# The chemical biology of antimicrobial bioactivity derived from kānuka (*Kunzea robusta*)

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# Abstract

Natural products are sourced from the environment where immense potential for bioactive compounds exist. Traditional medicine has explored these compounds, with many discoveries based on medicinal plants still in use today. The native flora in Aotearoa (New Zealand) contains many endemic species that are yet to be extensively investigated, for example *Kunzea robusta* or kānuka. Kānuka is considered a taonga species to Māori, the indigenous people of Aotearoa, due it to extensive use in Māori traditional medicine. Unlike its close relative mānuka, the bioactive potential of kānuka has yet to be extensively explored. Therefore, the aims of this thesis were to determine its medicinal potential by investigating the antifungal, antibacterial, and anti-P. acnes bioactivities of kānuka oil. Chemical profiles were also investigated, and statistical analyses correlated the chemical composition of kānuka oil extracts to the aforementioned bioactivities, thus identifying potential lead drug compounds.

**Methods:** Using 99 samples of kānuka oil extracted from leaves collected from various locations in Te Tairawhiti via steam distillation, chemical profiles were determined using gas chromatography mass spectrometry (GC-MS) analysis. The growth of *Candida albicans*, methicillin-resistant *Staphylococcus aureus* (MRSA) and *Propionibacterium acnes* was evaluated in the presence of all kānuka extracts to represent each bioactivity. Multivariate statistical analysis was then used to identify the compounds within the kānuka extracts that positively correlate with the three aforementioned bioactivities.

**Results:** Bioassays were conducted using minimum inhibitory concentration (MIC) of $5 \times 10^{-5}$ % (v/v) for antifungal and anti-MRSA bioactivity and a MIC of $500 \times 10^{-5}$ % (v/v) for anti-P. acnes bioactivity. Seasonal and geographical variation for all three bioactivities was detected. Antifungal bioactivity assays revealed median percent growth of treated *C. albicans* cells to range between 38-54% with more variation in growth observed in season one (spring).

The most potent extract in this experiment was seen in season one from land block H; however, in season two (autumn) land block H was the least potent (96%) and land block F was the most potent (32%). Antibacterial bioactivity assays revealed median percent growth of treated MRSA to range between 35-79% with variation consistent in both seasons. The most potent effects were seen in season one from land block H (28%) with the less effective coming from land block E where in some cases there was no effect on treated cell conditions. Finally, anti-P. acnes bioactivity assays revealed median percent growth of treated *P. acnes* cells to range between 62-73% with more variation observed in season two. The most potent effects were seen in season one from land block B (~43%) with the less effective coming from season two land block H (89%). GC-MS analysis distinguished α-pinene as the most abundant compound (~34%) and multivariate analysis identified lead compounds such as α-Murolene, isoamyl isovalerate for antifungal activity and compounds such as limonene and nerolidol for anti-MRSA and anti-P. acnes bioactivity. The unidentified compound, unknown[9] was particularly interesting as it was the only compound to positively correlate to all three bioactivities.

**Conclusion:** Kānuka oil extracted from Te Tairawhiti exhibits antifungal, anti-MRSA and anti-P. acnes properties that show seasonal and geographical variation. The results from this study provide scientific validation for the medicinal benefits of kānuka oil that can be further explored for pharmaceutical potential.

# Dedication

This thesis is dedicated to my father Omeka Vincent Hurunui who passed away unexpectedly during this study. Thank you for all the sacrifices you made for me, for the lessons you taught me, for always supporting me in whatever I wanted to pursue and most of all for your unconditional love.

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# Abbreviations

| | |
|---|---|
| AIDS | Acquired immune deficiency syndrome |
| CAS | Chemical abstracts service |
| DNA | Deoxyribonucleic acid |
| DMSO | Dimethyl sulfoxide |
| ESRI | Environmental Science and Research Institute |
| GCMS | Gas chromatography mass spectrometry |
| HIV | Human immunodeficiency virus |
| IFI | Invasive fungal infections |
| KH | Kānuka honey |
| LB | Luria-Bertani |
| LUQ | Left upper quadrant |
| LLQ | Left lower quadrant |
| MDR | Multiple drug resistant |
| MGO | Methylglyoxal |
| MH | Mānuka honey |
| MIC | Minimum inhibitory concentration |
| MRSA | Methicillin-resistant *staphylococcus aureus* |
| MVA | Multivariant analysis |
| OD | Optical density |
| PCA | Principal component analysis |
| PLSR | Partial-least-squares-regression |
| RT | Retention time |
| RUT | Right upper quadrant |
| RLQ | Right lower quadrant |

| | |
|---|---|
| SI | Similar index |
| SC | Synthetic Complete |
| SS | Statistically significant |
| T0 | Time 0 |
| TS | Tryptic Soy |
| UMF | Unique mānuka factor |
| WRS | Weighted regression co-efficient score |

# 1.0 Chapter 1: Literature Review

## 1.1 Introduction to Kānuka

Since the early 1800's, botanists such as Hooker, Cheeseman and Harris have documented extreme variation in New Zealand's kānuka population comprised of *Kunzea ericoides* (Cheeseman, 1906; de Lange et al., 2004; Harris, 1996; Hooker, 2011). Recent taxonomy studies investigating the New Zealand population of *K. ericoides* have revealed as many as ten different kānuka species that are all endemic to New Zealand (de Lange, 2014). This identification has been achieved by comparing and investigating morphology, cytology, and DNA sequences (de Lange et al., 2005; de Lange, 2014). There are over 20 macroscopic and microscopic features such as growth habitat, bark and leaves that have determined these species (de Lange, 2014). Novel species identified include *K. triregensis, K. amathicola, K. salterae, K. serotina, K. robusta, K. tenuicaulis* and *K. toelkenii* (de Lange, 2014). Other identified species endemic to New Zealand include *K. ericoides, K. linearis* and *K. sinclairii.*

Some of these species can be easily identified via geographic origin; for example, *K. triregensis* and *K. sinclairii* species can only be found on the Three Kings Islands and Great Barrier Island, respectively (de Lange, 2014; Harris, 1996). *K. tenuicaulis* is the most diverged species and appears to be endemic to geothermal habitats. *K. robusta* is the tallest species in the genus and also the most abundant and widespread with high prevalence in the East Coast region (de Lange, 2014; Stephens et al., 2005). For some species, the differences are less obvious and more evidence to classify species is achieved by investigating molecular characteristics such as chromosome differences and scanning electron microscopy to identify other microscopic features such as differences in branchlet indumentum (de Lange et al., 2004;

de Lange, 2014). For instance, the species with large leaves such as *K. sinclairii* have large and small chromosomes whereas the species with small leaves have only small chromosomes. Additionally DNA sequences of large leaf taxa are also distinct from small leaved taxa (de Lange et al., 2004; de Lange et al., 2010).

## 1.2 The Relationship of Kānuka and Mānuka

New Zealand is home to native oil-producing trees mānuka and kānuka that are also known as *Leptospermum scoparium* and *Kunzea ericoides*, respectively (Perry et al., 1997). They are both flowering plant species in the myrtle family, Myrtaceae, and are both commonly referred to as tea trees (Chen et al., 2016). Both are distributed throughout New Zealand as small-to-large trees or shrubs in subalpine zones and lowland environments (Perry et al., 1997). Kānuka is considered endemic to New Zealand and mānuka is considered not only the most resilient native tree in the New Zealand woody flora but also the most abundant and most widely distributed (Häberlein et al., 1994; Perry et al., 1997).

At first glance both plants have a similar appearance and can easily be mistaken for one another (Stephens et al., 2005). Upon closer observation, there are distinct features (Park, 2014). They differ in terms of their height, seed capsule size, foliage texture and flowers (Prakash et al., 2008). Mānuka trees have more dispersed flowers and larger seed capsules that remain on the tree for several years (Saunders, 2017). Comparatively, the flowers on kānuka trees grow in clusters, their seed capsules are much smaller and typically shred within the year (Patel et al., 2013; Saunders, 2017). The easiest way to tell the two apart is by touching the foliage of both trees (Centre., 2015; Conservation., 2017). Mānuka has prickly leaves whereas kānuka leaves are soft (Essien et al., 2019). Mānuka typically grow as shrubs ~4-8 metres tall capable of growing in all types of soil included waterlogged areas (Essien et al., 2019; Park, 2014). In contrast, kānuka can potentially grow as trees ~6-20 metres tall that require well-

drained soil and are more likely to live longer compared to mānuka (Chen et al., 2016; Park, 2014). Further to these physical differences, they also have genetic, molecular and chemical differences (Maddocks-Jennings et al., 2005).

## 1.3 Drug Discovery

### 1.3.1 Pipeline of drug discovery

Drug discovery is the identification of novel compounds and/or druggable targets (Harvey, 2008; Knight et al., 2003). These drug targets, for example in the case of an antimicrobial drug, are usually essential components of a disease-causing microbe that are required for its survival (Atanasov et al., 2021; Gershell et al., 2003). For instance, effective drug targets may include surface proteins, genes and ribonucleic acid (RNA) that are required for cell viability (Hughes et al., 2011). A diverse range of materials can be sourced from nature to produce medicines with therapeutic benefit (Chandrababu et al., 2020; Devi, 2020). This includes parts of plants and trees as well as animals and microorganisms (Yuan et al., 2016). Successful drug discovery can be a laborious and expensive process, on average costing billions of dollars and taking a decade or more to get to the market (Enzmann et al., 2019).

One approach to drug discovery starts with identification and validation of a drug target associated to a disease of interest (Aslam et al., 2020; Atanasov et al., 2021; Devi, 2020; Koehn et al., 2005). The next step is to utilise high-throughput screening methods to identify ‘hits’; this step eliminates hundreds of thousands of compounds and only the most efficacious drug candidates remain (Abdel-Razek et al., 2020; Devi, 2020). Hits are then validated to identify ‘likely drug candidates or ‘lead compounds’ (Devi, 2020). These lead compounds then undergo optimisation which involves testing the safety and efficacy of the drug (Abdel-Razek et al., 2020; Li et al., 2009). Those that are promising in the preclinical stage will then move forward

with clinical trials seeking final approval to enter the market by an appropriate drug administration such as the U.S. Food and Drug Administration (FDA) (Fisher et al., 1999; Koehn et al., 2005)). The target needs to be safe, effective, and meet clinical and commercial needs (figure 1.1) (Enzmann et al., 2019; Watkins, 2011).

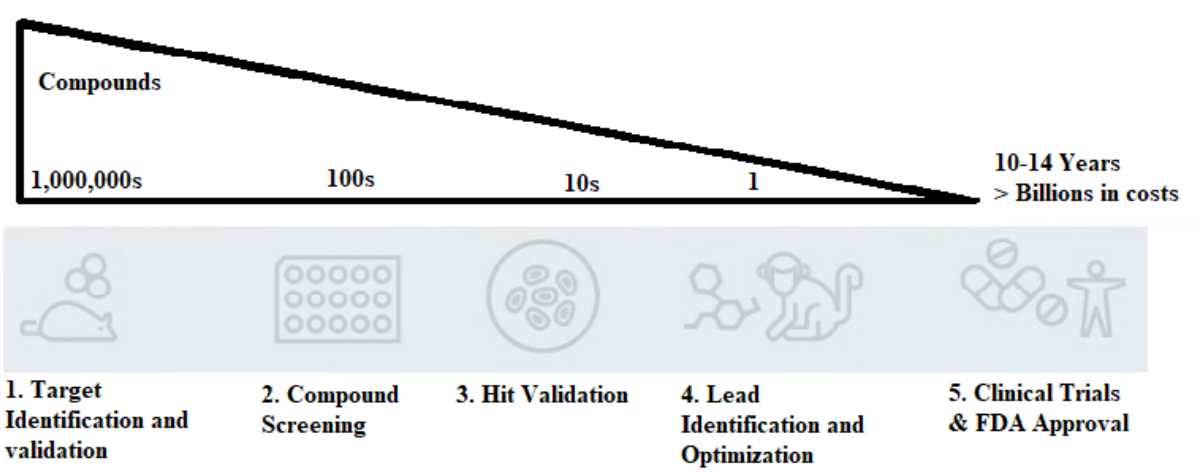



**Figure 1. 1: One form of the drug discovery pipeline.**

### 1.3.2 History of Drug Discovery and Natural Products

Prehistoric records show that natural products were used to treat illness or disease in many regions and cultures around the world (Koehn et al., 2005; Petrovska, 2012). For example, Ebers Papyrus recorded over 700 drugs in Egyptian culture (2900 B.C.), primarily plant-based, that were infused into pills and ointments to treat various conditions (Aboelsoud, 2010; Li et al., 2009). Traditional Chinese medicine (TCM) utilised natural products derived from herbs, spices and plants that are well documented in Marteria Medica as early as 1100 B.C (Patwardhan et al., 2005; Tang et al., 2008). The antimalarial drug Artemisinin has been used in TCM for over 2,000 years to treat fever and is still used today as a frontline treatment of malaria (Miller et al., 2011; Normile, 2003; Patwardhan et al., 2005).

Exploration like this paved the way for the development of early drug discovery using natural products which have since been studied, validated, and are still used to treat medical conditions today (Dias et al., 2012; Gershell et al., 2003). The most pronounced discovery that revolutionised medicine by saving millions of lives particularly during World War II and arguably responsible for the "greatest therapeutic impact event of all time" (Kardos et al., 2011) was the discovery of the antibiotic Penicillin (Gaynes, 2017; Markel, 2013). Penicillin is derived from common moulds known as *Penicillium,* and it was the first medication to be effective against many common and fatal infectious diseases caused by *Staphylococci* and *Streptococci* (Dias et al., 2012; Hughes et al., 2011). The clinical benefits of Penicillin were "large and immediate" (Kardos et al., 2011) with widespread global use resulting in an increase to life expectancy (Gershell et al., 2003; Kardos et al., 2011).

During the early 19$^{th}$ and 20$^{th}$ century, natural products were extensively studied for potential sources of novel human therapy, and by 2002 approximately 50% of new compounds were directly or partly sourced from natural products (Devi, 2020; Gruber et al., 1996; Koehn et al., 2005). Despite this success, the last 20 years has seen a reduction in the use and targeting of natural products by the pharmaceutical industries (Lahlou, 2013; Rishton, 2008). The major contribution of this shift is extensive production time and resource constraints that arise when producing drugs from natural extracts (Galanie et al., 2015; Potterat et al., 2006; Terrett et al., 1995). Biochemical analysis and high-throughput screening along with the availability of gene-editing, CRISPR and bioengineering have accelerated modern drug discovery processes and dampened natural extract screening operations (Enzmann et al., 2019; Liu et al., 2004; Singh et al., 2020). Synthetic *in vitro* bioassays and *in silico* modelling of molecular interactions have been regarded to be cost effective, easy to implement and predicted to discover lead drug compounds much more effectively than screening natural products (Mishra et al., 2008; Rishton, 2008). However, problems soon surfaced, when it was obvious production rates were

low despite increased funding into research and resources as well as the predicted benefits of high-throughput screening (Enzmann et al., 2019; Mishra et al., 2008).

Thus the demand for new drugs persists and is rapidly increasing, particularly due to the rise in multidrug-resistant organisms (Harvey et al., 2015; Hughes et al., 2011). The future of drug discovery is likely to consist of both natural extract operations alongside high-throughput screening, which can be achieved through incorporating methods such as prefractionation and chemical conditioning (Lahlou, 2013; Rishton, 2008). It is undeniable that the quality, quantity and structural diversity of compounds found in nature is vast and its potential is yet to be fully elucidated (Abdel-Razek et al., 2020; Aslam et al., 2020; Atanasov et al., 2021). Many plant species as well as organisms such as bacteria, fungi and sponges remain untapped sources of medicinal value that emphasise the importance of continued drug discovery using natural products (Cragg et al., 2013; Li et al., 2009).

### 1.3.3 Rongoā Māori (Traditional Māori Medicine)

Like many parts of the world, New Zealand to has its own history of traditional medical practices using natural resources (Mark et al., 2017). Rongoā Māori or traditional Māori medicine is a healing system used by Māori, the indigenous people of Aotearoa (New Zealand) (Beresford et al., 2006). Rongoā Māori utilised resources such as rongoā rākau (plant medicines) to successfully treat a variety of ailments (Johnson, 2012; Mark et al., 2010). For example, kawakawa (*Macropiper excesum*) leaves were refined to a paste used to treat toothaches or inflammation to the mouth and cheek region (Beresford et al., 2006; Koopu et al., 2020; Mark et al., 2017). Harakeke (*Phormium tenax*) leaves and roots were roasted and pulped to treat inflamed joints, ringworm and skin irritations (Mark et al., 2012; Matau, 2008).

Essentially different preparations of rongoā were undertaken depending on what illness or condition it was targeted to treat (Ahuriri-Driscoll et al., 2008; Nicolson, 2017).

Mānuka and kānuka are well recognised rongoā rākau, and, from a rongoā Māori perspective, have proven to be successful at treating a range of medical conditions (Ahuriri-Driscoll et al., 2008; Johnson, 2012). For example, Māori soaked the leaves and bark in bath water to produce vapour to treat eye, mouth and throat problems as well as colds, coughs and inflammation of the breast (Lis-Balchin et al., 2000; Wyatt et al., 2005). The leaves were chewed to treat dysentery, and also made into concoctions that were used to reduce fevers and treat urinary tract infections (Beresford et al., 2006; Porter et al., 1999). The bark and seed capsules were used in a variety of ways to treat and cure many inflammatory ailments such as diarrhoea as well as illnesses caused by bacterial, fungal or viral infections (Carter et al., 2016; Chen et al., 2016; Häberlein et al., 1994). Kānuka was applied to wounds caused by fire and sunburn, and was also used as a sedative to help relieve stress (Jones, 2000; Lis-Balchin et al., 2000).

Kānuka is yet to be as widely studied as mānuka. Although aroma therapists have used kānuka for years, there is still inadequate information available regarding the safety and efficacy of this natural product (Johnson, 2012; Lis-Balchin et al., 2000). To compare the difference in data availability of mānuka and kānuka, a Pubmed search of mānuka displayed over 423 publications compared to 136 publications for kānuka (PubMed, 2019, 2019). The under-representation of kānuka research alongside traditional evidence of the potential medicinal benefits of kānuka suggest further research is critical (Ahuriri-Driscoll et al., 2008; Beresford et al., 2006).

## 1.4 Antifungal Activity

### 1.4.1 Fungal infections

The Kingdom Fungi contains over 6 million species that are among the most widely distributed organisms in the world, and therefore have a profound impact on the environment and its inhabitants (Fisher et al., 2020; Köhler et al., 2015). Approximately 200 fungal species are known to be pathogenic to humans and approximately 8000 species are known to be pathogenic to plants (Campoy et al., 2017; Fisher et al., 2020). Fungal pathogens pose an increasing threat to global human health and global food security as the incidence of drug resistant fungal pathogens continues to rise at an unprecedented rate (Garber, 2001; Rodrigues et al., 2020). Global vegetation that is affected by fungal pathogens is approximately 20% worldwide with another 10% of crops affected after harvest (Fisher et al., 2020; Fisher et al., 2018). Fungicides have been developed to counteract this issue; however, the problem persists with rapid resistance developing to many of these chemicals (Fisher et al., 2020; Fisher et al., 2018).

Human population growth and advances in medical care mean more individuals are living longer despite having weakened immune systems, and it is these individuals that are most at risk of fungal infections, presenting huge challenges for the healthcare system (Fisher et al., 2020; Köhler et al., 2015). Fungal pathogens are either primary pathogens or opportunistic pathogens that can cause superficial, subcutaneous and systemic infections (Kim et al., 2020; Rodrigues et al., 2020). Superficial fungal infections are caused most often by opportunistic fungi such as *Candida albicans* that colonise the keratinised layer of the skin resulting in conditions such as oral and vaginal thrush, and if not treated promptly can spread to systemic infections (Brown et al., 2012; Fisher et al., 2020; Thiel, 2010). Subcutaneous fungal infections affect the dermis and subcutaneous tissues (Chiller, 2019; Denning et al.,

2015). Dermatophytes such as *Epidermophyton* and *Microsporum* are the most common fungi causing these infections, which include well-known conditions such as athlete's foot and ringworm (Brown et al., 2012; Paswan et al., 2019). Systemic infections, also known as invasive fungal infections (IFI), are often life-threatening with high rates of mortality with the infection spreading to critical structures such as the blood or essential organs (Badiee et al., 2014; Kim et al., 2020). IFI are primarily due to infections caused by *Candida, Cryptococcus, Aspergillus,* and *Pneumocystis* species (Brown et al., 2012; Chakrabarti et al., 2005; Fisher et al., 2020). The most predominant fungal species known to cause infection in a hospital setting is *C. albicans* with IFI due to *C. albicans* (invasive candidiasis) affecting 1 in 10,000 hospitalised persons in the USA (Linton et al., 2007; Paswan et al., 2019).

IFI due to *Aspergillus* have recently been identified alongside severe COVID-19 infections; for example, over 30% of COVID-19 patients in intensive care in France also tested positive for invasive aspergillosis (Marion et al., 2020; Scharping, 2021; Verweij et al., 2020). Treating patients with these two deadly infections is a huge challenge facing clinicians today particularly with the fungal pathogens being resistant to currently available antifungal drugs, hence many of these critically ill patients will die (Benedict et al., 2018; Verweij et al., 2020). The rise in antifungal drug resistance and cost to maintain effective treatment is becoming increasingly problematic; the United States spends a staggering $2.6 billion per year just to treat infections caused by *Candida* spp. (Fisher et al., 2018; Loeffler et al., 2003; Pfaller, 2012). It is clear that fungal pathogens are a current worldwide health and food security issue with unavoidable high costs and with endemics close approaching, the need for new and effective antifungal treatments is critical in the medical and agricultural sectors (Pierce et al., 2019).

### 1.4.2 Antifungal drugs

In recent years there has been a global upsurge in the incidence of fungal infection and disease (Brown et al., 2012; Kim et al., 2020). Billions of people are infected worldwide and over 1.5 million people are dying each year due to fungal infections; these statistics are comparable to some of the leading causes of death such as tuberculosis, malaria, HIV and breast cancer (Denning et al., 2015; Fisher et al., 2018). The need for effective diagnosis and treatment is critical to addressing this health issue today (Eliopoulos et al., 2002; Fisher et al., 2018). Antifungal drugs licensed to treat human disease differ in terms of their molecular target, of which there are only five unique molecular targets for currently approved antifungal drugs (Campoy et al., 2017; Kong et al., 2020). The types of antifungal drugs include azoles, echinocandins and amphotericin B (AmB), which from these types, fluconazole is the most commonly prescribed antifungal drug and in many countries it is the only available treatment (Farha et al., 2019; Kong et al., 2020). However, azoles do present with problems in treating some fungal infections such as *Aspergillus fumigatus,* which has developed a high prevalence of resistance to *A. fumigatus* in humans (Campoy et al., 2017; Farha et al., 2019). As a consequence, treatment has failed and mortality rates have increased to almost 100% in these individuals (Fisher et al., 2018; Paassen et al., 2016).

AmB is the standard treatment for systemic mycoses or life-threatening fungal infections from species such as *Candida, Cryptococcus* and *Aspergillus* (Dismukes, 2000; Fisher et al., 2018). However, these drugs are known for their adverse side effects such as chills, fever and in some cases more serious side effects such as renal tubular damage (Campoy et al., 2017; Fostel et al., 2000). Echinocandins are capable of treating invasive *Candida* infections and are commonly used as an alternative to amphotericin B and azoles, or in combination with azoles to give a more effective treatment (Munro, 2013; Walsh, 2002).

Fungal pathogens are eukaryotic, which means fungi share many similar molecular features to human host cells, which means antifungal drugs must specifically target fungal cells and not impact human cells (Bassetti et al., 2015; Eliopoulos et al., 2002; San-Blas et al., 2008). It is clear that further research into improving antifungal drug therapies is paramount if we are to provide effective treatments against fungal infections and antifungal resistance (Campoy et al., 2017; Fostel et al., 2000).

### 1.4.3 Mechanisms of antifungal drug resistance

Antifungal resistance is a feature that arises when fungi become insensitive to a drug that was previously effective (Cowen et al., 2015; Denning et al., 2015; Paswan et al., 2019). It is a process based on parallel evolution and natural selection where species can acquire genetic mutations that allow them to achieve resistance (Balkis et al., 2002). Three common genetic mechanisms to evolve resistance can occur in fungal species (Heitman, 2011; Kim et al., 2020; Wilson et al., 2002). Firstly, mutations that result in conformational changes to the drug target site will in turn result in the drug being unable to bind and have an effect (Badiee et al., 2014; Balkis et al., 2002; Fisher et al., 2018). Secondly, changes to promoter regions can result in the up-regulation of the drug target sites and the drug dosage becoming ineffective at treating the infection (Balkis et al., 2002; Fisher et al., 2018). Finally, resistance can evolve via the up-regulation of efflux pumps that work by pumping out any drug that enters the cell (Fisher et al., 2020; Fisher et al., 2018; Fostel et al., 2000).

One of these mechanisms seen in resistance of *A. fumigatus* to antifungals is the evolution of tandem repeats in a promoter region with upstream single-nucleotide polymorphisms (SNPs) that give rise to an azole-resistant phenotype; individuals infected with

this resistant strain have poor prognosis with death resulting in 90% of individuals (Balkis et al., 2002; Fisher et al., 2020; Paassen et al., 2016). Moreover, a study from the Netherlands shows that more than 25% of patients presenting with *Aspergillus* strain infections carry the allele conferring this resistance (Fisher et al., 2018; Paassen et al., 2016).

Major contributing factors to resistance include the regulated overuse of antifungal agents, as well as their long-term use in vulnerable patients. (Balkis et al., 2002; Cowen et al., 2015). For instance, clinical azole-resistance can arise in patients with HIV that are on long-term treatment of prophylactic drugs for preventative measures (Balkis et al., 2002; Fisher et al., 2020; Kim et al., 2020). Fungal genomes are highly plastic and undergo rapid population growth, these attributes mean mutations and generation of antifungal-resistant alleles evolve quickly at a rate that far surpasses the discovery rate of new antifungal treatments (Heitman, 2011; Pierce et al., 2019; Rodrigues et al., 2020). For example, *Candida auris* poses a current global threat to hospital acquired systemic infections as it withstands standard hospital room decontamination procedures and is now resistant to all current forms of antifungal drugs (Dismukes, 2000; Fisher et al., 2020; Kong et al., 2020). The development of antifungal resistance means fungal pathogen control is short-lived (Kong et al., 2020; Pierce et al., 2019; Rodrigues et al., 2020). The discovery of new modes of action, development of novel drug targets and understanding how specific mechanisms develop resistance are critical to discovering safe and effective antifungal drugs (Cowen et al., 2015; Kong et al., 2020; Rodrigues et al., 2020). It is apparent that more research into antifungal treatments is an urgent health issue that needs to be addressed before things spiral out of control (Chiller, 2019; Cowen et al., 2015).

### 1.4.4 Candida albicans

*Candida albicans* is an opportunistic pathogenic yeast that can be both neutral and harmful to human health (Calderone et al., 2001; Mayer et al., 2013). It exists as a commensal microorganism in the gut, oral cavity and vaginal mucosa and can therefore cause disease at multiple locations throughout the body (Calderone et al., 2001; Kullberg et al., 2015). Candidiasis is the third leading cause of hospital acquired infection in the USA and worldwide has a striking 35% mortality rate (Pagliano et al., 2020; Vallabhaneni et al., 2016). The cost to treat these patients in the USA is approximately $2-4 billion dollars per year (Wilson et al., 2002; Zaoutis et al., 2005). Patients that are immunocompromised due to conditions such as Acquired immune deficiency syndrome (AIDS) or Human immunodeficiency virus (HIV) are at higher risk of infection with an estimated 70% of AIDS patients in the USA also developing oropharyngeal candidiasis (Brown et al., 2012; Horton et al., 2020).

*C. albicans* invades its host cell via induced endocytosis or active penetration; these mechanisms are achieved with the assistance of a wide range of virulence factors (Calderone et al., 2001; Lockhart et al., 2016; Mayer et al., 2013). Expression of adhesin and invasin proteins aid with host infiltration, production of biofilms on medical devices, and expression of virulence factors that cumulatively facilitate *Candida* pathogenesis (Chen, 2020; Prasad, 2017). The incidence and burden of *Candida* infections is predicted to increase, which will result in more deaths and more costs to the health systems worldwide (Calderone et al., 2001; Horton et al., 2020). The need for further research and development of antifungal drugs is thus essential to addressing or at less mitigating some of the pressures of infections due to *Candida* species (Microbiology, 2017; Uppuluri et al., 2017).

## 1.5 Antibacterial Activity

### 1.5.1 Bacterial pathogens

The majority of bacterial species are harmless or even beneficial to humans via commensal and mutualistic symbioses (Alter et al., 2011; Le Guennec et al., 2020). In humans, bacteria can be found on the skin, in the nose, mouth and throughout the digestive and genitourinary systems (Kho et al., 2018; Wilson et al., 2002). Bacteria are acquired from birth and in the first years of life through diet, environment, and interactions with other living organisms (Alter et al., 2011; Pushkareva, 2020). Bacteria that are pathogenic are those that can cause human disease and illness; for example, *Mycobacterium tuberculosis* is one of the top ten most deadly diseases in the world causing more than 10 million new cases and 2 million deaths per year worldwide surpassing incidence and mortality caused by HIV/AIDS (Tang et al., 2016; Viela et al., 2020; Yates et al., 2016). *Streptococcus, Haemophilus,* and *Pseudomonas* species are common causes of pneumonia that is a significant global health issue particularly in children (Brooks, 2020; Chellammal, 2014; Craig et al., 2009; Tandon et al., 2008).

Bacterial pathogens also commonly cause skin infections such as cellulitis, folliculitis, impetigo and boils (Hedrick, 2003; Sethi, 2020; Sukumaran et al., 2016). Bacterial skin infections are the 28$^{th}$ most common cause of hospitalisation in the USA, which are predominantly caused by *Staphylococcus* and *Streptococcus* species (Bassetti et al., 2020; Sethi, 2020; Stulberg et al., 2002). *Shigella*, *Campylobacter* and *Salmonella* species are common causes of mild to severe cases of foodborne illnesses (He et al., 2020; Jin et al., 2020; Oldfield III, 2001). *Campylobacter* species are the leading cause of diarrhoea in the USA and *Salmonella* is reported to cause over 2.4 million infections in the US per annum (Oldfield III, 2001; Tauxe, 2002). Foodborne illness is an important and increasing global health issue with

more than 200 diseases associated with bacterial infections (Jin et al., 2020; Koluman et al., 2013). *Chlamydia trachomatis, Neisseria gonorrhoeae, Treponema pallidum* are common causes of sexually transmitted infections that are routinely reported in primary care health worldwide (de Lima et al., 2014). Pathogenic bacteria can also cause other serious illnesses such as meningitis, diptheria, and tetanus. (Alter et al., 2011; Kollef, 2008). In developing countries, childhood/infant mortality and morbidity due to pathogenic bacterial infections is a serious health issue. Together, all of these scenarios highlight the need for improved treatments to combat pathogenic bacterial species (Alter et al., 2011; Osrin et al., 2004).

### 1.5.2 Antibacterial Drugs

Antibacterial drugs, commonly referred to as antibiotics, work by destroying or inhibiting the growth of bacteria and are often derived from natural products (Barzic et al., 2015; Leekha et al., 2011). Classification of antibiotics is determined by its chemical composition, mechanism of action and spectrum of activity (Calderón et al., 2007; Mugumbate et al., 2015). For instance, some commonly prescribed antibiotics include β-lactam antibiotics that work by targeting cell wall synthesis, tetracyclines that inhibit protein synthesis, fluoroquinolones and rifampin that target nucleic acid synthesis, and daptomycin that targets the membrane structure (Béahdy, 1974; Etebu et al., 2016). There are two major groups of antibiotics that either target Gram-negative bacteria or Gram-positive bacteria (Devasahayam et al., 2010; Mugumbate et al., 2015). Some broad-spectrum antibiotics such as ampicillin are able to target both Gram-negative and Gram-positive bacteria and thus can be used in critical situations when the underlying pathogenic bacteria is unknown (Projan et al., 2007; Simmons et al., 2010). Narrow spectrum antibiotics such as nafcillin target only Gram-positive bacteria (Caniça et al., 2019; Sugden et al., 2016). This is useful when the disease-causing microbe is

known, causing less collateral damage and slowing the inevitable burden of bacterial drug resistance (Giedraitienė et al., 2011; Melander et al., 2018).

Early discovery of antibacterial agents such as streptogramins, β-lactams, quinolones and tetracycline provided a huge relief to the burden of bacterial related diseases and infections in the 1930-1940s (Devasahayam et al., 2010; Zervosen et al., 2012). However, prolonged use, misuse and overuse of antibiotics over decades of use has led to the development of drug-resistant bacterial strains, many which are now resistant to all currently available treatments (Walsh, 2000; Zervosen et al., 2012). The World Health Organisation (WHO) has estimated that by 2050 more than 10 million deaths will result from bacterial infections, which will cost global health systems more than $10 trillion USD (Ferrante et al., 2019; Theuretzbacher, 2012). Thus there is an urgent need for global strategies to discover and develop new antibiotic drugs (Miller et al., 2019; Theuretzbacher, 2012).

### 1.5.3 Mechanisms of antibacterial drug resistance

First generation penicillin-based compounds are prime examples of successful antibacterial drugs that have declined in use due to antibacterial drug resistance (Giedraitienė et al., 2011; Hashim et al., 2020; Lowy, 2003). Penicillin works by inhibiting enzymes that synthesize the cross-linking proteins of the peptidoglycan layer that surrounds the bacterial cell wall (Lobanovska et al., 2017; Marko, 2020). Without this layer, the cell loses its ability to maintain the correct osmotic gradient resulting in cell lysis and death (Giedraitienė et al., 2011; Marko, 2020).

These first-generation penicillin compounds were introduced in 1941 and subsequently saved millions of lives paving the way for the antibiotic era (Aminov, 2010; Levy et al., 2004).

Almost immediately, bacteria started to develop resistance and by 1942 cases of penicillin-resistant *Staphylococci* were detected in hospitals and by 1960 over 80% of *Staphylococci* isolates sourced from the hospital and community were resistant to penicillin (Abraham et al., 1988; Kirby, 1944). Bacteria can acquire resistance to one or more types of drugs through numerous and complex mechanisms, such as the transfer of resistant genes from one bacteria to another (Brown et al., 2016; Devasahayam et al., 2010). Acquired antibiotic resistance can occur through genetic mutations, vertical and/or horizontal gene exchange via plasmids, transposons and/or integrons (Spratt, 1994; Tenover, 2006). For example, *E. coli* has developed complete resistance to rifampin via a single point mutation in the *rpoB* gene (Rice et al., 2007). Bacterial can also obtain innate resistance through spontaneous development as a means of survival.

Bacteria also utilise biochemical mechanisms to help defend against antibiotic invasion; for instance, bacteria that produce β-lactamase enzymes are resistant to the commonly used β-lactam antibiotics (Jacoby et al., 2005). These enzymes enable the bacteria to hydrolyse the pharmacophore of the drug and deactivate its antimicrobial properties (Mims et al., 2004). This resistance is common and has dramatically limited the use of these previously effective antibiotics (Mugumbate et al., 2015). Bacteria are also capable of other biochemical mechanisms to reduce exposure to antibiotics via cell wall modifications, efflux pumps, reduced drug uptake, changes to the drug target site and upregulation of the drug target site (Alekshun et al., 2007; Wickens et al., 2005). (Giedraitienė et al., 2011).

### 1.5.4 Methicillin-resistant *Staphylococcus aureus* (MRSA)

MRSA is a type of *Staphylococcus* (staph) bacteria that has become resistant to many of the antibiotics used to treat ordinary staph infections (Stefani et al., 2012). With *S. aureus* being a Gram-positive, nonmotile bacterium, it is the most clinically relevant of all staph species. MRSA was initially a leading cause of hospital-acquired infections, but now community-acquired infection is just as prevalent (Abdel-Razek et al., 2020). MRSA can gain entry into the human host through breaks in the skin barrier caused by conditions such as surgical interventions, wounds, burns, acne and eczema. The level of MRSA infection varies from mild skin infections that are manageable to more aggressive infections that can become progressively worse if treated unsuccessfully (Vitko et al., 2013). Areas of soft tissues and/or the bloodstream can become infected, leading to fatal conditions such as sepsis that are difficult to treat. People at high risk of infection are those who are immunocompromised, have invasive medical devices such as pacemakers or live with pre-existing chronic diseases such as diabetes (Stefani et al., 2012).

The reported global prevalence of MRSA shows variation in demographic distribution due to factors such as living conditions, population size and country resources (Lee et al., 2018). The burden of MRSA shows low prevalence in Scandinavian countries to greater prevalence in parts of South America such as Brazil and Chile as well as parts of Asia such as Japan, Sri Lanka and South Korea (Lee et al., 2018).

*S. aureus* strains gain resistance by acquiring the mobile genetic element SCCmec (staphylococcal cassette chromosome mec) via horizontal gene transfer. This *gene* encodes for *mecA* and *mecC* responsible for modifications to the penicillin-binding protein and thus creates resistance to most β-lactams antibiotics and other classes of antibiotics (Chambers & DeLeo, 2009; Gerlach et al., 2018).

Millions of people are infected with MSRA per year and the cost to keep up with the demand to treat this infection is a significant concern. The mean cost to treat someone infected with MRSA in the USA is US$9100 and this often not successful (Hübner, Hübner, Hopert, Maletzki, & Flessa, 2014). Therefore, it is paramount that better treatments be developed to reduce the burden and impact of MRSA on global world health.

## 1.6 Anti-P. acnes Activity

### 1.6.1 Acne vulgaris

Acne vulgaris (acne) is the most common skin disease that causes inflammation of the pilosebaceous follicles and this is most prominent during adolescence (Jeong et al., 2017; Rivera, 2008). Common clinical presentations of this disease appear on the face, neck, chest and back regions that are classified as either non-inflammatory lesions such as open and closed comedones or inflammatory lesions such as papules, pustules and nodules (Purvis et al., 2006; Rivera, 2008). The prevalence of acne is widespread affecting approximately 85-100% of people at some stage in life and some cases persisting into late adulthood, over 95% of people affected will develop some form of scarring (Collier et al., 2008; Jeong et al., 2017). Persistence and scarring from acne can affect a person's quality of life and lead to mental health problems and psychological disorders (Purvis et al., 2007; Ramrakha et al., 2016) (Purvis et al., 2006; Ramrakha et al., 2016).

The pathogenesis of acne is a multifactorial process that includes four major processes such as sebaceous gland hyperactivity, hyper colonisation of *Propionibacterium acnes* in the sebaceous duct, alterations to keratinisation within the follicle and release of inflammatory cytokines such as TNFα, interleukin (IL)-1β, IL-8 and IL-6 into the skin. The major

determinant of acne formation, development and inflammation is heavily influenced by the presence of *P. acnes* and thus many anti-P. acnes treatments attempt to inhibit the growth of *P. acnes*.

### 1.6.2 Anti-P. acnes drugs

Topical retinoids, topical and oral antibiotics, antimicrobial agents and hormonal therapies are common and acceptable forms of treatment for acne (Gollnick et al., 1998). The type of treatment used differs for each individual and depends on factors such as acne severity, age, pregnancy, pre-existing medical conditions and whether the condition is relapsing (Karadag et al., 2021). For instance, retinoids are derivatives of vitamin A and work by reducing hyperproliferation and inflammation of the epithelium. The three main types of topical retinoids include tretinoin, adapalene and tazarotene, with tretinoin being the most widely used. Approximately 75% of people that use retinoids will experience side effects that include dryness, burning, irritation, peeling and possibly light sensitivity. Retinoids are the first line of treatment for comedones and non-inflammatory mild to moderate forms of acne. However, its high risk of side effects warrant close monitoring by clinicians and optimising treatment concentrates to meet individual needs (Walsh et al., 2016).

Benzoyl peroxide (BPO) is the most widely used antimicrobial agent to treat mild acne conditions (Otlewska et al., 2020). It inhibits the growth of *P. acnes* by producing reactive oxygen species into the sebaceous follicle region (Taylor et al., 2004). BPO is more effective when taken as a combination therapy alongside either tretonin or topical antibiotics. However, 1-3% of patients will develop an allergic reaction to this treatment, and other patients will find it unfavourable due to it bleaching the skin (Hsiao et al., 2020).

Topical antibiotics such as clindamycin, erythromycin and tetracycline are also commonly used to treat inflammatory acne at a mild to moderate level (Karadag et al., 2021). However, with the increase in antibiotic resistance of *P. acnes*, topical antibiotics are rarely given as a monotherapy and more often prescribed in combination with BPO or tretonin (Walsh et al., 2016). Oral antibiotics are also an option for patients that suffer adverse effects to topical treatments; these systemic antibiotics have both antimicrobial and anti-inflammatory properties with the most widely used being tetracycline, doxycycline, minocycline and erythromycin (Hsiao et al., 2020). Current research suggests topical antibiotics have greater efficacy and reduced side effects such as dyspepsia, candidiasis, dental problems and ulceration (Leyden, 2001).

Although a range of therapeutic options currently exist to treat acne, low compliance and re-occurrence continue to be major issues and are usually due to side effects and the suitability or choice of treatment for individual patients. In some cases all treatment options have been exhausted and little can be done for these individuals (Karadag et al., 2021). It is therefore necessary to continue to explore other avenues for acne treatment.

### 1.6.3 Impact of anti-P. acnes drug resistance

The most common disadvantage of topical and oral antibiotics is the high risk of developing resistance to *P. acnes* particularly when the antibiotics are used as a monotherapy (Leyden, 2003; Paswan et al., 2019). Strains of *P. acnes* with resistance to erythromycin have been reported in over 50% of patients treated with oral erythromycin and approximately 40% of patients treated with clindamycin (Karadag et al., 2021; Otlewska et al., 2020). These two

antibiotics are still commonly prescribed because side effects are infrequent and when present are usually minor (Bojar et al., 2004; Karadag et al., 2021).

Cross resistance has also been demonstrated in some strains of *P. acnes* with resistance to both erythromycin and clindamycin (Walsh et al., 2016). Often clinicians do not factor in drug resistance when deciding to treat patients long-term with antibiotics, therefore it becomes common and acceptable to prescribe long-term therapy with antibiotics especially in combination with other drugs and as a result the burden of anti-P. acnes drug resistance continues to escalate (Bojar et al., 2004; Leyden, 2001). Antibiotic resistance of *P. acnes* is most commonly a consequence of chromosomal point mutations and less often via transfer of resistance to and from other species of bacteria. Treating *P. acnes* over long periods of time can have off-target effects and result in other bacteria in or near the treated vicinity developing drug resistance (Beylot et al., 2014). For instance, *Staphylococcus epidermidis* has been reported to be resistant to clindamycin in more than 30% of acne patients treated with clindamycin (Paswan et al., 2019; Shaheen et al., 2013). The impact of resistance to *P. acnes* results in failure of treatments and recurrent onset of disease states (Otlewska et al., 2020). The continued and rising issue of drug resistance reinforces the need for exploration of new drug therapies to treat acne (Shaheen et al., 2013).

### 1.6.4 *Propionibacterium acnes*

*Propionibacterium acnes* is a pleomorphic rod, non-motile, non-spore forming Gram-positive bacillus bacteria classified in the Actinobacteria phylum (Abate, 2013; Paswan et al., 2019). This organism is typically slow growing in an aerobic environment with optimal growth conditions achieved anaerobically at 37˚C (Najafi et al., 2004). It exists as part of the normal

human skin microbiota in the sebaceous follicles as it prefers a moist, oily environment (Perry et al., 2011). *P. acnes* strains are able to grow as biofilms within sebaceous follicles, which aids its resistance to common antimicrobial agents (Bojar et al., 2004; Coenye et al., 2007).

*P. acnes* is key to regulating skin homeostasis and balancing the microbiota to ensure harmful pathogens do not colonise skin sites (Perry et al., 2006). However, *P. acnes* is also an opportunistic pathogen involved in chronic inflammatory skin diseases such as acne and rheumatoid arthritis. Additionally this species can be involved in some other disease states such as endocarditis, osteomyelitis and prostate cancer (Jeong et al., 2017). The problem of antibiotic resistance applies to *P. acnes* as antibiotic use continues to escalate. Resistant strains of *P. acnes* were first reported in the 1970s and this problem persists decades later (Perry et al., 2006). This and many other reasons reinforce the importance to discover and develop antibacterial agents to inhibit *P. acnes* growth (Bojar et al., 2004).

## 1.7 Bioactivity-Guided Metabolomics

Metabolomics is a helpful tool in drug discovery that uses sophisticated analytical technology alongside statistical multi-variant methods to extract and interpret data for the identification and quantification of critical cellular metabolites (Idle et al., 2007; Mokhtari et al., 2018). The typical workflow of metabolomics involves collection of samples, preparation of samples, sample analysis using methods such as gas chromatography-mass spectrometry (GC-MS), liquid chromatography mass spectrometry (LC-MS) and/or nuclear magnetic resonance (NMR) in tandem with a bioactivity assay (Emwas et al., 2019; Worley et al., 2013). These platforms allow for the separation, detection and characterisation of small molecules that

can then be applied to user-friendly statistical software packages for data processing and interpretation (Lindon et al., 2011).

The abundance and presence of certain metabolites provides a direct measure of the underlying biochemical activity in cells (Lindon et al., 2011; Wishart, 2019). For example, a recent metabolomics study harnessed both LC-MS and NMR technologies to investigate seasonal variation of antibacterial activity in leaf extracts of *Leucosidea sericea* (Sehlakgwe et al., 2020). Statistical analysis via principle component analysis (PCA) revealed seasonal variation in chemical composition wherein the winter extracts had the greatest antibacterial activity compared to autumn, spring and summer extracts (Sehlakgwe et al., 2020). This confirmed that winter provided optimal environmental conditions and the most favourable time to harvest (Sehlakgwe et al., 2020). This study therefore endeavours to perform a similar investigation using similar methods to help understand seasonal variation in the antifungal, antibacterial and anti-P. acnes activity of kānuka leaf extracts.

## 1.8 Mānuka and Kānuka Oil Chemical Composition

The chemical composition of New Zealand mānuka oil demonstrates geographical variation (Douglas et al., 2004; Porter et al., 1999). Sesquiterpene is widespread in mānuka oil throughout the country and most abundant in the South Island. The far north has mānuka oil high in pinene content and the Marlborough Sounds and the East Cape have a particularly high content of triketones. The East Cape region is particularly unique as it contains high levels of β-triketones (~33%) compared to mānuka oil sourced from other regions in New Zealand that have β-triketone levels below 5%. The bioactive potential of this compound has been established with antimicrobial, antibacterial, antifungal and antiviral properties (Perry et al., 1997). The antibacterial activity of mānuka oil includes efficacy at treating MRSA more

effectively than mānuka honey and other tea tree oils. It has been hypothesised that this uniqueness rises from a combination of environmental, genetic, and epigenetic factors such as soil conditions, temperature, and the relatively isolated habitats of mānuka plants in New Zealand. New Zealand mānuka oil contains at least 3 different chemotypes: those with high pinenes (particularly α-pinene), those with high triketones, and those with high sesquiterpenes which relate some degree to their appearance, size and geographical location (Perry et al., 1997). The variation in chemical makeup of mānuka oil extracted from different locations is also reflected in their bioactivity profiles (Porter et al., 1998).

The chemical composition of New Zealand kānuka oil is less understood (Wyatt et al., 2005). Variation with respect to geographical location has been documented, albeit it was based on only a few samples from each location (Perry et al., 1997). For example, α-pinene has been identified as the major component in *K. ericoides* but the amount of this compound present in kānuka plants sourced from northern and southern parts of New Zealand can vary between 52-77% of the oil (Perry et al., 1997). Further, the presence of minor compounds can also differ in these regions. Low level detection of β-triketone, leptospermol and citral has been reported in northern regions, while low level detection of β-pinene, 1,8-cineole, limonene, β-terpinene and linalool has been reported in southern regions (Perry et al., 1997).

Phytochemical analysis of *K. ericoides* by GC-MS showed oxygenated sesquiterpenoids to be the main component (Wyatt et al., 2005). The presence of terpenes such as globulol demonstrates potential antifungal properties, as well as pentacyclic triterpenoids that have antiviral, anticancer and anti-inflammatory properties (Wyatt et al., 2005). In conclusion, it appears that α-pinene is a key component present in both mānuka and kānuka essential oils (Perry et al., 1997); however extensive sampling of more than a few plants per species has not been investigated.

## 1.9 Thesis Aims

This thesis will focus on validating the potential medicinal properties of kānuka extracts for pharmaceutical and/or nutraceutical use. Specifically, this work will investigate the seasonal variation in antifungal, antibacterial and anti-P. acnes activities of kānuka oil via *C. albicans,* MRSA and *P. acnes* as model organisms. Since seasonal variation has been demonstrated for mānuka oil, I hypothesize that there will also be seasonal variation in kānuka oil for these three bioactivities. Specifically, my thesis aims are the following:

Aim 1: To detect the chemical composition of kānuka oil extracted from 99 *K. robusta* plants at two different seasons.

Aim 2: To quantify anti-fungal, anti-MRSA and anti-P. acnes activity of the 99 kānuka oil samples.

Aim 3: To correlate the chemical profiles and bioactivities in order to identify the compounds positively correlated with each bioactivity.

# 2.0 Materials and Methods

## 2.1 Introduction

Drug discovery requires the identification of novel compounds and/or druggable targets, which often focuses on natural products given the extensive diversity of compounds in nature (Harvey, 2008; Knight et al., 2003). Prehistoric records show that natural products were used to treat illness or disease in regions and cultures around the world (Koehn et al., 2005; Petrovska, 2012). Many plant species and organisms such as bacteria, fungi and sponges remain unexplored and thus untapped sources of medicinal value, emphasising the importance of continued drug discovery using natural products (Cragg et al., 2013; Li et al., 2009).

Rongoā Māori or traditional Māori medicine is a healing system used by Māori, the indigenous people of Aotearoa (New Zealand) (Beresford et al., 2006). Mānuka and kānuka are well recognised rongoā rākau, and from a rongoā Māori perspective, have proven to be successful at treating a range of medical conditions (Ahuriri-Driscoll et al., 2008; Johnson, 2012). Although mānuka oil and mānuka honey have been developed into a billion dollar sector in the nutraceutical industry (Traynor, 2015), kānuka oil and kānuka honey has yet to be as widely studied as mānuka. Although aroma therapists have used kānuka oil for years, there is still inadequate information available regarding the safety and efficacy of this natural product (Johnson, 2012; Lis-Balchin et al., 2000).

Here, in a collaboration with Hikurangi Bioactives Limited Partnership, seasonal variation of kānuka oil samples extracted from *K. robusta* in Te Tairawhiti were investigated in an effort to characterise and unlock their medicinal potential. The model organisms *C. albicans,* MRSA *and P. acnes* were used to investigate antifungal, antibacterial and anti-P.

acnes activities, respectively. Multivariate analysis of the chemical profiles and each bioactivity was then conducted to identify the key compounds mediating each bioactivity.

## 2.1. Strains

The strains of fungi and bacteria used throughout my thesis were established strains purchased from culture collections (Table 1.1).

| Species | Strain Identification | Source |
|---|---|---|
| *Candida albicans* | NZRM-1212, American Type Culture Collection (ATCC) 10231, | New Zealand Reference Culture collection, medical Section (NZRM) |
| Methicillin-resistant *Staphylococcus aureus* (MRSA) | #3529, ATCC 43300, C Thornsberry F-182 | New Zealand Reference Culture collection, medical Section (NZRM) |
| *Propionibacterium acnes* | NZRM-1212, American Type Culture Collection (ATCC) 6919, | New Zealand Reference Culture collection, medical Section (NZRM) |

**Table 1. 1: Microbial strains used in this study.**

Species, strain identification and source information of species used in this thesis.

## 2.2 The in vitro study of C. albicans

### 2.2.1 Strain and media for C. albicans

Lyophilized culture of *Candida albicans* ATCC10231 was sourced from the Environmental Science and Research Institute (ESRI) in New Zealand. All experiments were conducted under strict aseptic conditions. Yeast extract peptone dextrose (YPD) agar was used to subculture *C. albicans* and isolate single colonies that were then grown in synthetic complete (SC) media in the proceeding experiments.

**Synthetic complete (SC) media amino acid composition:**

3 g adenine, 2 g alanine, 2 g asparagine, 2 g aspartic acid, 2 g cysteine, 2 g glutamic acid, 2 g glutamine, 2 g glycine, 2 g histidine, 2 g inositol, 2 g isoleucine, 10 g leucine, 2 g lysine, 2 g methionine, 0.2 g paraaminobenzoic acid, 2 g phenylalanine, 2 g proline, 2 g serine, 2 g threonine, 2 g tryptophan, 2 g tyrosine, 2 g uracil and 2 g valine

**SC broth composition:**

0.1% (w/v) monosodium glutamate without amino acids or ammonium sulphate (Sigma-Life Sciences), 0.17% (w/v) Yeast Nitrogen Base (Formedium), 0.2% (w/v) amino acid mixture to suit (Formedium) and 2% (w/v) glucose (Sigma-Aldrich).

**YPD agar media composition:**

2% (w/v) yeast extract (Formedium), 2% (w/v) peptone (Formedium), 0.012% (w/v) adenine (Formedium), 2% (w/v) agar granulated bacteriological grade (Formedium) and 2% glucose (Sigma-Aldrich).

### 2.2.2 Activation of C. albicans

The lyophilized *C. albicans* strain was supplied in a double-layered vacuum glass tube. Once the seal of the vacuum tube was broken, 1 mL SC broth was added into the glass tube. The culture content was resuspended using a pipette until the dried *C. albicans* cells were fully dissolved. The culture was then purified by streaking on YPD agar and incubating at 25°C for 24 hours under aerobic conditions. Additionally, 500 µl of the reconstituted biomass was transferred into a 2 mL cryogenic vial containing equal amounts of volume of 40% (v/v) glycerol, which was then mixed vigorously and stored in a -80°C freezer.

### 2.2.3 Routine cell culture

For routine antifungal activity testing, one fresh colony of *C. albicans* on agar was inoculated in 2 mL of SC media and incubated at 25°C shaking for 16 hours. The optical density ($OD_{600}$) of the culture was then measured using a spectrophotometer (Jenway Genova MK3), whereby cells were then diluted in SC media to generate a cell density of $OD_{600}$ = 0.1 required for the antifungal bioactivity assay.

## 2.3 The in vitro study of MRSA

### 2.3.1 Strain and media for MRSA

Lyophilized culture of MRSA was sourced from the Environmental Science and Research Institute (ESRI) in New Zealand. All experiments were conducted under strict aseptic conditions. Luria-Bertani (LB) agar was used to subculture MRSA and isolate single colonies that were grown in LB media in the proceeding experiments.

**LB agar:** 0.5% (w/v) yeast extract (Formedium), 1% (w/v) tryptone (Formedium), 0.5% (w/v) sodium chloride (Thermo Fisher Scientific), 2% (w/v) agar granulated bacteriological grade (Formedium).

**LB broth:** 0.5% (w/v) yeast extract (Formedium), 1% (w/v) tryptone (Formedium), 0.5% (w/v) sodium chloride (Thermo Fisher Scientific).

### 2.3.3 Activation of MRSA

A frozen aliquot of MRSA was supplied by Prof David Ackerley at my university. The culture was activated by streaking on LB agar and incubated at 37°C for 16 hours until single

colonies were distinct. This strain was archived as a frozen stock in 40% (v/v) glycerol and stored at -80°C.

### 2.3.4 Routine cell culture

For routine anti-MRSA activity testing, one fresh colony from cell culture plate of MRSA was inoculated in 5 mL of LB broth and incubated at 37°C shaking at 200 RMP for 16 hours. The optical density ($OD_{600}$) of the culture was then measured using a spectrophotometer (Jenway Genova MK3), whereby cells were then diluted in LB liquid media to generate a cell density of $OD_{600}$ = 0.1 required for the anti-MRSA bioactivity assay.

## 2.4 The in vitro study of P. acnes

### 2.4.1 Strain and media preparation for P. acnes

Lyophilized culture of *P. acnes* was sourced from the Environmental Science and Research Institute (ESRI) in New Zealand. All experiments were conducted under strict aseptic conditions. Luria-Bertani (LB) agar was used to subculture *P. acnes* and isolate single colonies that were grown in LB media in the proceeding experiments. Trypticase soy agar (TSA) agar was used to subculture *P. acnes* and isolate single colonies that were grown in TSA media in the proceeding experiments.

**TS broth composition:**

30 g of TS powder (BD BBL™) was dissolved in 1 L of distilled water.

**TS agar composition:**

TS powder (BD BBL™) 15 g was dissolved in 500 mL of distilled water until dissolved, followed by 10 g of agar granulated bacteriological grade (Formedium).

### 3.4.2 Activation of P. acnes

The lyophilized culture of *P. acnes* was supplied in a double-layered vacuum glass ampule. Once the seal of the vacuum ampule was broken, 1 mL of TSB was added into the glass tube. The culture content was resuspended using a pipette until the *P. acnes* cells were fully dissolved. The culture was then purified by streaking on TS agar and incubating at 37°C for 3 days under inside an anaerobic chamber containing an anaerobic test strip and a GasPak™ anaerobic sachet (BD BBL™). Additionally, 500 µl of the reconstituted biomass was transferred into a 2 mL cryogenic vial containing equal amounts of volume of 40% (v/v) glycerol, which was then mixed vigorously and stored in a -80°C freezer.

### 3.4.3 Routine cell culture

For routine anti-P. acnes activity testing, one fresh colony from a petri dish of *P. acnes* colonies was inoculated in 15 mL TSB in a 50 mL conical tube and incubated at 37°C for 48 hours within an anaerobic chamber. The optical density ($OD_{600}$) of the culture was then measured using a spectrophotometer (Jenway Genova MK3), whereby cells were then diluted in TSB media to generate a cell density of $OD_{600} = 0.1$ required for the anti-P. acnes bioactivity assay.

## 2.5 Kānuka Oil Preparation

Kānuka oil was extracted by Hikurangi Bioactives Limited Partnership using steam distillation. Extracts were diluted in dimethyl sulfoxide (DMSO) (Sigma-Aldrich) to generate stock solutions for biological assays. All stock solutions were stored at -20°C.

## 2.6 Liquid-Based Bioactivity Assays

Antifungal, anti-MRSA and anti-P. acnes bioactivity was assessed by measuring growth of *C. albicans*, MRSA and *P. acnes*, respectively, in liquid media in the presence and absence of kānuka extracts at varying concentrations. Each strain was grown on the appropriate agar media and one colony was sub-cultured into 2 mL of the appropriate liquid media and incubated as mentioned above. Optical density (OD) of this culture was then measured using Jenway Genova MK3 spectrophometer at 660 nm using 1:10 dilution, where 100 µL of each culture was added to 900 µL of the appropriate media. Each overnight culture was then diluted in the appropriate media to generate a culture with a final OD reading of 0.1 that will be used as inoculum for the three bioassays.

### 2.6.1 Growth inhibition of C. albicans

Growth of *C. albicans* was measured in a 96-well plate (Biofill) where each well contained 190 µL of liquid media, 9 µL of cells at an OD 0.1, and 1 µL of kānuka extract to give each well a total volume of 200 µL. Cells were incubated statically at 30°C for 48 hours, $OD_{590}$ readings were taken every hour using the Envision 2102 Multi-label plate reader (Perkin Elmer). Growth inhibition was calculated via residual growth, a ratio of optical density values in treated cells compared to untreated cells at mid-log (OD = 0.22). Optical density values were normalised by subtracting the initial OD at time 0 (T0).

### 2.6.2 Growth inhibition of MRSA

Growth of MRSA was measured in a 96-well plate (Biofill) where each well contained 99 µL of liquid media, 100 µL of cells at an OD 0.1, and 1 µL of kānuka extract to give each well a total volume of 200 µL. Cells were incubated shaking at 200 RPM at 37°C for 6 hours where $OD_{590}$ readings were taken every hour using the Envision 2102 Multi-label plate reader

(Perkin Elmer). Growth inhibition was calculated via residual growth, a ratio of optical density values in treated cells compared to untreated cells at mid-log (OD = 0.5). Optical density values were normalised by subtracting the initial OD at time 0 (T0).

### 2.6.3 Growth inhibition assays of P. acnes

Growth of *P. acnes* was measured in a 96-well plate (Biofill) where each well contained 99 µL of liquid media, 100 µL of cells at an OD 0.1, and 1 µL of kānuka extract to give each well a total volume of 200 µL. Cells were incubated in an anaerobic chamber at 37°C for 48 hours where $OD_{590}$ readings were taken manually approximately every 5 hours using the Envision 2102 Multi-label plate reader (Perkin Elmer). Growth inhibition was calculated via residual growth, a ratio of optical density values in treated cells compared to untreated cells at mid-log (OD = 0.35). Optical density values were normalised by subtracting the initial OD at time 0 (T0).

## 2.7 Compound Profiling

All kānuka samples were processed for gas chromatography-mass spectrometry (GC-MS) analysis using a Shimadzu QP2010 Plus system (Kyoto, Japan), equipped with an AOC20i autosampler. Samples were prepared by adding 10 µL of kānuka oil to 990 µL ethyl acetate spiked with phenol (internal standard) at a concentration of 3.2 mg/mL. Separation was achieved using a Restek (Belafonte, CA) Rxi-5SilMS column (30 m x 0.25 mm x 0.25 µm) using He as the carrier gas at a flow rate of 1.43 mL/min (linear velocity 43.4 cm/s). Sample (1 µL) was injected into the GCMS with the injector port at 270°C with a split ratio of 50:1. The initial column temperature was 50°C, held for 2 min followed by a temperature ramp of 10°C/min to a final temperature of 300°C, held for 5 min, giving a full run time of 32 min. Compounds were detected by a single quadrupole electron impact (EI) mass spectrometer

operating at 70 eV. The mass spectrometer transfer line was maintained at 305°C while the ion source was held at 200°C. Ions were detected in the range of *m/z* 42 – 600 with 0.3 s scan speed, beginning at 4.00 min. Compounds were annotated by comparison to the NIST-11 mass spectral library, with positive identification by comparison of retention time and fragmentation pattern with those of authentic standards. Shimadzu's GSM Solutions Realtime and Post-Run analysis software were used for data acquisition and processing, respectively. Retention time (RT) is helpful with compound identification and measures the time taken for a compound to pass through the chromatography column. The GC-MS report provided information on all possible compound names and their corresponding CAS (chemical abstract service). CAS numbers (Cas No.) are unique numeric identifiers designated to only one substance and can be confirmed using Scifinder. The most common name for each compound is given as the compound name and the corresponding numerical CAS number. The percent (%) area is the overall percent peak area, the greater the percentage the higher the peak area and thus the more abundant the compound.

## 2.8 Multivariate Statistical Analysis

Compound profiles and bioactivities were compared using a partial-least-squares-regression (PLSR) analysis incorporated in UNSCRAMBLER (version X10.4, CAMO, Oslo, Norway) as previously described (Mokhtari et al., 2018; Palermo et al., 2009). The data was arranged in an X × Y matrix, in which the response variable, Y (bioactivity), corresponded to the growth inhibition of each strain being investigated against the kānuka extracts, and the predictor variable, X, corresponded to the largest peak area for each compound. All of the X variables were log-transformed, mean-centred, and scaled to 1 standard deviation. All of the Y variables will also be mean-centred. The PLSR analysis used the nonlinear-iterative-partial-least-squares (NIPALS) algorithm. Compound lists for each kānuka extract were generated

using the weighted regression coefficient and validated using random cross-validation. The Martens uncertainty test based on cross-validation used factors 1 and 2 to identify the compounds statistically significant to the model.

# 3. Chapter 3: Results

## 3.1 Chemical Profiling of 99 Kānuka Oil Extracts

The chemical profiles of kānuka extracts have previously been investigated at random geographical locations within New Zealand (Perry et al., 1997; Perry et al., 1997; Porter et al., 1999; Porter et al., 1998). However, these publications demonstrate inconsistencies within and between results, the studies are relatively old, and the study populations were relatively small. This thesis presents unpublished data on kānuka oil sourced from the East Coast region of New Zealand in an attempt to support the potential use of kānuka oil in the pharmaceutical industry and thus develop a commercial entity for iwi/hapū Māori living in this region. To get a better representation of the chemical diversity of kānuka oil extracts, this thesis endeavoured to comprehensively identify the chemical composition of 99 kānuka oil samples sourced over two seasons from Te Tairawhiti on the East Coast of New Zealand. An investigation of this number of samples across two seasons has not yet been conducted.

Using GC-MS to separate compounds in each kānuka oil sample and the NIST database to identify the compounds, there was an overall total of 85 compounds across all 99 samples (Table 1.2). The presence and abundance of each compound varied in terms of the season of harvest as well as between and within individual plant samples. The ten most abundant compounds were α-pinene, unknown[16], ledol, γ-terpinene, unknown[10], γ-elemene[aa], p-

cymene, nerolidol and 1,8-cineole and α-cubebene[bbbbb] (Figure 3.1). The most predominant compound was α-pinene representing nearly 50% of the total compound content with only moderate levels of the other top ten constituents which is consistent with other studies that have also looked a chemical composition of kānuka oil extracts (Lis-Balchin, Hart et al., 2000; N. B. Perry, Van Klink et al., 1997; N. Porter, P. Smale et al., 1998).

| **RT** | **Compound Name** | **Cas No.** | **% Area** | **Season 1** | **Season 2** |
|---|---|---|---|---|---|
| 4.84 | Isoamyl acetate | 123-92-2 | 0.576 | Yes | Yes |
| 5.73 | α-thujene | 2867-05-2 | 1.108 | Yes | Yes |
| 5.87 | α-Pinene | 80-56-8 | 34.443 | Yes | Yes |
| 6.22 | Benzene acetic acid, 2-pentyl ester | 39180-01-3 | 0.004 | Yes* | Yes*** |
| 6.54 | β-thujene | 28634-89-1 | 0.027 | Yes | No |
| 6.61 | β-Pinene | 127-91-3 | 0.012 | Yes* | No |
| 6.81 | Myrcene | 123-35-3 | 0.052 | Yes | No |
| 7.06 | 4-Carene[d] | 29050-33-7 | 0.020 | Yes*** * | No |
| 7.06 | 4-Hexen-1-ol, acetate | 72237-36-6 | 0.070 | Yes | Yes |
| 7.06 | α -Phellandrene | 99-83-2 | 0.005 | Yes** | No |
| 7.06 | (*E*)-3-Hexenyl acetate | 3681-82-1 | 0.013 | Yes** | Yes* |
| 7.14 | Methacrolein | 78-85-3 | 0.020 | Yes*** | Yes |
| 7.27 | 4-Carene[dd] | 29050-33-7 | 0.102 | Yes | Yes* |
| 7.40 | *p*-Cymene | 99-87-6 | 3.517 | Yes | Yes |
| 7.48 | Limonene | 5989-27-5 | 1.559 | Yes | Yes |
| 7.53 | 1, 8-Cineole | 470-82-6 | 2.804 | Yes | Yes |
| 7.59 | (*E)*-β-Ocimene[i] | 3779-61-1 | 0.012 | Yes** | No |
| 7.77 | (*E)*-β-Ocimene[ii] | 3779-61-1 | 0.415 | Yes | Yes |
| 7.88 | Isoamyl butyrate | 106-27-4 | 0.188 | Yes | Yes |
| 7.97 | γ-Terpinene | 99-85-4 | 4.854 | Yes | Yes |
| 8.21 | Unknown[1] | - | 0.033 | Yes | Yes |
| 8.46 | Unknown[2] | | 1.277 | Yes | Yes |
| 8.61 | Linalool | 78-70-6 | 1.375 | Yes | Yes |
| 8.68 | Isoamyl isovalerate | 659-70-1 | 0.625 | Yes | Yes |
| 8.93 | Thujone | 546-80-5 | 0.085 | Yes | No |
| 9.07 | α-Campholenal | 4501-558-0 | 0.425 | Yes | Yes |
| 9.305 | Benzyl isopentyl ether | 122-73-6 | 0.042 | No | Yes |
| 9.68 | Pinocarvone | 30460-92-5 | 0.052 | Yes | Yes |
| 9.718 | Unknown[3] | - | 0.001 | No | Yes*** |
| 9.89 | Terpinen-4-ol | 562-74-3 | 0.161 | Yes | Yes |
| 9.971 | Benzene,1,1'-(1-butene-1,4-diyl)bis-,(Z)- | 70388-65-7 | 0.0056 | No | Yes |
| 10.11 | Unknown[4] | - | 0.148 | Yes* | Yes |
| 10.19 | Myrtenal | 564-94-3 | 0.011 | Yes* | No |

| | | | | | |
|---|---|---|---|---|---|
| 10.39 | Verbenone | 1196-01-6 | 0.015 | Yes | Yes* |
| 10.48 | Unknown[5] | - | 0.009 | No | Yes |
| 4 | | | | | |
| 10.87 | Isopentyl hexanoate | 2198-61-0 | 0.014 | Yes*** | Yes*** |
| | | | | * | |
| 10.87 | Carvone | 2244-16-8 | 0.005 | Yes* | No |
| 11.02 | 2-phenylethyl acetate | 103-45-7 | 0.1052 | Yes | Yes |
| 11.43 | Unknown[6] | - | 0.002 | Yes* | No |
| 12.23 | γ -Elemene[a] | 29873-99-2 | 0.005 | Yes** | No |
| 12.40 | α-Cubebene[b] | 17699-14-8 | 0.195 | Yes | No |
| 12.72 | Geranyl acetate | 141-12-8 | 0.042 | Yes | No |
| 12.79 | α-Cubebene[bb] | 17699-14-8 | 0.554 | Yes | Yes |
| 12.93 | Pentanoic acid, phenylmethyl ester | 10361-39-4 | 0.125 | Yes | Yes*** |
| 12.98 | β-elemene | 515-13-9 | 0.005 | Yes** | No |
| 13.26 | Guaia-3,9-diene | 489-83-8 | 0.826 | Yes | Yes**** |
| 13.36 | (-)-Aristolene | 6831-16-9 | 0.003 | Yes* | Yes* |
| 13.40 | (-)-Caryophyllene | 87-44-5 | 0.659 | Yes | Yes*** |
| 13.55 | β-Phenylethyl butyrate | 103-52-6 | 0.069 | Yes | Yes* |
| 13.58 | α-Guaiene[c] | 3691-12-1 | 0.035 | Yes | No |
| 13.66 | Aromandendrene | 489-39-4 | 0.956 | Yes | Yes |
| 13.71 | 1,3,6-Heptatriene, 2,5,5-trimethyl- | 29548-02-5 | 0.001 | Yes* | No |
| 13.78 | α-Cubebene[bbb] | 17699-14-8 | 0.644 | Yes | No |
| 13.84 | Humulene | 6753-98-6 | 0.292 | Yes | No |
| 13.94 | Alloaromadendrene | 25246-27-9 | 0.840 | Yes | Yes |
| 14.06 | Unknown[7] | - | 0.504 | Yes | No |
| 14.14 | α-Muurolene | 10208-80-7 | 0.022 | Yes*** | Yes** |
| 14.20 | Phenylethyl isovalerate | 140-26-1 | 0.202 | Yes | Yes |
| 14.20 | Pentanoic acid, 2-phenylethyl ester | 7460-74-4 | 0.002 | Yes* | No |
| 14.24 | Unknown[8] | - | 0.008 | Yes* | No |
| 14.24 | α-Cubebene[bbbb] | 17699-14-8 | 0.032 | Yes | No |
| 14.29 | α-Guaiene[cc] | 3691-12-1 | 0.550 | Yes | Yes** |
| 14.38 | γ-Elemene[aa] | 29873-99-2 | 3.597 | Yes | Yes |
| 14.47 | Unknown[9] | - | 0.094 | Yes | No |
| 14.57 | γ -Muurolene | 30021-74-0 | 0.136 | Yes | No |
| 14.67 | Unknown[10] | - | 3.627 | Yes | Yes |
| 14.71 | Unknown[11] | - | 0.039 | Yes | Yes* |
| 14.80 | α-Cubebene[bbbbb] | 17699-14-8 | 2.114 | Yes | Yes |
| 14.86 | Isolongifolene, 4,5,9,10-dehydro- | 156747-45-4 | 0.021 | Yes | No |
| 14.94 | Unknown[12] | - | 0.018 | Yes | No |
| 14.98 | Geranyl butanoate | 106-29-6 | 0.004 | Yes** | No |
| 15.08 | Unknown[13] | - | 0.122 | Yes* | Yes |
| 15.12 | Nerolidol | 40716-66-3 | 3.472 | Yes | No |
| 15.29 | Palustrol | 5986-49-2 | 1.444 | Yes | Yes |
| 15.38 | Unknown[14] | - | 0.131 | No | Yes |
| 1 | | | | | |
| 15.47 | Caryophyllene oxide | 1139-30-6 | 1.219 | Yes | Yes |
| 15.55 | Unknown[15] | - | 1.413 | Yes | Yes |

| | | | | | |
|---|---|---|---|---|---|
| 15.60 | Unknown[16] | - | 10.756 | Yes | No |
| 15.76 | Ledol | 577-27-5 | 8.847 | Yes | Yes |
| 15.87 | Dihydrophytol | 645-72-7 | 1.412 | Yes | No |
| 15.97 | Di-epi-.alpha.-cedrene-(I) | 21996-77-0 | 0.133 | Yes | Yes |
| 16.00 | Phenylethyl hexanoate | 6290-37-5 | 0.032 | Yes** | Yes** |
| 16.13 | Unknown[17] | - | 0.364 | Yes | Yes* |
| 17.20 | Pulegone | 15932-80-6 | 0.167 | Yes | No |
| 17.25 | Thunbergol | 25269-17-4 | 0.002 | Yes | No |

**Table 1. 2: GCMS analysis separates 85 compounds in the kānuka extracts.** Each compound was identified via comparison with NIST database based on similarity index greater than 85, and otherwise indicated as unknown. All separated compounds were annotated for retention time (RT), CAS number, average percent area (abundance) in all samples containing the corresponding compound, and presence/absence in samples collected in different seasons. * indicates the number of samples from that season that contain that corresponding compound (e.g., * = 1, ** = 2, *** = 3 and **** = 4; we chose a cut off of 4 samples or less to show this detail for simplification, those without a * i.e., Yes = 4+ samples) see the graph below for further detail. Unknown compounds are distinguished with superscript numerals. Superscript letters are used to indicate compounds at different retention times with the same name (e.g., 4-Carene[d] and 4-Carene[dd]).

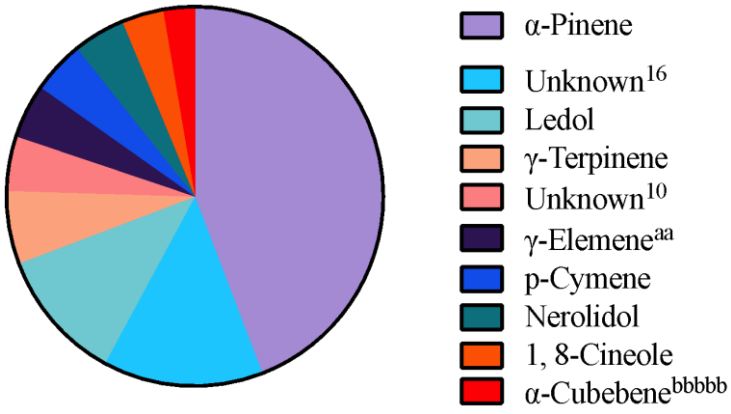



**Figure 3. 1: The ten most abundant compounds in kānuka extracts.** Pie graph representing the top 10 most abundant compounds detected in the GC-MS analysis of the 99 kānuka samples. This analysis was extrapolated from the percent of peak area in the GC-MS report.

Complementary to the most abundant compounds, there were 20 compounds that only appeared in less than five of the 99 samples. For instance, β-Pinene was present in very low levels (0.012%) of only one sample from season 1, and similarly 1,3,6-Heptatriene, 2,5,5-

trimethyl-, Carvone, Myrtenal, Pentanoic acid, 2-phenylethyl ester, Thunbergol and Unknown[6] were also present in low levels (~0.125-0.001%) in only one sample from season one (spring). These results suggest that there are fine differences in the chemical composition of kānuka oil with respect to seasonal variation and variation between plants and within plants from the same population.

| **Compound Name** | **Season 1** | **Season 2** | **Total** |
|---|---|---|---|
| α-Pinene | 52 | 44 | 96 |
| Limonene | 52 | 42 | 94 |
| Unknown[10] | 52 | 41 | 93 |
| α-Cubebene[bbbbb] | 52 | 36 | 88 |
| Alloaromadendrene | 52 | 35 | 87 |
| (-)-Caryophyllene | 51 | 3 | 54 |
| Caryophyllene oxide | 51 | 12 | 63 |
| Guaia-3,9-diene | 51 | 4 | 55 |
| Palustrol | 51 | 28 | 79 |
| α-Campholenal | 51 | 43 | 94 |
| Aromandendrene | 50 | 26 | 76 |
| Ledol | 49 | 44 | 93 |
| Unknown[7] | 50 | 0 | 50 |
| Humulene | 49 | 0 | 49 |
| Linalool | 49 | 45 | 94 |
| α-Cubebene[bb] | 49 | 0 | 49 |
| α-Cubebene[bbb] | 49 | 0 | 49 |
| 1, 8-Cineole | 48 | 40 | 88 |
| Dihydrophytol | 48 | 1 | 49 |
| Unknown[17] | 48 | 1 | 49 |
| γ-Elemene[aa] | 48 | 36 | 84 |
| *p*-Cymene | 47 | 44 | 91 |
| α-Guaiene[cc] | 47 | 2 | 49 |
| α-thujene | 47 | 35 | 82 |
| γ-Terpinene | 47 | 38 | 85 |
| α-Guaiene[c] | 46 | 0 | 46 |
| Unknown[16] | 45 | 0 | 45 |
| Unknown[2] | 45 | 36 | 81 |
| α-Cubebene[b] | 45 | 35 | 80 |
| Isoamyl isovalerate | 41 | 40 | 81 |
| (*E)*-β-Ocimene | 40 | 31 | 71 |

| | | | |
|---|---|---|---|
| Isoamyl butyrate | 37 | 37 | 74 |
| Pulegone | 37 | 0 | 37 |
| β-Phenylethyl butyrate | 37 | 1 | 38 |
| Myrcene | 36 | 0 | 36 |
| γ -Muurolene | 36 | 0 | 36 |
| 2-Phenylethyl acetate | 34 | 14 | 48 |
| Pentanoic acid, phenylmethyl ester | 32 | 3 | 35 |
| Phenylethyl isovalerate | 31 | 0 | 31 |
| Nerolidol | 30 | 0 | 30 |
| Unknown[9] | 29 | 0 | 29 |
| 4-Carene[d] | 20 | 1 | 21 |
| Isoamyl acetate | 18 | 23 | 41 |
| Terpinen-4-ol | 18 | 32 | 50 |
| 4-Hexen-1-ol, acetate | 14 | 23 | 37 |
| Geranyl acetate | 10 | 0 | 10 |
| Pinocarvone | 10 | 18 | 28 |
| Isolongifolene, 4,5,9,10-dehydro- | 9 | 0 | 9 |
| Di-epi-.alpha.-cedrene-(I) | 8 | 5 | 13 |
| Unknown[11] | 8 | 1 | 9 |
| β-thujene | 8 | 0 | 8 |
| Unknown[15] | 7 | 52 | 59 |
| Thujone | 6 | 0 | 6 |
| Unknown[1] | 6 | 19 | 25 |
| Unknown[12] | 6 | 0 | 6 |
| Verbenone | 5 | 1 | 6 |
| α-Cubebene[bbbb] | 5 | 0 | 5 |
| 4-Carene[dd] | 4 | 0 | 4 |
| Isopentyl hexanoate | 4 | 3 | 7 |
| Methacrolein | 3 | 16 | 19 |
| α-Muurolene | 3 | 2 | 5 |
| (*E*)-3-Hexenyl acetate | 2 | 1 | 3 |
| (*E)*-β-Ocimene | 2 | 0 | 2 |
| Geranyl butanoate | 2 | 0 | 2 |
| Phenylethyl hexanoate | 2 | 2 | 4 |
| Unknown[4] | 2 | 45 | 47 |
| α -Phellandrene | 2 | 0 | 2 |
| β-elemene | 2 | 0 | 2 |
| γ -Elemene[a] | 2 | 0 | 2 |
| (-)-Aristolene | 1 | 1 | 2 |
| 1,3,6-Heptatriene, 2,5,5-trimethyl- | 1 | 0 | 1 |
| Benzeneacetic acid, 2-pentyl ester | 1 | 3 | 4 |
| Carvone | 1 | 0 | 1 |

| | | | |
|---|---|---|---|
| Myrtenal | 1 | 0 | 1 |
| Pentanoic acid, 2-phenylethyl ester | 1 | 0 | 1 |
| Thunbergol | 1 | 0 | 1 |
| Unknown[13] | 1 | 29 | 30 |
| Unknown[14] | 1 | 46 | 47 |
| Unknown[6] | 1 | 0 | 1 |
| Unknown[8] | 1 | 6 | 7 |
| β-Pinene | 1 | 0 | 1 |
| Benzene,1,1'-(1-butene-1,4-diyl)bis-,(Z)- | 0 | 11 | 11 |
| Benzyl isopentyl ether | 0 | 39 | 39 |
| Unknown[3] | 0 | 3 | 3 |
| Unknown[5] | 0 | 14 | 14 |

**Table 1. 3: Annotation of each identified compound in kānuka oil samples.** The number of samples from each season that contain each compound identified among all 99 kānuka oil samples (ref to table 1.2).

Seasonal variation results in not only a different yield of the same compounds but variation in the presence/absence of particular compounds. Four compounds (Benzene,1,1'-(1-butene-1,4-diyl)bis-,(Z)-; Benzyl isopentyl ether; and 2 unknown compounds) are detected in season two but not in season one. Likewise, 31 compounds were detected from season one but not in season two (Humulene; α-Cubebene, α-Cubebene, α-Guaiene; Pulegone; Myrcene; γ-Muurolene; Phenylethyl isovalerate; Nerolidol; Geranyl acetate; Isolongifolene, 4,5,9,10-dehydro-; β-thujene; Thujone; α-Cubebene; 4-Carene; (E)-β-Ocimene; Geranyl butanoate; α-Phellandrene; β-elemene; γ-Elemene; 1,3,6-Heptatriene; 2,5,5-trimethyl-; Carvone; Myrtenal; Pentanoic acid; 2-phenylethyl ester; Thunbergol; β-Pinene; and 1 unknown compound). Of the 17 unknown compounds, these are all compounds with distinct GC-MS spectra, and albeit these cannot be identified within this thesis, these compounds further contribute to the seasonal variation. These results highlight the complexity of producing a standardised, reproducible natural product extract, in this case the seasonal and plant-to-plant variation in the chemical profile of 99 kānuka oil extracts.

## 3.2 Antifungal Bioactivity of Kānuka Extracts Against C. albicans

The antifungal bioactivity of kānuka oil has previously been investigated, however studies in this field are limited by small population sampling, inconsistent results and the lack of research in this area within the last 20 years (Lis-Balchin et al., 2000). The sparse available research reports both negative and positive correlations between kānuka oil and antifungal activity (Chen et al., 2016; Essien et al., 2019). For instance, kānuka oil exhibits little to no antifungal activity against filamentous fungi such as *Aspergillus niger, Aspergillus ochraceus* and *Fusarium culmorum*. Moreover, kānuka oil has been reported to have less antifungal activity compared to other tea tree oils such as mānuka and melaleuca oils (Lis-Balchin et al., 2000); however, another study reported stronger antifungal activity in kānuka oil compared to mānuka oil against fungal species such as *Malassezia furfur, Trichosporon mucoides Candida albicans* and *Candida tropicalis* (Chen et al., 2016). This thesis attempts to evaluate natural variation in antifungal properties of kānuka oil, via our 99 kānuka oil samples, against the common fungal pathogen *Candida albicans*.

### 3.2.1 Normal growth of C. albicans

To begin this investigation, it was first necessary to identify the growth exponential phase (mid-log phase) of *C. albicans*. This phase is important to identify as it is the time point to compare a kānuka treatment condition to an untreated condition. Figure 3.2 shows the normal growth of *C. albicans* wherein the curve is sigmodial in nature with an initial lag phase (~0-9 hours), a mid-log phase (~15 hours) and a plateau phase indicating maximal growth (~24

hours), which is consistent with other reputable studies (Kesavan et al., 2005; Lopez-Abarrategui et al., 2016).

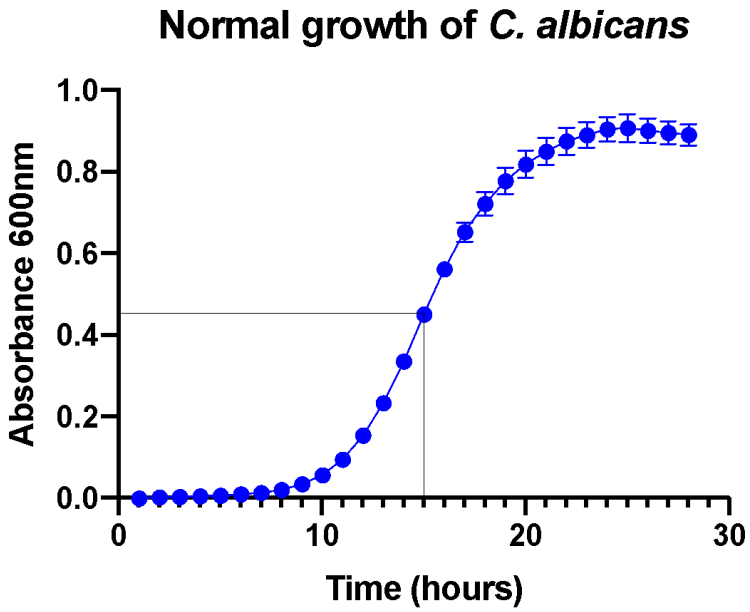



**Figure 3. 2: Normal growth of *C. albicans* in SC liquid media.** Cells were grown as technical triplicates in SC media. Initial lag phase (0-10 hours) followed by an exponential phase (12-24 hours) and a linear phase (>24 hours). Lines in the graph indicate the mid-log phase.

### 3.2.2 Optimisation of kānuka extracts for growth assays with C. albicans

A 96-well microtiter plate was used to study the antifungal activity of the kānuka samples against the growth of *C. albicans* in liquid media; this bioassay was recommended as a sensitive assay with good reproducibility and repeatability (Patton et al., 2006). To identify a concentration of kānuka extract that will reveal variation in the 99 extracts, dose-dependent growth curves were constructed to show the inhibitory effect of a subset of the extracts. Kānuka samples in this study were selected at random (J9, B13, F18, B6, F3 H1 and for comparison two sample of mānuka oil extracts were also tested (M1 and M2). The growth of *C. albicans* in the presence and absence of varying amounts of these extracts ($5 \times 10^{-4}$ % , $1 \times 10^{-4}$ %, $5 \times 10^{-5}$ %, $3.33 \times 10^{-5}$ %, $2.5 \times 10^{-5}$ % (v/v)) was measured via optical density (OD) every hour

for 30 hours. At the time point previously identified as mid-log phase (15 hours) and relative to the DMSO vehicle control, antifungal activity against *C. albicans* at each concentration was measured. Cells treated with 5 x $10^{-4}$ % (v/v) of each kānuka oil extract demonstrated a cytotoxic phenotype as all treated cells did not grow (Figure 3.2a). Cells treated with 1 x $10^{-4}$ % (v/v) of each kānuka oil extract demonstrated significantly reduced and delayed growth relative to control, whereby treated cells did not show growth until 18 hours, a time point where untreated cells were well within the exponential phase (Figure 3.2b). Cells treated with 3.33 x $10^{-5}$ % (v/v) or 2.5 x $10^{-5}$ % (v/v) of each kānuka oil extract exhibited a near normal phenotype with growth of treated cells similar to untreated cells (Figures 3.2c, 3.2d). In contrast, cells treated with 5 x $10^{-5}$ % (v/v) of each kānuka oil extract demonstrated a cytostatic phenotype as treated cells showed variable levels of growth defect relative to untreated cells. In general, cells exposed to mānuka oil at each of these concentrations demonstrated greater growth inhibition compared to its kānuka oil counterpart, which is consistent with previous studies that also demonstrated greater antifungal potency of manuka oil compared to kānuka oil (Lis-Balchin et al., 2000). Together, these results indicate 5 x $10^{-5}$ % (v/v) as an informative concentration that will be used to reveal variation in antifungal activity of all 99 kānuka oil samples.

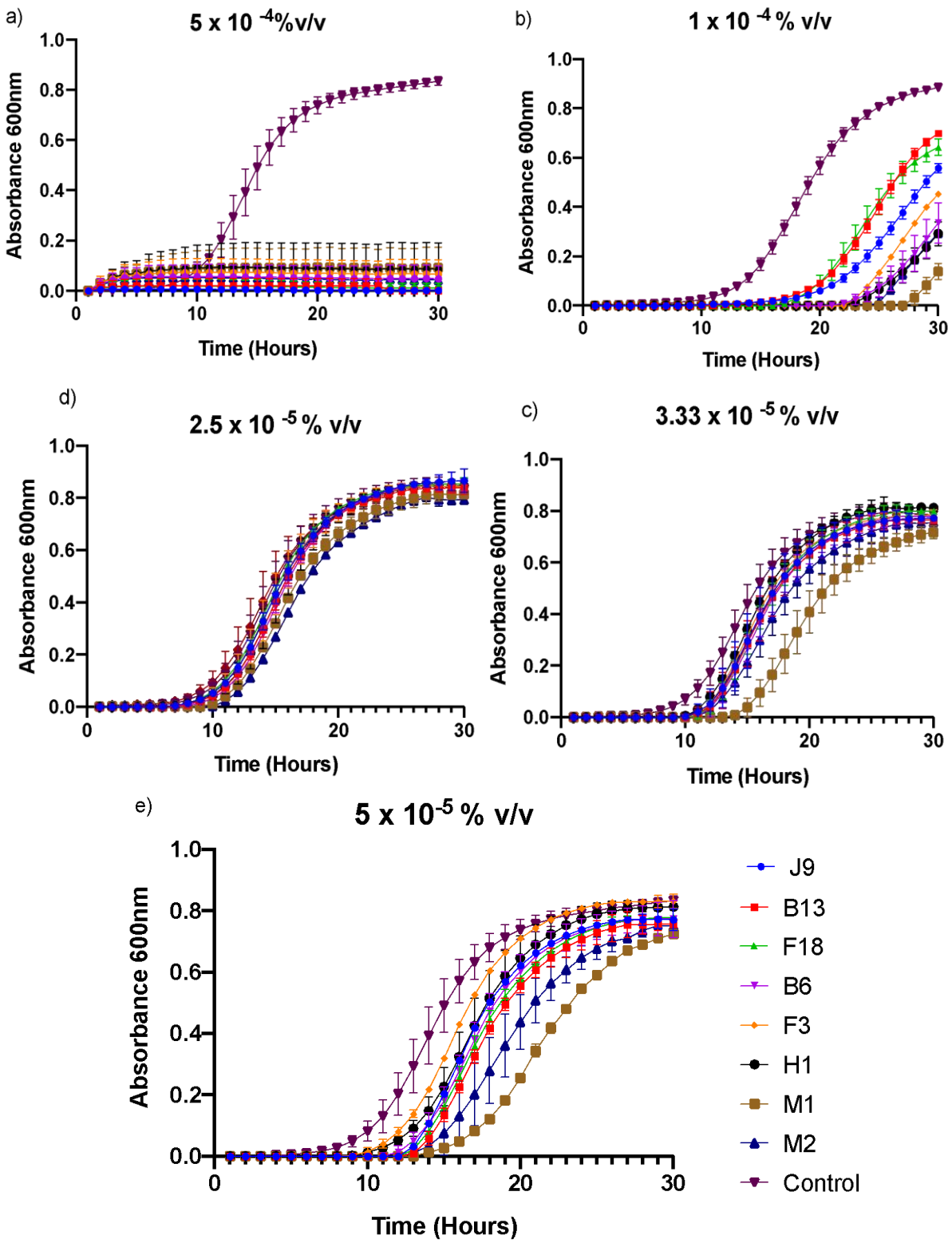
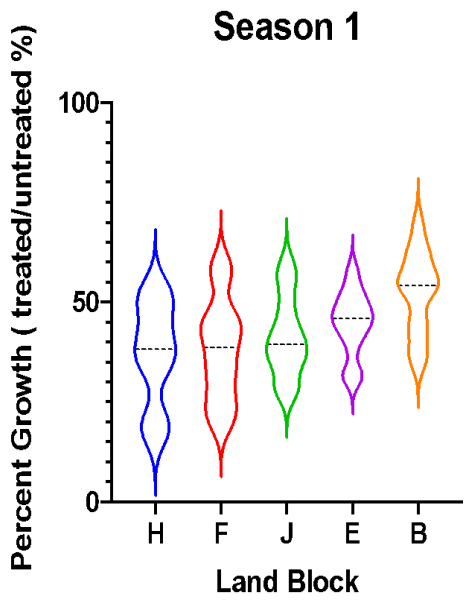



**Figure 3. 3: Liquid dose-response assay of *C. albicans* treated with a subset of kānuka oil extracts.** Cells were grown in triplicate tech with and without kānuka oil for 30 hours at $25^0$C and growth was measured every hour. Data shown as mean ± SD. **(a-e)**

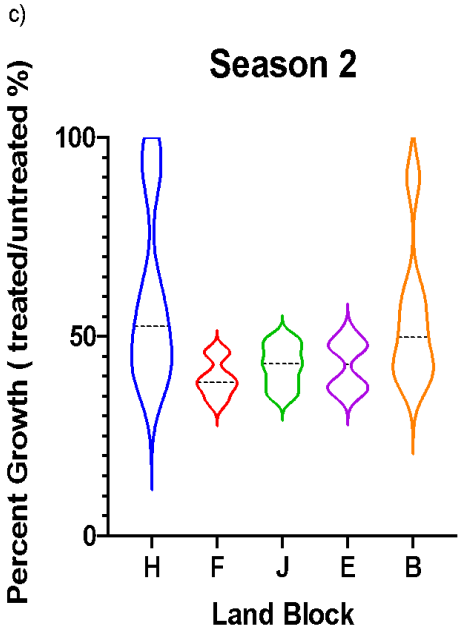
### 3.2.3 Screening and analysis of antifungal activity with respect to land block and season

Using the identified optimal concentration 5 x 10 -5 % (v/v), a screen of antifungal activity against C. *albicans* was performed on all 99 samples of kānuka oil extracts in this study. The 99 samples of kānuka oil extracts differ individually by being collected from kānuka trees in five different geographic locations (land blocks H, F, J, E and B) at two different time points (seasons 1 and 2). Season one contains 54 samples representing kānuka leaves harvested during spring, while season two contains 49 samples representing kānuka leaves harvested during autumn. Residual growth, a ratio of growth in treated and untreated cells, was measured at mid-log for all 99 kānuka oil extracts. Cells that express residual growth closer to 100% are those that grow the same or similar to control cells and those that express percent growth near 0% are those that show reduced growth relative to control cells. There was extensive variation among the samples within each land block with 17.65 - 90% residual growth (Figure 3.3). In general, land block H demonstrated more potent antifungal activity compared to all other land blocks. Likewise, there was extensive variation among the samples within each land block with respect to season; for example, land blocks F, J and B in season one demonstrated a wider variation in antifungal activity in comparison to season two where all samples showed a smaller range of variation (Figures 3.3a, 3.3c). Although the median percent growth of all samples is not dramatically different, these results indicate that individual samples from different land blocks as well as samples from the same land block but different seasons exhibited different profiles of antifungal activity (Figures 3.3a, 3.3c; Tables 3.3b, 3.3d).

a)




c)




b)

| | H | F | J | E | B |
|---|---|---|---|---|---|
| Number of values | 8 | 12 | 10 | 11 | 12 |
| | | | | | |
| Minimum | 17.65 | 20.60 | 28.18 | 31.48 | 35.30 |
| 25% Percentile | 23.31 | 26.62 | 33.70 | 39.41 | 43.74 |
| Median | 38.26 | 38.64 | 39.53 | 46.06 | 54.22 |
| 75% Percentile | 50.14 | 46.09 | 50.14 | 51.10 | 61.27 |
| Maximum | 52.26 | 58.80 | 59.09 | 57.70 | 69.44 |
| Range | 34.61 | 38.19 | 30.90 | 26.22 | 34.14 |

d)

| | H | F | J | E | B |
|---|---|---|---|---|---|
| Number of values | 8 | 12 | 11 | 8 | 7 |
| | | | | | |
| Minimum | 38.95 | 32.75 | 34.78 | 35.24 | 38.95 |
| 25% Percentile | 42.00 | 35.75 | 38.14 | 37.17 | 41.84 |
| Median | 52.66 | 38.63 | 43.27 | 42.97 | 49.83 |
| 75% Percentile | 83.61 | 44.13 | 47.11 | 47.60 | 61.43 |
| Maximum | 96.93 | 46.47 | 49.48 | 50.87 | 90.00 |
| Range | 57.99 | 13.72 | 14.70 | 15.63 | 51.05 |

**Figure 3. 4 Residual growth of *C. albicans* treated with all 99 kānuka oil extracts.** Residual growth of *C. albicans* treated with kānuka oil at a concentration of 5 x $10^{-5}$ % (v/v) was measured at mid-log phase (15 hours). **(a)** Percent growth of *C. albicans* treated with kānuka samples sourced from season 1 and from different land blocks. **(b)** Table of values interpretated from graph a. **(c)** Percent growth of *C. albicans* treated with kānuka samples sourced from season 2. **(d)** Table of values interpretated from graph c.

### 3.2.4 Multivariate analysis of kānuka oil antifungal activity

Many studies have previously demonstrated that partial least squares regression (PLSR) analysis is a reliable and informative tool to correlate compound profiles with bioactivity (Coutinho et al., 2016; Killeen et al., 2013; Mokhtari et al., 2018). Here I used PLSR analysis to correlate the chemical profiles of 99 samples of kānuka extracts with antifungal activity as determined by growth inhibition of *C. albicans*. This multivariate analysis tool measures the

degree of correlation between identified compounds (independent variable) and the bioactivity under investigation (dependent variable), in this instance, anti-fungal activity. PLSR analysis generates a weighted regression co-efficient score (WRS) for each compound ranging from -13 to +6, where the higher scores reflect positive correlation with bioactivity and lower scores reflect negative correlation with bioactivity.

| CAS No. | SI (%) | Compound | WRS | No. of samples | Compound No. |
|---|---|---|---|---|---|
| 0-00-0 | <85 | Unknown[12] | 3.3831 | 6 | 2 |
| 30021-74-0 | 87-90 | α-Murolene | 2.86604 | 5 | 1 |
| 0-00-0 | 71-89 | Unknown[9] | 2.745129 | 29 | 3 |
| 659-70-1 | >90 | Isoamyl isovalerate | 2.702568 | 74 | 6 |
| 29050-33-7 | 77 | 4-Carene[d] | 2.652694 | 21 | 4 |
| 0-00-0 | ~80 | Unknown[4] | 2.573518 | 47 | 5 |
| 17699-14-8 | > 90 | α-Cubebene[bbb] | 1.96117 | 49 | 7 |
| 0-00-0 | 82 | Unknown[7] | 1.217554 | 50 | 8 |

**Table 1. 4: Top weighted regression co-efficient scores (WRS) for compounds exhibiting antifungal activity against *C. albicans*.** This table displays compounds with the highest WRS scores including compounds that demonstrate statistical significance ($P < 0.05$) based on the Martens Uncertainty Test (highlighted in green). CAS No. = Chemical Abstracts Service Number; SI level = similarity index as per match with GC-MS database. Frequency of each compound among the 99 samples is indicated. Compound number refers to the location of each compound in Figure 3.5.

The PLSR analysis identified six compounds as being most positively correlated with antifungal activity (Table 1.4). Similarity index (SI) was used as a means to identify compounds based on similarity of the GC-MS spectra to compounds in the NIST database, where an SI value greater than 85 is considered a good indication of compound identification and an SI level that falls below 85 is considered an unreliable indication of compound identification (Costa et al., 2007; Hertz et al., 1971). Of the six compounds positively correlated with antifungal activity, three were identified based on SI and three could not be identified

based on SI. Unknown[12] (Figure 3.5b) has the highest WRS score suggesting this compound is strongly correlated with anti-fungal activity in comparison to other detected compounds. However, this compound is difficult to interpret as it was unable to be identified, its abundance was only 0.018% of the range of % area and it was only present in a total of 6/99 samples. Moreover, the remaining compounds in this list (Table 1.4) also have very low abundance (i.e. <1% of the range of percent area). For instance the abundance of α-Murolene, Unknown[9], Isoamyl isovalerate, 4-Carene[d], Unknown[4], α-Cubebene[bbb] and Unknown[7] are 0.022%, 0.094%, 0.625%, 0.020%, 0.148%, 0.0845%, 0.052%, 0.202%, 0.644% and 0.504%, respectively. Thus although these compounds have high WRS scores, they only make up a small fraction of the overall chemical composition of the kānuka extracts (Table 1.2). These results suggest that perhaps only a very small concentration of these compounds are required to produce effective antifungal activity.

The overall presence of these compounds in each sample ranges from very low presence in unknown12 and α-murolene that are present in 5 and 6 of the total 99 samples, respectively, to mid-range presence of 21-74 out of the total 99 samples in the remaining compounds (Tables 1.3, 1.4). α-cubebene[bbb] and unknown[7] are interesting compounds as they have been identified as statistically significant in this analysis, they also have SI values of >90 and 82, respectively, which suggests a confident value for correct compound identification of only α-cubebene[bbb]. 4-Carene[d], Unknown[12] and Unknown[4] also fall short of being confidently identified; however, all other compounds have SI values of 87 or above.

To visually represent the results of the PLSR analysis, a principal component analysis (PCA) was conducted to show the relationship between chemical composition and antifungal activity. Figure 3.5a correlates each tested sample to antifungal bioactivity. All samples that lie within the right upper quadrant (RUQ) are most closely associated with antifungal activity of

the kānuka extracts. There is also a clear separation of samples from season 1 and samples from season 2. Season 2 samples appear to cluster in the left upper quartile (LUQ) suggesting these samples share a positive correlation with factor 2 but a negative correlation with factor 1, however 5 samples from this group have a positive correlation with both factors 1 and 2. Season 1 samples also appear to cluster together and are most positively associated to factor 1, with 21 samples demonstrating a positive correlation to both factors (seven samples in land block B, four samples in land block B, four samples in land block F, three samples in land block H, two samples in land block E, and one sample in land block J). Overall this analysis indicates that more samples from season 1 are positively correlated with antifungal activity more so than samples from season 2.

Figure 3.5b demonstrates the correlation of all compounds to antifungal activity. Compounds that associate closely to bioactivity in the RUQ are suggested to play an important role in the antifungal properties of these extracts. Six compounds were located within the RUQ; however, these compounds were not statistically significant within the analysis. Two compounds (α-Cubebene[bbb] (7) and Unknown[7] (8)) were statistically significant, suggesting these compounds are positively correlated to antifungal activity, albeit only with a positive correlation to factor 1.

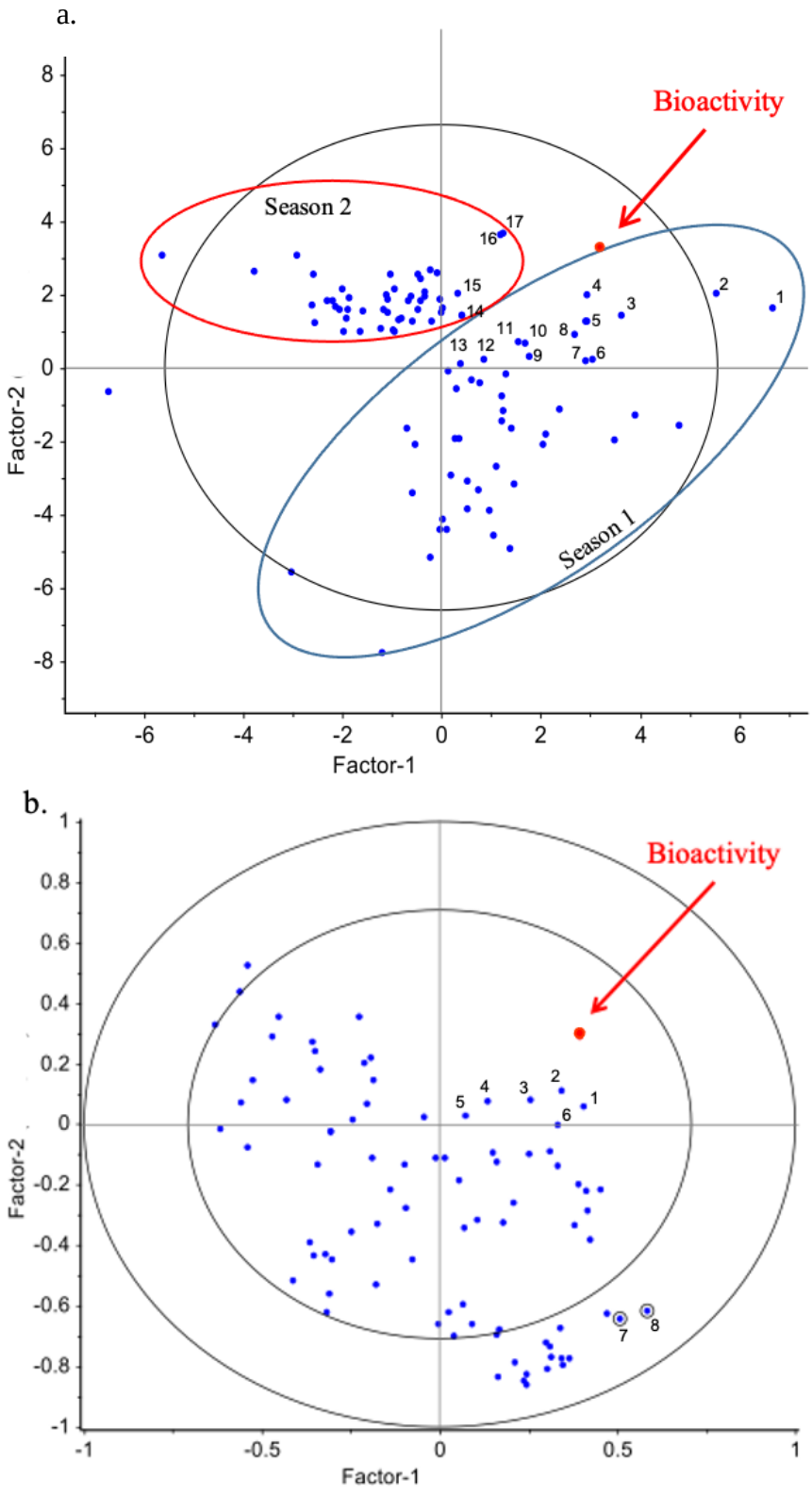



**Figure 3. 5: Multivariate analysis correlates chemical profiles and antifungal bioactivity.** Partial least-squares regressions of (A) the kānuka oil samples relative to antifungal bioactivity and (B) kānuka oil compounds relative to antifungal bioactivity. Bioactivity (red dot) was defined as the growth inhibition of *C. albicans* wherein the percentage of growth inhibition

was calculated via the optical density (OD) of the treated cells at mid-log-phase relative to the untreated cells.

## 3.3 Antibacterial Bioactivity of Kānuka Extracts Against Methicillin-resistant Staphylococcus aureus (MRSA)

The antibacterial bioactivity of kanuka honey has previously been investigated, however studies in this field mainly focused on comparing kānuka honey with the well-established and already commercialised mānuka honey. There is a desperate need for literature to be updated with investigations of antibacterial activity of kānuka oil against antibiotic-resistant bacteria (Lis-Balchin et al., 2000). For example, MRSA is a leading cause of antibiotic-resistant bacterial infections in both hospital and community settings (Lee et al., 2018). To realise this potential, this thesis attempts to evaluate natural variation in antibacterial properties of kānuka oil, via our 99 kānuka oil samples, against the antibiotic-resistant pathogen MRSA.

### 3.3.1 Normal growth of MRSA

To begin this investigation, it was first necessary to identify the growth exponential phase (mid-log phase) of MRSA. This phase is important to identify as it is the time point to compare a kānuka treatment condition to an untreated condition. Figure 3.2 shows the normal growth of MRSA, wherein the mid-log phase can be detected at 4 hours and saturation at 8-9 hours, which is comparable with the normal growth of MRSA seen in other studies (Jung et al., 2009; Wong et al., 2019).

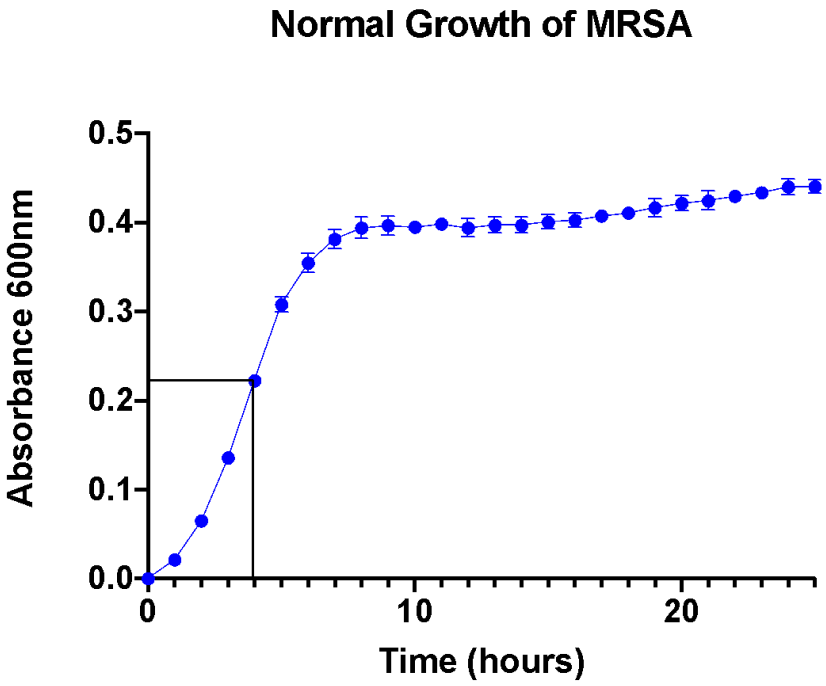



**Figure 3. 6 Normal growth of MRSA in LB liquid media.** Cells were grown in triplicate in LB media. Initial lag phase (0-1 hours) followed by an exponential phase (1-8 hours) and a linear phase (>10 hours). Lines in the graph indicate the mid-log phase.

### 3.3.2 Optimisation of kānuka extracts for growth assays with *MRSA*

A 96-well microtiter plate was used to study the antibacterial activity of the kānuka samples against the growth of MRSA in liquid media; this bioassay was recommended as a sensitive assay with good reproducibility and repeatability (Vitko et al., 2013). To identify a concentration of kānuka extract that will reveal variation in the 99 extracts, dose-dependent growth curves were constructed to show the inhibitory effect of a subset of the extracts. Kānuka samples in this study were selected at random (J9, B13, F18, B6, F3 H1 and for comparison two sample of mānuka oil extracts were also tested M1 and M2). The growth of MRSA in the presence and absence of varying amounts of these extracts (2500 x $10^{-6}$ %, 200 x $10^{-6}$ % , 33 x $10^{-6}$ %, 16 x $10^{-6}$ % and 50 x $10^{-6}$ % (v/v)) was measured via optical density (OD) every hour for 25 hours (Figure 3.7).

At the time point previously identified as mid-log phase (4 hours) and relative to the DMSO vehicle control, antibacterial activity against MRSA at each concentration was measured. Cells treated with these concentrations of each kānuka oil extract demonstrated significantly reduced and delayed growth relative to control (Figure 3.7). In general, cells exposed to mānuka oil at each of these concentrations demonstrated greater growth inhibition compared to its kānuka oil counterpart, which is consistent with previous studies that also demonstrated greater antibacterial potency of manuka oil compared to kānuka oil (Lis-Balchin et al., 2000). Together, these results indicate $50 \times 10^{-6}$ % (v/v) as an informative concentration that will be used to reveal variation in antifungal activity of all 99 kānuka oil samples.

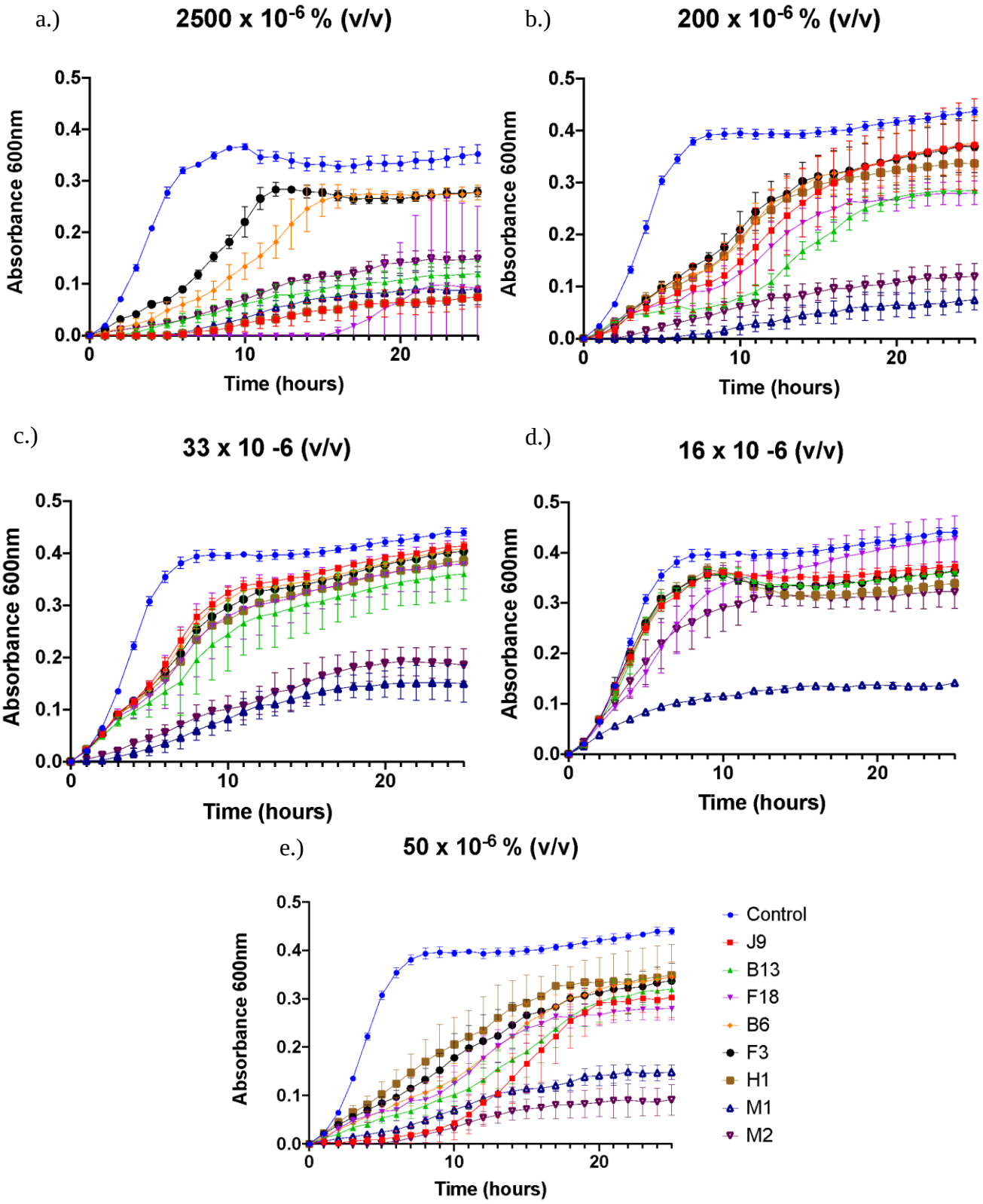
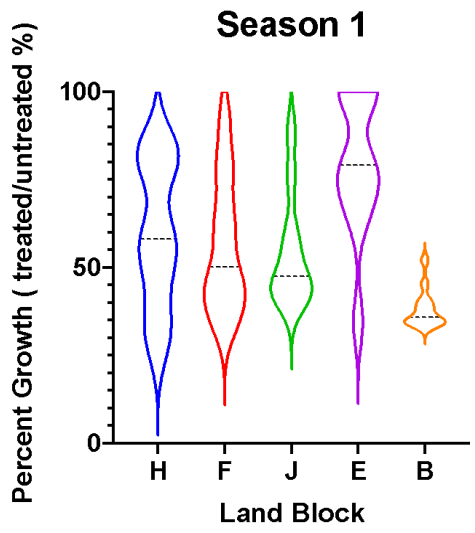



**Figure 3. 7: Liquid dose-response assay of MRSA treated with a subset of kānuka oil extracts.** Cells were grown in triplicate tech with and without kānuka oil for 25 hours at $37^0$C and growth was measured every hour. Data shown as mean ± SD. **(a-e)**

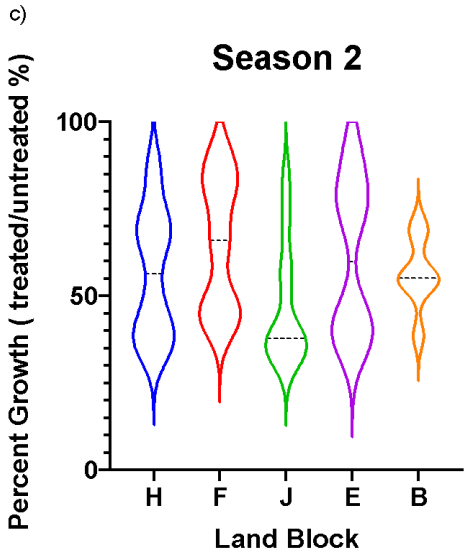
### 3.3.3 Screening and analysis of anti-MRSA activity with respect to land block and season

Using the identified optimal concentration 50 x $10^{-6}$ % (v/v), a screen of antibacterial activity against MRSA was performed on all 99 samples of kānuka oil extracts in this study. The 99 samples of kānuka oil extracts differ individually by being collected from kānuka trees in five different geographic locations (land blocks H, F, J, E and B) at two different time points (seasons 1 and 2). Season one contains 54 samples representing kānuka leaves harvested during spring, while season two contains 49 samples representing kānuka leaves harvested during autumn. Residual growth, a ratio of growth in treated and untreated cells, was measured at mid-log for all 99 kānuka oil extracts. Cells that express residual growth closer to 100% are those that grow the same or similar to control cells and those that express percent growth near 0% are those that show reduced growth relative to control cells. There was extensive variation among the samples within each land block. The spread of variation was greatest in land block H followed closely by land block F, J and E with the exception of land block B that showed notably less variation in percent growth compared to all other locations. This result is similar to what we saw with *C. albicans* and also consistent with existing studies that demonstrate variation in antibacterial activity in kānuka extracts sourced from the same as well as different geographical locations (Christoph et al., 2000; Harkenthal et al., 1999; Lis-Balchin et al., 2000). Likewise, there was extensive variation among the samples within each land block with respect to season; for example, land block B in season one demonstrated less variation in anti-*MRSA* activity in compared to season two. Although the median percent growth of all samples is not dramatically different, these results indicate that individual samples from different land blocks as well as samples from the same land block but different seasons exhibited different profiles of antifungal activity (Figure 3.8).

a)




c)




b)

| | H | F | J | E | B |
|---|---|---|---|---|---|
| Number of values | 8 | 12 | 10 | 11 | 12 |
| | | | | | |
| Minimum | 28.13 | 32.76 | 39.38 | 34.58 | 33.16 |
| 25% Percentile | 39.52 | 40.97 | 43.27 | 68.83 | 34.40 |
| Median | 58.19 | 50.14 | 47.49 | 79.18 | 35.89 |
| 75% Percentile | 81.24 | 73.03 | 64.24 | 100.0 | 40.44 |
| Maximum | 84.99 | 93.17 | 88.89 | 100.0 | 52.08 |
| Range | 56.86 | 60.41 | 49.51 | 65.42 | 18.91 |

d)

| | H | F | J | E | B |
|---|---|---|---|---|---|
| Number of values | 8 | 12 | 11 | 8 | 7 |
| | | | | | |
| Minimum | 36.21 | 41.22 | 32.92 | 37.30 | 38.59 |
| 25% Percentile | 37.27 | 44.64 | 33.61 | 39.07 | 53.29 |
| Median | 56.30 | 66.11 | 37.93 | 59.82 | 55.18 |
| 75% Percentile | 71.56 | 82.81 | 59.19 | 81.43 | 67.16 |
| Maximum | 86.57 | 92.15 | 83.69 | 92.77 | 70.82 |
| Range | 50.35 | 50.93 | 50.77 | 55.47 | 32.23 |

**Figure 3. 8: Residual growth of MRSA treated with all 99 kānuka oil extracts.** Residual growth of MRSA treated with kānuka oil at a concentration of 5 x $10^{-5}$ % (v/v) was measured at mid-log phase (4 hours). **(a)** Percent growth of MRSA treated with kānuka samples sourced from season 1 and from different land blocks. **(b)** Table of values interpretated from graph a. **(c)** Percent growth of MRSA treated with kānuka samples sourced from season 2. **(d)** Table of values interpretated from graph c.

### 3.3.4 Multivariate analysis of kānuka oil anti-MRSA activity

Many studies have previously demonstrated that partial least squares regression (PLSR) analysis is a reliable and informative tool to correlate compound profiles with bioactivity (Coutinho et al., 2016; Killeen et al., 2013; Mokhtari et al., 2018). Here I used PLSR analysis to correlate the chemical profiles of 99 samples of kānuka extracts with anti-MRSA

activity. This multivariate analysis tool measures the degree of correlation between identified compounds (independent variable) and the bioactivity under investigation (dependent variable), in this instance, anti-bacterial activity. PLSR analysis generates a weighted regression co-efficient score (WRS) for each compound ranging from -13 to +6, where the higher scores reflect positive correlation with bioactivity and lower scores reflect negative correlation with bioactivity.

The PLSR analysis identified 17 compounds as being positively correlated with anti-MRSA activity (Table 1.5). Similarity index (SI) was used as a means to identify compounds based on similarity of the GC-MS spectra to compounds in the NIST database, where an SI value greater than 85 is considered good indication of compound identification and an SI level that falls below 85 is considered an unreliable indication of compound identification (Costa et al., 2007; Hertz et al., 1971). Of the 17 compounds positively correlated with antifungal activity, 10 were identified based on SI and 3 could not be identified based on SI.

Terpinen-4-ol has the highest WRS score suggesting this compound is strongly correlated with anti-MRSA activity in comparison to other detected compounds. This compound is present in approximately half of the overall sample population, for instance, 18/53 samples in season 1 and 32/46 samples in season 2; however, it is present in relatively low abundance (~0.161% of the range of percent area). Limonene also has a high WRS score and it is present in almost all samples (94/99) and the 11$^{th}$ most abundant compound detected in kānuka extracts in this study. Likewise, Caryophyllene oxide, Linalool, Unknown$^{2}$, Nerolidol and especially γ-Terpinene have relatively high abundance with 1.2- 4.9% of the chemical composition (Table 1.2). We can be confident with the analysis of Verbenone, γ-Terpinene, 4-Carene$^{dd}$ and Unknown$^{2}$ were each significantly correlated with anti-MRSA activity. All other compounds were present at relatively low abundance (<1%). Enigmatically, 1,3,6-Heptatriene,

2,5,5-trimethyl was positively correlated with anti-MRSA bioactivity; however, it is only present in 1 sample of the total 99 samples in this study. (E)-β-Ocimene[i], 4-Carene[dd] , Verbenone, Unknown[8] and Isolongifolene, 4,5,9,10-dehydro- are also placed in the top 10 WRS scoring compounds, yet these compounds are only sparsely represented in 2-9 samples. These results indicate that there are compounds, both rare and common among the 99 samples, required to produce effective anti-MRSA activity.

The overall presence of these compounds in each sample ranges from very low presence in 1,3,6-Heptatriene, 2,5,5-trimethyl-; (E)-β-Ocimene[i]: Verbenone; Unknown[8] and Isolongifolene, 4,5,9,10-dehydro- that are present in 1, 2, 6, 7 and 9 of the total 99 samples, respectively, to mid-high range presence of 29-97 out of the total 99 samples in the remaining compounds (Tables 1.3, 1.5). Terpinen-4-ol , Limonene, Verbenone, γ-Terpinene, 4-Carene[dd] and Unknown[2] are interesting compounds as they have been identified as statistically significant in this analysis, they also have SI values of 88, 90, 88, 95, 94 and 91, respectively, which suggests confident values for correct compound identification.

To visually represent the results of the PLSR analysis, a principal component analysis (PCA) was conducted to show the relationship between chemical composition and anti-MRSA activity. Figure 3.9a correlates each tested sample to anti-MRSA bioactivity. All samples that lie within the RUQ are most closely associated with anti-MRSA activity of the kānuka extracts. Like with antifungal activity, there is a clear separation of samples from season 1 to samples from season two when it comes to anti-MRSA activity. Season two samples cluster together with samples in the RUQ and the right lower quartile (RLQ). Conversely, season one samples cluster together and are spread across the RUQ, left upper quartile (LUQ) and the left lower quartile (LLQ). Interestingly, land block E samples from both seasons were the most positively correlated to anti-MRSA bioactivity, with sample 3 (E25) being the most correlated sample to

anti-MRSA bioactivity. Overall, this analysis indicates that more samples from season two are positively correlated with anti-MRSA activity more so than samples from season one, which is the opposite pattern than what was observed for antifungal activity.

| CAS No. | SI (%) | Compound | WRS | No of Samples | Compound No. |
|---|---|---|---|---|---|
| 562-74-3 | 88 | Terpinen-4-ol | 6.30 | 50 | 1 |
| 0-00-0 | 86 | Unknown[13] | 4.40 | 30 | 2 |
| 5989-27-5 | 90 | Limonene | 4.38 | 97 | 3 |
| 40716-66-3 | 92 | Nerolidol | 3.14 | 30 | 4 |
| 103-45-7 | 94 | 2-phenylethyl acetate | 2.84 | 48 | 9 |
| 29548-02-5 | 74 | 1,3,6-Heptatriene, 2,5,5-trimethyl- | 2.72 | 1 | 5 |
| 1139-30-6 | 88 | Caryophyllene oxide | 2.57 | 63 | 10 |
| 0-00-0 | 84 | Unknown[9] | 2.57 | 29 | 6 |
| 78-70-6 | 93 | linalool | 2.41 | 94 | 7 |
| 0-00-0 | 82 | Unknown[8] | 2.40 | 7 | 8 |
| 3691-12-1 | 84 | α-Guaiene[c] | 2.37 | 46 | 11 |
| 156747-45-4 | 76 | Isolongifolene, 4,5,9,10-dehydro- | 2.36 | 9 | 12 |
| 3779-61-1 | 90 | (E)-β-Ocimene[i] | 2.28 | 2 | 13 |
| 1196-01-6 | 88 | Verbenone | 1.56 | 6 | 15 |
| 99-85-4 | 95 | γ-Terpinene | 1.52 | 85 | 16 |
| 29050-33-7 | 94 | 4-Carene[dd] | 0.87 | 4 | 17 |
| 0-00-0 | 91 | Unknown[2] | 0.28 | 81 | 18 |
| 99-87-6 | 93 | *p*-Cymene | -0.53 | 91 | 19 |
| 30021-74-0 | 82 | γ -Muurolene | -0.73 | 36 | 20 |
| 489-83-8 | 91 | Guaia-3,9-diene | -1.18 | 55 | 21 |

| | | | | | |
|---|---|---|---|---|---|
| 6753-98-6 | 92 | Humulene | -2.04 | 49 | 22 |

**Table 1. 5: Top weighted regression co-efficient scores (WRS) for compounds exhibiting antibacterial activity against MRSA.** This table displays compounds with the highest WRS scores including compounds that demonstrate statistical significance ($P < 0.05$) based on the Martens Uncertainty Test (highlighted in green). CAS No. = Chemical Abstracts Service Number; SI level = similarity index as per match with GC-MS database. Frequency of each compound among the 99 samples is indicated. Compound number refers to the location of each compound in Figure 3.9.

Figure 3.9b demonstrates the correlation of all compounds to anti-MRSA activity. Compounds that associate closely to bioactivity in the RUQ are suggested to play an important role in the anti-*MRSA* properties of these extracts. From this analysis, 15 compounds fall within the RUQ, suggesting these could be potentially important in anti-MRSA bioactivity. However, only 8/15 in the RUQ were statistically significant within the analysis (Table 1.5). Additionally, there were 8 SS compounds in the LUQ and one SS compound in the right lower quartile (RLQ). Of all compounds, compound 1, 3 and 4 appear to be the most interesting compounds as these are most closely associated to bioactivity and also show SS.

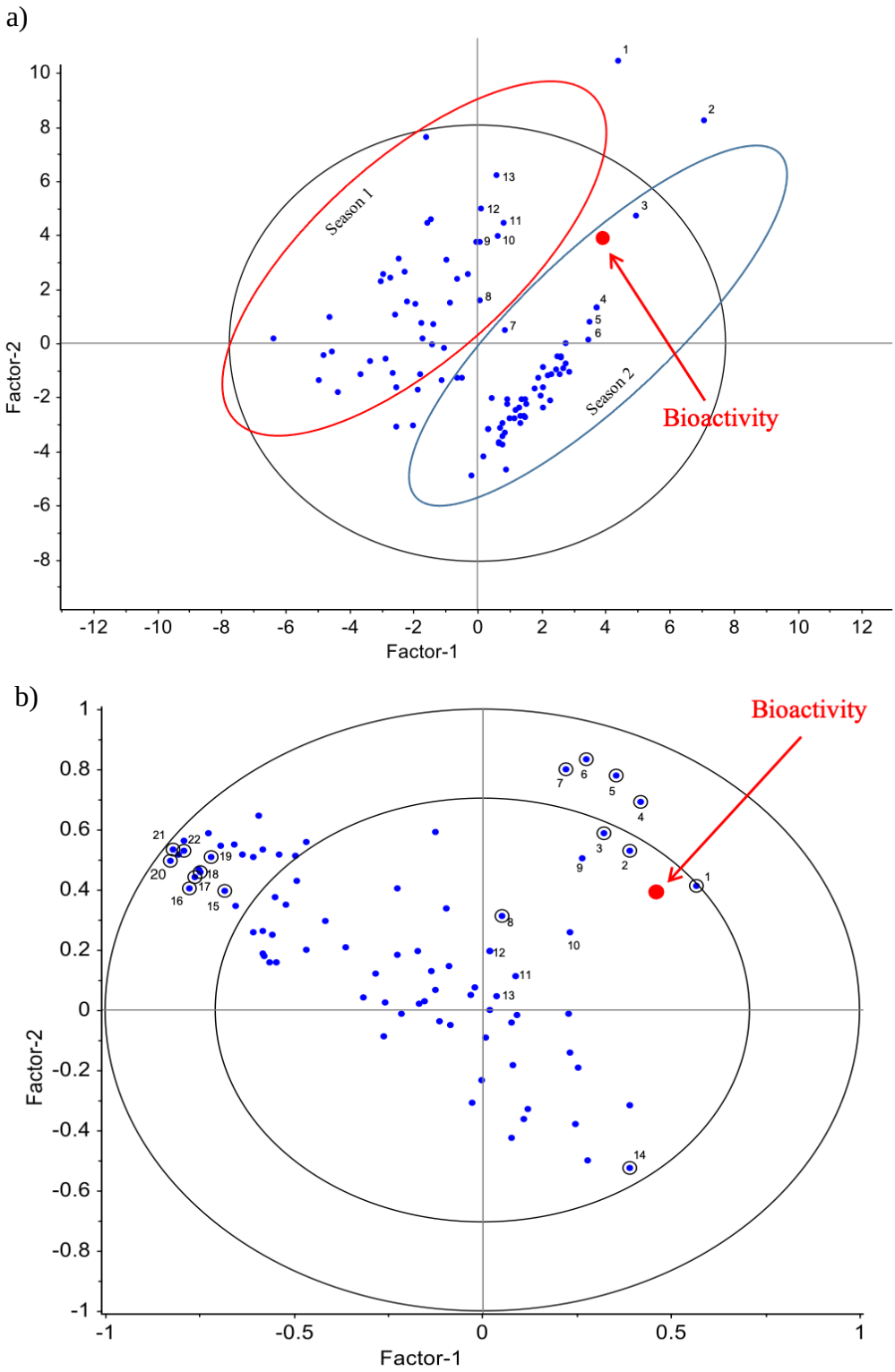



**Figure 3. 9: Multivariate analysis correlates chemical profiles and anti-MRSA bioactivity.** Partial least-squares regressions of **(a)** the kānuka oil samples relative to anti-MRSA bioactivity and **(b)** kānuka oil compounds relative to anti-MRSA bioactivity. Bioactivity (red dot) was defined as the growth inhibition of MRSA wherein the percentage of growth inhibition

was calculated via the optical density (OD ) of the treated cells at mid-log-phase relative to the untreated cells.

## 3.4 Anti-P. acnes Bioactivity of Kānuka Extracts Against *Propionibacterium acnes*

The anti-P. acnes bioactivity of kānuka honey has previously been investigated (Braithwaite et al., 2015; Fingleton et al., 2014; Holt et al., 2012; Semprini et al., 2016; Semprini et al., 2019). However, there are not any studies that investigate the anti-P. acnes activity of kānuka oil. This thesis attempts to evaluate natural variation in anti-P. acnes properties of kānuka oil, via our 99 kānuka oil samples, against the common fungal pathogen *Propionibacterium acnes*.

### 3.4.1 Normal growth of *P. acnes*

To begin this investigation, it was first necessary to identify the growth exponential phase (mid-log phase) of *P. acnes*. With growth of this being obligatory anaerobic, growth was only measured every five hours to minimise the number of times anaerobiosis in the anaerobic chamber was temporarily disrupted. Identifying the mid-log phase is important to identify as it is the time point to compare a kānuka treatment condition to an untreated condition. Figure 3.10 shows the normal growth of *P. acnes* wherein exponential growth occurs for 60 hours with mid-log at 32 hours, which is consistent with other reputable studies (Kesavan et al., 2005; Lopez-Abarrategui et al., 2016).

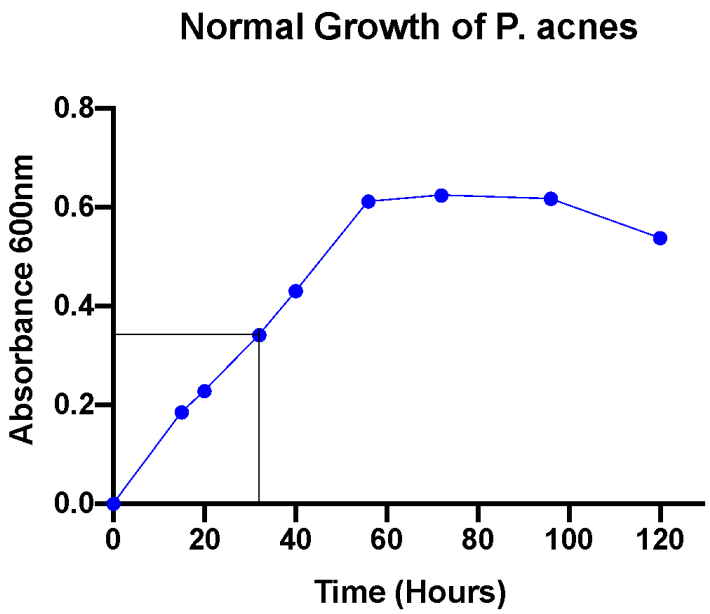



**Figure 3. 10: Normal growth of *P. acnes* in TS liquid media.** Cells were grown in as biological triplicates in TS media. No notable lag phase followed by an exponential phase (1-56 hours). Lines in the graph indicate the mid-log phase.

### 3.4.2 Optimisation of kānuka extracts for growth assays with *P. acnes*

A 96-well microtiter plate was used to study the anti-P. acnes activity of the kānuka samples against the growth of *P. acnes* in liquid media; this bioassay was recommended as a sensitive assay with good reproducibility and repeatability (Vitko & Richardson, 2013). To identify a concentration of kānuka extract that will reveal variation in the 99 extracts, dose-dependent growth curves were constructed to show the inhibitory effect of a subset of the extracts. Kānuka samples in this study were selected at random (J9, B13, F18, B6, F3, H1 and for comparison two sample of mānuka oil extracts were also tested (M1 and M2). The growth of *P. acnes* in the presence and absence of varying amounts of these extracts (500 x $10^{-6}$ %, 200 x $10^{-6}$ %, 100 x $10^{-6}$ %, 66 x $10^{-6}$ %, 50 x $10^{-6}$ % and 16 x $10^{-6}$ % (v/v)) was measured via optical density (OD) at varies time points over 120 hours.

At the time point previously identified as mid-log phase (32 hours) and relative to the DMSO vehicle control, anti-P. acnes activity against *P. acnes* at each concentration was measured (Figure 3.11). Cells treated with 16 x $10^{-6}$ % (v/v) of each kānuka oil extract were not impacted with this treatment and not significantly different from the control. Cells treated with all other concentrations demonstrated significantly reduced and delayed growth relative to control. At 500 x $10^{-6}$ % (v/v), there was the most sample-to-sample variation in growth inhibition. As the concentration of kānuka decreased less than 500 x $10^{-6}$ % (v/v), the sample-to-sample variation was less apparent; for instance at 200 x $10^{-6}$ % (v/v) there was no longer statistically significant differences in growth inhibition between cells treated with kānuka samples H1, B6 and F3, while there was still a clear difference between samples J9, B13 and F18. In comparison, both mānuka extracts at 500 x $10^{-6}$ % (v/v) exhibited a cytotoxic effect on *P. acnes* as cells did not grow in this condition, suggesting that mānuka extracts may have greater anti-P. acnes activity than kānuka extracts. These results indicate that 500 x $10^{-6}$ % (v/v) kānuka oil extract as an informative concentration that will be used to reveal variation in anti-P. acnes activity of all 99 kānuka oil samples (Figure 3.11).

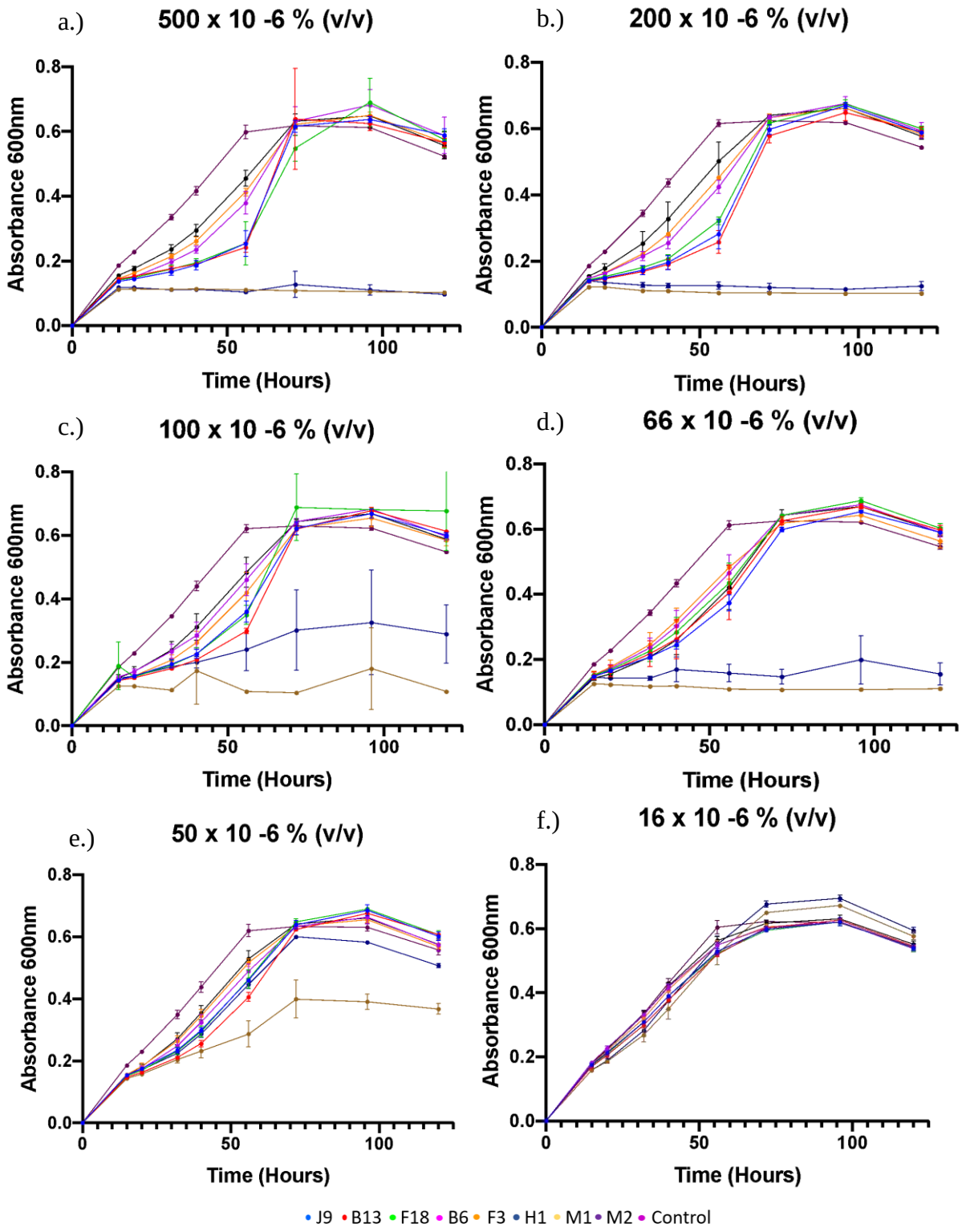
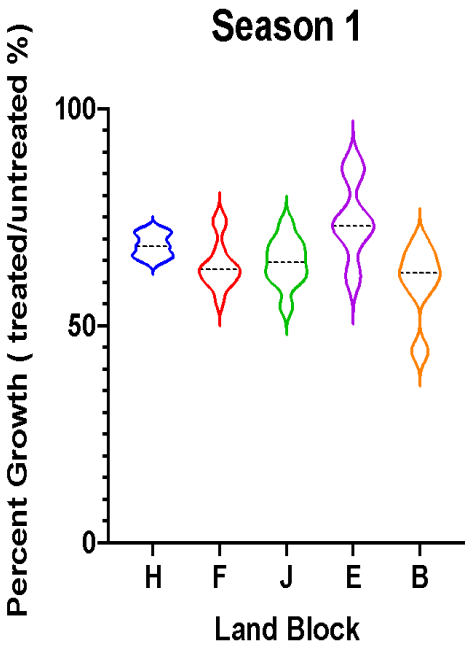



**Figure 3. 11: Liquid dose-response assay of *P. acnes* treated with a subset of kānuka oil extracts.** Cells were grown in triplicate with and without kānuka oil for 120 hours at $37^0$C and growth was measured every five hours. Data shown as mean ± SD. **(a-f)** Growth of cells treated with varying concentrations of kānuka oil extract. Data shown as mean ± SD.

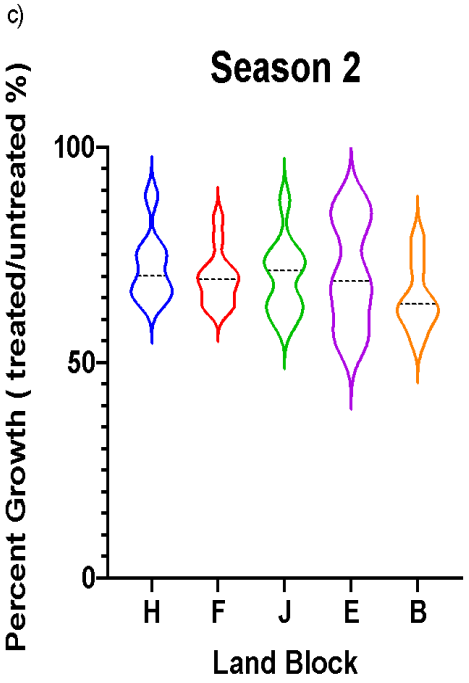
### 3.4.3 Screening and analysis of anti-P acnes activity respective to land block and season

Using the identified optimal concentration 500 x $10^{-6}$ % (v/v), a screen of anti-P. acnes activity against *P. acnes* was performed on all 99 samples of kānuka oil extracts in this study. The 99 samples of kānuka oil extracts differ individually by being collected from kānuka trees in five different geographic locations (land blocks H, F, J, E and B) at two different time points (seasons 1 and 2). Season one contains 54 samples representing kānuka leaves harvested during spring, while season two contains 49 samples representing kānuka leaves harvested during autumn. Residual growth, a ratio of growth in treated and untreated cells, was measured at mid-log for all 99 kānuka oil extracts. Cells that express residual growth closer to 100% are those that grow the same or similar to control cells and those that express percent growth near 0% are those that show reduced growth relative to control cells. There was extensive variation among the samples within each land block (Figure 3.12), albeit it was not as extensive as described for the antifungal and anti-MRSA activities. The spread of variation was greatest in land block E followed closely by land blocks F and J. Likewise, there was extensive variation among the samples within each land block with respect to season (Figure 3.12); for example, most land blocks (e.g., land block H) in season one demonstrated less potency in anti-P. acnes activity compared to season two. Although the median percent growth of all samples is not dramatically different, these results indicate that individual samples from different land blocks as well as samples from the same land block but different seasons exhibited different profiles of anti-P. acnes activity (Figure 3.12).

a)




c)




b)

| | H | F | J | E | B |
|---|---|---|---|---|---|
| Number of values | 8 | 12 | 10 | 11 | 12 |
| | | | | | |
| Minimum | 64.92 | 55.78 | 54.46 | 60.54 | 43.04 |
| 25% Percentile | 66.18 | 60.10 | 60.64 | 70.08 | 57.65 |
| Median | 68.36 | 63.00 | 64.62 | 72.98 | 62.14 |
| 75% Percentile | 71.29 | 66.94 | 69.20 | 85.04 | 65.80 |
| Maximum | 72.05 | 74.89 | 73.60 | 87.20 | 70.22 |
| Range | 7.134 | 19.11 | 19.14 | 26.66 | 27.18 |

d)

| | H | F | J | E | B |
|---|---|---|---|---|---|
| Number of values | 8 | 12 | 11 | 8 | 7 |
| | | | | | |
| Minimum | 63.76 | 62.12 | 58.40 | 54.42 | 55.33 |
| 25% Percentile | 66.01 | 63.92 | 62.75 | 58.52 | 60.40 |
| Median | 70.31 | 69.30 | 71.49 | 69.01 | 63.59 |
| 75% Percentile | 76.03 | 72.43 | 74.57 | 84.33 | 72.57 |
| Maximum | 88.75 | 83.73 | 87.74 | 86.88 | 78.77 |
| Range | 24.99 | 21.61 | 29.34 | 32.46 | 23.44 |

**Figure 3. 12: Residual growth of *P. acnes* treated with all 99 kānuka oil extracts.** Residual growth of *P. acnes* treated with kānuka oil at a concentration of 500 x $10^{-6}$ % (v/v) was measured at mid-log phase (30 hours). **(a)** Percent growth of *P. acnes* treated with kānuka samples sourced from season 1 and from different land blocks. **(b)** Table of values interpretated from graph a. **(c)** Percent growth of *P. acnes* treated with kānuka samples sourced from season 2. **(d)** Table of values interpretated from graph c.

### 3.4.4 Multivariate analysis of kānuka oil anti-P acnes activity

Many studies have previously demonstrated that partial least squares regression (PLSR) analysis is a reliable and informative tool to correlate compound profiles with bioactivity (Coutinho et al., 2016; Killeen et al., 2013; Mokhtari et al., 2018). Here I used PLSR analysis

to correlate the chemical profiles of 99 samples of kānuka extracts with anti-P acnes activity. This multivariate analysis tool measures the degree of correlation between identified compounds (independent variable) and the bioactivity under investigation (dependent variable), in this instance, anti-P acnes activity. PLSR analysis generates a weighted regression co-efficient score (WRS) for each compound ranging from -13 to +6, where the higher scores reflect positive correlation with bioactivity and lower scores reflect negative correlation with bioactivity.

The PLSR analysis identified 13 compounds as being most positively correlated with anti-P acnes activity. (Table 1.6). Similarity index (SI) was used as a means to identify compounds based on similarity of the GC-MS spectra to compounds in the NIST database, where an SI value greater than 85 is considered good indication of compound identification and an SI level that falls below 85 is considered an unreliable indication of compound identification (Costa et al., 2007; Hertz et al., 1971). Of the 13 compounds positively correlated with anti-P acnes activity, 8 were identified based on SI and 5 could not be identified based on SI.

Limonene is the compound with the highest WRS score, suggesting this compound strongly correlates with anti-P acnes activity. This compound is present in almost all of the samples in this study (97/99) and it is the 11$^{th}$ most abundant compound detected in all kānuka extracts. Nerolidol, 1, 8-Cineole, Palustrol and unknown$^{16}$ also have relatively high abundance in terms of the overall chemical compositions of all extracts with percent area values above 1%, where most compounds are present in significantly low abundance (<1%). Benzene, 1,1'-(1-butene-1,4-diyl)bis-, (Z)- , Unknown$^{11}$, Phenylethyl hexanoate and Verbenone are also positively correlated to bioactivity, yet these were identified in only a small subset of samples (between 4-11 of the 99 samples). Inversely, there were three compounds with negative WRS

scores (γ -Muurolene, Guaia-3,9-diene and Humulene), suggesting these compounds are negatively associated with anti-P acnes bioactivity and may be inhibiting anti-P acnes properties of these kānuka extracts.

To visually represent the results of the PLSR analysis, a principal component analysis (PCA) was conducted to show the relationship between chemical composition and anti-P acnes activity. Figure 3.13a correlates each tested sample to anti-P acnes bioactivity. All samples that lie within the RUQ are most closely associated with anti-P acnes activity of the kānuka extracts. Like with antifungal and anti-MRSA activity, there is a clear separation of samples from season one to samples from season two when it comes to anti-P acnes activity. Season two samples cluster together with samples in the RUQ and the right lower quartile (RLQ). Conversely, season one samples cluster together and are spread across the LUQ and LLQ. Most of season one samples correlate positively to factor 2 and thus negatively to bioactivity. Furthermore this model highlights sample 1 from land block J and samples 2 and 3 from land block E as the samples that lie closest to bioactivity. Of the 11 samples that fall into the RUQ, 4 are from land block E, 3 from J and 2 from H and F respectively. Overall, season two has many more samples positively correlated with anti-P acnes activity compared to season one (11 compared to 0 samples). ((Samples from season 1 are negatively correlated to anti-P acnes bioactivity which could suggest that these compounds are diminishing some of the potential anti-P. acnes properties of other compounds such as those in the RUQ)). Overall, this analysis indicates that more samples from season two are positively correlated with anti-P acnes activity more so than samples from season one, which is the opposite pattern than what was observed for antifungal activity yet the same pattern observed for anti-MRSA activity.

Figure 3.13b demonstrates the correlation of all compounds to anti-P acnes activity. Compounds that associate closely to bioactivity in the RUQ are suggested to play an important

role in the anti-MRSA properties of these extracts. From this analysis, 10 compounds fall within the RUQ, suggesting these could be potentially important in anti-P acnes bioactivity. However, none of these compounds were statistical significance correlated with bioactivity. Additionally, there were 13 statistically significant compounds in the LUQ, suggesting these compounds are negatively correlated to bioactivity. Of all compounds, compounds 1, 2, 3 and 4 appear to be the most interesting compounds as these are most closely associated to bioactivity and also statistically significant ($P < 0.05$) based on Martens Uncertainty Test (Figure 3.13).

| CAS No. | SI (%) | Compound | WRS | No of Samples | Compound No. |
|---|---|---|---|---|---|
| 5989-27-5 | 90 | Limonene | 2.232233 | 97 | 11 |
| 0-00-0 | 80 | Unknown[14] | 1.878925 | 47 | 1 |
| 70388-65-7 | 74 | Benzene, 1,1'-(1-butene-1,4-diyl)bis-, (Z)- | 1.483357 | 11 | 2 |
| 40716-66-3 | 92 | Nerolidol | 1.314253 | 48 | 3 |
| 470-82-6 | 93 | 1, 8-Cineole | 1.23435 | 88 | 4 |
| 122-73-6 | 79 | Benzyl isopentyl ether | 1.188297 | 39 | 5 |
| 0-00-0 | 84 | Unknown[11] | 1.145801 | 9 | 6 |
| 0-00-0 | 84 | Unknown[9] | 1.088614 | 29 | 7 |
| 6290-37-5 | 93 | Phenylethyl hexanoate | 1.082813 | 4 | 8 |
| 1196-01-6 | 88 | Verbenone | 1.018672 | 6 | 12 |
| 7460-74-4 | 87 | Pentanoic acid, 2-phenylethyl ester | 1.002286 | 35 | 9 |
| 4501-558-0 | 91 | α -Campholenal | 0.9370998 | 94 | 10 |
| 17699-14-8 | 90 | α-Cubebene[bb] | 0.0554604 | 49 | 14 |
| 30021-74-0 | 82 | γ -Muurolene | -0.3350128 | 36 | 15 |
| 87-44-5 | 96 | (-)-Caryophyllene | -0.3939772 | 54 | 16 |
| 0-00-0 | 87 | Unknown[17] | -0.395795 | 49 | 17 |
| 489-83-8 | 85 | Guaia-3,9-diene | -0.4287927 | 55 | 18 |
| 6753-98-6 | 91 | Humulene | -0.4459769 | 49 | 19 |
| 489-39-4 | 90 | Aromandendrene | -0.8657221 | 76 | 20 |
| 25246-27-9 | 78 | Alloaromandendrene | -1.06783 | 87 | 21 |
| 5986-49-2 | 92 | Palustrol | -1.11389 | 79 | 22 |
| 0-00-0 | 84 | Unknown[16] | -1.282645 | 97 | 23 |

**Table 1. 6: Top weighted regression co-efficient scores (WRS) for compounds exhibiting anti-P. acnes activity against *P. acnes*.** This table displays compounds with the highest WRS scores including compounds that demonstrate statistical significance ($P < 0.05$) based on Martens Uncertainty Test (highlighted in green). CAS No. = Abstracts Service Number; SI level = similarity index as per match with GC-MS database. Frequency of each compound among the 99 samples is indicated. Compound number refers to the location of each compound in Figure 3.13.

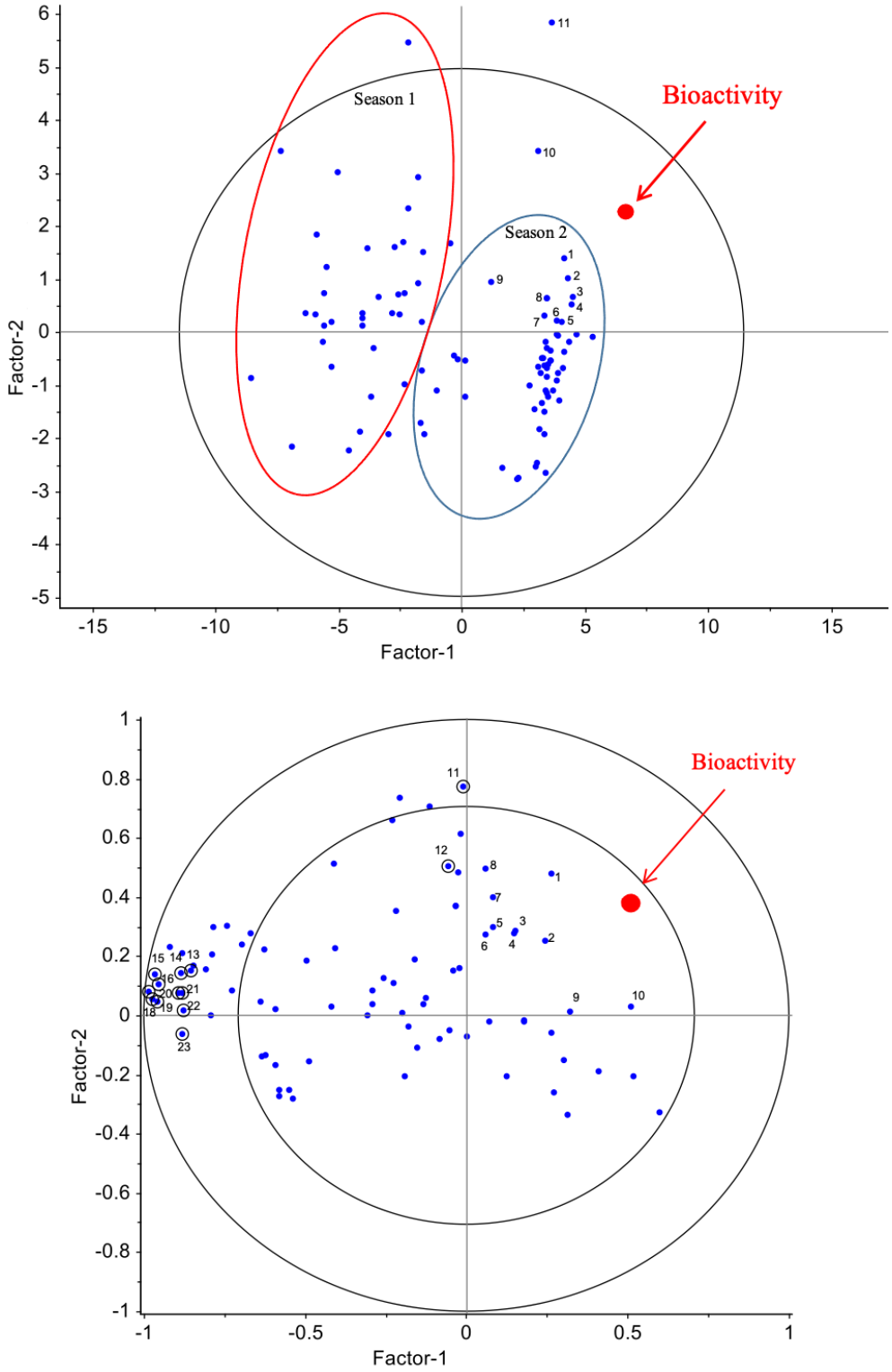



**Figure 3. 13: Multivariate analysis of GCMS analysis with anti-P acnes bioactivity.** Multivariate analysis reveals the correlation of compounds detected in the 99 samples of kānuka oil and/or samples with anti-P acnes bioactivity. **(a)** This graph represents all tested samples and how well they correlate with anti-P acnes activity. **(b)** This graph demonstrates the correlation of all identified compounds to anti-P. acnes activity. Blue dots = samples and compounds, red dots = anti-fungal bioactivity.

# 4.0 Chapter 4: Discussion

This thesis focused on evaluating natural variation in antifungal, anti-MRSA and anti-P acnes bioactivities of 99 kānuka extract oil samples sourced from Te Tairāwhiti (East Coast). Maximum variation at mid-log was revealed with 50 x $10^{-6}$ % v/v, 50 x $10^{-6}$ % v/v and 500 x $10^{-6}$ % v/v as ideal concentrations to evaluate growth of *C. albicans,* MRSA and *P. acnes,* respectively. These results rank the kānuka extract oil samples as being most potent with anti-P acnes activity, followed equally by anti-MRSA and antifungal activity. Following this, a full screen of all extracts was performed at the optimal concentration to evaluate variation in kānuka extract oil with respect to land block and season. Chemical composition was then determined by GC-MS analysis revealing 85 compounds, of which α-pinene was the most abundant. PLSR analysis correlated chemical profiles with the samples and bioactivities. A clear demarcation between seasons was observed in all investigated bioactivities of this study. Interestingly, antifungal activity was more potent in season 1, while anti-MRSA and anti-P acnes activity was more potent in season 2. Compounds positively and negatively correlated with each bioactivity were identified, which included compounds that could not be confidently identified based on similarity with a GC-MS spectra database. Some of these compounds have already been associated with each bioactivity, thus reflecting the interest in further research of the unsuspected compounds and/or synergy of compounds in the kānuka extracts.

## 4.2 Bioactivity of Kānuka Honey vs Bioactivity of Kānuka Oil

### 4.2.1 Antifungal bioactivity

This thesis exhibits extensive antifungal activity of kānuka oil extracted from kānuka leaves. Overall kānuka oil extracts inhibited the growth of *C. albicans* at an average of ~46-62% at a MIC of 50 x $10^{-6}$ % (v/v), where the maximum inhibition recorded at this concentration was ~82%. This is the first study of correlating antifungal activity with chemical composition of oil sourced from kānuka leaves, but similarly antifungal activity has been shown for kānuka honey (Brady et al., 2004). Like mānuka honey, kānuka honey has demonstrated antifungal bioactivity that was thought to be attributed to high levels of MGO (Holt et al., 2012). However, the antifungal activity of kanuka oil was not explicitly due to MGO since MGO was not detected in the chemical profiles.

This is the first study that has looked at the influence of geographical location and seasonal variation together across a large kānuka oil study population. The results in this thesis demonstrate geographical and seasonal variation in antifungal properties of kānuka oil extracts, which is consistent with results from other studies that looked at fewer samples per species (Perry et al., 1997; Perry et al., 1997; Porter et al., 1999). Overall land block H demonstrated the most potent antifungal activity with residual growth at 18% (Figure 3.4b), however this activity is not repeated in season 2 as land block F has the most potent antifungal activity in this season with residual growth at 32% compared to land block H which has residual growth of 39%. The range of residual growth (17-69%) from season one in spring suggests kānuka oil extracts have greater antifungal activity at this time compared to season two (32-96%) in autumn.

### 4.2.2 Anti-MRSA bioactivity

This report shows potent antibacterial activity of kānuka oil extracted from kānuka leaves. Overall kānuka oil extracts inhibited the growth of MRSA at an average of ~21-65% at a MIC of 50 x $10^{-6}$ % (v/v), where the maximum inhibition recorded at this concentration was ~72%. Like mānuka honey, kānuka honey has demonstrated antibacterial properties albeit the investigation into the compounds responsible for these properties was heavily understudied (Maddocks-Jennings et al., 2005). It has largely been an unexplored area of research over the past decade, with the few studies available focusing on the biological analysis of kānuka honey, mānuka oil, or mānuka honey (Albaridi, 2019; Braithwaite et al., 2015; Johnston et al., 2018).

This is the first study that has looked at the influence of geographical location and seasonal variation together across a large kānuka oil study population. The results in this thesis demonstrate geographical and seasonal variation in anti-MRSA properties of kānuka oil extracts, which is consistent with results from other studies that looked at fewer samples per species (Perry et al., 1997; Perry et al., 1997; Porter et al., 1999). Overall, land block H demonstrated the most potent anti-MRSA activity with the lowest detected residual growth at 28% (Figure 3.8b), however this activity is not repeated in season 2 (autumn) as land block J has the most potent anti-MRSA activity with residual growth at 33% compared to 36% residual growth in block H. The range of residual growth from season 2 (autumn) suggests kānuka oil extracts have greater anti-MRSA activity at this time compared to season 1 (spring).

### 4.2.3 Anti-P Acnes bioactivity

This thesis shows potent anti-P acnes activity of kānuka oil extracted from kānuka leaves. Overall kānuka oil extracts inhibited the growth of *P. acnes* at average of ~28-38% at a MIC of 500 x $10^{-6}$ % (v/v), where the maximum inhibition recorded at this concentration was

~57%. These results were consistent with the anti-P acnes properties of mānuka honey and existing applications containing mānuka honey for the treatment of acne and related skin conditions (Männle et al., 2020). However current literature for use of medical-grade kānuka honey as an alternative treatment for acne was not as strong (Semprini et al., 2016). For instance, 90% kānuka honey and 10% glycerin provided a mild improvement in acne, but these results were not significantly better than antibacterial soap alone (Männle et al., 2020; Semprini et al., 2016). Thus, kānuka honey has demonstrated anti-inflammatory and anti-bacterial properties but its effectiveness on *P. acnes* is poorly understood. Therefore, this thesis endeavoured to explore these properties as well as investigated potential lead compounds within kānuka oil that may be responsible for these properties. This is the first study that has looked at the influence of geographical location and seasonal variation together across a large kānuka oil study population.

The results in this thesis demonstrate geographical and seasonal variation in anti-P acnes properties of kānuka oil extracts, which is consistent with results from other studies that looked at fewer samples per species (Perry et al., 1997; Perry et al., 1997; Porter et al., 1999). Overall there is little difference between land blocks and season. Samples from both seasons and all locations resulted in a range of residual growth values from 43-64% (Figure 3.12). The range of residual growth from each season is almost identical, where season one (spring) had 43-87% residual growth range compared to 54-88% residual growth in season two (autumn). In essence, there is no single sample, season or land block that will generate superior anti-P acnes activity.

## 4.3 Chemical Diversity

Chemical diversity is influenced by environmental factors such as geographical location, soil quality, pest and predators, competition for resources and space from neighbouring plants as well as atmospheric pollution and weather exposure (Douglas et al., 2004; Iriti et al., 2009). A subset of kānuka plants from Te Tairāwhiti (East Coast region) in New Zealand have previously been sequenced and found to be genetically distinct from other species of kānuka in different parts of New Zealand, and they also show differences in their chemical composition and have subsequently been named as a distinct species of kānuka, *K. robusta* (de Lange, 2014). This thesis investigates the chemical diversity of 99 oil samples sourced from the East Coast *K. robusta* kānuka lineage. The chemical profiles of all lineages of kānuka are not well understood with minimal sampling of only a few plants from each lineage (de Lange, 2014; de Lange et al., 2004; Maddocks-Jennings et al., 2005). This thesis provides an initial exploration into the chemical and biological variation as per land block and season, which could guide future investigations of other kānuka lineages.

### 4.3.1 Kānuka oil chemistry vs mānuka oil chemistry

The composition of kānuka and mānuka oil have previously been investigated (de Lange, 2014; de Lange et al., 2004; Maddocks-Jennings et al., 2005; Perry et al., 1997; Perry et al., 1997). These two oils have many overlapping similarities with some very distinct and important differences. Unlike kānuka oil chemistry, mānuka oil chemistry is well understood with thorough identification of its bioactive components (Mavric et al., 2008; Saunders, 2017). Three major groups of compounds have been found in mānuka oil: sesquiterpenes (>60%) such as cubebene, elemene, caryophyllene and calamenene and present in smaller quantities are monoterpenes (<3%) such as limonene and linalool and α-pinene (~1%). Triketones are the third major group of compounds identified in mānuka oil and are of particular interest as they

are said to be the major contributors of the antimicrobial properties of manuka including killing the antibiotic-resistant MRSA (Douglas et al., 2004; Porter et al., 1999; Research, 2000). The concentration of this component that can be extracted from mānuka oil samples is extremely variable, which raised concerns in regards to commercialisation (Douglas et al., 2004; Owens et al., 2013; Saunders, 2017). Intriguingly, high levels of triketones were restricted to the East Coast and Marlborough Sound regions in New Zealand, suggesting promising antimicrobial potential of East Coast kānuka extracts tested in this study.

On the other hand, few studies have explored the chemical composition of kānuka oil. Studies such as (Sartorelli et al., 2007) have identified the major chemical component of kānuka oil as the monoterpene α-pinene (>50%). Modest levels of sesquiterpenes such as calamenene and ledol have also been detected. This correlates well with the findings from this thesis that identified α-pinene as the dominant compound in kānuka extracts tested here (~34%) and modest rates of ledol (~1.86%) (Table 1.2). Indeed, Table 1.7 illustrates a lot of overlap when the chemical composition of kānuka oil extracts is compared with mānuka oil extracts tested in another study (Porter et al., 1999).

| RT in Porter and Wlkins et al. 1999 | RT in this thesis | Compound Name | Kānuka oil in this thesis (% Area) | Kānuka oil in Porter and Wlkins 1999 (% Area) | Mānuka oil in Porter and Wlkins 1999 (% Area) |
|---|---|---|---|---|---|
| 4.50 | 5.73 | α-thujene | 1.108 | 0.62 | 0.03 |
| 4.66 | 5.87 | α-Pinene | 34.443 | 55.21 | 1.31 |
| 5.56 | 6.61 | β-Pinene | 0.012 | 0.63 | 0.12 |
| 5.88 | 6.81 | Myrcene | 0.052 | 0.1 | 0.24 |
| 6.64 | 7.40 | *p*-Cymene | 3.517 | 3.41 | 0.16 |
| 6.89 | 7.48 | Limonene | 1.559 | 3.94 | 0.10 |
| 6.83 | 7.53 | 1, 8-Cineole | 2.804 | 3.94 | 0.22 |
| 7.43 | 7.59 | (*E)*-β-Ocimene$^{i}$ | 0.012 | 0.33 | Nd |
| 7.69 | 7.97 | γ-Terpinene | 4.854 | 2.53 | 0.16 |
| 8.77 | 8.61 | Linalool | 1.375 | 1.52 | 0.10 |
| 8.97 | 8.68 | Isoamyl isovalerate | 0.625 | ND | 0.13 |
| 11.15 | 9.89 | Terpinen-4-ol | 0.161 | 0.24 | 0.04 |
| 18.56 | 12.98 | β-elemene | 0.005 | ND | 0.55 |
| 21.88 | 14.14 | α-Muurolene | 0.022 | 0.20 | 0.77 |
| 17.31 | 14.80 | α-Cubebene$^{bbbbb}$ | 2.114 | 0.21 | 3.95 |
| 23.65 | 15.12 | Nerolidol | 3.472 | 1.79 | 0.24 |
| 24.76 | 15.76 | Ledol | 8.847 | 1.86 | 0.23 |

**Table 1. 7: Comparison of compounds identified in kānuka oil this thesis with published identifications of compounds in kānuka and mānuka oil.** All compounds were separated using GC-MS analysis for which the retention time (RT) and percent area (abundance) are shown for all studies. ND = not detected <0.03%.

In Table 1.7, (E)-β-Ocimene$^{i}$ has a 1 letter suffix that indicates there are other compounds in our GC-MS analysis that were also detected with this name (e.g., there are two compounds with this name in Table 1.2). However since (*E)*-β-Ocimene$^{i}$ has a retention time (RT) that more closely matches the RT of published literature, we can be confident that this compound has been correctly identified in our analysis (Porter et al., 1999). The other detected compound in our analysis (i.e., table 1.2, (*E)*-β-Ocimene$^{ii}$) needs further validation to obtain the correct compound identification. A similar principal can be applied to α-Cubebene$^{bbbbb}$, which is one of five annotations in this study with the same name. We can be more confident that α-Cubebene$^{bbbbb}$ is actually α-Cubebene as the RT for this compound matches more closely

to the RT in the literature. Those compounds also identified with this name will require further validation to give correct compound identification.

This thesis shows diverse variation in the chemistry of 99 kānuka oil samples sourced from Te Tairāwhiti (East Coast region) New Zealand. It is clear that chemical composition is not driven by geographical location. The results from this study show clear variation in chemistry between samples from the same block and samples from different blocks. Similarly, the chemical composition of kānuka and mānuka oil extracts has been shown to vary not only between different species but also within the same species (Saunders, 2017), which further validates results from this thesis.

### 4.3.2 Chemical diversity and antifungal bioactivity

This thesis was the first study to conduct bioactivity-guided compound identification on kānuka oil antifungal bioactivity. The results from this thesis suggest eight individual compounds may be more important than others in terms of the antifungal activity of the kānuka oil extracts. These compounds are α-Murolene, Isoamyl isovalerate, 4-Carene[d], α-Cubebene[bbb] as well as four unknown compounds (table 1.4). α-Murolene is a commonly identified compound in both kānuka and mānuka oils as well as other essential oils (Douglas et al., 2004; Lahlou, 2003; Perry et al., 1997; Porter et al., 1999; Porter et al., 1999). For example, α-Murolene is also present in *Pinus roxburghii* Sarg., a plant extract that demonstrates strong antifungal properties to the same extent as commercial antifungal drugs (Kaushik et al., 2013). 4-Carene[d] and α-Cubebene[bbb] compounds will require further investigation to validate their identification, however the identification of isoamyl isovalerate is likely to be correct as the RT in this study matches previously published literature (Table 1.7). Isoamyl isovalerate and α-Cubebene have previously been identified in mānuka oils but not kānuka oils. However, 4-Carene does not seem to be commonly identified in either mānuka or kānuka, yet it has been

detected in other essential oils that possess antifungal properties such as *Santolina chamaecyparissus* (Salah-Fatnassi et al., 2017; Smeriglio et al., 2017).

Considering our results, it is clear that kānuka oil, like kānuka honey and/or mānuka honey, exhibit antifungal activity against fungal pathogens. However, combining the results from our chemical analysis with existing research of kānuka/mānuka honey and oil chemical analysis, we can see that the underlying compounds are different in the oil compared to honey, and that variations exist between the oil samples investigated in this study as well as previous studies of kānuka oil extracts (Christoph et al., 1999; Porter et al., 1999). It is also worth noting a vast number of unidentified compounds were detected in this study (Table 1.4); these compounds are interesting as they could be responsible for specific antifungal bioactivity but further research is needed to identify these compounds and further characterise kanuka oil as an extract. Furthermore, season one (spring) samples are most closely correlated to antifungal activity in comparison to season two (autumn) samples, suggesting this may be the preferred season to harvest in order to produce kānuka oil with stronger antifungal properties.

### 4.3.3 Chemical diversity and anti-MRSA bioactivity

The PLSR multivariate analysis tool used in this study identified 13 compounds that positively correlate with anti-MRSA bioactivity. It is interesting to note the highest scoring WRS compounds in this analysis are all different to the highest scoring WRS compounds for antifungal properties, suggesting that there may be different underlining molecular pathways responsible for each bioactivity. The top identified compound that also shows statistical significance is Terpinen-4-ol, which has previously been detected in other studies of plant essential oils including kānuka oil (Porter et al., 1999). Terpinen-4-ol is common in many plant-based essential oils and at a relatively high abundance. A clinical study on the topical use of tea tree oil isolated from *Melaleuca alternifolia* revealed that Terpinen-4-ol was the main

contributor of antibacterial bioactivity as well as other antimicrobial properties (Brun et al., 2019). However, when they compared the antimicrobial effects of this compound alone versus the antimicrobial effects of the crude extract, the crude extract was more effective, suggesting it may be a combination of compounds mediating antimicrobial activity. More specifically, Terpinen-4-ol has been found to be an effective antibacterial agent against MRSA and coagulase-negative staphylococci with no toxicities reported. These results corroborate the thesis results herein that found kānuka oil has high antibacterial activity against *MRSA* and that Terpinen-4-ol was the most correlated compound to anti-MRSA bioactivity.

The next most positively correlated compound to anti-MRSA activity that also showed statistical significance was Limonene. This compound has been investigated in a number of studies that have identified it as a strong individual constituent (IC) in plant essential oils as it consistently elicits strong anti-bacterial activity. Limonene is potent again strains of MRSA biofilm formation (Espina et al., 2015). The next correctly identified compound in this study with a high positive correlation to anti-MRSA activity was Nerolidol, which has been identified as a common constituent in essential oils such as black pepper, Cananga and myrrh oils where these three oils were found to markedly reduce the formation of MRSA biofilm formation (Lee et al., 2014). Furthermore when Nerolidol was tested as an IC alongside these three oils, all conditions led to an almost complete inhibition of the haemolytic activity of MRSA (Lee et al., 2014). These findings in existing literature further support the findings in this thesis; thus we can be more confident that these compounds play important roles in the anti-MRSA bioactivity of kānuka oil. Furthermore four compounds were unidentified in this analysis and three compounds were detected at multiple retention times; these compounds were also considered important as they showed high positive correlation to anti-MRSA bioactivity and therefore will be important to further investigate in future directions. In conclusion, results from this study suggest season two samples are more closely correlated to anti-MRSA activity than season one

samples, suggesting this may be the preferred season to harvest in order to produce kānuka oil with stronger anti-MRSA properties.

### 4.3.4 Chemical diversity and Anti-P Acnes bioactivity

This thesis is the first study to conduct bioactivity-guided identification of compounds underlying anti-P acnes bioactivity of kānuka oil. In this study, the top compounds that positively correlated with anti-P acnes bioactivity were Limonene; Benzene, 1,1'-(1-butene-1,4-diyl)bis-,(Z)-; Nerolidol; 1, 8-Cineole; Benzyl isopentyl ether; Phenylethyl hexanoate; Verbenone; Pentanoic acid, 2-phenylethyl ester; α -Campholenal and α-Cubebene[bb] (Table 1.6). Limonene and 1, 8-Cineole have previously been identified in New Zealand mānuka and kānuka oils (Maddocks-Jennings et al., 2005; Perry et al., 1997). Limonene is particularly interesting as it was also detected as the third lead compound for anti-MRSA bioactivity.

Studies have shown limonene as an effective treatment against *P. acnes* that also demonstrated anti-inflammatory and non-toxic properties against immune cells; this supports the use of Limonene as a promising skin care agent that can be used to treat skin diseases such as acne (Kubo et al., 1994; Yoon et al., 2010). Verbenone, α -Campholenal and α-Cubebene are also commonly identified constituents in plant essential oils including kānuka and mānuka, however literature is lacking on their potential individual antimicrobial properties (Magin et al., 2006; Zu et al., 2010). Verbenone is a dominant component in Rosemary essential oil which have been shown to exert strong anti-P acnes effects against *P. acnes* (Jafari-sales et al., 2020). Furthermore four compounds were unidentified in this analysis and one compound was identified at multiple retention times; these compounds were also considered important as they showed high positive correlation to anti-P acnes bioactivity and therefore it will be important to further investigate these compounds in future directions.

It is interesting to note the top ten WRS compounds for anti-P acnes activity are all different to the top ten WRS compounds for antifungal properties with the exception of unknown[9]. This compound is particularly interesting as it is the only compound that is present in all bioactivities with a high WRS score, suggesting this compound could be a key driver of all three antimicrobial bioactivities studied in this thesis. In conclusion, results from this study suggest season two samples are more closely correlated to anti-P acnes activity than season one samples, suggesting this may be the preferred season to harvest in order to produce kānuka oil with stronger anti-P acnes properties.

## 4.4 Alpha-Pinene

Alpha-pinene is a class of terpene, and in addition to kanuka oil, can be identified in other plant essential oils such as pine, tea tree oil, rosemary, and cannabis (Silva et al., 2012). Alpha-pinene is an important compound to consider here as it has been detected as the dominant compound in all of the kānuka oil samples tested in this study. The overall abundance of α-pinene is ~34.5% in the samples in this study (table 1.2). This abundance far outweighed the abundance of all other detected chemical constituents. This finding aligns with previous studies that have also investigated the chemical composition of kānuka oil extracts (Perry et al., 1997; Perry et al., 1997; Porter et al., 1999), where they detected α-pinene in the range of 50-70% of kānuka oil chemical composition.

Alpha-pinene is present as two enantiomers (-)-α-pinene and (+)-α-pinene. The negative enantiomer exhibits antiviral properties and has been shown to be an effective treatment and prevention for infectious bronchitis virus (Yang et al., 2011). The positive enantiomer has been shown to have potent antifungal activity against the pathogenic *C. albicans* and *Cryptococcus neoformans* and antibacterial activity against MRSA (Silva et al., 2012). This is interesting as previous literature that has investigated kānuka chemical

composition identified (+)-α-pinene as the predominant enantiomer (Perry et al., 1997). Other features of alpha-pinene include its anti-inflammatory properties via prostaglandin E1 and acetylcholinesterase inhibitor activity that aids in memory (Russo, 2011; Silva et al., 2012). However, despite α-pinene being detected as the most abundant compound in this thesis, it was not identified as a compound that positively correlated to any of the three bioactivities. This suggests that perhaps it is a synergistic relationship of α-pinene with other positively correlated compounds that are driving kānuka oil bioactivity. On the contrary, it could be that α-pinene is not at all responsible for any of the bioactivity in this study and could potentially be dampening the bioactivity of other chemical constituents.

## 4.5 Conclusion

In conclusion, 99 novel kānuka extracts were sourced in Ruatoria by Hikurangi Bioactives Limited Partnership and this thesis investigated the chemical and biological properties of these extracts. Firstly, *C. albicans* was used as a model organism to demonstrate anti-fungal bioactivity of these extracts. The MIC was identified at $5 \times 10^{-5}$ % (v/v) and residual growth demonstrated a median percent growth of treated *C. albicans* cells to range between ~38-54% with more variation in growth observed in season one (spring). The most potent extract in this experiment was seen in season one from land block H, however in season two (autumn) land block H was the least potent (96%) and land block F was the most potent (32%). Secondly, MRSA was used as a model organism to demonstrate the antibacterial bioactivity of these extracts, wherein MIC was surprisingly identical to antifungal bioactivity (i.e. $50 \times 10^{-6}$ % (v/v)). Residual growth showed the median percent growth of treated MRSA to range between 35-79% with comparable variation in both seasons. The most potent effects were seen in season 1 from land block H (28%) with the less effective coming from land block E where in some cases there was no effect on treated cell conditions. Finally, *P. acnes* was used as a

model organism to demonstrate the anti-P acnes bioactivity of these extracts. The MIC was identified at a much lower concentration, 500 x $10^{-6}$ % (v/v), compared to the two aforementioned bioactivities, suggesting these extracts have much more stronger anti-P. acnes properties compared to antifungal and antibacterial properties. Residual growth showed the median percent growth of treated *P. acnes* cells to range between 62-73% with more variation observed in season 2. The most potent effects were seen in season 1 from land block B (43%) with the less effective coming from season 2 land block H (89%). In conclusion, differences in growth inhibition could not be explained by geographic location or seasonal variation.

Chemical analysis of all 99 samples of kānuka oil was determined using GC-MS. Compounds were identified based on comparisons of GC-MS spectra with databases, and α-pinene was the most dominant compound accounting for ~34% of the total chemical composition. Other compounds with significant overall abundance included Ledol ~8.8%, γ-Terpinene ~4.8%, Nerolidol ~3.5% and 1, and 8-Cineole ~2.8%. It was also an interesting observation to note the overall differences in the presence of these compounds in the extracts: α-pinene was in 96/99, Ledol 93/99, γ-Terpinene 85/99, Nerolidol 30/99 and 1, 8-Cineole 88/99. Nerolidol was unique as it was only present in very few samples and only from samples from season 1.

The biological data was then related to the chemical data using multivariate statistics, specifically the PLSR multivariant analysis. This analysis identified those samples and compounds that were more positively correlated to bioactivity. For antifungal bioactivity, the most positively correlated samples were from land block B and the compounds that demonstrated high positive correlation were α-Murolene and Isoamyl isovalerate. For anti-MRSA bioactivity, the most positively correlated samples were from land block E and the compounds that demonstrated high positive correlation were Terpinen-4-ol, Limonene and Nerolidol. For anti-P acnes bioactivity, the most positively correlated samples were again from

land block E and the compounds that demonstrated high positive correlation were Limonene and Nerolidol, 1,8-Cineole. Unknown[9] is of particular interest as it was the only compound to have a high positive corelation to all three bioactivities. Overall, the 99 tested kānuka extracts in this study were highly bioactive with identification of lead compounds with known antimicrobial properties and some that remain unknown this gives promising potential for future research into these potentials that will benefit the pharmaceutical and nutraceutical sectors.

## 4.6 Future Directions

Unfortunately, due to time limitations of my thesis, there were still many experiments I would have liked to have done. Based on the experimental results and discussion, a few recommendations can be proposed for further work.

### 4.6.1 Further characterisation of kānuka bioactivity

In this thesis, key compounds where identified for three bioactivities and the identity of these compounds were based on the similarity index generated in the GC-MS analysis. It would be ideal to confirm the identity of these key compounds by ordering commercial standards of each of these compounds and running them though a GC-MS analysis. Comparing the GC-MS spectra of these commercial compounds to the spectra of the compounds identified in the kānuka extracts will directly determine correct or incorrect identification. If identification is correct, the bioactivity of the compound can then be investigated using the same assays used in this thesis. These experiments will directly determine if these compounds alone or a combination of these compounds in the kānuka oil is driving the bioactivity.

### 4.6.2 Identification of unknown compounds

Interesting compounds that were not able to be correctly identified in this study can be future characterised used bioassay-guided fractionation that is commonly used in natural product drug discovery (Dasyam et al., 2014). In particular, I would like to investigate unknown[9] as this was the only compound identified as important in all three bioactivities and thus maybe a key driver of all three antimicrobial bioactivities investigated in this thesis. Using this approach, the kānuka extracts can be fractionated and the resulting fractions can be monitored for bioactivity using the same methods as described in this thesis. Those fractions that display the bioactivities of interest can then undergo nuclear magnetic resonance (NMR) to identify the number of compounds in each fraction. The above steps can then be repeated until one compound is isolated and identified. This experiment is critical because, although unknown[9] is not the most abundant compound, it is possibly the most important for bioactivity.

### 4.6.3 Validation of season variation and geographical variation

This thesis demonstrated seasonal and geographical variation for *K. robusta*. This was achieved with 99 samples across two seasons. It would thus be interesting in further studies to look at more samples of *K. robusta* from more seasons and try to more accurately relate the bioactivity to season. Likewise it would be interesting to look at seasonal and geographical variation of extracts from other kānuka species around New Zealand to identify the best species to develop for commercialisation of certain bioactivities.

### 4.6.4 Identification of mechanism of action

This thesis identified three bioactivities for kānuka extracts, but the mechanism of action of these bioactivities was not determined in this thesis due to time constraints. Mechanism of action is important for drug discovery and development (Dasyam et al., 2014).,

For instance, pathogenic fungi and bacteria have evolved resistance to currently available antibiotics by overcoming the drug's mechanism of action (Rossiter et al., 2017). It would be most interesting to use chemical genetics to identify mechanisms of action by which kānuka extracts and their key compounds realise each bioactivity. (Dasyam et al., 2014; Mokhtari et al., 2018). Mechanisms of action that are distinct from the currently available antibiotics will provide a point of novelty to further investigate the pharmaceutical potential of the kānuka extracts.

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# 6. Appendix

## 6.1 PCA Plot Scores for Anti-Fungal Bioactivity

| Sample | Factor-1 | Factor-2 |
|---|---|---|
| B05#1 | 6.69 | 1.65 |
| H14#1 | 5.55 | 2.05 |
| B02#1 | 4.80 | -1.58 |
| J01#1 | 3.91 | -1.28 |
| B04#1 | 3.61 | 1.44 |
| H01#1 | 3.49 | -1.98 |
| E28#1 | 3.05 | 0.22 |
| F01#1 | 2.94 | 1.27 |
| B14#1 | 2.93 | 1.97 |
| H07#1 | 2.92 | 1.26 |
| B10#1 | 2.91 | 0.19 |
| F06#1 | 2.67 | 0.90 |
| E10#1 | 2.39 | -1.15 |
| F12#1 | 2.09 | -1.82 |
| F10#1 | 2.06 | -2.08 |
| B13#1 | 1.76 | 0.29 |
| B15#1 | 1.68 | 0.66 |
| E5#1 | 1.56 | 0.71 |
| H04# | 0.1.45 | -3.19 |
| B11#1 | 1.40 | -1.66 |
| B09#1 | 1.38 | -4.92 |
| H06#1 | 1.30 | -0.15 |
| E27#1 | 1.24 | -1.17 |
| B05a#2 | 1.23 | 3.67 |
| J09#1 | 1.22 | -1.46 |
| E29#1 | 1.22 | -0.75 |
| H05#1 | 1.19 | 3.63 |
| E25#1 | 1.11 | -2.69 |

| | | |
|---|---|---|
| F13#1 | 1.05 | -4.56 |
| J13#1 | 0.97 | -3.90 |
| E26#1 | 0.84 | 0.23 |
| B08#1 | 0.77 | -0.40 |
| F18#1 | 0.75 | -3.33 |
| E24#1 | 0.61 | -0.34 |
| B01#1 | 0.53 | -3.83 |
| F15#1 | 0.53 | -3.09 |
| J14a#1 | 0.42 | 1.42 |
| J14a#2 | 0.42 | 1.42 |
| F02#1 | 0.39 | 0.11 |
| E3#1 | 0.35 | -1.91 |
| B06a#2 | 0.33 | 2.05 |
| J12#1 | 0.30 | -0.57 |
| F03#1 | 0.27 | -1.95 |
| J08#1 | 0.18 | -2.93 |
| E23#1 | 0.14 | -0.08 |
| F16#1 | 0.10 | -4.41 |
| F15#2 | 0.02 | 1.63 |
| F05#1 | 0.02 | -4.14 |
| B02a#2 | -0.02 | 1.53 |
| H02#1 | -0.03 | -4.43 |
| F13#2 | -0.03 | 1.87 |
| F02#2 | -0.08 | 2.57 |
| F14#2 | -0.20 | 1.26 |
| J02#1 | -0.23 | -5.15 |
| E01a#2 | -0.23 | 2.67 |
| H02a#2 | -0.33 | 1.95 |
| F01#2 | -0.34 | 2.05 |
| H16#2 | -0.41 | 2.42 |
| E02a#2 | -0.41 | 1.83 |
| B15#2 | -0.48 | 1.60 |
| B01#2 | -0.49 | 2.54 |
| J10#1 | -0.54 | -2.10 |

| | | |
|---|---|---|
| E05#2 | -0.59 | 1.28 |
| J05#1 | -0.60 | -3.40 |
| F06#2 | -0.63 | 1.93 |
| H04a#2 | -0.68 | 1.82 |
| J11#1 | -0.70 | -1.63 |
| H01#2 | -0.82 | 1.37 |
| F16#2 | -0.86 | 1.32 |
| F10#2 | -0.95 | 0.97 |
| J09#2 | -0.96 | 2.14 |
| F03#2 | -0.98 | 1.04 |
| E23#2 | -1.03 | 2.54 |
| B04a#2 | -1.09 | 1.52 |
| J10#2 | -1.10 | 1.85 |
| J12a#2 | -1.13 | 2.00 |
| J24a#2 | -1.17 | 1.58 |
| H03#1 | -1.21 | -7.78 |
| F05#2 | -1.23 | 1.06 |
| J11a#2 | -1.59 | 1.55 |
| J02#2 | -1.65 | 0.99 |
| H05#2 | -1.85 | 1.91 |
| J13#2 | -1.89 | 1.60 |
| B08a#2 | -1.92 | 1.36 |
| H06#2 | -1.98 | 0.98 |
| E03#2 | -2.00 | 2.17 |
| E10a#2 | -2.05 | 1.59 |
| H08a#2 | -2.14 | 1.67 |
| E06a#2 | -2.20 | 1.81 |
| J08a#2 | -2.31 | 1.82 |
| F09#2 | -2.57 | 1.21 |
| J03#2 | -2.58 | 2.56 |
| H11#2 | -2.62 | 1.72 |
| F12#2 | -2.91 | 3.08 |
| F09#1 | -3.03 | -5.58 |
| E16a#2 | -3.78 | 2.62 |

| | | |
|---|---|---|
| J01a#2 | -5.63 | 3.08 |
| E2#1 | -6.72 | -0.66 |
| B06#1 | -7.77 | -11.22 |

**Table 1. 8: WRS of all samples in MVA of *C. albicans.***

Cumulative and individual explained variance of factor 1 and factor 2, this data reflects what is demonstrated in the PCA plots in section 3.2.4. The weighted regression coefficient scores correlating anti-fungal bioactivity with all 99 kānuka extract samples used in this study. The letter and 2 digits represent the sample name e.g. J01.The hash tag and number represent the season that sample corresponded too e.g. #1 = season 1.

## 6.2 PCA Plot Scores for Anti-MRSA Bioactivity

| Sample | Factor-1 | Factor-2 |
|---|---|---|
| E25#1 | 7.508025 | 12.62972 |
| E5#1 | 7.090959 | 8.248352 |
| E26#1 | 4.93385 | 4.705965 |
| H01#1 | 4.376426 | 10.42741 |
| J14a#1 | 3.732579 | 1.311868 |
| J14a#2 | 3.732579 | 1.311868 |
| E05#2 | 3.49493 | 0.776296 |
| H01#2 | 3.450713 | 0.156095 |
| J12a#2 | 2.869269 | -1.04321 |
| F10#2 | 2.762616 | -0.76381 |
| B02a#2 | 2.742208 | 0.009797 |
| F05#2 | 2.698564 | -0.92462 |
| B06a#2 | 2.615151 | -0.51849 |
| F14#2 | 2.609096 | -0.48847 |
| H06#2 | 2.585364 | -1.14838 |
| F15#2 | 2.490865 | -0.47769 |
| F16#2 | 2.448617 | -0.9501 |
| J02#2 | 2.310791 | -1.15008 |
| E16a#2 | 2.255095 | -2.10332 |
| F03#2 | 2.212373 | -1.20654 |
| F13#2 | 2.052456 | -0.9001 |
| E06a#2 | 2.033016 | -2.38992 |
| B15#2 | 2.02935 | -1.60763 |
| E10a#2 | 1.986065 | -1.94252 |
| H02a#2 | 1.880188 | -1.26126 |
| E02a#2 | 1.796034 | -1.66658 |
| H16#2 | 1.541739 | -2.22784 |
| J13#2 | 1.496132 | -2.72464 |
| E23#2 | 1.482293 | -2.09146 |
| F09#2 | 1.45222 | -2.68238 |
| F01#2 | 1.376173 | -2.0852 |

| | | |
|---|---|---|
| J10#2 | 1.326759 | -2.68979 |
| J03#2 | 1.32497 | -2.96641 |
| J24a#2 | 1.289673 | -2.38896 |
| F06#2 | 1.18695 | -2.47588 |
| H04a#2 | 1.134337 | -2.76533 |
| B04a#2 | 1.009577 | -2.79572 |
| F02#2 | 0.945655 | -2.05266 |
| J09#2 | 0.915365 | -2.24894 |
| J01a#2 | 0.910622 | -4.66755 |
| E03#2 | 0.85665 | -3.31656 |
| E23#1 | 0.847337 | 0.484703 |
| E10#1 | 0.815708 | 4.439001 |
| H05#2 | 0.785356 | -3.76526 |
| H11#2 | 0.778496 | -2.96518 |
| E01a#2 | 0.767988 | -3.4217 |
| B01#2 | 0.722429 | -3.14304 |
| J08a#2 | 0.684224 | -3.69291 |
| J11a#2 | 0.657923 | -3.64761 |
| E28#1 | 0.63914 | 3.948365 |
| E2#1 | 0.580176 | 6.198611 |
| B08a#2 | 0.451812 | -2.00488 |
| H05#1 | 0.329308 | -3.16927 |
| B05a#2 | 0.318701 | -3.14993 |
| H08a#2 | 0.176864 | -4.16968 |
| H04#1 | 0.099855 | 4.976841 |
| E27#1 | 0.077487 | 3.768686 |
| J11#1 | 0.059499 | 1.601634 |
| F10#1 | -0.02996 | 3.769426 |
| F12#2 | -0.18648 | -4.89894 |
| F12#1 | -0.28956 | 2.575479 |
| F02#1 | -0.4785 | -1.26805 |
| J12#1 | -0.62419 | -1.2792 |
| F09#1 | -0.62916 | 2.388978 |
| B04#1 | -0.87508 | 1.511154 |

| | | |
|---|---|---|
| J13#1 | -0.96878 | 3.090837 |
| F06#1 | -1.02707 | -0.17691 |
| E24#1 | -1.11686 | -1.38608 |
| E3#1 | -1.36294 | 0.727446 |
| F03#1 | -1.42057 | -0.02477 |
| F13#1 | -1.43618 | 4.565593 |
| B02#1 | -1.57293 | 4.43647 |
| J02#1 | -1.59776 | 7.632375 |
| J10#1 | -1.72638 | 0.171541 |
| J05#1 | -1.74662 | 0.731939 |
| B08#1 | -1.78485 | -1.16006 |
| B15#1 | -1.86918 | -1.72236 |
| F15#1 | -1.95614 | 1.472527 |
| H06#1 | -2.02133 | -3.05683 |
| F18#1 | -2.20117 | 1.530201 |
| B05#1 | -2.26256 | 2.659606 |
| H03#1 | -2.45765 | 3.146984 |
| B13#1 | -2.52766 | -3.06183 |
| B14#1 | -2.54039 | -1.61182 |
| B11#1 | -2.55702 | 1.056043 |
| E29#1 | -2.62876 | -1.09698 |
| F16#1 | -2.72238 | 2.409553 |
| J09#1 | -2.86255 | -0.56979 |
| J01#1 | -2.94855 | 2.545879 |
| F05#1 | -3.023 | 2.273572 |
| H07#1 | -3.36074 | -0.65108 |
| J08#1 | -3.66094 | -1.12754 |
| F01#1 | -4.36261 | -1.79481 |
| B10#1 | -4.54633 | -0.3106 |
| H14#1 | -4.61577 | 0.974598 |
| B01#1 | -4.82216 | -0.44196 |
| H02#1 | -4.94102 | -1.34855 |
| B09#1 | -6.38049 | 0.172188 |
| B06#1 | -17.5964 | 6.002018 |

**Table 1. 9: WRS of all samples in MVA of *MRSA*.**

Cumulative and individual explained variance of factor 1 and factor 2, this data reflects what is demonstrated in the PCA plots in section 3.3.4. The weighted regression coefficient scores correlating anti-bacterial bioactivity with all 99 kānuka extract samples used in this study. The letter and 2 digits represent the sample name e.g. J01.The hash tag and number represent the season that sample corresponded too e.g. #1 = season 1.

## 6.3 PCA Plot Scores for Anti-MRSA Bioactivity

| Sample | Factor-1 | Factor-2 |
|---|---|---|
| J01a#2 | 5.314622 | -0.0914 |
| E16a#2 | 4.671584 | -0.02891 |
| E10a#2 | 4.517148 | 0.667936 |
| J12a#2 | 4.458881 | 0.526532 |
| E06a#2 | 4.388679 | -0.1823 |
| H01#2 | 4.299541 | 1.00791 |
| F09#2 | 4.177377 | -0.37307 |
| E05#2 | 4.156145 | 1.38821 |
| J03#2 | 4.108207 | -0.66606 |
| F05#2 | 4.072382 | 0.19674 |
| H05#2 | 3.937729 | -1.28831 |
| J24a#2 | 3.924866 | -0.07112 |
| J08a#2 | 3.920493 | -0.77737 |
| F16#2 | 3.867186 | -0.03515 |
| E03#2 | 3.857563 | -0.91034 |
| H06#2 | 3.842306 | 0.209095 |
| J13#2 | 3.734048 | -1.10303 |
| E5#1 | 3.66866 | 5.850747 |
| J02#2 | 3.634487 | -0.34321 |
| E23#2 | 3.621553 | -0.54015 |
| F03#2 | 3.595895 | -0.53624 |
| F10#2 | 3.531354 | -0.60541 |
| H11#2 | 3.521061 | -1.22443 |
| J14a#1 | 3.481823 | 0.644348 |
| J14a#2 | 3.481823 | 0.644348 |
| B01#2 | 3.480612 | -0.84064 |
| B06a#2 | 3.474697 | -0.30274 |
| B04a#2 | 3.44875 | -1.1434 |
| F06#2 | 3.441405 | -0.66661 |
| H04a#2 | 3.424109 | -1.10687 |
| F12#2 | 3.409772 | -2.64625 |

| | | |
|---|---|---|
| H02a#2 | 3.385906 | -0.18783 |
| J11a#2 | 3.372015 | -1.91396 |
| F01#2 | 3.359979 | -0.63099 |
| J10#2 | 3.353191 | -1.50589 |
| B02a#2 | 3.341874 | 0.316425 |
| F14#2 | 3.327508 | -0.48428 |
| B15#2 | 3.257597 | -1.32117 |
| F15#2 | 3.247909 | -0.4882 |
| E02a#2 | 3.187666 | -0.77737 |
| H16#2 | 3.177911 | -1.82131 |
| E26#1 | 3.132635 | 3.427849 |
| F13#2 | 3.086134 | -0.64233 |
| H08a#2 | 3.071751 | -2.45722 |
| E01a#2 | 2.999914 | -2.53275 |
| J09#2 | 2.946184 | -1.45918 |
| F02#2 | 2.778253 | -0.99301 |
| H05#1 | 2.282181 | -2.7321 |
| B05a#2 | 2.241448 | -2.76552 |
| B08a#2 | 1.654865 | -2.5587 |
| E23#1 | 1.187242 | 0.946897 |
| E25#1 | 0.991381 | 8.368621 |
| J12#1 | 0.161364 | -0.52709 |
| F02#1 | 0.144241 | -1.21352 |
| H06#1 | -0.18196 | -0.52021 |
| E24#1 | -0.31594 | -0.4325 |
| J11#1 | -0.46985 | 1.688684 |
| B13#1 | -1.00269 | -1.10654 |
| E2#1 | -1.49356 | 7.234996 |
| B15#1 | -1.50367 | -1.91884 |
| E3#1 | -1.58391 | 1.516865 |
| F06#1 | -1.60278 | -0.72434 |
| F03#1 | -1.61307 | 0.202924 |
| B08#1 | -1.65685 | -1.70804 |
| F09#1 | -1.76337 | 2.915475 |

| | | |
|---|---|---|
| J10#1 | -1.76879 | 0.914409 |
| H01#1 | -2.14799 | 5.455719 |
| E28#1 | -2.16183 | 2.330804 |
| B14#1 | -2.30374 | -0.98262 |
| J05#1 | -2.31674 | 0.741295 |
| E10#1 | -2.35315 | 1.704233 |
| B04#1 | -2.54064 | 0.341775 |
| F12#1 | -2.55592 | 0.719924 |
| E27#1 | -2.74435 | 1.602253 |
| J09#1 | -2.83233 | 0.367969 |
| E29#1 | -2.96502 | -1.92938 |
| F10#1 | -3.39611 | 0.657245 |
| F15#1 | -3.58889 | -0.30465 |
| H07#1 | -3.6977 | -1.22357 |
| H04#1 | -3.84661 | 1.582868 |
| F18#1 | -4.01241 | 0.359782 |
| J13#1 | -4.01559 | 0.2672 |
| B11#1 | -4.05158 | 0.121412 |
| J08#1 | -4.13607 | -1.87863 |
| F01#1 | -4.56245 | -2.2213 |
| B05#1 | -5.02336 | 3.027032 |
| H02#1 | -5.27209 | -0.64829 |
| F16#1 | -5.31336 | 0.201789 |
| F13#1 | -5.48117 | 1.223922 |
| B01#1 | -5.5882 | 0.127385 |
| F05#1 | -5.60715 | 0.743823 |
| B10#1 | -5.65742 | -0.18937 |
| B02#1 | -5.88389 | 1.833721 |
| H03#1 | -5.93499 | 0.346914 |
| J01#1 | -6.32248 | 0.370317 |
| H14#1 | -6.91901 | -2.14855 |
| J02#1 | -7.36447 | 3.413838 |
| B09#1 | -8.55105 | -0.85442 |
| B06#1 | -26.0498 | -4.92354 |

**Table 1. 10: WRS of all samples in MVA of *P. acnes.***

Cumulative and individual explained variance of factor 1 and factor 2, this data reflects what is demonstrated in the PCA plots in section 3.4.4. The weighted regression coefficient scores correlating anti-P. acnes bioactivity with all 99 kānuka extract samples used in this study. The letter and 2 digits represent the sample name e.g. J01.The hash tag and number represent the season that sample corresponded too e.g. #1 = season 1.