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The Chemical and Biological Characterisation of Propolis Sourced from Te Urewera

A thesis

submitted partial fulfilment

of the requirements for the degree

of

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Shaun Toroa Te Rire McNeil



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Abstract

Propolis is a sticky resinous substance produced and used by bees for various structural and defensive purposes. Propolis is a potent source of biologically active compounds. Bees produce this substance using resin from nearby flora. For this reason, the composition of propolis in determined by the flora near the hive.

Te Urewera, home to Ngāi Tūhoe, is the largest native forest in Aotearoa. This forest has remained primarily untouched by invasive introduced flora, and therefore holds a relatively unique botanical composition. Because of this, it was speculated that propolis sourced from Te Urewera may be the first of a new kind of propolis. There have been no previous studies carried out on Te Urewera propolis. This study aimed to explore the chemical composition and biological activities of extracts from Te Urewera propolis.

There is a small amount of literature on propolis from Aotearoa. There had been no other studies carried out on propolis sourced from vast native Aotearoa bush such as Te Urewera. All other studies on propolis from Aotearoa identified their samples as being of the poplar class, a type of propolis common throughout the temperate zone. The literature described the biological activities and chemical compositions of various propolis from different locations internationally. The literature indicated that there was a consistent range of biological activities for all propolis, despite differing compositions. The literature also contained information relevant to the process of extracting biologically active compounds from crude propolis samples.

The first step in this project was to develop a method suitable to generate ethanolic Te Urewera Propolis Extract (TUPE). This extract would be necessary for all further experiments. A seven day maceration in absolute ethanol followed by low temperature centrifugation was determined to be effective for both the extraction of biologically active compounds, and the removal of wax from the extract. A sample of commercially produced propolis was also extracted in parallel. Data gathered in this method development was sufficient to determine that the commercial sample was of the standard poplar composition, and that TUPE differed completely from the commercial sample.

Following the successful generation of TUPE, the chemical composition of the sample was determined using Gas Chromatography – Mass Spectrometry (GCMS) and Liquid

Chromatography – Mass Spectrometry (LCMS) techniques. This analysis found that TUPE was composed primarily (50.6%) of diterpenes. Torulosol, a labdane diterpene was the most highly concentrated constituent present (38.5 mg g^{-1}) , followed closely by ferruginol (32.2 mg g^{-1}). The findings of this work confirmed that TUPE was not of the poplar class which hereforeto had been considered to be the only class of propolis found in Aotearoa. Instead, TUPE most closely resembled Greek propolis due to a shared high concentration of diterpenes. However, although TUPE was similar to Greek propolis, the concentrations of the specific diterpenes indicated that TUPE was in fact a new class of propolis.

The antiproliferative and cytotoxic biological activities of TUPE were investigated. This was carried out on HL60 cell cultures using the combination of an MTT assay and the generation of a trypan blue exclusion dose response curve. It was found that TUPE demonstrated an antiproliferative effect and was cytotoxic against HL60 cell cultures at concentrations greater than 12.5 μg mL^{-1}. The data also indicated that the cytotoxicity of TUPE was due to the induction of apoptosis in HL60 cells. This indicates that there is potential for further investigation into how TUPE can be used as an anticancer treatment.

The antioxidant activity of TUPE was determined using the CUPRAC method. TUPE was found to have a Trolox equivalent antioxidant capacity (TEAC) of 1481 ± 145 (mg_{Trolox} g^{-1}). This antioxidant capacity was comparable to that of highly potent poplar propolis samples found in the literature. This antioxidant activity indicates that TUPE may be useful for the treatment of diseases linked to oxidative stress such as diabetes.

The inhibitory effect of TUPE on superoxide production in HL60 cells undergoing respiratory burst was investigated. This was carried out using a colorimetric assay which utilised the reduction of water soluble tetrazolium 1 (WST-1) by superoxide to give a detectable chromophore. This experiment found that TUPE was able to inhibit superoxide production in HL60 cells in a logarithmic dose dependant manner. This inhibition of superoxide production indicates that TUPE may be useful in treating damage caused by inflammation responses.

TUPE was also tested for its antibacterial activity against two strains of *Staphylococcus aureus*, MSSA87 and MRSA 4549. This testing revealed that TUPE was able to

effectively inhibit the growth of both strains of *S. aureus* in a dose dependant manner. This antibacterial activity may have use in various areas including clinical settings.

This study found that propolis sourced from Te Urewera was unique in its composition and was in fact a new class of propolis. A method for the extraction of TUPE was successfully determined, and the antiproliferative, cytotoxic, antioxidant, antibacterial, and superoxide inhibitory activities of this sample were confirmed. This study provided a strong base of knowledge from which future studies on TUPE can be built. It was recommended that the biological activities identified in this study be expanded upon through testing with various other methods. It was also recommended that other biological activities such as antifungal and antiviral effects be tested. The pharmacokinetics of TUPE and the efficacy of treatment in whole organisms should also be tested using *in vivo* models.

TUPE is only able to be sourced from native bush and it is hoped that this work will incentivise protection and regrowth.

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This thesis is dedicated to my late father, Whiti Te Rire. You may not be here to read this today, but you helped me write it. I feel you and all of my Tīpuna over my shoulder every day guiding me in what I do. Many of the most important lessons in my life came from you, the two most relevant to my work today are the lessons of compassion and determination. Throughout our time together you never hesitated to remind me how much you cared. You were proud of me and you cared about me. It wasn't just because I was walking down a good path, but rather because I was me and that was enough. When things are tough, which they often are, I still know to be compassionate to myself, it's what you would have done for me. You also taught me to fight hard for what I believe in, to be determined. In the end, you fought an impossible fight with strength I have never seen elsewhere, all to stay here with us. Although your fight was lost, I am inspired to carry it on. I do what I do today in the hopes that I can find solutions to spare other people from going through what we all did. Even though you didn't get to stay with us, I will fight to help others stay with the ones they love. You were a good Dad.

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The support from Brenda Tahi and Manawa Honey NZ has been excellent. It was through Brenda that Te Urewera propolis samples were obtained and funding for this project was

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None of this would have been possible without the help of Laura, Jenny, and Annie as well. The work you all do to keep our labs running smoothly is something I can't thank you enough for.

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List of Abbreviations

Abbreviation	Meaning
AGE	Advanced Glycated End products
akt	Protein Kinase B
ATCC	American Type Culture Collection
BSTFA	*N*, *O*-Bistrifluoroacetamide
CAPE	Caffeic Acid Phenethyl Ester
CUPRAC	Cupric Reducing Antioxidant Capacity
DMSO	Dimethyl sulfoxide
DPPH	2,2-diphenyl-1-picrylhydrazyl
ELISA	enzyme-linked immunosorbent assay
FBS	Foetal Bovine Serum
GCMS	Gas Chromatography Mass Spectrometry
HBSS	Hank's Balanced Salt Solution
HPLC	High Performance Liquid Chromatography
IC50	Half maximal inhibitory concentration
IL-1B	Interleukin 1B
IMDM	Iscove's Mod
LDH	Lactate Dehydrogenase
MGO	Methylglyoxal
MRSA4549	Methicillin Resistant *Staphylococcus aureus* 4549
MSSA87	Methicillin Sensitive *Staphylococcus aureus* 87

Abbreviation	Meaning
MSTFA	*N*-Trimethylsilyl-*N*-methyl trifluoroacetamide
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NIST17	National Institute of Standards and Technology 17
PC1	Physical Containment Level 1
PC2	Physical Containment Level 2
PMA	Phorbol 12-myristate 13-acetate
ROS	Reactive Oxygen Species
scCO2	Super critical CO2
SOD	Superoxide Dismutase
TAC	Total Antioxidant Capacity
TEAC	Trolox Equivalent Antioxidant Capacity
TMS	Trimethylsilyl-
TNF-α	Tumour Necrosis Factor alpha
TUPE	Te Urewera Propolis Extract
UV	Ultraviolet
WST-1	Water Soluble Tetrazolium 1

Chapter 1

Introduction

1.1 The Significance of Propolis.

Propolis is a resinous substance produced by bees in a process independent of honey production. Within a colony of bees, some worker bees will be allocated the role of propolis production. These propolis specialists will seek out exposed resins and waxes from nearby plants during the warm part of the day. The bees immerse their legs in the resins and waxes and return to the hive coated in the materials. Once the bee has returned to the hive, the resin and wax is removed from the legs with the help of secretions from the bee's mandibular glands. Thus, propolis is formed.[1]

Propolis is soft and sticky when warm, while hard and brittle when cool. This property gives propolis useful mechanical functionality for bees. Propolis is also generally bioactive which adds chemical functionality to the substance. Bees utilize this combination of mechanical and chemical functionality for structural, defensive, and sanitary purposes. Some examples of this include[2]:

- Sealing holes or cracks in hive/cavity.
- Strengthening the borders of the comb.
- Weatherproofing hives.
- Making hive entrances defensible.
- Embalming hive intruders.

Propolis has been used by humans for millennia. In ancient Egypt, people used propolis to embalm their dead. In ancient Greece, propolis was used to treat ulcers, external wounds, and respiratory irritation. Two notable Greek physicians who recorded the use of propolis were Hippocrates and Pedanius Dioscorides. The use of propolis carried over into ancient Rome; Romans utilized propolis in the same manner as in Greece, however, by this time it was commonly used as an aid for splinter removal. Propolis was used less extensively during medieval times but human understanding of its medicinal properties survived in folk medicine.[3] This folk knowledge was formalized in the notable early modern medicine text, *The Complete Herbal* by Nicolas Culpeper.[4]

The arrival of the 19th century brought great improvements to the scientific method and the study of propolis flourished. This remedy which had been used extensively for so long was able to be put under the metaphorical microscope and become further understood. However, this area of research is far from being completely understood. Propolis is still an area of intense focus for many natural products chemists due to the significant potential for the discovery and development of new pharmaceuticals from this ancient remedy.[3]

1.2 Propolis Classes

In similarity to how honeys are grouped into their floral origin, propolis is also sorted into classes based on its botanical origin. All classes of propolis share the same macroscopic properties such as being sticky, containing a mixture of wax and balsam, and the way in which it is produced by bees. All propolis, regardless of class, is generally biologically active across a range of bioactivities. However, classes of propolis differ in their exact chemical composition. For example, the component which may be the primary source of antimicrobial activity in one class of propolis is likely completely absent in a different class of propolis which is still just as antimicrobial.

This combination of variation and consistency across classes means, from a natural products perspective, that the discovery of a new class of propolis is also the discovery of a potent source of bioactive molecules.

From a research perspective, this means that when working with propolis samples of unknown class, methods which may be viable for other propolis classes may not be suitable for propolis of a new class. A biological assay which may look at the expression of a protein which is known to be reduced by another propolis class to indicate anti-inflammatory activity may not be reduced at all by a different class of propolis. However, the new sample of propolis may also hold anti-inflammatory properties which act through the modulation of a completely different cellular pathway. The same is true for chemical profiling methods. A method of derivatization which may be effective at improving the volatility or ionization patterns of the constituents of one class, may be ineffective at improving the volatility or ionization patterns of the constituents of another.

It is crucial to be aware of the variation between propolis classes throughout all aspects of propolis research. From the initial reviewal of literature through to laboratory practice, it must be kept in mind that each class of propolis must be treated differently. On this issue, Professor Vassya Bankova, a world expert on propolis studies, once stated:

"It became clear that comparing propolis samples from different regions of the world might be the same as comparing extracts of two plants that belong to different plant families"[5]

1.3 Aotearoa and Propolis.

There have been relatively few studies conducted which have included propolis samples from Aotearoa. Studies which included chemical characterisation, and therefore determination of class, found that samples they investigated were poplar class propolis.[6-8] Poplar propolis is characteristically sourced from a mixture of trees found in Europe including *Populus spp.* and *Pinus spp*. In general, the trees from which poplar propolis is sourced were imported to Aotearoa during the early colonisation of the country from 1769 through to 1840. This introduced flora can be found nearly everywhere in Aotearoa today and there are few forests left which remain untouched by this arboreal colonisation.

The previously mentioned studies on propolis sourced from Aotearoa either listed source locations as bush which is not endemic or did not include a specific location. There have been no studies on propolis sourced from definitively endemic bush. Due to the unique composition of endemic Aotearoa bush, there is great potential for the discovery of a new class of propolis here.

1.4 Te Urewera.

Te Urewera is a forest situated on rugged hills in the eastern Bay of Plenty and the northern Hawke's Bay regions of Aotearoa. Te Urewera is occupied by Ngāi Tūhoe, an iwi from the Mataatua waka who have strong ideas surrounding kaitiakitanga (guardianship) of the forest. Tūhoe believe in living synergistically with the environment and reject ideas of land ownership in place of guardianship. Through Tūhoe's stance that the bush is something which is not meant solely for human exploitation, the trees which were introduced to the rest of the country for arboricultural purposes were never introduced widely into Te Urewera. Figures 1.1 and 1.2 show a photograph taken within Te Urewera and the location of Te Urewera on a map of Aotearoa respectively.

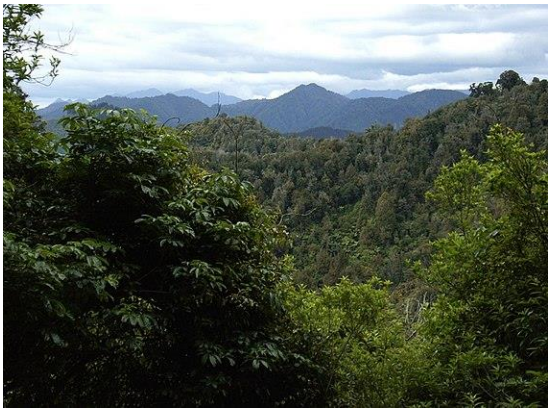


Figure 1.1. Image of Te Urewera Bush.[9]



Figure 1.2. Te Urewera highlighted in red on a map of Aotearoa.[10]

Apiculture in Te Urewera began in 1830 upon the introduction of the European honeybee to Aotearoa. Since then, apiculture within Te Urewera has continued to expand. The propolis produced in Te Urewera has never been investigated before.

1.5 Aims and Outline of the Thesis

This study aimed to carry out a chemical and biological characterisation of propolis sourced from Te Urewera. It was believed that there was significant potential for this propolis to be the first of a new class.

Chapter 2 is a survey of the available international and domestic scientific literature regarding chemical and biological activity investigations on propolis. Chapter 3 provides details on some of the general methods used in this study. Chapter 4 describes the development of a method to extract bioactive compounds from Te Urewera propolis using an ethanolic maceration. Chapter 5 details the determination of the chemical characterisation of Te Urewera propolis utilising both gas chromatography – mass spectrometry (GCMS) and liquid chromatography – mass spectrometry (LCMS) methods. Chapter 6 is an investigation into the cytotoxic and antiproliferative effect of Te Urewera propolis extract (TUPE) on HL60 cell cultures. Chapter 7 determined the antioxidative capacity of TUPE using a simple colorimetric assay, which provided quantitative data for comparison against other literature. Chapter 8 was an investigation into the inhibitory effect of TUPE on superoxide generation in HL60 cells undergoing respiratory burst.

Chapter 9 reports on the antibacterial of TUPE when tested against a two strains of *Staphylococcus aureus* using an agar well diffusion assay. Finally, the conclusions which can be drawn from this study as a whole and recommendations for future research are given in Chapter 10.

The findings of this research will hopefully provide a strong base for further research into useful biological activities of this propolis sample. It is also hoped that the findings of this research may raise awareness of the unique nature of Aotearoa's native bush and promote its protection and regrowth.

Chapter 2

Literature Review

In this chapter, the literature surrounding propolis research is discussed. As this literature is very extensive only selected sections will be dealt with in detail. This includes: a description of some relevant propolis classes, a summary of all the past research on propolis sourced from Aotearoa, information relevant to the extraction of bioactive molecules from propolis, and finally propolis products which are commercially available in Aotearoa will be listed.

2.1 Classes of Propolis

Propolis is organised into classes based on botanical origin in much the same way as honey is organised into floral origin. Opinions vary on the number of propolis classes, some would argue that there are between five to ten propolis classes while others believe there are closer to thirty. This difference in opinions arises as there is no strict definition of what a class is. The chemical composition of propolis classes varies heavily between classes, but the biological effects remain similar. The origin, composition, and confirmed biological activities of some propolis classes will be discussed. This will provide some general knowledge on propolis, give the chemical composition of other propolis classes for comparison against the Te Urewera sample, and justify what activities should be explored in this new sample.

Poplar propolis is discussed first as it is the most common class of propolis and it conforms to most of the traits common to all propolis classes. Brazilian green propolis will be discussed in detail as it is a good example of non-poplar propolis and it is an exception to some of the traits common to all other propolis classes.

2.1.1 Poplar

Poplar is considered to be the most common class of propolis. The majority of commercially available propolis products are of the poplar class unless otherwise specified. Currently the literature so far categorises propolis from Aotearoa to be of the poplar class. [6-8, 11]

2.1.1.1 Origin

The primary botanical origin of poplar propolis is, as the name suggests, from *Populus* spp. This includes but is not limited to: *P. alba*, *P. nigra*, and *P. tremula*, Figure 2.1.[11]



P. alba *P. nigra* *P. tremula*

Figure 2.1 Various Populus species.[12-14]

Poplar trees are commonly found in temperate zones including Europe, North America, and non-tropical regions of Asia, Figure 2.2.[5] In temperate zones poplar trees account for a significant portion of indigenous scrubland. As seen in Figure 2.2, Aotearoa is located within the temperate zone. Introduced *Populus*. sp. flourishes in Aotearoa for this reason.

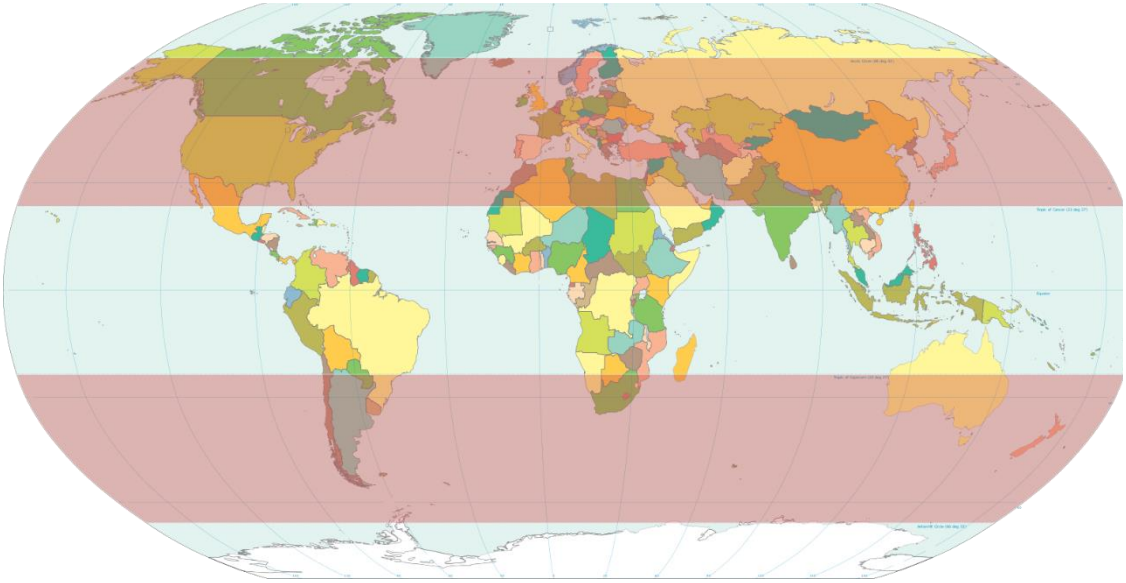


Figure 2.2 World map with temperate zone indicated in red.[15]

Poplar trees are considered to be of great importance ecologically due to their contribution to community level interactions within the forests in which they are present. The collection of propolis from poplar trees is an example of this. Beyond bees, it has been

observed that the entire composition of arthropods within a forest community is determined by whether or not the poplar trees present are genetically susceptible to the formation of galls by aphids. If the aphids are able to form galls on poplar trees, then this interaction supports a range of birds and insects which depend on the aphids. If the poplar are resistant to the aphids, a different range of birds and insects will be present. In short, poplar trees are common to temperate forests and are a keystone member of their communities. They contribute to a wide range of community level interactions including propolis production.[16]

Eva Crane, a world renowned expert on bees, was the first to attempt to determine the botanical origin of any propolis, this was carried out in a temperate region during the late 1970s. Crane first identified which bees were collecting propolis and wrote a list of the plants from which it was suspected they collected resins. However, due to difficulties in observation with bees collecting resin from high locations in trees, Crane was unable to narrow the list of plants down to determine exact origins.[17] Later work by a Russian botanist, Popavko, finally identified the origin of the propolis from the temperate zone to be from poplar trees by comparing the flavonoids in propolis originating in temperate zones to the bud exudate from poplar trees, Figure 2.3. Thus, poplar propolis was the first class of propolis identified, and it was determined that it was sourced from bud exudate located in the upper branches of the tree.[17]



Figure 2.3 Poplar bud showing exudate.[18]

One of the secondary botanical sources of poplar class propolis, *Pinus* spp., is also of significant relevance in the context of Aotearoa. Aotearoa is home to a large amount of

plantation forests, 89% of which are comprised of *Pinus radiata*.[19] Pinocembrin is one of the key compounds which identifies poplar propolis, and is also found commonly in *Pinus* spp.[20] The current view that Aotearoa only contains propolis from the poplar class is likely due to the overwhelming presence of *Pinus radiata*.

2.1.1.2 Chemical Composition

As poplar propolis is the most common class of propolis in the world, it has also been the most thoroughly investigated. Over 300 compounds have been recorded in poplar propolis so far. The compounds most strongly associated with poplar propolis are flavonoids.[11] Poplar propolis, like all other propolis classes, is known to vary in quantitative composition, but remains fairly constant in qualitative composition. This is due to bees collecting exudate from the same sources, but at different proportions depending on the relative abundance of each source nearby.[21]

Qualitatively, poplar propolis extract is made up of a range of polyphenols including phenolic acids and flavonoids. The polyphenols generally used to identify a propolis extract as being poplar in origin are the flavonoids pinocembrin, chrysin, galangin, pinobanksin, Figure 2.4[22].

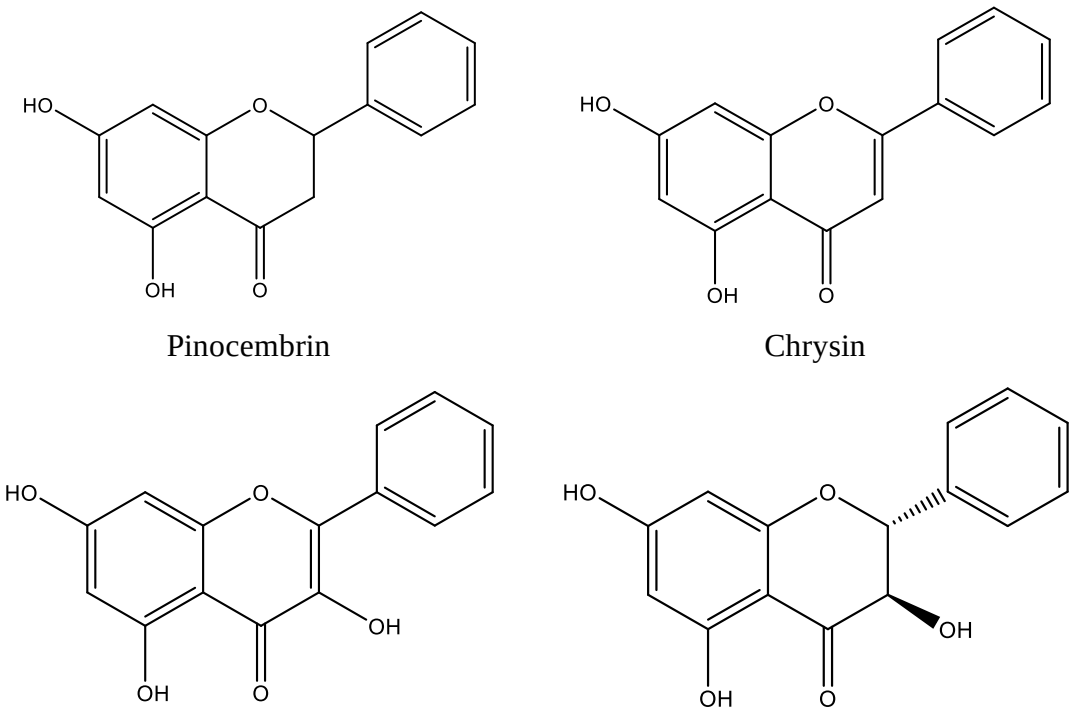


Figure 2.4 Molecular structures of common poplar propolis extract constituents.

The molecules previously mentioned are not the only polyphenols in poplar propolis extracts. The following polyphenols are also found: caffeic acid, caffeic acid phenethyl ester (CAPE), caffeic acid cinnamyl ester, coumaric acid, ferulic acid, isoferulic acid, quercetin, quercetin 3-methyl ester, quercetin 7-methyl ester, quercetin 5,7-dimethyl ester, apigenin, kaempferol, kaempferide, isorhamnetin, lueteolin, luteolin 5-methyl ester, galangin 5-methyl ester, methoxychrysin, naringenin, liquiritigenin, pinostrobin, pinobanksin 5-methyl ester, pinobanksin-3-*O*-propionate, pinobanksin-3-*O*-pentenoate, pinobanksin-3-*O*-hexanoate, and other pinobanksin derivatives.[11, 23]

2.1.1.3 Biological Activity

There are numerous reviews on the bioactivities of propolis. Generally, the articles cited in this review primarily describe poplar propolis.[24, 25]

Ethanolic extracts of poplar propolis have been confirmed to have *inter alia* antibacterial, antifungal, antiviral, antioxidant, anti-inflammatory, immunomodulating (in autoimmune disease models), antitumor, antimutagenic, antidiabetic, hepatoprotective, antiulcer, and cardioprotective properties.[11, 24-26] These properties have been tested through a range of methods across many samples. There is no one molecule which is solely responsible for any of these activities. As previously mentioned, the repeated failure to identify single components responsible for the activities indicates that the biological activities of propolis are caused by synergistic interactions of many compounds present in the extract.[5, 11]

However, it can be said that the flavonoid components of poplar propolis extracts do seem to play a major role in many of these biological activities. Several studies have found that the concentration of flavonoids within propolis extract samples has an approximate direct impact on the strength of activity.[26-28]

Even though none of the biological activities of poplar propolis can be attributed to single compounds, compounds of biological interest can be identified from propolis extracts. Although it is not solely responsible for the range of anticancer properties demonstrated by poplar propolis extracts, caffeic acid phenethyl ester (CAPE), Figure 2.5, is a molecule of specific interest in cancer research. Since its identification in poplar propolis, CAPE

has been tested alone and found to be effective for a range of anticancer effects such as: the suppression of human neurofibromatosis, tumour xenographs in mice, the upregulation of the p53 tumour suppressor protein and the suppression of metastasis inducing protein kinase B (akt) .[29-31]

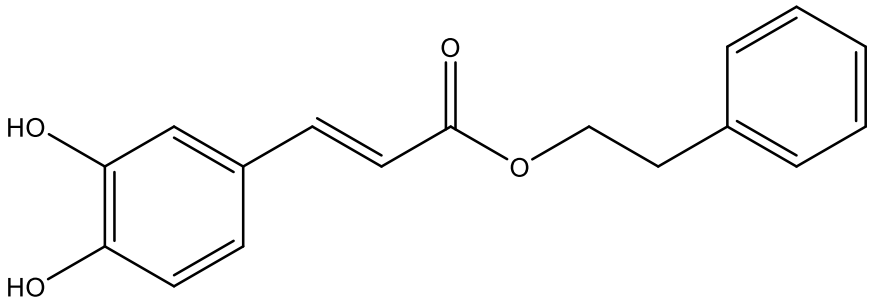


Figure 2.5 Structure of CAPE.

Although the biological activities of propolis extracts are due to biochemically complex synergistic effects, there are still observable trends with these effects. The antibacterial activity of poplar propolis extracts is a good example of this. Although poplar propolis extracts have been shown to possess a reliably strong antibacterial activity, it has also been shown that they are consistently less effective against gram negative bacteria compared to gram positive bacteria.[25, 32, 33] It has been hypothesised that the poplar propolis extract may have some interaction with the outer membrane of gram negative bacteria which affects its activity.[34]

2.1.2 Brazilian Green Propolis

Brazilian green propolis, Figure 2.6, is a good example of a propolis class completely separate from poplar propolis, for this reason, it will be discussed in detail here. There are many different classes of propolis which originate in Brazil, Brazilian red, Brazilian yellow, and Brazilian brown are also only found in Brazil.



Figure 2.6 Crude Brazilian green propolis. [35]

2.1.2.1 Origin

As the name suggests, Brazilian green propolis was originally discovered in Brazil. Brazilian green propolis is considered to be the "highest quality" class of propolis produced in Brazil as it has the highest artepillin C content, see below. *Baccharis dracunculifolia*, Figure 2.7, was identified to be the botanical source of Brazilian green propolis. This was achieved through comparison of the plant resin's chemical composition to that of the propolis. Observations of both the morphological and anatomical features of *B. dracunculifolia* and its interactions with both native and European honeybees bees were also used to confirm the origin of Brazilian green propolis.[36]



Figure 2.7 *Baccharis dracunculifolia*, the botanical source of Brazilian green propolis.[37]

Brazil is a potent source for propolis of classes other than poplar due to the fact that there are extensive native forests with no trees of the *Populus* genus present in these forests. As previously mentioned, in the temperate zone and in general throughout Europe, *Populus* trees are abundant and bees will preferentially source plant resins from *Populus*. In Brazil, that is not possible and, therefore, bees will look to other plants such as *B. dracunculifolia* for plant resin.[36]

Brazilian propolis is a good example of the conditions required for a non-poplar class of propolis to be produced. Firstly, there must be a lack of *Populus* sp. and other secondary poplar class propolis sources such as *Pinus* spp. Secondly, there must be other plants available from which the bees can source useful resin for the production of propolis.

2.1.2.2 Composition

As expected, Brazilian green propolis has a different chemical composition to poplar propolis.

The defining trait of Brazilian green propolis is the presence of Artepillin C, Figure 2.8 in high concentration. Artepillin C is a derivative of *p*-coumaric acid, it is also known as 3,5-diprenyl-4-hydroxycinnamic acid. In plants, *p*-coumaric acid, like all phenolic acids, is biosynthesised through the shikimate pathway following the breakdown of glucose.

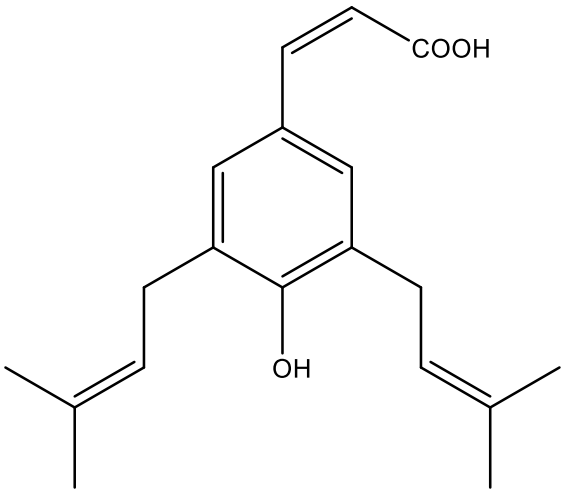


Figure 2.8 Structure of artepillin C, the characteristic component of Brazilian green propolis.

A study carried out in 2020 by Xu *et al*[38] gives the most detailed chemical composition of Brazilian green propolis yet. This study found that the chemical composition of Brazilian green propolis contained many of the same molecules as poplar class. However, the difference between the classes was principally due to the presence of artepillin C which made up 36% of Brazilian green propolis extract but is completely absent from poplar propolis. The Brazilian green propolis extract was also missing many compounds which were found in poplar propolis.

The compounds found in the Brazilian green propolis extract [38] were: *p*-coumaric acid, dicaffeoylquinic acid, an isomer of dicaffeoylquinic acid, quercetin, pinobanksin, 4-methoxy pinobanksin, kaempferol, quercetin-methyl ether, 5-isoprenyl caffeic acid, 3-isoprenyl-*p*-coumaric acid, an isomer of 3-hydroxy-2,2-dimethyl-8-prenylchromane-6-propenoic acid, galangin, kaempferide, pinobanksin-3-*O*-acetate, quercetin-dimethyl ether, 5-isoprenyl caffeic acid-*p*-coumaric acid ester, an isomer of diisoprenyl-*p*-coumaric acid, artepillin C, 3-prenyl-4-(dihydrocinnamoyloxy)-cinnamic acid, (*E*)-3-[2,3-dihydro-2-(1-methylethenyl)-7-prenyl-5-benzofuranyl]-2-propenoic acid, and a range of unidentifiable triterpenes.

2.1.2.3 Biological Activity

Like all classes of propolis, the extract of Brazilian green propolis boasts a wide range of biological activities. For Brazilian green propolis extracts a range of biological activities have been tested including but not limited to anti-inflammatory, antioxidant, anticancer, neuroprotective, antiinfluenza, and antimicrobial activities.[39-44]

However, Brazilian green propolis does have a distinctive trait, which separates it from other propolis classes which is that most of its biological activities can be attributed to a single compound. This compound is artepillin C. As previously mentioned, the presence of artepillin C in high concentrations is the defining chemical trait of Brazilian green propolis. Artepillin C is also an extremely potent bioactive molecule across a range of biological activities.

The biological activities of artepillin C alone has been an area of much study and it has been found to have antimicrobial, anti-inflammatory, antioxidant, anticancer, immunomodulatory, neuroprotective, and gastroprotective properties across a range of studies.[45]

2.1.3 Other Classes

Poplar and Brazilian green propolis have been discussed in detail. However, they are far from the only propolis classes which can be found. In Brazil alone, there are at least three distinct classes of propolis other than green propolis.[36]

Other classes include Russian birch propolis which is high in flavones and flavonols which are different to the ones found in poplar propolis.[5] The botanical source of Greek propolis is *Cupressus sempervirens* and other *Pinus* spp.[46] the composition of Greek propolis consists of a wide range of diterpenic acids.[46-49]
Pacific propolis from Japan, Hawai'i, and Taiwan all have the same botanical source, *Macarenga tanarius*. The chemical composition of Pacific propolis notably comprises a range of prenyl flavonoids.[5]

Australian stingless bees produce a propolis, the extract of which consists primarily of terpenic acids (30%) and phenolic acids (7%).[50, 51]

2.2 Propolis in Aotearoa.

There has been a limited amount of research conducted on Aotearoa propolis samples in the past. To date, there have been only seventeen total studies performed which have used Aotearoa propolis samples.

The first study to investigate propolis sourced from Aotearoa was conducted in 1995 at the University of Waikato (UoW). This was performed by Markham *et al*[6]. This is the only study in all scientific literature on propolis from Aotearoa to include specific locations from which the samples were sourced.

Eleven of the seventeen studies sourced their propolis from Mānuka Health New Zealand (Te Awamutu, Aotearoa).[8, 29, 52-60] Mānuka Health farms their bee products from mānuka rich bush around Aotearoa. These samples were all part of the BIO30 or BIO100 range which are available commercially; The BIO30 and BIO100 ranges of propolis products do not list any specific source locations. Four of the studies used Comvita Limited (Paengaroa, Aotearoa) as their propolis source.[51, 61-63] Comvita also sources their propolis from mānuka rich bush, but do not disclose any exact locations. The remaining study was that of Kumazawa *et al*[7] which did not disclose either a specific location within Aotearoa or a commercial source for their propolis sample.

2.2.1 Studies on Non-Commercial Propolis Sourced in Aotearoa.

The first study conducted on propolis from Aotearoa was by Markham *et al*[6] in 1995. This was a collaborative study between the New Zealand Institute for Industrial Research and Development and the Chemistry Department at the University of Waikato. This study investigated propolis sourced from the Waikato, Bay of Plenty, Coromandel, Auckland, Northland, and Taranaki regions of Aotearoa. The specific locations of the sampling sites in this study were not defined. Of these locations the Coromandel region is the most likely to resemble native Aotearoa bush. However, it is still unlikely that this propolis has a solely native botanical origin due to extensive pine forestry within the Coromandel bush. The aim of the study was to obtain a chemical profile of the ethanol extracts of propolis samples using high performance liquid chromatography (HPLC) and GC-MS methods. In all of the samples tested, the same primary constituents were identified.

The constituents Markham *et al*[6] identified via HPLC analysis , Figure 2.9, included: cinnamic acid, pinobanksin, pinocembrin-3-acetate, 1,1, -dimethyl-allylcaffeic acid, caffeic acid phenethyl ester (CAPE), chrysin, galangin, pinocembrin 7-methyl ether, chrysin 7-methyl ether, and galangin 7-methyl ether. This study also noted that there were other constituents of the ethanolic extract of propolis (EEP) which were too low in concentration to identify.

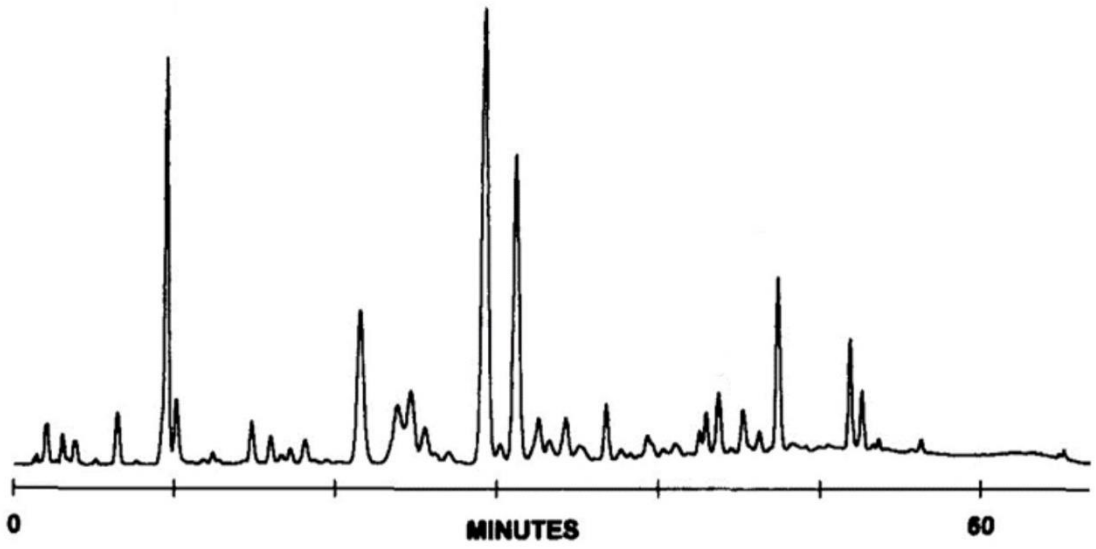


Figure 2.9 HPLC chromatogram of Aotearoa EEP. (Reproduced with permission from Markham et al).[6]

The aromatic constituents of the Aotearoa propolis identified via GC-MS included: styrene, benzyl alcohol, benzoic acid, 2-phenylethanol, 3-phenylpropanoic acid, ethyl 3-phenylpropanoate, cinnamic acid, vanillin, ethyl cinnamate, 5-phenyl-*trans*-3-pentenoic acid, ethyl 5-phenyl-*trans*-3-pentanoate, vanillic acid, *p*-coumaric acid, 5-phenyl-*trans*-*trans*-2,4-pentadienoic acid, ferulic acid, ethyl ferulate, 3-methylbut-2-enyl ferulate, and 3-methylbut-3-enyl ferulate.

The fatty acid constituents of all Aotearoa propolis samples identified via GC-MS in this study[6] included: myristic acid, ethyl myristate, palmitic acid, ethyl palmitate, linoleic acid, oleic acid, stearic acid, ethyl linoleate, ethyl oleate, and ethyl stearate.
Although there was no biological testing carried out in the original study by Markham *et al*[6], the authors did acknowledge that propolis is generally biologically active.

A study by Kumazawa *et al*[7] in 2004 investigated the chemical composition and antioxidant potential of ethanolic propolis extracts from around the world. A sample of

Aotearoa propolis was included in this study, the authors noted that this particular sample was obtained at the Japanese propolis conference as an ethanolic extract. No exact location was given for the origin of the sample.

Chrysin was found to be the largest constituent in Aotearoa propolis by Kumazawa *et al*[7] with a concentration of 101.9 g/mg_{EEP}. Pinocembrin closely followed with a concentration of 99 mg g^{-1}_{EEP}. Apigenin, pinobanksin-3-acetate, tetochrysin, and galangin were all present in the sample with concentrations between 78.3 mg g^{-1}_{EEP} and 58.2 mg g^{-1}_{EEP}. Other compounds present in the sample with concentrations between 1 mg g^{-1}_{EEP} and 36.6 mg g_{EEP}^{-1} were caffeic acid, *p*-coumaric acid 3,4-dimethoxycinnamic acid, quercetin, pinobanksin-5-methyl ether, kaempferol, pinobanksin, cinnamylideneacetic acid, CAPE, and cinnamyl caffeate.

The study by Kumazawa *et al*[7] also found Aotearoa propolis to be a very effective free radical scavenger. This was tested using a 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay system. Of the 16 samples tested, the Aotearoa propolis was one of the four most effective at DPPH free radical scavenging with a free radical scavenging ability of over 60%.

The antioxidant activity of the Aotearoa propolis EEP was evaluated by Kumazawa *et al*[7] using the β-carotene-linoleic acid system. Aotearoa propolis was found to have an antioxidant activity which was similar to all other propolis samples analysed.

The studies which utilised Mānuka Health New Zealand's BIO30 or BIO100 samples or their precursors[8, 29, 52-60] generally differed from the studies of Markham *et al*[6] and Kumazawa *et al*[7] by being less focused on the complete chemical composition of the samples. Nine of these studies used BIO30 or BIO100 propolis extracts to test biological activities. These studies either did not discuss the chemical composition of the extracts at all and considered the samples to be a mixture of unknowns[52, 55, 56], or they only commented on a single compound found in the extracts and attributed all of their findings to that single chemical[8, 29, 53, 57, 58, 60]. These biological studies were generally investigating the antiproliferative and antioncogenic effects of the samples, CAPE was always discussed as being the component responsible for these effects. There was one study which investigated the antioxidant effect of these samples.[55]

There were two studies which did not test biological activities. One of these studies was restricted to investigating the wax composition of the crude propolis used in the production of BIO30 and BIO100 propolis products.[59] The other study was about the use of supercritical systems to separate individual classes of components of propolis extracts, for example essential oils, flavonoids etc and did not discuss the chemical composition at all.[54]

Of the four studies using propolis sourced from Comvita Limited, three were focused on biological activity.[61-63] The remaining study was centred on the GCMS fingerprint of an Australian stingless bee propolis sample with Comvita Limited's propolis as a reference with some discussion on anti-inflammatory potential.[51] These were all primarily interested in propolis as a complete mixture giving rise to biological activity compared to the studies carried out using Mānuka Health's BIO30 and BIO100 samples. The biological studies identified antimicrobial activity against selected pathogenic bacteria[61, 63], and observed the vasomodulatory effects of the propolis extracts on human and porcine arteries.[62] The study which investigated the chemical composition of another propolis sample did not describe the composition of Comvita's propolis as it was only used as a standard and was not discussed in much detail.[51]

2.3 Extraction

Crude propolis is lipophilic, brittle when cold and sticky when warm.[26] Crude propolis is not particularly easy to deal with or convenient for any research or potential therapeutic applications. For this reason, the active components of propolis are generally extracted from the crude form using solvent extractions. In many cases, the solvent is removed resulting in a dry propolis extract which is a concentrated, biologically potent, mixture of the bioactive molecules found in propolis.

This section of the literature review contains discussion on the different methods for the extraction of propolis. There will be specific focus on choice of solvent and the use of extraction aids. This will also include discussion from a green chemistry perspective.

2.3.1 Green Chemistry.

Green chemistry is a set of twelve principles put forward by Paul Anastas which focus on making chemical processes both safe and environmentally sustainable.[64] These principles can be seen in Appendix 1.

Propolis is currently considered a waste product by many beekeepers. Therefore, the production of useful products from propolis is inherently green. The reduction or prevention of waste from the apicultural industry through utilisation of a former waste product satisfies the 1st principle of green chemistry and as propolis is renewable the 7th principle is also satisfied.

As the utilisation of propolis is an inherently green process, it is worth taking the green chemistry of all factors involved into consideration thus maximising this already strong green potential.

2.3.2 Solvents.

Solvent choice in arguably the most important choice when developing a method for propolis extraction. The polarity of the solvent used in the extraction of bioactive compounds from crude propolis determines what compounds will be extracted. A range of solvents have been used in the past for the extraction of propolis. Ethanol, water, and supercritical fluids as solvents will be discussed and compared based on their efficacy

and how green they are. Currently, ethanolic extractions are the most common in industry followed by supercritical CO_2. Other solvents have been used, however, the applications of these are generally limited to research.

2.3.2.1 Water.

Water has been investigated as a solvent for extracting biologically active compounds from propolis. However, the use of water in industry is uncommon. Studies on the effectiveness of different solvents for extracting natural products from crude propolis have shown that water was significantly less effective than ethanol. A study on Brazilian, Chinese, and Japanese propolis by Miyataka *et al* [65] found the average yields of the water extracts to be 10.8% (*w/w*), 2.7% (*w/w*), and 1.8 % (*w/w*) respectively. In comparison, ethanol extracts of the same samples of gave average yields of 47.5% (*w/w*), 56.2% (*w/w*), and 64.1% (*w/w*) respectively. Hayashi *et al*[66] found that 70% ethanol extract of the Brazilian propolis gave a yield of 44.5% (*w/w*) while a water extract gave a yield of 11.1% (*w/w*). This same trend was observed in a study by Cunha *et al*[67]. Park and Ikegaki[68] did not report yields but did find that water extracts of propolis had significantly less antioxidative effect than ethanol extracts of propolis, Figure 2.10.

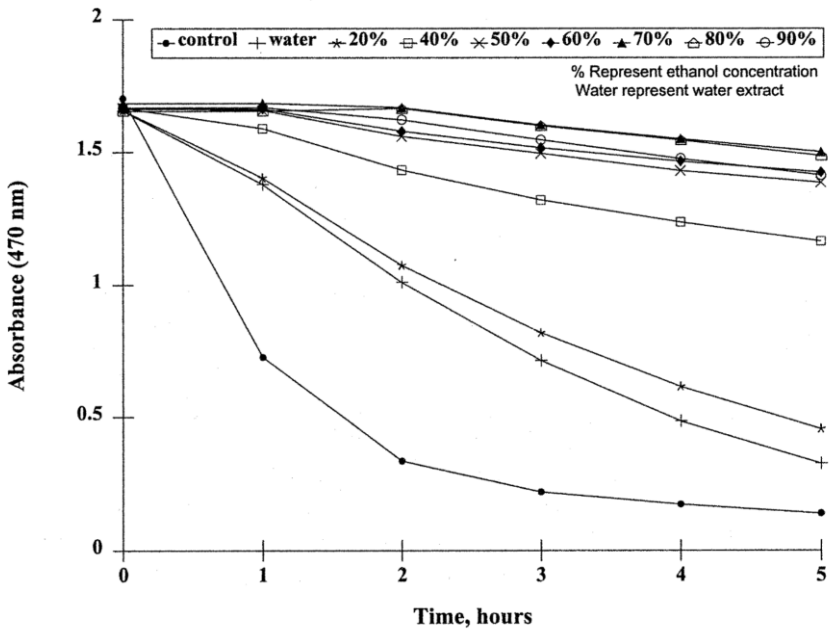


Figure 2.10 A Comparison of antioxidative potential in water and aqueous ethanol extracts of propolis in which absorbance is a direct proxy for the antioxidative effect. (Reproduced from Park and Ikegaki[68]).

Water extracts offer less practicality in a research environment compared to an absolute ethanolic extract. The use of GCMS equipment requires a sample dissolved in volatile

solvents. Water is not volatile and, therefore, aqueous extracts of propolis cannot be directly analysed via GCMS, rather the sample would need to be dried and reconstituted using a volatile solvent. Water is also significantly more difficult to remove to give a dry extract than absolute ethanol.

Water is often regarded as the ideal green solvent. It appeals to every principle of green chemistry that a green solvent could appeal to.

To summarise, water is in some regards a very green solvent for the extraction of bioactive compounds from propolis. However, it is not very effective and has been demonstrated to give lower yields by mass and also have lower activities.

2.3.2.2 Ethanol.

Ethanol has been proven to be a very effective solvent for propolis extractions[69, 70]. It is for this reason that ethanol is the most common choice of solvent for propolis extractions.

Bioactive compounds can be extracted from propolis using ethanol at a range of different concentrations. The ratio of ethanol to water used as a solvent for extraction has been shown to affect the yield of propolis extractions with a higher ethanol content generally resulting in a better yield.[67] However, the toxicity of the extract also increases with increasing ethanol concentration. For this reason, hydroethanolic extracts are useful at a range of concentrations. A pure ethanolic extract (100%) is useful for chemical research purposes as it will extract bioactive molecules very effectively and does not require resuspension in a water free solvent for GCMS analysis. Commercially,70% hydroethanolic extracts are common as solutions containing 70% ethanol or under may be sold as food products. These 70% hydroethanolic extracts are similar in extraction efficacy to pure ethanolic extracts. Extracts with ethanol concentrations lower than 70% are sometimes useful for biological research as the lower ethanol concentrations enable *in vitro* testing to be carried out without further dilution.

Although ethanol is a very effective solvent for the extraction of bioactive molecules from propolis, it is not a green solvent. There are a range of attributes ethanol holds which contribute to it not being a green solvent.

Ethanol presents problems due to flammability. It also has adverse physiological side effects.

These effects on the greenness of ethanolic extracts are reduced through the use of hydroethanolic mixtures of ethanol and water. However, as previously mentioned, this can affect the efficacy of extraction.

To summarise, ethanol is a very effective solvent for propolis extraction. In both absolute and hydroalcoholic forms it offers strong efficacy. However, the use of ethanol is a limiting factor in the green potential of any propolis extraction. Absolute ethanol will be from here on be referenced as the standard with which other solvents will be compared for propolis extraction.

2.3.2.3 Supercritical CO_2

Supercritical fluid extractions are extractions which are carried out using a fluid with temperatures and pressure near the critical point. Supercritical fluids are generally highly diffusive and have reduced viscosities, densities, and surface tensions. However, these can all be modified by changing variables such as temperature and pressure slightly. Supercritical fluid extractions have been used extensively for the extraction of bioactive compounds from biological sources.[71] Supercritical CO_2 ($scCO_2$) is an example of a green solvent which has been explored for use in propolis extraction.

The costs for $scCO_2$ extraction, however, is high in comparison to ethanol maceration. This high cost of $scCO_2$ extraction alongside the low extraction capacity have meant that this method is second to ethanol maceration in industry.

In early 2020 a study by Saito *et al*[72] was published which directly compared $scCO_2$ extractions of propolis. This study found that $scCO_2$ extraction of propolis was less effective for some analytes but more effective for others. The total yield of $scCO_2$ extraction of red Brazilian propolis was only 16.3% while an ethanol extraction was able to achieve a yield of 45% for the same sample. $scCO_2$ was also less effective at extracting phenols from propolis than ethanol. However, it was found that $scCO_2$ extraction was more effective than ethanol extraction when it came to recovery of flavonoids from the sample, Figure 2.11.

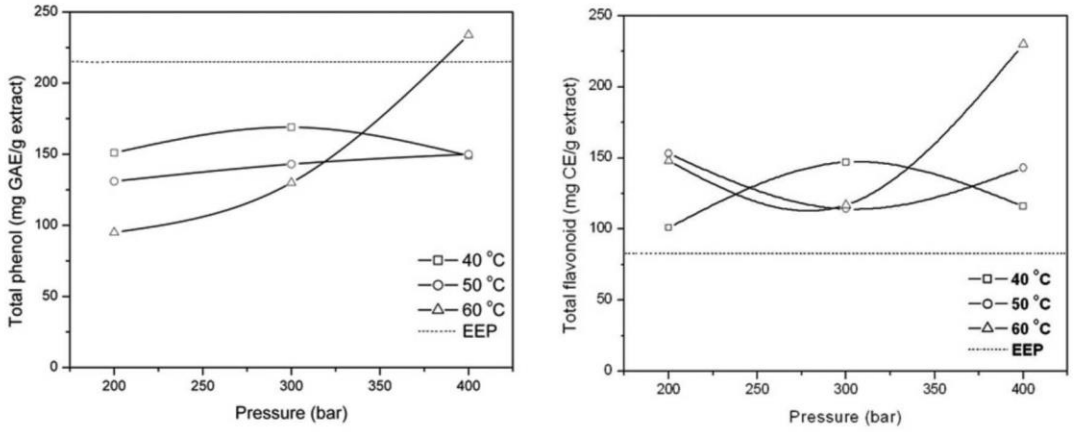


Figure 2.11 Comparison of phenols and flavonoids recovered from Brazilian red propolis using scCO2 and ethanol extraction. (Reproduced from Saito et al[72]).

2.3.2.4 Comparison of Solvent Efficacies

Ethanol is still arguably the most effective solvent for the extraction of bioactive molecules from propolis. In the majority of cases, it proves to extract the best overall percentage yield by mass from propolis. Extracts made with ethanol also generally prove to be the most biologically active. $scCO_2$ showed a comparable efficacy to ethanol. $scCO_2$ is able to attain yields in approximately the same range as ethanol although often slightly lower, Figure 2.11, or was able to be more effective than ethanol for some analytes while being less effective for others. Water was shown to have very limited efficacy to the point where its low efficacy even affected how green it is. Ethanol was the most effective solvent, $scCO_2$ was slightly less effective on average, and water was by far the least effective solvent.

2.3.2.5 Summary of Solvents.

From the information gathered in the literature it was determined that for this study absolute ethanol would be used as a solvent. $scCO_2$ was considered to be an appealing solvent for use in extraction, however, for ease of access, supercritical carbon dioxide was not investigated further. Water was not chosen over ethanol due to its inefficacy.

A 70 % hydroethanolic solution was initially chosen as a solvent for extraction due to its reflection of food grade ethanol. However, absolute ethanol was later chosen over a hydroalcoholic solution for ease of GCMS analysis and solvent removal.

Although ethanol was chosen for use in this study, following reviewal of the literature it is recommended that for any future projects where $scCO_2$ extraction methods are available, comparison of ethanolic maceration and $scCO_2$ extraction is recommended.
If this process were to be scaled up for commercial extractions, hydroalcoholic solutions would be another good choice of solvent. A hydroalcoholic solution of 70% food grade ethanol would likely give an extraction yield comparable to that of absolute ethanol and would be easier to deal with legally if it were being sold as a tincture.

2.3.3 Extraction Parameters

Beyond solvent choice, there are other factors which need to be considered for propolis extraction. One of which is the use of extraction aids. In this context, an extraction aid is anything which can be used to assist in the transfer of analyte from the crude propolis into the extraction solvent. Here the use of heat and sonication will be discussed.

2.3.3.1 Heat

Many authors have reported using heat to aid in their extractions of bioactive products from propolis. Methods which include the use of heat to aid in extraction often include shorter maceration times compared to methods which do not use any extraction aids.
In the preparation of EEP, it has been shown that heating an extraction above room temperature does not significantly improve the yield.[73] Another study using 24 hour extraction periods compared extracts of propolis at both 25 ^{o}C and 50 ^{o}C with a range of solvents and found no significant difference on extraction efficacy between the two temperatures.[74] These studies, among many others, indicate that the heating of propolis extractions does not make any significant difference to extraction yields. With all of this in mind, it is interesting to note that there are still some authors who report using heat in their extraction methods despite there being large amounts of data indicating it is unnecessary. This is especially important to consider in propolis extraction because it is common to extract mixtures for weeks or even months. It requires a large amount of energy to provide heating for such a long period of time. The use of heat in an extraction for such an extended period of time is not ideal from a green chemistry perspective. Also, when dealing with a sample with a completely unknown composition there is potential that heating could enable some components to degrade.

Ultrasonic assisted extractions are common for the preparation of EEP. Ultrasonic assistance for an ethanol-based extraction of propolis can significantly reduce the amount

of time an extraction takes. In the study published by Trusheva *et al*[69], it was shown that a 30 minute ultrasonic assisted extraction was just as effective as a 72 hour maceration extraction. In that study, the 30 minute ultrasonic assisted extraction was shown to give a 53% yield while the 72 hour maceration gave a 55% yield. Ultrasonic assisted extractions are able to be performed more quickly than maceration alone. However, a maceration at room temperature has no energetic cost and, therefore, definitely satisfies the 6th principle of green chemistry. Ultrasonic assisted extraction does have an energy cost and, therefore, is less green than simply leaving the extraction mixture to macerate.

2.3.3.2 Summary of Extraction Parameters

The use of heat to aid extraction of bioactive compounds is an unnecessary energetic expense and should be avoided. The use of ultrasonic assistance does enable extractions to be performed more quickly. However, when there is no need to rush the extraction process a maceration is just as effective. There have also been no studies conducted so far on the effect sonication has on the composition of extracts. When dealing with completely unknown samples such as a potentially new class of propolis, caution should be taken to avoid any accidental alteration or destruction of sample.

2.4 Commercially Available Propolis Products

In Aotearoa, there are already a range of propolis products available for over-the-counter purchase. There are two major brands which produce these products: Comvita Limited and Manuka Health New Zealand. The commercially available propolis products are available in a wide range of forms. Both Manuka Health and Comvita produce liquid extracts, these can either be hydroalcoholic, or alcohol-free using glycerol. However, the most common forms in which propolis is purchased is either as capsules, or as toothpaste. Propolis capsules are sold by both Manuka Health and Comvita. These products come in a range of strengths with products being marketed based on how much crude propolis they are equivalent to. These products contain dry propolis extract bulked out by common bulking agents. These products say that they are sourced from New Zealand propolis. Toothpaste is the other common form in which propolis is sold. Red Seal has toothpaste which contains propolis available in most supermarkets in New Zealand. The propolis toothpaste claims to contain New Zealand propolis. Propolis extract is listed on the ingredients list of Red Seal propolis toothpaste, no more information is given. Propolis has been shown to hold antibacterial activity against the plaque causing *Streptococcus mutans*.[75]

Propolis can also be sourced as an oral spray, tablets, chewable treats, or as ointments. These products are not as common as the previously described ones in New Zealand.

Chapter 3

Methods and Materials

This chapter describes the general methods used in this study. However, more specific detail is given in each chapter for methods specific to that chapter. Information is also given here on the materials used.

All experiments described in this thesis were carried out at the University of Waikato (UoW). Chemical bench work was carried out in E.3.24 (PC1). GCMS and LCMS analyses were carried out in rooms E.3.09 and D.3.14 respectively. All work involving mammalian cell cultures was carried out in the E.3.11 and E.3.13 laboratories under physical containment level 1 (PC1) and physical containment level 2 (PC2) conditions respectively. Work involving bacterial cell cultures was carried out in C.2.08 under PC2 conditions. All cell work was carried out using aseptic techniques. This included thorough cleaning of workspaces using 70% ethanol and also 1% trigene when necessary.

3.1 Materials

3.1.1 Preparation of Common Solutions

Table 3.1 Composition of common solutions.

Solution	Composition of Solution
Complete Iscove's modified Dulbecco's medium (IMDM)	40 mL incomplete IMDM media (catalogue no 12440053) 10 mL foetal bovine serum (FBS) Store at 4 ^{o}C
70% Ethanol (for extraction)	350 mL ethanol (chromatography grade, catalogue no 459828) 150 mL water (Type 1)
5% Formic acid in water	Formic acid (98-100% reagent grade, sourced from Scharlau, catalogue no AC10852500) Water (Type 1)

3.1.2 Propolis Samples

Three batches of propolis samples from Te Urewera were provided by Manawa Honey NZ. Batch one (Te Urewera Propolis Batch 1) was sourced from hives near Ruatāhuna. Batches two and three (Te Urewera Propolis Batch 2/3) were sourced from the wider Te Urewera.

A commercial sample of propolis was provided by an undisclosed beekeeper near Taihape for comparison.

3.1.3 Reagents

Most of the reagents used in this study were sourced from Sigma Aldrich. This includes ethanol (catalogue number 459828), methanol (catalogue number 105021), sclareol (catalogue number 49944), *N*-Methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) (catalogue number M7891), pyridine (catalogue number 270970), acetic anhydride (catalogue number 320102), Antioxidant Assay Kit (catalogue number MAK334), phorbol-12-myristate-13-acetate (PMA) (catalogue number P1585), WST-1 (catalogue number 5015944001), and superoxide dismutase (SOD) (catalogue number S5395).

IMDM (catalogue number 12440053), FBS, CyQYANT™ MTT Cell Viability Assay Kit (catalogue number V13154), Difco™ Nutrient Agar, Tryptic Soy Broth, and all other generic biological consumables were sourced from Thermo Fisher Scientific.

Formic acid was sourced from Scharlau (catalogue number AC10852500). Hydrochloric acid was sourced from UNIVAR (catalogue number A1367).

HL60 cells were sourced from the American Type Culture Collection (ATCC). *S. aureus* strains were acquired from another laboratory group in the UoW courtesy of Linda Peters.

3.1.4 Instrumentation

The details on the instrumentation used are covered completely in the methods sections for each chapter.

Eppendorf Research Plus™ pipettes were used throughout this study. Cells were incubated in a Hera Cell 240 Incubator. Samples/cell solutions/reagents for use in

biological experiments were thawed using a Grant GD100 water bath. Two centrifuges were used in this study. These were a Heraeus Megafuge 1.0 and a Heraeus Multifuge X3r.

3.2 Cell Culture Methods

The culturing of HL60 cells was carried out following the ATCC guidelines. The specific methods used are described below.

3.2.1 Revival of Cryopreserved HL60 Cells

Aliquots of HL60 Cells were revived from cryopreservation at -80 ^{o}C. The frozen aliquots (1 mL) of cells were thawed with haste using a Grant GD100 water bath at a temperature of 37^{o}C. The thawed HL60 Cells were added to complete IMDM (5 mL) in a sterile 15 mL falcon tube. The resultant solution was centrifuged at 400 rpm for 5 minutes using a Heraeus Megafuge 1.0 centrifuge. The supernatant media was discarded leaving 1 mL of media with the cell pellet behind. Complete IMDM (4 mL) was added to the 15 mL falcon tube and the solution was mixed to relative homogeneity by drawing up and expelling solution using the serological pipette 4-6 times. The homogenous cell solution (5 mL) was transferred into a sterile T25 flask and transferred into a Hera Cell 240 incubator.

3.2.2 Subculturing of HL60 Cells

HL60 cells were incubated in a Hera Cell 240 incubator at 37 ^{o}C with 5% CO_2 in an appropriate volume of complete IMDM media for their flask. The media was changed every 2-3 days or sooner if the phenol red in the media indicated that it was due to be changed by showing a colour change from red to orange. Media changes were achieved by transferring the cells from the flask into a 15 mL falcon tube. The cell solution was centrifuged at 400 rpm for 5 minutes in a Heraeus Megafuge 1.0. The supernatant media was removed until 1 mL of residual media and the cell pellet remained. Complete IMDM was added to the falcon tube until a volume appropriate to the flask was obtained. The resultant cell solution with fresh media was transferred back into the original flask and returned to the incubator. The concentration of cells was maintained between 10^5 cells mL^{-1} and 10^6 cell mL^{-1}. If the concentration of cells was nearing 10^6 cells mL^{-1} the culture would be split.

3.2.3 Splitting of HL60 Cells

HL60 cell cultures were split when the concentration of cells exceeded 10^6 cells mL^{-1}. This was achieved by removing the media from the cells as described above to form a cell pellet. This cell pellet would be resuspended in complete IMDM (~1 mL). This highly concentrated cell solution would be aliquoted into a suitable number of cell culture flasks before being diluted to an appropriate concentration using complete IMDM.

3.2.4 Counting of HL60 Cells

The cell concentrations of HL60 cell cultures were determined using trypan blue staining and haemocytometer methods. Trypan blue (10 μL) was added to a strip of parafilm to form a droplet. The cell solution within the well on the 6 well plate was stirred using a sterile pipette tip, agitated by gentle rocking of the plate, and mixed by pumping the solution up and down using a pipette to reach homogeneity. This homogenous solution (10 μL) was transferred to the droplet of trypan blue. The droplet was mixed thoroughly by pumping with a pipette and the stained cell solution (10 μL) was transferred to a haemocytometer for counting.

The number of viable unstained cells in each quadrant of the haemocytometer were counted using inverse microscopy. The averages of these values were taken and multiplied by 20000 to give the concentration of viable cells in cells mL^{-1}.

Chapter 4

Extraction Method Development

4.1 Introduction

Propolis samples are notorious both in the literature and in the views of apicultural research groups of being difficult to deal with. Crude propolis alone is not amenable to commercial or research use as it is too sticky to crush into any form of powder which will last at room temperature. The crude extract is also not completely soluble in any solvents, which will not also destroy the complex secondary metabolites for which it is marketed. For this reason, the bioactive components of propolis must be extracted from the crude samples.

Generally, this includes some form of maceration of the crude propolis with either ethanol, methanol, water, or a mixture of these solvents. The use of ethanol is the most common. However, this still does not give a clean sample. When ethanol is used to extract bioactive analytes from crude propolis, wax can also be co-extracted. Wax is generally of little interest to researchers as it does not have much bioactivity and can cause precipitates to form in solutions of the sample at certain temperatures. For this reason, waxes are often removed from the sample.

Finally, it is common to remove all of the solvent from propolis extracts to allow for accurate preparation of sample solutions with known concentrations of dry extract per unit volume.

Based on the literature described in Chapter 2, a few key points for the development of an extraction method were determined. They are listed as follows:

- 70% ethanol was an effective solvent which would be reflective of any commercially available products which may be produced in the future.
- A sample to solvent ratio of 1 g: 10 mL was effective.
- Maceration time would need to be no less than 48 hours and it was not detrimental to extract for longer.
- Following extraction, the solvent would need to be removed. Rotary evaporation should be sufficient to achieve this.

This information gave a general idea of what methods have been shown to extract bioactive molecules effectively from other propolis samples. The macro composition of the sample matrix is well conserved across propolis classes. Therefore, the initial extraction of analytes from the matrix should remain fairly similar.

The preservation of sample integrity was determined to be a priority. This sample had never been analysed before. Therefore, if any of the analytes in the sample were being altered or degraded by the extraction process, there would be no other standards with which to compare to identify the issue. For this reason, no heat or ultrasonic assistance would be utilised during extractions.

The method described below was also developed with more than just efficacy in mind. The 12 principles of green chemistry put forward by Anastas and Eghbali[64] were taken into consideration during the development of the method. These can be seen in Appendix 1.

The aim of the research described in this chapter was to develop an effective method for the preparation of high quality dry Urewera propolis extract. It was hypothesised that methods based on those seen in the literature for the extraction of other propolis classes would also be effective for the extraction of Te Urewera propolis.

4.2 Methods

4.2.1 Extraction Method 1

Crude propolis samples were frozen using a commercial freezer (-4 ºC) before being ground into a powder using a mortar and pestle. Powdered crude propolis (~2.000 g) was weighed accurately into a conical flask. Hydroalcoholic solution (20 mL, 70% ethanol 30% type 1 water *v/v*) was measured using a measuring cylinder and added to each flask. A stir bar was added to each flask before sealing with parafilm and covering the entire flask with tin foil. The samples were left to macerate with stirring for 168 hours. The resultant solution was transferred into a refrigerator (4 ºC) for a small amount of time before being centrifuged at 4000 rpm using a Heraeus Multifuge™ X3R. A portion of the supernatant was transferred into a glass vial and stored in cool conditions for later analysis.

A measured volume of the remaining extract was added to a round bottomed flask and dried using rotary evaporation (35 ºC) and the residual mass recorded. The dry extract was stored in refrigerated (4 ºC) conditions for later use.

4.2.2 Extraction Method 2

Crude propolis samples were frozen using a commercial freezer (-4 ºC) before being ground into a powder using a mortar and pestle. Powdered crude propolis (10.0 ± 0.5 g) was weighed accurately into a conical flask. Absolute ethanol (100.00 mL, chromatography grade) was aliquoted using a volumetric flask and added to the conical flasks. A stir bar was added to each flask before sealing with parafilm and covering the entire flask with aluminium foil. The samples were left to macerate with stirring for 168 hours. The resultant solutions were transferred to 50 mL falcon tubes and centrifuged at 4000 rpm for 5 minutes using a Heraeus Multifuge™ X3R. The supernatant was transferred into new 50 mL falcon tubes and centrifuged again at 4000 rpm for 5 minutes. Some of the supernatant was transferred into glass vials and stored in refrigerated conditions for later analysis.

The remaining extract was transferred to a round bottom flask and dried using rotary evaporation (35 ºC). The dry extract was stored in cool conditions for later use.

4.2.3 Extraction Method 3

Crude propolis samples were frozen in a commercial freezer (-4 ^{o}C) before being ground into a powder using a mortar and pestle. Powdered crude propolis samples (10.0 ± 0.5 g) were weighed accurately into a conical flask. Absolute ethanol (100.00 mL, chromatography grade) was aliquoted using a volumetric flask and added to the conical flasks. A stir bar was added to each flask before sealing with parafilm and covering the entire flask with tin foil. The samples were left to macerate with stirring for 168 hours. The solution was transferred into 50 mL falcon tubes, left in the freezer overnight, and centrifuged at 4000 rpm for 10 minutes. The supernatant was transferred to new falcon tubes and left in a freezer(-4 ^{o}C) for 24 hours. The chilled extracts were centrifuged at 4000 rpm for 15 minutes at -10 ^{o}C. Some of the supernatant was transferred into glass vials and stored in cool conditions for later analysis.

The remaining supernatant was dried by gradually adding the solution to a 7 mL vial placed in a sample concentrator set to 70 ^{o}C with constant nitrogen gas flow. The resultant resin was refrigerated (4 ^{o}C) for later use.

4.2.4 Retroactive Refinement Method

Previously produced dry unrefined propolis extracts from extraction methods one and two were dissolved in absolute ethanol (Chromatography Grade, ~1 mL per 0.2 g dry propolis). The resultant solution was chilled in the freezer (-4 ^{o}C) before being centrifuged at 4000 rpm for 15 minutes at -10 ^{o}C. The supernatant from this was transferred to a glass vial before the ethanol was removed using a 70 ^{o}C sample concentrator under constant nitrogen gas flow. The resultant dry, purified extract was stored refrigerated (4 ^{o}C) for later use.

4.3 Results and Discussion

4.3.1 Extraction Method 1

Extraction method 1 was developed based on the ideas previously stated in the introduction and the review of literature seen in Chapter 2. This extraction was carried out on a relatively small scale as it was the first attempt at extraction. It was found that this method was able to recover 168.9 mg of dry propolis extract per gram of crude Te Urewera propolis.

The first extraction method indicated visually that Te Urewera propolis was different to the commercial propolis, Figure 4.1. During the extraction, the flasks were noted as having a visible difference in both hue and tone. A difference in tone alone could possibly be explained by the flasks both containing the same major chromophores with different concentrations. But a difference in hue indicates that the major chromophores in the two solutions absorb light at different wavelengths and are therefore different compounds.

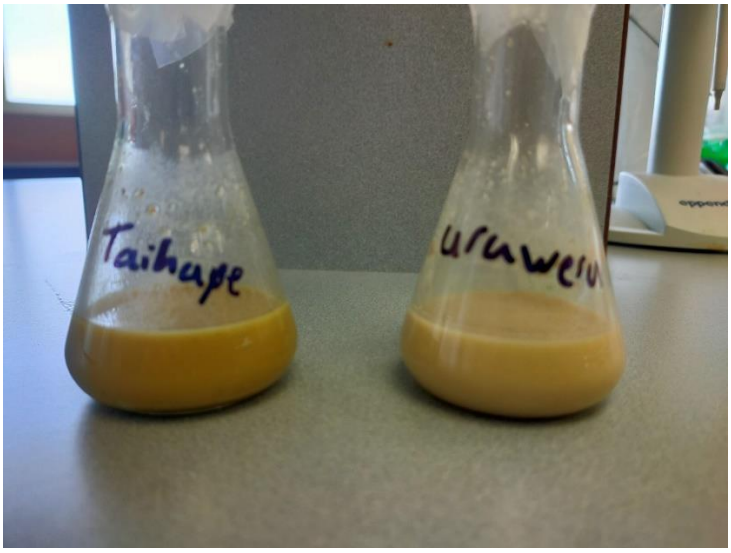


Figure 4.1 Visual comparison of commercial propolis (left) and Te Urewera propolis (right) solutions after stirring.

Following centrifugation, the samples remained different in colour. The centrifuged commercial extract solution was a brownish yellow clear liquid and the centrifuged Te Urewera extract solution was a clear yellow liquid. Upon rotary evaporation the resultant dry extracts were both light brown solids. These solids were sticky but also held a texture which was somewhat waxy.

Resuspensions of the solutions in ethanol (100%, chromatography grade) were analysed using GCMS, Figure 4.2 and Figure 4.3. The total ion chromatograms differed completely and the mass spectral data for each sample gave no indication that the two samples held any major shared compounds. The exact composition of the Te Urewera propolis extract will be discussed in more depth in Chapter 5. The commercial Aotearoa propolis sample was determined to be of standard poplar composition based on the GCMS data gained in this extraction. [6, 11, 21, 22]

The information gained from this extraction was deemed sufficient to prove that Te Urewera propolis was completely different from the commercially sourced propolis sample, which itself was in the poplar class. For this reason, the commercially sourced propolis was not investigated further.

This extraction method was effective and it was decided that the extraction would be carried out again on a larger scale to gather enough extract to use for bioactivity testing.

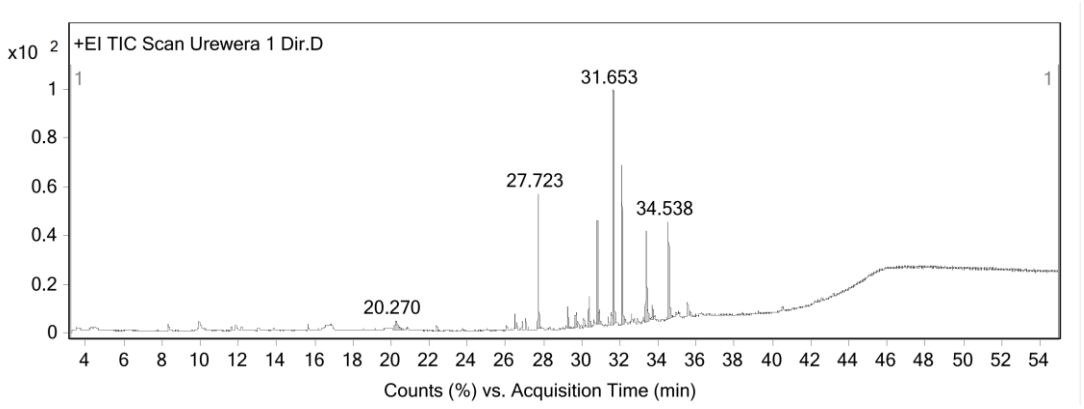


Figure 4.2 GCMS chromatogram of TUPE acquired through extraction method 1.

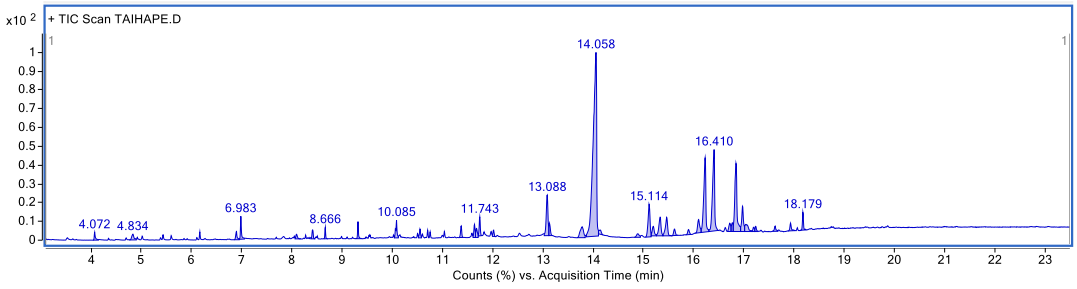


Figure 4.3 GCMS chromatogram of commercial propolis extract acquired through extraction method 1.

4.3.2 Extraction Method 2

Extraction Method 2 was a modification of extraction method 1. It involved the change of solvent from 70% ethanol to 100% for improved ease of solvent removal. This also unintentionally involved the removal of the chilling step before centrifugation.

Quantitatively, this method appeared to be successful. The mass of the material extracted was determined after the removal of the solvent. This extraction initially seemed more effective than method 1 as method 2 was able to recover 238.1 mg of dry propolis extract per gram of crude propolis used compared to method 1, 168.9 mg g^{-1}.

However, qualitatively this method had limited success. The centrifugation gave extracts which initially did not appear dissimilar to the extracts obtained in extraction method 1. However, issues began to arise just before solvent removal. The centrifuged solvent was set aside in the refrigerator overnight before work was resumed the next day. The following morning, it was noted that the extract was mostly solid. This was different to the extract generated from extraction method 1, which did not solidify after refrigeration overnight. After the extracts from extraction method 2 were placed on the bench at room temperature for some time, they once again became clear coloured liquids. The samples were dried using rotary evaporation. The resulting dry extract was a waxy brown solid which was not sticky. Which was again a very different texture to the dry extracts obtained using extraction method 1.

The dry extract was resuspended in absolute chromatography grade ethanol for GCMS analysis. The GCMS data obtained for this extraction method was also very different from the data obtained from extraction method 1, Figure 4.4. The primary constituents of method 2 extracts were long chain hydrocarbons. The primary constituents of the extracts obtained using extraction method 1 were not long chain hydrocarbons. The chromatograms also both showed many new peaks which were not present in the chromatograms obtained using the previous method and which were unidentifiable.

It was clear from the physical observations of the extract, the dry extract, and the GCMS data that this extract had extracted not only potential bioactive compounds of interest but also large quantities of beeswax. This extraction method was repeated to identify if this co extraction of wax was due to operator error. A sample of commercial propolis was also extracted using this method to determine if the wax observed in this extraction was due

to the propolis sample used or if it was the method. Both of these repeats identified that the issue lay with the method and were not due to error or sample variation.

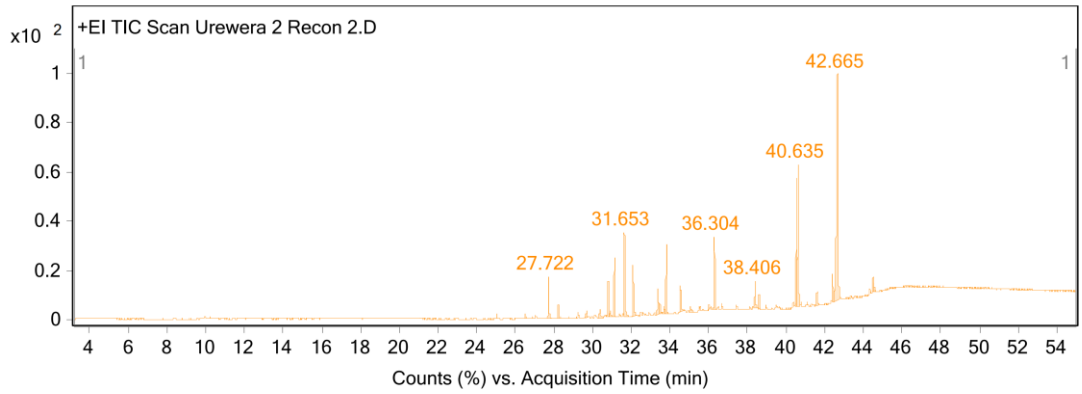


Figure 4.4 GCMS chromatogram of TUPE obtained using extraction method 2.

4.3.3 Extraction Method 3

Extraction method 3 was applied only to Te Urewera propolis as extraction method 1 had confirmed that Te Urewera propolis was sufficiently different to poplar propolis to warrant further investigation. Extraction method 3 was designed to address the issues observed with extraction method 2. This method was based on the previous methods with a few major changes to remove the unwanted, extracted wax.

In this method, Crude extracts were transferred to 50 mL falcon tubes following maceration and placed in the freezer overnight before centrifuging at -10^{o}C. This process was repeated on the supernatant before collecting the final extract solution. At the solvent removal step, the rotary evaporation process used in the previous extractions was substituted for a gentler method. This was the use of evaporation under a stream of dry nitrogen in a sample concentrator. The sample concentrator was used because there was less potential for loss of analyte.

This method was able to extract 126.3 mg of pure dry propolis extract per gram of crude Te Urewera propolis used. This value is significantly lower than the values observed in the previous methods, which would have included co-extracted beeswax in various quantities.

This extraction gave a much higher quality dry propolis extract than the previous methods, there were numerous physical observations throughout the process which indicated this. This improvement in extract quality was confirmed through the GCMS analysis. The first physical indication that this method gave a high quality extract was observed after the first centrifugation of the sample. The pellet in the centrifugation of this sample was more clearly defined in comparison to the previous methods. This allowed for easier removal of the supernatant and gave a very clear extract. During the second round of centrifugation, a small pellet still formed in the bottom of the tubes. This indicated that the second round of centrifugation was necessary to further remove waxes. A third round of centrifugation did not result in a pellet forming and was deemed unnecessary. The next physical indication that this method produced a higher quality extract than the extraction method 2 was observed after solvent removal. The dry propolis produced by extraction method 3 was deep brown and very sticky in nature and did not at all have a waxy consistency, unlike the products of the previous two methods.

The GCMS analysis of the reconstituted dry propolis extract from extraction method 3, Figure 4.5, confirmed that this sample was free of the previously observed waxes. The extract generated using this method was also much easier to reconstitute in ethanol than the previous extracts and was deemed suitable to work with for the remainder of the project.

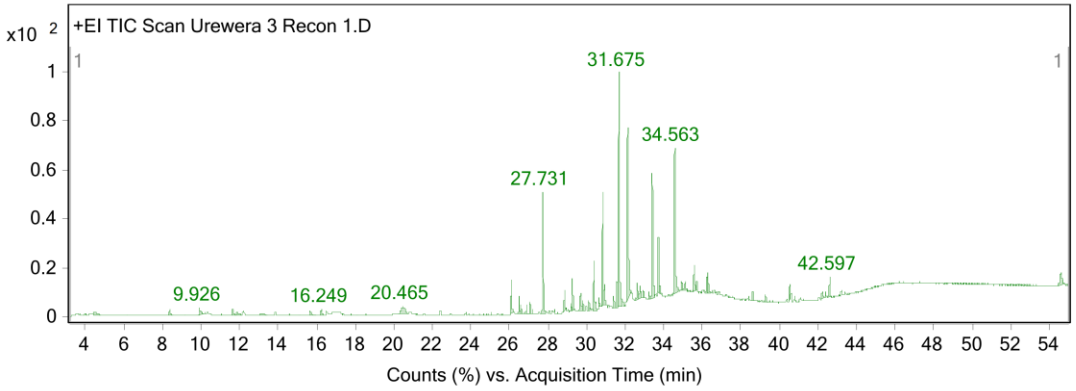


Figure 4.5 GCMS chromatogram of TUPE produced using extraction method 3.

4.3.4 Retrospective Refinement of Products from Extractions 1 and 2

Extracts from Methods 1 and 2 were resuspended in ethanol and subjected to the freezing and centrifuging process used in Method 3. The resultant products had the same physical and chromatographic properties as the product of Method 3, Figure 4.6.

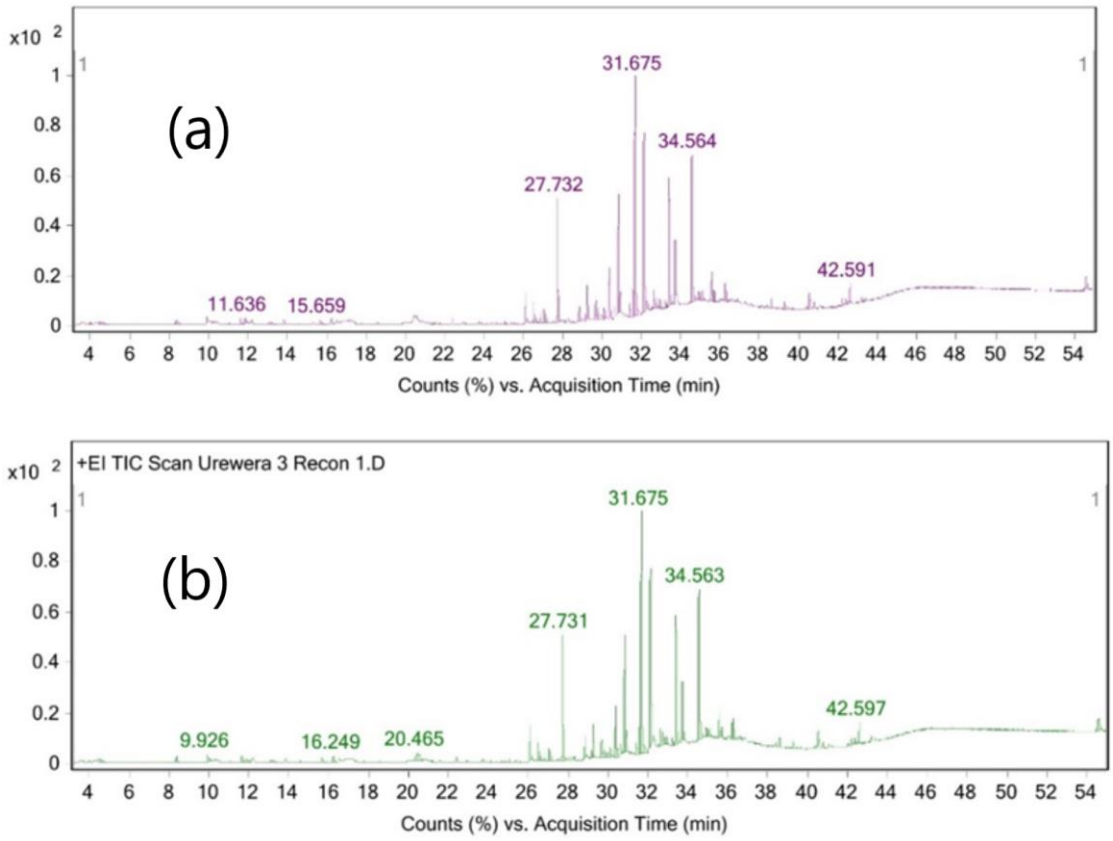


Figure 4.6 GCMS chromatograms of the resuspended refined TUPE (a) and of the TUPE acquired through extraction method 3 (b) for comparison.

4.3.5 Comparison to Literature

The scientific literature describing propolis research all involves either the extraction and quantitation of classes of compounds in propolis or involves the use of propolis extracts in biological or chemical assays. However, the results of this method development highlight a gap in some of the propolis literature.

These extractions found that unless maceration samples were chilled prior to and during the centrifugation process, a low quality extract would be acquired. This low quality extract was high in wax content, which introduced solubility issues into a sample which was already difficult to solubilise. This could easily be overcome when carrying out the

total phenolic content assays described in some literature. However, a low quality dry propolis sample which contains large amounts of wax would be useless in biological assays due to the solubility issues.

The gap in the literature, thus highlighted, is that the articles which describe extraction, often do not generally apply the resultant extracts in bioactivity studies.[67-69, 72, 76] Articles which use propolis extracts for cell line experiments generally use commercially made propolis extracts.[29, 52, 53, 55, 60, 77-79] The few articles which extract their own propolis samples and carry out cellular biology experiments are ambiguous in their wording of how propolis was delivered to cell solutions.[74, 80-83] There are very few which actually describe the removal of wax.[38, 84]

It is possible that both of the propolis samples used in this experiment were abnormally high in wax content which thus required an unusual step of chilled extraction. However, it seems unlikely that both of the samples used in this experiment were abnormally high in wax content. The samples used in this experiment were collected using industry standard propolis mats by different beekeepers in different locations.

The second explanation is that commercial propolis extractions involve some form of wax removal step and research articles that describe propolis extraction methods are producing waxy dry extracts which are very different from the commercial samples generally used in cellular research.

Chapter 5
Chemical Characterisation of Te Urewera Propolis

5.1 Introduction

Natural products chemistry is a hybrid field of chemistry and biology which focuses on secondary metabolites found in nature. There are three focuses in this field, the determination of how the natural products are produced, the chemical characterisation of mixtures of natural products, and the assessment of the biological activity of natural products. The chemical characterisation of mixtures of natural products is the central pillar of the three. Without sufficient knowledge of the chemical composition, biological activities cannot be meaningfully assessed, and the origin of natural products cannot be determined. The chemical characterisation of natural products or mixtures of natural products should be performed as early as possible in any form of natural product research.

As described in the literature, propolis samples from around the world, including some locations in Aotearoa, have previously been characterised chemically. However, this is the first ever scientific study on Te Urewera propolis.

The standard methods for determining the composition of propolis extracts are GCMS and LCMS.[5, 6, 8, 22, 28, 46, 48, 53, 85] However, each method has its limitations. Previous studies on other propolis samples have found that sample derivatisation is often required to improve the volatility of analytes within the samples by removing hydrogen bonding groups. Common derivatisation methods for propolis extracts include acetylation and trimethylsilylation. Methylation has also been used to derivatise propolis extracts, but this is less common.

Acetic anhydride and dry pyridine can be used for derivatisation by acetylating any alcohol groups present in the sample. In this derivatisation reaction, acetic anhydride donates an acetyl group which turns alcohol groups into acetyl esters. Acetic acid is formed as a by-product of this reaction, dry pyridine is used both as a solvent and as a catalyst, Figure 5.1.

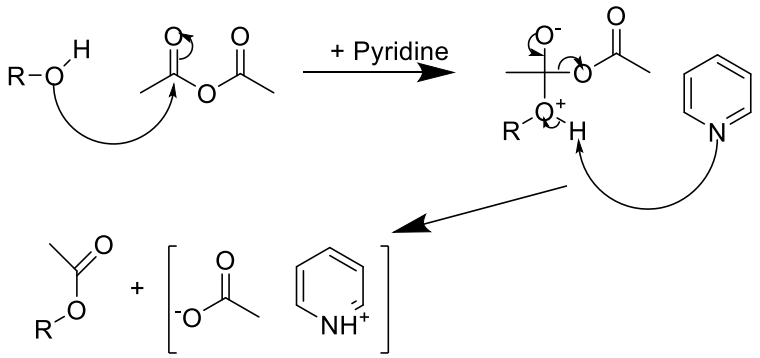


Figure 5.1 Mechanism of the acetylation of alcohols by acetic anhydride and dry pyridine.

The derivatisation of alcohols in samples into trimethylsilyl (TMS) ethers is very common, especially for use in GCMS analysis. Per-*O*-TMS derivatised samples are particularly useful in GCMS as they not only give more informative fragmentation patterns in the mass spectrometric data, but they also increase the volatility of analytes by weakening intermolecular forces with their large TMS groups. The most common method of silylation in propolis literature involves the use of *N,O-bis*(TMS)trifluoroacetamide (BSTFA).[22, 46, 47, 50, 86] However, it is not the only method available for the TMS derivatisation of propolis samples. *N*-Methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) is a relatively uncommon derivatising agent, but it is also effective in forming TMS derivatives, Figure 5.2.

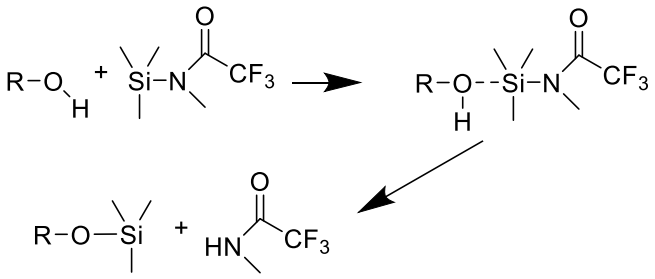
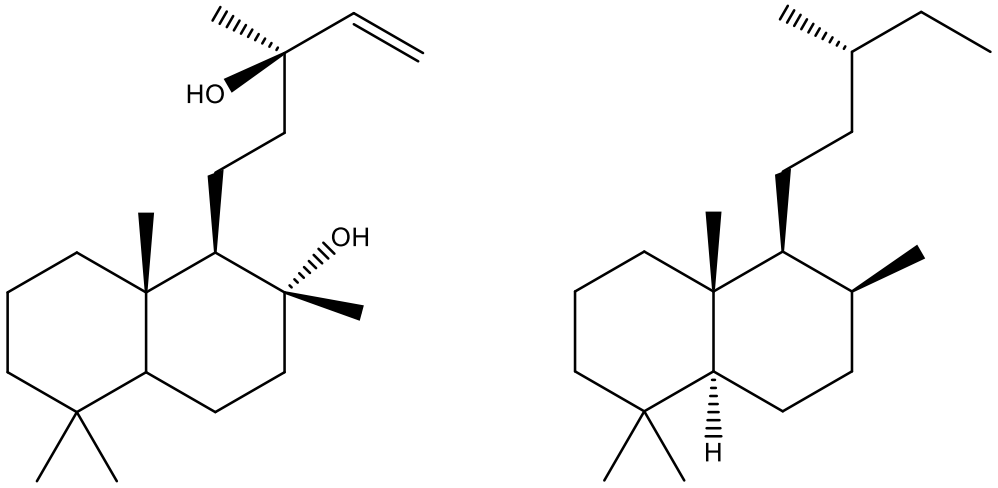


Figure 5.2 Reaction scheme for TMS derivatisation of alcohols by MSTFA.

To gather quantitative data using GCMS, a standard must be used. For this project, *n*-dodecane was chosen as an internal standard. Most of the analytes in this sample were diterpenes, for which standards could not be readily obtained and, thus, it was decided that a proxy would be used. The relative response factors of both *n*-dodecane and the diterpene proxy needed to be determined. The diterpene proxy used to determine this relative response factor was sclareol. Sclareol was chosen as it is a labdane diterpene, much like the other diterpenes observed in TUPE. The structure of sclareol and the

structure of labdane, a standard labdane diterpene, can be seen below for comparison, Figure 5.3.



Sclareol Labdane

Figure 5.3 Structures of sclareol and labdane.

The aim of this research was to determine the chemical composition of Te Urewera propolis utilising acetyl and TMS derivatisation methods in conjunction with GCMS and LCMS analysis.

5.2 Methods

5.2.1 Quantitative Sample Preparation

Samples for quantitation were produced by dissolving precisely measured masses of high quality propolis extract in a solution composed of a precisely known mixture of ethanol (100%, chromatography grade) and *n*-dodecane (>99%) as an internal standard. Three quantitative samples were produced using propolis extracts from Te Urewera propolis batch 1. These solutions were transferred to GCMS vials for GCMS and LCMS analysis.

5.2.2 Determination of Relative Response Factor

The relative response factor of labdane diterpenes compared to *n*-dodecane was determined by dissolving three precise masses of sclareol (analytical grade) in a solution composed of a precisely known mixture of ethanol (100%, chromatography grade) and *n*-dodecane (>99%, chromatography grade). These solutions were transferred to GCMS vials for GCMS analysis.

5.2.3 Acetylation of Dry Propolis Extract

High quality dry propolis extract (50 mg) was added to a 7 mL vial with a well-sealed cap and dry pyridine (500 µL) was added. The contents of the vial were stirred until dissolution of the dry propolis was complete. Acetic acid (500 µL) was added and the solution was left to stir overnight. Hydrochloric acid (1 mL, 1 M) was added and left to stir for 1 hour. The solution was transferred to a 15 mL falcon tube. Chloroform (0.5 mL) was added to the falcon tube and shaken with vigour (10 minutes). The solution was centrifuged (5 minutes, 14000 rcf) and the bottom layer of liquid was transferred to a 2 mL microcentrifuge tube. The chloroform extraction process was repeated twice to give approximately 1.5 mL of extracted solution. The microcentrifuge tube was placed in a freezer (-4 ^{o}C) overnight. The layer of ice on the surface of the solution within the microcentrifuge tube was pierced using a glass pipette and the solution below was transferred to a GCMS vial for analysis.

5.2.4 Trimethyl Silylation of Dry Propolis Extract

Dry high quality propolis extract was reconstituted in ethanol to a known concentration. An aliquot appropriate to deliver 1 mg of dry propolis was added to a 7 mL vial. The

solvent was removed from the sample under a stream of dry nitrogen at 70 ^{o}C. MSTFA (270 μL) was added to the 7 mL vial and the solution was heated for 10 minutes at 70 ^{o}C. Dry pyridine (30 μL) was added to the vial and heated for a further 10 minutes at 70 ^{o}C. The solution was transferred to a GCMS vial equipped with an 8 mm 200 μL low volume insert for GCMS analysis.

5.2.5 Gas Chromatography Mass Spectrometry

GC-MS Analysis was carried out using an Agilent Technologies 7890B gas chromatography system connected to an Agilent Technologies 7000D triple quadrupole mass spectrometry system. The gas chromatograph was equipped with a J&W HP-5ms Ultra Inert 30 m x 250 μm x 0.25 μm GC Column.

The temperature program for the oven had an initial temperature of 50 ^{0}C which increased at a rate of 20 ^{o}C min^{-1} until a temperature of 250 ^{o}C was reached and held for 10 min. The temperature continued to increase at a rate of 20 ^{o}C min^{-1} until a temperature of 320 ^{o}C was reached and held for a further 25 minutes. This gave a total run time of 48.5 min.

The liquid injection tool on the autosampler was used with a volume of 10 μL. The split ratio was 30:1. The inlet temperature was set to 250 ^{o}C . Helium was used as a carrier gas with a flow rate of 1 mL min^{-1}.

5.2.6 Liquid Chromatography Mass Spectrometry

LC-MS Analysis was carried out on a Waters SymmetryShield RP18 5 μm 3.0 x 250 mm column in a Dionex Ultimate 3000 liquid chromatography system connected to a Bruker amaZon X mass spectrometry system.

Solvent A was 5% formic acid in Type 1 water. Solvent B was chromatography grade methanol. Elutions were carried out at 21 ^{o}C with a constant flow of 600 μL min^{-1}. The elution scheme was 35% B held for 16.5 min increasing to 45% B at 33 min and held until 90 min then increased to 80% at 90 min.

5.3 Results and Discussion

5.3.1 GCMS Analysis of TUPE

The standard curve of peak area versus concentration for sclareol, Figure 5.4, showed a linear trend over the expected concentration range with an R^2 value of 0.9718.

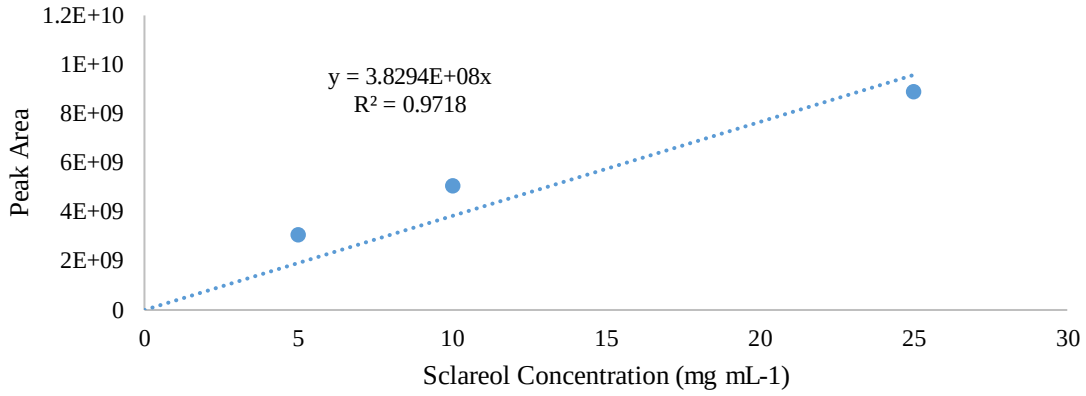


Figure 5.4 Standard curve of sclareols peak area vs concentration.

The relative response factor of sclareol to *n*-dodecane was calculated using equation 5.1. Through this equation, the relative response factor sclareol against *n*-dodecane was determined to be 1.303 ± 0.122 (*n*=3, 95% confidence).

$$Relative\ Response\ Factor = \frac{(Peak\ Area\ of\ Sclareol)/(Concentration\ of\ Sclareol)}{(Peak\ area\ of\ n{-}dodecane)/(Concentration\ of\ n{-}dodecane)} \quad (5.1)$$

Figure 5.5 shows the total ion chromatogram from one of the non-derivatised quantitative samples of Te Urewera propolis. There were 42 peaks which were consistently observed across all three samples. Of the 42 peaks identified, only 20 returned National Institute of Standards and Technology 17 (NIST17) library search results with a confidence greater than 75%. Table 5.1 summarises the data collected from the three repeat GCMS analyses.

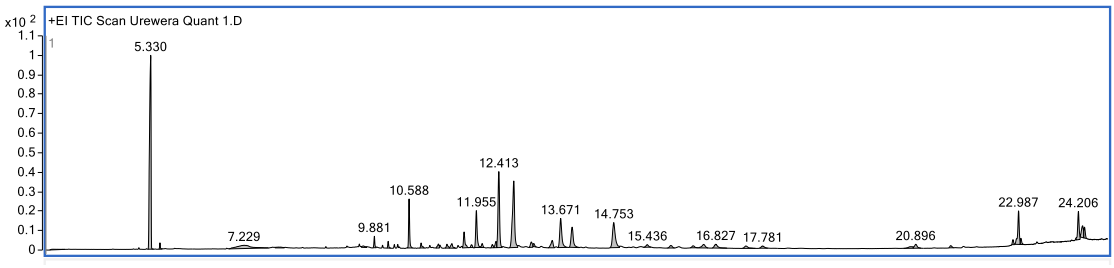


Figure 5.5 GCMS chromatogram of non-derivatised TUPE.

Table 5.1 Data acquired from GCMS analysis of quantitative samples.

Retention time (min)	Peak Area (%, n=3)	Base Peak (*m/z*)	Compound	NIST17 Library Search Score
5.3	23.2	56.9	Dodecane	95.17
5.5	0.38	54.9	1-Undecanol	95.09
9.9	1.07	72.8	*n*-Hexadecanoic acid	87.09
10.2	0.48	242.6	Thunbergen	75.19
10.3	0.27	90.8	Bicyclo[4.3.0]nonan-1-ol, 7,9-bis(methylene)-2,2,6-trimethyl-	65.7
10.4	0.29	122.7	Thunbergol	73.59
10.6	3.90	136.7	Epi-13-manool	91.75
10.8	0.65	54.9	*cis*-Vaccenic acid	78.08
11.2	0.58	80.8	2*H*-Pyran, 2-(7-heptadecynyloxy)tetrahydro-	72.31
11.2	0.28	80.8	Thunbergol or Thunbergol Isomer	73.14
11.4	0.57	92.8	2-methylene-4,8,8-trimethyl-4-vinyl-bicyclo[5.2.0]nonane	69.46
11.5	0.80	122.7	Sandaracopimarinal	75.02
11.7	2.12	80.8	Copalol	82.54
11.9	0.47	42.9	Thunbergol or Thunbergol Isomer	74.11
12.0	4.46	80.8	Verticiol or Verticiol Isomer	80.73
12.1	0.52	256.6	Pimarinol	79.06
12.3	0.45	270.6	Totarol	75.04
12.4	0.79	201.6	Podocarpa-8,11,13-triene-7β,13-diol, 14-isopropyl-	69.7
12.4	9.78	285.6	Ferruginol	86.31
12.7	11.67	120.7	Torulosol	81.13
13.1	0.84	130.7	Pinostrobin	68.13
13.1	0.46	120.7	Communic acid	69.93
13.5	1.29	96.8	13-Tetradecenal	75.78
13.7	5.19	80.8	Verticiol or Verticiol isomer	78.15
13.9	4.12	255.5	Pinocembrin	89.25
14.8	5.97	94.8	Agathadiol	89
15.4	0.48	267.5	Dihydro sandaracopimaric acid	60.06
15.9	0.57	284.6	Sugiol	73.94
16.1	0.32	27.9	$\Delta^{5,23}$-Stigmastadieneol	63.06
16.4	0.46	96.8	13-Tetradecenal	74.26
16.6	1.09	152.7	Naringenin	52.69
16.8	1.07	253.5	Chrysin	75.12
17.4	0.65	56.9	Octadecane, 1-chloro-	64.99
17.8	0.71	269.5	Galangin	71.14
20.9	0.70	96.8	Behenic alcohol	76.52
21.6	0.39	56.9	Heptacosane	65.67
22.9	0.55	206.5	*Z,Z*-11,13-Hexadecadien-1-ol acetate	61.39
23.0	4.16	96.8	Behenic alcohol	74.83
23.0	0.63	206.5	4-Ethyl-5-hexyl-2,2-bis(trifluoromethyl)-1,3-dioxolane	59.32
24.2	3.44	206.5	5-Nonadecen-1-ol	60.25

Retention time (min)	Peak Area (%, n=3)	Base Peak (*m/z*)	Compound	NIST17 Library Search Score
24.3	2.08	206.5	11,13-Dimethyl-12-tetradecen-1-ol acetate	56.15
24.3	1.24	206.5	1,3-Dioxolane, 4-ethyl-5-octyl-2,2-bis(trifluoromethyl)-, *trans*-	54.6

Of the 42 peaks identified which can be seen in Figure 5.5 and which were described in Table 5.1, 12 were identified to be diterpenes. Quantitative concentrations for these compounds were able to be calculated using the previously determined relative response factor, Table 5.2. The quantitative concentrations for the labdane diterpenes were calculated using Equation 5.5. These components are discussed further below, section 5.3.4.

$$Concentration\ of\ Analyte\ in\ Dry\ Propolis\ (mg\ g^{-1}) = \frac{(Peak\ Area\ Analyte)}{(Peak\ Area\ IS)\times RF} \times \frac{(Concentration\ of\ Internal\ Standard\ (mg\ ml^{-1})}{(Mass\ Dry\ Propolis\ in\ solution\ (g) \div Volume\ of\ Solution\ (mL))} \quad (5.2)$$

The concentrations for each diterpene were calculated for each repeat and then averaged. While the concentrations of diterpenes were able to be accurately quantified in high quality dry Te Urewera extract, response factors were unavailable for the other compounds present in the sample.

Table 5.2 Concentrations of diterpenes in TUPE.

Compound	Concentration ($mg_{(Compound)}/g_{(dry\ propolis)}$)	CI (95%)
Epi-13-manool	12.87	0.30
Sandaracopimarinal	2.65	0.29
Copalol	7.02	1.10
Pimarinol	1.72	0.36
Totarol	1.48	0.21
Podocarpa-8,11,13-triene-7β,13-diol, 14-isopropyl-	2.61	0.28
Ferruginol	32.29	1.41
Torulosol	38.52	1.78
Communic acid	1.51	0.47
Agathadiol	19.76	1.84
Dihydro Sandaracopimaric acid	1.57	0.10
Sugiol	1.89	0.16

CI calculated using Student's t test with 95% confidence (n=3), values calculated using n-dodecane as an internal standard, response factors of diterpenes were assumed to be the same as that of sclareol

Without appropriate response factors, the concentrations of other compounds in the sample could not be determined. However, using the peak areas in the total ion chromatogram of the non-derivatised GCMS analysis of Te Urewera propolis extract the macrocomposition of the extract could be estimated by assuming a response of 1, Table 5.3. This estimation identified that Te Urewera propolis was primarily composed of diterpenes in comparison to other classes of molecules. This was significant evidence for the identification of Te Urewera propolis as a new class of propolis. This is discussed below, section 5.3.5.

Table 5.3 Estimation of the macrocomposition of dry TUPE.

Class of Compound	Percentage Composition (%)
Diterpene	50.6
Flavonoid	9.8
Wax	16.4
Other	23.2

n=3, compositions based on response factor of 1 to n-dodecane

The acetylated extract gave little useful information on the composition of the propolis extract. The acetylation revealed the presence of some carbohydrates which were not observable in the non-derivatised sample. However, it was unable to exactly identify any of the carbohydrates with confidence. Notwithstanding the identifications offered by the NIST17 library, it is likely that the monosaccharides are glucose and fructose and the disaccharides are sucrose and maltose arising from contamination of the propolis with honey and/-or nectar. Other monosaccharides may be constituents of bud exudates; carbohydrates much larger than mono and disaccharides are unlikely to be extracted by 100% ethanol.[87] There have been very few scientific investigations into the origin of carbohydrates in propolis. The identification of these sugars was not pursued further.

Some acetylated derivatives of compounds found in the non-derivatised sample were also observed. This gave further evidence for the presence of these compounds, but the library identification results were not particularly confident. The total ion chromatogram for this sample can be seen in Figure 5.6 and results with confidence not less than 75% are shown in Table 5.4.

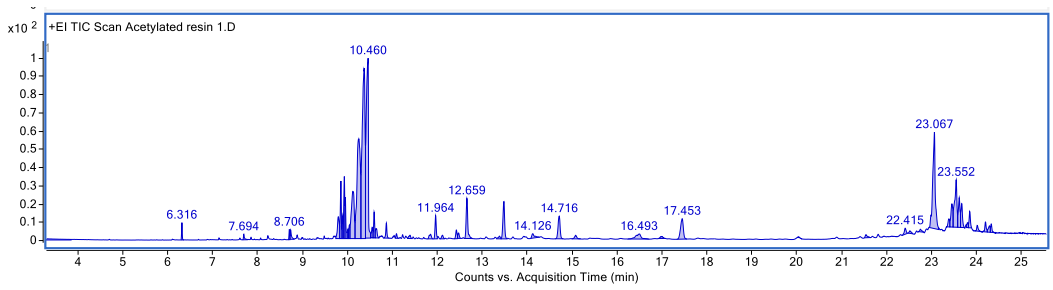


Figure 5.6 GCMS chromatogram of acetylated TUPE.

Table 5.4 Data acquired from GCMS analysis of acetylated TUPE.

Retention time (minutes)	Peak Area (%)	Base Peak (m/z)	Compound	Library Search Score
6.316	0.620726	42.9	1,3-Diacetin	96.96
8.706	0.392776	42.9	β-D-Fructofuranose, 1,3,4-tri-*O*-acetyl-2,6-anhydro-	83.45
8.735	0.633451	125.7	1,2,4-Benzenetriol, triacetate	78.42
9.855	3.217783	42.9	α-Ethyl-L-sorboside tetraacetate	78.4
9.931	3.345821	302.6	α-Ethyl-L-sorboside tetraacetate	78.48
9.954	1.923437	42.9	α-Ethyl-L-sorboside tetraacetate	82.95
10.044	0.763	42.9	β-D-Fructopyranose, pentaacetate	75.32
10.12	7.743972	42.9	β-D-Fructopyranose, pentaacetate	78.45
10.376	27.40729	43	α-D-Glucopyranose, pentaacetate	80.68
10.46	22.07669	114.9	β-D-Glucopyranose pentaacetate	92.19
10.55	0.746415	42.9	1,3,4,6-Tetra-*O*-acetyl-β-D-mannopyranose	86.57
10.649	0.915292	42.9	D-Galactofuranose, pentaacetate	81.62
11.964	1.547814	80.8	Kolavelool	79.33
12.427	0.638057	136.7	Epimanool or Epimanool Isomer *	81.2
12.659	3.608448	285.6	Acetyl Ferruginol *	82.18
14.716	2.605293	80.8	Sandaracopimaral *	79.75
21.548	0.350862	168.6	Sucrose Octaacetate	76.15
23.067	13.23049	168.8	Sucrose Octaacetate	82.38
23.552	6.149251	168.7	Sucrose Octaacetate	76.23
23.674	2.083135	168.8	Maltose octaacetate or isomer	75.59

** Indicates that this compound is a derivative of a compound which was identified in other GCMS analysis*

The GCMS analysis of TMS derivatised Te Urewera Propolis samples provided much more useful information regarding the composition of Te Urewera propolis, Figure 5.7. GCMS analysis of the TMS derivatised sample identified TMS derivatives of some of the compounds identified in the analysis of non-derivatised TUPE. This data provided further evidence on the presence of some of the previously identified compounds. The only issue with the TMS derivatisation is that the mass spectral information was less reliable than expected. TMS derivatised samples often give more informative and more distinct mass spectral data than non-derivatised samples. However, it was observed in this experiment that although the derivatisation was thorough, the mass spectral data returned low NIST17 library search scores. Table 5.5 shows the some of the relevant peak information for this analysis; this table only contains peaks, which were potentially relevant for confirming previously identified compounds.

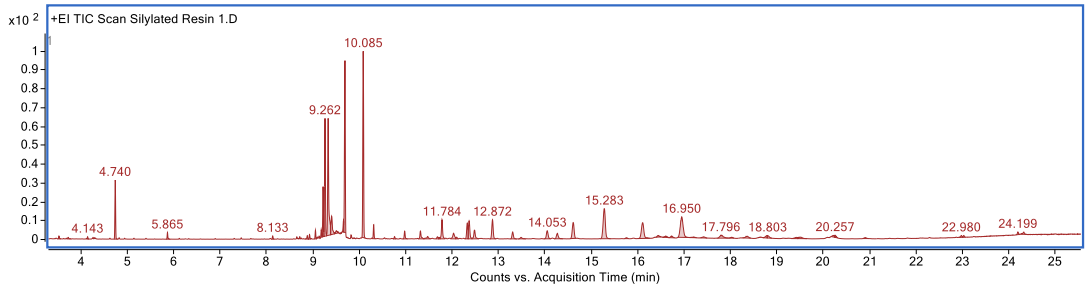


Figure 5.7 GCMS chromatogram of TMS derivatised TUPE.

Table 5.5 Data acquired from GCMS analysis of TMS derivatised TUPE.

Retention time (minutes)	Peak Area (%)	Base Peak (m/z)	Compound	Library Search Score
10.98	2.56	142.7	Epimanool TMS	81.63
11.69	0.89	273.5	TMS 2,3-Dehydroferruginol	54.34
11.72	0.66	256.7	18-[TMS]-(4α)-Kaur-16-ene or Isomer	69.71
11.78	8.41	357.5	Ferruginol TMS ether	70.48
12.03	3.66	72.8	Communic Acid TMS Derivative or Isomer	69.77
12.33	6.89	72.8	Communic Acid TMS Derivative or Isomer	84.97
12.37	9.29	120.7	Pimaric acid TMS derivative	84.12
12.87	9.63	142.7	18-[TMS]-(4α)-Kaur-16-ene or Isomer	63.64
13.49	0.92	142.7	Sclareol bis(TMS) ether	55.08
14.05	5.03	72.8	Sandaracopimaral	70.91
14.61	11.45	72.8	18-[TMS]-(4α)-Kaur-16-ene or Isomer	72.23
15.28	25.14	72.8	Communic Acid TMS Derivative or Isomer	71.73
15.75	0.66	72.8	2TMS derivative (3α,5β,20S)-Pregnane-3,20-diol	48.35
16.11	14.80	72.8	Communic Acid TMS Derivative or Isomer	76.36

5.3.2 LCMS analysis of non-derivatised TUPE

There were 28 distinct peaks in Te Urewera propolis identified by LCMS analysis, Figure 5.8 and Table 5.6.

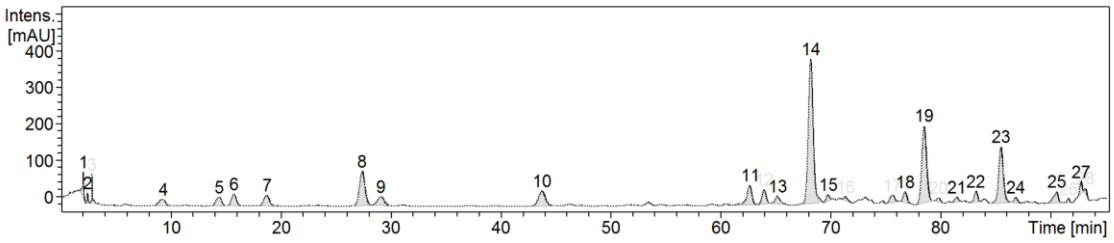


Figure 5.8 UV (280 nm) chromatogram of TUPE acquired during LCMS analysis.

Table 5.6 Peak data from LCMS analysis of TUPE

RT[min)	Area %	Base Peak (m/z)	UV maxima
2.1	4.58	327.98	n/a
2.4	1.59	330.43	236
2.9	1.91	315.93	246, 376
9.3	6.44	89.14	247, 326
14.4	6.85	89.14	247, 325
15.8	8.30	389.99	312
18.8	8.64	191.11	247, 323
27.4	27.01	327.76	280
29.1	8.59	593.07	290
43.7	14.72	564.84	294
62.6	14.30	163.04	248, 328
63.9	8.36	517.04	246, 328
65.1	5.62	345.02	247, 330
68.2	100.00	256.86	243, 294
69.7	2.75	327.63	247, 328
71.3	2.02	327.77	248, 325
75.6	4.83	327.56	270, 317
76.8	5.53	327.75	242, 296
78.5	51.82	255.14	270
79.8	2.43	327.02	269
81.4	2.59	327.32	302
83.2	4.82	327.41	248, 327
85.5	36.74	271.12	268, 360
86.8	2.50	327.73	246, 314
90.5	7.77	327.86	242, 291
91.6	1.42	327.50	283
92.7	3.93	327.71	257
93.1	1.87	301.34	241, 290

The data collected from this LCMS analysis was used to confirm the presence of some of the notable diterpenes identified using GCMS. The significance of this data is discussed below, section 5.3.4.

5.3.3 Non Derivatised Propolis Sourced From Other Locations Within Te Urewera

Propolis samples from two other locations in Te Urewera were extracted and analysed using GCMS. This was to verify that the composition of Te Urewera propolis was roughly consistent throughout the entire bush. The second and third samples of Te Urewera propolis were obtained nearer the edge of Te Urewera than the first. The GCMS data obtained for these samples indicated that it was of standard poplar composition. As standard poplar composition is already well documented, details of the chemical composition of these extracts will not be discussed in depth in this study.[6, 7, 11, 21, 22] The chromatogram of one of these samples obtained by GCMS is shown, Figure 5.9. This data showed that the composition of propolis throughout Te Urewera was not consistent. The outer borders of Te Urewera are occupied by pine forestry blocks, and beyond those blocks lies farmland containing poplar trees. The presence of poplar constituents in propolis sourced from nearer the edges of Te Urewera is possibly due to bees preferentially collecting resin from these introduced plants and travelling a distance to do so. In Te Urewera propolis batch 1, these plants will have been too far from the hive to be found by bees. Batches 1 and 2 were evidently still too close. This implies that if the unique propolis identified in this study is to be collected, it must not only be collected from hives which themselves are located within native bush, but from hives which are in native bush large enough that resin cannot be collected from introduced plant species. This also explains why Markham *et al*[6] were unable to find unique propolis from hives located in the Coromandel. It is likely that if a hive location in the Coromandel region to study propolis was to be selected, it would be located in one of the many areas of native bush available. However, the native coromandel bush is dotted with patches of introduced plants. Therefore, it is probable that the bees in those hives travelled beyond the native bush to preferentially collect resin from pine or poplar trees.

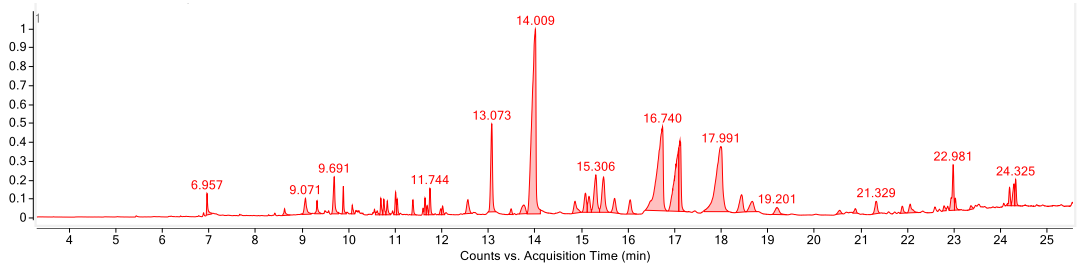


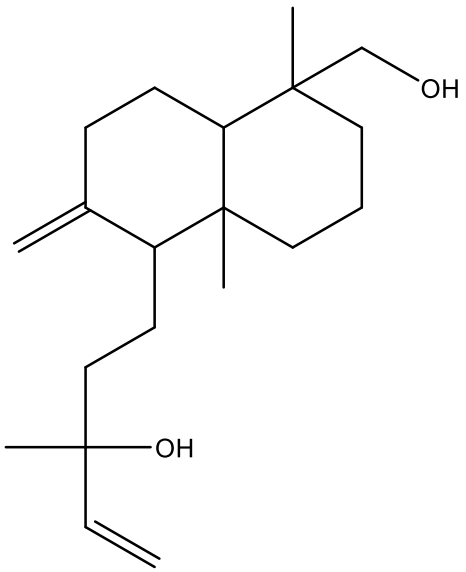
Figure 5.9 GCMS chromatogram of TUPE sourced from secondary location.

5.3.4 Main Components Observed in Te Urewera Propolis

5.3.4.1 Diterpenes

Te Urewera propolis extract was found to be composed primarily (51%) of diterpenes, Tables 1.2 and 1.3. This high concentration of diterpenes is uncommon in propolis extracts and has only been observed previously in propolis of the Mediterranean class sourced from Greece.[46, 88]

The primary component of TUPE was the labdane diterpene torulosol (**1)** with a concentration of 38.52 ± 1.78 mg g^{-1} dry extract. This was closely followed by ferruginol (**2**) with a concentration of 32.29 ± 1.41 mg g^{-1} dry extract.



(1)

1 was directly identified in the non-derivatised GCMS analysis of the Te Urewera Propolis extract with a fairly high library search score of 81. The molecular ion (306.5) was not observed but fragment ions were, *m/z* = 273 $[M\text{-}2OH]^+$, and *m/z* = 257 $[M\text{-}H_2O\text{-}CH_3OH]^+$, Figure 5.10. When the experimentally obtained mass spectral data for this peak is compared against reference mass spectral data the similarities are clear.

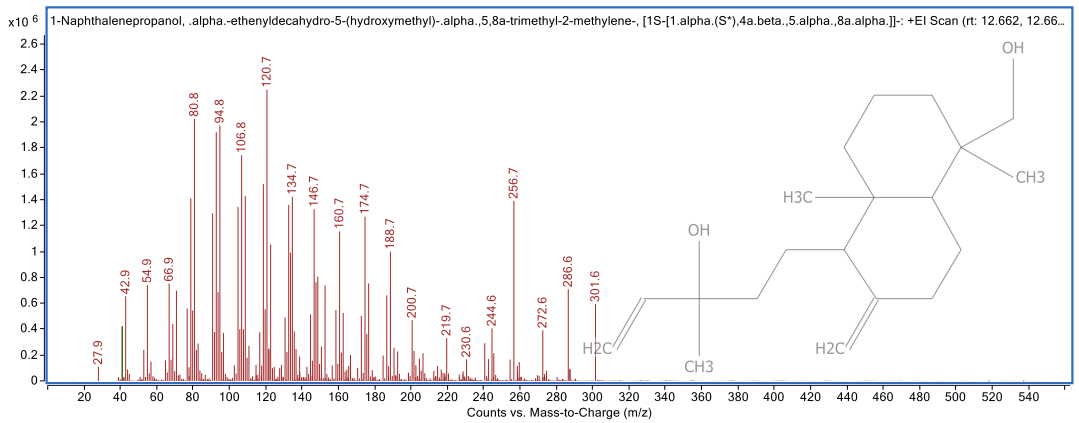


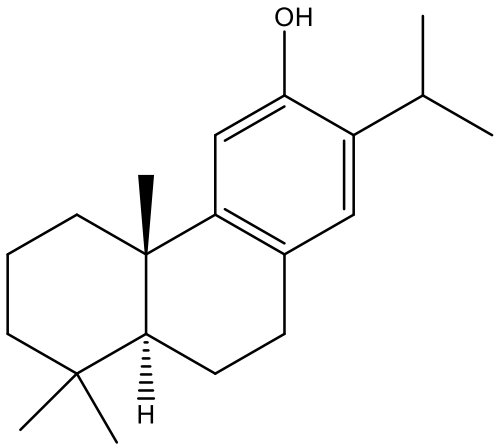
Figure 5.10 Mass spectrum of 1.

1 is also likely responsible for RT = 85.5 min in the LCMS data detailed in Table 5.6. The max m/z for this peak was 271 $(M\text{-}2H_2O)^+$. **1** has previously been shown to have a peak at *m/z*= 271 $(M\text{-}2H_2O)^+$ in positive ion mode electrospray ionisation mass spectrometry.[89]

1 has been observed in Greek, Algerian and Columbian propolis and was one of the primary constituents in Columbian propolis, whereas it was only a minor constituent in Greek and Algerian propolis.[46, 86, 90] The botanical origin of Columbian propolis has not yet been determined. However, the botanical origin of Greek propolis has been identified as *Cupressus sempervirens* as well as various *Pinus* spp.[46]

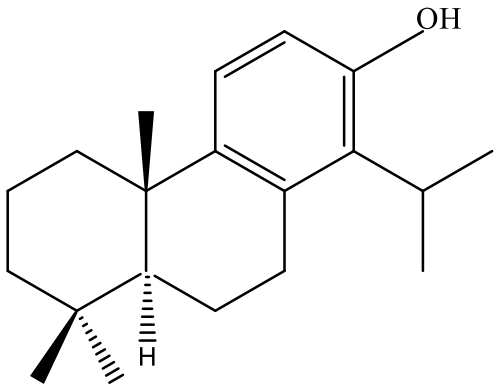
In Aotearoa, **1** has been identified in the heartwood of mountain pine (*Dacrydium bidwilli*), the heartwood of the yellow pine (*Dacrydium biforme*), and the manoao (*Dacrydium kirkii*).[91] All of these are part of the Podocarpaceae family and are characteristic of native Aotearoa bush such as Te Urewera.

2, was the second most highly concentrated diterpene in ethanolic Te Urewera propolis extract.



(**2**)

2 was directly identified with strong confidence of 86.31%. It is important to note that **2** is an isomer of totarol, (**3)**, with great structural similarity.



(**3**)

The easiest way to distinguish between the two compounds is the ratio between the peaks at 271 and 286. *m/z* = 286 is the molecular ion for both **2** and **3**. The m/z 271 is likely due to $[M\text{-}CH_3]^+$. The peak at 271 is proportionally stronger in comparison to the peak at 286 in **3** than it is in **2**. In **2**, these two peaks are similar in strength, Figure 5.11, whereas, in **3** they are unequal, Figure 5.12.

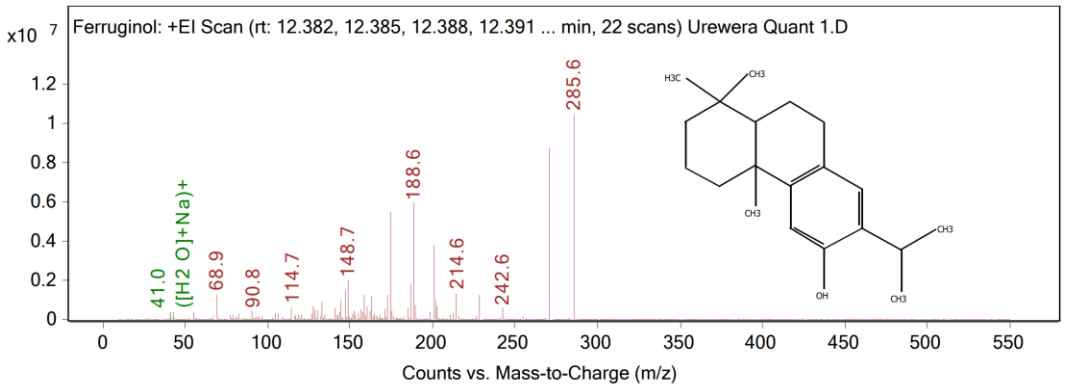


Figure 5.11 Mass spectrum of 2.

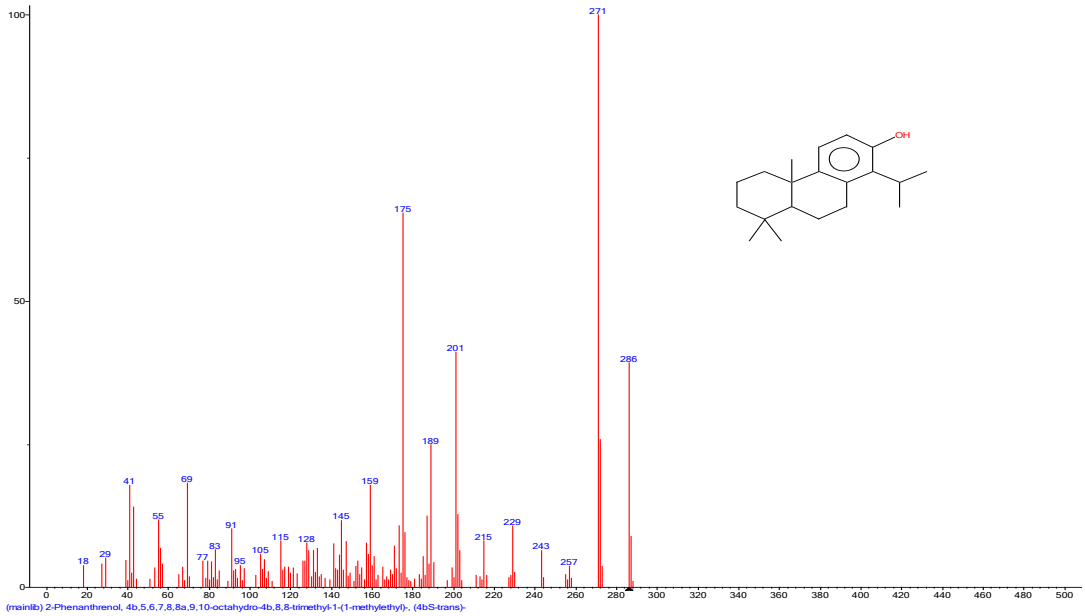


Figure 5.12 Reference mass spectrum of 3.

2 was successfully derivatised using acetylation. RT = 12.651 min in the acetylated sample was identified as acetyl ferruginol with a library search confidence of 82.18%. The TMS derivatisation of the sample identified a peak caused by derivatisation of **2**. This peak was ferruginol TMS ether, RT = 11.78 min in Table 5.5, Figure 5.13. [92, 93] Comparison of the mass spectrum of the experimentally obtained ferruginol TMS ether, Figure 5.13, to reference spectra in both the NIST database and literature confirms the library search result. The m/z of the peaks and their respective intensities aligns very strongly with reference spectra.[92, 93] The ion *m/z* 358 corresponds to M^+ and *m/z* 343 is $[M\text{-}CH_3]^+$.There were no peaks which were readily attributable to ferruginol in the LCMS chromatogram.

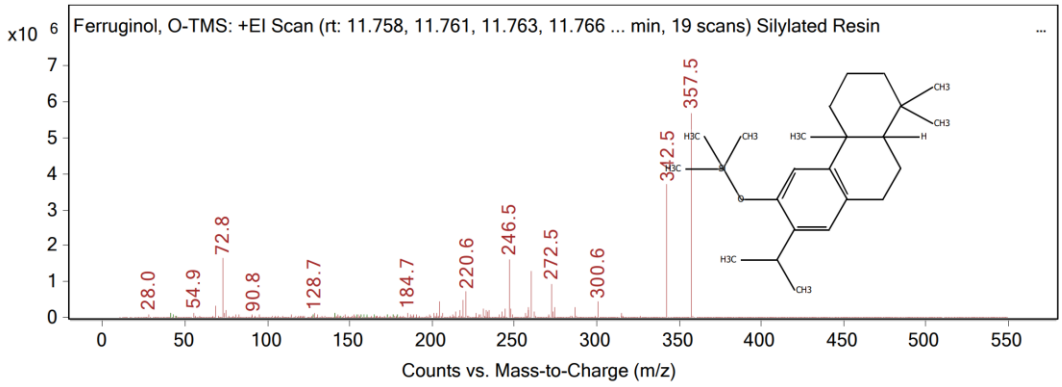
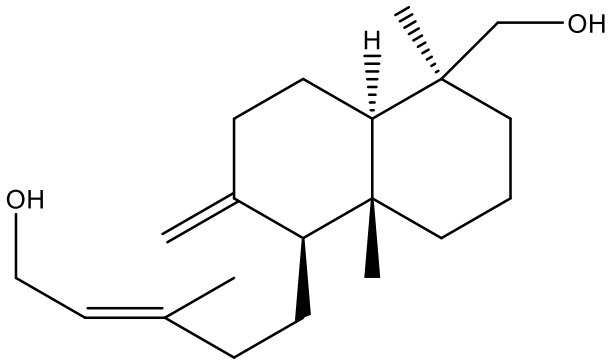


Figure 5.13 Mass spectrum of ferruginol TMS ether.

2 was first observed in Greek propolis in low concentrations. [46] **2** has also been reported in Brazilian red propolis. However, it is worth noting that the review which stated the latter failed to provide suitable sources for this statement.[94]

Within Aotearoa, the Miro tree (*Podocarpus ferruginea*) was the first identified botanical source of ferruginol. Since then, **2** has been reported in various plants from the *Podocarpaceae*, *Cupressaceae*, *Lamiaceae* and *Verbenaceae* families.[95] The Miro tree can be found within Te Urewera.

Agathadiol, **4**, was identified as the third most highly concentrated labdane diterpene in the ethanolic extract of Te Urewera Propolis.



(**4**)

4 appeared at RT=14.8 minutes in the chromatogram of the underivatized sample and was identified in the NIST17 library with a confidence of 86%, Figure 5.14. The molecular ion of **4** is absent in the experimental mass spectrum. There are, however, other identifiable ions: *m/z = 290* $[M\text{-}OH]^+$, *274* $[M\text{-}CH_2OH]^+$, *257* $[M\text{-}CH_2OH\text{-}H_2O]^+$.

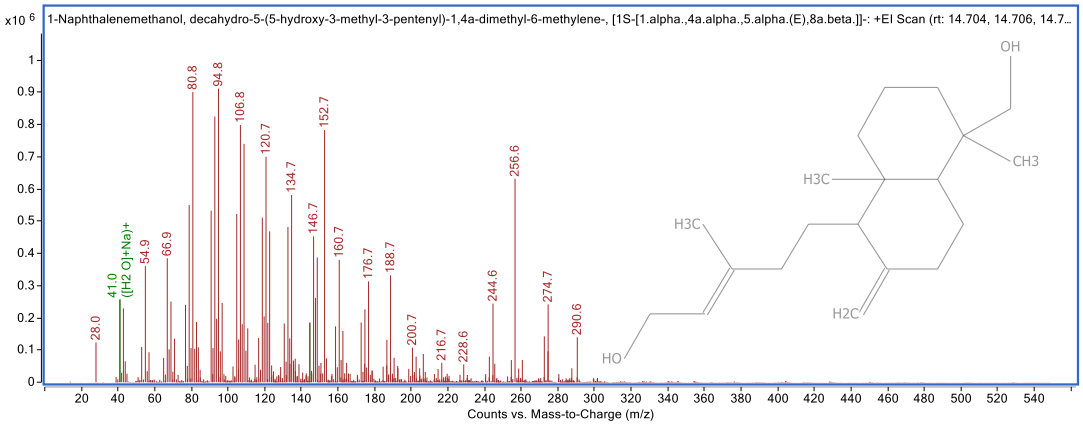


Figure 5.14 Mass spectrum for agathadiol.

4 did not appear in the GCMS analysis of the acetylated sample and its TMS derivative is not in the NIST library. However, it is possible that RT = 16.11 in Table 5.5, which the library search initially identified as *O*-TMS communic acid was in fact *O*-TMS agathadiol and the mass spectrum for this peak can be seen in Figure 5.15. It is possible that *m/z = 360* was caused by the (M^+-TMSOH) ion of O-TMS agathadiol. There were no peaks in the LCMS data which were able to be attributed to **4** directly based on the ESI mass spectral data and UV absorption maxima obtained. The use of a reference standard in future studies may help to determine which peak represents **4**.

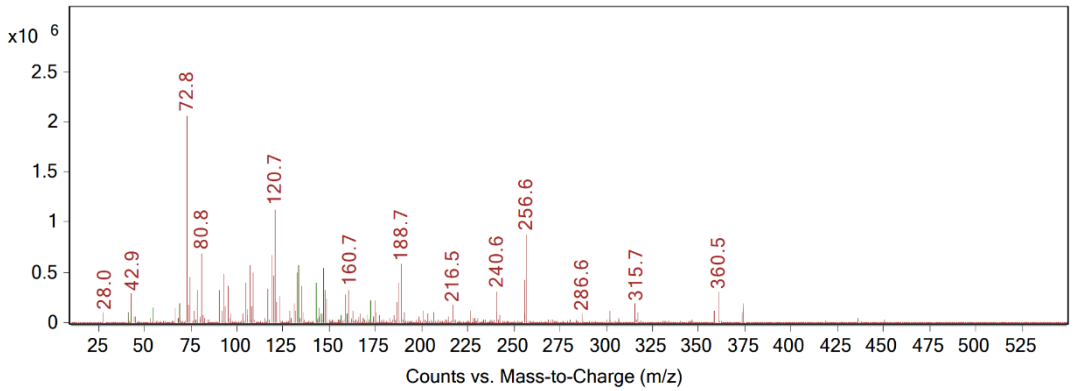
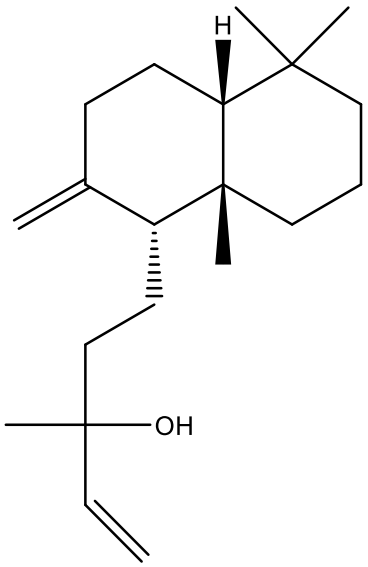


Figure 5.15 Mass spectrum of RT = 16.11 in the GCMS analysis of TMS derivatised TUPE, potentially agathadiol.

4 has previously been identified as a primary constituent in Columbian propolis. As previously discussed, the botanical origin of Columbian propolis has not yet been determined.[86] **4** was also found as a minor constituent in Greek propolis.[46]

There are no reported botanical sources of **4** in Aotearoa. However, agathic acid has been identified in Aotearoa. This was first isolated from the resin of the New Zealand Kauri tree (*Agathis australis*). Agathic acid has been shown to form **4** when reduced.[91, 96] The presence of **4** in TUPE may be explained due to either reduced Agathic acid from Kauri being introduced to propolis, or the collection of **agathic acid** from Kauri and its subsequent reduction. More study would be required to determine if either of these is the case, there is currently no relevant literature on this topic.

The diterpene found in the fourth highest concentration in the ethanolic extract of Te Urewera propolis was 13-epimanool, **5**.



(**5**)

5 was identified with 91.75% confidence in the GCMS analysis of the non-derivatised Te Urewera propolis extract, Figure 5.16, Table 5.1. *m/z* 272 in this figure is attributable to the ion $[M\text{-}H_2O\text{-}CH_3]^+$.

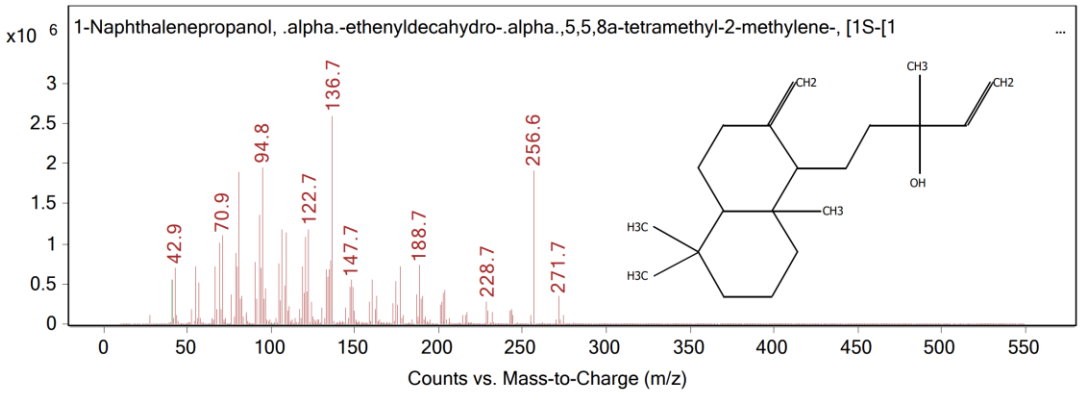


Figure 5.16 Mass spectrum of 5.

The acetylation of 5 was unsuccessful. Non-derivatised **5** was observed in the GCMS analysis of acetylated Te Urewera propolis extract at 12.4 minutes, Table 5.4, and there were no peaks which indicated that epimanool acetyl ester was present. There were also no peaks in the trimethylsilylated mixture which could easily be attributed to any TMS derivatives of **5**. It is possible that a TMS derivative of **5** is responsible for one of the unallocated peaks from the TMS derivatised analysis. However, none of the peaks gave fragmentation patterns which could be confidently attributed to O-TMS Epimanool. None of the peaks in the LCMS data could be confidently attributed to **5**. Issues with both identification of Epimanool TMS in the analysis of the TMS derivatised sample and the identification of **5** in the LCMS analysis of the sample could be remedied through the use of a reference standard of **5**.

5 has previously been observed in propolis samples from both Greece and Brazil. **5** was present to a similar extent in these samples as it was in Te Urewera propolis.[22, 46, 88, 97] With respect to indigenous flora of Aotearoa, **5** has been identified in the sticky mountain daisy (*Celmisia viscosa*), and yellow or pink pine (*Halocarpus biformis*) [98, 99].

Other than the four previously discussed labdane diterpenes identified in Te Urewera propolis, there were eight more diterpenes found in this sample. These were all present in lower concentrations. However, they can still offer some insight into the potential botanical origins of Te Urewera propolis.

3, RT = 12.3 minutes Table 5.1, is a labdane diterpene which is very similar in structure to ferruginol (see above). **3** was first isolated in the 1930s from the tōtara tree (*Podocarpus totara*) as small droplets which appear in the exposed flesh of the tree a few hours after cutting. Since then, the ability of tōtara timber to withstand rot has been attributed to the presence of **3**. The totara tree holds great significance to Māori due to its use in rongoa Māori and large mature specimens are found throughout Te Urewera.[100]

Sandaracopimarinarol (RT = 11.5 min), sugiol (RT = 15.9 min), dihydro-Sandaracopimaric acid (RT = 15.4 min) as well as **2**, and **5** have also been observed in the Japanese "Sugi" cedar tree (*Cupressus japonica*).[101] Sugi are present in Aotearoa but are an introduced plant species which would be unlikely to be found in the heart of Te Urewera.

There are no reports of 14-isopropylpodocarpa-8,11,13-triene-7β,13-diol (RT = 12.4) occurring in nature, . However, this molecule is similar to **3** but with the addition of an extra hydroxyl group. For this reason, it is possible that the 14-isopropyl-podocarpa-8,11,13-triene-7β,13-diol observed in Te Urewera propolis extracts was botanically sourced as **3** which has been converted to 14-isopropyl-podocarpa-8,11,13-triene-7β,13-diol.

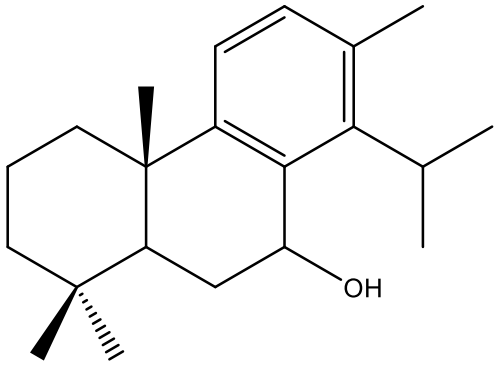


Figure 5.17 Structure of 14-isopropylpodocarpa-8,11,13-triene-7β,13-diol.

Copalol (RT = 11.7 min), Figure 5.18, within the context of Aotearoa has been identified in the resin of one of the trees which have the Māori name Monoao (*Halocarpus kirkii/ Dracophyllum subulatum/ Manoao colensoi*) (Podocarpaceae).[102]

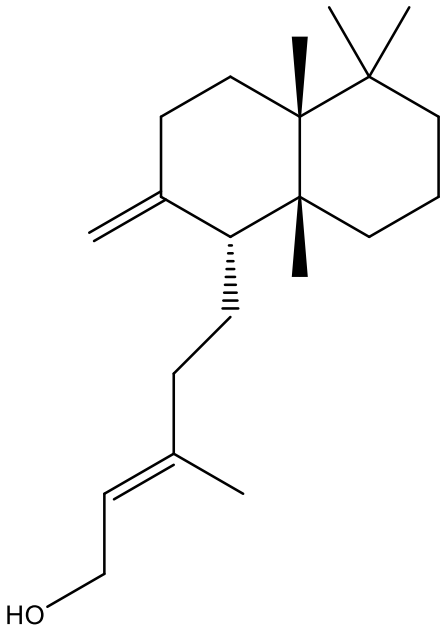


Figure 5.18 Structure of copalol.

The specific botanical origin of pimarinol (RT = 12.1), Figure 5.19, has not been determined. However, pimarinol has been found in the tall oil pitch produced during the pulping process of Aotearoa wood, which is principally the exotic *Pinus radiata*.[103]

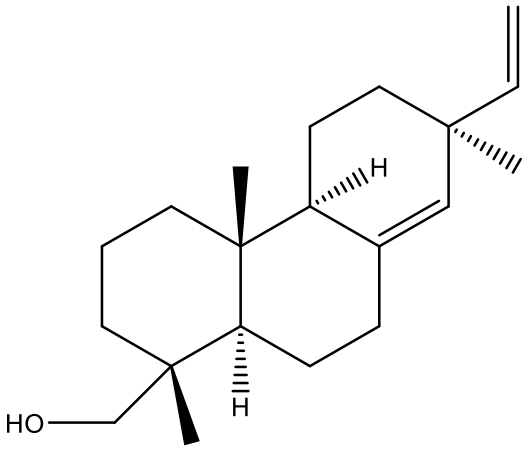


Figure 5.19 Structure of pimarinol.

Communic acid (RT = 13.1), Figure 5.20, is found in the leaf resin of the Kauri tree (*Agathis australis*).[91, 96]

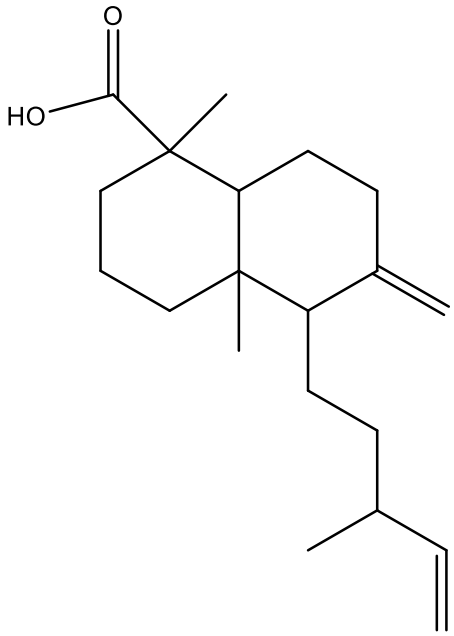


Figure 5.20 Structure of communic acid.

2,3-Dehydroferruginol RT = 11.69 was also identified in the GCMS analysis of TMS derivatised TUPE. This compound has not been identified in any plants native to Aotearoa, but has been identified in *Austrocedrus chilensis*, a plant native to Argentina which has not been found in Aotearoa.[104]

5.3.4.2 Flavonoids

Flavonoids are the primary components of poplar and other propolis classes. Flavonoids were not a major component of the Te Urewera propolis sample. However, pinocembrin, chrysin, and galangin were present as minor constituents in the ethanolic extract of Te Urewera propolis. High quality dry TUPE was estimated to be composed of approximately 10% flavonoids. This was much lower than the concentration of diterpenes in the sample which represented approximately 51% of the sample, Table 5.3.

5.3.4.3 Wax

There were a large number of peaks in all of the GCMS analyses, which corresponded to compounds with long hydrocarbon chains including alkanes, alcohols, and esters. The library search was unable to effectively find exact matches for many of these compounds. However, it was clear from the mass spectral fragmentation patterns that these were molecules which most likely represented the wax portion of propolis, despite the efforts made to remove this. The long hydrocarbon chains explain the very slight waxy and extremely hydrophobic nature of the dry propolis extract.

5.3.5 Comparison to Literature

Heretofore, it has been considered that Aotearoa only contained propolis of the poplar class. [6-8, 29, 51-63] The results of this study have found that a second, novel class of propolis exists in Aotearoa, Te Urewera propolis. This study found Te Urewera propolis to be primarily composed of diterpenes. Poplar propolis does not possess any major amount of diterpenes.[11] The diterpenes detected in this sample, Table 5.2, were also not detected in previous studies, which characterised propolis samples from Aotearoa.[6, 7] Te Urewera propolis most closely resembles the Greek Mediterranean propolis class which is also characterised by high concentrations of diterpenes.[46, 88] However, Te Urewera propolis is also distinctly different from Mediterranean propolis in the proportions of diterpenes.

For these reasons, it is probable that Te Urewera propolis is not only the first non-poplar propolis identified in Aotearoa, but that it is also an entirely new class of propolis. With the increasing trend of replanting large tracts of iwi-owned land with indigenous vegetation it seems likely that this new class could in the future become more common.

Fortunately, extensive work on the phytochemical composition of Aotearoa bush has already been carried out. This provides good initial data for the determination of the botanical origin of Te Urewera propolis. Based on the diterpenes discussed in section 5.3.4.1, it is likely that the botanical source of Te Urewera propolis is a mixture of plants from the Podocarpaceae family including the Miro tree, the Monoao, and the Yellow and Pink pine. There is also likely to be a significant contribution from the Kauri tree. All of these are endemic flora to Aotearoa which would be expected to be present in the heart of Te Urewera.

Chapter 6
Antiproliferative Effect of Te Urewera Propolis Extract.

6.1 Introduction

The first step in the study of any unknown sample *in vitro* is to investigate its cytotoxicity. If the cytotoxic concentrations are not known and an overly dilute sample is used, biological effects which the sample may hold can be missed as the sample was not presented to the cells in a significant concentration, which results in no useful information. If an overly concentrated sample is presented to the cells, the cells may instead be killed, which again provides no useful information. Knowledge on the cytotoxicity of a sample allows future *in vitro* experiments to be carried out at appropriate concentrations to gather useful data.

The methods used to determine the cytotoxicity of a sample can also be used to determine the antiproliferative activity. Antiproliferative activity is the ability of a sample to inhibit the growth of cells. Although it cannot be directly confirmed from *in vitro* work, a sample which has been observed to hold significant antiproliferative effect against cancerous lines may exhibit anticancer properties. As cancer is caused by unregulated cell growth, compounds with anti-proliferative effects may have potential as an anti-cancer treatment, although many more factors would be involved with development of such a treatment.

In addition to the determination of cytotoxic concentrations and antiproliferative effect, there is another useful piece of information to gather during this process. The determination of the mechanism of cell death induced by the sample can provide insight into how the sample interacts with the cells. There are two mechanisms for cell death, apoptosis, and necrosis. Apoptosis is programmed cell death where the cell dies in a controlled manner caused by a range of pathways within the cell. Apoptosis is characterised by blebbing[1] of the cell morphology and mitochondrial stress as the cell packages the cell constituents into vesicles. Apoptosis results in the release of these cell-constituent containing vesicles into the extracellular space rather than lysis of the cell

[1] Blebbing is a change in the cell membrane morphology which occurs during the process of apoptosis. Blebs appear as protrusions in the membrane. This is caused by contraction of the cytoskeletal actomyosin.

which is seen in necrosis. Necrosis is non-programmed cell death and involves unregulated destruction of the membrane. Apoptosis inducing compounds have the potential to be useful in the treatment of cancer as cancer cells are generally unable to undergo apoptosis.[105, 106]

There are a range of methods which can be used to investigate the cytotoxic and antiproliferative potential of samples *in vitro*. Arguably, the most simple is the trypan blue exclusion method.[107] One of the most commonly used is the MTT assay.[81, 108]

The trypan blue exclusion method utilises trypan blue, a cell staining solution which is unable to pass through intact cell membranes. The trypan blue exclusion method involves treating cells with a range of concentrations of sample and measuring the concentration of viable cells in solution by counting unstained cells under a microscope with a haemocytometer.[107] This method is useful for the generation of dose response curves which can be used to identify key stages of cell growth and the direct effect treatments have on viable cell numbers.

The MTT assay utilises the conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazolium dye, to formazan crystals through mitochondrial dehydrogenase enzymes. Formazan crystals are water insoluble but once solubilised are purple in colour. This purple colour allows for colorimetic determination of the conversion of MTT to formazan, and thus provides insight into the levels of mitochondrial stress of cells *in vitro*. Lowered levels of MTT production indicate mitochondrial stress which is indicative of cytotoxicity.[81, 108]

The cytotoxicity and antiproliferative effect of extracts of other propolis classes has been investigated in the literature. The antiproliferative effect of Aotearoa poplar propolis extracts has been tested against DLD-1 (human colorectal adenocarcinoma cells) using an MTT assay and the study found that the extract held antiproliferative activities. Fractionation of the sample was also carried out, this revealed that nearly all of the constituents in this extract independently held antiproliferative activities.[53] MTT and trypan blue exclusion methods have also been used together to evaluate the antiproliferative and cytotoxic potential of extracts of Indian stingless bee propolis. This study found through both methods that the propolis extracts they had tested held dose dependant antiproliferative and cytotoxic activities. It was noted in this study that

exposure of this extract at high concentrations to cell cultures was able to induce apoptosis within the first twenty four hours of exposure.[109]

The aim of the research described in this section was to gain insight into the cytotoxic and antiproliferative potential of TUPE. This would be achieved through the utilisation of both the trypan blue exclusion method and MTT assays. It was hypothesised, based upon propolis literature, that, TUPE would be cytotoxic at some concentration and also exhibit antiproliferative activity.

6.2 Methods

6.2.1 Trypan Blue Exclusion

HL-60 cells (passage 10, 11, and 12) were seeded into flat- bottomed, sterile, 6 well plates using complete IMDM (20% FBS) to give a final volume of 1980 μL per well with a concentration of 1.5 x 10^5 cells mL^{-1}. To each well TUPE (20 μL, in different concentrations) was added. The samples used were various concentrations of high quality dried ethanolic extract of Te Urewera propolis reconstituted in ethanol (100%, chromatography grade), and a vehicle control of ethanol (20 μL , 100 %, chromatography grade). The 6 well plates were incubated (5% CO_2, 37 ^oC) for the duration of the experiment excluding short periods during which the concentration was being determined. The media was not changed during the experiment to ensure that no cells were lost during the media changing process.

The concentration of cells in solution was determined using the trypan blue exclusion method. Trypan blue (10 μL) was added to a strip of parafilm to form a droplet. The cell solution within the well on the 6 well plate was stirred using a sterile pipette tip, agitated by gentle rocking of the plate, and mixed by pumping the solution up and down using a pipette to reach homogeneity. This homogenous solution (10 μL) was transferred to the droplet of trypan blue. The droplet was mixed thoroughly by pumping with the pipette and the stained cell solution (10 μL) was transferred to a haemocytometer for counting.

The concentrations were always measured in duplicate. The experiment was also repeated twice. The concentrations were measured upon initial seeding and approximately every 24 hours following that for 7 days.

6.2.2 MTT Assay

HL-60 cells (passage 14, and 15) were seeded into flat bottomed, sterile, 24 well plates using complete IMDM (20% FBS) to give a final volume of 990 μL per well with a concentration of 1.5 x 10^5 cells mL^{-1}. To each well TUPE (10 μL) was added. The samples used were various concentrations of high quality dried TUPE reconstituted in ethanol (100%, chromatography grade), and a vehicle control of ethanol (10 μL, 100%, chromatography grade). The 24 well plates were incubated (5% CO_2, 37 ^oC, 24 hours). Each treatment was carried out in duplicate.

The MTT Assay was carried out using a CyQUANT MTT Cell Proliferation Assay Kit. The recommended complete assay protocol provided with the kit was followed.

The media was removed from each of the wells in the 24 well plate after centrifugation (500 rcf, 10 min, 37 ^{o}C). Fresh complete media was added to make the volume up to 1000 μL per well. Each well was mixed to homogeneity and cell solution (100 μL) was transferred to a flat bottom, sterile, 96 well plate in duplicate. To each well MTT stock solution was added (10 μL, 12 mM). The plate was left to incubate (3 hours, 37 ^{o}C, 5% CO_2). SDS-HCl solution (0.01 M HCl, 0.1 g mL^{-1} SDS, 100 μL) was added to each well before being mixed by pumping the pipette. The plate was left to incubate (4 hours, 37 ^{o}C, 5% CO_2). The absorbance of each well at 570 nm was recorded using a Thermo Scientific MULTISKAN GO plate reader.

6.3 Results

6.3.1 Dose Response Trypan Blue Exclusion Curve

The trypan blue exclusion method was used to generate a curve for the viable cell count over seven days under a range of different exposures to TUPE, Figure 6.1. The dose response is presented as the cell number for each day relative to the cell number at which the wells were seeded. Exposure of HL60 cells cultures to Te Urewera propolis extract in concentrations of 62.5 µg mL^{-1} and higher caused the death of all cells after approximately 4 days and also showed that the cultures of still viable HL60 cells entered exponential growth between days five and six.

Numerous dead cells were observed using the trypan blue exclusion methods in all treatments excluding the vehicle control and the 12.5 µg mL^{-1} treatment which both had very few dead cells. There was no difference in the number of dead cells observed in the vehicle control compared to the 12.5 µg mL^{-1} treatment.

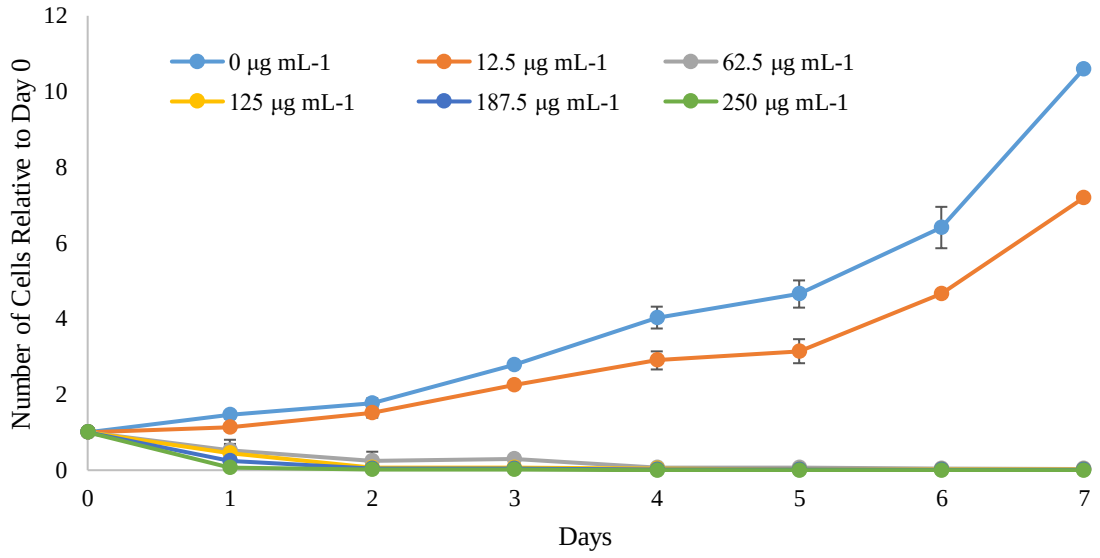


Figure 6.1 Growth curve of HL60 cells in response to TUPE measured using trypan blue exclusion. (Error bars showing ±S.E.M, *n*=2, in duplicate).

Figure 6.2 shows the difference in cell number within the first 24 hours of exposure to TUPE and highlights the difference in the effects of each treatment. Cultures exposed to ethanol alone and the lowest concentration of TUPE tested were still able to grow while exposure to higher concentrations resulted in decreases in cell number. Thus, the HL60 cultures were responding to TUPE exposure in a dose dependant manner.

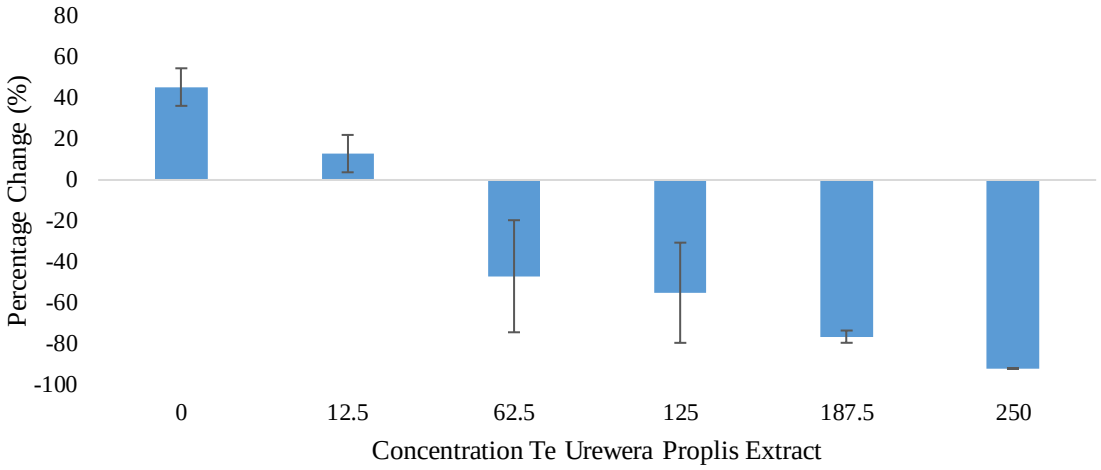
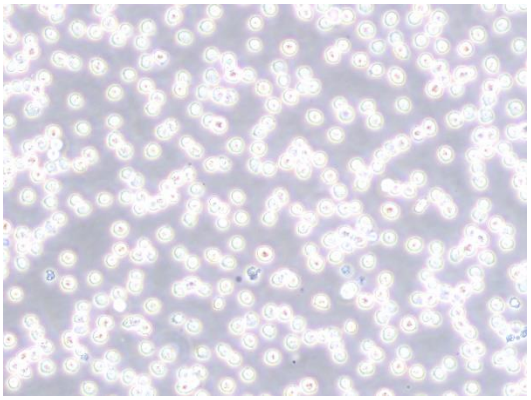
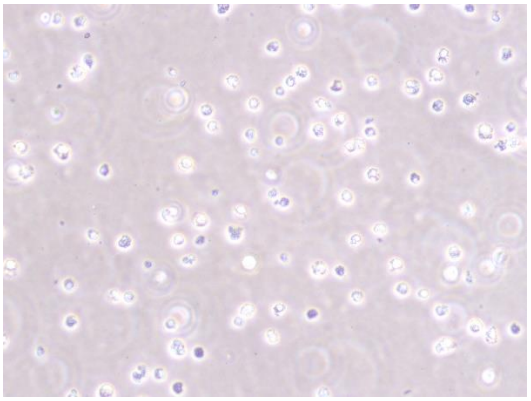


Figure 6.2 Change in HL60 cell number after 24 hours exposure to TUPE. (Error bars showing ±S.E.M, n=2, in duplicate)

In the time during which the dosage response curves were being developed, the cells were observed under the microscope regularly for changes in morphology. It was noted during these observations that HL60 cells exposed to high concentrations of TUPE may have been undergoing apoptosis, since visible blebbing occurring in some treated cells. Images of this can be seen in Figure 6.3.



Cells exposed to vehicle control. No blebbing visible.



Cells exposed to TUPE (62.5 µg mL^{-1}). Blebbing visible

Figure 6.3 Observed blebbing of HL60 cells exposed to high concentrations of TUPE.

6.3.2 MTT Results

Figure 6.4 shows the results from the MTT assay of TUPE. As the concentration of TUPE increased, mitochondrial dehydrogenase activity decreased in a logarithmic trend.

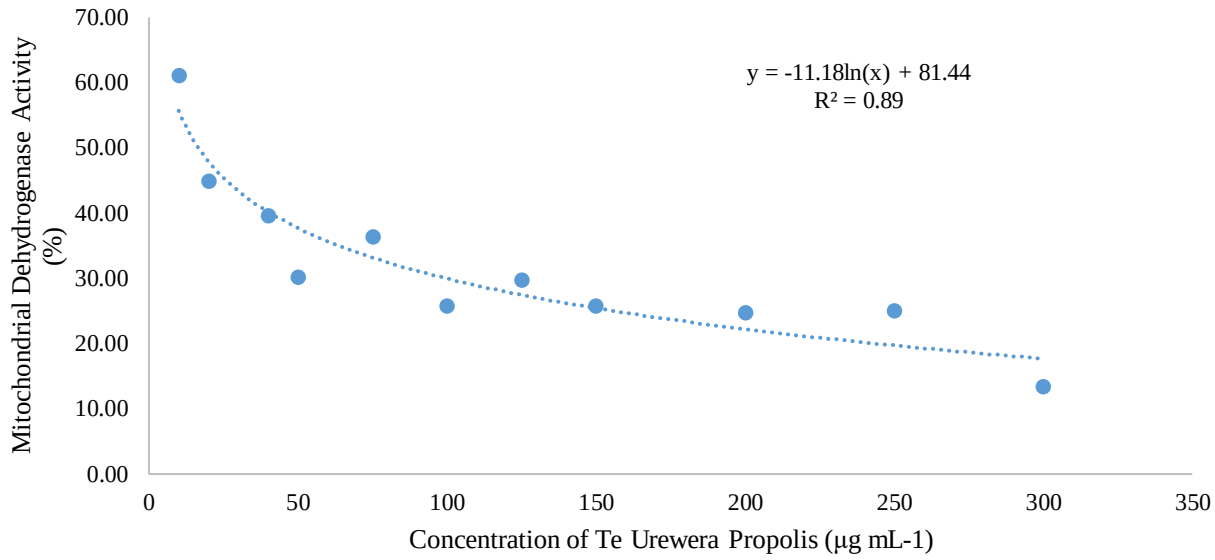


Figure 6.4 Mitochondrial dehydrogenase activity in response to exposure to TUPE.

6.4 Discussion

It is clear that TUPE demonstrates an anti-proliferative effect and at high enough concentrations is also cytotoxic.

The Trypan blue dose response curve, Figure 6.1, showed defined lag and exponential growth phases in the vehicle control. The cell numbers in dosed samples were affected by treatment within the first 24 hours, Figure 6.2. Slow growth was observed in the vehicle control and 12.5 µg mL^{-1} treatments for approximately 5 days. A clear exponential growth phase followed the lag phase. This exponential growth phase continued beyond the window observed in this experiment and a stationary phase was not observed. The clear growth phases displayed by the vehicle control indicated the suitability of 1% ethanol as a delivery agent. Only the 12.5 µg mL^{-1} sample, was able to still retain clear growth phases, although this was with lower growth rates and, therefore, lower cell density. Treatments at concentrations higher than this only showed steadily decreasing cell numbers until there were no viable cells left in solution.

Although the numbers of dead cells present in the cell solutions was not recorded during the data collection process, it was noted that there were many dead cells observed in the concentrations higher than 12.5 µg mL^{-1}. Observation of the morphology of the cells revealed blebbing, Figure 6.3. This indicates that there is potential that the cells were undergoing apoptotic cell death rather than necrotic cell death. The MTT assay was

carried out after identification of this likely apoptotic cell death so more evidence could be collected to support the claim that some TUPE concentrations killed the HL-60 cells via apoptosis.

The data from the MTT assay, Figure 6.4, showed a clear decrease in mitochondrial dehydrogenase activity over 24 hours upon treatment with TUPE. This decrease appears to be dose dependant but not in a linear manner. For the concentrations of TUPE greater than 12.5 μg mL^{-1}, this can be explained by a decrease in cell number because the concentrations to which they were exposed were cytotoxic and, therefore, reduced cell number. However, there was still a clear decrease in mitochondrial dehydrogenase activity in cells treated with concentrations less than 12.5 μg mL^{-1}. This indicates that although there was an increase in cell number during the incubation time, there was still a significant decrease in mitochondrial dehydrogenase activity which cannot be attributed to cell number alone and may be due to mitochondrial stress, which is closely linked to apoptosis.[110]

From the information discussed so far, it can be determined that Te Urewera propolis is not cytotoxic at concentrations up to and including 12.5 μg mL^{-1}. However, at concentrations between 12.5 μg mL^{-1} and 62.5 μg mL^{-1} and probably higher, TUPE not only inhibits growth but begins to kill cells.

This experiment was unable to determine an exact IC_{50} of TUPE against HL60 cells, but it can be estimated that the IC_{50} of TUPE against HL60 cells lay between 12.5 μg mL^{-1} and 62.5 μg mL^{-1}. This was comparable to results seen in the literature for the treatment of HL60 cells using ethanolic Brazilian propolis extracts which had an IC_{50} of approximately 50 μg mL^{-1}.[84]

Observations of cells during the generation of the trypan blue exclusion dose response curve and data from low concentrations in the MTT assay indicate that apoptosis may be occurring. Results similar to this have been observed in the literature for propolis extracts of other classes as described above in the introduction to this chapter.[109] This is not enough information to conclusively determine that TUPE induces apoptotic cell death in HL60 cells, rather it shows that it induces mitochondrial stress through the decreased activity of mitochondrial dehydrogenase. However, this information indicates that there may be potential in further investigation of the antiproliferative effects of TUPE.

Although it is probable that a range of the components of TUPE are responsible for the antiproliferative effect and cytotoxicity observed in this study, there is one compound which is likely to be responsible for a significant amount of this. Studies have shown that ferruginol is able to induce mitochondrial dependant apoptosis in cancer cells. The literature has shown that ferruginol held an IC_{50} of approximately 3.5 μg mL^{-1} against MDA-T32 thyroidal cancer cell .[111] Based on the information determined in Chapter 5, the concentration of ferruginol in the 12.5 μg mL^{-1} TUPE treatment was 0.40 μg mL^{-1}. The concentration of ferruginol in the 62.5 μg mL^{-1} TUPE treatment was 2.02 μg mL^{-1}. The IC_{50} of the HL60 cells treated with TUPE in this experiment was between these values. This supports the idea that ferruginol is most likely a significant contributor to the cytotoxic effect of TUPE. However, it is worth noting that different cell lines may differ in their responses to the same treatments. To directly compare, the cytotoxic effect of TUPE to that of Luo *et al*[111] would require testing on MDA-T32 cell cultures.

The findings of the current study can be generally explained due to the presence of **2** in TUPE. There are other constituents of TUPE which have also been reported to hold an antiproliferative effect, such as sugiol, communic acid, and **3**.[112, 113] However, the concentration of **2** in TUPE and the nature of cell death observed in this study indicate that **2** is likely the main contributor to these effects.

Chapter 7

Antioxidant Activity of Te Urewera Propolis

7.1 Introduction

Antioxidants are compounds which are able to prevent cellular damage caused by reactive oxygen species (ROS). ROS are produced from a wide range of standard cellular functions in aerobic cells, these are generated in and around the mitochondria during cellular respiration. ROS are key to signalling a range of cellular functions including apoptosis, proliferation, and differentiation. Cells have a range of antioxidative enzymes specifically designed to keep ROS concentrations in balance. However, when ROS production increases beyond the rate that antioxidative enzymes are able to remove them, cellular damage can occur. Primary metabolites such as lipids, nucleic acids, and proteins can react with ROS and can lose function. For this reason, high concentrations of ROS have been shown to cause harmful effects such as cell death and mutations.[114-116]

Propolis extracts from various geographic origins have been shown to have strong antioxidant activity.[7] However, TUPE has not yet been confirmed to demonstrate antioxidant capability. As antioxidants offer protection against cellular damage caused by ROS, it is worthwhile evaluating the antioxidant activity of TUPE.

There are a wide range of methods available for the testing of antioxidant activities. In this experiment the cupric ion reducing antioxidant capacity (CUPRAC) assay was used to assess the antioxidant capacity of TUPE.

The CUPRAC assay is a simple method in which the capacity of antioxidant compounds to reduce bis(neocuproine) copper (II) to bis(neocuproine) copper (I) is assessed. Bis(neocuproine) copper (I) is a chromatophore with a strong absorbance at 450 nm. This reaction is generally completed within 30 minutes and offers fast results. The results of the CUPRAC assay can be standardised through comparison against a Trolox standard.[117]

This experiment aimed to determine the antioxidant capacity of TUPE using the CUPRAC method. It was hypothesised that TUPE would hold some level of antioxidant activity based on the general antioxidant activity of all propolis extracts.

7.2 Methods

7.2.1 CUPRAC Assay

The CUPRAC assay used in this experiment was carried out using a commercially available CUPRAC assay kit (Sigma Aldrich, MAK334). The methods used in this study varied from the manufacturer's instructions only in that the ultrapure water used to make up the Trolox standards was substituted by ethanol (100%, chromatography grade) as this was the solvent in which the TUPE was dissolved.

To a fresh non-sterile flat-bottom 96 well plate Trolox standards (0, 300, 600, & 1000 μM, 20 μL) were added to wells in triplicate. Samples (0.1, 1, 5, 50, 100 mg mL^{-1} TUPE in ethanol (100%, chromatography grade), 20 μL) were plated in triplicate. The commercially provided reaction mixture was added to each well (100 μL). The plate was gently shaken to mix the reagents and incubated (10 min, room temperature). The absorbance of the wells at 570 nm was recorded using a Thermofisher Multiskan GO. No repeats were carried out due to resource limitations.

7.3 Results

7.3.1 Trolox Standard Curve

Figure 7.1 shows a scatterplot of the absorbances of the Trolox standards. This figure shows a very linear increase in absorbance in response to increasing Trolox concentration as expected. The slope from this figure, as $slope_{trolox}$mg mL^{-1}, is used to calculate the total antioxidant capacities of the propolis samples.

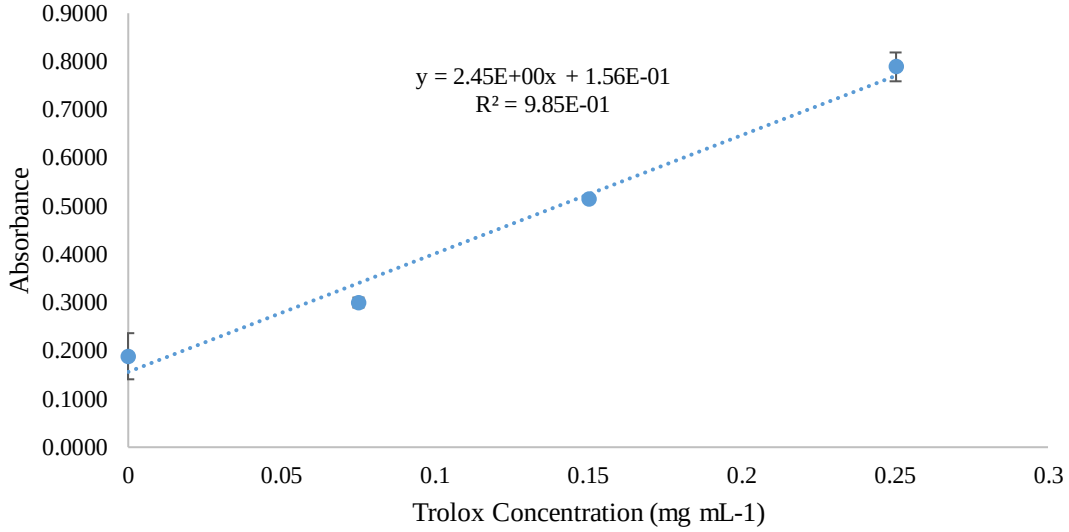


Figure 7.1 CUPRAC generated trolox standard curve. (Error bars showing S.E.M, *n*=1, from triplicate wells.).

7.3.2 TUPE Samples

The total antioxidant capacity of TUPE was calculated using equation 7.1. This was subsequently converted into a Trolox equivalent total antioxidant capacity (TEAC) per gram propolis using equation 7.2.

$$TAC\ (mg_{trolox}\ mL^{-1}) = \frac{(A_{570})_{sample}}{Slope_{trolox}((mg\ mL^{-1})^{-1})} \quad (7.1)$$

$$\frac{[TAC] \times mL}{g_{propolis}} = TEAC_{propolis}(mg_{trolox}\ {g_{propolis}}^{-1}) \quad (7.2)$$

The use of both equations 7.1 and 7.2 were combined to calculate the TEAC of TUPE. The TEAC values generated for each treatment can be seen in Table 7.1. The SEM for all values in this table were calculated using the data from the triplicate wells. The final TEAC of TUPE can also be seen in this table, this was generated by averaging the values

from all concentrations. The final TEAC of TUPE measured using the CUPRAC method was determined to be 1481 ± 145 mg_{trolox} g^{-1}.

Table 7.1 Calculated TEAC of TUPE.

TUPE Concentrations (mg mL^{-1})	TEAC (mg_{Trolox} g^{-1})	SEM (mg_{Trolox} g^{-1})
100	1031	154
50	941	17
5	2412	94
1	1538	316
Final Average TEAC	1481	145

7.4 Discussion

This experiment found that the ethanolic extracts of Te Urewera propolis held very potent antioxidant activity when measured via a CUPRAC assay. This value was comparable to other propolis samples in the literature. Turkish propolis extracts, which are of poplar type have been widely studied using the CUPRAC method.[118, 119]

Ozdal *et al*[119] determined ethanolic extracts of Turkish propolis to be 1184.94 ± 63.27 mg g^{-1} via the CUPRAC method. This Turkish propolis was deemed to be highly antioxidative from this result. Guzelmeric *et al*[118] investigated the antioxidant activity of 47 different Turkish propolis samples and none showed TEAC values of higher than 1019 mg TE g^{-1}. These values are very similar to Te Urewera propolis, which can, therefore, be regarded as highly antioxidative.

As TUPE is a complex mixture of secondary metabolites, it is unlikely that there is a single component in TUPE which is responsible for all of its antioxidant activity. As demonstrated in Chapter 5, TUPE contains high concentrations of diterpenes. Diterpenes, specifically those which have plant origins, have been reported as having antioxidant activity.[120] For this reason, it is probable that the high concentrations of diterpenes observed in TUPE are responsible for a large part of the antioxidant activity observed here.

Specifically, ferruginol has been observed to exhibit a highly potent antioxidant activity due to its ability to scavenge ROS by forming a temporary quinone methide group which eventually leads to the formation of dehydroferruginol, 7-hydroxy ferruginol, and sugiol.[121] Ferruginol was the second most highly concentrated compound identified in TUPE at a concentration of 32.29 ± 1.41 mg g^{-1}. The changes in ferruginol upon exposure to ROS could also help explain the presence of compounds like sugiol in TUPE, Chapter 5. Aside from ferruginol, the labdane diterpene, podocarpic acid, and many of its derivatives have been observed to exhibit antioxidant effects when measured using an ABTS assay.[122]

There were small amounts of flavonoids, such as pinocembrin and galangin, found in TUPE which have reported antioxidant activity in poplar propolis extracts.[23] But due to the low concentrations of these compounds in TUPE, it is unlikely they made any major contribution to the antioxidant activity. If these were somehow the primary antioxidant

compounds in TUPE, the Turkish propolis extracts would have had consistently stronger antioxidant capacity as they had higher concentrations of flavonoids. [118, 119]

As previously described, high concentrations of ROS are harmful to cells. The application of antioxidants such as TUPE has potential to aid in the treatment or alleviation of some diseases, for example diabetes.

The pathogenicity of diabetes stems from the hyperglycaemic induced chronic generation of harmful reactive species such as advanced glycated end products (AGE), methylglyoxal (MGO), and ROS. On top of the direct damage to cells caused by ROS, ROS also induces inflammation in cells exposed to hyperglycaemic conditions. This combination of direct damage from ROS and the inflammation are key contributors to the pathogenic effects of diabetes.[123-125] The potent antioxidative activity of TUPE observed in this experiment offers promising potential for the prevention of diabetic pathogenicity.

Chapter 8

The Effect of Te Urewera Propolis Extract on the Respiratory Burst

8.1 Introduction

Substances which reduce the response of the immune system to potentially harmful stimuli demonstrate anti-inflammatory properties. Anti-inflammatory drugs are used for pain relief and for the treatment of common symptoms of inflammation such as headaches, muscle pain, and period pain relief.[126]

Inflammation occurs through various different processes in the immune system. An example of an inflammatory response can be seen in the activation of granulocytes. When granulocytes are activated by a pro-inflammatory signal, they move chemotactically towards the stimulus and will aim to destroy the perceived threat. The mechanism of this destruction comes from the generation and delivery of superoxide to targets. Superoxide is highly reactive and generates products which are also highly reactive. This reactivity is responsible for the cytotoxic effect of granulocytes against their targets. This process is called the respiratory burst and is a key form of inflammation. [114, 127]

There are many methods available for the measurement of the modulation of inflammatory responses *in vitro*. The most common methods used to measure the inflammatory responses of cell cultures to stimuli involve the quantification of cytokines such as tumour necrosis factor α (TNFα), interleukin 1β (IL-1β), or nuclear factor κB (NFκB) using ELISA or immunofluorescence measurement.[79] However, there are other methods. One such method is the observation of superoxide production in granulocytes during the respiratory burst.

When testing the superoxide production, granulocytes are treated with a range of propolis concentrations alongside exposure to proinflammatory stimuli, caused by phorbol myristate acetate (PMA). This test measures the levels of superoxide produced and can provide insight into the anti-inflammatory effect of the propolis. These superoxide levels can be measured simply by adding water soluble tetrazolium salt one (WST-1), a cell impermeable tetrazolium salt, to the solution and measuring its reduction

colorimetrically.[127] The greater the reduction of WST-1, the greater the concentration of superoxide in the sample. A high concentration of superoxide indicates that inflammation is occurring and a reduced concentration of superoxide indicates that inflammation is being inhibited.

The anti-inflammatory activity of propolis extracts of different classes has been reported in the literature across many studies.[24, 79, 128-131] Extracts from propolis samples originating from Turkey were effective in reducing the respiratory burst and this effect was attributed to the strong antioxidant potential of these samples.[132, 133]

This experiment aimed to measure the effect on the respiratory burst and anti-inflammatory potential of TUPE. This would be achieved through the observation of superoxide concentration in propolis treated activated granulocytes undergoing respiratory burst. It was hypothesised that TUPE may hold anti-inflammatory activity based on the observation that other propolis extracts also hold this property.

8.2 Methods

8.2.1 Differentiation and Preparation of HL60 Cell Cultures

Into a sterile 15 mL falcon tube sterile DMSO (225 μL) and complete IMDM (13.775 mL) were added. To this tube a seed solution of HL60 (P13/14) in fresh complete IMDM (1 mL, 20% FBS) was added to give a final concentration of 15 x 10^5 HL60 cells mL^{-1}. The contents of the falcon tube were transferred to a T75 cell culture flask and incubated (37 ºC, 5% CO_2) for 4 days. After 4 days the media was refreshed with 1.5% DMSO in complete IMDM (20% FBS). The culture was incubated (37 ºC, 5% CO_2) for a further 4 days to allow complete differentiation of HL60 cells into mature granulocytes.

Before the cells were added to the superoxide assay their viability was checked. A minimum of 95% viability determined through trypan blue exclusion methods was required for the experiment to continue. The media was removed from the cell culture solution by centrifugation (700 rcf, 5 min) and the cells were resuspended in Hank's balanced salt solution (HBSS) (5 mL). This cell solution was centrifuged (700 rcf, 5 min) again to form a cell pellet and to remove HBSS. This cell pellet was resuspended in an appropriate volume of HBSS to give a concentration of 5.5 x 10^4 cells mL^{-1}. The resultant solution was the prepared differentiated cell solution.

8.2.2 Superoxide Assay of Differentiated HL60 Cells Treated With TUPE

Hank's balanced salt solution (HBSS) (49 μL) and TUPE (1 μL) were plated in sextuplicate. The concentration of the TUPE ranged from 50 mg mL^{-1} to 0.01 mg mL^{-1} across 11 concentrations. Superoxide dismutase enzyme (SOD) in HBSS (49 μL, 50 μg mL^{-1}) and ethanol (1 μL, 100%, chromatography grade) were plated in triplicate as a positive control of superoxide inhibition. HBSS (50 μL) was plated in triplicate as a negative control. HBSS (60 μL) was added to three of each of the propolis containing wells to serve as background controls. The prepared differentiated cell solution (50 μL) was added to all wells excluding the background control wells. The plate was incubated (15 min, 37 ºC, 5% CO_2). PMA in DMSO (5 mg mL^{-1}) diluted to 80 μg mL^{-1} in HBSS was added (2.5 μL) to each cell containing well. WST-1 (7.5 μL, as supplied by the manufacturer) was added to each cell containing well. The absorbances of the wells at 450 nm was recorded every 2 minutes at 37 ºC for 30 minutes. A normalising reference reading was taken at 630 nm with each measurement to later remove to account for interfering absorbances.

8.3 Results

The data was first checked to determine if there was a significant difference in relative superoxide concentrations between the negative and positive controls in this experiment. The positive control was shown to have significantly less superoxide than the negative control, but so did all of the treatments excluding the lowest concentration of TUPE, Figure 8.1.

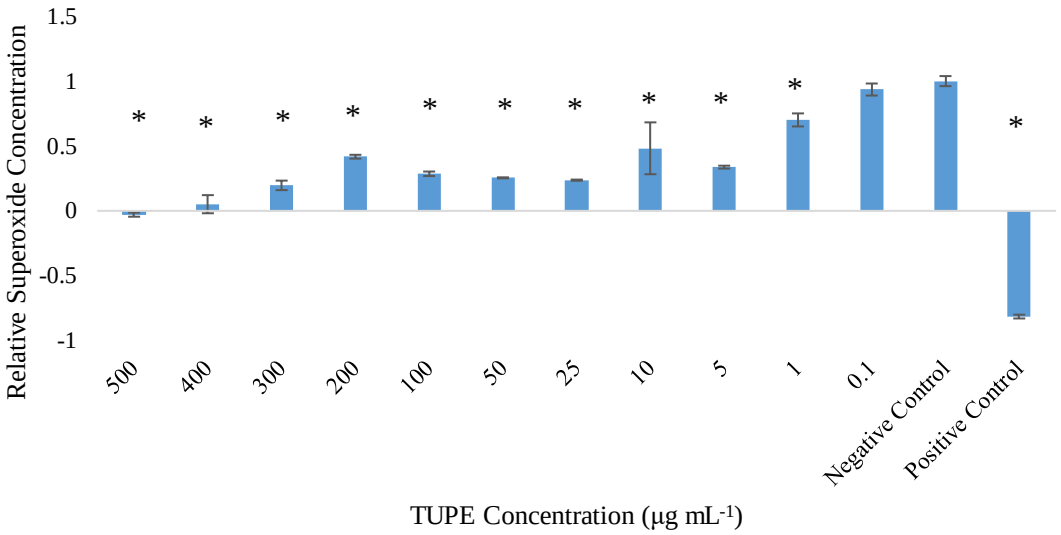


Figure 8.1 Comparison of superoxide concentrations across varying concentrations of TUPE. (Error bars showing ±S.E.M. * indicates $p<0.05$ compared to the control using Student's t test. $n=2$ plated in triplicate).

Figure 8.2 shows the levels of superoxide inhibition in relation to the concentration of TUPE. The values in Figure 8.2 were calculated by subtracting the normalising reference absorbance at 630 nm from the absorbance at 450 nm, and further subtracting the blanks from the samples. The values in this figure are given as percentages where the negative control was assumed to not inhibit superoxide production at all (0% inhibition) and the positive control was assumed to completely inhibit superoxide production (100% inhibition).

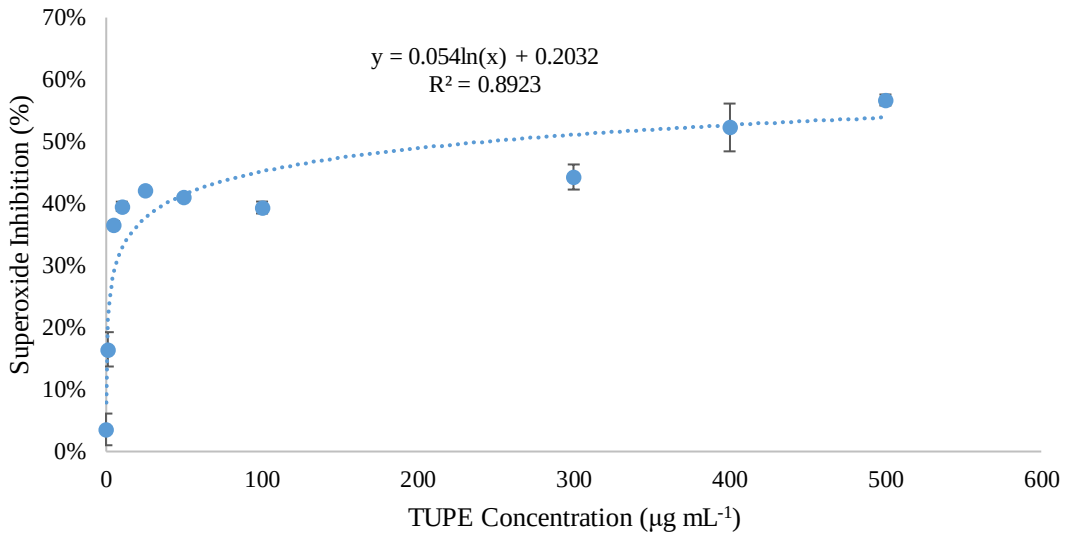


Figure 8.2 Superoxide inhibition by TUPE. (Error bars showing SEM, n=2 plated in triplicate, trendline equation presented in decimal value rather than percentage).

TUPE inhibited superoxide production with a logarithmic trend. Using the equation generated for this graph, the IC_{50} of TUPE on the generation of superoxide in differentiated HL60 cells undergoing respiratory burst was found to be approximately 243 µg mL^{-1}. This was calculated by simply taking the equation for the trendline seen in Figure 8.2 and substituting y with 0.5 and solving for x where x is equal to the IC_{50}.

8.4 Discussion

This experiment first confirmed that the positive control of SOD was able to reduce the concentration of superoxide in statistically significant manner ($p<0.05$). This was critical for the study as the IC_{50} determination was calculated using data relative to the difference between the positive and negative control, Figure 8.1.

Following the validation of the controls, TUPE was shown to inhibit superoxide production in HL60 cells undergoing respiratory burst in a dose dependant, logarithmic trend, Figure 8.2. From this data the IC_{50} of superoxide production was found to be approximately 243 $\mu g\ mL^{-1}$.

This study found TUPE to have a statistically significant ($p<0.05$) inhibitory effect on superoxide production in cells undergoing respiratory burst by measuring the reduction of WST-1. However, although the data is significant, this does not confirm that it inhibits the production of superoxide in the cells. Instead, when the data present in Chapter 7 is taken into consideration, it is possible that TUPE is directly acting on the superoxide produced by the cells. TUPE has been shown to scavenge ROS; superoxide is an example of an ROS. While this information complicates interpretation of the data collected in this chapter, it does not make it redundant. If TUPE is reducing superoxide levels by acting as an antioxidant directly on superoxide, this indicates that it is also carrying out an anti-inflammatory role. By destroying superoxide produced from the respiratory burst, the cytotoxic effect which causes the destruction of inflammatory targets and subsequent proliferation of inflammation is reduced.

Even with this confounding effect caused by direct antioxidant potential, it is possible that TUPE may still be reducing the initial production of superoxide. The inhibition of superoxide by TUPE observed in this experiment occurred in a logarithmic dose dependant manner. While the total antioxidant capacity (TAC) observed in Chapter 7 was dose dependant in a linear manner, the difference in trends may be explained by the cumulative effect of TUPE both inhibiting superoxide production as well as destroying superoxide produced. If the positive control was not able to inhibit superoxide production any more than the most concentrated TUPE treatments, i.e., the concentrations with inhibition values nearing the asymptote of the logarithmic trend, the observation of a logarithmic trend could simply be explained by total depletion of superoxide in the cell solutions at high concentrations of TUPE. However, because the positive control held an

inhibition level much greater than the asymptote of the TUPE treatments, this must not be true. Therefore, the logarithmic trend observed is true, rather than the trend being linear until a threshold of total superoxide depletion is met.

This data also could potentially be explained by the cytotoxicity of TUPE observed in Chapter 6. The increase in superoxide inhibition observed in this experiment could instead be a decrease in superoxide production caused by the destruction of the cells by TUPE. However, this is most likely not a true explanation for the trends observed in this experiment. Because superoxide production was still inhibited at concentrations lower than those observed to be cytotoxic in Chapter 6, the superoxide inhibition cannot therefore be explained by a cytotoxic effect.

The logarithmic response in superoxide inhibition observed in this experiment is similar to those seen in studies on Turkish propolis extracts. These studies on poplar propolis used a different line of cells, a different method for measuring superoxide concentrations, and a different propolis sample. However, the results were very similar in that superoxide concentrations were reduced in a logarithmic trend in response to propolis concentration. The superoxide inhibitory activity of Turkish poplar propolis might also potentially be caused by direct antioxidant action as discussed here.[132]

Chapter 9

Antibacterial Activity of Te Urewera Propolis Extracts

9.1 Introduction

All propolis exhibit antibacterial properties.[26, 32, 134-136] For bees, the antibacterial property of propolis is arguably the most useful of all of its biological properties. Bees are able to embalm hive intruders effectively due to the antibacterial property of the propolis they use. The propolis laid by bees in the entryway to hives disinfects the legs of bees through its antibacterial property, Figure 9.1. Without this, there would be a much greater chance that colonies would become sick through infection.



Figure 9.1 Propolis coated entry to beehive.[137]

The antibacterial activity of ethanolic extracts from poplar propolis are well documented. The flavonoid component of poplar propolis is mainly responsible for this effect.[24, 34] TUPE has a different chemical composition to other propolis classes. Therefore, even though propolis is considered to be universally antibacterial in nature, the antibacterial properties of TUPE need to be confirmed. The current research has shown that TUPE propolis contains various potentially antibacterial compounds, specifically some antibacterial diterpenes were found.

There are a range of methods available for measuring the antibacterial activity of propolis extracts. The most common in the literature is the broth dilution method, which involves culturing bacteria in a range of liquid media containing the treatment in various concentrations.[24, 32, 138] However this is not the only method available. The method selected for use in this experiment was the agar well diffusion method.

The agar well diffusion method for measuring antibacterial activity has been used extensively in the research of apicultural products and was used by Allen *et al*[139] to survey the antibacterial activity of various Aotearoa honeys which eventually led to the foundation of the mānuka honey industry. Agar well diffusion measures the zone of inhibition of bacterial growth in inoculated agar around a well containing a solution of interest. This method has previously been used for propolis samples, however, it is uncommon, possibly because it relies on diffusion through the agar and the propolis is dissolved in ethanol whereas the agar gel is water-based.[140] The agar disc dilution method is similar to the well dilution method, this method loads the liquid sample onto a disc rather than into a well. It is much more common within the literature for propolis research than the agar well diffusion method.[135, 141] It is worth noting that there are a number of problems associated with the well diffusion assay. This includes issues around defining where the zone of inhibition ends due to gradual fading of the cell culture, error in reading the callipers, and edge effects from the plate. In this experiment, measures such as the application of digital callipers, and well randomisation were applied to reduce the effect of these issues.

This study aimed to investigate the antibacterial activity of TUPE against two strains of *S. aureus* using the agar well diffusion method.

9.2 Methods

9.2.1 Revival of Bacterial Stocks

The bacterial stocks used in this experiment were obtained as cryopreserved beads with the bacteria on the surface. A single bead was transferred and streaked onto a nutrient agar plate. The agar plate was transferred into secondary containment and left to incubate (overnight, 37 ^{o}C). An inoculation hoop was used to subclone the bacteria onto a new agar plate as single colonies. The new agar plate was placed inside secondary containment and left to incubate (overnight, 37 ^{o}C). The plate was observed to see if single colonies had formed. If single colonies had formed the plate was transferred to a Tupperware™ container and refrigerated (2 – 4 ^{o}C, maximum of four days). This process was carried out for both the MRSA4549 *S. aureus* and MSSA87 *S. aureus* strains.

9.2.2 Preparation of Inoculated Agar

Nutrient broth agar was prepared according to manufacturer's instructions, made into 150 mL aliquots using an A grade 200 mL measuring cylinder, and autoclaved. The aliquots were stored at 55 ^{o}C.

Inoculum from a previously isolated single bacterial colony was collected using an inoculation hoop. The inoculation hoop was used to inoculate tryptic soy broth (10 mL). The tryptic soy broth was incubated with shaking (overnight, 37 ^{o}C). The absorbance of the inoculum solution was measured using a WPA CO 8000 Portable Cell Density Meter and diluted until an absorbance of 0.5 was observed at 490 nm.

The previously aliquoted nutrient agar broth was cooled to 50 ^{o}C. Inoculum solution (100 μL) was added to the aliquoted nutrient agar broth. The nutrient agar broth was shaken gently to ensure homogenous inoculation. The inoculated agar was poured into a large square assay dish (245 mm x 245 mm) on a level surface and left to set at room temperature. Once the agar had set, the plate was transferred to a refrigerator and inverted. This process was completed to give 3 plates each of inoculated agar for both MRSA4549 *S. aureus* and MSSA87 *S. aureus*.

9.2.3 Treatment and Measurement

64 holes (16 x 4) were punched into each agar plate using a cork borer (10 mm diameter). 100 μL of treatment was added to each well using a pipette, the locations of the treatments on the plate were randomised. The treatments used included a negative control of sterile water, phenol standards (2-6% w/v phenol in 10 % v/v aqueous ethanol) or samples (250-1250 μg mL^{-1} TUPE in 10% aqueous ethanol)[†]. Each treatment was plated in duplicate. The plates were left to incubate (18 hours, 37 ^{o}C). The plates were removed from the incubator and inverted. The diameter of the zone of inhibition around each well was measured twice using a digital calliper and recorded. This was repeated for all of the plates previously generated providing a final count of 3 plates for each *S. aureus* strain with each well plated in duplicate.

[†] The same solvent was used in the preparation of both standards and samples to mitigate solvent effects upon diffusion.

9.3 Results

All three MSSA87 *S. aureus* plates gave clear zones of inhibition with definite edges. These plates were easy to measure with digital callipers and were able to provide high quality data, Figure 9.2. Of the three MRSA4549 *S. aureus* plates generated, only one was able to provide useful data. The agar in two of the MRSA4549 *S. aureus* plates generated was visibly not level, these plates were discounted as they were unable to provide quality data.

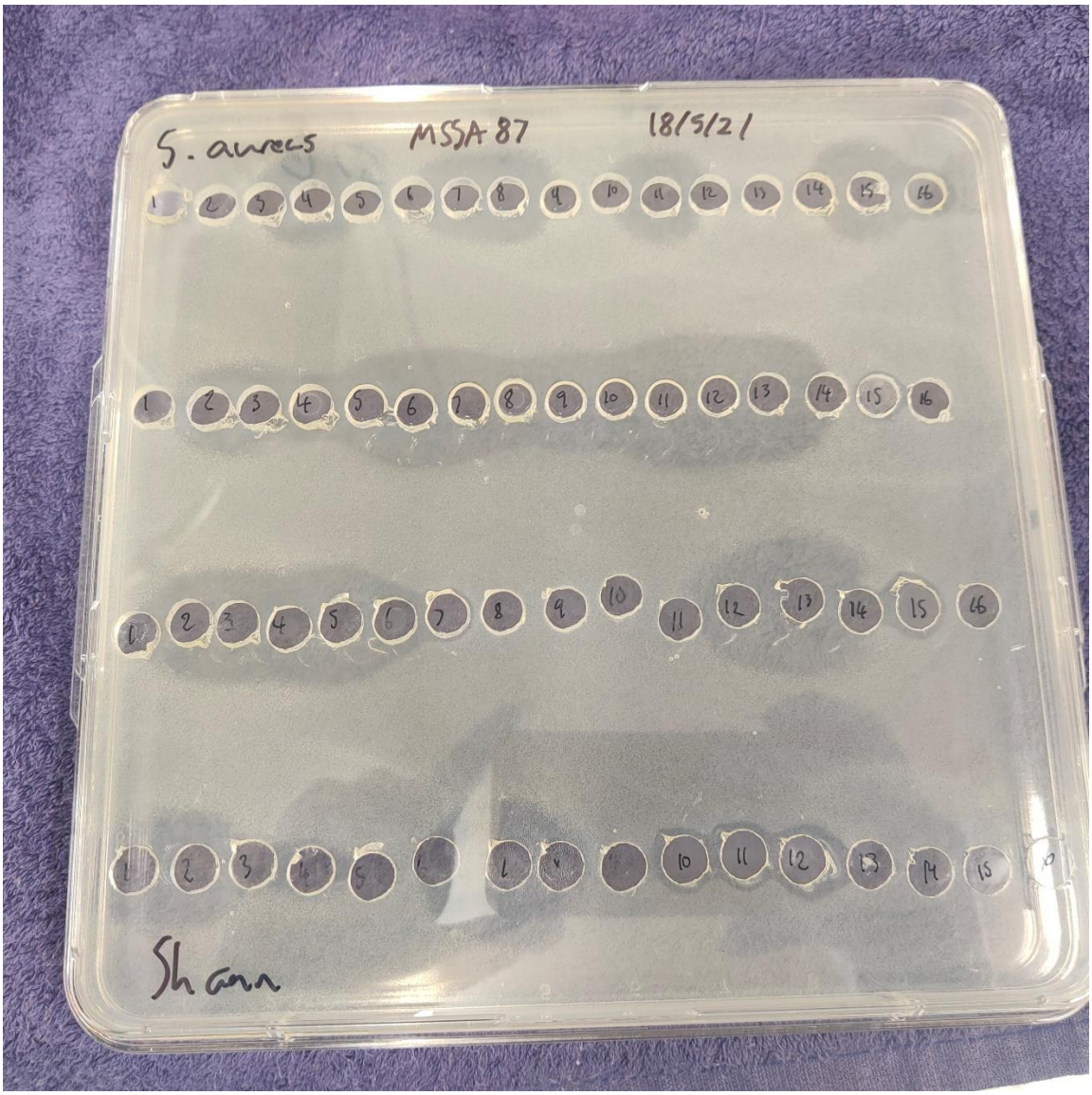


Figure 9.2 MSSA87 *S. aureus* minimum zone of inhibition plate.

A scatterplot showing the diameter of the zones of inhibition for the phenol standards against MSSA87 *S. Aureus* is shown in Figure 9.3. A positive dose dependant response

was exhibited by the diameter of the zone of inhibition relative to the phenol concentration.

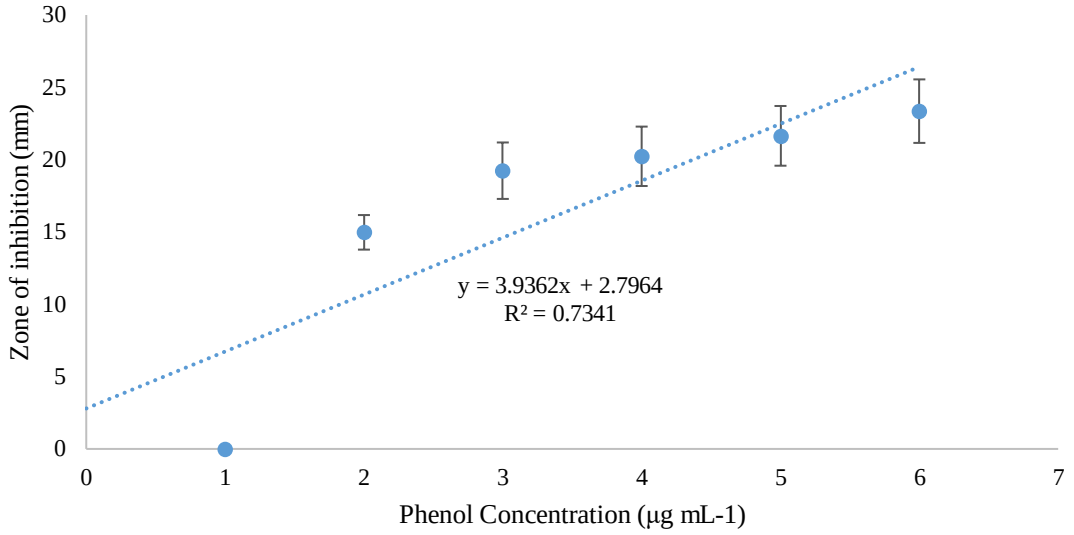


Figure 9.3 Diameter of zone of inhibition caused by phenol standards against MSSA87 *S. aureus.* (Vertical Error bars showing ±S.E.M, n=3, in duplicate, zone of inhibition presented as the diameter of the inhibitory region minus the diameter of the hole).

A scatterplot showing the diameter of the zones of inhibition for TUPE against MSSA87 *S. Aureus* is shown in Figure 9.4. A positive dose dependant response was shown by the diameter of the zone of inhibition relative to TUPE concentration.

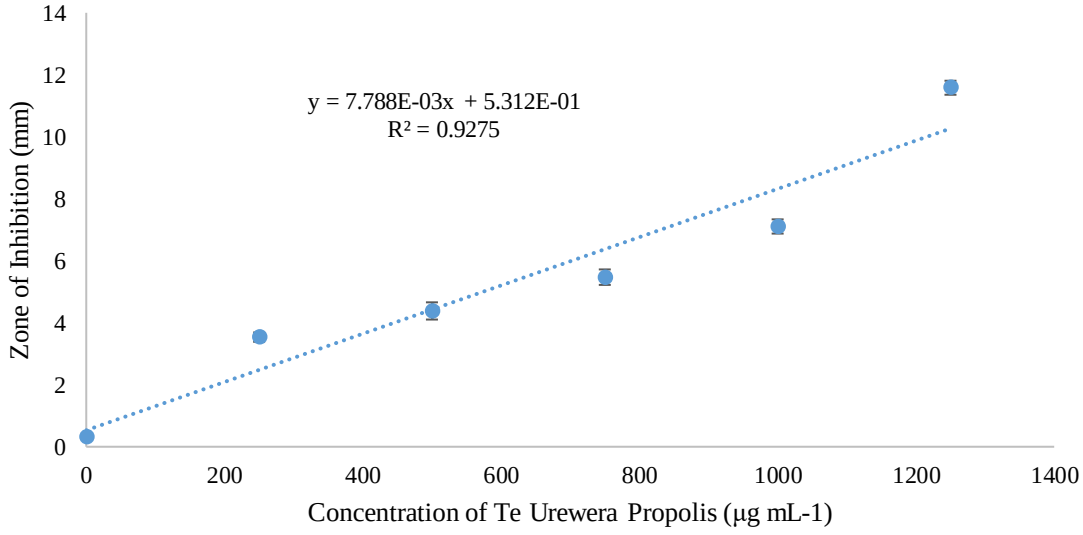


Figure 9.4 Diameter of zone of inhibition caused by TUPE Against MSSA87 S. aureus. (Error bars represent the S.E.M, *n*=3, in duplicate, zone of inhibition presented as the diameter of the inhibitory region minus the diameter of the hole).

A scatterplot showing the diameter of the zones of inhibition for the phenol standards against MRSA4549 *S. Aureus* can be seen in Figure 9.5. This figure showed a positive dose dependant response in the diameter of the zone of inhibition relative to phenol concentration.

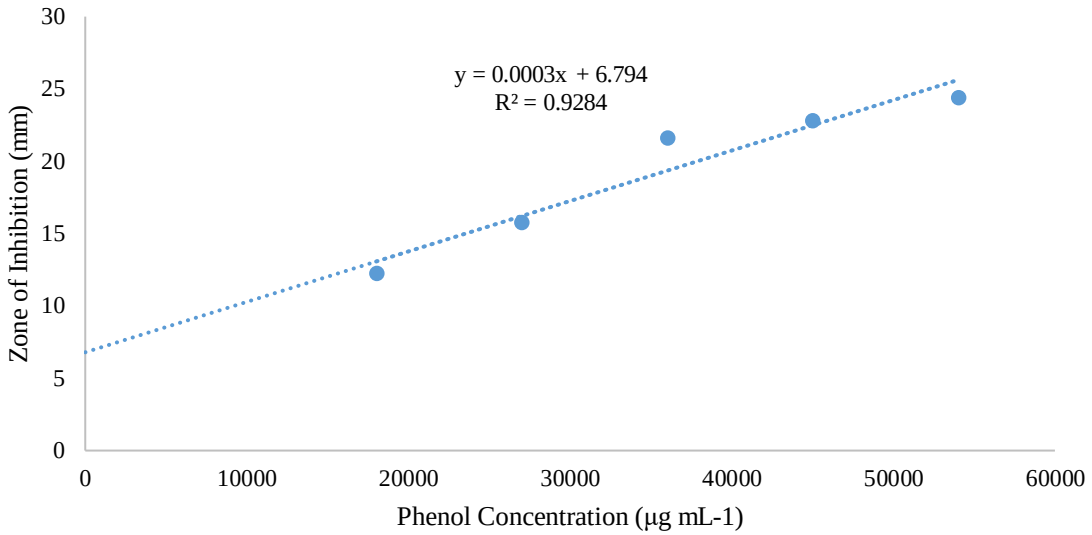


Figure 9.5 Diameter of zone of inhibition caused by phenol standards against MRSA4549 S. aureus. (*n*=1, zone of inhibition presented as the diameter of the inhibitory region minus the diameter of the hole).

A scatterplot showing the diameter of the zones of inhibition for TUPE against MRSA4549 *S. Aureus* can be seen in Figure 9.6. This figure showed a positive dose dependant response in the diameter of the zone of inhibition relative to Te Urewera propolis concentration.

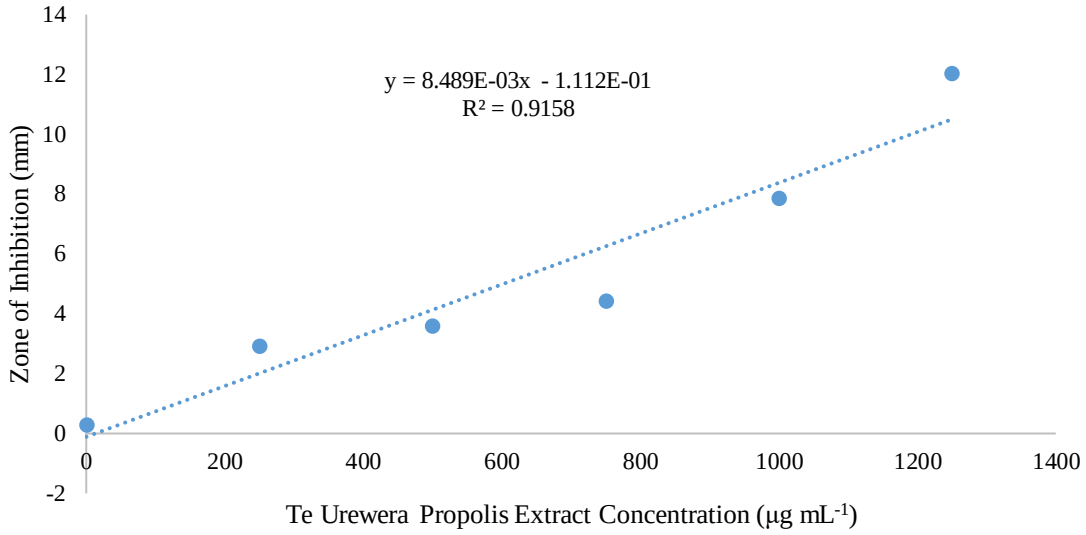


Figure 9.6 Diameter of zone of inhibition caused by TUPE Against MRSA4549 S. aureus. (*n*=1, zone of inhibition presented as the diameter of the inhibitory region minus the diameter of the hole).

9.3.1 Phenol Equivalent Antibacterial Activities

From the slopes observed in Figure 9.3 – 9.6, the phenol equivalent antibacterial activities can be calculated from the slopes, Equation 9.1.

$$Phenol\ Equivalent\ Antibacterial\ Activity = \frac{mm_{diameter}\left(\mu g\ mL_{sample}{}^{-1}\right)^{-1}}{mm_{diameter}\left(\mu g\ mL_{phenol}{}^{-1}\right)^{-1}} \quad \text{(Eq. 9.1)}$$

The relevant information for use in Equation 1 and the resultant phenol equivalent antibacterial activities of Te Urewera propolis extract against the two strains of *S. aureus* tested are summarized in Table 9.1.

Table 9.1 Antibacterial activity values.

S. aureus Strain	Treatment	mm (µg mL^{-1})$^{-1}$	Phenol Equivalent Antibacterial Activity
MSSA87			
	Phenol	2.432x10^{-4}	
	Te Urewera Propolis Extract	7.788x10^{-3}	32.02
MRSA4549b			
	Phenol	3.487x10^{-4}	
	Te Urewera Propolis Extract	8.489x10^{-3}	41.11

9.4 Discussion

The ethanolic extract of Te Urewera propolis is able to inhibit the growth of the two *S. aureus* strains tested in a dose dependant manner. This indicates there is potential for the use of ethanolic Te Urewera propolis in a clinical setting, after further research. This finding agrees with the generalisation found in literature that ethanolic extracts of propolis are antibacterial.[24, 34, 134]

There is a lot of literature explaining the antibacterial activity of poplar propolis[24]. But as Te Urewera propolis is significantly different in chemical composition to poplar propolis, this information is not applicable to this sample. Te Urewera propolis is different to, but still most similar in composition to Greek propolis. However, there has only been one study carried out on the antibacterial activity of Greek propolis extract. Unfortunately, the samples used in that study did not contain high diterpene concentrations, and are not comparable.[28] The agar well diffusion method has been used before on other propolis samples but there was no attempt to standardise the response against a control such as phenol.[140] Therefore, the results are again not comparable.

Some of the components of Te Urewera propolis identified in Chapter 5 have been previously reported to have antibacterial activity. A notable constituent of Te Urewera propolis with antibacterial activity is Ferruginol. Unfortunately, due to differences in methodology, the literature antibacterial activity of ferruginol could not be compared to the antibacterial activity of TUPE determined in the present study.[142]

Chapter 10
Concluding Remarks

10.1 Conclusions

10.1.1 Extraction Method Development

This study described the development of a method to produce high quality dry propolis extracts which were suitable for use in *in vitro* cell systems. A seven day ethanolic maceration procedure was deemed suitable for the extraction of the biologically active compounds present from the crude sample. The coextracted undesired wax portion was able to be removed via exposing the maceration solution to chilled (-10 ^{o}C) centrifugation. This method of wax removal from ethanolic propolis extracts had not yet been described in the literature to the best of the author's knowledge. The TUPE collected using this method was of high enough quality that it was deemed sufficient for use throughout the rest of the study.

The data obtained from GCMS analysis of TUPE and a sample of commercial propolis which was extracted in parallel confirmed that TUPE was distinctly different from poplar class propolis.

10.1.2 Chemical Characterisation

Chemical characterisation of TUPE through GCMS and LCMS methods confirmed that TUPE was a new class of propolis. TUPE was found to hold a high concentration of diterpenes. When compared to the literature there were few other classes of propolis with such high diterpene concentrations, none of which contained the same specific diterpenes at similar concentrations. The two primary constituents of TUPE were determined to be the labdane diterpenes torulosol (38.5 mg g^{-1}) and ferruginol (32.2 mg g^{-1}).

10.1.3 Antiproliferative Effect of TUPE

These experiments found firstly that the final concentration of 1% ethanol, which was required to bring Te Urewera propolis extract into solution, did not render the cells used in this experiment unviable. TUPE was found to be cytotoxic and antiproliferative at

concentrations greater than 12.5 μg mL^{-1}. Some evidence was also found which indicated that Te Urewera propolis extract may induce apoptotic cell death rather than necrotic cell death at cytotoxic concentrations. The induction of apoptosis by the extract could not be confirmed using the methods utilised in this study. Further study would be required to confirm this.

10.1.4 Antioxidant Effect of TUPE

TUPE was determined to hold potent antioxidant activity using the CUPRAC method. This study found TUPE to have a TEAC of 1487 ± 338 TE (mg_{Trolox} g^{-1}). This TEAC was high in comparison to literature TEAC values for other propolis classes such as poplar. Further research carried out on this promising antioxidant could include a lipid peroxidase assay and the common DPPH assay. This indicated that TUPE may have use in the treatment diseases which cause oxidative stress, such as diabetes. Further study is recommended to determine the effect of TUPE on diabetes.

10.1.5 Superoxide Inhibitory Effect of TUPE

The effect of TUPE on the superoxide concentrations of solutions of cells undergoing respiratory burst was tested. It was found that TUPE was able to decrease superoxide levels in the cell solutions at a statistically significant level ($p<0.05$). This inhibition occurred in a logarithmic dose dependant manner. However, the results of the experiment were unable to conclusively determine how this superoxide concentration was reduced due to potential interference caused by the antioxidant capacity of TUPE alone. Further testing *in vitro* was recommended before any wider conclusions about the anti-inflammatory effect of TUPE were drawn.

10.1.6 Antibacterial Effect of TUPE

The antibacterial effect of ethanolic Te Urewera propolis extracts using the agar well diffusion method on *Staphylococcus aureus* was tested. It was found that Te Urewera propolis extracts did have a dose dependant antibacterial effect against two strains of *S. aureus*, MSSA87 and MRSA4549.

10.2 Recommendations for Future Work

10.2.1 Chemical Composition of TUPE

This study identified TUPE extracted from Te Urewera propolis batch one to be a new class of propolis. However, batches two and three of Te Urewera propolis were found to be of standard poplar composition. Batch one was collected near the heart of Te Urewera while the others were collected from the periphery. It is recommended that more samples of propolis are sourced from Te Urewera from a range of hives spread throughout. GCMS analysis of extracts of the propolis samples could provide geographical information on what locations within Te Urewera will produce the unique propolis identified in this study.

10.2.2 Antiproliferative Effect of TUPE

Following on from the information deduced from this study, there is one obvious area which may benefit from more investigation. The antiproliferative experiment collected some information which indicated that TUPE might be inducing apoptotic cell death at high concentrations rather than necrosis. If Te Urewera propolis extracts were confirmed to induce apoptotic cell death, there may be potential for the extract to be used in cancer treatment, since the induction of apoptosis is one of the primary targets for anticancer therapies.[143]

A simple method to determine whether or not the cells are undergoing apoptosis rather than necrosis is to carry out a lactate dehydrogenase (LDH) assay. The LDH assay detects compromised cell membranes in cell solutions. In necrotic cell death, the cell membrane is compromised whilst in apoptotic cell death, the cell membrane is not compromised.[144] An LDH assay could be carried out to determine if there is difference in the integrity of cell membranes between cultures exposed to high concentrations of Te Urewera propolis extract, compared to low concentrations or a control. If no difference is observed, this could provide good evidence of the induction of apoptosis. An LDH assay was originally planned for this study. However, due to a malfunction in the freezer which contained the LDH assay this could not be completed.

Other methods for determining the method of cell death of HL60 cells exposed to Te Urewera propolis extracts could include enzyme-linked immunosorbent assay (ELISA) analysis of apoptotic proteins, or the use of flow cytometry.[144]

The antiproliferative effect of Te Urewera propolis could also be potentially developed into an anticancer effect through other research. This would include the determination of the cytotoxicity and antiproliferative effects of the extract on non-cancerous cell cultures. This data could then be compared to the cancerous HL60 cell culture used in this experiment. *In vivo* testing in animal models with cancer would also provide very strong information on the potential anti-cancer effects of this sample.

10.2.3 Antioxidant Effect of TUPE

Although the results from this study indicated that Te Urewera propolis holds a strong antioxidant activity using the CUPRAC method, this study has not completely characterised the antioxidant activity of the sample. Future research on the antioxidant activity of Te Urewera propolis should include the use of other antioxidant assays. The CUPRAC assay is one method available for determining the antioxidant activity of a solution. This method measures the free radical scavenging potential of samples. However, there are other antioxidant assays available which instead look at the ability of samples to prevent lipid peroxidation. While the ability to protect against free radicals directly is potentially indicative of useful antioxidant activity in cells, lipid peroxidation assays directly measure useful antioxidant activity. The thiobarbituric acid assay is an example of a lipid peroxidation assay which could be carried out.[145]

There would also be some benefit to be gained from running another free radical scavenging assay on TUPE. If this sample was analysed via the DPPH assay, the values of these could be compared to further validate the value generated from the CUPRAC assay. The DPPH assay is also very common within the literature for the determination of the antioxidant activity of propolis samples.[7, 146, 147]

If a lipid peroxidation assay, such as the thiobarbituric acid assay, were also to be carried out on ethanolic TUPE, a comprehensive image of the antioxidant activity could be determined. If another, more common, free radical scavenging assay such as the DPPH assay were to be carried out the antioxidant activity of TUPE could be compared to a wider range of propolis samples.

10.2.4 Superoxide Inhibitory Effect of TUPE

While this experiment did show that TUPE decreases superoxide levels in the cell solution of cells undergoing respiratory burst, there are still more questions to be answered. The potential confounding of data in this experiment caused by the antioxidant activity of TUPE observed in Chapter 7 makes interpretation of the mechanism difficult.

To gain more information on the anti-inflammatory effect of TUPE other anti-inflammatory assays should also be run, such as ELISA and immunofluorescence testing. It is the recommendation of this author that other methods for the analysis of anti-inflammatory effects be carried out before any significant conclusions be drawn about the wider effects of TUPE on inflammation *in vitro*. As always, it is also recommended that if further *in vitro* testing proved promising, *in vivo* experiments be carried out.

10.2.5 Antibacterial Activity of TUPE

Although the antibacterial results obtained in this study were strong indicators of antibacterial activity, they were not comprehensive. Firstly, to expand on the findings from this study, more *in vitro* studies could be carried out. This study had difficulty finding literature with which to compare data. The same method could be carried out but instead with a wide range of propolis extracts to give direct comparison of efficacy. It was originally planned for this study to include ethanolic poplar propolis extracts from Taihape as samples. However, due to a combination of logistical and time issues this could not be completed.

Alternatively, the lack of comparable studies could be overcome by using a different method. There is extensive data on the antibacterial effect of propolis extracts using the broth dilution method which could provide direct comparison.

In vitro testing of Te Urewera propolis extracts could also be used to potentially identify the contributions of each constituent. Chromatographic fractionation of Te Urewera propolis extracts could be carried out. These fractions could be tested to gain more information on which are responsible for antibacterial activity . It is worth noting though that for other propolis samples these studies generally are unable to pinpoint any molecules directly responsible for the activity. This has been assumed to be due to the constituents of other propolis classes working synergistically.[34] Even if this were also the

case for Te Urewera propolis, this would still provide useful information on the mechanisms behind this activity.

By determining the antibacterial activity of ethanolic Te Urewera propolis extracts against a wide range of bacteria there is potential that trends may arise. It had been observed that poplar propolis is more effective against gram positive bacteria than it is against gram negative bacteria.[25, 32, 33] This led to the discovery that it is probable that the antibacterial activity of poplar propolis is cell wall dependant and in general gave insight into the mechanism of the activity. If trends such as this were observed for Te Urewera propolis extracts, there would be potential for insight into the mechanisms of its antibacterial activity. Any insight into these mechanisms would prove useful for eventually promoting Te Urewera propolis extracts as clinical antibiotics.

In vivo experiments would need to be carried out before TUPE could be used in a clinical setting. These experiments would first need to aim to gather information on the pharmacokinetics of TUPE. This would provide information necessary to determine if the substance has use in whole organisms. Following this, it could provide useful information about delivery methods and areas in which to look for side effects. Following this, *in vivo* experimentation could include treatment of animal models in an induced diseased state which *in vitro* experiments indicate may be affected by Te Urewera propolis. An example of this could be in a rodent model with an induced *S. aureus* infection. This would provide direct evidence if the substance is useful for treating infections which would likely be the main use in a clinical setting. This could also give insight into any potential immunomodulatory effects.

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Appendix

Appendix A

