotropic cytokine like TNF-α, and is known for its role in amplifying acute inflammation. It also contributes to the chronic inflammatory state of injured tissue (Garcia-Closas *et al.*, 2014). It is produced by macrophages, activated T-cells, endothelial cells and fibroblasts and it induces inflammatory conditions such as rheumatoid arthritis (RA), febrility and atherosclerotic diseases in response to stimuli like LPS and collagen (Markom *et al.*, 2007; Zheng *et al.*, 2011). Rheumatoid arthritis is a chronic autoimmune disease which is characterized mainly by joint inflammation and synovial hyperplasia and can sometimes be enhanced by both IL-6 and TNF-α *in vivo* in the presence of antibiotic-killed *S. aureus,* as demonstrated in a recent study (Falk *et al.*, 2014). This study showed the RA was intensified due to increased concentrations of these two cytokines caused by bacterial components from antibiotic killing. Previous work by Chatterjee *et al* (2014) reported a slightly different observation of TNF-α production *in vivo*. Production of TNF-α was reported to be low in adipose tissue of humans but in contrast was high in an *in vitro* beta cell model with high insulin concentration in the media secretion (Chatterjee *et al.*, 2014). This study also demonstrated high levels of IL-6 to be correlated with high insulin in the adipose tissues. These two studies, including other similar studies (Chalaris *et al*., 2011; Chatterjee*et al*., 2014) reported that receptors

from immune cells contribute to the differences in the IL-6 production, in both *in vivo* and *in vitro* study models.

Interleukin-10 is an anti-inflammatory cytokine that is produced by myeloid dendritic cells and macrophages when microbial products such as LPS are recognized by the PRRs. The PRRs are important features that trigger proinflammatory and anti-inflammatory cytokine production in immune cells (Jeannin *et al*., 2008; Saraiva & O'Garra, 2010). Interleukin-10 was initially described as a helper-T (T_H2) cytokine but further evidence from different investigations have proven its production by T_H1 and T_H17 (Lang *et al.*, 2002; Rutz *et al.*, 2008) and T-regulatory cells (Li, W. Q. *et al.*, 2014). In another study by Kluth *et al* (2004), it was demonstrated that IL-10 was not only produced by T_H2 cells but also by the effector T_H1 cells. This resulted in the limitation of damage caused by exaggerated inflammation. In addition, O'Garra & Viera (2007) described the participation of B cells and NK cells in the expression of IL-10. Interleukin-10 modulates both innate and adaptive immunity in animals and humans when the PRRs recognize microbial products and send signals to the respective immune cells to function accordingly to reduce inflammation.

Both transcriptional and posttranscriptional mechanisms have been implicated for the inhibitory effect of IL-10 on the production of cytokines and chemokines in T cells, monocytes and macrophages. There are specific mechanisms that trigger IL-10 expression by different immune cells, dependent on whether they are from the innate or adaptive immune systems (Arteaga *et al*., 2015). This study reported that microbial products were recognized by PRRs on myeloid dendritic cells and macrophages of the innate immune system, which then signal to activate extracellular signal-regulated kinase (ERK) for IL-10 production. The ERK in myeloid dendritic cells is intermediately expressed while it is highly expressed in macrophages, resulting in intermediate and high IL-10 production, respectively, in these two immune cells.

Interleukin-10 plays a protective role in the suppression of gene expression of proinflammatory cytokines in both an autocrine and paracrine manner (Yao *et al.*, 2013). Yao and co-workers reported that administration of exogenous IL-10 increased the survival of mice bred with an absence of IL-10 that were given high levels of TNF-α compared to those receiving phosphate-buffered saline. This is consistent with a previous study that demonstrated IL-10 acting in an autocrine manner on monocytes induced by INF-γ. Interleukin-10 production was reduced in the human peripheral blood monocytes whilst TNF-α levels increased with

increasing concentrations of INF-γ (Donnelly *et al.*, 1995). This shows that a negative feedback loop was involved in these T_H1 cells which made them differentiate into self-peptide-specific IL-10 secreting cells, resulting in low IL-10 production. This negative feedback loop has also been demonstrated in another study by Sica *et al* (2000). Their data showed that peptide-induced IL-10 T-regulatory cells from the mice blocked the function of the dendritic cells, which were dependent on the IL-10, thus, having a negative feedback loop exerted by the T_H1 cells.

Nitric oxide is a proinflammatory molecule which is produced by inducible NO synthase. It is also a vasodilator and has been reported to regulate physiological processes such as blood flow and neurotransmission (Petkovic *et al.*, 2013). A review paper reported that nitrite, an oxidative product of NO, contributed to hypoxic vasodilation, physiological blood pressure control, and redox signalling signalling (Kim-Shapiro & Gladwin, 2014). Nitric oxide production, like TNF-α and IL-6, is enhanced in cells by LPS, the endotoxin of Gram negative bacteria, and TLR4 signalling. Contaminants in phosphate buffered solution (PBS) were demonstrated to produce higher levels of NO, which contributed to massive septic shock in mice compared to the ultrapure LPS-stimulated cells (Cauwels *et al.*, 2014). In addition to LPS inducing cells to produce NO, a recent study demonstrated high levels of ATP in epithelium producing high levels of NO in blood cells of healthy rabbits when incubated with hydroxyurea, a therapy for enhancing blood flow, while the ATP levels were low in the cells void of hydroxyurea (Lockwood *et al.*, 2014).

1.8. Pathogenesis of bacteria

Pathogenesis of bacteria is complex and is influenced by many host biological factors and the biological, morphological and molecular properties of pathogenic bacteria. Bacterial infections range from acute to chronic in nature and are mostly characterized by their complexity (Komor *et al.*, 2012). An acute bacterial infection may last a week whilst a chronic infection is persistent for months. These infections may trigger the innate immune system as well as the adaptive immune system for defence in hosts (Bui *et al*., 2015). Bacterial infections are intracellular or extracellular in nature and strategies for enhancement and persistence of infections vary between the Gram negative and Gram positive bacteria due to differences in their cell structural composition (Parolia *et al*., 2014).

1.8.1. Factors that enhance pathogenesis of bacteria

There are various factors that enhance pathogenesis of bacteria and these include the presence or absence of capsules and cell walls, toxins, adhesins and host/bacteria relationship, and the regulation of virulence factors, antibiotic resistance and the interaction of pathogenic bacteria with the innate immune system (Wilson *et al*., 2002). Mechanisms of bacterial proliferation and invasion of tissues of hosts vary between Gram negative and Gram positive bacteria due to differences in their cell structures (Parolia *et al*., 2014; Ribet & Cossart, 2015).

Bacteria possess capsules that prevent them from being easily recognized and opsonized by TLRs of phagocytic cells such as macrophages and neutrophils in the host. A previous study demonstrated that the introduction of *Pasteurella multicida* with acapsular genes into mice and chickens resulted in low level of bacterial infection (Chung *et al*., 2001). In contrast, the capsular genes in these animals triggered a severe infection. The PBA954 strain of *P. multocida,* which has substantial levels of capsules, was more virulent to the mice, demonstrating that the quantity of capsules produced by bacteria contributes to increased resistance to antibiotics. Similarly, a *Neisseria meningitidis* capsule-deficient mutant bound easily to the human antimicrobial peptide (LL-37) as demonstrated by Jones *et al* (2009). In addition, the capsular genes (for example, siaC and siaD) were highly expressed in the bacteria with low concentrations of LL-37. In another study it was shown that the bacterial capsular polysaccharide found on bacterial cell walls prevented antimicrobial peptides from reaching the target sites (Campos *et al.*, 2004). The literature clearly defines the role of capsules in bacterial resistance to antibacterial agents.

The cell walls of Gram positive bacteria play a part in their pathogenesis in which lipoteichoic acids (LTA) and peptidoglycan (PGN) are predominant. The LTAs are reported to enlarge the cell wall of bacteria, which prolongs the penetration of antibiotics into the deep target sites. The LTAs were demonstrated by Atilano *et al* (2010) to have affinity for cross-linking with the PGN. The mucosal pathogens modify the PGN residues that surround the cleavage site on the cell wall for lysozyme in order to avoid PGN degradation Davis & Weiser, 2011). The modifications and the cleavages are dependent on the type of penicillin-binding proteins (PBPs) present. The PBP1 enhances viability and proliferation of bacteria while PBP2 triggers the bacteria to develop resistance to antibiotics. Gram negative bacteria have low PGNs but have other cell structures like the pili, also known as

fimbriae, for adhesion to host cells which enhances their pathogenicity (Ribet & Cossart, 2015).

Bacteria also use adhesion for enhancing their pathogenesis. Adhesion is a very important pathogenic factor for bacteria to attach to specific host cells; a crucial step in establishing an infection. Gram negative bacteria use pili to attach to the cells of the hosts, thus causing infection as discussed in a review paper by Ribert and Cossart (2015). The pili are anchored to the bacterial outer cell membrane and their tips protrude out from the membrane with specific binding factors that enable them to adhere to the cells of the host. There are different types of pili and each one functions slightly different to the others in Gram negative bacteria. The type 1 and P pili of the uropathogenic *E. coli* strain are equipped with usher pores of the fimbriae donor (FimD) which transverse the bacterial outer membrane and act as a plug which maintains membrane integrity (Lillington *et al.*, 2015). There are subunits of the Fim with their respective N-terminals with folded proteins such as the mannose-binding proteins (Ribet & Cossart, 2015).. These provide extreme mechanical strength on the outer cell membrane for adhesion of bacteria (Craig *et al*., 2004).

In contrast, Gram positive bacteria assemble pili by involving sortase which cross-links monomers of pili to the polymers of the cell wall, forming covalent bonds with the peptidoglycan (Ton-That & Schneewind, 2004; Xiao *et al*., 2013). Actinomyces, a Gram positive microbe, was reported to be using this mechanism with pili types 1 and 2 for adhesion on teeth enamel, forming plaques, and on mucosal tissues at the base of the teeth (Ton-That & Schneewind, 2004). Type 1 pili specifically interact with the proline-rich proteins that coat the teeth while type 2 pili are sensitive to lactose and attach to polysaccharides on the cell walls. They are genetically regulated by surface protein genes. The sensitivities of the two pili vary among different Gram positive bacterial species.

Adhesion mechanisms that are used by pathogenic bacteria to attach themselves to host cells result in the formation of biofilms which cause chronic bacterial infections. Komor *et al* (2012) demonstrated development of biofilm by planktonic *Pseudomonas aeruginosa* administered to mice. The bacteria were observed under the electron microscope to be multiplying around the subcutaneous tumours and becoming aggregated with an influx of neutrophils at the site as demonstrated by the immunohistological analysis. This shows that the activation of TLRs of the mice monocytes was due to biofilm accumulation of *P. aeruginosa* with increasing time. In another study, two of the nine chemicals tested for their ability to cause stress in

skin samples from cystic fibrosis patients showed a 2-fold increase in accumulation of *S. aureus*, an opportunistic pathogenic bacterial species (Bui *et al*., 2015). Stress of the host cells can enhance accumulation of pathogenic bacteria. This was demonstrated by a previous study that showed stress molecules such as reactive oxygen species and reactive nitrogen species in high levels in the planktonic form of *S. aureus* producing biofilms and small colony variants Cushnie & Lamb, 2011). The formation of biofilms is also determined by the genetic and molecular aspects of the bacterial community and they have assisted the pathogenic bacterial species to develop resistance to antibiotics (Davey & O'toole, 2000).

Resistance of pathogenic bacteria and other pathogenic microbes is a worldwide concern as they develop mechanisms of resistance to antibiotics. The resistance of bacteria enable them to cause chronic bacterial infections. Lawal *et al* (2011) reported the types of mechanisms that are acquired by pathogenic bacteria to become resistance to drugs. Penicillin has been reported to be ineffective against Staphylococci that produce the enzyme β-lactamase, which degrades antibiotics such as penicillin G and penicillin. The persistence of resistance among bacterial species over many years has resulted in them becoming resistant to multiple drugs. For instance, the Staphylococcal species developed resistance to methicillin and vancomycin while *P. aeruginosa*, *Acinetobacter baumanii*, *E. coli* and *Klebsiella pneumonia* have also become multiple-drug resistant.

Another antibiotics resistant mechanism within pathogenic bacteria is by assimilating genes found on host's chromosomes. For example, AmpC β-lactamase, primarily from Gram negative bacteria, builds efflux systems that allow antibiotics to flow out of the target sites. Efflux proteins of bacterial cells contribute to their resistance and they are of two general types. The first type of efflux proteins can only act on a few or multiple antibacterial agents from the same class of drugs while the second type, the resistance nodulation division based efflux systems, bind to multiple structurally unrelated compounds which exhibit broad resistant phenotypes (Li & Nikaido, 2004). Another mechanism involves mutations of target genes for antibiotics, which can be transferred from one bacterial species to another through conjugation of plasmids, transposons and transduction of bacteriophages (Cushnie & Lamb, 2011). A plethora of antibiotic resistant genes in many pathogenic bacteria have been investigated and reported. A review article by Cushine and Lamb (2011) reported mutations of genes of pathogenic bacteria enabling the transfer of resistant genes from one bacterium to another. A previous review article reported transfer of *van*A

(vancomycin) resistant gene from *Enterococcus faecalis* to *Staphylococcus aureus* when investigated *in vitro* and on the skin of the mouse (Tenover, 2006). In another study, the *van*A plasmid DNA of vancomycin-resistant *Enterococcus faecalis* was detected in another strain of *E*. *faecalis* and vancomycin-resistant *S. aureus* in patients in Michigan (Zhu *et al.*, 2008). A combination of vancomycin and oxacillin was used against vancomycin-resistant *S. aureus* due to *S. aureus* strains harbouring a plasmid-borne Tn1546 element following conjugation from glycopeptide-resistant *Enterococcus* strain (Aman *et al.*, 2013). In another study, it was demonstrated that bacteriophage DNA was transferring antibiotic resistant genes to healthy humans (Quirós *et al.*, 2014). Similarly, bacteriophage DNA transferred antibiotic resistance genes from the microorganisms in polluted water which subsequently infected humans (Colomer-Lluch *et al.*, 2011). The resistant bacteria have also developed a strategy for altering cell membrane structure making it difficult for antibiotics to permeate to the target sites.

Infections from Staphylococci have been difficult to treat and also to contain the spread of resistance among *S*. *aureus* strains in public health establishments. The different strains have developed mechanisms that enhanced resistance to methicillin and to vancomycin treatments over many years. A study by Livorsi *et al* (2012) demonstrated the prevalence of *bla*Z genes in methicillin-susceptible *S*. *aureus* and its inoculum size that caused ineffectiveness of cefazolin. The authors also showed that the body target site for drug administration determined its effectiveness. Other genes like D-Ala-D-Ala found in *S*. *aureus* strains were demonstrated to enhance synthesis of peptidoglycan (Livorsi, 2012). The thickening of the peptidoglycan by the D-Ala-D-Ala genes made it difficult for vancomycin to reach the target site for treatment (Lowy, 2003).

Not only has *S*. *aureus* developed resistant strains, *Pseudomonas aeruginosa*, a common pathogen amongst immuno-compromised patients, has also become resistant to azithromycin. The resistance was due to mutations in the 23S ribosomal RNA encoding genes, found in different strains of *P*. *aeruginosa* from clinical samples (Marvig *et al*., 2012). These mutations blocked the drug from binding to the 50S ribosomal subunit and thus, causing resistance of *P*. *aeruginosa*. This drug has raised a need to review its use in the treatment of patients with cystic fibrosis since it is currently one of the recommended drugs for treating this inflammatory disease. *Escherichia coli* strains have also developed resistance to tigecycline as shown by the data when tested using urine, liver aspirate, rectum and blood (Spanu *et al*.,

2012). The tested *E*. *coli* strains were also resistant to other thirteen commonly used drugs for infections caused by *E*. *coli*.

1.9. Perspective and objectives of this study

The traditional medicinal uses of the leaves and bark of *Eucalyptus* species and their respective antimicrobial effects have been discussed, showing their importance to the search for target pharmacological agents to be used in the production of drugs that can replace those that have become ineffective. In addition, kinos from several Australian *Eucalyptus* species have also been documented to relieve burns, wounds, pains, fever and diarrhoea. In contrast, very limited antimicrobial studies have been conducted on kinos. Therefore, this study sets out to research the antimicrobial therapeutic potential of kinos from Australian and PNG *Eucalyptus* species *in vitro*. Kinos from the PNG *Eucalyptus* species were used in this study for comparative purposes.

The main objectives of this study were to investigate:

I. the anti-inflammatory effects and;
II. the antibacterial effects of kinos from Western Australian (WA) and PNG *Eucalyptus* species

Null hypothesis of this study

The main null hypothesis of this study is that neither kinos (from WA or PNG) *Eucalyptus* species will display significant anti-inflammatory or antibacterial activities.

CHAPTER 2

MATERIALS AND METHODS

2.1. Introduction

Resistance of pathogens to numerous drugs and the side effects of some drugs are a world-wide concern (Cushnie & Lamb, 2011). This concern triggers pharmaceutical scientists to investigate antimicrobial effects of plant extracts based on ethnomedicinal uses. Such antimicrobial studies require several methods to detect and confirm a specific antimicrobial effect of secondary metabolites in plants. Screening of crude extracts is the starting point of any investigations into antimicrobial effects of plants; followed by chemical isolation and re-testing of the specific antimicrobial activity *in vitro* and *in vivo* (Ncube *et al.*, 2008). Numerous methods for antimicrobial studies have been developed, modified and standardized over the years based on reproducibility of data from each of the protocols (Ali *et al.*, 2015). The main factors contributing to the development of different methods for the investigation of antimicrobial effects of plant extracts include differences in the chemical constituents, type of microorganisms or cells and whether the test models are *in vitro*, *ex vivo* or *in vivo*.

Like other investigations into the antimicrobial effects of plant crude extracts and phytochemical constituents, several methods were employed to assess the anti-inflammatory and antibacterial effect of kinos from the *Eucalyptus calophylla* and *Eucalyptus confertiflora* in this study. This chapter outlines and describes details of methods used to detect anti-inflammatory and antibacterial effects of kinos. It also outlines specific reagents that were used in each of the methods (Table 2.1). Viability of RAW264.7 murine macrophage cells after treatment with different concentrations of kinos was tested in acid phosphatase assays. Morphological features of the cells after treatment with kinos were observed and the images were taken with a phase contrast microscope. Enzyme-linked immunosorbent assays (ELISA) were used to detect secretions of pro-and anti-inflammatory cytokines in supernatants of LPS-stimulated RAW264.7 murine macrophage cells after co-incubating with crude kinos extracts and fractions. Similarly, NO levels in the supernatants of the kinos treated cells were measured using the Griess assay. Measurement of phagocytosis of Gram negative and Gram positive bacteria was conducted by flow cytometry. The antibacterial effect of kinos was measured in disc and agar well diffusion and broth microdilution methods.

In an attempt to detect major groups of compounds from the kinos, fractionation, UV light spectral analysis of kinos fractions and assays, thin layer chromatography (TLC) and qualitative tests of crude kinos were employed.

2.2. Kinos collection and preparation

Kinos, astringent reddish exudates from *Eucalyptus calophylla* (voucher number MW8), was collected from a mature Western Australian *Eucalyptus* tree in the spring. The kinos from *E*. *confertiflora* (voucher number UPNG MY1115), was also collected from a mature *Eucalyptus* tree in PNG: collected in the wet season. The WA kinos were collected from the same tree in Kings Park Botanical Garden and similarly, the PNG kinos were also collected from the same tree from the University of Papua New Guinea (UPNG) campus. The soil type of Kings Park is generally well-drained and loose sandy in texture whilst the soils of the UPNG campus are loam sandy and compact. The *Eucalyptus* species from WA was identified by Mr. Jeremy Thomas, the chief arborhorticulturist of Kings Park Botanical Gardens, Perth, Western Australia while the PNG *Eucalyptus* species was identified by one of PNG's well-known botanists, Mr. Pius Piskaut. The WA kinos were reddish-brown whilst PNG kinos were light-brown in colour, as shown in Figure 2.1. The kinos were ground with a mortar and pestle and sieved through number 16 meshes with 1.19 mm size.

The kinos were solubilized in 1x (v/v) phosphate buffered saline (PBS) (Sigma-Aldrich, MO, USA) with a pH of 7.4. The concentration of kinos stock solution was 10 mg/mL (w/v) for the cell culture experiments inclusive of cell viability, cell morphology characterization, enzyme-linked immunosorbent assays for pro-and anti-inflammatory cytokines, phagocytosis of bacteria and NO assays. The concentration of kinos stock for the disk and broth microdilution methods for the antibacterial activity study was 120 mg/mL. All kinos stocks were filter-sterilized with 0.22 µM Millipore filters (Merck Millipore Ltd, Cork, Ireland) and stored in -80^{0}C.

2.3. Materials

Table 2.1 presents all names of reagents used in this study and their respective manufacturers /suppliers. All reagents used were of an analytical grade.

Table 2.1. Reagents used in this study and their respective manufacturers/ suppliers.

Reagent	Manufacturer/Supplier
Aztreonam	Sigma-Aldrich, St Louis, MO- USA
4-Nitrophenyl phosphate (NPP) disodium salt hexahydrate	Sigma-Aldrich, St Louis, MO- USA
Amoxicillin-potassium clavulanate	Sigma-Aldrich, MO, USA
Bovine Serum Albumin	Fisher Biotec, Australia
Dichloromethane	Fisher Scientific UK Ltd, UK
Dimethyl Sulfoxide	Fisher Scientific UK Ltd, UK
Fluorecein isothiocynate isomer I	BioLab (AUST) Ltd - Australia
Foetal bovine serum	Bovogen Biologicals, Victoria, Australia
Mouse Interleukin -10 ELISA kit	R&D Systems, Minneapolis, MN - USA
Mouse Interleukin -6 ELISA kit	R&D Systems, Minneapolis, MN - USA
Mouse Tumour Necrosis Factor-alpha ELISA kit	R&D Systems, Minneapolis, MN - USA
Mueller Hinton agar	Oxoid Ltd., Hampshire, England
Mueller Hinton broth	Oxoid Ltd., Hampshire, England
Orthophosphoric acid	Fisher Scientific UK Ltd, UK

Table 2.1. continued

Reagent	Manufacturer/Supplier
Phosphate buffered tablets	Sigma-Aldrich, MO, USA
RPMI 1640 (1x) cell culture media	HyClone Laboratories Inc., Utah, USA
Sodium Chloride	Biolab (Aust) Ltd., VIC – Australia
Stretavidin conjugated horseradish-peroxidase	R&D systems, Minneapolis, USA
Sulphuric Acid	Fisher Scientific UK ltd, UK
Trypan Blue solution (0.4%)	Sigma-Aldrich, St Louis, MO - USA
Tween-20	Sigma-Aldrich, St Louis, MO - USA
UltraPure distilled water	Life Technologies, New York, USA

(A)

(B)

Figure 2.1. Appearances of powdered WA (A) and PNG (B) kinos.

2.4. Cells, cell culture and incubation conditions

RAW264.7 cells, a murine monocyte-macrophage cell line, was obtained from American Type Culture Collection (ATCC) TIB-71 (Manassas, VA) and maintained in Roswell Park Memorial Institute (RPMI) 1640 (1x) cell culture media with 2.05 mM L-glutamine (HyClone Laboratories Inc., Utah, USA) supplemented with 5% (v/v) foetal bovine serum (FBS). All cell culture experiments were also done in 5% (v/v) FBS RPMI media. The cells were incubated at 37°C in a 5% (v/v) CO_2 humidified incubator for all experiments. In order to reduce variability, only RAW264.7 ATCC TIB-71 macrophage cells were used in all experiments that investigated cell viability and the anti-inflammatory effects of kinos. In addition, RAW264.7 cells readily produced cytokines when stimulated with LPS from *Escherichia coli* 0111:B4 (Sigma-Aldrich, MO, USA). All cells were tested to ensure mycoplasma negative before use.

2.5. Morphology of RAW264.7 cells

Cells were seeded in 6-well Nunc plates at a density of 2.5 $x10^5$ cells/mL and were exposed to 0, 62.5, 125, 250 and 500 µg/mL kinos or lipopolysaccharide (LPS). There were two controls in this experiment: the untreated cells with and without LPS. The morphology of the cells after being exposed to these concentrations of WA and PNG kinos for 0, 6, 12 and 24 h, under normal incubation conditions, were observed and their images were taken using the Nikon DS-L3 phase-contrast microscope (Nikon Corporation, Japan) with a 40x objective and an attached Olympus E330 camera. The purpose of this experiment was to observe any changes in the morphology of the cells after being exposed to different concentrations of kinos at different time points.

2.6. Cell viability

Acid phosphatase (APH) assay was employed in this study to compare the viability of RAW264.7 cells with or without kinos exposure.

2.6.1. Acid phosphatase assay

The cell viability of the RAW264.7 cells was evaluated using the APH assay. The cells were seeded in a Nunc® 96-well plate at a density of 2 $x10^5$ cells/well in RPMI media with a volume of 150 µL each. The cells were stimulated with LPS and exposed to WA and PNG kinos at 37 °C in a 5% (v/v) CO_2 humidified incubator for

6, 12 and 24 h. The two controls for this cell viability assay were cells in the vehicle control, 1x PBS, and cells with LPS alone for each individual experimental setup. The cells were washed in 150 µL of sterile saline then incubated for 40 min in the described conditions in acid phosphate buffer. This buffer consisted of 100 mM (w/v) of sodium acetate at a pH of 5.2, 0.1% Triton X – 100 (v/v) and 2 mg/mL p-Nitrophenyl phosphate (NPP) (Sigma-Aldrich, St Louis, MO, USA). The absorbance of the NPP solubilized supernatants was read at 405 nm wavelength within 10 min using the EnSpire microtitre plate reader (Perkin Elmer, Norwalk, CT, USA).

2.7. Measurement of cytokines and nitric oxide as mediators of inflammation

The methods described below were used to measure pro- and anti-inflammatory cytokines and NO as mediators of inflammation.

2.7.1. Tumour necrosis factor alpha (TNF-α) ELISA

Induction of TNF-α in supernatants from $5x10^5$ cells/well RAW264.7 cells stimulated with 100 ng/mL LPS and exposed to 62.5, 125, 250 and 500 µg/mL kinos for 6 and 12 h was measured using the TNF-α kit from R&D systems (USA) by following the manufacturer's instruction. The 96 well Nunc® Elisa plate was coated with diluted 1:180 (v/v) capture antibody in 1x PBS with a pH of 7.28 (recommended 1x PBS pH for capturing cytokines) and incubated for 15 h at 37°C. The antibody-captured wells were washed with ELISA wash buffer (0.05% (w/v) Tween 20 in 1x PBS) after the incubation period. The wells were blocked with reagent diluent comprising of 1% (w/v) BSA dissolved in 1x PBS for 1½ h and washed for the second time before supernatants from the standards or samples were added to the wells. One hundred microliters each of five concentrations of standards or 100 µL of sample supernatants were added to these wells in triplicates and incubated for 2 h at room temperature. Concentrations of the standard for TNF-α were 200, 400, 600, 800 and 1000 pg/mL. Another wash was done after the incubation of the standards and the samples. One hundred microliters of the diluted (1:180 v/v) detection antibody was added to the aspirated wells and incubated at room temperature for another 2 h. This was followed by another wash with the wash buffer. One hundred microliters standard diluted (1:200 v/v) horseradish peroxidase-labelled streptavidin was added to the wells and incubated for 20 min in the dark. Then, 100 µL of substrate solution, 3, 3′, 5, 5′-tetramethylbenzidine (TMB) was added to the wells after they were

washed and aspirated. The wells were incubated for 20 min in the dark before 50 µL of 1N sulphuric acid (H_2SO_4) was added to the wells to stop the reaction. The absorbance was read at 540 nm using the EnSpire microtitre plate reader (Perkin Elmer, Norwalk, CT, USA).

2.7.2. Interleukin-6 (IL-6) and IL-10 ELISAs

The induction of IL-6 in supernatants from $5x10^5$ cells/well RAW264.7 cells stimulated with 100 ng/mL LPS and exposed to 62.5, 125, 250 and 500 µg/mL kinos for 6 and 12 h was measured using the IL-6 kit from the R&D systems (USA) by following the manufacturer's instruction. All steps for the protocol used in this ELISA were exactly the same as previously described for the TNF-α ELISA in Section 2.7.1. The only difference was in the concentrations of the standards, which were 100, 200, 300, 400 and 500 pg/mL for the IL-6 ELISA.

Similarly, all steps for the IL-10 ELISA were exactly the same as previously described for the TNF-α ELISA. In addition, the concentrations for the IL-10 ELISA were the same as those used in TNF-α ELISA. The IL-10 dilution factor (1:160 v/v) for the detection antibody was the only difference between the two ELISAs.

2.7.3. Nitric oxide assay

Nitrite, the stable end product of NO, was used as an indicator of NO production in the culture medium. The nitrite released in the culture medium was measured according to the Griess reaction (Fangkrathok *et al*., 2013; Srisook & Cha, 2005). RAW 264.7 cells seeded at a density of 5 $x10^5$ cells/well were cultured in 12-well plates in triplicates overnight. The cells were stimulated with LPS (100 ng/mL) and treated with 62.5 125, 250 and 500 µg/mL solubilized WA and PNG kinos for 24 h. There were two controls in this assay: untreated cells (negative control) and cells with LPS alone (positive control). After 24 h of incubation at 37°C in 5% CO_2 humidified incubator, the supernatants of the different treatments were collected and tested for NO production by the cells. The nitrite in the supernatants was measured by mixing 50 µL of supernatant with 50 µL of Griess reagent (1% (w/v) sulphanilamide and 0.1% (w/v) N-1-naphthly-ethylenediamine dihydrochloride in 5% (v/v) phosphoric acid (H_3PO_4)). The samples and the standard were incubated in the dark for 10 min before reading the absorbance at 595 nm using the microtitre EnSpire plate reader (Perkin Elmer). The concentration of nitrite in the supernatants of the kinos treated cells was determined from the standard curve of sodium nitrite

made up in RPMI 1640 media containing 5% (v/v) FBS and the results were expressed as µM NO_2.

2.8. Phagocytosis of Gram negative and Gram positive bacteria

2.8.1. Growing and labelling of bacteria

Two bacterial species were used in the phagocytosis assay and these included *E. coli* ATCC 25922 (Gram negative) and *S. aureus* ATCC 29213 (Gram positive). Mueller-Hinton broth (MHB) and Mueller-Hinton agar (MHA) were prepared according to their respective labels and autoclaved at 121^0C for 15 min. Twenty millilitres of the warm molten agar each was poured into sterile petri dishes under aseptic conditions. Frozen bacterial culture in 20% (v/v) glycerol was stabbed with a sterile needle and placed into 5 mL of sterile cation-adjusted MHB (CAMHB) to release the bacteria and incubated on the shaker at 180 rpm for 20 h at 37^0C. A sterile loopful of the 20 h bacterial culture was streaked on the MHA plates under aseptic conditions and incubated at 37ºC in an incubator. This was purposely done to ensure the bacterial species to be investigated was the species of interest for the phagocytosis assay.

2.8.2. Heat-inactivation and fluorescein isothiocynate (FITC)-labelling of bacteria

The 20 h old bacterial culture was suspended in 10 mL 0.22 µM filtered PBS with an optical density (OD) of 0.8 with 1 to 2 x 10^8 CFU/mL that was equivalent to 0.5 McFarland standards. The bacterial suspension was centrifuged at a gravity of 204.82 using Allegra X-12 series centrifuge rotor (Beckman Coulter, California, USA) for 5 min. The bacterial culture was re-suspended in 10 mL of 1x PBS and heat-inactivated in a 50 mL tube in a water bath at 65°C for 60 min. The tube was swirled every 10 min to allow even heat distribution in the culture. The heat-inactivated bacterial culture was sedimented in the centrifuge. The supernatant was removed and the bacterial pellet was re-suspended in 10 mL of 1x PBS and washed again by centrifuging at the same speed as previously described.

Fifty microliters of heat-inactivated bacteria was plated out on MHA and incubated at 37°C and checked for growth after 24 and 48 h of incubation at 37°C. The bacterial pellet was re-suspended in 1 mL 1x PBS and in 500 µL of FITC solution ((0.1% (w/v) in 50 mM sodium bicarbonate ($NaHCO_3$) and 100 mM sodium chloride (NaCl)) and incubated for 20 min. The FITC-labelled bacteria were washed twice in 5 mL 1x

PBS by centrifuging. The bacteria were observed under a fluorescent microscope to confirm the FITC-labelling of bacteria. The FITC-labelled bacteria were re-suspended in 5 mL 1x PBS and stored at 4°C and used for the phagocytosis assay.

2.8.3. Phagocytosis of heat-inactivated and FITC-labelled bacteria by kinos treated RAW264.7 macrophage cells

Macrophage cells (RAW264.7) at a density of $1x10^6$ cells per teflon pot were incubated in 5% FBS RPMI 1640 media for 2 h at 37°C in a 5% (v/v) CO_2 humidified incubator. Western Australian and PNG total kinos extracts at concentrations of 62.5, 125, 250 or 500 µg/mL were added to the teflon pots containing the cells and incubated for 24 h.

After 24 h of incubation of cells with total kinos extract, 100 µL of FITC-labelled bacteria was added to the assigned wells (0 h) and incubated at normal cell incubation conditions for 1 and 4 h. At the end of each time point, the cell suspension with the FITC-labelled bacteria was transferred to 1.5 mL ice-cold 1x PBS in 15 mL tubes on ice. The cells and FITC-labelled bacterial suspension were washed twice in 5 mL ice-cold 1x PBS and centrifuged at 4°C at a gravity of 204.82 in an Allegra X-12 series centrifuge rotor (Beckman Coulter, California, USA) for 5 min. The supernatants were removed after each wash.

The washed macrophage cells were fixed by re-suspending in 500 µL of 4% (v/v) paraformaldehyde in 1x PBS (thus, resulting in 2% paraformaldehyde fixing of the cells). Fifteen µL of propidium iodide (PI) (1 mg/mL) was added to the assigned 2 mL vials containing the paraformaldehyde fixed cells and the bacteria and incubated in the dark on ice for 30 min. The cells were washed twice with ice-cold 1x PBS and centrifuged at gravity of 117.39 in an Eppendorf centrifuge rotor (Victoria, Australia) for 5 min.

The cells were re-suspended in 1 mL of 1x PBS and kept on ice until the samples were analysed on the flow cytometer. The prepared samples were analysed on a FACSCalibur™ cytometer (BD Biosciences) using a 488 nm argon laser and 564-606 nm emission filter. The debris for each sample was gated using forward and side scatter.

2.8.4. Imaging of kinos treated RAW264.7 cells phagocytising heat-inactivated and FITC-labelled *E.coli* and *S. aureus* ATCC 29213

The imaging of cells treated with a 125 µg/mL of WA and PNG kinos was performed as a follow-up analysis of the flow cytometry data. The 125 µg/mL concentration of kinos at the 4 h incubation time was selected for the imaging of phagocytosis of *E. coli* and *S. aureus* in this experiment. This kinos concentration and the incubation time were selected because these were the optimal conditions for the phagocytosis of the bacteria as reflected by increases in the change in total FITC and the mean fluorescence intensity (MFI) from the flow cytometry data.

RAW264.7 macrophage cells at a density of $1x10^6$ cells in teflon pots containing 5% FBS RPMI 1640 media were incubated for 2 h at 37°C in a 5% (v/v) CO_2 humidified incubator before they were treated with 125 µg/mL of WA and PNG kinos. The cells were co-incubated with kinos for 23 h. A 1:1000 (v/v) diluted cell tracker dye; the deep red dye (Life Technologies, USA) in DMSO was added to the untreated and kinos treated cells and incubated for 45 min. The cells were washed once with 1x PBS and once with RPMI media containing 125 µg/mL of kinos. A volume of 1.5 mL of fresh RPMI media was added to the cells and co-incubated with 100 µL each of FITC-labelled *E. coli* and *S. aureus* in the dark at the previously described incubation conditions for 4 h. The cells and the bacteria were harvested at the end of the 4 h incubation time point and washed twice with ice-cold 1x PBS by centrifuging the cells at gravity of 204.82 in an Allegra X-12 series centrifuge rotor (Beckman Coulter, California, USA) for 5 min. The cells and FITC-labelled bacteria were fixed with ice-cold 2% paraformaldehyde and incubated on ice for 30 min. They were then washed once with 1x PBS by centrifuging at the same speed and time as previously described. The 2% paraformaldehyde fixed cells were re-suspended in 100 µL of 1x PBS and 15 µL from this stock was mounted with anti-fade reagent on the glass slide with coverslips.

The mounted cells and FITC-labelled bacteria were then observed under the fluorescent microscope with a Perkin Elmer Nuance multi-spectrometric imaging system camera mounted onto it. The images were taken with 20x magnification objective lenses.

2.9. Diffusion and broth microdilution methods

2.9.1. Bacterial species, medium and bacterial incubation conditions

Four reference bacterial species; *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and two strains of *Staphylococcus aureus*, ATCC 13709 and ATCC 29213 were studied. Mueller Hinton pre-prepared agar plates were obtained from the Pathwest Laboratory Medicine WA (Nedlands, Western Australia, Australia) for the disc and agar well methods while MHB (Oxoid Ltd, Hampshire, England) was used for the microdilution method. Before the experiments, the strains were grown in MHB supplemented with 50 mg calcium ions and 25 mg magnesium ions overnight at 37°C. This yielded an inoculum of approximately $5x10^8$ CFU/mL, which was diluted according to inoculum size for the disc diffusion and microdilution methods, as recommended by the Clinical and Laboratory Standards Institute (CLSI), M7-A7 guidelines (Wilker *et al*., 2006).

The four reference bacterial species were grown in the supplemented MHB overnight and diluted to a 0.5 McFarland inoculum size for the disc method as recommended by CSLI (Wilker *et al*., 2006) and Wiegand *et al* (2008) with an optical density ranging between 0.08 and 0.13, at a wavelength of 625 nm. The 0.5 McFarland was further diluted 1:10 in sterile MHB to obtain a concentration of 10^7 CFU/mL. The final inoculum on MHA and in MHB for the disc diffusion and broth dilution methods, respectively, was approximately 10^4 CFU/mL.

2.9.2. Disc and agar-well diffusion methods

A sterile glass spreader was used to spread 50 µL of the 0.5 McFarland bacterial cultures on the MH agar plates within 15 min of adjusting it with MHB. The inoculated agar plates were allowed to stand for 15 min to allow the bacteria to settle. The 6 mm disk of 30 µg of amoxicillin and clavulanic acid (AMC) in the ratio 2:1 (Sigma-Aldrich, MO, USA) was used as a positive control when testing for the antibacterial effect of WA and PNG kinos on the *E. coli* and the two strains of *S. aureus* (ATCC 29213 and ATCC 13709). These three test bacterial species are sensitive to the antibiotic cocktail AMC as outlined in the 2014 Australian Medicines Handbook. Similarly, *P. aeruginosa* is sensitive to the antibiotic, aztreonam, and 6 mm discs of 35 µg/disc concentration were used in this setup.

Western Australian and PNG kinos concentrations of 0.05, 0.5, 1, 2, 3, 6.25, 12.5, 25 and 50 mg/mL in 1x PBS were tested for their antibacterial activity using the disc

and agar well diffusion methods. The agar well diffusion method was used to further test for the susceptibility of the two Gram negative bacteria (*E. coli* and *P. aeruginosa*) to the kinos since they were not inhibited by the kinos when tested in the disc diffusion method.

The MHA plates inoculated with the bacterial culture containing 6 mm sterile paper discs impregnated with the 25 µL of kinos and the respective antibiotic discs were inverted and incubated at room temperature for 15 min. The antibacterial effect of different kinos concentrations and the antibiotics were tested in triplicates. The saturated 1x PBS, vehicle control of 6 mm discs, also in triplicates, were also inoculated in the agar plates. The inoculated agar plates were incubated at 37°C for 20 h as recommended by the CLSI protocol, M7-A7 (Wilker *et al*., 2006). There were also control agar plates with one set of non-inoculated agar plates used as a sterility check, while the other plates were inoculated only with bacterial culture simultaneously to the treated plates at these incubation conditions. The inhibition zones were observed and measured in millimetres with a 30 cm ruler and recorded after the incubation period.

In addition, the MHA wells were prepared by having the solid agar dug out with a sterile 6 mm cork borer and 75 µL kinos were dispensed into these wells and the inoculated agar plates were allowed to stand for 15 min. The AMC and aztreonam discs and vehicle control were also applied to the wells in the agar plates. The inoculated agar plates were incubated at 37°C for 20 h. There were also control agar plates incubated along with the plates as described for the disc method above. The inhibition zones were observed and measured in millimetres with a 30 cm ruler and recorded after the incubation period.

2.9.3. Broth microdilution method

The two strains of *S. aureus,* ATCC 13709 and 29213, were tested further for their susceptibility to the high kinos concentrations (1, 3, 6.25 and 12.5 mg/mL) and these concentrations were selected for the broth microdilution method because they were effective in the inhibition of the growth of the two bacteria when tested in the disc method.

Fifty microliters of diluted 0.5 McFarland bacterial cultures for the two strains of *S. aureus* as described in Section 2.8.2, was dispensed into the 96 Nunc® plate within 15 min of inoculum preparation. Similarly, 50 µL of the following double strength kinos concentrations; 1, 3, 6.25 and 12.5 mg/mL in 1x PBS were added to the

inoculated wells in triplicate. Control wells contained only 100 µL of MHB, or 50 µL MHB and 50 µL of the bacterial inoculum, 50 µL each of amoxicillin-potassium clavulanate (Sigma-Aldrich, MO, USA) and 1x PBS were also in triplicates. In addition, there were wells with MHB and double strength kinos but without bacteria in order to detect any effect of the colour of the kinos on the absorbance readings in the test wells. Both samples and controls were incubated at 37°C for 20 h and at the end of each incubation period, the absorbance of the wells was read at a wavelength of 625 nm using the EnSpire microtitre plate reader (Perkin Elmer).

Following the absorbance readings, 25 µL of the 20 hr old *S*. *aureus* in kinos extracts were plated out on MH agar and incubated for 20 h to observe for any bacterial growth and this was done in three individual experiments. In order to quantify the bacterial growth preceding the initial two experiments, 25 µL of the kinos exposed *S*. *aureus* inocula were streaked on the agar and incubated for 20 h for any bacterial growth. The colony forming units were counted and recorded accordingly.

2.10. Preliminary chemical screening of kinos

2.10.1. Acid-base sub-fractionation of kinos

The WA and PNG total kinos in 1x PBS were fractionated with an acid-base (liquid-liquid) method using 0.1 mM (v/v) hydrochloric acid (HCl) and 0.5 mM (v/v) sodium hydroxide (NaOH) (See Figure 2.2 for details of the method). Kinos solution (20 mL) was acidified with 0.1 mM HCl to a pH of 4 and mixed thoroughly with 20 mL of dichloromethane (DCM) in a separating flask by vigorously shaking for 1 h. The mixture was allowed to stand for 30 min before acidic aqueous fraction was collected (fraction 1). The DCM fraction was mixed with 20 mL of 0.5 mM NaOH (pH 10). Again shaken vigorously for 1 h and allowed to settle for 30 min. The two layers (the basic aqueous and DCM layers) were collected to give fractions 2 and 3, respectively. Three kinos fractions (fraction 1, fraction 2 and fraction 3) were collected from this whole fractionation process. Three fractions each for the WA and the PNG kinos were dispensed into aliquots of 1 mL in glass vials and protected from light. The aliquots were freeze-dried in the freeze-dryer (Dynavac engineering Pty Ltd., Victoria, Australia) at -35°C and 1 mbar pressure for 24 h. All freeze-dried samples were stored in -80°C.

The dried samples (kinos fractions) were reconstituted in 1x PBS (pH 7.4) before they were tested for their anti-inflammatory effect. The three fractions for each kinos

type were further tested for their anti-inflammatory effect following the protocols previously described for IL-6 ELISA. These were compared to the crude kinos extracts. Each experiment was conducted three times in triplicates.

Further phytochemical screening of kinos was done to assess the presence of the general classes of compounds in the kinos extracts. The following methods for detecting phenolic compounds and tannins were adopted from von Martin *et al* (2012) and Hagerman (2002) with some minor modifications. The methods used to detect the total phenolics and total tannins in kinos were adopted from von Martius *et al* (2012) that was initially derived from (Bajaj, 1977) and (Hagerman *et al.*, 2002). In addition, qualitative and spot tests were employed to test for the presence of alkaloids, flavonoids and triterpenoids saponins.

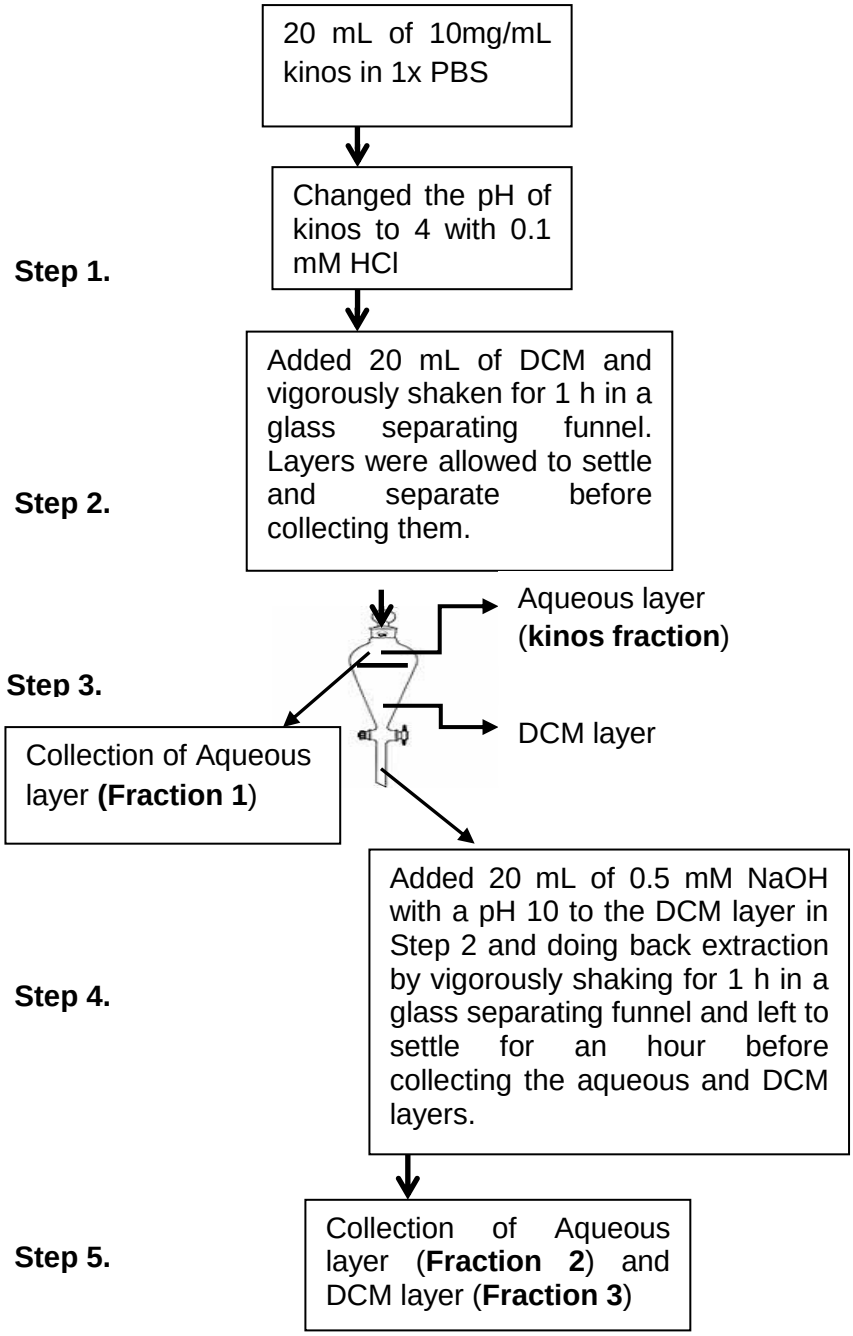


Figure 2.2. A flowchart showing the different steps involved in the acid-base sub-fractionation of kinos.

2.10.2. Determination of total phenolics in kinos

All solutions and extracts were freshly prepared and protected from light for each assay. The method from von Martin *et al* (2012) was followed with certain modifications to accommodate smaller volumes but keeping proportions comparable. In brief, kino was freshly powdered and one hundred milligrams was measured into a 50 mL centrifuge tube. Deionised water (50 mL) was added to the kinos powder and mixed thoroughly and dissolved completely by incubating in a 100 °C water bath for 30 min, followed by cooling to room temperature. Then 0.1 mL of the kino solution was transferred into a 15 mL centrifuge tube containing 8.4 mL deionised water followed by addition of 1 mL 29% sodium carbonate solution and 0.5 mL Folin-Ciocalteu reagent. The tube was inverted several times in order to mix the contents thoroughly and left at room temperature for 30 min before its absorbance was read at 760 nm against a blank containing reaction components with only deionised water. The results were expressed as percentage tannic acid by comparing against a fresh tannic acid standard (STD) curve between a range of 2.5 and 50 mg/mL. Fresh dilutions of tannic acid (Sigma-Aldrich, Victoria, Australia) in deionised water, as for the test samples and also incubated for 30 min at 100 °C. The STD curve was prepared and run at the same time as the test samples. A linearity of the two experiments for phenolic assay was on average, $r^2 = 0.9906$.

2.10.3. Determination of total tannins in kinos

The kino solution was prepared as described in the determination of total phenolics in kinos (Section 2.10.2). Kino solution (10 mL), acidic sodium chloride solution (25 mL) and 25% (w/v) of gelatine solution (15 mL) were mixed in a 50 mL centrifuge tube by shaking for 15 min. The solution was then centrifuged at 250 x g for 5 min. Then a 0.25 mL of the supernatant was used to analyse the phenolic content as described in the determination of the total phenolic contents. The total tannins were determined by subtracting the absorbance readings of the supernatants (the non-tannin fractions) from those for the total phenolics.

2.10.4. Thin layer chromatography of kinos for alkaloids using Dragendorff reagent

Methanol and dichloromethane (10 µL) kinos powdered extracts of WA and PNG were spotted on normal phase thin layered chromatography (TLC) plate coated with 0.22 mm layers of silica gel $60F_{254}$ (Merck, USA) and sprayed with the Dragendorff reagent. Bulleyaconiyine A (Sigma-Aldrich, Victoria, Australia) was used as the alkaloid standard used in the setup. The Dragendorff reagent contained bismuth

nitrate and potassium iodide. The Dragendorff's spray reagent was made of 1 mL of the stock solution containing equal volumes of solution (a) and solution (b) (1:1), 2 mL of glacial acetic acid and 40 mL of milli-Q water. The presence of alkaloids in those kinos extract spots should have been indicated by the dark orange in colour.

Preparation of Dragendorff's reagent

Solution (a) had 0.85 g bismuth (III) nitrate (Bi $(NO_3)_3.5H_2O$ dissolved in 10 mL glacial acetic acid and 40 mL water. The warm water bath was used to help heat the solution to dissolve the (Bi $(NO_3)_3.5H_2O$). Solution (b) had 8 g of potassium iodide (KI) dissolved in 30 mL milli-Q water. The stock solution Dragendorff's reagent was made of equal volumes of solution (a) and solution (b).

2.10.5. Thin layer chromatography of kinos for flavonoids using anisaldehyde-sulphuric acid reagent

Methanol and dichloromethane (10 µL) powdered extracts of WA and PNG kinos were spotted on a normal phase thin layered chromatography (TLC) plate coated with 0.22 mm layers of silica gel $60F_{254}$ (Merck, USA) and sprayed with Anisaldehyde reagent and heated using a hair blow drier and evaluated under UV light with a wavelength of 365 nm. Quercetin (Sigma-Aldrich, Victoria, Australia) was used as standard for the flavonoids. The yellow colouration of the spots was indicative of presence of the flavonoids in the kinos extract.

Preparation of anisaldehyde-sulphuric reagent

The anisaldehyde-hydrochloric acid reagent was prepared by adding 0.5 mL anisaldehyde, 10 mL glacial acetic acid, 85 mL methanol and 5 mL concentrated sulphuric acid together and mixing thoroughly before use. The different components of this reagent were added in the order mentioned.

2.10.6. Qualitative flavonoid tests

Two qualitative tests were used for further testing for the presence of flavonoids in the kinos. These two methods were adopted from (Edeoga *et al.*, 2005).

The first test involved 0.5 mg of kinos powder in 2 ml of milli-Q water was thoroughly mixed and then followed by the addition of 2 mL of 1 % aluminium solution into the mixture and mixed thoroughly. The presence of flavonoids was indicated by the yellowish colouration of the solution.

Similarly, the second test involved 0.5 mg of kinos powder in 2 mL of ethyl acetate was thoroughly mixed and then followed by adding 2 mL of 1 % aluminium solution

into the mixture and mixed thoroughly. The presence of flavonoids was indicated also by the yellowish colouration of the solution.

2.10.7. Qualitative triterpenoid saponin test

One milligram of kinos was mixed in 4 mL of milli-Q water and boiled in a water bath and filtered. Then, the filtrate was mixed with 2 mL of milli-Q water and shaken vigorously for a stable persistent froth which indicated the presence of triterpenoid saponins. This was also further tested by mixing with 2 drops of olive oil and shaken vigorously and observed for the emulsion. This method was also adopted from Edeoga *et al* (2005).

2.11. Statistical analysis

One-way and two-way analysis of variance (ANOVA) were performed to compare the means of three or more samples followed by Dunnett's or Bonferroni's multiple comparison post-test to compare samples of interest with a control sample as described in the figure legends of the different figures for the thesis. In addition, multivariate statistics and paired student t-tests were performed to compare the means of two samples where appropriate. Error bars represent the standard error of the mean. P-values of <0.01 and 0.05 were considered statistically significant. All statistical analyses were performed using Graphpad Prism version 6.0 (La Jolla, CA, USA) and Statistical Analysis System SAS version 9.2 (SAS institute, Cary, NC, USA). All statistical analyses for all data presented with statistical descriptions in this study were confirmed by the statistician of Curtin University's Faculty of Health Sciences, Dr. Richard Parsons.

CHAPTER 3

EFFECTS OF KINOS ON CELL MORPHOLOGY AND CELL VIABILITY

3.1. Introduction

The medicinal properties of plants and other sources of bioactive compounds have certainly been discovered serendipitously to some extent. Some previous studies have reported surprising but important discoveries in isolating bioactive chemical compounds that had several antimicrobial activities in their investigations (Sadgrove, 2014; Seto, 2012). The study by Sadgrove *et al* (2014), isolated cisopsoralen, xanthyletine and osthole (coumarins) from one of the essential oil samples of *Geijera parviflora* Lindl. (*Rutaceae* family) that were dissolved in hydrosol. However, other coumarins were not present in the specimen that was analysed earlier for the chemical compositions. The three coumarins were prominent in inhibiting the fungal and bacterial species tested. Similarly, rapamycin, an antibiotic was isolated from the *Streptomyces hygroscopicus* (a fungal species) and was demonstrated to prevent cancerous cell growth. It was later investigated to inhibit cell growth of fungal species, plants and animals as reported in a review article (Seto, 2012). Eleutherobin, a marine diterterpene glycoside was isolated among the other five new antimitotic compounds that were found to be arresting cells in mitosis by stabilizing microtubules (Roberge *et al.*, 2000). The combinations of different sensitive assays for screening compounds lead to isolation of novel compounds. These are a few examples of investigations that show that some isolation of bioactive chemical compounds and the antimicrobial activities detected occur serendipitously. However, more often, they have been investigated intentionally, initiated through claimed therapeutic effects from indigenous groups. Indeed, this ethnological approach has been employed herein.

The cytotoxicity of plant extracts, claimed to possess remedial properties, are of primary concern, particularly when antimicrobial properties are being investigated. Here the aim is to identify pathogen-killing properties with minimal damage to the hosts' cells. Tests for cytotoxicity of crude extracts and purified chemical compounds are normally initiated by *in vitro* studies (Huang *et al*., 2011; Palombo & Semple, 2001). The goal of cytotoxic testing is to evaluate the ability of representative host cells to survive when exposed to extracts or compounds over a period of time (Akiyama *et al.*, 2001). Different tests that evaluate cytotoxicity *in vitro* include the lactate dehydrogenase leakage assay, protein assay and the neutral red assay. Other tests which are classified as viability assays are also routinely employed to evaluate toxicity and these include the acid phosphatase assay (APH) and the methyl tetrazolium (MTT) assay (Huang *et al.*, 2011). These tests enable the

determination of maximum non-toxic concentrations of any pharmacological agent in order to exclude those concentrations that have deleterious side-effects, leading to cell death. This allows future antimicrobial investigations to use concentrations that are conducive for cell growth and proliferation and can produce reliable and reproducible data.

Studies have been published investigating the cytotoxic effects of crude extracts and essential oils from *Eucalyptus* plants (Döll-Boscardin *et al.*, 2012; Saethre, 2007). A study that investigated cytotoxicity of essential oils of *Eucalyptus benthamii* and their terpenes on tumour cells showed inhibition of Jurkart cell proliferation due to toxicity, as tested by the MTT assay (Döll-Boscardin *et al.*, 2012). A similar study demonstrated the resistance of J774A murine macrophages to different concentrations of essential oils from the leaf extracts from a different *Eucalyptus* species, *Eucalyptus globulus*. Even the essential oils from *E. citriodora*, *E*. *tereticornis* and *E*. *globulus* had no toxic effects on mice at a dose of ≤100 mg/kg (Silva *et al*., 2003). Not only have the essential oils of the leaves of *Eucalyptus* species have been investigated for cell viability, cytotoxicity and morphology, other previous studies have demonstrated cells to be less viable with cell death due to toxicity of plant extracts and the changes in the morphologies of the cells. Similarly, it was demonstrated that the ethanolic leaf extracts of *Artemisia nilagirica* and *Murraya koenigii* to be cytotoxic to different macrophage cells at 300 and 100 µg/mL respectively (Naik *et al*., 2014). The cells rounded up at these extract concentrations and were shown to have damaged DNA in the fluorescent microscopic pictures. The decreases in the number of viable macrophage cells, the toxicity of the cells and morphological changes due to the effects of *Citrus aurantium* L. extracts (CME) were also investigated by Kang *et al* (2010). The cells were spread out in non-LPS stimulated and non-CME treated cells but the spreading decreased with its increasing concentrations. The pseudopodia formations of the LPS and CME treated cells also decreased with these high doses. In addition, the viability of the cells also decreased with the high doses of a CME. The several studies discussed above show that any antimicrobial agent may decrease cell number, cause cell-death and change the morphologies of the cells with increasing concentrations.

In contrast, cytotoxicity and the effect of *Eucalypt* kinos on cell morphology have not been reported in any literature. Therefore, the aims of investigations in this chapter were to evaluate the cytotoxicity and morphological changes resulting from WA and PNG kinos administration on RAW264.7 murine macrophages. The results will also

provide a range of working kinos concentrations to further investigate kinos bioactivities in subsequent chapters of this thesis.

3.2. Results

3.2.1. Effects of WA and PNG kinos on the morphology of LPS-stimulated RAW264.7 cells

The morphology of the untreated cells remained the same at the 6, 12 and 24 h time points (Figure 3.1 a, b and c). The LPS-stimulated cells (100 ng/mL) had slightly spherical and flattened morphology with larger diameters compared to the untreated cells, and these morphological changes became more distinct with increasing incubation time (Figure 3.1 d, e and f). In addition, the LPS-stimulated cells also developed small vacuoles that increased in number and size with increasing incubation time.

Macrophages exposed to WA and PNG kinos became slightly elongated. This effect increased with both increasing concentration and incubation time. Macrophages exposed to WA kinos (Figure 3.1 g-l) displayed greater vacuolation, both in number and size of vacuoles compared to LPS-treated cells, with particularly obvious differences in the 125 µg/mL treatment for 12 and 24 h (Figure 3.1 k and l, respectively). Furthermore, the cells incubated for 24 h displayed relatively large vacuoles. In contrast, treatment with PNG kinos (Figure 3.1, m-r) did not increase vacuole number and size beyond that of LPS-treated cells for 12 h, except for the 125 µg/mL treatment for 24 h, where the cells displayed vacuolation comparable to 24 h LPS-treated cells with the addition of some cells containing relatively larger vacuoles.

3.2.2. Effects of WA and PNG kinos on cell viability of LPS-stimulated RAW264.7 cells

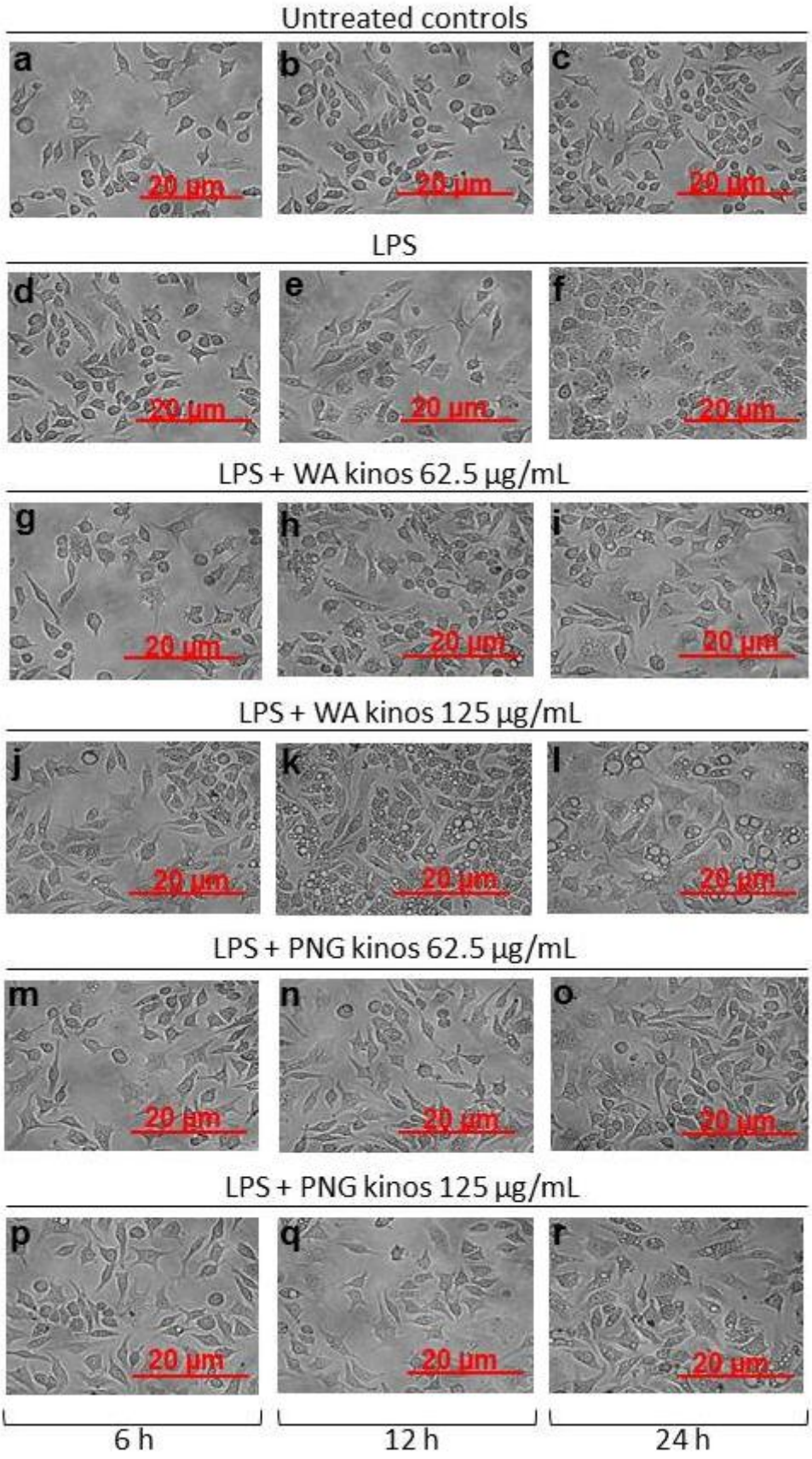
The treatment of LPS-stimulated RAW264.7 cells with varying concentrations of WA and PNG kinos showed a general trend in reducing cell number, which increased in magnitude with increasing kinos concentration and incubation time (Figure 3.2 and 3.3). Most notable was the effect of kinos at a concentration of 500 µg/mL for both WA and PNG sources. The effects were especially at 12 and 24 h, suggesting cytotoxicity, a reduction in proliferation or a combination of both at this highest kinos concentration.

The concentration of 62.5 µg/mL did not cause a significant change in cell viability, compared to LPS treatment, except for the PNG kinos at 12 h (Figure 3.3 B). Concentrations of 125 and 250 µg/mL resulted in statistically significant reductions in cell viability. The exceptions were 125 µg/mL WA kinos incubated for 6 h, which

resulted in an increase in cell number (Figure 3.2 A), and PNG kinos at 6 h incubation, displaying non-significant changes (Figure 3.3 A).

Figure 3.1. Morphological features of LPS-stimulated RAW264.7 macrophages treated with WA and PNG kinos. [See next page for images]

Images showing the morphology of untreated RAW264.7 cells (a-c), LPS-stimulated cells (d-f), LPS-stimulated cells treated with WA kinos at 62.5 (g-i) and 125 µg/mL (j-l), and LPS-stimulated cells treated with PNG kinos at 62.5 (m-o) and 125 µg/mL (p-r). Each treatment was incubated for 6 h (left column), 12 h (middle column) and 24 h (right column). Images were acquired using a phase contrast microscope. Magnification was 400X and the scale was 20 µm.


Untreated controls
a
b
c
LPS
d
e
f
LPS + WA kinos 62.5 µg/mL
g
h
i
LPS + WA kinos 125 µg/mL
j
k
l
LPS + PNG kinos 62.5 µg/mL
m
n
o
LPS + PNG kinos 125 µg/mL
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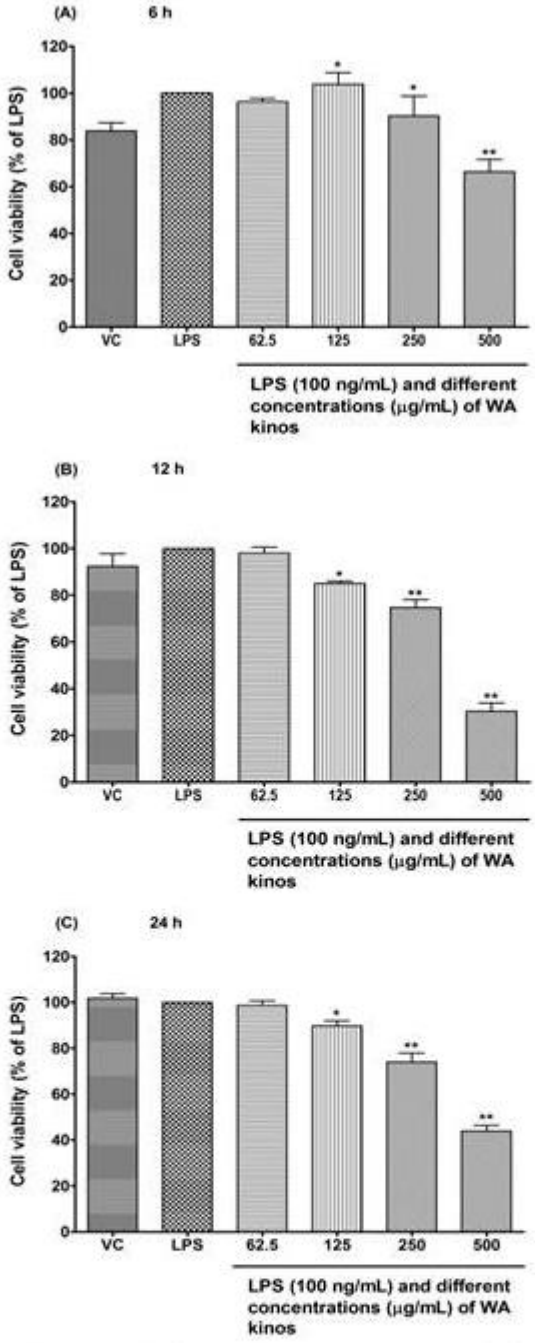


Figure 3.2. Effect of WA kinos on the viability of LPS-stimulated RAW264.7 macrophage cells over time.

LPS-stimulated RAW264.7 macrophages were treated with WA kinos with a concentration range of 62.5-500 µg/mL for 6 (A), 12 (B) and 24 h (C). Cell viability was measured with the APH assay. Controls were LPS-treated macrophages and PBS for the vehicle control (VC). Data presented are from thee individual experiments, n = 3 (± SEM). The statistically significant levels are at **P<0.001 and *P<0.05. The error bars for the LPS are included but they are too small to visualize.

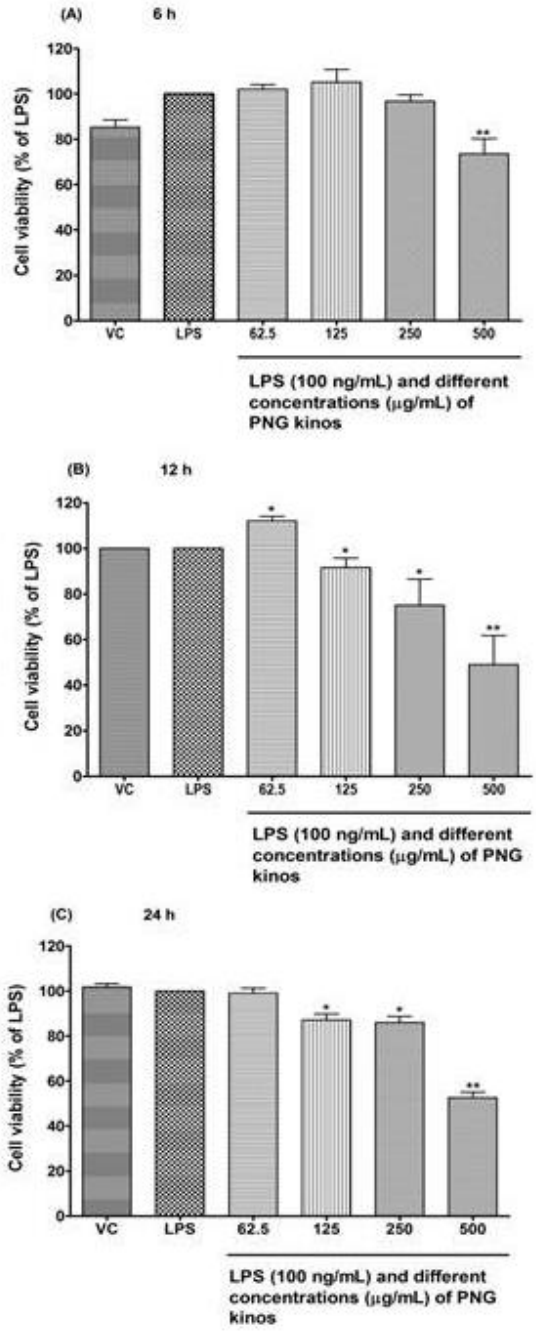


Figure 3.3. Effect of PNG kinos on the viability of LPS-stimulated RAW264.7 macrophage cells over time.

LPS-stimulated RAW264.7 macrophages were treated with PNG kinos with a concentration range of 62.5-500 µg/mL for 6 (A), 12 (B) and 24 h (C). Cell viability was measured with the APH assay. Controls were LPS-treated macrophages and PBS for the vehicle control (VC). Data presented are from thee individual experiments, n = 3 (± SEM). The statistically significant levels are at **P<0.001 and *P<0.05. The error bars for the LPS are included but they are too small to visualize.

3.3. Discussion

Cell morphology and viability studies *in vitro* offer important, primary information in investigations on pharmacological agents. The work in this chapter was carried out to determine basic information on the macrophage cell response to kinos, offering clues to possible effects, such as the activation of the primary function of macrophages and cytotoxic effects. In addition, it enabled the determination of the working concentration range for subsequent investigations including ELISA assays on cytokine profiles and phagocytosis assays.

The normal morphological shape of a cell is usually recognized as a good indicator for the functional state of the cell. The images (Figure 3.1) show that the morphology of LPS-stimulated RAW264.7 cells became slightly elongated after their exposure to WA and PNG kinos, and this effect increased with both increasing concentrations of kinos and incubation time. This slight elongation of cells by pharmacological agents has been demonstrated previously by McWhorter *et al* (2013). McWhorter and co-workers showed that the LPS and INF-gamma stimulated macrophage cells demonstrated slight elongation as opposed to the IL-4 and IL-13 stimulated cells, in a dose-dependent manner. It was suggested in their study and in other similar studies (Barros *et al.*, 2013; Tsikas, 2005) that the increased degree of cell elongation correlated to anti-inflammatory (M2) activation. However, pro-inflammatory activation (M1) was observed in this study as indicated by the rounding and vacuolation of the macrophage cells. These two cell characteristics have also been demonstrated for the LPS-stimulated (M1) macrophages by Chung *et al* (2005).

A study by Petricevich *et al* (2008), demonstrated increased number and size of macrophages after being exposed to scorpion toxins. They further demonstrated increasing phagocytic ability of these cells, while another study showed cell morphological changes such as rounding of cells and formation of vacuoles to be a marker of cellular activation and differentiation (Ogura & Kitamura, 1998). A recent study also demonstrated increased phagocytic indices and extent of vacuole formation in macrophages treated with an ethanolic extract from *Bougainvillea xbuttianna* (Cheenpracha *et al.*, 2010). Hence, it can also be suggested that the vacuolation of macrophage cells is a sign of stress after being treated with the WA and PNG kinos, showing they have been activated to produce cytokines. Studies have shown MAPK and NF-κB, two important cellular signals, to be activated after exposure to stimuli such as LPS, toxins and natural products in macrophages,

resulting in the release of high levels of inflammatory cytokines (Martin *et al*., 2005; Tsang *et al*., 2015). Finally, the marked rounding and vacuolation of the WA kinos exposed LPS-stimulated cells suggest that there is more intense activation in these cells than those cells treated with PNG kinos.

The cell viability data demonstrated that both WA and PNG kinos affected RAW264.7 cell proliferation to varying degrees depending on concentration and incubation time. In addition, potential cytotoxicity was detected at the highest kinos concentration. Therefore, the 500 µg/mL concentration was not interpreted in subsequent analyses due to possible complications associated with cytotoxicity. Many other studies (Jeong *et al.*, 2014; Liu *et al*., 2012; Nogueira *et al*., 2014; Pomari *et al*., 2014) have also demonstrated decreasing viability of cells with increasing concentrations of pharmacological agents despite the differences in the type of method used to test for viability. The cell viability data from a study by Nogueira *et al.* (2014) is consistent with this study. It demonstrated that the viability of monoblastic leukaemia macrophage cells decreased with increasing concentrations of tea tree oil, although there were differences in the type of macrophage cells. In a similar study, Pomari *et al* (2014) demonstrated that cell viability of LPS-stimulated RAW264.7 cells treated with four different concentrations of six plant extracts also decreased in a dose and time-dependent manner. Despite the low viability of the cells treated with two of the extracts (from *Echinacea angustiflora* and *Eleutherococcus senticosus*), the non-LPS stimulated RAW264.7 macrophages displayed up-regulation of the investigated inflammatory genes.

In a study by Liu *et al.* (2012), the viability of LPS-stimulated porcine alveolar macrophages resulted in a reduction in cell number with increasing plant extract concentration. Despite the reduction in cell number, only non-LPS-stimulated porcine alveolar macrophages induced appreciable amounts of IL-1β after being exposed to two of the seven plant extracts tested. Furthermore, Jeong *et al* (2014) also showed cell viability of RAW264.7 macrophage cells decreasing with increasing concentration of extracts from fungus. The fungal extracts alone were not able to activate the cells to produce prostaglandin E2, nitric oxide, IL-1β and TNF-α.

Therefore, the treatment of macrophages with kinos resulted in a trend of reduction in cell number that increased with increasing incubation time and kinos concentration. Only the highest concentration of kinos appeared to be toxic, dramatically reducing cell numbers. In addition, the effects of kinos on macrophages were apparent through clear morphological changes, which are consistent with the

activation of macrophages. Consequently, the treatment of macrophages with concentrations of kinos at or below 250 µg/mL should maintain their viability and normal function, free of toxicity.

3.4 Conclusion

In conclusion, the data herein, supported by relevant literature, demonstrate that kinos, a crude plant extract, have a variety of effects on macrophages including proliferation, viability and morphology. These effects are dose and time dependent, despite differences in the source of plant. In addition, the reduction of viability and proliferation of LPS-stimulated macrophage cells treated with plant extracts does not necessarily mean that their ability to produce cytokines is hampered however, this is to be tested in the following Chapters.

CHAPTER 4

ANTI-INFLAMMATORY EFFECTS OF CRUDE KINOS EXTRACTS, INCLUDING SOME PRELIMINARY ANALYSES OF SUB-FRACTIONS OF THE EXRTACTS

4.1. Introduction

A need for new, effective and affordable drugs to treat medical conditions (for example, inflammatory conditions) and microbial diseases in the developing world is a global health concern (Awouafack *et al.*, 2013). Factors such as affordability, compliance and drug ineffectiveness, due to the growing problem of drug resistant pathogens, result in the wide use of plant remedies for treating medical conditions in the developing world (Awouafack *et al*., 2013). Alternative medicines have been the lead targets for the isolation of chemical constituents for the development of new drugs and about 80% of drugs for the relief of microbial infections, cardiovascular diseases, cancer and inflammatory conditions are of plant origin (Pan *et al*., 2013).

Inflammation is a pathophysiological response to any infection encountered by the body and is driven largely by macrophages. In addition, inflammation has been implicated in many acute and chronic diseases like cancer, cardiovascular, and neurologic diseases that are related to ageing (Qureshi *et al.*, 2011). There are many mediators of inflammation including TNF-α, IL-6, IL-1, IL-11 and NO. TNF-α is a key cytokine, with a pleiotropic function on immunological processes, influencing the activities of other pro-inflammatory cytokines during an acute inflammation process (Gonçalves, *et al* , 2013; Roy & Chatterjee, 2014b). However, overproduction of cytokines can lead to negative physiological effects in the hosts. The overproduction of mediators for inflammation has prompted numerous studies to investigate the anti-inflammatory effect of crude extracts and their chemical constituents from many plant species (Cheenpracha *et al.*, 2010; Hillis, 1952). *Eucalyptus* species have also been known to relieve inflammatory conditions for centuries by first Australian communities (Maiden, J., 1892; Maiden, J. H., 1890; Scalbert, 1991). The effect of crude extracts and essential oils from the leaves and flowers of *Eucalypts* on cytokine production in stimuli-induced cells has mainly been investigated *in vitro* (Serafino *et al*., 2008).

To date, no investigations on the anti-inflammatory effect of kinos from the *Eucalyptus* plants have been conducted to confirm their ability to reduce inflammatory reactions. Therefore, this is the first study to scientifically establish whether kinos can modulate cellular inflammatory responses. As macrophages play a key role in inflammation, this *in vitro* study used LPS-stimulated RAW264.7 macrophage cells to investigate the effect of WA and PNG kinos on the production of pro-and anti-inflammatory cytokines and soluble factors.

Lipopolysaccharide was used to activate macrophages in order to mimic the process of inflammatory induction and response to a pathogen. The production and secretion of TNF-α, IL-6 and NO (all pro-inflammatory factors) and IL-10 (an anti-inflammatory cytokine) by RAW264.7 macrophages was measured from culture supernatants in response to co-incubation with either WA or PNG kinos. Macrophages stimulated with LPS were incubated with or without a range of WA or PNG kinos concentrations for 6 and 12 h. ELISAs were used to detect TNF-α, IL-6 and IL-10 while the Griess colorimetric assay was used to detect the production of NO in the supernatants of kinos treated cell cultures.

The second part of this study was aimed at fractionating the crude kinos extracts and testing the effects of the kinos fractions on the secretions of pro-inflammatory cytokines by the LPS-stimulated macrophages.

Testing the bioactivity of a natural product and preliminary chemical analyses are the two main approaches to any antimicrobial study of a new natural product. A spectrum of procedures from simple phytochemical screenings to highly complex high performance liquid chromatography and liquid chromatography-mass spectroscopy (LCMS) are undertaken to detect classes of chemical constituents potentially responsible for the observed antimicrobial activity. The screening of the crude plant or animal extracts have to be done accurately before any chemical compounds responsible for the bioactivity are isolated and their chemical structures elucidated (Butler, 2004). Preliminary screening of plant extracts for the detection of bioactive compounds was the approach of some of the previous studies (Edeoga *et al*., 2005; Musyimi & Ogur, 2008). Spectroscopic methods are also used in detecting UV light absorbing compounds which are dependent on the pigmentations of different plant parts (Cerovic *et al.*, 2002). The absorbance scanning profiles of the bioactive compounds from the plant extracts detected from small to large scale pharmacologically relevant assays are done to direct isolation and characterization of these compounds using high-level techniques including HPLC and LC-MS (Chanwitheesuk *et al.*, 2007; Strege, 1999; Zahin *et al.*, 2010),

Therefore, the second part of this part of the study involved subfractionation of the kinos using a simple acid-base extraction method to test the fractions for bioactivity in an attempt to semi-identify the active group of the compounds of the three fractions. The acid-base extraction method was employed since it is a sequential

liquid-liquid extraction method that can fractionate compounds from the complex mixtures in the kinos extracts. Each of the three fractions for WA and PNG kinos was tested for their anti-inflammatory effects. The levels of IL-6 production were tested in LPS-stimulated macrophages. Further basic/general phytochemical analyses of the crude kinos extracts were performed to detect the presence of main groups of chemical compounds.

4.2. Results

4.2.1. Enhancement of TNF-α secretion in LPS-stimulated cells after treatment with kinos

The LPS-stimulated RAW264.7 cells, as expected, significantly increased TNF-α production at both time-points measured (6 and 12 h) relative to the untreated controls (Figure 4.1). When RAW264.7 cells were treated with kinos from both sources, the general trend was an elevated production of TNF-α compared to LPS-stimulation alone. The WA kinos resulted in a statistically significant elevation of TNF-α for all concentrations at 12 h and for the 250 µg/mL at 6 h ($p<0.05$; Figure 4.1 A). The highest concentration of kinos, 500 µg/mL, had levels comparable to LPS alone. Data from Chapter 3 demonstrated that kinos, at this concentration (see Chapter 3, Figure 3.2), caused significant cell death, which explains this deviation from the general trend. The 6 h time-points demonstrated a dose-dependent response for TNF-α stimulation whereas by 12 h, the level of TNF-α appears to have reached a maximal stimulation of 2-fold at even the lowest concentration of kinos (62.5 µg/ mL). This is in the light of preliminary assays testing up to 24 h, where there were reductions in the TNF-α level (data not shown).

For PNG kinos treated macrophages, TNF-α production increased steadily only for 125 and 250 µg/mL (*$P<0.05$). The concentrations of 62.5 and 500 µg/mL were the same as for the LPS stimulated cells. Again as for WA kinos, the sample with the highest concentration sustained massive cell death. In the whole, both kinos sources enhanced TNF-α production in LPS-stimulated macrophages in a dose and time dependent manner, with the suggestion that the WA kinos were more potent between these two preparations.

4.2.2. Reduction of IL-6 secretion in LPS-stimulated cells after treatment with kinos

The exposure of LPS-stimulated RAW264.7 cells to different concentrations of WA and PNG kinos revealed a dose-dependent reduction of IL-6 secretion compared to the expected increase in IL-6 production by LPS stimulation alone (Figure 4.2 A and B). Discounting the 500 µg/ mL concentration due to significant cell death, the greatest IL-6 suppression was attained at 250 mg/mL, demonstrating an 8-fold decrease in cells treated with WA kinos for the 6 h incubation while there was a 4-fold decrease for the PNG kinos. Additionally, there was a 4-fold decrease for the IL-6 secretion by both types of kinos for the 12 h incubation time.

The WA kinos induced greater inhibition of IL-6 secretion than the PNG kinos at the 6 h time point. The IL-6 response was brief as lower IL-6 levels were seen after 12 h of incubation with LPS and both WA and PNG kinos (Figures 4.2A and B). It is possible that the LPS could have been broken down by the lysosomes in the cells and hence, there were low levels of IL-6 in culture media after 12 h of incubation.

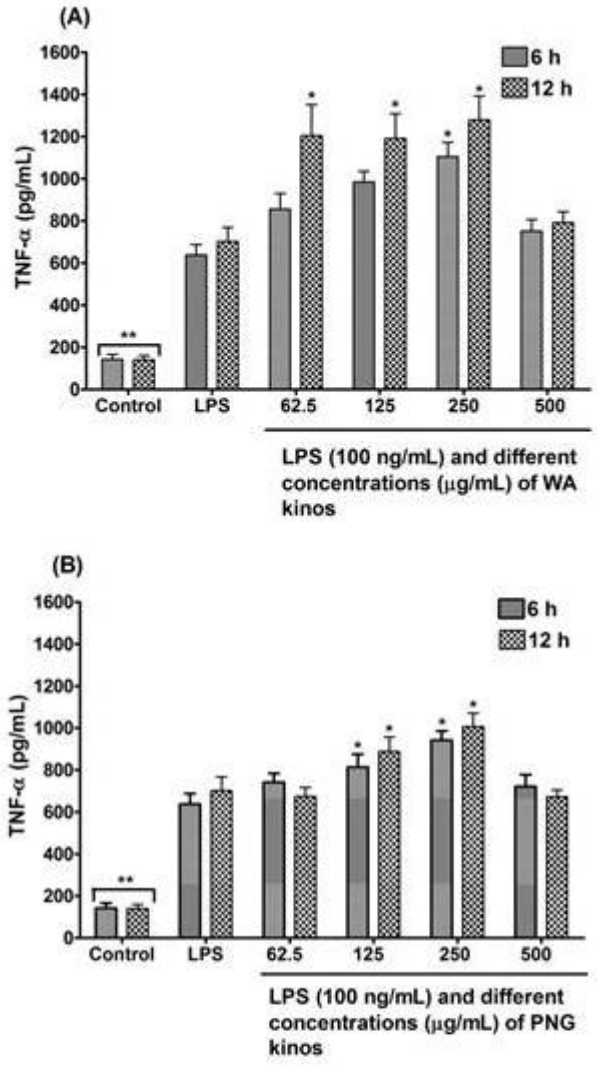


Figure 4.1. Effects of WA and PNG kinos on TNF-α secretion by LPS-stimulated RAW264.7 cells after 6 and 12 h of incubation.

RAW264.7 cells were incubated with 100 ng/ mL lipopolysaccharide (LPS) alone or either with kinos from WA (**A**) or PNG (**B**) for 6 or 12 h. TNF-α levels from media were quantitated with TNF-α ELISA. Data are presented as mean ± SEM, n = 3 individual experiments, each experiment done in triplicates on the day. Statistical analysis was measured using Two-way ANOVA where *P<0.05 compared to the positive control (LPS alone).

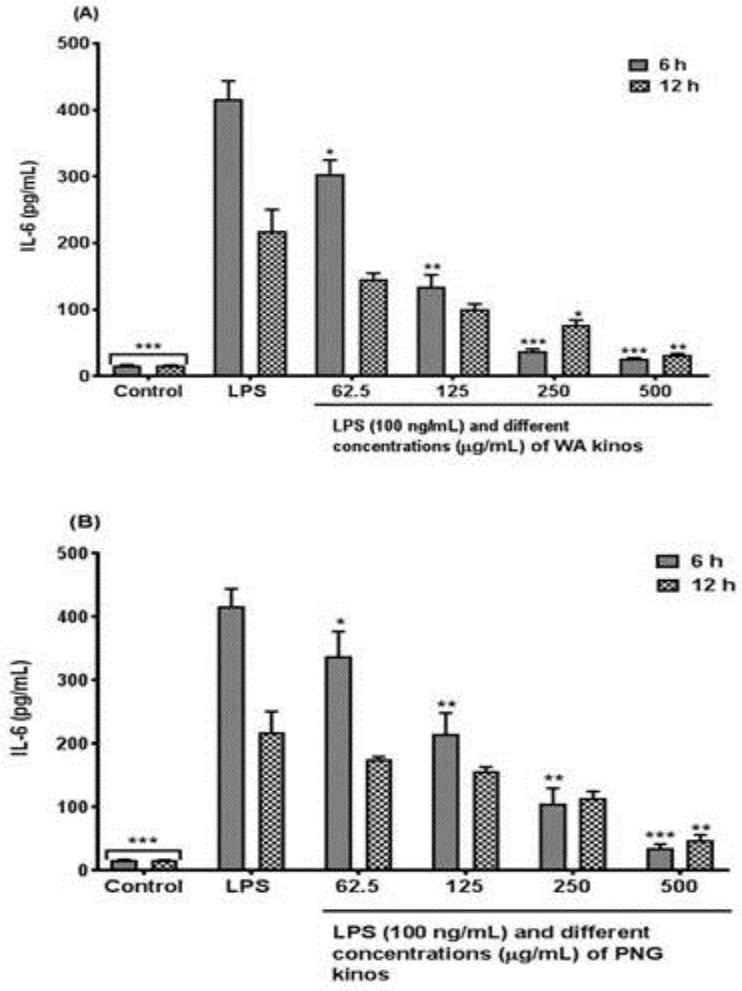


Figure 4.2. Effects of WA and PNG kinos on IL-6 secretion by LPS-stimulated RAW264.7 cells after 6 and 12 h of incubation.

RAW264.7 cells were incubated with 100 ng/ mL lipopolysaccharide (LPS) alone or either with kinos from WA (**A**) or PNG (**B**) for 6 or 12 h. IL-6 levels from media were quantitated with IL-6 ELISA. Data are presented as mean ± SEM, n = 3 individual experiments, each experiment done in triplicates on the day. Statistical analysis was measured using Two-way ANOVA where ***P< 0.001, **P<0.01 and *P<0.05 compared to the positive control (LPS alone).

4.2.3. Secretion of IL-10 by LPS-stimulated RAW264.7 cells after treatment with kinos

The secretion of IL-10, an anti-inflammatory cytokine, was increased by LPS stimulation of RAW264.7 cells, relative to the untreated controls (Figure 4.3). The addition of WA kinos resulted in the significant elevation of IL-10 secretion at both 6 and 12 h compared to LPS-only stimulated macrophages (Figure 4.3 A). The highest secretion of IL-10, 10-fold increase ($P<0.001$), was attained with the lowest concentration of kinos, at 62.5 µg/ mL for both time-points. Interestingly, there was an inverse relationship between kinos dose and IL-10 secretion.

The inclusion of PNG kinos to LPS-stimulated macrophages exhibited the opposite to WA kinos, with the reduction of IL-10 secretion compared to the LPS-alone stimulated RAW264.7 cells (Figure 4.3 A and B). The response, however marginal, seems to also be inversely proportional to the kinos concentration.

4.2.4. Nitric oxide (NO) expression is suppressed by kinos

Nitric oxide production by LPS-stimulated RAW264.7 cells was significantly reduced in a dose-dependent manner after being exposed to different concentrations of WA and PNG kinos for 24 h. With the exception of the 500 µg/ mL samples, the greatest NO inhibition was attained with 250 µg/ mL for both kinos sources (statistical significance of $p<0.001$), with a 10-fold reduction by WA and 5-fold reduction by PNG kinos (Figure 4.4 A and B, respectively). Interestingly, the potency at a concentration of 62.5 µg/ mL was notable, resulting in a 2.5-fold and 1.5-fold reduction in NO expression for WA and PNG kinos, respectively.

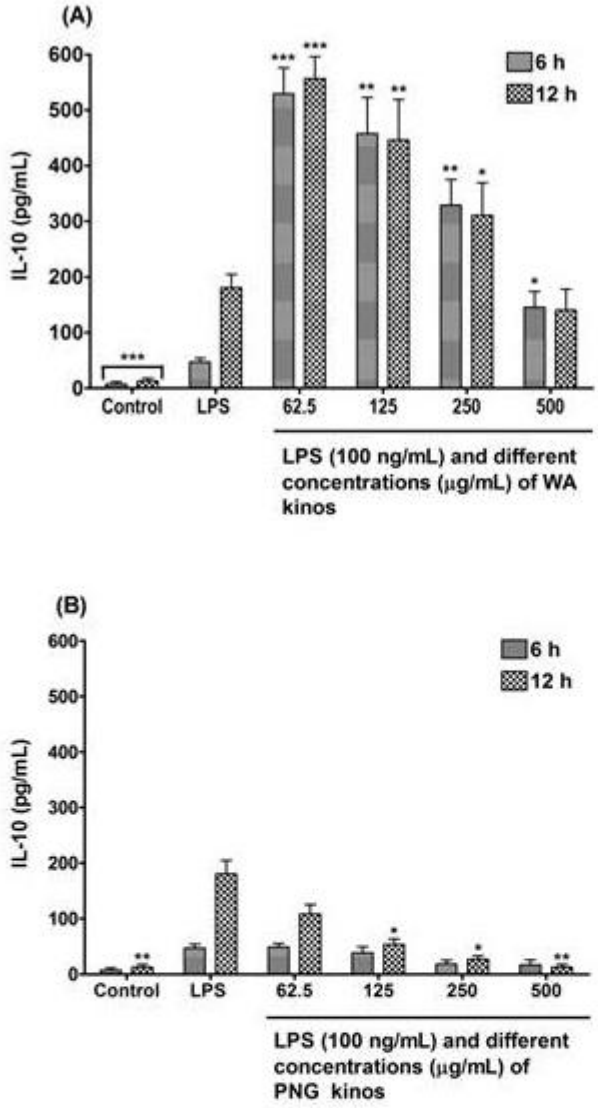


Figure 4.3. IL-10 secretion by LPS-stimulated RAW264.7 cells after treatment with WA and PNG kinos for 6 and 12 h.

RAW264.7 cells were incubated with 100 ng/ mL lipopolysaccharide (LPS) alone or either with kinos from WA (**A**) or PNG (**B**) for 6 or 12 h. IL-10 levels from media were quantitated with IL-10 ELISA. Data are presented as mean ± SEM, n = 3 individual experiments, each experiment done in triplicates on the day. Statistical analysis was measured using Two-way ANOVA where ***P< 0.001, **P<0.01 and *P<0.05 compared to the positive control (LPS alone).

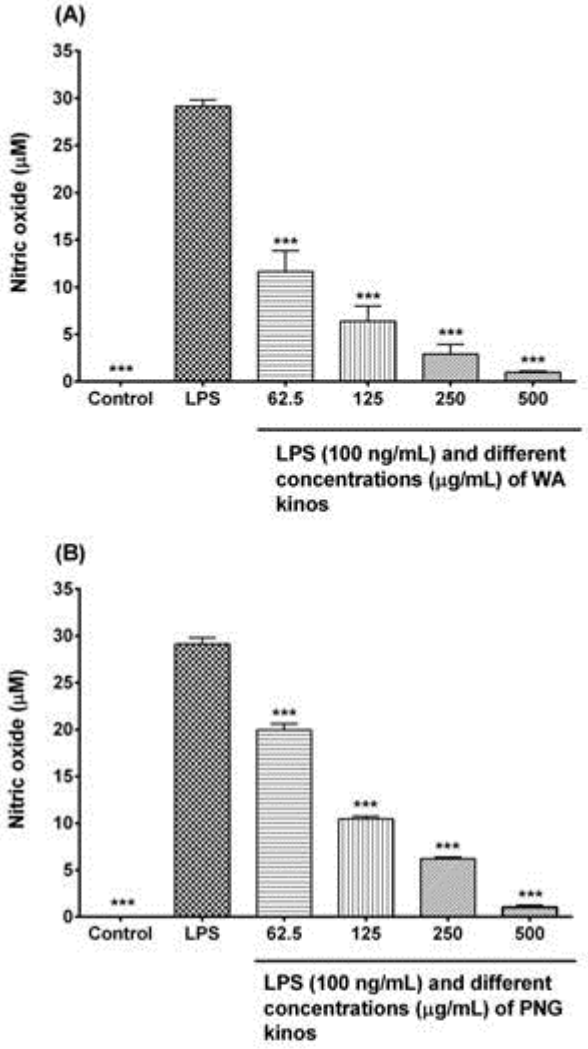


Figure 4.4. Nitric oxide secretion by LPS-stimulated RAW264.7 cells after treatment with WA and PNG kinos for 24 h.

RAW264.7 cells were incubated with 100 ng/ mL lipopolysaccharide (LPS) alone or either with kinos from WA (**A**) or PNG (**B**) for 24 h. Nitric oxide levels from media were quantitated with Griess assay. Data are presented as mean ± SEM, n = 3 individual experiments, each experiment done in triplicates on the day. Statistical analysis was measured using Two-way ANOVA where ***P< 0.001 compared to the positive control (LPS alone).

4.2.5. UV light absorption spectral scans of WA and PNG kinos and sub-fractions

The kinos from both locations (WA and PNG) were fractionated and subsequently scanned. Crude (TE), the kinos extract, subfraction 1, subfraction 2 and subfraction 3 were analysed for their UV spectrum. Appendices A2-A5 show individual scans for the total (crude) kinos extract (TE), fraction 1, fraction 2 and fraction 3. Three peaks were apparent in all the samples tested: peak 1 ~102-119 nm, peak 2 ~276-293 nm and peak 3 ~ 321-326. The peaks were quite broad and this accounted for the peak wavelength variations, as identified by the software. Peak 1 was due to phosphate buffered solution and peaks 2 and 3 were due to the natural chemical constituents in kinos. In addition, the absorbance levels cannot be compared as samples were diluted independently to enable the best wavelength scan. In all the samples, peaks 2 and 3 were detected but interestingly, their relative peak heights varied depending on the fraction.

4.2.6. IL-6 secretion by LPS-stimulated RAW264.7 cells after being exposed to WA and PNG kinos sub-fractions

The IL-6 secretion by RAW264.7 macrophages treated with the crude WA kinos (TE) was significantly (p<0.01) different to the LPS-alone treatment and the three fractions (Figure 4.5 A). The three fractions did not result in a change in IL-6 secretion. For the PNG kinos, all the treatments displayed a reduction in IL-6 secretion (Figure 4.5. B). However, only the basic fraction (F2) did not show a statistically significant reduction, while the crude (TE), acidic (F1) and DCM (F3) fractions resulted in statistically significant differences. For the PNG kinos, the acidic and DMC fractions appear to have retained activity to suppress IL-6 while none of the fractions for the WA kinos appear to have retained this effect.

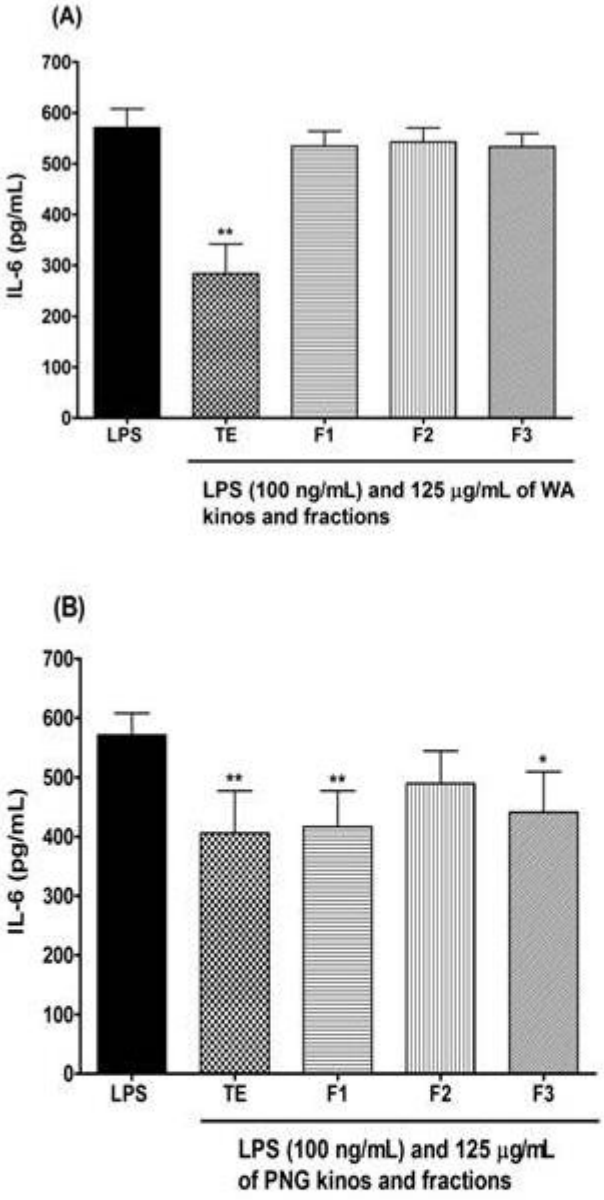


Figure 4.5. Effects of WA and PNG kinos and their fractions on secretion of IL-6 by LPS-stimulated RAW264.7 macrophage cells after 6 h of incubation.

RAW264.7 cells were incubated with 100 ng/mL lipopolysaccharide (**LPS**) alone or with WA (A) or PNG (B) kinos and their respective fractions. **TE**-total kinos extract (crude), **F1**-fraction 1 (acidic), **F2**-fraction 2 (basic) and **F3**-fraction 3 (DCM). IL-6 levels from media were quantitated with IL-6 ELISA. Data are presented as means ± SEM, n = 3 individual experiments. Statistical analysis was measured using One-way ANOVA where **P<0.01 and * P<0.05 compared to the positive control (LPS alone).

4.2.7. Determination of major classes of chemical compounds from WA and PNG kinos

Flavonoids in the WA and PNG kinos were detected on the TLC analysis (kinos spotted on thin layer chromatography plate and sprayed with anisaldehyde-sulphuric acid reagent) and qualitative (flavonoid) tests while triterpenoid saponins were detected by the qualitative assay (Table 4.1). Phenolics and tannins were detected in kinos when assessed using the quantitative assays. The PNG kinos has 5% and 10% total phenolics and tannins, respectively, more than the WA kinos (Table 4.2). However, no alkaloids were detected in the extracts although they were tested.

Table 4.1. Main groups of chemical compounds from the phytochemical screening of WA and PNG kinos.

Name of chemical constituent	WA kinos	PNG kinos
Alkaloids	n.d	n.d
Flavonoids	detected	detected
Saponins	detected	detected
Triterpenoids	detected	detected

nd: not detected

Table 4.2. Mean total phenolics and total tannins in 100 mg of kinos.

Kinos sample	Total phenolics		Total tannins of the total phenolics	
	(mg/100 mg)	(%)	(mg)	(%)
WA	51.95	51.95	33.93	65.3
PNG	56.69	56.69	42.61	75.2

The data presented is from triplicate weight of kinos. Each value is a mean of two separate assessments. The total tannins are given in total mg amount in the 100 mg of kinos and also as a percentage of the total phenolics in kinos.

4.3. Discussion

Inflammation is a pathophysiological process mediated by cytokines, which controls the cellular conditions of any injured tissues. Tumour necrosis factor-alpha, IL-6 and NO are major pro-inflammatory molecules that regulate inflammation. However, an overproduction of the pro-inflammatory molecules can be detrimental to the tissues (Lee *et al*., 2012). Hence, a physiological balance occurs in the tissues by the secretion of anti-inflammatory cytokines such as IL-1 receptor antagonist, IL-4, IL-6, IL10, IL-11 and IL-13 by the respective immune cells (Opal & DePalo, 2000). However, the synergistic effect of the pro-inflammatory cytokines overrides the immunoregulatory function of the anti-inflammatory cytokines. Kinos have been used for centuries by first Australian communities for the relief of inflammatory conditions. Therefore, this study was aimed at investigating the effects of kinos on TNF-α, IL-6, IL-10 and NO secretion in LPS-stimulated RAW264.7 murine macrophages.

The data clearly shows that kinos from both sources affect the levels of secretions of TNF-α, IL-6, IL-10 and NO in LPS-stimulated RAW264.7 macrophages. There was a significant reduction in the pro-inflammatory molecules (IL-6 and NO) secretion and the secretion of the anti-inflammatory cytokine, IL-10, was significantly enhanced by the WA kinos. Conversely, PNG kinos treatment resulted in the significant reduction of IL-10. Both WA and PNG kinos-treated RAW264.7 macrophage cells showed enhanced TNF-α production. This is in contrast to other studies demonstrating a reduction in TNF-α production by phytochemical-exposed, LPS-stimulated macrophages: as a consequence of de-activation of the transcriptional process which down regulated mRNA (Liu *et al.*, 2012; Surh *et al.*, 2001). Similarly, TNF-α production was also significantly reduced by mycelial extract from *Lentinus polychrous* (an edible mushroom) both i*n vitro* and *in vivo* (Fangkrathok *et al.*, 2013). Other studies also demonstrated anti-inflammatory effects from various natural products (Liu & Lin, 2013; Manjula *et al*., 2006). Manjula *et al* (2006) showed that bioassay-guided crude ethyl acetate extract from *Commiphora molmol aka mukul* gum inhibited phosphorylation of all the mitogen-activated protein kinases (MAPK) including extracellular signal-regulated kinase *(ERK),* JUN N-terminal kinase (JNK) and p38 MAPK. Consequently, TNF-α protein synthesis was suppressed in a concentration-dependent manner.

Nonetheless, the data from this study is in agreement with others who have shown an increase in TNF-α production by LPS-stimulated macrophages after exposure to kinos. Yang *et al* (2009) also demonstrated an increase in TNF-α secretion in peritoneal macrophages from mice treated with the aqueous extracts of *Mori fructus*. Nitric oxide levels were also increased in these cells. Yang and co-workers further identified TLR4 (Toll-like receptor 4) expression and NF-κβ activation to be the mechanism for the increase in TNF-α and NO: high TLR4s on the macrophages enhanced helper-T cell type1 (Th1) immunity. Similarly, the kinos treated cells secreted high levels of TNF-α in the current study, which strongly suggests an induction of the MAPKs and NF-κβ activation through expression of inhibitory kinase, since these two cell signalling pathways are the major factors in TNF-α and NO production stimulated by LPS (Oh *et al.*, 2014; Yu *et al.*, 2011). With NO, the kinos blocked the two cell signalling pathways which resulted in NO reduction. It is possible that the increased TNF-α seen in response to kinos-treated LPS-stimulated macrophages in this study was also driven by NF-κβ activation. Production of TNF-α was increased by WA and PNG kinos-treated macrophages in a dose-dependent manner from 62.5, 125 and 250 µg/mL concentrations but reduced at 500 µg/mL concentration. Insufficient numbers of viable cells at the 500 µg/mL WA and PNG kinos levels (see Chapter 3, Figures 3.2 and 3.3) may account for the reduced TNF-α production.

Interleukin-6 is also a pivotal pro-inflammatory cytokine and is regarded as an endogenous mediator of LPS-induced fever and rheumatoid arthritis (Molloy *et al*., 1993; Nilsberth *et al*., 2009). It is released by macrophages in response to TNF-α, IL-1β and LPS at the site of infection (Bae *et al*., 2012). Interleukin-6 can function as a transitional cytokine that is involved in changes from acute to chronic inflammation. It induces acute phase proteins in acute inflammation and exerts stimulatory effects by producing monocyte chemoattractant protein 1 (MCP1) in chronic inflammation at the site of infection (Gabay, 2006). In contrast to the increased levels of TNF-α by the kinos, IL-6 secretion levels in LPS-stimulated macrophages were reduced in a concentration and time-dependent manner. The lower levels of IL-6 at 12 compared to 6 h, in the studies herein, may have been due to either disintegration of IL-6 or overproduction of IL-6 at 6 h, acting in an autocrine manner (Gabay, 2006). The most likely mechanism contributing to the high levels of IL-6 within the first 6 h followed by the lower levels between 7-12 h of incubation is the activation of IL-6 (classic signalling) and the soluble IL-6 receptor (IL-6R) complex known as IL-6-trans-signalling (Hedrich & Bream, 2010; Rose-John, 2012).

It is possible that IL-6-trans-signal mediated glycoprotein (gp) 30 may have acted in an autocrine manner that reduced IL-6 secretion in the kinos treated macrophages between the 7-12 h incubation period. In contrast, the IL-6-trans-signal activated the gp 30 to act in a paracrine manner within the first 6 h of incubation, resulting in high levels of IL-6 secretion in the cells. Studies have demonstrated that macrophages are some of the few immune cells that express IL-R6 on the cell surface to bind to the gp30, thus activating both autocrine and paracrine cell signalling pathways, resulting in different levels of IL-6 secretions at different times (Rose-John, 2012; Scheller *et al*, 2011).

The other cell signalling pathway that may have contributed to IL-6 reduction in this study is down-regulation of the IL-6 /signal transducer and activator of transcription 3 (IL-6/STAT3) by the kinos. Previous studies also observed differences in IL-6 production due to incubation time (Ai *et al.*, 2013), the type of immune cells used, whether the study was *in vitro* or *in vivo* (Ley *et al.*, 2014) and concentration of LPS (Ai *et al.*, 2013; Thorley *et al.*, 2007). Ai and co-workers (2013) demonstrated that different cytokines either increased or decreased with increasing incubation time but were increased with increasing concentrations of LPS. The study by Fitting and co-workers (2004) showed variations in the levels of TNF-α secretion among different cells when tested in an *ex vivo* study model. The LPS-stimulated bronchoalveolar cells secreted increased levels of TNF-α compared to the splenocytes, peritoneal and bone marrow cells in the three different incubation times. Thorley *et al* (2007) demonstrated increases in secretions of TNF-α, IL-1β and IL-6 with increasing concentrations of LPS-stimulated primary human alveolar type II cells and macrophages.

The mechanisms that may have caused IL-6 to be reduced by exposure to kinos in the experiments herein are described above but, this does not necessarily account for the lower levels of IL-6 before and after 6 h of incubation. It is possible that the IL-6 may have been disintegrated and phagocytized by the macrophages. Blander (2007) reported in a review article, that the TLR4 of the dendritic cells recognized the infected apoptotic cells and internalized them, resulting in low IL-6, which could also be the case in this study. However, if IL-6 was still being produced at the initial rate by the cells, this would not fully explain the reduction. The LPS may have also been internalized at 12 h in this study and was therefore not at a substantial quantity to stimulate the cells to secrete the same level of IL-6 as at the 6 h incubation. A previous study by Dobrovolskaia & Vogel (2002) reported LPS being internalized

when the TLRs intensely triggered the macrophages and therefore provides evidence to support this proposed model.

Several studies have shown reduced TNF-α, IL-6 and NO production induced by LPS-stimulated macrophages when they were exposed to plant extracts (Bae *et al.*, 2012; Del *et al.*, 1993; Scheller *et al.*, 2011). Like the present study, Bae *et al* (2012) showed a significant reduction in NO by dehydrogeijerin isolated from *Heracleum moellendorffii*, using high performance chromatography. Two recent studies (Huang *et al.*, 2014; Jeong *et al.*, 2014) investigated chemical constituents from lichen and a fungus using two different LPS concentrations to stimulate RAW264.7 cells and showed anti-inflammatory effects. Jeong *et al* (2014) demonstrated reduction of proinflammatory cytokines by LPS-stimulated (500 ng/ mL) macrophages after being treated for 24 h with an ethanol extract of fungus. Huang *et al* (2014) investigated usnic acid purified from a lichen, and used an ethanol extract to treat 10 ng/ mL LPS-stimulated macrophages, also for 24 h. Nonetheless, the anti-inflammatory effects were not as robust as those reported by Jeong *et al* (2014). Differences between the results of these two studies as well as the one described herein may be accounted for by use of different concentrations of LPS as well as by the use of different extraction methods.

Nitric oxide is a mediator of inflammation and is produced by inducible nitric oxide synthase (iNOS) in LPS-stimulated macrophages. Nitric oxide contributes to host defence against viruses and bacteria. Excessive production of NO by macrophages can lead to adverse conditions such as chronic inflammatory diseases therefore, and its production has to be adequately regulated (Clancy *et al*., 1998; Martin *et al*., 1994; Rimbach *et al.*, 2000). LPS-stimulated macrophages activate iNOS to produce oxygen (O_2), which in turn reacts with NO, generating peroxynitrite ($ONOO^-$). However, overproduction of $ONOO^-$ leads to cytotoxicity which causes injury to the host. In the process, nitrite is produced in substantial quantities. Many *in vitro* and *in vivo* studies have looked for potential pharmacological agents to reduce NO. A study by Rimbach *et al* (2000) demonstrated that fermented papaya preparations reduced NO in LPS-stimulated RAW264.7 macrophages. Similarly, n-hexane and the dichloromethane fractions of *Sargassum micracanthum* (brown algae) suppressed iNOS in a dose-dependent manner which resulted in the reduction of NO (Yoon *et al.*, 2009). An ethanol extract of *Poria cocos*, a fungus, reduced NO, IL-1β, TNF-α and prostaglandin E2 (PGE2) via suppression of NF-κB in LPS-stimulated RAW264.7 macrophages (Jeong *et al*., 2014). Similar results were demonstrated in

this study; NO production was significantly reduced by LPS-stimulated RAW264.7 macrophages in the presence of the WA and PNG kinos. There could have been inhibition of gene expression of inducible NO synthase (iNOS) and cyclooxygenase-2 (COX-2) by the kinos, resulting in the reduction of NO production. A previous study demonstrated the inhibition of expression of these two genes to reduce NO production (Huang *et al*., 2014). The kinos may contain chemical constituents that are similar to the NO-reducing agents in the other studies; however, further studies are required to determine the mechanisms by which NO was reduced by the kinos.

Nitric oxide reduction by phytochemicals has also been demonstrated in *in vivo* studies. A study by Pinheiro *et al* (2013) established significant NO reduction by ethanol, hexane and ethyl acetate (EtoAc) fractions from *Couroupita guianensis* leaves in a dose-dependent manner, shown by the licking response in Swiss mice. Fangkrathok *et al* (2013) reported mycelial extract from edible mushroom to reduce NO, intracellular O_2^- and TNF-α production by LPS-stimulated RAW264.7 macrophages. The mycelial extracts also reduced paw swelling in male Sprauge-Dawley rats after carrageenan injections in a dose-dependent manner: neutrophil accumulation at the site of the swelling was prevented. Another study found that *Pluchea indica* ethanol leaf extracts exhibited a potent effect against NO production and PGE2 release by LPS-activated RAW264.7 macrophages (Buapool *et al.*, 2013). These leaf extracts were also effective against the same pro-inflammatory mediators in an animal model. They further demonstrated the ethanol *P*. *indica* leaf extract suppressed nuclear translocation of the NF-kB p65 subunit and iNOS promoter activity. NF-kB was investigated because it is a ubiquitous transcriptional factor that regulates expression of genes involved in inflammatory responses such as iNOS and COX-2 (Cheon *et al.*, 2009; Hseu *et al.*, 2005; Israf *et al.*, 2007).

Two distinct patterns in the secretion of IL-10, an anti-inflammatory cytokine, were observed in this study. The IL-10 levels were secreted in large quantities by the WA kinos-treated LPS-stimulated macrophages but were significantly reduced by the PNG kinos. It is acknowledged that low macrophage viability at the 500 µg/ mL concentration would account for the low IL-10 secretion seen at this concentration. The IL-10 secretions increase with incubation time in this study, which is consistent with previous studies (Mehrota *et al*., 1998; Schildberger *et al*., 2013). Schildberger and co-workers (2013) demonstrated high levels of IL-10 induction in monocytes stimulated with LPS and respiratory syndrome virus (Hou *et al.*, 2012) with 8 to 24 h of incubation. The low levels of IL-10 secretion in the supernatants of macrophages

treated with PNG kinos is consistent with a previous study that demonstrated the significant reduction of IL-10 secretions in J774A.1 macrophage cells by the leaf extracts of *Campomanesia velutina* (Michel *et al.*, 2013). The RAW264.7 macrophages, in the absence of LPS, secreted basal levels of IL-10, whilst LPS stimulation resulted in an appreciable increase of IL-10 when treated with WA kinos but not with PNG kinos. Several factors could have contributed to the differences in the IL-10 secretion: a) the differences in the chemical composition of WA and PNG kinos and b) the high levels of TNF-α secretion by the PNG kinos dampened the IL-10 secretion in cells, thus, very low levels were detected. Another factor suggested by Salez and co-workers (2000) could be due to microenvironmental factors, such as the LPS (stimulus) being broken down fast by the PNG kinos which might have suppressed the IL-10 protein expression in the RAW264.7 cells. In addition, the p38 MAPK pathway of the LPS stimulated macrophages could have been blocked by the PNG kinos and therefore, reduced levels of IL-10 being secreted.

Screening of plant extracts for antimicrobial activities involve phytochemical analyses and testing them for antibacterial, anti-inflammatory, anti-fungal, anti-oxidant and anti-viral activities. The phytochemical and antimicrobial activity tests encompass simple to complicated assays when investigating natural products (Butler, 2004). All anti-inflammatory tests were undertaken *in vitro* in this study to investigate for possible chemical constituents in kinos for the alleviation of inflammatory conditions. These chemical constituents could potentially replace those synthesized anti-inflammatory drugs that are no longer effective against these conditions and those that have side effects. Analyses of UV light spectra, anti-inflammatory effects and phytochemical screenings are discussed below.

UV light spectra of the crude kinos extracts and their respective fractions (Appendices 2-5) showed peaks that were around 278 nm and 326 nm. The range of wavelengths of the kinos fractions were consistent with those of tannins detected in the kinos of several *Eucalyptus* species, being plants from the same genus as the samples in this study. Furthermore, both the tannic acids (ellagic acid) and flavonoids (aromadendrin and kaempferol) that were isolated from the kinos of *Eucalyptus hemiphloia* were within the wavelength UV light range of 226 nm and 380 nm (Hillis & Carle, 1963). The wavelength range of 278 and 326 nm for the kinos sub-fractions in this study and that of other few studies (Hillis & Carle, 1963; Maiden, 1891;1892; von Martius *et al*., 2012) shows that no compounds with the UV-visible light range of ≥ 400 and ≤ 800 nm were detected in these different

studies, This is further supported by the phytochemical screening of the WA and PNG kinos using a modified method of von Martius *et al* (2012) that detected the total phenolics and total tannins that are not within the visible light wavelength range in this study. The detection of these chemical compounds is consistent with previous studies (Maiden, 1890; 1892; von Martius *et al*., 2012). The wavelength range between 200-600 nm was also evident for the flavonoids in other studies that investigated these chemical compounds (Pan *et al*., 2013; Wang & Baldwin, 1998). Further preliminary chemical analysis of the WA and PNG kinos suggests the presence of flavonoids and triterpenoid saponins. However, alkaloids were not detected in any kinos extracts. The saponins are suggested to be present in the kinos within the wavelength range of 278 and 324 nm is consistent with the saponins with a wavelength of 280 nm that was measured in a previous study (Zhang *et al.*, 2014). The total phenolics and tannins are more abundant in PNG kinos than the WA kinos. This suggests that the species of *Eucalyptus* and their geography can contribute to differences in the levels of chemical constituents in kinos.

Kinos, like other plant products, have been reported to have a range of phenolic compounds. Several previous studies (Alves *et al.*, 2013; Koolen *et al.*, 2013; Sreelatha *et al.*, 2011) have demonstrated that some, but not all phenolic compounds possess anti-oxidant, anti-inflammatory, and antibacterial effects. It appears that the position of the main functional groups on the structure of the chemical compound influences these activities (Alves *et al*, 2013). Pereira and co-workers (2007) also demonstrated isolated phenolic compounds to be synergistically effective against several bacterial species and two fungal species. This strongly suggests that the phenolics (example, flavonoids), tannins and triterpenoid saponins present in the kinos extracts in this study may have contributed to the reduction of IL-6 levels in the LPS-stimulated macrophages. This is consistent with the previous studies that demonstrated antimicrobial activities of these chemical compounds in *Eucalyptus* (Akin *et al.*, 2012; Rauha *et al.*, 2000). Flavonoids, not only from *Eucalyptus* but from other plant species also, have been demonstrated to possess antimicrobial activities. They exert antibacterial, antiviral, anti-inflammatory and antifungal effects when isolated and tested either *in vitro* or *in vivo* (Lin *et al*., 2014; Parolia *et al*., 2014; Pomari *et al*., 2014; Silva *et al*., 2003). Interleukin-6 was significantly reduced by both the WA and PNG crude kinos extracts, as reported earlier in this chapter, and it was also reduced when tested in parallel with the PNG fractions but not WA fractions. The IL-6 data suggests that the presence of the

phenolics (example, flavonoids), tannins, and triterpenoid saponins in the crude kinos extracts could have acted in an additive or synergistic manner. The fact that the WA kinos fractions did not result in IL-6 reduction suggests that there are differences between the PNG and WA kinos, and these most probably depend on a number and quantity of different chemical constituents.

Furthermore, it is possible that, kinos extract was protonated by the HCl which may have extracted the macromolecules including carbohydrates, lipids and some carboxylic acids like tannic acids in the first kinos aqueous layer (Fraction 1) during this chemical compound extraction process. In addition, the DCM fraction (fraction 3) could have precipitated small peptides which could have also been present in this fraction. The suggestion of the detection of these compounds is consistent with the detection of carboxylic acids and phenols with the strong and weak acids, respectively in the study that investigated the antioxidant activity of compounds isolated from pyroligneuos acid from *Rhizophora apiculata* (Loo *et al.*, 2008). In contrast, the kinos extract was deprotonated with the NaOH (a base) when it was added to the DCM layer (fraction 3) with the kinos. Polar compounds like the flavonoids and the triterpenoid saponins may have been extracted in the DCM layer (Fraction 3). The polarity of solvents used in the extraction of compounds from natural sources determines the type of compounds isolated based on their polarity (Ghasemzadeh *et al.*, 2011). This observation was also apparent in another previous study that detected saponins in different methanol (polar solvent) African plant extracts (Ncube *et al*., 2012). Similarly, flavonoids were extracted from the methanol extracts of two varieties of *Zingiber officinale* and more potent than the extracts that were extracted with less polar solvents (Ghasemzadeh *et al.*, 2011). In the acid-base extraction method that was employed in the fractionation of kinos extracts; suggesting the DCM, the fraction 3 extracting some polar compounds since it was more polar than the two aqueous kinos fractions. The suggestions of the different groups of compounds in the kinos fractions are consistent with the detection of phenols (example, flavonoids), tannins and triterpenoid saponins in the crude kinos using TLC, assays and the qualitative methods. The UV light scanning profiles also were within the wavelengths of the tannins and the flavonoids. Hence, it can be suggested that the carboxylic acids were detected in the kinos aqueous layer (Fraction 2) and the flavonoids and the triterpenoid saponins in the DCM layer (fraction 3) were presumably the compounds that reduced the secretion of IL-6.

It is possible that reduction of IL-6 by the kinos fractions was due to inactivation of the MAP kinase and NF-κB signalling pathways thus, reducing IL-6 protein and gene expression. Previous studies (Lee *et al.*, 2012; Zamora-Atenza *et al.*, 2014) reported crude plant extracts and their fractions deactivating the inflammatory signalling pathways, the NF-κB or the MAPK, which resulted in the reduction of IL-6 and other pro-inflammatory cytokines.

Antioxidants in plant extracts contributing to antimicrobial effects was also demonstrated in a study by Diaz *et al* (2012). Diaz and co-workers (2012) showed that plant extracts with high phenolic and flavonoid contents showed increasing levels of antioxidants and anti-inflammatory effects in LPS-and IFN-γ-activated J7774A.1 macrophages (Diaz, 2012). The presence of phenolics and tannins in the WA and PNG kinos is also consistent with the study by von Martius *et al* (2012) that also detected these two classes of chemical compounds in the kinos of several *Eucalyptus* species. This study and that of von Martius *et al* (2012) provide substantial evidence of the presence of phenolics and tannins in the kinos of the *Eucalypts* and also confirm them to possess antimicrobial properties. Previous studies (Amakura *et al*., 2002; Ferreira, 2007; Silva *et al*., 2003) also demonstrated antimicrobial activities of the phenolics, tannins and flavonoids which provide support for this study that these chemical compounds synergistically were responsible for the reduction of the IL-6 and NO levels in the LPS-stimulated macrophages and the inhibition of the two reference strains of *S*. *aureus*. In addition, saponins have also been demonstrated by previous studies to possess antimicrobial properties. A study by Karimi *et al (*2011) demonstrated saponin-rich fractions of leaf extracts of *Labisa pumila* Benth to inhibit Gram positive bacteria. In another study, the saponins were found to be effective against Gram negative bacteria (Khanna & Kannabiran, 2008). This suggests that the triterpenoid saponins in the kinos may also have contributed to the anti-inflammatory and antibacterial effect of the kinos extracts.

4.4. Conclusion

Production of TNF-α by LPS-stimulated RAW264.7 macrophages increased after exposure to the WA and PNG kinos in this study whilst IL-6 and NO production were reduced in a concentration-dependent manner. Whilst the mechanisms mediating this response are unknown and it is likely that the mechanisms triggering increased TNF-α production are different to those responsible for the reduction of IL-6 and NO.

These data suggest that LPS-induced macrophages treated with kinos create a microenvironment that enables them to function in an autocrine manner. Increased TNF-α would likely preserve the macrophages ability to respond to pathogens, an effect that may be enhanced by the synthesis of other cytokines. In contrast, decreased IL-6 and NO, as well as increased IL-10 may function to dampen overt inflammatory responses and skew T cell responses from T_H1 cells towards T_H2 cells. These data show that WA and PNG kinos can modulate the production of cytokines by LPS-stimulated macrophages *in vitro* and that they should be further investigated for their effects on inflammatory signalling pathways and their effects on other cytokines in *in vitro*, *ex vivo* and *in vivo* studies prior to development of anti-inflammatory therapeutic agents. The data support the claim for the medicinal use of kinos from *Eucalyptus* species by first Australians. The promising anti-inflammatory results from this *in vitro* study contribute to the list of potential anti-inflammatory extracts for development of drugs to alleviate inflammation.

The simple phytochemical tests and assays used herein suggest phenolics (example, flavonoids), tannins and triterpenoid saponins as the major phytochemical constituents in kinos. The overall effect of these compounds contributes to the antimicrobial and anti-inflammatory effects detected in kinos. The chemical constituents of PNG kinos could be separated and still exert biological effect but this was not seen from WA kinos, suggesting that PNG and WA kinos from these *Eucalyptus* species are not identical, and that the WA kinos bioactivity may rely on several constituents that were separated by the acid/base method and detected by the phytochemical qualitative and quantitative tests. Nonetheless, further more extensive analyses are needed to confirm which constituents are contributing to the observed anti-inflammatory effects.

CHAPTER 5

EFFECTS OF KINOS ON PHAGOCYTOSIS OF BACTERIA BY RAW264.7 MACROPHAGE CELLS

5.1. Introduction

The treatment of infections and inflammatory conditions by agents from plants continues to play an essential role in healthcare, and plant remedies are used by different cultures in many countries (Butler, 2004), though it is only in the 21st century that this has been extensively documented in Australia (Williams, 2010). For example, decoctions, infusions, tinctures and essential oils from the leaves, bark and fruit of *Eucalyptus* plants are used in the first Australian communities (Williams, 2010; 2011; Lassak & McCarthy, 2001) and other communities around the world (Musyimi & Ogur, 2008; Sartorelli *et al*., 2007; Silva *et al*., 2003; Takahashi *et al*., 2004) for treating various bacterial, fungal and inflammatory conditions. Leaves, bark and fruit are not the only parts of the *Eucalyptus* species claimed to have medicinal effects by the first Australians, who also use kinos for relieving bacterial and inflammatory conditions (Maiden, 1892; Lassak & McCarthy, 2001). Plant remedies are the main targets for investigations into various antimicrobial activities in the hope of producing potential drugs to replace those that are no longer effective against infections. The crude extracts and essential oils from *Eucalyptus* plants have been tested widely for antibacterial and antifungal activities (Gilles *et al*., 2010; Musyimi & Ogur, 2008; Safaei-Ghomi, 2010), but there are limited studies on their anti-inflammatory effects (Serafino *et al*, 2008). Monoterpenes such as citrionellol, citronellal and 1, 8-cineole are the major group of compounds present in most *Eucalyptus* species (Gilles *et al* 2010; Musyimi & Ogur, 2008) and these compounds have been effective against pathogenic bacteria and fungal species both *in vitro* and *in vivo*.

The few anti-inflammatory studies on the essential oils of the *Eucalyptus* species so far have only focused on pro-inflammatory cytokines, with limited studies on phagocytosis. In addition, the first part of this study also investigated the effects of crude kinos on pro-inflammatory cytokines and molecules. It demonstrated reductions in IL-6 and NO (data presented in Chapter 4) in LPS-induced murine macrophages. Hence, this study aimed to investigate the phagocytic effect of kinos from *E. calophylla* and *E*. *confertiflora* in RAW264.7 murine macrophages *in vitro*.

5.2. Results

5.2.1. Kinos enhance phagocytosis of heat-inactivated *E. coli* and *S. aureus*

The effect of kinos on phagocytosis of Gram negative (*E*. *coli*) and Gram positive (*S*. *aureus*) bacteria was investigated by co-incubating these two heat-inactivated bacteria with RAW264.7 macrophage cells that had been exposed to kinos for 23 h. The FITC-labelled bacteria were co-incubated with the 23 h kinos treated cells for 1 and 4 h. The cells were stained with propidium iodide (PI) at the end of the incubation period. Details of the protocol are described in Section 2.8.3 of Chapter 2. The number of non-phagocytized and phagocytized cells was counted by their fluorescence through the flow cytometer. Figure 5.1 shows how the cell populations were measured through gating using the FlowJo analysis program version 10 (USA). The gating helped to identify and group the different macrophage and bacterial cell populations of the multi-complex flow cytometry data. As shown in Figure 5.1, the upper left quadrant is the PI stained cell population, while the bottom left quadrant is the unstained cell population. The upper right quadrant contains both PI (cell) and FITC (bacterial cell) populations while the right bottom quadrant has only the FITC (bacterial cell) population.

In order to determine the level of effect kinos treatments had on phagocytic activity of RAW264.7 murine macrophages, the percentage of the total, FITC fluorescence (FITC-labelled bacteria) and PI-stained macrophages were detected simultaneously. Figures 5.2 and 5.3 graphically show the effects of the pre-incubation of the macrophages with WA and PNG kinos on the phagocytosis of *E. coli* and *S. aureus*, respectively. The total fluorescent readings demonstrated there was a general trend of increased phagocytosis of bacteria due to the effect of kinos. However, no statistical significance was attained. Unexpectedly, WA kinos demonstrated higher phagocytic activity for *E. coli* (Figure 5.2 A) at 1 h.

Further analysis of data revealed results that were expected. When the data was analysed for the mean fluorescent intensity (MFI) per macrophage, predictable and definitive results emerged (Figures 5.4 and 5.5). The 4 h treatment for *E. coli* showed enhanced phagocytic activity of the macrophages by both WA and PNG kinos (Figure 5.4 A and B). The exception was the 1 h treatment with 250 µg/mL of WA kinos (Figure 5.4 A), which was also significant, and the 4 h treatment with 250 µg/mL for PNG, which did not display a change compared to control. The highest activity was from the 125 µg/mL for both WA and PNG kinos, reaching between 4-6-fold increases. Treatment of *S. aureus* by WA and PNG kinos (Figure 5.5)

demonstrated a similar pattern of macrophage activation; statistically significant increases in phagocytic activity were only seen for the 4 h treatments with the exception of the 1 h treatment for the WA kinos at 250 µg/mL. Interestingly, although small, there was a statistically significant reduction of phagocytic activity at 1 h for the 125 µg/mL kinos concentration, except for the WA kinos on *E. coli*.

In order to visualise the phagocytic activity, FITC-labelled bacteria were incubated with macrophages and treated with the 125 µg/mL WA and PNG kinos concentration (Figure 5.6). Bacteria at different stages of phagocytosis can be observed.

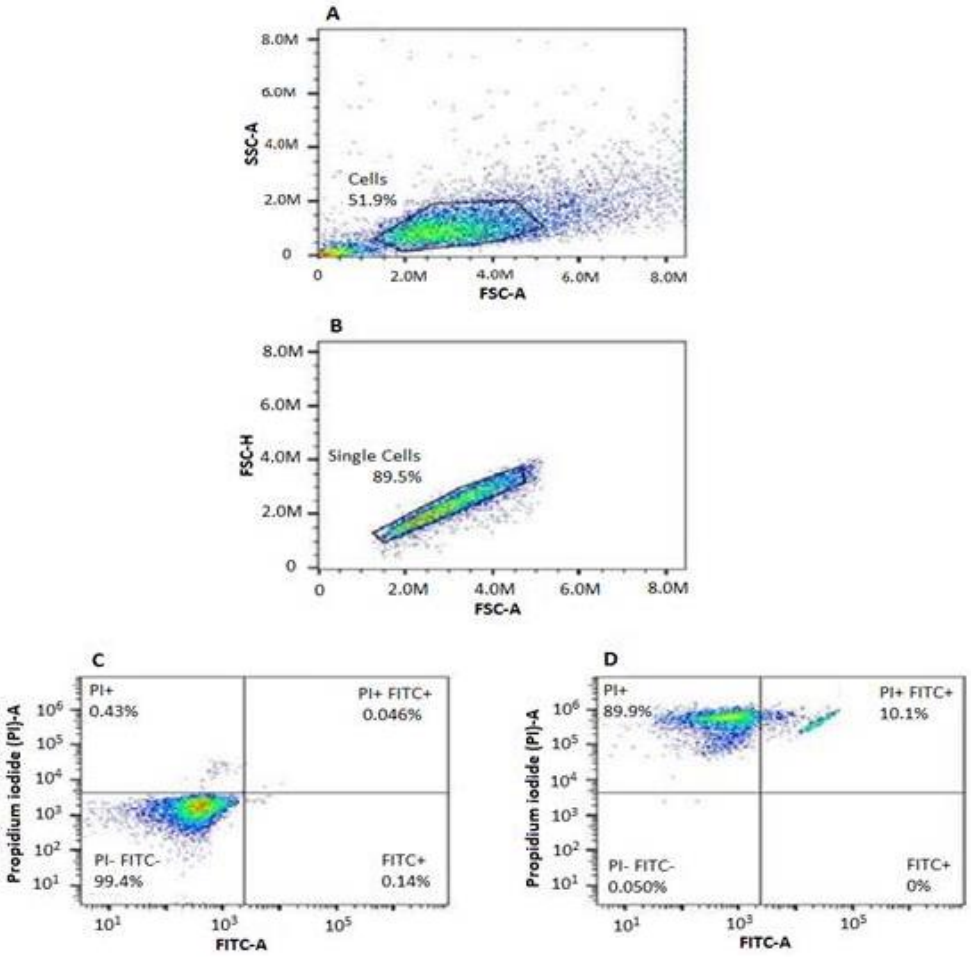


Figure 5.1. Analysis of flow cytometry data

Shown is the representative gating strategy. Macrophage cell population was identified on FSC and SSC (**A**), and sub-gated for doublet discrimination (**B**). Region for phagocytosis were set based on unstained sample (**C**) with low background for PI and FITC (PI- FITC-). Gate was applied to stained samples (**D**) showing macrophages alone (i.e. no phagocytosis), the PI+ region (upper left quadrant), and macrophages with bacteria (phagocytosis), the PI+ FITC+ region (upper right quadrant).

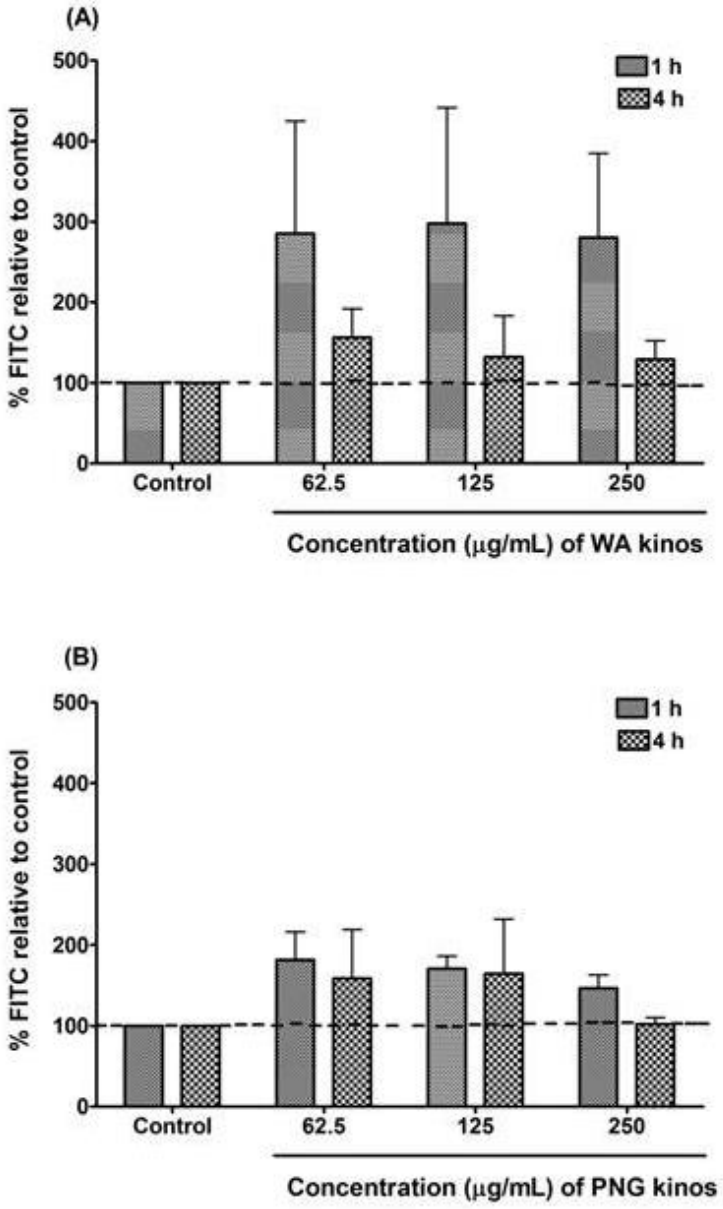


Figure 5.2. Effect of WA (A) and PNG (B) kinos on phagocytosis of heat-inactivated FITC-labelled *E. coli* by RAW267.4 cells.

Percentage change of FITC fluorescence (bacteria) bound to macrophages compared to control (PI stained cells and *E. coli* only) (i.e. upper right quadrant of cytometric analysis). *E. coli* were incubated for 1 or 4 h with macrophages that had been pre-treated with either WA (A) or PNG (B) kinos at different concentrations, as indicated. Data presented are the means of 3 independent experiments. Error bars are ±SEM.

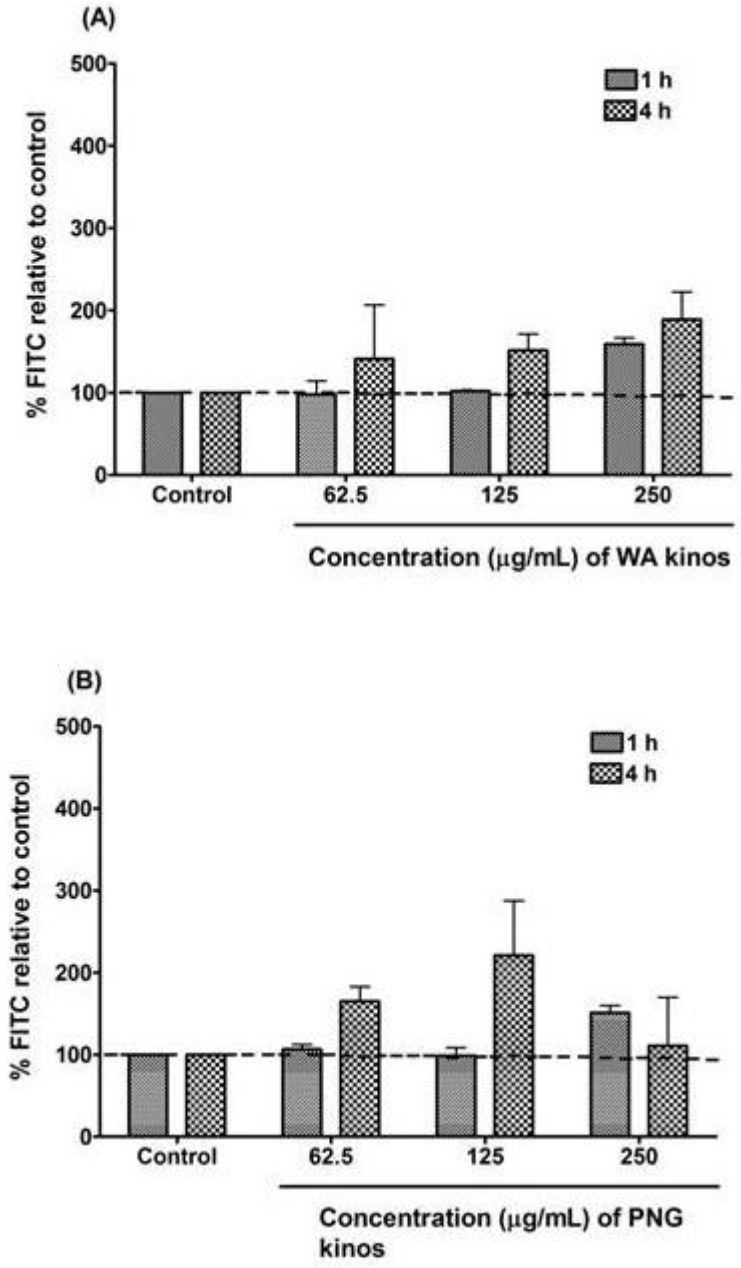


Figure 5.3. Effect of WA (A) and PNG (B) kinos on phagocytosis of heat-inactivated FITC-labelled *S. aureus* by RAW267.4 cells.

Percentage change of FITC fluorescence (bacteria) bound to macrophages compared to control (PI stained cells and *S. aureus* only) (i.e. upper right quadrant of cytometric analysis). *S. aureus* were incubated for 1 or 4 h with macrophages that had been pre-treated with either WA (A) or PNG (B) kinos at different concentrations, as indicated. Data presented are the means of 3 independent experiments. Error bars are ±SEM.

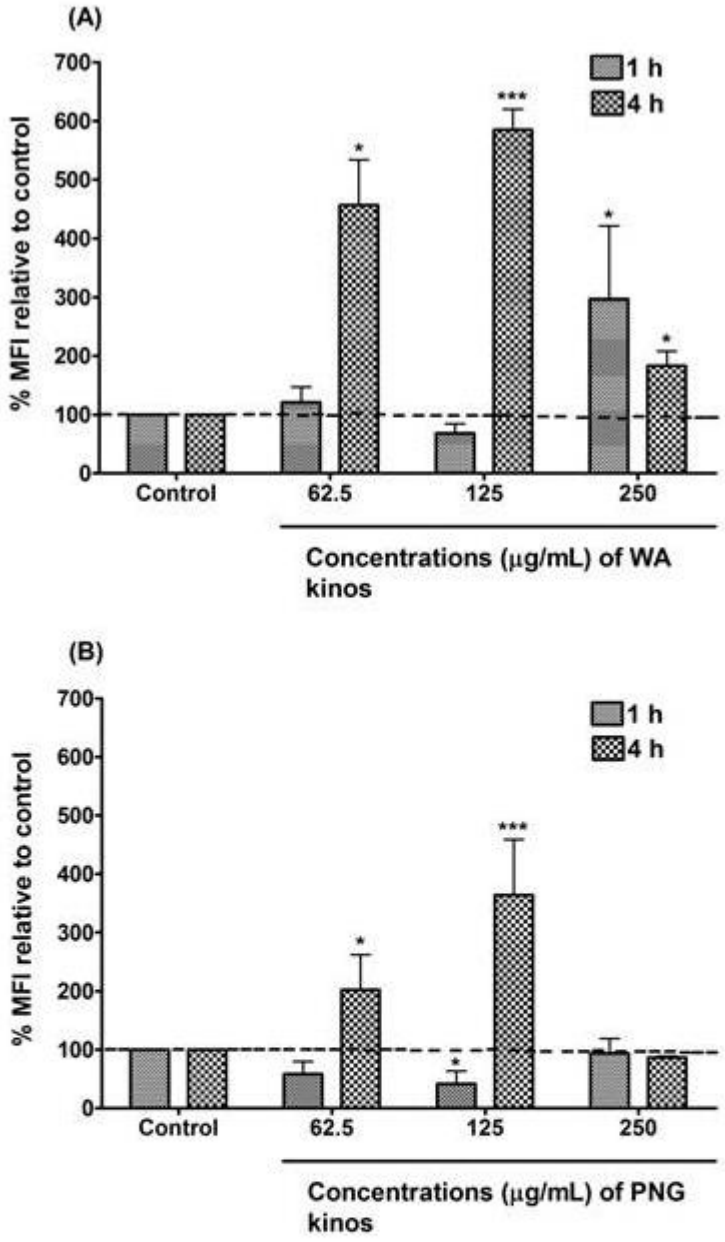


Figure 5.4. Effect of WA (A) and PNG (B) kinos on phagocytosis of heat-inactivated FITC-labelled *E. coli* by each RAW 264.7 cells.

Percentage change of mean fluorescence intensity (MFI; bacteria) per macrophage compared to control (PI stained cells and *E. coli* only) (i.e. upper right quadrant of cytometric analysis). *E. coli* were incubated for 1 or 4 h with macrophages that had been pre-treated with either WA (A) or PNG (B) kinos at different concentrations, as indicated. Data presented are the means of 3 independent experiments. Error bars are ±SEM, with some being too small to be visualized. Statistical significance was attained by treatments compared to respective control; *P<0.05 and ***P<0.001.

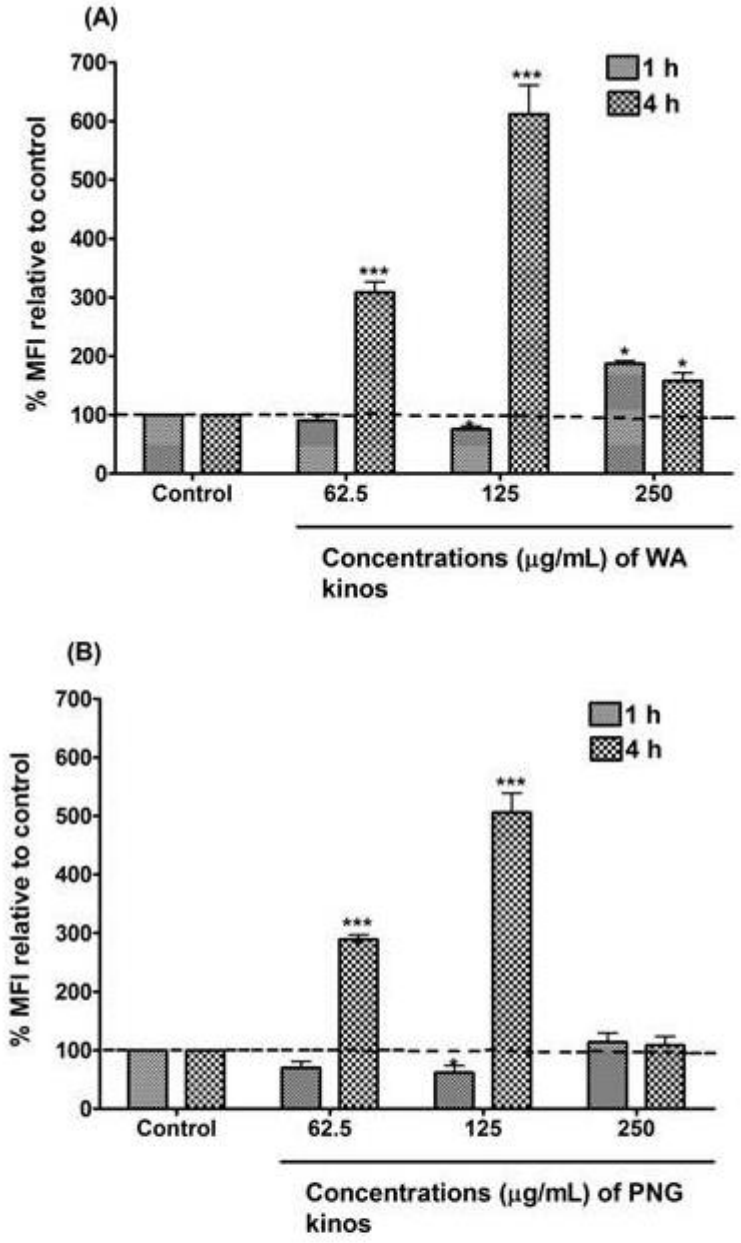


Figure 5.5. Effect of WA (A) and PNG (B) kinos on phagocytosis of heat-inactivated FITC-labelled *S. aureus* by each RAW 264.7 cells.

Percentage change of mean fluorescence intensity (MFI; bacteria) per macrophage compared to control (PI stained cells and *S. aureus* only) (i.e. upper right quadrant of cytometric analysis). *S. aureus* were incubated for 1 or 4 h with macrophages that had been pre-treated with either WA (A) or PNG (B) kinos at different concentrations, as indicated. Data presented are the means of 3 independent experiments. Error bars are ±SEM, with some being too small to be visualized. Statistical significance was attained by treatments compared to respective control; **P*<0.05 and ****P*<0.001.

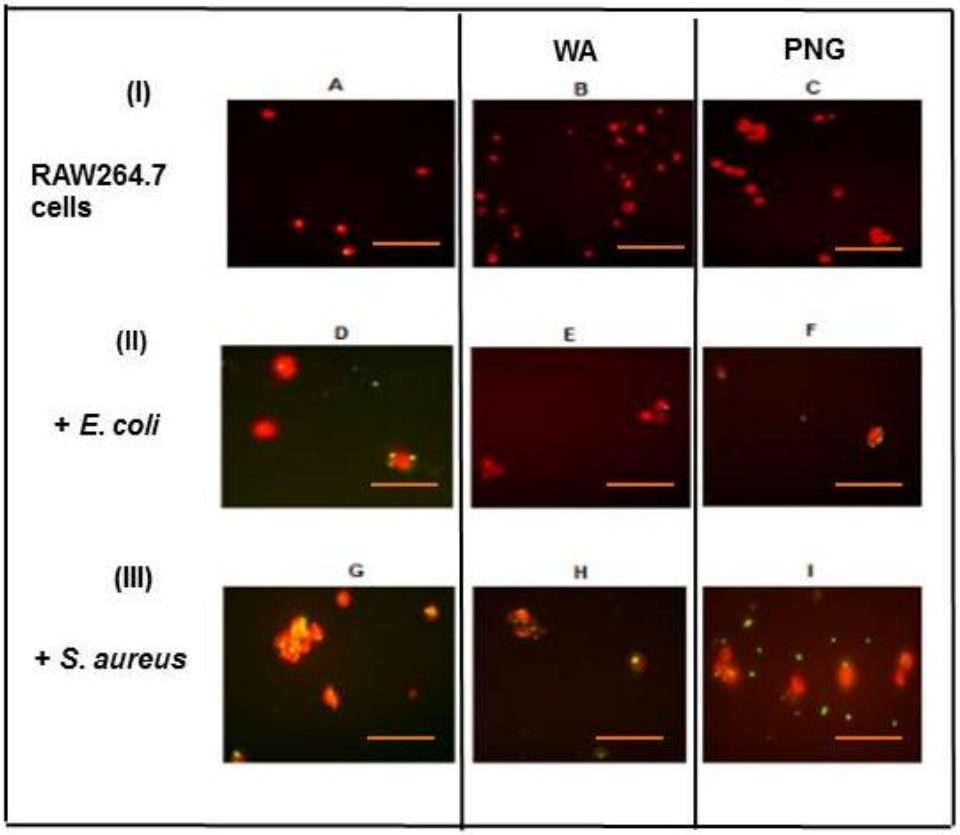


Figure 5.6. Effect of kinos on phagocytosis of heat-inactivated FITC-labelled *E. coli* and *S. aureus* by RAW 264.7 cells after being exposed to 125 µg/mL WA and PNG kinos for 4 h. The bar size for each of the images for the phagocytosis of the bacteria by the cells is 20 µm.

Images were taken with fluorescent microscope with Perkin Elmer Nuance multi-spectrometric imaging system camera. The cells are red in colour while the bacteria are green in colour. **Panel (I)-**cells + WA and PNG kinos only, **Panel (II)-**cells +WA and PNG kinos + *E. coli* and **Panel (III)-**cells + WA and PNG kinos + *S. aureus*. **A**-untreated cells, **B**- cells +WA kinos, **C**- cells + PNG kinos, **D**-Untreated cells + *E. coli*, **E**- cells + WA kinos +*E. coli*, **F**- cells + PNG kinos +*E. coli*. **G**- Untreated cells and *S. aureus*, **H**- cells + WA kinos + *S. aureus*, **I**- cells + PNG kinos +*S. aureus*.

5.3. Discussion

Phagocytosis is part of the second line of defence for the innate immune system against invading microorganisms during the early stages of infection (Akira, 2011; Greub, 2013). Pathogen recognition receptors (PRRs) recognise and stimulate phagocytosis of microbes (Bonilla *et al*, 2013) and also initiate an acute inflammatory response in tissue through the release of cytokines (Akira *et al*., 2006). In Chapter 4, kinos treatment of mouse macrophages demonstrated a reduction in the production of inflammatory molecules IL-6 and NO, and WA kinos induced IL-10, an anti-inflammatory cytokine. In this Chapter, kinos are investigated quantitatively and qualitatively for effects on the phagocytic activity of mouse macrophages in response to two bacterial types: *E. coli*, Gram negative, and *S. aureus*, Gram positive.

Flow cytometry data was presented in two formats, total fluorescence of sample and MFI (mean fluorescence intensity) of each count i.e. each macrophage. The data expressed as total fluorescence gave unexpected results particularly for WA kino with *E. coli* with the results being highly variable without statistical significance. However, when the MFI was plotted, predicted results were seen: kinos treatment increased phagocytosis of both bacterial types and the effect was dose and time dependent. In addition, the data was statistically significant. It is postulated that the discrepancies between these analyses, of the same data, are due to non-specific binding of bacteria to macrophages, particularly at the early time point (see Figure 5.7). The total fluorescence value consists of non-specific signal (background noise) and specific signal (activated macrophages which phagocytized bacteria). This “background noise” was greater for the WA kinos and at the early time point. The MFI data suggests that there was a population of macrophages that were actively phagocytizing bacteria and their activity was increased by pre-treatment with kinos i.e. the mean fluorescent value of individual macrophages increased because each macrophage was phagocytizing more bacteria. This activation mechanism was masked by the high non-specific signal. The possible reasons for non-specific binding of bacteria to macrophages could have been from components in the kinos itself or components in the serum.

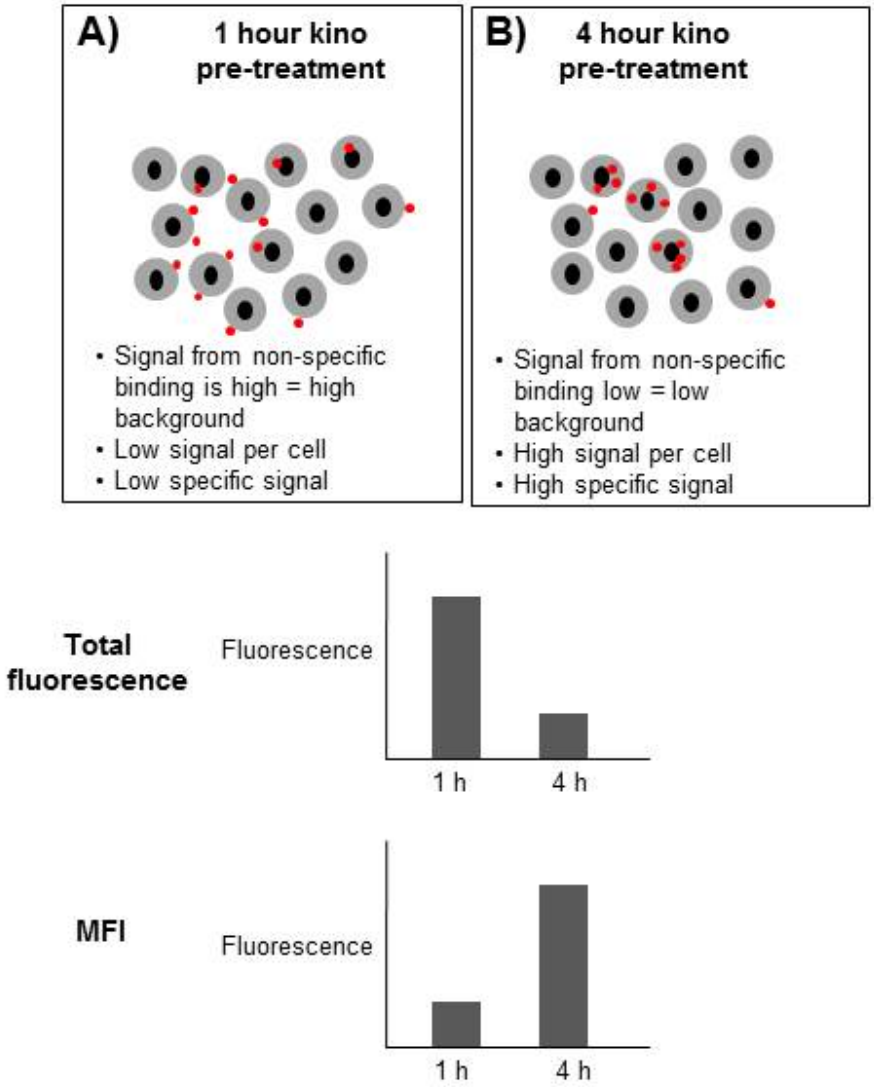


Diagram 5.7. Proposed model for discrepancies between 'total fluorescence' and 'MFI'. A) Fluorescence at 1 hour, primarily due to non-specific binding of bacteria to macrophages and low level of specific signal due to phagocytosis: the mean signal per macrophage is low. B) High specific signal due to phagocytosis activity with low non-specific signal: the mean signal per macrophage is high. Note – non-specific binding represented by bacteria (red) in cell (grey-black) surface and phagocytosis represented by bacteria inside cell. C) Hypothetical representation of data plotted by 'total fluorescence and MFI (mean fluorescence intensity).

The data therefore suggests that macrophage receptors that recognise antigens were activated by kinos, and the effect was dose-dependent: at least it was up to 125 µg/mL. This is supported by a previous study which demonstrated the expression of surface toll-like receptors (TLRs) such as TLR4/MD2, TLR1/TLR2, and TRL6/TRL2 on plasma membranes of macrophages enabled the detection and recognition of microbial products (Sanjuan *et al.*, 2007). The effective enhancement of bacterial phagocytosis by the kinos-treated macrophages at 4 hours of incubation, strongly suggest an activation of the receptors and thus, the bacteria were phagocytized to a greater degree. The optimal kinos concentration was confirmed qualitatively with fluorescence imaging.

The range of kinos concentrations tested showed an effect of too high a concentration at 250 µg/mL, with the favourable effects on phagocytosis diminishing (for WA kino) or disappearing completely (for PNG kino). Possible reasons for the loss of phagocytic activity at this concentration could have been, in part, due to lower macrophage viability (80%, see Chapter 3) and subsequent general cell damage, or inhibitory components in kinos could have reduced TLR concentrations. This notion is also supported in a study by (Bass *et al.*, 2010) who demonstrated a reduction in pro-inflammatory cytokine secretion in neutrophils by treating them with DNase and exposure to *Pseudomonas aeruginosa*. In addition, the kinos may directly affect the bacteria. It can be strongly suggested that bacterial DNA has to be intact in order to enhance the surface cell receptors to be activated for engulfment, ingestion and digestion of bacteria (Bass *et al*., 2010). This reasoning as to why the high kinos concentration did not enhance phagocytosis is supported by the study by Harun *et al* (2015) which was evident in the low concentrations of plant extracts enhancing this immunomodulatory process but not the high concentrations.

The different mechanisms of phagocytosis in macrophages have been described in a review by Aderem and Underhill (1999). It was reported in this review that different receptors such as a) fragment crystallizable region receptors, b) complement receptors and c) mannose receptors contribute to differences in the ability of phagocytic cells to phagocytize antigens. However, it is not only the receptors of the phagocytic cells that play a role in the process of phagocytosis, structural features of microbes also contribute to the phagocytic differences (Aderem & Underhill, 1999; Henneke *et al*., 2005; Malley *et al*., 2003).. For example, Gram negative bacteria possess lipopolysaccharide, a predominant mediator of inflammation while peptidoglycan and lipoteichoic acid (also induce inflammation) are common cell wall

features of Gram positive bacteria (Kuroki *et al.*, 2007; Malley *et al.*, 2003; Wilson *et al.*, 2002). Malley and colleagues (2003) demonstrated TLR4s of the cell surface of the *Streptococcus pneumoniae* that allowed them to be recognized and phagocytized by the macrophages and this resulted in secretion of an appreciable level of pro-inflammatory cytokines to provide immunity to the mice. Another similar study demonstrated the pili of *E. coli* (Gram negative bacterium) enhancing its susceptibility of being phagocytized using a selective mechanism that did not allow D-mannose to inhibit its binding to the phagocytes (Silverblatt *et al.*, 1979). From the literature, it can be strongly suggested that the TLR4s on the cell surface of *S. aureus* and the lipopolysaccharide of the *E. coli* enabled the binding to the kinos treated macrophages in this current study. On the molecular level, it is possible that the kinos may have activated the NF-κB in the cells to enhance the phagocytosis of *S. aureus* as reflected by high MFI increases compared to the *E. coli*. A recent study demonstrated activation of NF-κB in RAW264.7 cells as a mechanism that enhanced the phagocytosis of *S. aureus* (Zhu *et al*., 2014). It was possible that the WA kinos treated macrophage cells could have activated the cellular signaling pathway more than that of the PNG kinos treated cells which resulted in the differences in the phagocytic effects on the two bacterial species.

There was a difference in the level of phagocytic activity induced by WA and PNG kinos: the WA kino treatment resulted in generally higher phagocytic activity of the macrophages, particularly for the Gram negative bacteria. These differences in the phagocytic activity could have been due to differences in the quantities of the chemical constituents in the respective kinos. The phenols (example flavonoids), tannins and triterpenoid saponins were detected in kinos despite them being obtained from two different *Eucalyptus* species. Other similar studies have reported differences in the anti-microbial activities due to differences in the chemical constituents in plants and also in different parts of the same plant. However, there were no differences in the main group of compounds detected in the kinos in this study, although they were from two different *Eucalyptus* species. The differences in chemical compositions in plants are due to variations in biosynthetic pathways from which different chemical constituents are produced (Cowan, 1999; Hammer *et al.*, 1999).

5.4. Conclusion

The WA and PNG kinos have an effect on the capacity of RAW264.7 macrophage cells to phagocytize Gram negative and Gram positive bacteria in a dose- and time-dependent manner. The Gram positive bacteria are easily phagocytized by the kinos treated macrophages. The WA kinos were more effective in increasing the phagocytosis of *S. aureus* than the PNG kinos. The data for the phagocytosis and binding of bacteria to the kinos treated macrophages (immune responses by macrophages in innate immunity) further validate the claim for the medicinal use of kinos in treating wounds, burns, pain, swelling and diarrhoea by the first Australians.

CHAPTER 6

ANTIBACTERIAL EFFECTS OF KINOS

6.1. Introduction

Resistance of bacteria to multiple drugs is a current worldwide concern, motivating researchers to investigate potential drugs from different sources. Plants and other organisms have been targeted for the isolation and identification of bioactive compounds using folk medicines as a basis for making selections for such investigations.

Bacteria have developed mechanisms besides their cell structural components that enhance their persistence in hosts (Wilson *et al*., 2002). The resistant bacteria have also developed strategies to alter cell membrane structures to prevent antibiotics to permeate through the membrane to the target sites. Mechanisms of bacterial proliferation and invasion of tissues of organs of hosts vary between Gram negative and Gram positive bacteria. Differences in cell structures contribute to these variations (Parolia *et al*., 2014; Ribert & Cossart, 2015). Encoding sequences such as AmpC β-lactamases of Gram negative bacteria break the beta lactam structure of some antibiotics such as penicillin, rendering them inactive. The efflux systems in these bacteria allow antibiotics to flow out of the target sites and this is one contributing factor to bacterial resistance. Efflux proteins of bacterial cells also contribute to their resistance by moulding cell structures into forms that do not allow the antibiotics to reach the inner target sites by enhancing the outflow of antibiotics (Li & Nikaido, 2004). Another factor involves mutations of target genes of the antibiotics and is caused by conjugation of plasmids and transduction of bacteriophages from one bacterial species to another (Giedraitienė *et al*., 2010).

In the hope of finding ways to reduce the world-wide concern over bacterial resistance, investigations into plants, such as *Eucalyptus* plants have been conducted using indigenous medicinal uses as the basis for investigations into antimicrobial activities. Several studies have investigated antibacterial effects of crude extracts of *Eucalyptus* species and their essential oils using disc and broth microdilution methods recommended by the then NCCLS, currently known as the CLSI (Wilker *et al*., 2006).

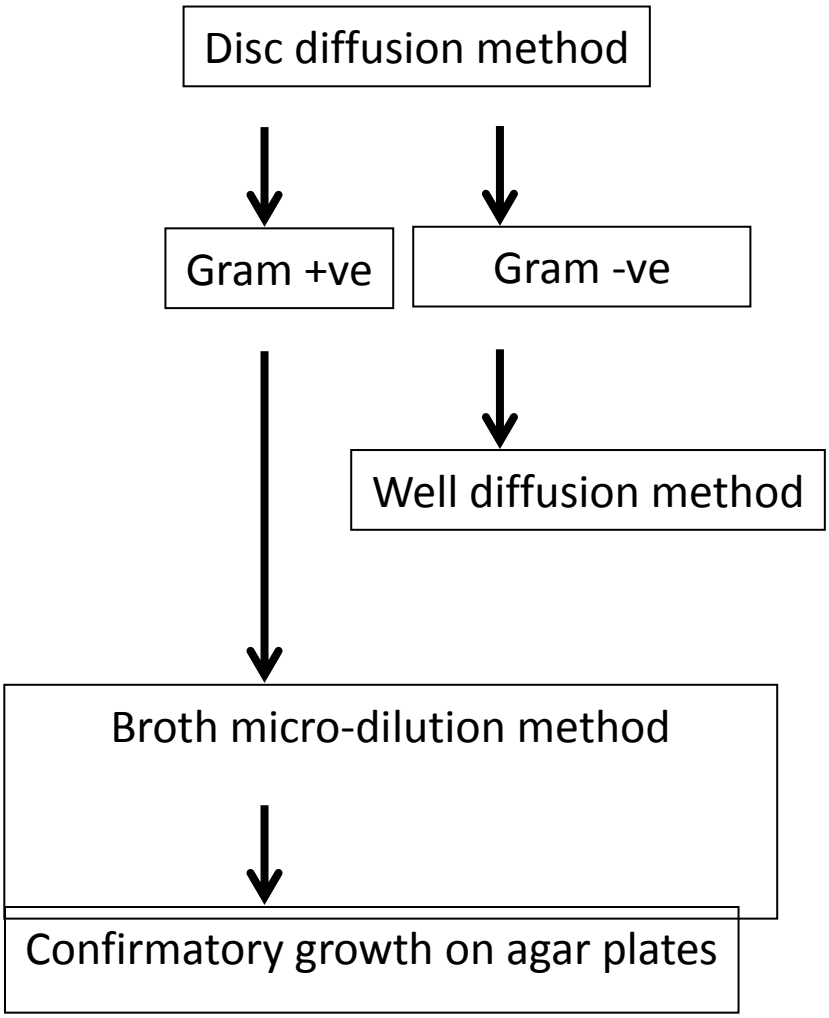
One of the earliest antibacterial studies demonstrated variability in the effect of essential oils of Australian *Eucalyptus* species on *S. aureus*, *Salmonella typhi* and *Mycobacterium phlei* (Atkinson & Brice, 1955). Another antibacterial study (Low *et*

al., 1974) demonstrated synergistic effects of essential oils of four *Eucalyptus species* on *S. aureus* and *S*. *typhi*. A recent study by von Martius *et al* (2012) demonstrated the tannins from the kinos of fifteen Australian *Eucalyptus* species to be more effective against Gram positive bacteria than Gram negative bacteria. However, no further studies have been conducted on antibacterial or other antimicrobial effects of kinos from Australian *Eucalyptus* species.

Essential oils and extracts of *Eucalyptus* species not only from Australia, but also from other countries, have been investigated for antimicrobial activity. For example, an investigation on the antibacterial effect of essential oils of the leaves of *Eucalyptus globulus* in Algeria (Bachir & Benali, 2012) showed antimicrobial activity against both Gram negative bacteria (*E. coli*) and Gram positive bacteria (*S. aureus*). In another study in Nigeria, methanol extracts of *Eucalyptus camaldulensis* and *Eucalyptus torellianna w*ere demonstrated to be effective against four non-tuberculosis mycobacterial species (Lawal *et al.*, 2011). Multi-drug resistant bacterial species other than Gram negative bacteria were inhibited by the essential oils from the fruits of *Eucalyptus globulus,* as demonstrated by a study in Germany (Mulyaningsih *et al*., 2011). The non-inhibition of Gram negative bacterial species by plant extracts and other pharmacological agents is due to multilayers of cell envelopes which include outer membrane, peptidoglycan cell wall and cytoplasmic membrane, preventing the permeability of chemical compounds into the target sites (Silhavy *et al*., 2010).

This part of the research was aimed at investigating antibacterial activity of WA and PNG kinos to validate the claim by the first Australians for medicinal uses of kinos in alleviating diarrhoea and inflammatory conditions such as wounds, burns and pains (Maiden, 1890; Lassak & McCarthy, 2001; Williams, 2011). The disc diffusion method was initially used to detect the antibacterial activity of the kinos on two Gram negative (*E. coli* and *P*. *aeruginosa*) and two Gram positive (two strains of *S. aureus*: ATCC 13709 and 29213) bacteria.

Sequence of experimental procedures



6.2. Results

6.2.1. Antibacterial effect of WA and PNG kinos using disc and agar well diffusion methods

The two Gram positive bacterial strains were susceptible to kinos. The *S. aureus* 13709 and 29213 strains were inhibited by WA kinos concentrations of ≥12.5 mg/mL and PNG kinos concentrations of ≥ 6.25 mg/mL. The growth inhibition zones for the two bacteria ranged between 7 and 10 mm for WA kinos (Table 6.1) while they were between 7 and 13 mm for the PNG kinos (Table 6.2). These inhibition zones were lower than the susceptibility range of ≥ 35 and ≥ 30 mm for *S. aureus* ATCC 13709 and *S. aureus* ATCC 29213, respectively, for the recommended antibiotic, amoxicillin-clavulanic acid (AMC), as outlined in the CSLI-M100-S17 document. Furthermore, the concentrations were very high, yet there were only relatively small inhibition zones, also indicating very low antibacterial activity. AMC was selected to be the positive control for the disc diffusion method since it is one of the recommended antibiotics for Staphylococcal infections, as outlined in the 2014 Australian Medicine Book.

The two Gram negative bacterial species, *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were not inhibited after being exposed to WA or PNG kinos after 20 h of incubation at 37°C using the disc diffusion method (Appendix A6 and A7). The two Gram negative bacteria were further tested for their susceptibility using the agar well diffusion method in order to confirm the absence of bacterial inhibition by the kinos when tested with the disc diffusion method. The two Gram negative bacteria were also not inhibited by the kinos with the agar well diffusion method (Appendix A8 and A9) confirming the above results. The agar well diffusion method was not used to detect the antibacterial effect of kinos with Gram positive bacteria since they were inhibited by the kinos when initially tested in parallel with the Gram negative bacteria using the disc diffusion method.

Table 6.1. Zones of growth inhibition (mm) of *S. aureus* ATCC 13709 and *S. aureus* ATCC 29213 due to effect of WA kinos tested in disc diffusion method.

Different treatment/ concentration (mg/mL) of kinos	Growth inhibition zone (mm) ±SEM	
	***S. aureus* ATCC 13709**	***S. aureus* ATCC 29213**
AMC 30 µg/disc	35 (±0.00)	35 (±0.00)
VC	NI	NI
Control	EG	EG
0.05	NI	NI
0.5	NI	NI
1	NI	NI
2	NI	NI
3	NI	NI
6.25	NI	NI
12.5	7 (±0.00)	7 (±0.00)
25	8 (±0.00)	8 (±0.00)
50	9 (±0.00)	9 (±0.00)
100	10 (±0.00)	10 (±0.00)

AMC- amoxicillin-clavulanic acid, VC-vehicle control, Control-untreated bacterial culture, NI- no inhibition, EG- excellent growth, n=3, SEM-standard error of mean

Table 6.2. Zones of growth inhibition (mm) of *S. aureus* ATCC 13709 and *S. aureus* ATCC 29213 due to effect of PNG kinos tested in disc diffusion method.

Different treatment/ concentration (mg/mL) of kinos	Growth inhibition zone (mm) ±SEM	
	***S. aureus* ATCC13709**	***S. aureus* ATCC29213**
AMC 30 µg/disc	35 (±0.00)	35 (±0.00)
VC	NI	NI
Control	EG	EG
0.05	NI	NI
0.5	NI	NI
1	NI	NI
2	NI	NI
3	NI	NI
6.25	8 (±0.17)	7 (±0.00)
12.5	9 (±0.00)	8 (±0.00)
25	11 (±0.17)	10 (±0.00)
50	12 (±0.33)	12 (±0.00)
100	13 (±0.33)	13 (±0.17)

AMC- amoxicillin-clavulanic acid, VC-vehicle control, Control-untreated bacterial culture, NI- no inhibition, EG- excellent growth, n=3, SEM-standard error of mean

6.2.2. Antibacterial effect of WA and PNG kinos extract using broth microdilution method

In order to confirm the sensitivity of the Gram positive bacteria to kinos and determine the minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs), *S. aureus* ATCC 13709 and ATCC 29213 were further tested for their susceptibility to different concentrations (1, 3, 6.25 and 12.5 mg/mL) of WA and PNG kinos using the MH broth microdilution method. The Gram negative bacteria (*E coli* ATCC 25922 and *P. aeruginosa* ATCC 27853) were not further tested as they demonstrated a lack of sensitivity to kinos with the disc and agar well diffusion methods (see Section 6.2.1). As expected, the amoxicillin potassium clavulanate (AMPC) treatment, being the positive control treatment, significantly reduced bacterial growth ($P<0.001$) for both Gram positive bacterial strains, compared to the untreated controls (Figures 6.1 and 6.2).

Treatment of *S. aureus* 13709 with WA and PNG kinos resulted in the inhibition of growth to a level equal to or greater than the control treatment (AMPC; Figure 6.1). The exceptions were the untreated control, as expected, and the WA kinos at a concentration of 3 mg/mL and PNG kinos at 12.5 mg/mL, when compared to AMPC treatment. The growth inhibition of *S. aureus* 29213 followed a similar pattern, demonstrating growth inhibition equal to or greater than the AMPC positive control samples: the exception being the negative controls and the PNG 12.5 mg/mL treatment.

It is worth noting that a complicating factor in the detection of bacterial growth by spectral absorbance. After treatment with the kinos, particularly those sourced from PNG, colouration at the higher concentrations was significant, and even though the absorbance readings of only kinos extracts were subtracted from the absorbance reading for each treatment of the bacteria with kinos, the 12.5 mg/mL was too dark and still interfered with absorbance readings (Figures 6.1 B and 6.2 B). The readings for the 12.5 mg/mL PNG kinos treatment may incorrectly reflect the levels of bacterial growth. For that reason, the results for the microdilution method were confirmed by plating out a small aliquot of each treatment into agar plates. Further 20 µL of the kinos treated bacterial culture were streaked out to quantify the bacterial growth. The results are shown in the appendix: *S. aureus*, strain 13709, with WA and PNG kinos, Appendix A10 and A11; *S. aureus*, strain 29213, with WA and PNG kinos, Appendix A12 and A13.

From Tables 6.3 and 6.4, it can be seen that bacterial growth was certainly inhibited by WA and PNG kinos, including at the highest kinos concentration. This data demonstrates that the inhibitory concentrations $(ICs)_{\geq 90\%}$ resulting from WA kinos treatment for *S. aureus* 13709 and *S. aureus* ATCC 29213 were 1 and 1-3 mg/mL, respectively (Table 6.3), and for PNG kinos $IC_{\geq 90\%}$ values were the same, 1 and 1-3 mg/mL, respectively (Table 6.4). The MBCs for both strains, *S. aureus* 13709 and *S. aureus* 29213, treated by WA and PNG kinos were 3 and 6.25 mg/mL, respectively: both bacterial strains having the same sensitivity to both kinos sources.

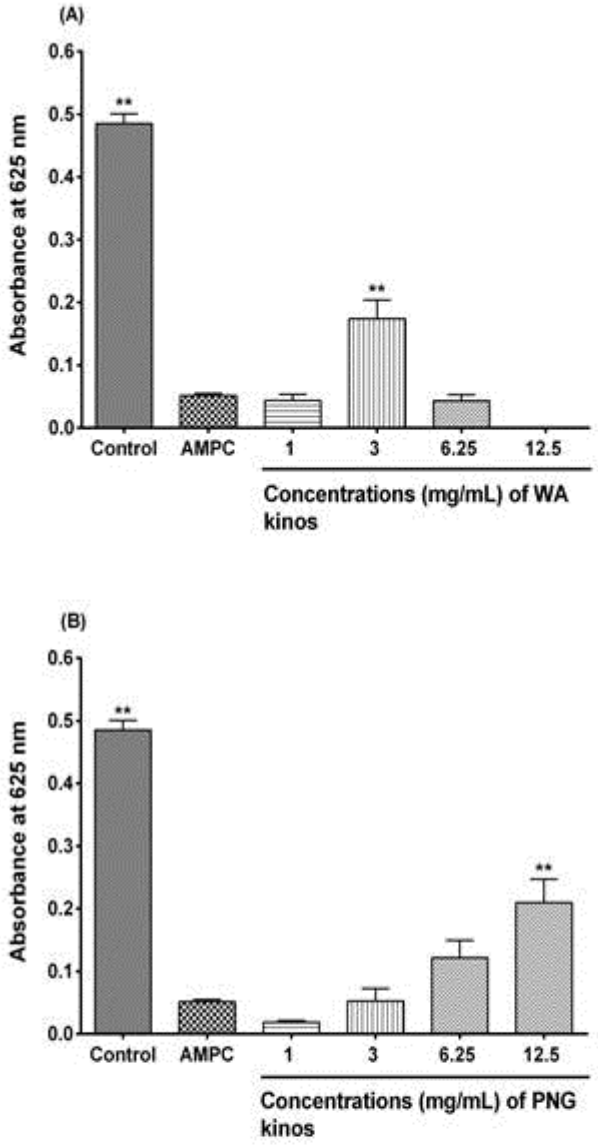


Figure 6.1. Effects of WA (A) and PNG (B) on *S. aureus* ATCC 13709 tested in mircodilution method.

Control represents untreated bacterial inoculum. AMPC (a positive control)-0.125 µg/mL of amoxicillin potassium clavulanate. The absorbance data presented are in ± SEM of 3 individual experiments done in triplicates. The significance levels of the data are: **P<0.01 compared to AMPC.

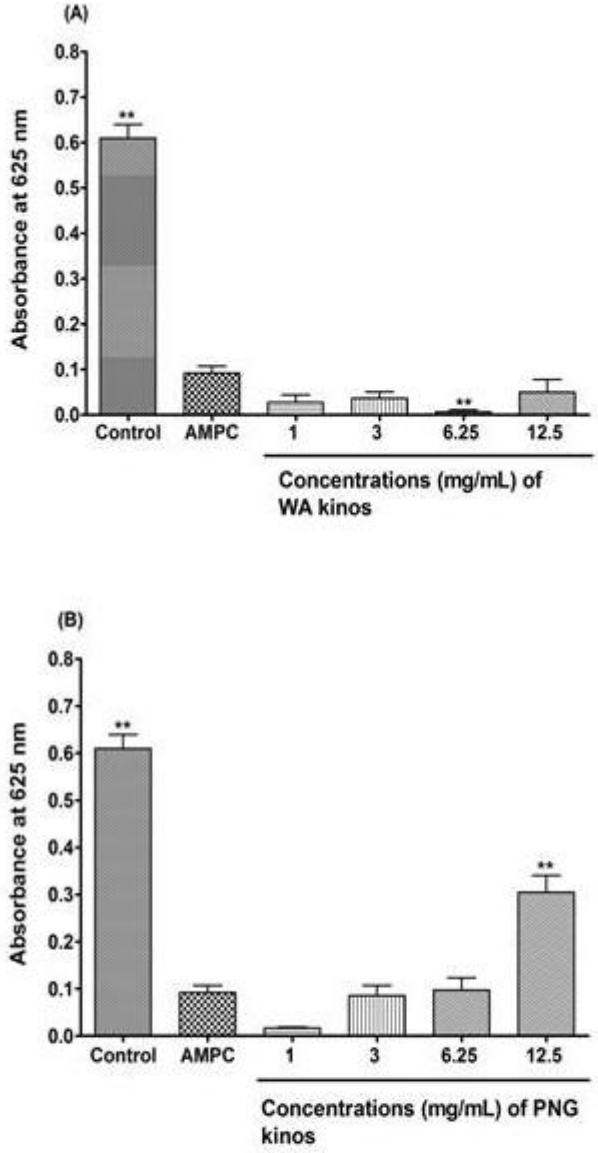


Figure 6.2. Effects of WA (A) and PNG (B) on *S. aureus* ATCC 29213 tested in mircodilution method.

Control represents untreated bacterial inoculum. AMPC (a positive control)-0.125 µg/mL of amoxicillin potassium clavulanate. The absorbance data presented are in ± SEM of 3 individual experiments done in triplicates. The significance levels of the data are: **P<0.01 compared to AMPC.

Table 6.3. Growth inhibition (mm) of *S. aureus* ATCC 13709 and *S. aureus* ATCC 29213 after treatment with WA kinos for 20 h and plating and streaking out 20 µL of kinos treated bacterial culture from the broth microdilution method.

Different treatment/ conc. (mg/mL) of kinos	Growth inhibition (in colony number) of:	
	***S. aureus* ATCC 13709**	***S. aureus* ATCC 29213**
Control	EG	EG
AMPC	50I	50I
1	90I	90I
3	CI	90I
6.25	CI	CI
12.5	CI	CI

Control-untreated bacterial culture, AMPC-amoxicillin potassium-clavulanate, EG-excellent growth, 50I-50% inhibition, 90I- 90% inhibition and CI-complete inhibition

Table 6.4. Growth inhibition (mm) of *S. aureus* ATCC 13709 and *S. aureus* ATCC 29213 after treatment with PNG kinos for 20 h and plating and streaking out 20 µL of kinos treated bacterial culture from the broth microdilution method.

Different treatment/ conc. (mg/mL)	Growth inhibition (in colony number) of:	
	***S. aureus* ATCC 13709**	***S. aureus* ATCC 29213**
Control	EG	EG
AMPC	50I	50I
1	90I	90I
3	CI	90I
6.25	CI	CI
12.5	CI	CI

Control-untreated bacterial culture, AMPC-amoxicillin potassium-clavulanate, EG-excellent growth, 50I-50% inhibition, 90I- 90% inhibition and CI-complete inhibition

6.3. Discussion

The emergence of multi-drug resistant bacteria has become a major cause of failure in treating many bacterial infections (Gibbons, 2005). Antibacterial multidrug resistance was demonstrated to be caused by mutations in genes of the bacteria which alter the target sites on the bacterial cells for the antibacterial agent to be effective (Alekshun & Levy, 2007). Another mechanism is through genes such as AmpC β-lactamase in Gram negative bacteria which would break the beta lactam structure of some antibiotics such as penicillin, rendering them inactive whilst the efflux systems enabling antibiotics to flow out of the bacteria. The resistance of bacteria to numerous antibiotics has triggered many researches into testing phytochemicals for their therapeutic potential against bacterial and other infections.

Reference bacteria were treated with WA and PNG kinos and subjected to several analyses to determine the effects of kinos on their growth and survival. The two Gram positive bacterial species, *S. aureus* ATCC 13709 and 29213, were inhibited by a concentration range between 6.25 and 12.5 mg/mL of both WA and PNG kinos using the conventional disc diffusion method which can detect moderate to low antibacterial activity of any pharmacological agent. However, the Gram negative strains, *E. coli* ATCC 25922 and *P*. *aeruginosa* ATCC 27853 were not inhibited by either kinos source. An additional assay was used to confirm the lack of response by the Gram negative bacteria, the agar well diffusion method, which confirmed the lack of response. Although the kinos from this study did not inhibit the Gram negative bacteria, other studies have demonstrated growth inhibition of *S*.*aureus* and *P*. *aeruginosa* as well as *E. coli* with plant extracts (Akinyemi *et al.*, 2005; Mills-Robertson *et al.*, 2012; Navarro *et al.*, 1996). These studies have demonstrated various solvent and aqueous plant extracts to be potent against both Gram negative and Gram positive bacteria. Differences in the antibacterial activity of kinos and other plant extracts show that sources of phytochemicals and their purity determine the significance of the levels of any antimicrobial activity. The bacterial species investigated by Mills-Robertson *et al* (2012) showed their susceptibility to high concentrations in micrograms per millilitre of the tested plant extracts, with varying MICs and MBCs.

The bioactivity of the kinos was further determined using the broth microdilution method because antimicrobial agents may not have diffused well through the solid agar but easily diffused through the broth to exert their effect on bacterial cells. The inocula of two strains of *S. aureus* treated with the range of high concentrations of

kinos showed absorbance values that indicated some form of bacterial inhibition, but the percentage of inhibition could not be established due to turbidity caused by the kinos. The nitrotetrazolium salt indicator method would have also been utilised by following the protocol described in Eloff (1998) since it would have allowed given starting low bacterial and extract concentrations. Hence, the interference of absorbance by the colour of the kinos extract would have been resolved by accurately measuring the percentage bacterial inhibition (Eloff, 1998). However, the nitrotetrazolium salts would have also formed deposits that would have damaged the bacterial cells (Tominaga *et al.*, 1999) and therefore, the data obtained for the inhibition of bacteria by the kinos would not have been representative. Hence, the interference by the colour of kinos with the observations of absorbance using the colorimetric assay for the broth microdilution method was overcome by employing the conventional plating method: namely, by plating out 20 µL each of the kinos treated bacterial inocula on MH agar plates and incubating for 20 h to observe any growth of colony forming units. The ICs and MBCs of the kinos were determined by both the plating and streaking of the bacterial inoculum from the kinos treated bacterial culture. This approach of confirming the MBCs of plant extracts was also used by a previous study that investigated the antimicrobial activities of plant extracts against methicillin-susceptible and methicillin-resistant *S. aureus* when the pigmentation of the plant extracts obscured the visual bacterial growth turbidity inspection (Chuah *et al.*, 2014). The absorbance of certain kinos concentrations was higher than the AMPC despite corrections with the absorbance of the respective concentrations of kinos only. Despite the absorbance of 3 mg/mL WA kinos, and 6.25 and 12.5 mg/mL PNG kinos for the *S. aureus* ATCC 13709 being higher than the positive control, AMPC, there was complete inhibition of this bacterial growth as observed in both plated and streaked MHA agar plates (Appendix A9 and A10). Similarly, there was complete inhibition of the growth of *S. aureus* ATCC 29213 with 12.5 mg/mL of PNG kinos despite its absorbance being higher than the AMC. There was complete inhibition of the two *S. aureus* strains with concentrations greater than 1 mg/mL of both WA and PNG kinos.

The Gram positive bacterial species were investigated so far, to be more susceptible to antibacterial agents such as plant extracts than the Gram negative bacteria (Akinyemi *et al.*, 2005; Cowan, 1999; Diao *et al.*, 2013). It was demonstrated in the study by Diao *et al* (2013) that Gram positive bacteria were more sensitive than Gram-negative bacteria to the essential oils from *Zanthoxylum schinifolium*. The data from the study of Diao *et al* (2013), further suggested that differences in the

outer layers of the cell membrane of the two groups of bacteria contributed to differences in the bacterial susceptibility to the essential oils. The susceptibility of the Gram positive bacteria may have been due to the greater percentage of peptidoglycan component of their cell walls which is not a regulator of the antibacterial agents' entry into the intracellular component of the bacterial cells. Thus, allowing the kinos to permeate into the cells and exert their antibacterial effect.

The overall data of this study demonstrate no inhibitory effects of both WA and PNG kinos from the *Eucalyptus* species on the two test reference Gram negative bacterial species; *E. coli* ATCC 25922 and *P*. *aeruginosa* ATCC 27853 using both disc and agar well diffusion methods. A study by von Martius *et al* (2012) demonstrated a similar trend in the antibacterial effect of the kinos extracts from nineteen Australian *Eucalyptus* species not being potent against *E. coli* ATCC 11775 and *P*. *aeruginosa* ATCC 9027 but they were bioactive against Gram positive bacterial species. It also showed that there was no association between relative quantities of hydrolysable and condensed total phenolics and tannin contents of the kinos and their bioactivity in the study by von Martius and co-workers (2012). The kinos were not potent against the Gram negative bacterial species in this study and that of von Martius *et al* (2012) despite differences in the type of Gram negative bacterial species tested, the kinos preparation methods, concentrations and the volume of kinos used in the diffusion method. It can be suggested that the kinos from *Eucalyptus* species are not significantly inhibitory against Gram negative bacterial species. The non-inhibition of *P*. *aeruginosa* by the Australian kinos may not correlate with the medicinal use of kinos on alleviation of infections from wounds and burns as claimed by the first Australians (Lassak & McCarthy, 2001; Williams, 2011) since it is a nosocomial bacterial species that causes infections in wounds. The *P. aeruginosa* ATCC 27853 was a multi-drug resistant strain that was tested in this study which was also not susceptible to the kinos. Thus, it was totally not inhibited by the kinos despite the application of very high doses. The *P. aeruginosa* and *S. aureus* are two bacterial species that are known to cause infections such as abscesses, carbuncles and wound sepsis. The two strains of *S. aureus* ATCC 13907 and *S. aureus* ATCC 29213 that were investigated for their susceptibility to kinos in this study are methicillin sensitive strains and were inhibited by the kinos. Similarly, another study demonstrated the *S. aureus* ATCC 29213 being inhibited by the diterpenoid obtained from acetone extract of hairy roots of *Salvia sclarea* (Różalski *et al.*, 2007). It can be suggested that there could have been methicillin components in the kinos

mixture or there were a synergistic effect of phenols (example, flavonoid), tannins and triterpenoid saponins in kinos inhibited the two strains of *S. aureus*. However, bacterial species can be susceptible to one or two drugs but they can be resistant to other drugs. It was demonstrated in a study by Singh and co-workers that the *S. aureus* ATCC 29213 was resistant to amikacin (aminoglycoside) and ciprofloxacin (fluoroquinolone) although it was a methicillin susceptible bacterial strain (Singh *et al.*, 2010). In contrast, many studies have demonstrated *P. aeruginosa* to be resistant to multiple drugs by different structural mutations (Livermore, 2002; Mah *et al.*, 2003). In addition, a study by Rizek *et al* (2014) also demonstrated *P*. *aeruginosa* to possess multiple carbapenemase genes that enhance antibiotic resistance. The lack of susceptibility of *P. aeruginosa* to the kinos extracts could be attributed to the fact that this bacteria are naturally resistant to many antibiotics due to the permeability barrier afforded by its outer membrane (Lino & Deogracious, 2006).

In another study on antibacterial activities of phytochemicals, Hemandez *et al* (2000) found no bioactivity against the two test Gram negative bacterial species, *E. coli* ATCC 25922 and *P*. *aeruginosa* ATCC 27853. However, other studies have shown several solvent-extracted leaf extracts of plant species traditionally used in Indian folk medicine (Bhattacharjee *et al.*, 2011) and methanol root bark and several isolated flavonoids (Kuete *et al.*, 2007) to inhibit the growth of *E. coli* and *P*. *aeruginosa* at varying antibacterial intensities.

The non-inhibition of *E. coli* and *P*. *aeruginosa* by the WA and PNG kinos in this study may have possibly been due to the presence of lactamases since these enzymes reduce the bioactivity of antibacterial agents (Lin *et al*., 2005; Tenover, 2006). In addition, the two Gram negative bacteria may have acquired efflux pumps that may have extruded the kinos out of the bacterial cells and therefore, they could not reach the target sites and exert their effects. The efflux pumps of the test bacterial species were demonstrated to be the contributing factor to the non-inhibition of the bacteria by the plant extracts in a previous study (Stavri *et al.*, 2007). There is also a possibility that the two test Gram negative bacterial species may have multiple genes through gene mutations which limited the kinos from reaching the intracellular targets. The three bacterial resistant mechanisms that are described here for the possible contributing factors to the non-inhibition of *E. coli* and *P*. *aeruginosa* by kinos have also been suggested as the mechanisms for the bacterial resistance in another similar study (Tenover, 2006).

From the data of the antibacterial effect of kinos on the two strains of Gram positive bacterial species, it can be suggested that the differences in the type of methods used in the detection of the pharmacological agent contributes to the intensity of inhibition of their growth. There were no large bacterial growth inhibition zones with the range of high kinos concentrations with the disc diffusion method and this could have been due to kinos not diffusing efficiently through the MH agar to reach the bacterial plasma membranes to exert their antibacterial effect. In contrast, the kinos could have been well solubilized in the MH broth in the microdilution method, and that allowed their active ingredients to diffuse well and reach all bacterial cells, thus, inhibiting bacterial growth more effectively than in the disc diffusion method. It has been demonstrated in a previous study (Chuah *et al.*, 2014) that there are advantages and disadvantages of the broth microdilution and disc and agar well diffusion methods and therefore, it was suggested that a combination of visual observation, optical density measurement and colorimetric assay methods should be the approach to take when testing for antibacterial effects of plant extracts. This study also used this approach to confirm the ICs and MBCs of kinos, which ascertain their antibacterial effects and therefore, it can be stated that the antibacterial data are reflective of their real bacteriostatic and bactericidal effects.

6.4. Conclusion

Antimicrobial studies aimed at scientifically investigating claimed uses of plant extracts and products from indigenous medicine often do not validate the claims. However, the experiments in this chapter validate, in part, claims for the antibacterial use of WA kinos by the first Australians: growth inhibition by kinos. The Gram positive bacteria demonstrated sensitivity whereas the Gram negative did not. Although the kinos concentrations were not physiologically attainable systemically, they would be suitable for topical application. Interestingly, the geographical difference for the kinos sources did not have an effect on the antibacterial properties and potencies.

Studies involving screening of plants for their antibacterial activities should use the broth microdilution method to detect MICs and MBCs, which are suggested to be the best indication of potential antimicrobial effects of any pharmacological agents *in vitro*. The antibacterial data for the two strains of *S. aureus* for the high concentrations of kinos *in vitro* suggest further investigation into their antibacterial activities *in vivo* by using purified chemical constituents.

CHAPTER 7

GENERAL DISCUSSION

7.1. General discussion

This research is a comparative study of anti-inflammatory, phagocytic and antibacterial effects of kinos from *E*. *calophylla* (WA) and *E*. *confertiflora* (PNG), contributing to investigations of *Eucalyptus* plant extracts for antimicrobial activities. It is worth noting that this study is the first to investigate anti-inflammatory and phagocytic effects of kinos from the Australian *Eucalyptus* species and the third investigation of antibacterial effect. For the kinos from PNG *Eucalyptus* species, it is the first anti-inflammatory, phagocytic and antibacterial study. Several methods were employed in this study to obtain data presented in each of the respective chapters of the thesis. Treatments of LPS-stimulated RAW264.7 murine macrophages involved a range of kinos concentrations and appropriate incubation times. The analyses included documentation of morphological characteristics and an evaluation of macrophage viability. Cytokine profiling was determined with ELISAs and a specific Griess reagent test for NO. Antibacterial properties of kinos were evaluated with specific growth assays and phagocytic activity was determined via FACS. In addition, phytochemical spot, qualitative and quantitative tests were performed to detect some of the main groups of chemical constituents of kinos.

Previous investigations on the anti-inflammatory effect on the essential oils of the leaf and fruits of the *Eucalyptus* species demonstrated anti-inflammatory effects *in vivo* (Santos & Rao, 2000; Silva, *et al*., 2003). These studies showed reductions of carrageenan-induced oedema increased capillary permeability, neutrophil migration into rat peritoneal cavities induced by carrageenan, and vascular permeability. Another study (Serafino *et al*., 2008) investigating the essential oils from the *Eucalyptus* species found them to be effective against cytokine production in LPS-stimulated human monocyte derived macrophages *in vitro* and also showed a phagocytic effect on *S. aureus.* The reduction in IL-6 by kinos in LPS-stimulated murine macrophages is consistent with the study by Sefarino *et al* (2008) despite the difference in the source of macrophages. The kinos also reduced NO secretions in the macrophages in this study. Despite the differences in the methods, the part of the *Eucalyptus* plant tested for the anti-inflammatory effect and whether the study is *in vitro* or *in vivo*, the data so far, can strongly suggest that the *Eucalypts* do possess anti-inflammatory effects.

The RAW264.7 macrophage cells produced vacuoles after being exposed to kinos and they increased with time of exposure and were also kinos-dose dependent. The cells exceeded 80% viability when exposed to the kinos and it also increased with

time. The enhancement of secretion of IL-10 and the reduction of the IL-6 and NO by the WA kinos extracts in the macrophages were most probably due to the synergistic effects of the phenolics (example, flavonoids), tannins and triterpenoid saponins detected in these extracts. This establishes and confirms the claim by the first Australians treating pain, wounds and rheumatic arthritis using kinos from Australian *Eucalypts*. In contrast, the levels of IL-10 were reduced by the PNG crude kinos extracts while the IL-6 and NO secretions by the cells were reduced like those of the WA kinos. The difference in the secretion of IL-10 by the WA and PNG kinos in the cells is most probably due to differences in the quantity and the effect of the chemical constituents, although phenolics (example flavonoids), tannins and triterpenoid saponins were also present in the PNG kinos. Furthermore, the enhancement of TNF-α secretion by these compounds in the kinos could have interacted antagonistically interfering with the IL-10 secretion, thus reducing the IL-6 significantly compared to the LPS, the positive control. Interestingly, the effects of WA kinos appear to have been abrogated when fractionated, whereas the kinos fractions for PNG retained some activity: further attesting to the potential multifactorial nature of the biological effect.

There have been investigations on the antibacterial effects of *Eucalyptus* species in previous studies that demonstrated the antibacterial effects of the essential oils from leaves and fruits (Ghalem & Mohamed, 2008; Takahashi *et al*., 2004; Sartorelli *et al*., 2007; Low *et al*., 1974) and also of kinos (von Martius *et al*., 2012) from *Eucalyptus plants*. It was demonstrated by von Martius *et al* (2012) that the kinos extracts from fifteen Australian *Eucalyptus* species to be active against Gram positive bacteria but not the two Gram negative bacteria (*E. coli* and *P. aeruginosa*). The inhibitory effect of the kinos on the Gram positive bacteria (*S. aureus* ATCC 13709 and *S. aureus* ATCC 29213) and the non-inhibitory effect on the Gram negative bacteria (*E. coli* and the *P. aeruginosa*) in this study are consistent with the antibacterial data from the study by von Martius and colleagues (2012). This study is however, more definitive than the study by von Martius and colleagues because both disc and agar well diffusion methods were used to detect the antibacterial effect of kinos on the Gram negative bacteria. The Gram positive bacteria were tested in broth microdilution method and their susceptibility to the kinos was confirmed by plating and streaking out of kinos treated bacterial culture of *S. aureus* ATCC 13709 and *S. aureus* ATCC 29213 on agar plates and observing any bacterial growth after 20 h of incubation in this study. The data from the confirmatory test confirmed the MICs that were obscured by the colouring of kinos in the broth

microdilution method and also determined MBCs of the kinos. The data for the antibacterial effect of kinos in this study confirms the previous antibacterial study and also provides a strong basis for further *in vivo* investigations into mechanisms that could have allowed Gram positive bacteria to be susceptible to the kinos.

In addition to the general anti-inflammatory and antibacterial effects, kinos were also effective at enhancing phagocytosis of both Gram negative (*E. coli*) and Gram positive (*S. aureus*) bacteria, which increased with increasing kinos concentrations. Interestingly, the enhanced activity was seen mostly as an increase in phagocytic activity per individual macrophage.

There were several limitations to the different experiments that were carried out in this study. Any one of the currently known drugs to relieve inflammation was not used in the treatment of LPS-stimulated RAW264.7 murine macrophage cells in parallel with the kinos for the supernatants to be tested in the ELISAs since no specific chemical compounds have been isolated from the kinos to date. Therefore, the anti-inflammatory effect of drugs would not have been comparable to the effects of the complex mixture of chemical constituents in the kinos extract. In addition, the levels of pro- and anti-inflammatory cytokines were lower than the LPS, positive control in this study. The regression of the standard curves for each experiment was on average with $r = \geq 0.98$. This is statistically accepted for the levels of the cytokines to be derived from. Furthermore, the levels of the standard drugs have been tested by many previous anti-inflammatory studies that demonstrated secretion of low levels of pro-inflammatory cytokines in the supernatants of different pharmacological agent treated cell cultures compared to the LPS. The general data indicated the assays were effective. Other types of macrophage cells were not used in the experiments to determine the anti-inflammatory effects of the kinos in order to minimize variability since there was already one major variation; the geography of the two *Eucalyptus* species from which the kinos were sourced. In addition, there have not been any previous studies on the anti-inflammatory effect of kinos making this study original and novel and therefore, the literature could not provide any suggestions for lead up experiments.

In conclusion, the ability of kinos to induce an anti-inflammatory profile in mouse macrophages and enhanced phagocytic activity, possess antibacterial properties against Gram positive bacteria, although limited to the *in vitro* context, provides evidence substantiating some of the claims for the medicinal uses of kinos by the first Australians.

Possible future investigations:

i. Investigate various mechanisms responsible for the anti-inflammatory and antibacterial effect of kinos in several immune macrophage cells and bacteria respectively, in *ex vivo* and *in vivo* study models.

ii. Isolate, purify, characterize and identify individual chemical constituents of the phenolics (flavonoids), tannins and triterpenoid saponins responsible for anti-inflammatory, phagocytic and antibacterial effect of kinos in animals.

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APPENDICES

RE: Seeking Permission to use Figure on interactions of innate and adaptive immunity

Wednesday, 9 September, 2015 6:30 AM

Mark as Unread

From:

"martha mungkaje" <mmungkaje@yahoo.com>

To:

"Shizuo Akira" <a-office@biken.osaka-u.ac.jp>

Full Headers Printable View

Dear Professor Shizuo Akira,

Thank you very much for allowing me to use your Figure on 'interactions and adaptive immunity'.

Best regards,

Martha

On Tue, 8/9/15, Shizuo Akira <a-office@biken.osaka-u.ac.jp> wrote:

Subject: RE: Seeking Permission to use Figure on interactions of innate and adaptive immunity

To: '"martha mungkaje"' <mmungkaje@yahoo.com>
Received: Tuesday, 8 September, 2015, 3:44 PM

Dear Dr. Martha Mungkaje,

Thank you so much for your email. I allow you to use the
following my figure.

Best
regards,

Shizuo Akira

-----Original Message-----
From: martha mungkaje [mailto:mmungkaje@yahoo.com]

Sent: Tuesday, September 08, 2015 2:12

PM
To: 'Professor Shizuo Akira'
Subject: Seeking Permission to use Figure on
interactions of innate and adaptive immunity

Dear Professor Akira,

My name is Ms Martha Yahimbu Mungkaje and I am Papua New Guinean PhD student. My PhD project's title is "Anti-inflammatory and antibacterial effects of kinos from Australian and Papua New Guinean Eucalyptus species". Please refer to the attachments for the cover page of my thesis and the abstract.

I am kindly asking you if I could use your Figure that clearly explained the interactions of innate and adaptive immunity from your article "Akira, S. (2011). Innate immunity and adjuvants. Philosophical Transactions of the Royal Society, 366, 2748-2755. http://dx.doi.org/:10.1098/rstb.2011.0106".

I would like to use it to explain what happens between innate and adaptive immunity in
my literature review section of my thesis if you grant me permission to use your Figure.

Thank you for your time.

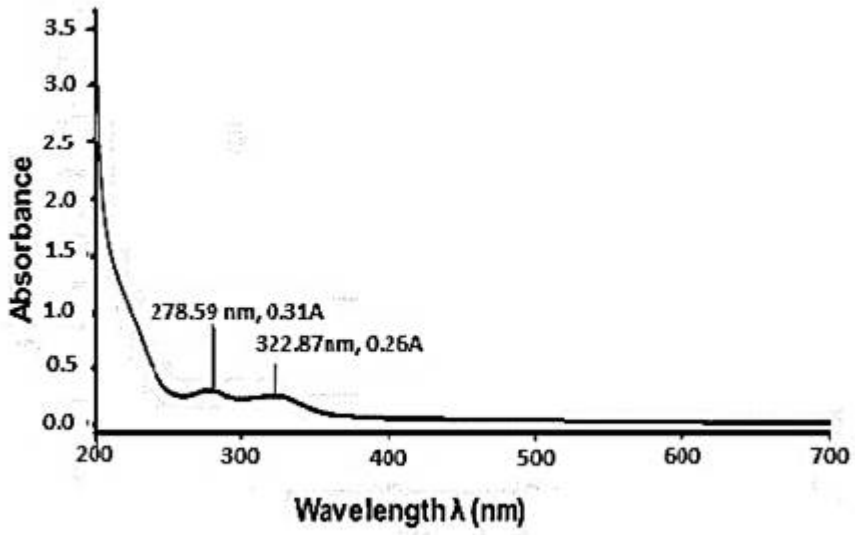
Best Regards,

Martha

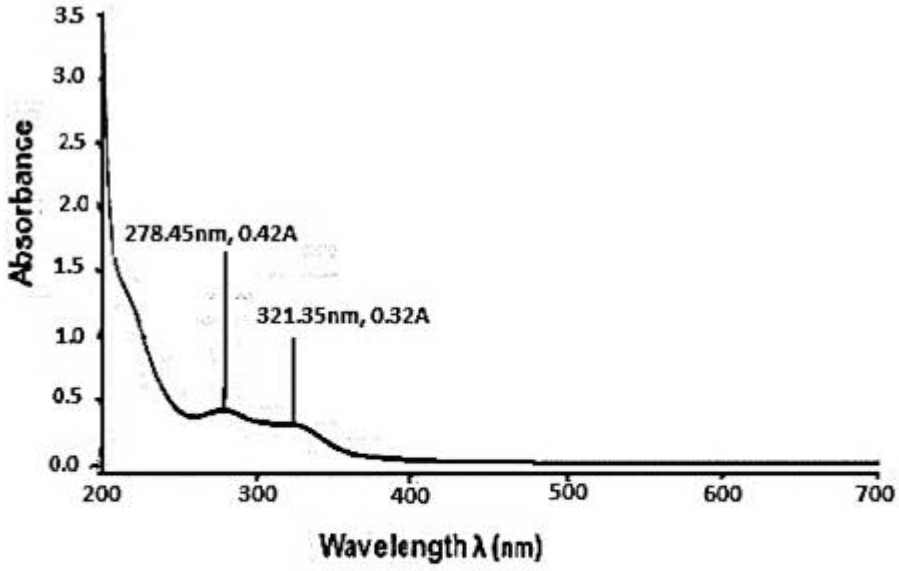
……

Appendix A1. Correspondences between the author of the journal article that contains the figure that was reproduced in this thesis for Figure 1.1 and the PhD candidate.

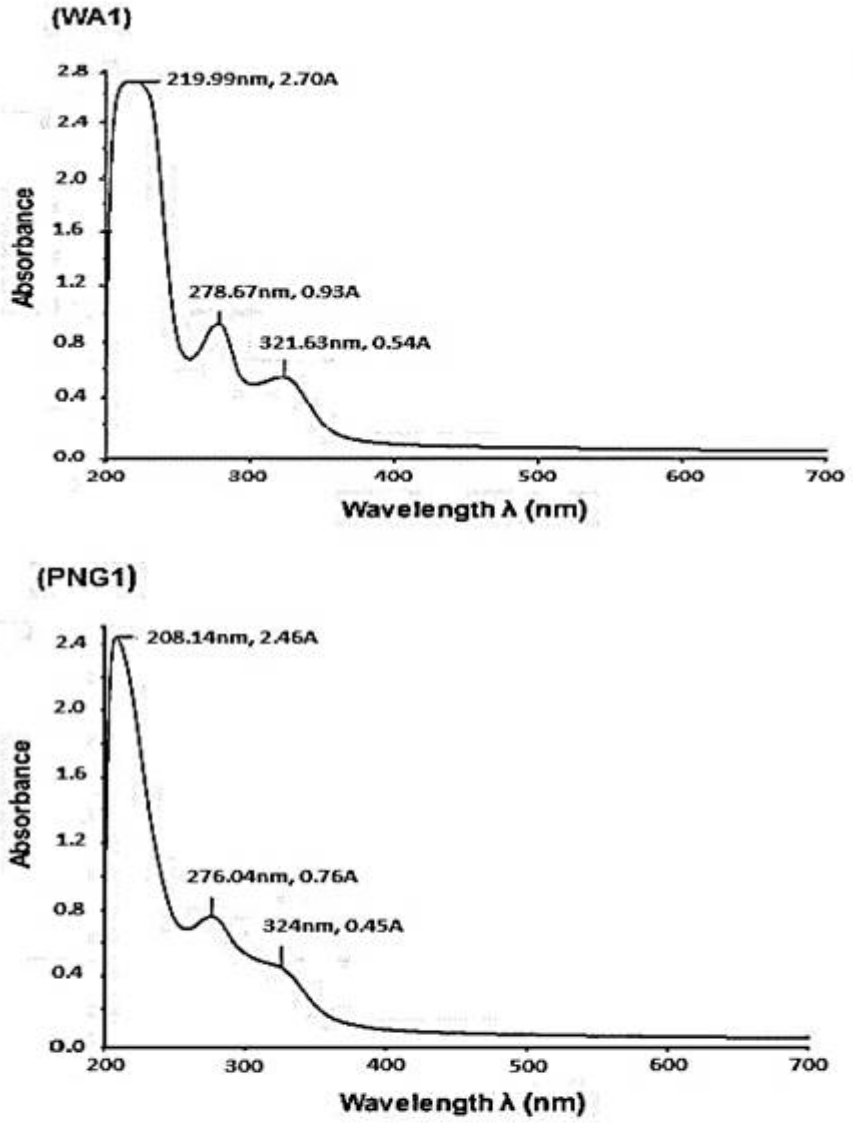
(WA TE)



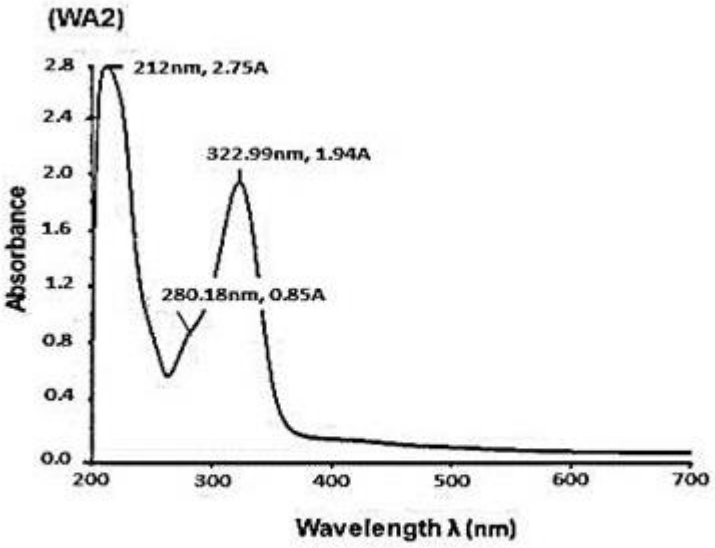
(PNG TE)

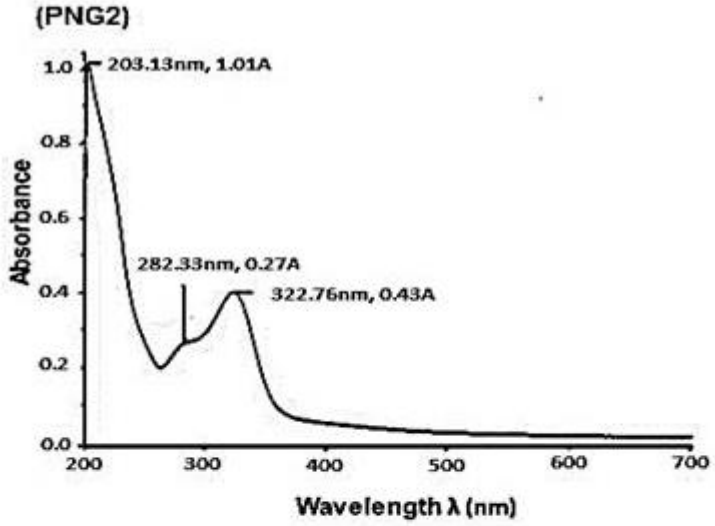


Appendix A2. UV light spectral profiles of WA and PNG total (crude) kinos extract (TE)

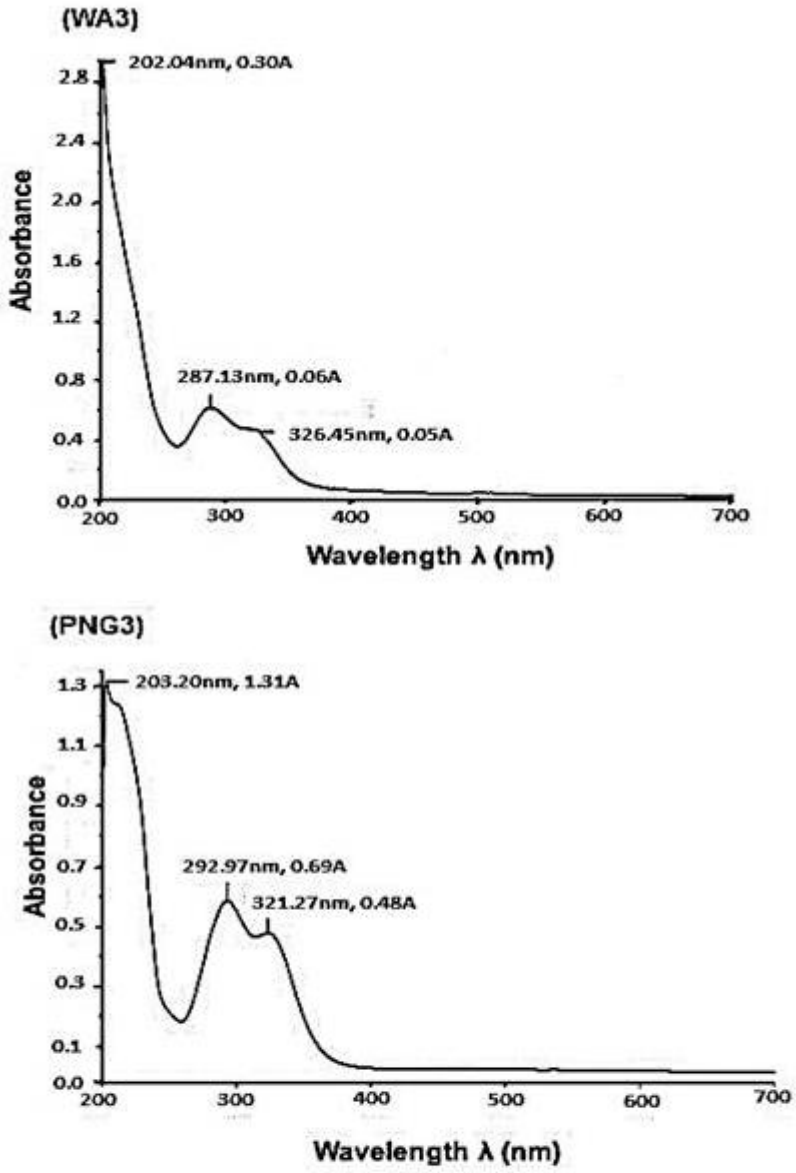


Appendix A3. UV light spectral profiles of fraction 1 of WA (WA1) and fraction 1 of PNG (PNG1) kinos





Appendix A4. UV light spectral profiles of fraction 2 of WA (WA2) and fraction 2 of PNG (PNG2) kinos



Appendix A5. UV light spectral profiles of fraction 3 of WA (WA3) and fraction 3 PNG (PNG3) kinos

Appendix A6. Zones of growth inhibition (mm) of *E. coli* ATCC 25922 due to the effect of WA and PNG kinos tested in disc diffusion method.

Different treatment/Concentration (mg/mL) kinos	Growth inhibition	
	WA kinos	**PNG kinos**
AMC 30 µg/disc	20 (±0.00)	20 (±0.00)
VC	NI	NI
Control	EG	EG
0.05	NI	NI
0.5	NI	NI
1	NI	NI
2	NI	NI
3	NI	NI
6.25	NI	NI
12.5	NI	NI
25	NI	NI
50	NI	NI
100	NI	NI

AMC-amoxicillin-clavulanic acid, VC-vehicle control, Control-untreated bacterial culture, NI- no inhibition, EG-excellent growth, n=3, SEM-standard error of mean

Appendix A7. Zones of growth inhibition (mm) of *P. aeruginosa* ATCC 27853 due to the effect of WA and PNG kinos tested in disc diffusion method.

Different treatment/Concentration (mg/mL) kinos	**Growth inhibition zone (mm) ±SEM *P. aeruginosa* ATCC 27853 with:**	
	WA kinos	**PNG kinos**
ATM 35 µg/disc	25 (±0.00)	25 (±0.00)
VC	NI	NI
Control	EG	EG
0.05	NI	NI
0.5	NI	NI
1	NI	NI
2	NI	NI
3	NI	NI
6.25	NI	NI
12.5	NI	NI
25	NI	NI
50	NI	NI
100	NI	NI

ATM-aztreonam, VC-vehicle control, Control-untreated bacterial culture, NI- no inhibition, EG-excellent growth, n=3, SEM-standard error of mean

Appendix A8. Zones of growth inhibition (mm) of *E. coli* ATCC 25922 due to the effect of WA and PNG kinos tested in MH agar well diffusion method.

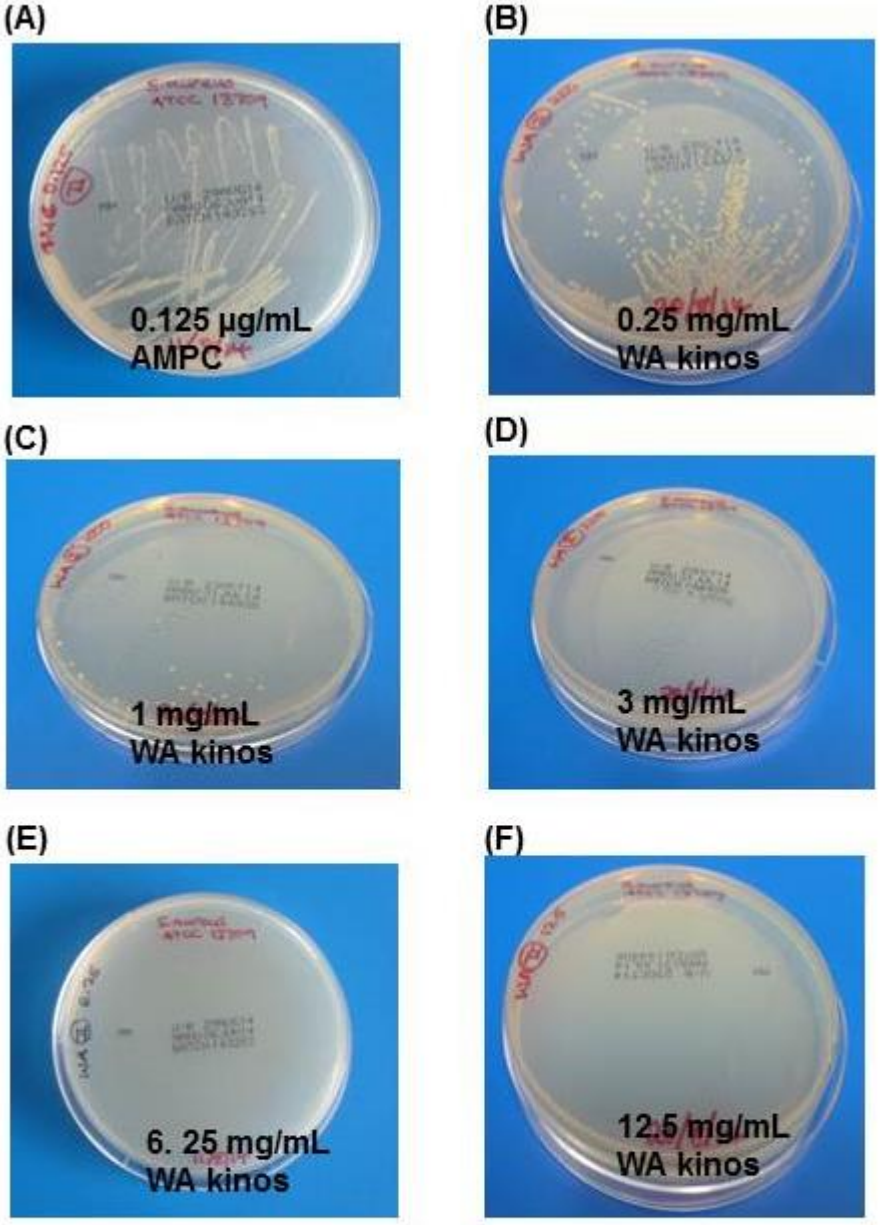
Different treatment/Concentration (mg/mL) kinos	**Growth inhibition zone (mm) ±SEM for *E. coli* ATCC 25922 with:**	
	WA kinos	**PNG kinos**
AMC 30 µg/disc	20 (±0.00)	20 (±0.00)
VC	NI	NI
Control	EG	EG
0.05	NI	NI
0.5	NI	NI
1	NI	NI
2	NI	NI
3	NI	NI
6.25	NI	NI
12.5	NI	NI
25	NI	NI
50	NI	NI
100	NI	NI

AMC-amoxicillin-clavulanic acid, VC-vehicle control, Control-untreated bacterial culture, NI- no inhibition, EG-excellent growth, n=3, SEM-standard error of mean

Appendix A9. Zones of growth inhibition (mm) of *P. aeruginosa* ATCC 27853 due to the effect of WA and PNG kinos tested in MH agar well diffusion method.

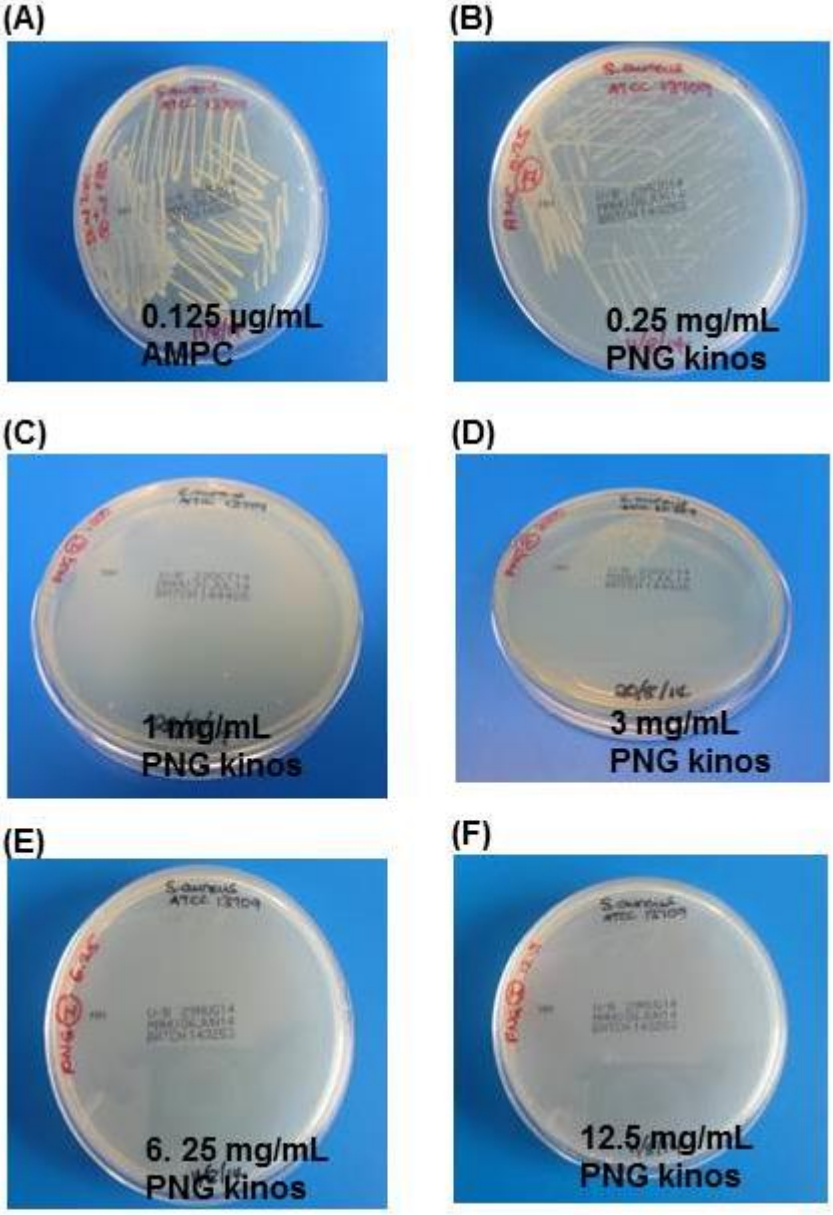
Different treatment/Concentration (mg/mL) kinos	**Growth inhibition**	
	WA kinos	**PNG kinos**
ATM 35 µg/disc	25 (±0.00)	25 (±0.00)
VC	NI	NI
Control	EG	EG
0.05	NI	NI
0.5	NI	NI
1	NI	NI
2	NI	NI
3	NI	NI
6.25	NI	NI
12.5	NI	NI
25	NI	NI
50	NI	NI
100	NI	NI

ATM-aztreonam, VC-vehicle control, Control-untreated bacterial culture, NI- no inhibition, EG-excellent growth, n=3, SEM-standard error of mean



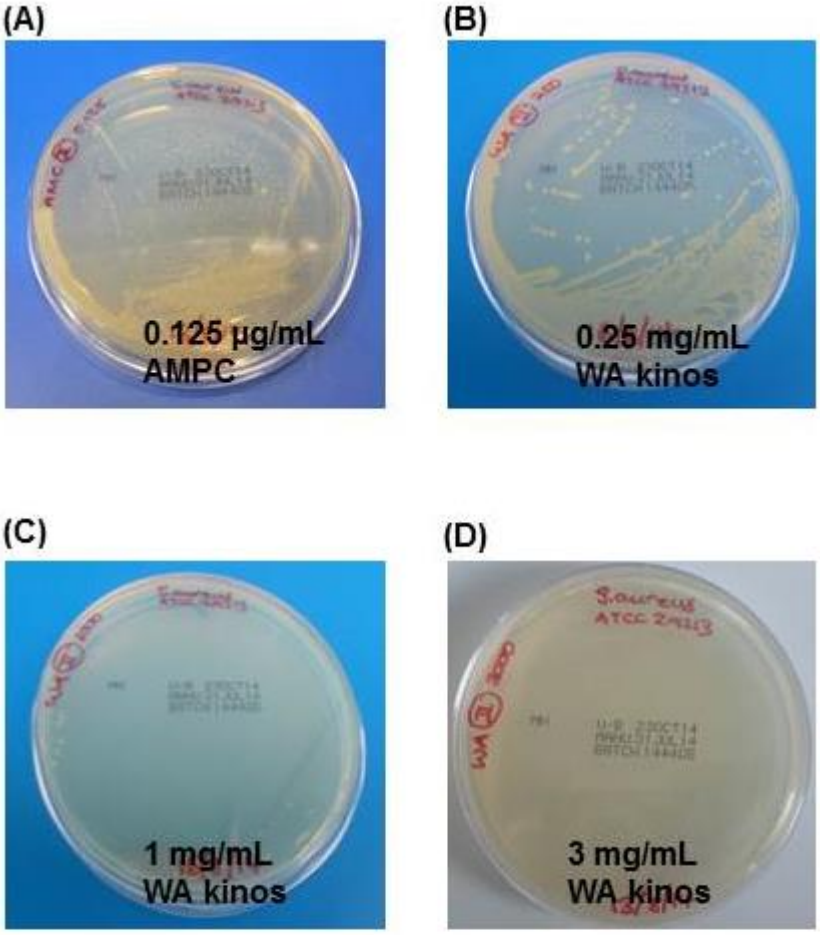
Appendix A10. Images showing inhibition of *S. aureus* ATCC 13709 by different concentrations (mg/mL) of WA kinos from 20 µL of 20 h old inoculum from the broth dilution method

A-0.125 µg/mL of amoxicillin-potassium clavulanate,
B-inhibition by 0.25 mg/mL kinos, **C-**inhibition by 1 mg/mL kinos,
D-inhibition by 3 mg/mL kinos, **E-**inhibition by 6.25 mg/mL kinos,
F-inhibition by 12.5 mg/mL kinos



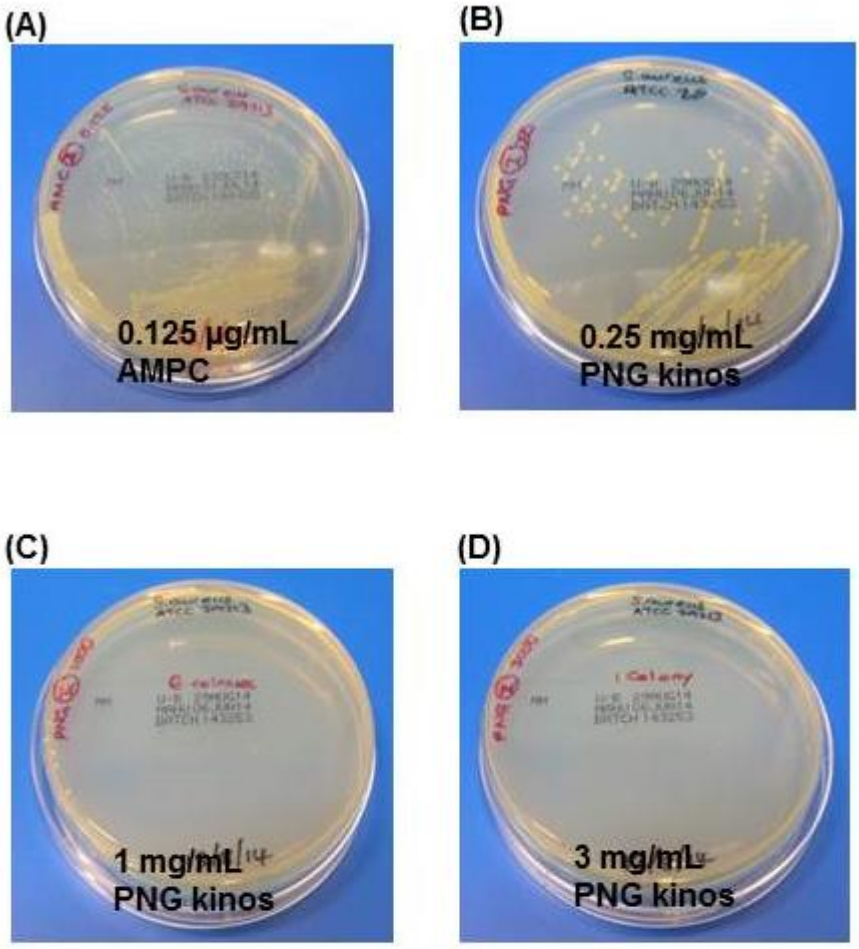
Appendix A11. Images showing inhibition of *S. aureus* ATCC 13709 by different concentrations (mg/mL) of PNG kinos from 20 µL of 20 h old inoculum from the broth dilution method

A-0.125 µg/mL of amoxicillin-potassium clavulanate,
B-inhibition by 0.25 mg/mL kinos, **C-**inhibition by 1 mg/mL kinos,
D-inhibition by 3 mg/mL kinos, **E-**inhibition by 6.25 mg/mL kinos,
F-inhibition by 12.5 mg/mL kinos



Appendix A12. Images showing inhibition of *S. aureus* ATCC 29213 by different concentrations (mg/mL) of WA kinos from 20 µL of 20 h old inoculum from the broth dilution method

A-0.125 µg/mL of amoxicillin-potassium clavulanate,
B-inhibition by 0.25 mg/mL kinos,
C-inhibition by 1 mg/mL kinos,
D-inhibition by 3 mg/mL kinos



Appendix A13. Images showing inhibition of *S. aureus* ATCC 29213 by different concentrations (mg/mL) of PNG kinos from 20 µL of 20 h old inoculum from the broth dilution method

A-0.125 µg/mL of amoxicillin-potassium clavulanate,
B-inhibition by 0.25 mg/mL kinos,
C-inhibition by 1 mg/mL kinos,
D-inhibition by 3 mg/mL kinos