The Antibacterial Activity of *Humulus lupulus* against Mycobacteria

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By

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List of abbreviations

Commonly used abbreviations within this study are listed in alphabetical order below:

ANOVA	Analysis of variance
atm	Atmospheres
bTB	Bovine tuberculosis
C	Catechin
CCC	Counter current chromatography
CFU	Colony forming units
cfu/mL^{-1}	Colony Forming Units per millilitre
CO_2	Carbon dioxide
diH_2O	Deionised water
DNA	Deoxyribonucleic acid
EC	Epicatechin
ECG	Epicatechin gallate
EGCG	Epigallocatechin gallate
FDA	Food and Drug Administration
Fe	Iron
FIC	Fractional Inhibitory Concentration
g	gram
h	hour
H_2	Hydrogen
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid

HPA	Health Protection Agency
HPLC	High performance liquid chromatography
L	litre
LB	Lysogeny broth
LC-MS	Liquid chromatography – Mass Spectrometry
M	Molar
MDR-TB	Multidrug Resistant Tuberculosis
mg	Miligrams
MIC	Minimum inhibitory concentration
min	minute
mL	millilitre
MRSA	Methicillin-resistant *Staphylococcus aureus*
MS	Mass Spectrometry
MTB	*Mycobacterium tuberculosis*
MTC	*Mycobacterium tuberculosis* complex
N/A	Not available
n	Number of replicates
N_2	Nitrogen
NaOH	Sodium hydroxide
NB	Nutrient Broth
NCIMB	National Collection of Industrial, Food and Marine Bacteria
NCTC	National Collection of Type Cultures
nm	nanometre

NMR	Nuclear magnetic resonance
NP	Not Present
NTM	Non tuberculosis mycobacteria
O_2	Oxygen
OD	Optical density
p	Value of significance
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
ppb	Parts per billion
ppm	parts per million
PSI	Pounds per Square Inch
r	r-value (Pearson product-moment correlation coefficient)
r^2	Coefficient of determination
RF	Retardation Factor
RNA	Ribonucleic acid
SD	Standard deviation
SDW	Sterile Distilled Water
SE	Standard error of the mean
sec	second
SEM	Scanning electron microscope
SF	Supercritical Fluid
SFE	Supercritical Fluid Extraction
TB	Tuberculosis

TEM	Transmission electron microscope
TLC	Thin layer chromatography
TPTZ	2,4,6-tris(2-pyridyl)-1,3,5-triazine
TSA	Tryptone soya agar
TSB	Tryptone soya broth
UHW	University Hospital of Wales
UK	United Kingdom
USA	United States of America
UV	Ultraviolet
Vis	Visible
v/v	Volume per volume
w/v	Weight per volume
w/w	Weight per weight
WHO	World Health Organisation
× *g*	Gravitational force (Relative centrifugal force)
°C	Degrees Celsius
µg	Micrograms

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Abstract

One third of the world's population is estimated to be infected with *M. tuberculosis*, a pathogen which causes more human death and misery than any other bacterial disease. Whilst treatment is available, resistance to commonly used antimicrobials is a growing problem. Thus there is an urgent need to identify new compounds that can kill drug resistant isolates and are able to potentiate the activity of currently available antibiotics.

The plant kingdom is a rich source of antibacterial compounds and a plant which has attracted particular interest is *Humulus lupulus*, more commonly known as the hop, which has been used as an antibacterial in beer for hundreds of years. Its antibacterial properties are thought to be due to the combined action of alpha and beta acids and polyphenols such as xanthohumol although the precise nature of their interactions and relative importance has yet to be determined.

An optimised agar antimicrobial assay was developed and employed based on *Mycobacterium smegmatis*, to characterize the antibacterial activity of fifty commercially available hop varieties with a view to identifying novel antibacterial compounds.

Surprisingly, no correlation was found between alpha and beta acid content and antibacterial activity. Chemical analysis of the most (Citra) and least (Galena) active hop variants using a combination of bioactivity based thin layer chromatography, mass spectrometry and HPLC revealed differences in the relative amounts of antimicrobial compounds such as humulone (alpha acid), lupulone (beta acid) and xanthohumol but failed to identify the presence of novel antibacterial compounds.
Whilst no new antimicrobial compounds were identified, the Citra hop extract was able to potentiate the activity of the antibiotics imipenem and ciprofloxacin against clinical isolates of *M. abscessus*, a fast growing member of the mycobacterium family which infects individuals suffering from cystic fibrosis. The Citra hop extract also inhibited the growth drug resistant isolates of *M. abscessus* suggesting that it may have activity against other antibiotic resistant mycobacteria such as *M. tuberculosis*

With regards to the mode of action, scanning electron microscopy revealed distinct changes to the outer cell structure of the bacteria, suggesting that hops contain compounds that interact with the bacterial cell membrane and/or cell wall. These changes were more profound in the presence of sub-inhibitory concentrations of imipenem, a compound which also targets the cell wall.

Overall hops were shown to contain compounds which inhibited the growth of mycobacterium and were able to potentate the activity of antibiotics currently used to treat these pathogens. These findings suggest hops may be a fruitful source from which to isolate next generation compounds with which to treat increasingly drug resistant strains of mycobacteria.

“In wine there is wisdom, in beer there is strength, in water there is bacteria”

- German Proverb

Chapter One

General Introduction, Aims and Objectives

1.0 General Introduction

1.1 Summary

Primarily a self-funded studentship, this thesis explored the potential of hops (*Humulus lupulus*) as an antimicrobial against clinically relevant Gram positive, Gram negative and Mycobacterial organisms. Hops are primarily used in the brewing of beer and influence the final flavour and aroma of beer as well as having an inhibitory effect on beer spoilage bacteria.

To determine if the hop plant did possess antibacterial activity, agar based methods were used to determine the minimum inhibitory and bactericidal concentrations of 50 commercially available hop variants. Previous research has suggested that the hop alpha and beta acids were directly responsible for the levels of antibacterial activity, however no correlation was observed. Chemical evaluation of the most active and least active hop extracts revealed differences in the known antimicrobial components, although not directly antimicrobial a novel compound was isolated.

Some plant species and their extracts are known to potentiate the activity of antibiotics and in this thesis several methods of determining antibacterial synergy are investigated. Furthermore, the potential mode of action of both the hop extracts and antibiotics alone and in combination was investigated using scanning electron microscopy.

1.2 The history of medicinal plants

The pharmacological properties of plants and plant extracts have been documented since ancient times. A Sumerian clay tablet dated over 4000 years old from Nagpur, India listed 12 herbal medicines and remedies for drug preparation which were derived from over 250 various plant species (Rooney, 2009).

It is estimated that the ancient Egyptians used over 700 different formulas of plant extracts and honey in a systematic and controlled way (Gurib-Fakim, 2006). There is also evidence that plants have been used for their medicinal qualities in China since 5000 B.C, indeed the *Pen T'Sao* the Chinese book on roots and grasses written around 2500 B.C describes over 365 plant derived preparations for medicinal use (Petrovska, 2012; Wang, 2000).

The Greeks and Romans further utilised the plants around them, Hippocrates (460-370 B.C) who is considered the father of medicine, described the importance of preventive medicine and is often regarded as the author of the following quote:

"*Let food be thy medicine and medicine be thy food"* (Kagan-Zur, 2014)

In the first century (circa 77 A.D) Discorides studied the properties of over 1000 plants and produced a synopsis of their chemical properties within the *Materia Medica*, a medicinal plant catalogue, which became the prototype for early pharmacopoeias (Cowan, 1999). The *Materia Medica* gave descriptions of the plant locations, their appearance and perhaps most importantly the recipes of medicinal preparations with potential therapeutic effects (Petrovska, 2012). Moving forward to around 1200 – 1500 AD the international trade in herbs, spices and plants from Asia, and the discovery of America in 1498, prompted an exponential increase in the availability of medical plant preparations (O'Hara *et al*., 1998).

It was not until the 18th and 19th centuries that that development of pharmacologically active pure natural products increased. One of the first of these was the opium poppy (*Papaver somnifernum*); originally used for its psychoactive and anaesthetising properties since Neolithic and Bronze ages, by extraction of the dried sap from the un-ripened seed pods. It was from this plant only 200 years ago that the compound morphine was isolated and is still in use today (Rauter *et al*., 2002; Sumner, 2000).

This was one of many discoveries that began an era in which potential bioactive compounds from plants could be purified, studied and administered in precise dosages. As such natural products provided a foundation for the discovery of many of today's longstanding medicinal treatments (Newton *et al*., 2000). As a consequence, over the last 20 years natural products or analogues inspired by them form the basis of 90% of therapeutics, approximately two thirds of the world's population relying on plants as their primary source of medicine (Cragg and Newman, 2013; McChesney *et al*., 2007; Newman and Cragg, 2007; Newton *et al*., 2000)

The use of plants is not restricted solely to developing medicines however, over the last 30 years there has been a growth of nutraceuticals (foods that aid health) and the use of natural compounds in food and drink preservation. This market is estimated to surpass revenues of $220 billion worldwide in 2014. (King, 2014; Hammer *et al*., 1999; Martindale *et al*., 1996).

It is estimated that there are at least half a million species of flowering plants in the world. Of these, fewer than 5 % have been investigated for their medicinal potential. However, over the last 25 years there has been a surge in the research of natural products and as such modern medicine is becoming increasingly receptive to the identification of novel compounds such as antimicrobials, from plants. This rise in the interest of natural products has coincided with an increase of drug resistant strains of bacteria and a rapid extinction of plant species (Borris, 1996; Lewis and Elvin-Lewis, 1995; Moerman, 1996). This interest has seen extracts of plants such as garlic, ginseng, valerian and soy, exceed sales of over $500 million in the US alone (Lewis and Elvin-Lewis, 1995).

1.3 *Humulus lupulus*

With the discovery of antibiotics came the announcement from the US secretary general that 'now the golden age of antibiotics is upon us, the war against bacterial infections is over' (Rooney, 2009). Their effectiveness and hence popularity among the world's population has led to years of over use and misuse which has left a vast array of drug resistant micro-organisms and antibiotics which are no longer effective (Gould, 2009). The cost of developing a new antimicrobial combined with the likelihood of bacterial resistance means that here are no new antibiotics in the developmental pipeline; in fact, no new classes of antibiotics have been developed since the 1980's (Alanis, 2005).

Plants and their derivatives represent a diverse resource from which discovering new, efficient and safe antibiotics is a priority. Increasingly, research is looking into early pharmacopeia's for plants or extracts which were once effective but fell out of favour with the introduction of antibiotics (Borris, 1996). The plant *Humulus lupulus*, known as the hop, is one such plant. The hop plant has been utilised throughout history for its flavouring, aroma and antibacterial qualities, primarily in the process of brewing beer (Hieronymus, 2012).

The genus *Humulus* is part of the *Cannabacae* family of plants, including hemp and cannabis. Three species of the hop exist; *Humulus lupulus, Humulus scandens,* and *Humulus yunnanensis*; the latter two of these do not produce resinous cones and therefore are of no use in brewing (Hieronymus, 2012; Stavri *et al*., 2004).

The first usage of the hop plant is difficult to accurately determine; workers widening a drainage ditch in Kent during 1970, found an Anglo-Saxon boat abandoned circa 949 A.D. Upon inspection, the cargo contained huge quantities of hops. This is believed to be the earliest record of hop growing and trade (Hieronymus, 2012; Wilson, 1975). Whilst this predates any previous mention of hops used in the brewing of beer it is presumed that the transport of such large quantities was destined for the brew kettle (Hieronymus, 2012).

Widely recognised as the first recorded history of the use of hops in brewing, is the writings of Abbess Hildegard of St. Rupertsberg in *Physia* (circa 1150-1160). She wrote:

"If thou desirest to make a beer from oats and hops boil it also … as such a beer purges the stomach of the drinker and easest his chest, its bitterness prevents in the latter putrefecation and gives a longer durability" – Adapted from (Arnold, 2005)

This early recording not only mentions the use of hops in beer, but recognises its importance as an antibacterial, increasing the longevity of the final brew. By the 18th century the use of hops as a household remedy as well as its addition in beer, was clear to the general public. Markets where hops were imported and sold from different regions were popular places (Figure 1.1) (Neve, 1991).

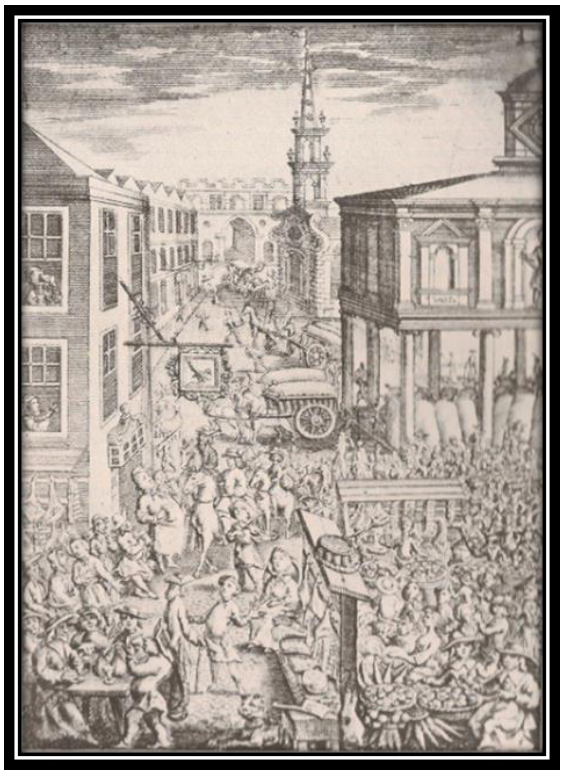

Figure 1. 1 Engraving of a central London hop market from 1779

Reproduced with permission from (Neve, 1991)

1.3.1 Hop cultivation

The origins of the hop plant and the growth of the original plants is a matter of speculation. It is assumed that the plants were originally isolated from the wild and were grown in private gardens. The original gardens would have contained a mixture of several genotypes and the plants which had the highest yield would have been selected as a source for new plants. This selection process favoured different hop variants in different parts of the respective country; therefore each garden eventually had different hop variants with a diverse range of bitterness and aroma properties. Head brewers therefore were able to identify differences in the hops based on the taste of the beer; this lead to the selection of hops based on their suitability for the brew (Arnold, 2005; Barth *et al*., 1994; Hieronymus, 2012; Neve, 1991).

Although this selection process led to around 60 different hop varieties being available around Europe circa 1900, there was a demand for bitterer, higher aroma hop varieties. Diseases were also becoming a problem and causing destruction to hop cultivars; indeed diseases such as downy mildew, hop mosaic virus, hop stunt viroid, powdery mildew and verticulum wilt were leading to a shortage of hops (Barth *et al*., 1994).

The risk of losing the hop plant in Europe to disease inspired several hop breading programmes. The first of these began in 1904 at Wye College in Kent and focused on improving hops by hybridizing several cultivars (Moir, 2000). Selecting hop varieties based on disease resistance and hybridizing existing German and English varieties led to twenty three sets of different seeds being obtained in the first year (Moir, 2000).

This research is ongoing; based around Hüll, Germany, the hop research laboratory is provided with €1.8 million annually from the German government. This meant that between 2001 and 2007 over 15,000 hops were screened for resistance to disease (Hieronymus, 2012). Hop varieties which exhibited disease resistance were examined for the chemical constituents; varieties which had different levels of bitter acids differed in their ability to resist disease (Bajaj, 1989).

1.3.2 The hop

One of the advantages of growing hops is the quick development of oil and polyphenol rich resinous cones (Ceh *et al*., 2007). The plant is a perennial on a permanent rootstock, consisting of an underground structure of both rhizomes (with buds) and true roots (without buds). The roots penetrate the soil to a depth of 15 feet or more and in the first year of growth the hop plant concentrates on building an extensive root system and few flowers are produced. In the second year prolific growth is usually expected (Hieronymus, 2012; Simmonds, 1897; Stavri *et al*., 2004).

When supported with poles, the hop plant naturally twines to a height of six metres or more (Figure 1.2). The hop plant is dioecious and thus has separate male and female plants; only the females produce the cone shaped 'hops' used in brewing (Figure 1.2). The male plant serves as a polliniser, but is not essential for the female plant to produce the hop cones (Neve, 1991).

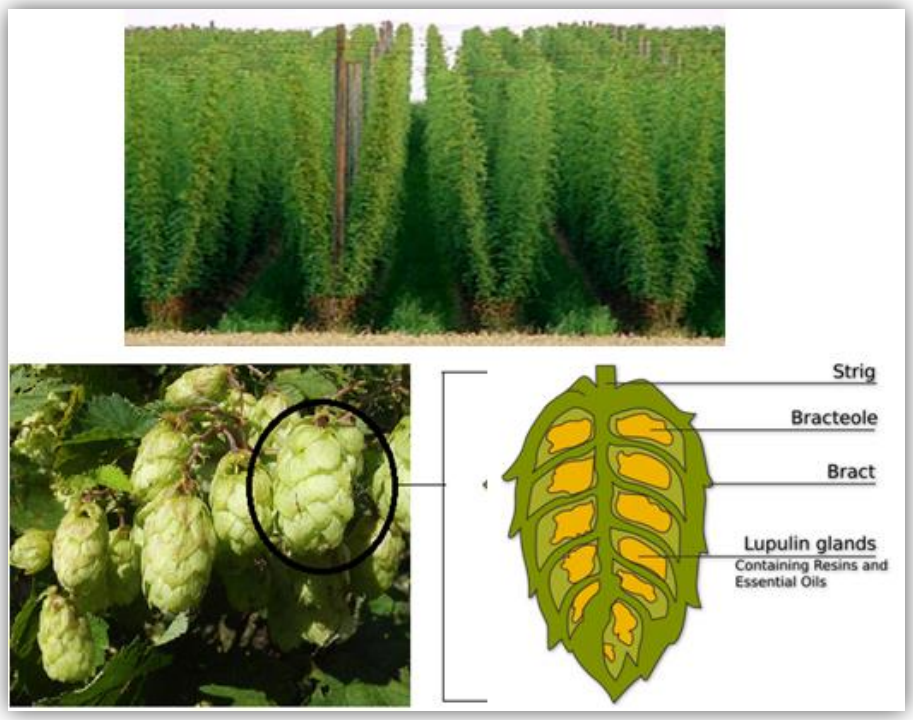

Figure 1. 2 The hop plant grown traditionally on supporting twines (top) and detailing of the individual hop cones (left) with a cross section (right) – adapted from (Hieronymus, 2012)

The hop cones of the female plant are the only part of the plant which is utilised in the brewing industry and this is the focus of the brewer. The cone or strobili develops from inflorescences (cluster of flowers). A strig extends through the centre of the strobili and bears a pair of bracts (outer leaves) and four bracteoles (inner petals) at each node (Neve, 1991). Lupulin glands develop at the base of the bracteoles. The lupulin glands contain hard and soft resins, hop oils and polyphenols. The soft resins include the alpha and beta acids, both of which contribute to the bitterness of beer (Neve, 1991).

1.3.3 Chemical components of hops

Research has been focused on identifying the bioactive ingredients of hops and to elucidate the underlying molecular mechanisms by which they exert the bioactive properties (Van Cleemput, 2009a). Much of this research has concentrated on the polyphenolic components of hops, such as xanthohumol and 8-prenylnaringenin, which have been shown to have multiple bioactive properties (Chadwick *et al*., 2006; Gerhäuser, 2005; Stevens and Page, 2004). Moreover, increasing evidence has shown that the hop bitter acids, namely the alpha and beta acids, also exhibit several bioactive properties (Acworth, 2012; Everard, 2012; Van Cleemput, 2009).

The following Table (2.1) details the average composition of lupulin glands of air dried hops.

Components	% m/m
α - acids	2 – 17
β – acids	2 – 10
amino acids	0.1
Ash/Salts	10
Cellulose - lignin	40 -50
Monosaccharides	2
Oils and fatty acids	1 -5
Pectins	2
Polyphenols and tannins	3 -6
Proteins	15
Volatile oil	0.5 – 3 (v/m)
Water	8 -12

Table 1. 1 The average chemical composition of dried hops.

Sources: (Acworth *et al*., 2012; Cornelison, 2008)

From a brewer's point of view, the hop acids, the alpha (humulones) and beta (lupulones), are the most important class of hop compounds (Gerhäuser, 2005). The relative amounts of the alpha and beta acids depend entirely on both the hop variety and the conditions of growth. Therefore, even hops of the same variety can have widely different levels of bitter acids if grown in different regions (Van Cleemput *et al*., 2009a).

The term alpha acid is used as a single product, but it in fact refers to a number of compounds which are structurally similar but are significantly different. The alpha acids are a mixture of the analogues humulone, cohumulone and adhumulone that differ only in the nature of the acyl side chain (R). The beta acids are a similar mixture of analogues lupulone, colupulone and adlupulone. Both the alpha and beta acids can exhibit tautomerism and exist as a mixture of readily interconvertible structures (Almaguer *et al*., 2014; Baker *et al*., 2008)

Isomerisation, the changing of the arrangement of molecules of both the alpha and beta acids occurs during exposure to heat or light. The analogue therefore, is a precursor to its isomerisation products; in the case of humulone it is transformed to *cis* and *trans* isohumulone (Jaskula *et al*., 2008). The process of isomerisation involves contraction of the six-membered alpha acid ring (through acyloin rearrangement) to form the five-membered iso-alpha-acid ring with two chiral centres, resulting in *cis* and *trans* diastereomers (Acworth *et al*., 2012; Jaskula *et al*., 2008) .

Recent research suggests that it is the hop bitter acids which account for the antibacterial qualities of hops (Cornelison, 2008; Larson *et al*., 1996; Van Cleemput *et al*., 2009a). This action has been attributed to the interference of the prenyl group, characteristic of the side chains of the hop acids. This bioactivity is of importance for killing micro-organisms during wort boiling, which ultimately leads to sterile beer (Arnold, 2005; Stevens and Page, 2004)

Prior to the development of successful breeding programmes, the average alpha acid content of UK sourced hops rarely exceeded 4%. With the need for more successful preservatives and bittering agents that figure has been increased. A variety of hop named Northern Brewer, a seedling of the Golding variety derived from the USA, was the first to combine high alpha acid content (>8% w/w on average) with a good European aroma (Hieronymus, 2012; Neve, 1991; Woodske, 2012).

The development of breeding programmes has allowed higher alpha acid (AA) varieties to be developed such as Target (the most commonly used hop in the UK), Chinook *(*12.7 % AA), Simcoe (12.2 % AA) and Magnum (14.5 % AA). This increase and need for alpha acids led to the development of alpha acid oil which eliminated the need for hop processing altogether and now over 85 % of the world's hops are now processed into pellets, extracts or other products; improving delivery, packaging, ease of use and storage (Simpson and Smith, 1992; Woodske, 2012).

The analogues of the alpha acids *humulone, cohumulone* and *adhumulone* represent 35−70 %, 20−65 %, and 10−15 % (w/w), of the total levels found within hops respectively. The relative amount of *humulone* is dependent on hop variety, while the amount of cohumulone and adhumulone is fairly constant throughout all varieties, even when there are changes in the environment. High levels of cohumulone and adhumulone are associated with lower quality hops and are not favoured by the brewer (Haseleu *et al*., 2009; Moir, 2000).

In contrast, the hop beta acids are considered of lower importance to the brewer as they have been shown to contribute less to the flavour of the final product (De Keukeleire, 2000). It has been recognised that most hop varieties contain approximately equal levels of lupulone and colupulone, which are extremely sensitive to oxidation (Zanoli and Zavatti, 2008). These compounds differ from the other beta acids in that they are very bitter and are usually present in quantities of a few mg L^{-1}. Beta acids are of little interest to the brewer as far as the flavouring and aroma characteristics are concerned. The alpha acids are more important for beer bittering than the beta acids. However, neither class remains unchanged in the finished product (De Keukeleire, 2000; Simpson and Smith, 1992).

1.3.4 Hop harvesting and storage

Hops in the northern hemisphere are usually harvested in August and September and are immediately dried to a water content below 10 %, vacuum packed and kept between 2-3 °C to increase longevity (De Keukeleire, 2000). It has been shown that if the hop material is not stored under these conditions the alpha acid concentration can breakdown rapidly during storage by as much as 70 % within 6 months (Baker *et al*., 2008; Malowicki and Shellhammer, 2005a; Neve, 1991).

The degradation of hop compounds is not only related to the storage conditions but also differs by variant and the environmental conditions that the plant was exposed to during growth. Despite the limited stability of dried hops, they are non-homogenous and have a low bulk density. However, only about 5% of the total annual harvest is used without further processing with around 60% converted into pellets (Hieronymus, 2012; Malowicki and Shellhammer, 2005a).

The process of pelleting the hops involves the inflorescences being cut, milled, homogenised and pressed into granules. The density of the granules, when tightly pressed and stored in correct conditions, increases their stability significantly (Nance and Setzer, 2011). For this reason pelleted versions of hops (1.3 right) are commonly used over their unaltered counterparts (Figure 1.3 left) in the brewing process (Hieronymus, 2012)

Figure 1. 3 The difference between whole unprocessed hops (left) and those which have undergone pelleting (right). Taken from (Brewmaster, 2014)

1.3.5 The brewing of beer

The primary use of hops is in the brewing of beer. Beer is a fermented aqueous drink based on four main ingredients; water, malted barley (grains), hops and yeast. Its production, known as brewing, has been understood since antiquity. Indeed, the Mesopotamians are famous for recognising the nutritional benefits of beer and also for noticing the side effects of an 'exhilarated, wonderful and blissful feeling' (Hieronymus, 2012). In the following Figure (Figure 1.4) the traditional brewing process is summarised.

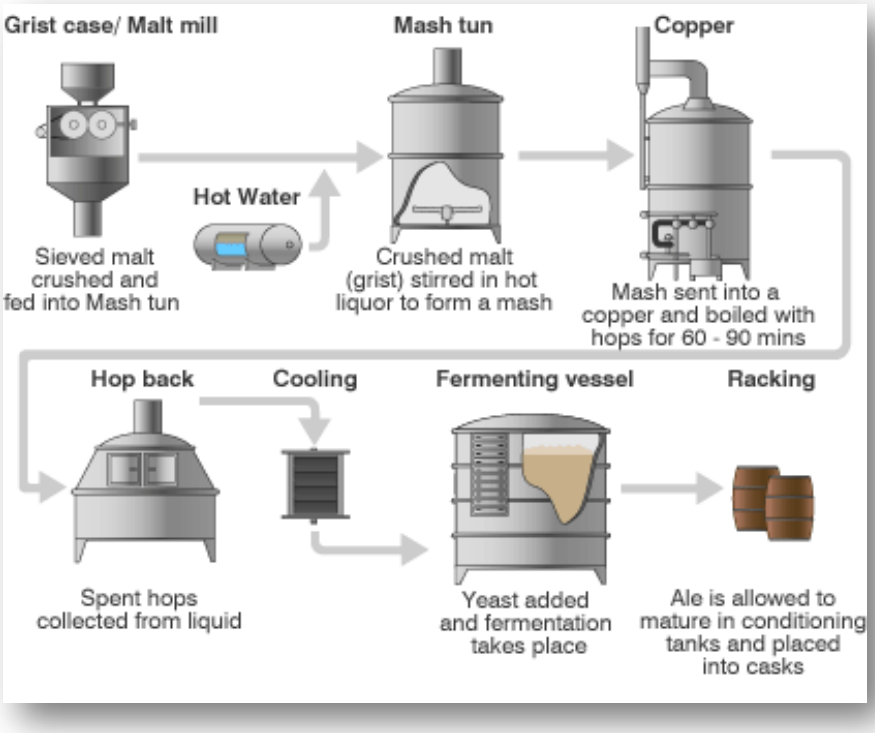

Figure 1. 4 Schematic of the traditional brewing process detailing the addition of hops. Adapted from (Poston, 2006)

The steps involved in the brewing of beer include; malting, milling, mashing, brewing, cooling, fermentation and maturation. The first, malting, is the preparation of barley and it is used as a starch base for the wort. It is the addition of wort and its subsequent germination during heating which leads to the beer having a pale or dark colouring.

The second stage, milling, mills the various grains (of which barley comprises around 40%). This ensures that the grains and their subsequent sugars will be more easily absorbed into the water of the mash tun. (Arnold, 2005; Jackson, 1998)

The next stage, mashing, turns the malt into a sweet liquid by converting starches to sugars that can be fermented. The previously milled grain is added to the brew and heated to around 75 °C in the mash tun. It is at this point that the grain and water create a cereal mash to dissolve the starch into water, transforming it into sugar. The water itself is then strained through the bottom of the mash and is now termed, “the wort” (Arnold, 2005; Jackson, 1998).

The next step, brewing, is the most important process. The simplest brew house layout has two principal vessels. The first, the mash tun for mashing, and the second, the brew kettle is where the infusion is boiled with hops. On average this process takes between 60 and 90 minutes; strong beers can require longer (Jackson, 1998). The main function of this step is to introduce the aroma and bitterness properties of the hops to the brew. Some beers are only made with the addition of only one hop variant, others multiple. The hops don’t contribute alcohol, however, the aromas and flavours are powerful. As such, even in large breweries the hops are usually often added by hand and in relatively small amounts (kilos) compared to the malt (tonnes). Hop variants intended to impart dryness or bitterness are added early in the boil, with some having a second addition mid boil to impart a stronger flavor (Jackson, 1998; Stevens and Page, 2004).

When the brewing is completed the hop leaves and sediment are removed. This is either done through centrifugal force or through a strainer. In some cases the wort is filtered through layers of hops, imparting further hop aromas and oils. Commonly, aroma hops are added in the maturation vessel. In the UK, this is known as dry hopping and hops within a muslin sack are added to the cask in which the beer matures (Jackson, 1998)

Finally, the beer is fermented, allowing for the sugar to be converted into alcohol. Once fermented, the beer is transferred to a conditioning tank where the taste ripens and the liquid clarifies which is usually completed within a few days. The beer, almost ready is filtered and bottled (Hornsey, 2003).

1.3.6 The medicinal qualities of hops

Although hops main use is in beer, whole hops and their extracts have been developed into a diverse range of products around the world. These products include non-alcoholic beverages, sleeping aids, aphrodisiacs and as treatments for a variety of maladies (Woodske, 2012). The traditional use of hops as a mild sedative stems from the observation of sleepiness and fatigue in hop pickers apparently due to the transfer of hop resin from their hands to their mouths (Foster and Tyler, 2000). In 1998 the German commission approved hops for the treatment of mood disturbances, such as restlessness and anxiety (Bundesinstitut für Arzneimittel und Medizinprodukte (Germany), 1998). Hops are also known to possess estrogenic activity, first recognised when female hop pickers had frequent menstrual disturbances during the early days of hop cone harvesting. In Germany, hop baths were frequently used as a treatment for gynaecological disorders (Chadwick *et al*., 2006; Milligan *et al*., 2000).

Beer was originally produced without the addition of hops, however it is thought that during the summer months 'unhopped' beer was seen to spoil much faster than its 'hopped' counterpart (Filmer, 1982). Due to this, hops were added to many beers. Circa mid-19th century drinking water was heavily contaminated and unsanitary due to the lack of sterilisation techniques and understanding. During this time beer was considered a safer alternative to water; due to the facts that the wort is boiled, thus destroying the bacteria, combined with the antibacterial effects of the hops during the brewing process and subsequent storage. The alcohol level of beer at this time was typically between 2-6% (v/v) and it has been shown that an alcohol concentration of this level would have no effect on the majority of pathogenic bacteria (Jackson, 1998; Rooney, 2009)

For the brewing industry beer spoilage bacteria have been problematic for centuries (Sakamoto 2003). Beer is generally a poor and hostile environment for most microorganisms as the typical pH (3.8-4.7) is unfavourable for bacterial growth. However this allows for the growth of specific organisms such as Lactobacillus and yeasts (Langezaal *et al*., 1992; Sakamoto and Konings, 2003a).

Haas and colleagues (1994) investigated the antibacterial role of isomerised alpha and beta hop resins against *Streptococcus salivarius, Staphylococcus aureus*, *Bacillus megaterium, Escherichia coli* B, and *Bacillus subtilis.* The study showed that the isomerised alpha and beta acid resins inhibited the growth of all of the organisms at concentrations from 0.01% to 0.03% (w/v). The inhibition of growth is generally thought to be due to primary membrane leakage by the interaction between the hydrophobic areas of alpha and beta acid molecules with the bacterial cell wall (Haas and Barsoumian, 1994; Teuber and Schmalreck, 1973).

A further study by Stavri *et al* (2004) investigated the anti-mycobacterial effectiveness of hop extracts and hypothesised that activity was associated with the alpha and beta acids. After a non-polar extraction process and preparative thin layer chromatography (TLC) on silica gel, several bands were isolated, all of which had activity against *Mycobacterium tuberculosis* at concentrations ranging from 8 to 16 µg mL^{-1} (Stavri *et al*., 2004). Originally, it was hypothesised that each separation on TLC resulted in a singular molecular species. However, nuclear magnetic resonance (NMR) indicated that in fact the separated fractions were a mixture of different components. As these components were difficult to separate using conventional techniques, individual components could not be identified as having inhibitory activity against mycobacteria (Stavri *et al*., 2004).

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A study by Serkani and colleagues investigated the antibacterial effect of polar hop extracts against 37 drug resistant and susceptible isolates of *Mycobacterium tuberculosis*. The research found that both drug sensitive and resistant strains of the bacterium were inhibited by the presence of the hop extract (Serkani *et al*., 2012), further establishing that the hop plant is potentially a useful resource for undiscovered novel antimicrobials.

1.4 *Mycobacterium*

Mycobacteria are members of the genus *Mycobacterium* which are slow growing, aerobic, rod shaped bacteria. The organism is made distinctive by a staining property known as acid-fastness. This property is due to the presence on the mycobacterial cell wall of unique lipids called mycolic acids, found only within this genus. It was first discovered by Robert Koch during his investigations into tuberculosis and acid fast staining allowed for the identification of the organisms within tuberculosis lesions (Black, 2004; Mims and Goering, 2013).

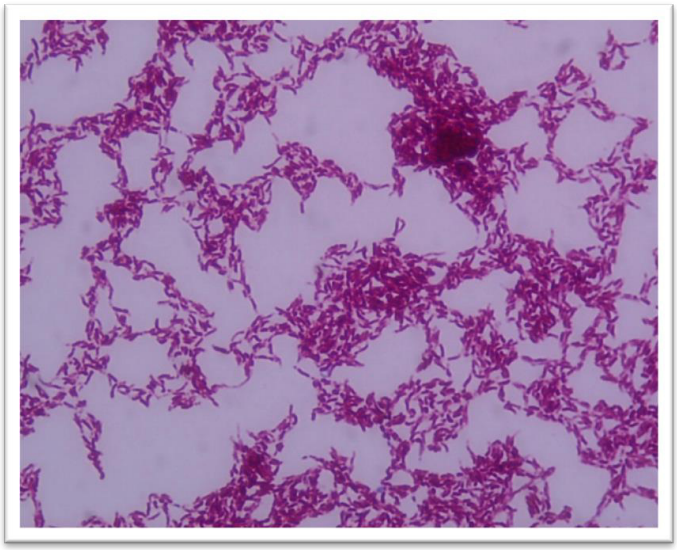

Figure 1. 5 Acid fast/ Ziehl Neelson stain of *M. smegmatis* 8159 as first demonstrated by Robert Koch

Mycobacteria are also categorised by other factors such as the production of specific pigments. For example, species such as *M. tuberculosis* and *M. smegmatis* do not produce a pigment, whereas, photo chromogenic species such as *M. kansasii* form a pigment only when cultured in light. Scotochromogenic species such as *M. gordonae* can form pigments when cultured in the dark (Mims and Goering, 2013).

Mycobacteria are pleomorphic and may undergo branching or filamentous growth. In general, *Mycobacteria* can be separated into two major groups, slow growers and fast growers. The difficulty in culturing slow growing bacteria in the laboratory has been a problem since their discovery. *Mycobacteria* do not grow readily on standard agar and due to the slow growth of the organism media can become contaminated (Madigan, 2003).

Slow growing species such as *Mycobacterium tuberculosis* have a doubling time of twenty hours and others can take up to ten weeks for cultures to become visible. Whereas faster growing species such as *Mycobacterium smegmatis* have a doubling time of 2 hours and colonies are visible within 72 hours. This shorter growth time and the advantage of being non-pathogenic allow for *M. smegmatis* being a useful tool within the laboratory as a simulant for *Mycobacterium tuberculosis* (Chaturvedi *et al*., 2007a; Madigan, 2003)

1.4.1 *Mycobacterium tuberculosis*

It is currently estimated that one third of the world's population is infected with the bacterium M. tuberculosis (Dooley *et al*., 2013). Tuberculosis (TB) on average is responsible for over 2 million deaths worldwide per annum, more than any other bacterial infection (WHO, 2010). The bacterium is successful as it is able to reside dormant within its hosts immune cells undetected; only causing symptoms when the patient is immunocompromised. As such, co-infection within HIV positive patients is increasing (Murray *et al*., 2014).

If left untreated, each person with an active TB infection will, on average, infect 10-15 other people per year (Murray *et al*., 2014; Pauli *et al*., 2005). The Health Protection Agency (HPA) has found that over the last 5 years there has been a 5% rise in the number of new cases of TB in the UK, with the majority of cases in London presenting 6,000 new cases of TB in 2011 (HPA, 2012).

Mycobacterium tuberculosis is only one of many similar organisms within the *Mycobacterium tuberculosis* complex (MTC). The group comprises of closely related organisms such as *M. africanum, M. microti, M. caprae, M. pinnipedi, M. bovis* along with the widely used vaccine strain *M. bovis* bacillus Calmette-Guerin (BCG) (Brosch *et al*., 2002; Cole *et al*., 1998).

The genetic similarities between the members of the MTC complex group of bacteria show an unusually high degree of conservation of housekeeping genes. It has been shown that members of the MTC group share around 85-100 % of their DNA (Frothingham *et al*., 1994). Paleopathogenic evidence suggests that tuberculosis afflicted humans as early as 3700 BC in Egypt and 2000 BC in Europe. The mutation frequency of *M. tuberculosis*, which is similar to that of other bacteria, has led to the speculation that MTC isolates may have diverged from a common ancestor 15,000 years ago, as demonstrated in the following Figure (2.5) (Raviglione *et al*., 2006).

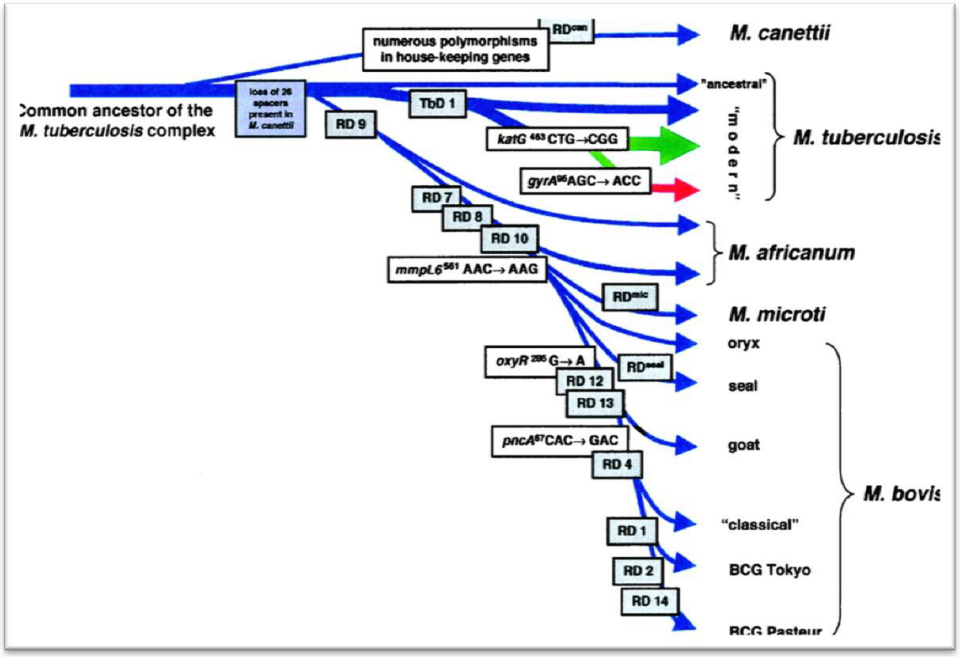

Figure 1. 6 Common lineage of the *Mycobacterium tuberculosis* complex (MTC) showing differences in the RD genes from one common ancestor and the subsequent development of different species. Source: (Brosch *et al*., 2002)

Polymorphism in *M. tuberculosis* complex bacteria has been found to be associated with repetitive DNA, such as transposable elements and short perfect or imperfect repeats. The direct repeat locus (DR locus) is currently the only well studied single locus in the MTC (Cole, 2002; Cole *et al*., 1998). The DR locus is composed of 36 bp DR copies which are interspersed by non-repetitive short sequences of about equal length known as spacers. The absence or presence of one or more of the spacers form the main differences in *M. tuberculosis* and *M. bovis* and this property has been exploited for identification of the bacteria within the MTC group (Fang *et al*., 1999; van Embden *et al*., 2000).

Due to the relationship between the genomics of *M. bovis* and *M. tuberculosis,* it was thought that human tuberculosis evolved from the bovine disease by adaption of an animal pathogen to a human host (Stead *et al*., 1995)**.** However work carried out by Brosch *et al*, (2002) shows that *M. bovis* has undergone numerous deletions relative to MTB through its evolution. It has been speculated that the successive loss of DNA may have contributed to clonal expansion and the appearance of more successful pathogens in certain new hosts as shown in Figure 2.6 (Brosch *et al*., 2002).

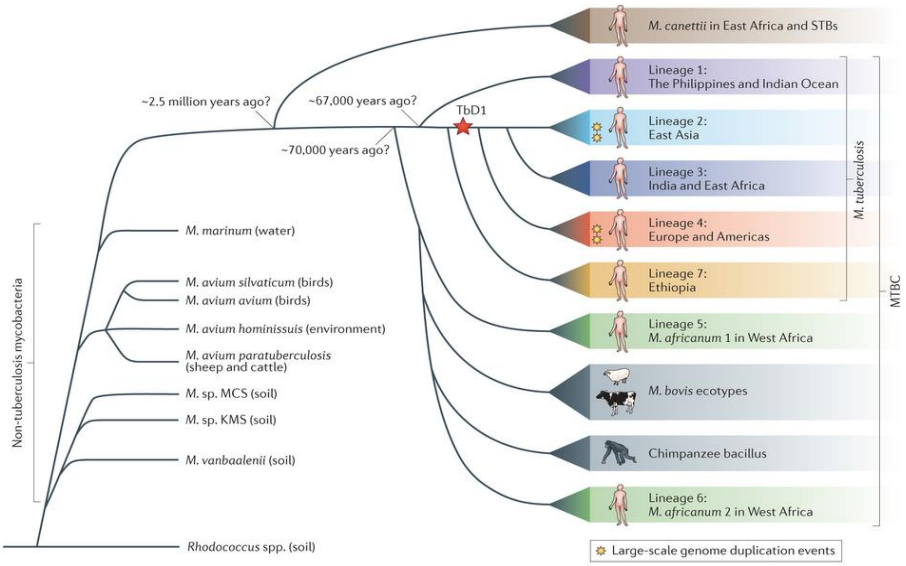

Figure 1. 7 Lineage of other non-tuberculosis mycobacteria (NTM) in comparison with MTB, detailing the range of hosts. Source: (Galagan, 2014)

1.4.2 Human infection

In primary infection, the mycobacteria, unlike other bacteria, do not avoid uptake by macrophages, instead they survive within the macrophage by blocking fusion of the phagosome with late endosomes and lysosomes. This is crucial for pathogenic mycobacteria as they can survive for long periods within these host cells (Armstrong 1971). Uptake of mycobacteria by macrophages requires recognition of the bacteria through receptor molecules such as complement receptors, (CR's), mannose receptor, scavenger receptors and immunoglobulin (Fc) receptors. Whilst precise uptake of the bacteria via receptors *in vivo* remains to be established, one of the most important receptors *in vitro* is CR3 (Ferguson *et al*., 2006; Velasco-Velázquez *et al*., 2003).

Phagocytosis of mycobacteria by CR3 requires the accumulation of cholesterol at the site of entry; the CR3-mediated uptake is specific for mycobacteria because cholesterol depleted macrophages can still internalise other microorganisms. The role of cholesterol remains unknown, but it has been suggested that it may aid in the rearrangement of the membrane-cytoskeletal network during phagocytosis or be involved in signal-transduction reactions following the engagement of CR3 (Peyron *et al*., 2000; Velasco-Velázquez *et al*., 2003).

1.4.3 Evasion of host immune defences

To prevent destruction within the macrophage, mycobacteria have developed several methods to prevent phagosome maturation utilising both the host and mycobacterial molecules (Pieters and McKinney, 2013). One such host molecule is coronin 1 (P57), which is expressed exclusively within the leucocytes and utilises cholesterol within the cell to associate with mycobacterial phagosomes. P57 has been shown to function in actin-cytoskeleton remodelling, phagocytosis, cell division and polarity and vesicular trafficking from the endoplasmic reticulum to the golgi apparatus (Pieters and McKinney, 2013). P57 is retained exclusively on phagosomes of macrophages infected with live mycobacteria, whereas in cells with dead mycobacteria, P57 rapidly dissociates from phagosomes leading to fusion of phagosomes to lysosomes and destruction of the bacteria. This suggests an essential role of P57 in the protection of the mycobacteria within the cell (Ferrari *et al*., 1999; Tailleux *et al*., 2003).

Another important defence mechanism is the inhibition of phosphatidylinositol 3-phosphate (PI3P). Biosynthesis of PI3P is required for phagosome maturation, and is a membrane trafficking regulatory lipid essential for phagolysosome biosynthesis. PI3P is synthesised by PI3-Kinase on endosomal and phagosomal membranes, and has been observed to move on the phagosomal membrane in temporally scheduled waves (Roth, 2004; Vergne *et al*., 2003; Wurmser *et al*., 1999). PI3P functions as a ligand for many proteins that may be involved in phagosome maturation such as hepatocyte growth factor (HGF) regulated tyrosine kinase substrate (Hrs), which has a key role in the regulation of traffic in the endosomal pathway (Raiborg *et al*., 2002; Chin *et al*., 2001; Komada and Soriano, 1999).

Mycobacteria are able to cause a reduction in the waves of PIP3 which results in the depletion of Hrs, and in turn halts phagosome maturation. This eventually leads to dissociation of Hrs with phagosomes preventing the mycobacteria being destroyed (Vieira *et al*., 2004)

1.4.4 Non-tuberculosis mycobacterium

There are other pathogens within the mycobacterial community which are not part of the MTC group. Non tuberculosis mycobacterial disease (NTM) was first recognised in a small minority of patients with TB in the 1950's (Cook, 2010). But it was not until disseminated *Mycobacterium avium* complex (MAC) was recognised as the most common opportunistic bacterial infection in AIDS patients that the low prevalence problem of pulmonary NTM disease attracted attention. Opportunistic NTM infections are acquired from environmental (water, soil) reservoirs and are not transmitted between humans or animals and humans. They notably cause disease in patients who are immunosuppressed and/or have pre-existing lung abnormalities (Al Jarad *et al*., 1996; Mims and Goering, 2013; Thomson and Yew, 2009).

Bacteria that fall within this group are MAC, *M. kansasii*, *M. xenopi, M. malmoense, M. simiae, M. abscessus, M. chelonae* and *M. fortuitum.* NTM that cause pulmonary disease have been shown to vary greatly between geographic regions (Cook, 2010).

MAC is the predominant pathogen in most regions worldwide. *M. kansasii* disease is relatively more common in the USA and the UK. *M. xenopi* is more common in the USA, Canada, the UK and some regions of Europe. *Mycobacterium malmoense* disease is common in the UK and northern Europe but is uncommon in the USA. It has been suggested that the differences in these geographical regions has been associated with variations in environmental conditions which favour the predominant NTM populations (Tan *et al*., 2013; Thomson and Yew, 2009).

Infection with NTM can vary between regions but it has been shown that NTM commonly contaminate domestic water supplies, swimming pools and hospitals. Hospitals particularly suffer from nosocomial infections with NTM namely *M. absessus, M. fortuitum* and *M. gordonae*. Infections associated with these bacteria are associated with the skin and soft tissue acquired during surgical procedures; there are even reports of NTM infection from bronchoscopes (Wallace *et al*., 1992).

1.4.5 *Mycobacterium smegmatis*

Mycobacterium smegmatis was first identified in 1884 from syphilitic chancres and was originally thought to be the agent that caused syphilis, but was eventually identified as a common environmental mycobacterium. *M. smegmatis* rarely causes disease in humans, with less than 30 infections ever recorded. Infection, if caused, is via skin or soft tissue infections following traumatic surgery (Best and Best, 2009). Its common use is within research facilities as a simulant organism of *M. tuberculosis*. The organism is successful as it can be handled within a category II laboratory and grows at a much faster rate than that of *M. tuberculosis*. A recent study also found that antibiotic sensitivities were similar between *M. smegmatis* and *M. tuberculosis* (Chaturvedi *et al*., 2007a).

1.4.6 *Mycobacterium abscessus*

M. abscessus is a rapid growing mycobacteria (RGM), which was first described in 1953. It was not until the 1990's however, that *M. abscessus* was recognised as an important human pathogen responsible for a wide spectrum of soft tissue infections and disseminated infection in immuo-compromised patients (Griffith *et al*., 2007; Moore and Frerichs, 1953; Nessar *et al*., 2012). Importantly *M. abscessus* is considered the prominent *Mycobacterium,* along with *Mycobacterium avium,* involved in infections with cystic fibrosis patients. The major concern of *M. abscessus* infection is its increasing drug resistance to first and second line antimicrobials, which has led to an increased mortality rate (Cullen *et al*., 2000; Jeon *et al*., 2009; Sanguinetti *et al*., 2001).

The current recommended regime for the treatment of *M. abscessus* infection relies on a combination of clarithromycin, with an aminoglycoside such as amikacin and an injectable antibacterial such as imipenem (Griffith *et al*., 2007). However, it has been shown that these combinations are becoming increasingly unsuccessful, highlighting the need for novel antimicrobials to combat the disease and potentiate the activity of redundant antibiotics.

Recently *M. abscessus* has been sub-classified into three sub-species on the basis of *rpoB* sequences: *M. abscessus sensu stricto*, *M. massiliense* and *M. bolletii*. Recent research has shown that differentiation between these species is problematic as each species shares ribosomal sequences (Tan *et al*., 2013).

It has been shown that although similar in genomic terms, the bacterial subspecies differs in antibiotic resistance (Cole *et al*., 1998). This leads to the treatment of *M. abscessus* and its subspecies notoriously difficult to treat. Treatment using multiple antibiotics with several modes of action can reduce the microbial population but can lead to the selection of resistant organisms (Jeon *et al*., 2009). Furthermore, *M. abscessus* has been shown to be resistant to many of the anti-tuberculosis drugs in clinical use today. Indeed the major pathogenicity factor of mycobacteria is the ability of the bacterium to be intrinsically resistant or acquire resistance to antibiotics (Fairhurst *et al*., 2002).

Acquired resistance as a result of genotypic changes within mycobacterial clinical isolates is generally thought to be the result of spontaneous mutations affecting the key targets of antibiotics but resistance may result from alteration in the function of more than one gene. Alteration in chromosomal gene function represents the main mechanism of acquired resistance in clinical strains of mycobacteria species; however, resistance has been observed in species which lack the absence of mutations within drug target genes suggesting other mechanisms can be involved (Cole, 2002; Rattan *et al*., 1998). The intrinsic resistance of mycobacteria is well understood, with the major contribution being the mycobacterial cell wall which consists of a complex matrix of fatty acids and lipids (following Figure 2.7) that can hinder the penetration of antimicrobial compounds (Cohn *et al*., 1997; Cole, 2002).

It is well documented that the cell envelope acts synergistically with antibiotic inducible internal systems in competing against the effect of drugs. The internal system, known as the intrinsic resistome, includes efflux pumps, antibiotic-modifying/inactivating enzymes, target modifying enzymes and genes conferring metal resistance. One of many inactivating enzymes that has been observed in *M. abscessus* and *M. tuberculosis* is β-lactamase, which can assist the bacterium in nullifying the effect of β-lactam antibiotics such as imipenem (Raviglione *et al*., 2006; Tailleux *et al*., 2003).

The intrinsic resistance of MTB to most antibiotics is generally attributed to the low permeability of the mycobacterial cell wall because of its specific lipid-rich composition and structure. This low permeability limits drug uptake and seems to be one of the main factors involved in resistance. Along with cell permeability, which limits drug uptake, active efflux systems also provide resistance by extruding the drug molecules that enter the cell, which therefore lowers the intracellular concentration of a given drug. In mycobacteria the two main efflux pumps associated with drug resistance are the lfrA efflux pump of *M. smegmatis* and the Rv1634 efflux pump of *M. tuberculosis* which are able to remove drugs from the cell before the drug can take effect (Brennan, 2003; Niederweis, 2003).

1.4.7 Mycobacterial cell wall

The cell wall of mycobacteria differs from that of other bacteria and due to this has a distinctive staining property known as acid fastness. This property is due to the presence of the lipid rich wall as shown in Figure 1.8. The cell wall of mycobacteria has been recognised as providing the bacilli with an effective innate immune defence against lysososomal degradation. In particular, the *Mycobacterium tuberculosis* PI3P analog glycosylated phosphatidylinositol lipoarabinomannan (ManLAM) has been reported to block phagosome maturation; the LAM binding site is also highlighted in Figure 2.7 (Black, 2004; Mims and Goering, 2013; Nguyen and Pieters, 2005).

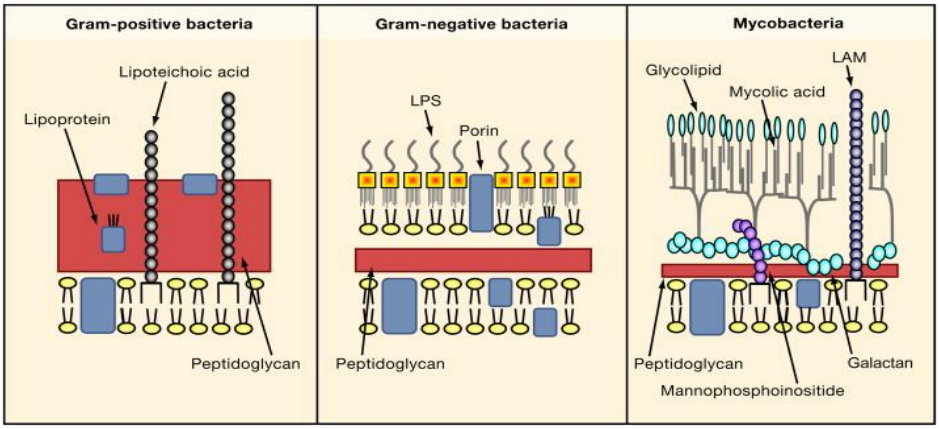

Figure 1. 8 Differences in Bacterial Cell Wall Morphology. Adapated from (Akira, 2006)

ManLAM has several different effects within the macrophage, such as blocking the PI3P-dependant pathway, which halts phagolysosome biogenesis, and inhibiting calcium uptake in the macrophage cytosol, which is necessary for the accumulation of PI3P on the phagosomal membrane (Fratti *et al*., 2003; Sturgill-Koszycki *et al*., 1996).

One of the most important macrophage combating molecules is interferon gamma (IFN-γ), which is secreted by activated T cells and natural killer cells during infection. IFN-γ signalling is used in many cellular processes, such as antigen presentation, leukocyte-endothelium cell interactions, cell growth and apoptosis, and phagosome-lysosome fusion. Indeed, it has been shown that both mice and humans with IFN-γ genetic defects are extremely susceptible to mycobacterial diseases (Adams and Hamilton, 1992; Murray *et al*., 2014; Stout, 1993).

In addition to the adaptive immune response, macrophage activation can be mediated through activities of the toll-like receptors (TLR's), of which TLR-2 and TLR-4 have key roles. Activation of the TLRs via cell wall constituents leads to the activation of mitogen-activated protein kinases (MAPKs). The MAPKs initiate signalling cascades that activate transcription factors, which lead to the synthesis of tumour necrosis factor (TNF-α), chemokine's and other inflammatory mediators. These signalling cascades boost antibacterial and inflammatory immune responses of macrophages including phagosome maturation. However, these pathways are exploited by pathogenic mycobacteria to promote mycobacterial survival within the macrophages. *Mycobacterium tuberculosis* in particular uses ManLAM to inhibit activation of MAPK in human monocytes (Means *et al*., 1999; Schaible *et al*., 1998; Schorey and Cooper, 2003).

Mycobacteria, and especially those of the MTC, has evolved many different strategies for evading the host immune systems to either cause infection, or to lie dormant within the cell until conditions are favourable for the bacteria to be reactivated. Reactivation of the bacteria relies upon the host defence systems to be lowered in cases such as malnutrition or in immunocompromised patients such as those with HIV/AIDS (Lawn, 2005; Murray *et al*., 2014).

1.4.8 HIV Co-Infection

The high prevalence of tuberculosis co-infection with HIV/AIDS remains a common complication, with an estimated 10 million people being infected. Most instances of co-infection are seen in the developing nations, with sub-Saharan Africa exceeding 1000 infected per 100,000 population. Worldwide, TB is the most common cause of death among patients with HIV/AIDS, killing 1 in every 3 individuals (Espinal *et al*., 2001; Lawn, 2005; Murray *et al*., 2014)

HIV enters macrophages and $CD4^+$ T cells by the adsorbtion of glycoproteins on its surface to receptors on the target cell followed by fusion of the viral envelope with the cell membrane and the release of the HIV capsid into the host cell. Shortly after this the enzyme *reverse transcriptase* liberates the single stranded RNA genome from the attached viral proteins and copies it into complementary DNA molecules. This process leads to the depletion of *M. tuberculosis* specific CD4 lymphocytes and functional impairment of CD4 lymphocyte-macrophage interactions resulting in impaired granuloma formation. This ultimately leads to failure to restrict *M. tuberculosis* replication (Mims and Goering, 2013; Gandhi *et al*., 2010; Friedland *et al*., 2007; Lawn, 2005).

The interaction between TB and HIV is bidirectional. The activation of mononuclear cells during the host response to TB leads to accelerated HIV replication, which may increase HIV load at anatomical sites involved with TB and systemically (Friedland *et al*., 2007). The bacteriological response to anti tuberculosis treatment is not significantly affected by HIV status. Standard therapy (Table 1.2), is used to treat the patients within the first months and cure rates are similar to those without HIV infection. However, mortality rates remain high for HIV infected individuals and can be associated with developing countries with little or no resources for MTB infection, poor compliance or lack of patient management (Rieder *et al*., 2009). In patients with advanced immunodeficiency (define by a CD4 count of less than 100/µl) anti tuberculosis treatment is complicated by the fact that finding Mycobacterium within clinical specimens may result from infection of *M. avium-intracellulare* or other NTM which require different treatment methods (Mims and Goering, 2013; Gandhi *et al*., 2010; Friedland *et al*., 2007; Lawn, 2005).

Anti-HIV treatment, namely highly active antiretroviral therapy (HAART), leads to dramatic reductions in the incidence of opportunistic infections, including TB as pathogen-specific immune responses are restored. Concurrent administration of HAART and anti-tuberculosis drugs is complicated by the fact that rifamycins (rifampicin, rifapentine and rifabutin) tend to reduce serum concentrations of non-nucleoside reverse transcriptase inhibitors and protease inhibitors, which may lead to increased replication of the virus (Rieder, 2009).

1.4.9 Testing for tuberculosis

The tuberculin or Mantoux skin test (TST) is the primary standard method for the determination of TB infection within individuals (Mims and Goering, 2013) (Goering, 2008). Tuberculin was first discovered by Robert Koch from a glycerine extract of *M. tuberculosis* and was initially, but unsuccessfully, used as a remedy for MTB infection. It wasn't until Clemens Von Pirquet discovered that patients who had been vaccinated against smallpox, but who were again injected, developed severe reactions, that the importance of the tuberculin skin test could be noticed. The glycerol extract of *M. tuberculosis* is still in use today and is known as purified protein derivative (PPD). This extract is derived from proteins on the surface of the mycobacterial cell, which are non-specific to mycobacteria. By using the same methods of injection, previous exposure to MTB can be determined (De Keyser *et al*., 2014; Mims and Goering, 2013; Rieder *et al*., 2009; Rooney, 2009)

Infection with TB leads to the development of an allergy to the tuberculin protein. Therefore, during testing, 5 tuberculin units (TU) of PPD are injected into the inner surface of the forearm. If the patient has had infection with TB, the tuberculin protein produces a hypersensitivity reaction namely, inflammatory swelling, within 72 hours (Rieder *et al*., 2009).

The TST is classified based on the size of the induration (palpable, raised, hardened area of swelling) within 72 hours. And is based on 3 scales;

1. **An induration of 5 or more millimetres is considered positive in:**
    - HIV infected individuals.
    - Patients who have had recent contact with TB infected individuals.
    - Patients with abnormal chest radiographs consistent with prior TB.
    - Patients with organ transplants.
    - Patients who are immunosuppressed.

2. **An induration of 10 or more millimetres is considered positive in:**
    - Recent immigrants (< 5 years) from high prevalence countries.
    - Injecting drug users.
    - Mycobacterial laboratory workers.
    - Children <4 years of age.

3. **An induration of 15 millimetres or more is considered positive in:**
    - Any persons – including those that have no known risk factors for TB.

(Adapted from National centre for HIV/AIDS, Viral hep, STD and TB prevention)

In some cases, the TST may produce no skin reaction at all even in patients who are known to be infected with *M. tuberculosis,* which can lead to false negative results (Goering 2008). This however, does not exclude the active MTB infection, as the TST can be suppressed by a number of factors, such as malnutrition, viral infections, HIV infection, measles, chickenpox, glandular fever, cancer, corticosteroids and similar drugs (Madigan 2009). A false positive result may be caused by non-tuberculosis mycobacteria or previous administration of the BCG vaccine and in some cases, vaccination with the BCG vaccine can lead to false positive results for many years afterwards. This is due to the use of nonspecific proteins derived from *M. tuberculosis* being recognised by the immunised host's immune system (De Keyser *et al*., 2014; Mims and Goering, 2013; Rieder *et al*., 2009).

In some patients with a latent TB infection, the immune response will gradually wane over time and may show a negative TST. However, the initial skin test, although negative, may stimulate the bodies' reactions to tuberculin in further tests. To combat false positives, a two-step testing method is used for adults who will be re-tested periodically (e.g. health care workers) so that any future positive tests can be related to a new infection. The two step testing relates to two separate TST; if the first TST is negative a second test is given 1-3 weeks later, and if the second is negative the patient is uninfected. If positive, the patient is considered previously infected (De Keyser *et al*., 2014; Morán-Mendoza *et al*., 2013)

1.5 Treatment of tuberculosis

Treatment of TB relies on a combination of isoniazid, rifampicin, pyrazinamide, ethambutol and streptomycin. The combinations and duration of course are summarised in Table 2.1.

TB patients	TB treatment regimens	
	Initial phase	Continuation phase Daily
New patients never previously treated	2 HRZE	4RH or 6HE
Previously treated for longer than 1 month	2 HRZES/ 1 HRZE	5HRE or 5HREZ
Patients with drug resistant TB	Patient specific regimens	

Table 1. 2 Standard treatments for TB

KEY – H = isoniazid; R = Rifampicin; Z= pyrazinamide; E = ethambutol; S = streptomycin

The number indicates the duration in months that drug combinations should be administered

(Adapted from Rieder *et al.*, 2009)

Drugs are most frequently administered daily. The number before the drug combination shows for how many months a drug combination is given. For example, 2HRZE indicates that isoniazid, rifampicin, pyrazinamide and ethambutol are given in a single dose daily for 2 months (Rieder *et al*., 2009). Drugs are most frequently administered daily. The number before the drug combination shows for how many months a drug combination is given. For example, 2HRZE indicates that isoniazid, rifampicin, pyrazinamide and ethambutol are given in a single dose daily for 2 months (Reider, *et al.,* 2009). Isoniazid is a prodrug that is activated by a bactericidal catalase-peroxidase enzyme in MTB termed KatG. KatG, promotes the coupling of isonicotinic acyl with NADH to form the NADH complex which binds to the enoyl-acyl carrier protein reductase. This blocks the action of fatty acid synthase. Fatty acid synthase is needed for the synthesis of mycolic acids within the mycobacterial cell wall leading to cell death (Carel *et al*., 2014; North *et al*., 2013).

Isoniazid is normally given by mouth and highly effective concentrations of the drug are achieved in all tissues including the cerebrospinal fluid. There is no cross resistance with other drugs. Dosage varies upon treatment program but generally for daily use 5 mg kg^{-1} up to a maximum of 300 mg is given in a single dose. When used intermittently (three times per week) the standard treatment is 10 mg kg^{-1} (Dooley *et al*., 2013; Espinal *et al*., 2001).

Rifampicin inhibits bacterial DNA-dependant RNA synthesis by inhibiting bacterial DNA-dependant RNA polymerase thus preventing extension of RNA products beyond a length of 2-3 nucleotides; it is bactericidal and acts on both intracellular and extracellular organisms. There is no cross resistance with other anti-TB drugs. Highly effective concentrations are obtained in all tissues and moderate levels are found within the cerebrospinal fluid. Dosage varies upon individual circumstances but in general, for daily or intermittent use, 10 mg kg^{-1} up to a maximum 600 mg is the accepted regimen. Rifampicin has several adverse effects, especially in older patients or those with HIV/AIDS, leading to recurrence of hepatitis infections (Cole, 2002; Espinal *et al*., 2001; Zhang *et al*., 1992).

Pyrazinamide is also an effective general bactericidal prodrug. Pyrazinamide diffuses into *M. tuberculosis* where the enzyme pyrazinamidase converts pyrazinamide to the active form pyrazinoic acid. Pyrazinoic acid slowly leaks out of the cell and converts to protonated conjugate acid, which easily diffuses back into the bacilli and accumulate. The overall effect is fatty acid synthesis is reduced by pyrazinoic acids binding to the ribosomal protein S1 (RpsA) and inhibiting trans-translation. In daily use, dosage is restricted to mg kg^{-1}, and for intermittent, 35 mg $kg^{-1}$is used and is taken orally. There are many adverse effects with the use of pyrazinamide, such as liver damage and pain in the joints (Gie 2003; Storia 2008; WHO 2003).

Streptomycin is a protein synthesis inhibitor. It binds to the small 16S rRNA of the 30S subunit of the bacterial ribosome, interfering with the binding of formyl-methionyl-tRNA to the 30S subunit. This leads to codon misreading leading to frame shift mutations, defective protein synthesis and ultimately cell death. Streptomycin is not absorbed from the intestine so is most often administered by intra-muscular injection, where it readily diffuses into most body tissues. Dosage is commonly 15 mg kg^{-1} to a maximum of 1 g, as it is excreted almost entirely through the kidney; the dose has to be lowered in patients with poor renal function and in older age groups. There are several adverse reactions; streptomycin can cross the placenta and cause deafness in unborn children and, as such, use of streptomycin during pregnancy is not recommended (Gie 2003; Storia 2008; WHO 2003).

Ethambutol is a bacteriostatic drug, and is mainly used to prevent the emergence of drug resistance to the other drugs mentioned and its mode of action relies upon the disruption of arabinogalactan. Arabinogalactan attaches to the mycolic acids of the mycobacterium cell wall to form mycolyl-arabingalactan peptidoglycan in the cell wall. Ethambutol inhibits the enzyme arabinosyl transferase, which is essential for arabinogalactan synthesis and leads to increased permeability of the cell wall. It is given orally at a dosage of 20 mg kg^{-1} for daily use and for intermittent 30 mg kg^{-1} three times per week. Ethambutol has serious adverse reactions and as such, large doses are no longer given. The main side effect is progressive loss of vision caused by retrobulbar neuritis. It has been reported that some patients notice decreased vision within the first few days of treatment. If unnoticed and the treatment continues, the likelihood of the patient going blind is extremely high. Eye damage is exaggerated in patients with renal failure and as such, treatment is not recommended for these patients (Rieder *et al*., 2009)

As mentioned, ethambutol is used mainly to prevent the emergence of drug resistant MTB and forms the basis of combination therapy against MTB. If anti-TB drugs were given alone, resistant bacteria would replace the sensitive organisms. However, in combination therapy, resistant species can be killed earlier in treatment with the other drugs, preventing an abundance of resistant species in the host (Gie 2003; Storia 2008; WHO 2003).

1.6 The burden of drug resistance

Ever since the discovery of antibiotics and their use against bacteria, resistance has been a problem. In most cases, the time between antibiotic deployment and the antibiotic resistance is less than 10 years. In the following Figure (2.8) a timeline of the deployment of several antibiotics is shown against a timeline of observed bacterial resistance.

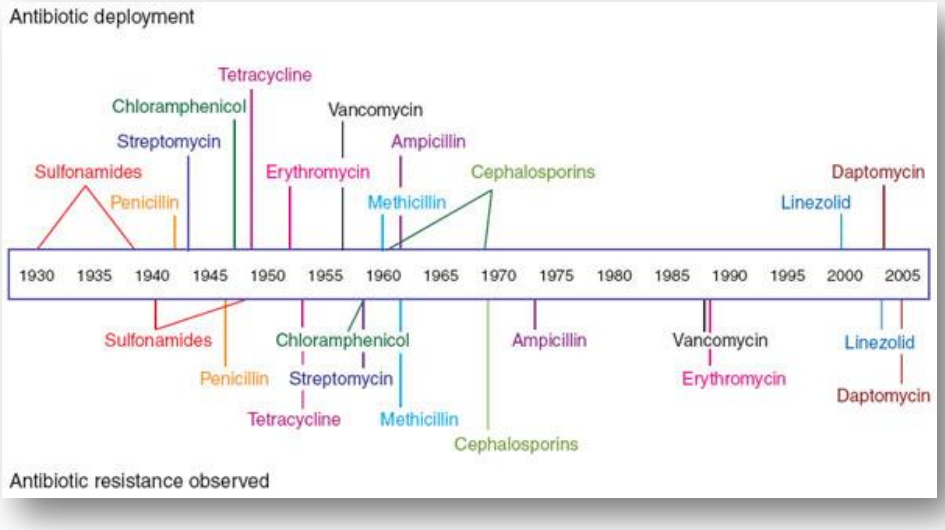

Figure 1. 9 Timeline of antibiotic deployment and subsequent antibacterial resistance. Adapted from (Meziane-Cherif and Courvalin, 2014)

The first antibiotic that was discovered to be inhibitory towards the growth of *M. tuberculosis* was streptomycin. Streptomycin was first introduced during 1943 and consequently the first resistant strain was observed by Pyle in 1947. In 1948 the British Medical Research Council (MRC) published a report which stated that streptomycin therapy for pulmonary TB had similar mortality rates for treated and untreated patients. Among patients who had been treated with streptomycin the majority who had died had experienced relapse that was the result of an antibiotic resistant strain (Gandhi *et al*., 2010).

Resistance mechanisms are either intrinsic or acquired. In the case of MDR TB, acquired resistance is seen in genetic mutations, encoding either the drug target or the enzymes involved in drug activation. The basis of resistance in some anti-TB agents is still not fully understood, as in the case of streptomycin. Streptomycin resistance emerges through a change in the bacterial binding site for streptomycin in the ribosome; this process has only been seen in half the strains studied to date (Espinal *et al*., 2001; Zhang *et al*., 1992).

Mutations in the *pncA* gene (encoding pyrazinamidase), are thought to be a major mechanism of PZA resistance in MTB, but resistant strains containing the wild type gene have been described. Moreover, the primary targets of activated isoniazid are enzymes involved in the biosynthesis of cell wall mycolic acids. Mutations associated with changes in these targets have been shown to contribute to isoniazid resistance (Stewart *et al*., 2003; Zhang *et al*., 1992).

Resistance to anti-TB drugs continued to be recognised as a clinical problem through the latter part of the 21st century. As a result, MDR and XDR TB are now becoming a major threat to health worldwide, accounting for almost 3 % of all newly reported cases of TB (Sayer, 2009). There have been several reports speculating the emergence of totally drug resistant TB (TDR-TB) in India, Iran and Italy (Miglori, 2007; Rowland, 2012; Udwadia *et al*., 2012; Velayati *et al*., 2009).

Multidrug resistant TB is classed as MTB that is resistant to at least isoniazid and rifampicin. In comparison, extensively drug resistant TB (XDR-TB) is MDR-TB that is also resistant to one or more of; quinolones, kanamycin, capreomycin and/or amikacin. The principles for treatment are the same for both types; however, XDR-TB is associated with a much higher mortality rate because of the reduced number of effective treatment options. Epidemiology of XDR TB is not very well understood, but it is generally believed that it transmits easily between populations which are more susceptible to MTB infection (Miglori, 2007; Rowland, 2012; Udwadia *et al*., 2012; Velayati *et al*., 2009).

It has been hypothesised that without the development of new antimicrobials there will be an exponential increase in the numbers of bacterial related deaths due to resistant strains of antibiotics. Currently, strains of MRSA and *C. difficile* continue to be a major cause of healthcare associated infection (HAI) which is proving difficult to treat. (Gould, 2009)

To encourage the development of the new antimicrobials, the UK government has recently published a review of current drug resistance and the predicted effects on mortality rates worldwide by 2050 if no new classes of antibiotics are developed (Figure 2.9).

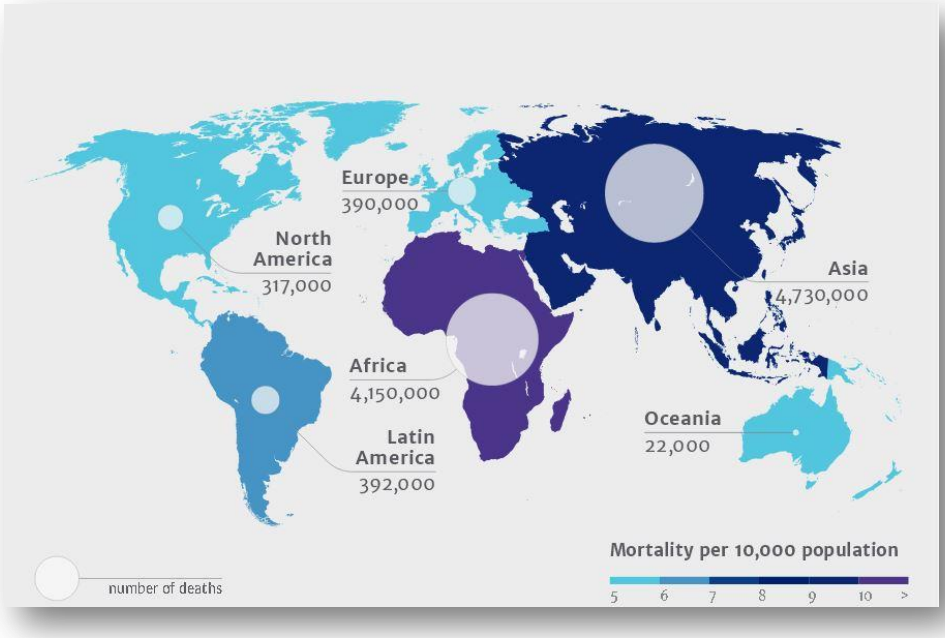

Figure 1. 10 Predicted mortality rates due to antibiotic resistant bacterial infection by 2050 (O'Neil, 2014)

It is apparent from Figure 1.10 that without the development of antibacterials, or indeed finding ways to potentiate the activity of redundant antibiotics, then mortality rates of over 10 million per annum could be expected by 2050 (O'Neil, 2014).

Comment [DH1]: hould this sentence be here?

1.7 Using natural products to potentiate the activity of antibiotics

When antibiotics first became available over a century ago their popularity led to their overuse and misuse; as such, over the last 20 years it has become clear that their effectiveness is reducing as pathogens evolve resistance against them. Bacteria can acquire multiple drug resistance, which requires the need of antibiotics with multiple modes of action. Due to increased drug resistant strains of bacteria such as *M. tuberculosis* and methicillin resistant *Staphylococcus aureus* there has been renewed interest in natural products as potential sources of novel antibiotics (Cragg and Newman, 2013; Coates and Hu, 2007; Pauli *et al*., 2005)

Natural products are a novel source of resistance modifying agents (RMAs); these are sets of compounds which have multiple modes of action combating the bacterium's intrinsic and acquired resistance mechanisms. The discovery and development of RMA's could pave the way to reduce the spread of resistant bacteria and to 'recycle' well established antimicrobials, which are often cheaper and less toxic than newly derived antimicrobials (Abreu *et al*., 2012; Lewis and Elvin-Lewis, 1995).

Antimicrobial synergy implies the beneficial interaction between two antimicrobial drugs. Exceeding additive effects can take several forms. One compound may potentiate the activity of the other at a biochemical level; one may assist the other in order to penetrate the bacterial cell; one may protect the other from destruction; or the two compounds may act on separate sections of the bacterial population (Acar, 2000)

The use of natural products with commonly used antimicrobials is a well-established principle and is the reason for combination therapy in TB (Rieder *et al*., 2009). By using two or more agents that have different targets in the bacterial cell, resistance can be reduced. Synergy between these compounds could result in greater efficacy using two agents than individually and may lead to lower host exposure to potentially toxic compounds (Acar, 2000; Hemaiswarya *et al*., 2008).

Plants are known to produce a number of low molecular weight (MW <500) compounds which have been shown to be antibacterial, albeit in some cases considerably weaker than antibiotics produced by bacteria and fungi (Abreu *et al*., 2012)

A study undertaken by Stermitz and colleagues reviewed the interaction between berberine and 5^{o} methoxyhydnocarpin, both of which are produced by berberry plants. Berberine, a hydrophobic alkaloid that intercalates into DNA, is ineffective as an antibacterial because it is readily extruded by pathogen encoded multidrug resistance pumps (MDPs). Hence, the plant produces 5^{o} -methoxyhydnocarpin that blocks the MDP pump. This combination is a potent antibacterial agent (Lewis and Ausubel, 2006; Stermitz *et al*., 2000).

Another way of overcoming resistance mechanisms such as β-lactamase enzymes; modification of target sites and efflux pump inhibition is to use a combination of antibiotics with multiple mechanisms of action. Inhibitors of beta lactamases have been long known and therefore are administered with antibiotics as co-drugs (Chambers *et al*., 1995). This has been especially seen in overcoming the resistance of penicillinase, by administering clavulinc acid with the drugs sulbactam and tazobactam (Lee *et al*., 2003)

However, tuberculosis and other mycobacterial infections continue to rise and are currently considered a primary health threat. Coupled with the long treatment regime is the lack of new antibiotics with different modes of action (O'Neil, 2014; Stewart *et al*., 2003)

Current antimycobacterial treatment is the same as that over 40 years ago, requiring a minimum of 6 months of multidrug chemotherapy. Novel targets are being identified alongside developing better drugs for known targets. Synergistic interaction between plant derived natural products and anti-tuberculosis drugs is gaining attention from the scientific community, but reports of synergistic combinations are still limited. Totarol, ferulenol and plumbagin were observed to increase the potency of isonicotonic acid hydrazide by fourfold against *Mycobacterium* sp. A napthoquinone 7-methyljuglone, isolated from the roots of *Euclea natallensis,* in combination with isoniazid or rifampicin, resulted in a four to six fold reduction in the MIC of the synthetic drugs (Mossa *et al*., 2004).

Comment [DH2]: Repetition of reporst

Several methods of detecting synergy are known but it has been reported that the accurate prediction of a synergistic, additive, indifferent or antagonistic combination between a drug and a natural product is crucial. The recognised gold standard is the checkerboard method where combinations of two antimicrobials in concentrations equal to, above and below their minimal inhibitory concentrations for the test organisms are assayed (Acar, 2000).

The drug combination in which growth is inhibited is taken as the effective MIC of the combination. Taking the effective MICs of each test compound in combination allows for the fractional inhibitory concentration (FIC) to be calculated. The FIC values can then accurately predict synergism, additive, indifferent or antagonism (Hall *et al*., 1983a).

Therefore, whilst screening plants for antibacterial activity is promising, there is a need for identifying potential resistance modifying agents, which alone may show little antibacterial activity but in combination with antibiotics can increase the susceptibility of resistant organisms. Little research has been undertaken in identifying plant and antibiotic combinations active against species of mycobacterium. This could be due to limited understanding of the mechanism of action of drugs and plant products or the lack of plant extracts inhibitory towards the growth of these organisms.

1.8 Aim and objectives of this study

1.8.1 Thesis Aim

The aim of this these was to determine if hop extracts have inhibitory activity towards clinically relevant bacteria such as *Mycobacteria*, identify active compounds and determine if these compounds can potentiate the activity of antibiotics against drug resistant organisms.

1.8.2 Thesis objectives

The objectives of this thesis were to:

- Develop and optimise a hop extraction process.
- Develop assays to determine the antibacterial activity of hop extracts against a range of clinically relevant bacteria.
- Identify differences in antibacterial activity between hop variants.
- Identify compounds responsible for antibacterial activity.
- Determine the inhibitory effect of hop extracts against multidrug resistant *Mycobacteria.*
- Develop and optimise assays to determine synergy, co-action, indifference and antagonism between antibiotics and hop extracts
- Examine the ability of hop extracts to potentiate the activity of antibiotics against multidrug resistant *Mycobacterium*
- Investigate the antibacterial mode of action of hop extracts and antibiotics using scanning electron microscopy.

Chapter 2

General Materials and Methods

2.0 General Materials and Methods

2.1 General Materials

2.1.1 Sources of chemicals and reagents

All chemicals and reagents were purchased from Sigma Aldrich Ltd, UK, unless otherwise stated in the text.

All solvents were from purchased from Thermo Fischer Scientific Ltd, UK unless otherwise stated in the text.

2.1.2 Biological culture media

Middlebrook 7H10 agar and Middlebrook 7H9 broth (for the growth of mycobacterium) and Luria broth (LB) and Luria agar (LA) for the growth of all other organisms were purchased from Thermo Fischer Scientific Ltd, UK. These agars and broths were made according to the manufacturer's instructions and sterilised at 121 °C for 15 minutes at 15 pounds per square inch (psi) pressure (Prestige, 9 L classic bench top autoclave, LabShop, UK)

2.1.3 Diluents

Deionised water (diH_2O) was obtained from an ELGA purelab option BP15 dispenser (ELGA labwater, UK). The inorganic content of the water at 25°C was between 1-15 MΩ-cm with a total organic carbon content of <30 ppb. It was sterilised at 121 °C for 15 minutes at 15 psi (Prestige, 9 L classic bench top autoclave, LabShop, UK) to form sterile distilled water (SDW).

Phosphate buffered saline (PBS) was prepared by dissolving 2 tablets in 500 mL of diH_2O once mixed, the solution contained 0.01 M phosphate buffer at pH 7.4 (composed of 0.0027 M potassium chloride and 0.137 M sodium chloride). Solutions were sterilised by autoclaving at 121 °C for 15 minutes at 15 psi (Prestige, 9 L classic bench top autoclave, LabShop, UK).

2.2 General Methods

2.2.1 Revival of bacterial cultures from Microbank™ Cryo-protective beads

To generate a working culture, vials containing Cryo-protection beads (Pro-Lab Diagnostics Ltd, UK) of the appropriate bacterium were removed from storage at -80 °C and defrosted on ice for 15 minutes. Once partially defrosted a single bead was removed using a sterile 10 µL inoculation loop (Fisher Scientific, UK) and inoculated into the appropriate broth for the appropriate time (Section 2.2.2).

2.2.2 Production of a bacterial suspension

Bacterial cultures were grown at 37 °C in broth within a shaking incubator (Thermo Fischer, UK) at 150 revolutions per minute (RPM) for 72 hours (*M. bovis* var. BCG; *M. abscessus* (all strains) and *M. smegmatis*); 18 hours (*Escherichia coli* NCTC 1093T; *Bacillus subtilis* NCTC 10073; *Staphylococcus aureus* NCTC 13277 and methicillin resistant *Staphylococcus aureus* NCTC 10442.

2.2.3 Determination of the concentration of bacterial suspensions

Centrifuge tubes containing 20 mL of LA or Middlebrook broth were inoculated from a bacterial slope culture using a 10 µL inoculation loop. The inoculated broths were then incubated ta 37 °C for the desired amount of time (2.2.2) in a shaking incubator at 150 RPM. After incubation the broths were centrifuged at 2600 x g for 15 min (Avanti J-20 XP, Beckman Coulter Inc. UK). The supernatant was removed and the pellet re-suspended in sterile PBS to produce a stock solution with an optical density (OD) of 0.8 as determined by a spectrophotometer (Ultrospec 3100 Pro, Amersham Biosciences Ltd., USA) set a wavelength of 600 nm (OD600). The neat bacterial culture stock was diluted with PBS to produce a range of OD600 between 0.1 – 0.8.

Simultaneously, a viable count of the colony forming units (CFU)/mL for each dilution was performed using the drop counting method by Miles *et al.,* (1938). The method by Miles *et al.,* (1938) was performed as follows. A 10 µL aliquot of each culture dilution was pipetted onto a sector of a circular TSA plate in triplicate and incubated for 24 h. Following incubation, colonies were counted on each sector that contained between 3 - 30 countable cfu's per spot and the average of the three spots was calculated.

The log cfu/mL^{-1} was then calculated by the following equation:

$$\text{Log cfu/mL}^{-1} = \frac{\text{Mean cfu}}{\text{Dilution Factor}}$$

The results were then recorded into an Excel® spread sheet and an OD_{600} versus CFU/mL graph was constructed for each bacterium. This produced a linear equation to predict the cfu/ml in a sample based on the optical density of the solution at 600 nm. This was repeated for each bacterium six times.

2.2.4 Preparation of a working culture

Bacteria were cultured as described in Section 2.2.3 and diluted in PBS to an OD_{600} indicative of a concentration of 1 x 10^9 cfu/mL^{-1}. This working culture was then further diluted in PBS using serial dilution to achieve the cfu/mL^{-1} required for subsequent assays.

2.2.5 Production of a bacterial slope culture

Bacterial slopes are routinely used as a long-term working culture (Reed, 2007). Firstly, 60 ml of the appropriate agar solution was prepared as per manufacturer's instructions and cooled to 60° C in a water bath (Thermo Fischer, UK). After cooling, 20 ml of molten agar was aseptically dispensed into each, of three, 50 mL centrifuge tubes (Fischer scientific, UK). Each tube was then positioned at a 45° gradient so that the agar had the greatest surface area and then allowed to set.

The agar slope was then inoculated with a 10 µL loop of the desired bacterial suspension (Section 2.2.2) and incubated statically at 37° C until colonies were visible. The bacterial suspension had been checked for purity, using the streak plate method (Section 2.2.6). Isolated colonies were then Gram stained (Section 2.2.8.1) or acid fast stained (Section 2.2.8.2) dependant on test bacteria. Cultures were then stored for periods no longer than 21 days at 2° - 5° C.

2.2.6 Streak plate method for determination of culture purity

To ensure bacterial purity and isolate individual colonies, the streak plate method was used. The method comprises of removing 10 µL of a bacterial suspension (Section 2.2.2) and transferring this to an appropriate agar plate. A series of streaks then spreads the concentrated suspension across an agar plate in order to obtain individual colony forming units (CFU's) on the last streak. After the procedure was completed the agar plate was incubated at 37 °C for the appropriate amount of time and colonies were inspected for uniformity.

2.2.7 Staining methods to determine purity and identity of micro-organisms

The Gram stain, developed by Hans Christian Joachim Gram in 1884 is the primary identification tool used in microbiology (Reed, 2007). The Gram stain and the Ziehl-Neelson (acid fast stain) are used to differentiate between Gram positive, Gram negative and *Mycobacterium* species. The Gram stain bases bacteria into two groups; bacteria that retain the initial crystal violet stain (purple) are said to be Gram positive, whereas those that are decolourised and stain red with safranin/carbol fuschin are said to be Gram negative (Beveridge, 2001). As previously described *Mycobacteria* possess mycolic acids in the outer cell membrane and are therefore not stained by the Gram stain (Reed, 2007). In this case the Ziehl Neelson stain is used (Section 2.2.8.2). (Beveridge, 2001)

2.2.8 Preparation of bacterial cultures for both Gram stain and Ziehl-Neelson stain

Colonies were isolated from a bacterial suspension and grown on solid media using the streak plate method. A single colony of the chosen organism was then mixed with 50 µL of sterile distilled water (SDW) on the centre of a sterile glass slide (Fisher-Scientific, UK). This turbid suspension was then spread across the slide using a fresh 10 µL inoculation loop, the slide was then allowed to air dry within a laminar flow cabinet for 5 minutes, then it was passed through a Bunsen burner 3 times to heat fix the colony to the slide.

2.2.8.1 The Gram Stain

1. The single bacterial colony is heat fixed
2. The slide is flooded with 2 % crystal violet (in 20 % ethanol / SDW solution) for no longer than 60 seconds.
3. The crystal violet was then poured off and the slide flooded with Grams iodine, for no longer than 60 seconds.
4. Slides were then rinsed with SDW to remove excess iodine.
5. Acetone was then added drop wise, and was left for a maximum of 3 seconds.
6. The slide was then rinsed with SDW
7. 2.5 % safranin (95 % ethanol/SDW) was then added as a counter-stain which was then left for 10-15 seconds and was then rinsed with SDW to remove excess safranin.
8. The smear was then left to air dry within a laminar flow cabinet, before being examined under oil immersion x 100 magnification.

2.2.8.2 Ziehl-Neelson Stain for *Mycobacterium*

1. The single bacterial colony is heat fixed
2. The inoculated glass slide is then flooded with carbol fuchsin and held over a breaker of steaming SDW at 60°C .
3. The slide was then steamed for 5 minutes whilst carbol fuschin was added to ensure that the slide remained flooded.
4. The slide was then rinsed with dH_2O to remove excess carbol fuchsin.
5. The slide was then decolourised with 95% ethanol containing 5% hydrochloric acid.
6. Slide was then immediately rinsed with dH_20.
7. The slide was then counter-stained with methylene blue, and left for 20-30 seconds.
8. The slide was then rinsed with dH_2O to wash away excess methylene blue.
9. The slide was then allowed to air dry within a laminar flow cabinet.
10. After the staining procedure, the slides were examined directly under oil immersion at x 100 magnification (Reed, 2007).

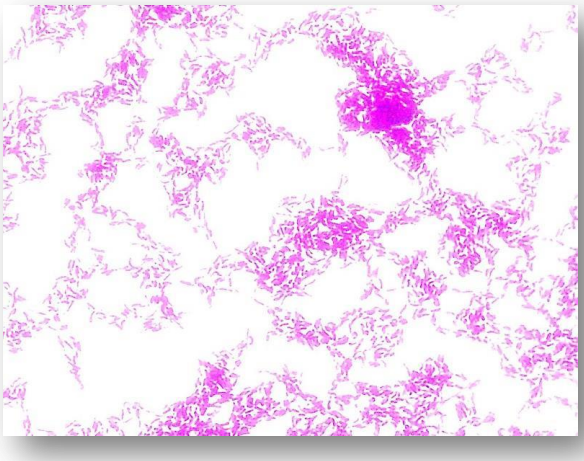

Figure 2. 1 Demonstration of a Ziehl-Neelson (acid fast) stain of *Mycobacterium smegmatis* 8159

2.3 Statistical Analysis

All results presented in this study are a mean of at least three separate repeats unless otherwise stated. All statistics were performed using IBM SPSS Statistics version 20.

2.3.1 Independent samples t-test

To determine the statistical difference in the means between two groups, the parametric independent students t-test was used if the following assumptions were met (Cohen, 1988).

1. The observations of the data were independent from each other.
2. The results follow normal distribution and pass normality tests.
3. The data sets to be compared show homogeneity of variance.

To assess normality, a histogram plot of the data was inspected to determine if a normal bell shaped curve was observed with minimal skewedness. The Q-Q plots and de-trended Q-Q plots were alos reviewed in conjunction with the results of the Kolomogorov-Smirnov and Shapiro-Wilk tests for normality.

Levines test of the equality of variances was conducted to determine if the data sets showed homogeneity of variance.

If any of the assumptions were violated, an appropriate transformation of the data was attempted (Log_{10}, square root, etc) to normalise the data or the equivalent non-parametric test will be used (e.g. Mann-Whitney U).

To determine the effect of size between the two groups, or the magnitude of difference, the eta-squared value (N^2) was determined by the following equation:

$$\eta^2 = \frac{t^2}{t^2 + (n1 + n2 - 2)}$$

Where t= t value from t-test, $n1$ = sample size of group 1 and $n2$ – sample size of group 2. To interpret the N^2 value the guidelines set out by Cohen (1988) shown in Table 2.1 were implemented.

Effect Size	N^2
Small	0.10-0.29
Moderate	0.30 – 0.49
Large	>0.5

Table 2. 1 Interpretation of effect size of N^2 between two statistically different groups (Cohen 1988).

2.3.2 One Way Analysis of Variance (ANOVA)

The parametric one-way ANOVA test was used to determine if there was a significant difference in the means between two or more groups of data if the assumptions described in Section 2.3.1 were met.

If a significant difference between groups was found, Tukeys honestly significant difference (HSD) test was used to determine which groups were significantly different from each other.

2.3.3 Pearson's Correlation

Correlation analysis was used to determine the strength and direction of a linear relationship between two variables. Pearson's correlation of parametric correlation was used if the following assumptions were met (Cohen 1988):

1. The relationship between two or more data sets is linear.
2. Observations in the data sets were independent.
3. Each data set in the analysis followed normal distribution.
4. The data sets showed homoscedasticity.

The strength of the relationship between variable was determined by the guidance set of by Cohen (1988). The coefficient of determination (R^2) was calculated by squaring the Pearson product-moment correlation coefficient (r) obtained between two variables.

Chapter 3

Determination of the antibacterial effects of hops against clinically relevant bacteria

3.0 Determination of the antibacterial effects of hops against clinically relevant bacteria

3.1 Introduction

Hops (*Humulus lupulus*) have been recognised for centuries as an important means of flavouring beer (Cornelison, 2008). Recent research has focussed on using hop extracts to improve the aroma, the stability of beer foam and as a preservative in a diverse range of food products such as meat products (Hieronymus, 2012). Indeed, the reason for using hops in beer is due to the fact that they contain antibacterial compounds such as humulone (alpha acid) and lupulone (beta acid). Both acids are released into the beer during the brewing process and increase both the shelf life of the product and the taste of the beer. The bitterness of the beer is proportional to the concentration of alpha acids, it is for this reason that a range of hop variants have been developed to allow brewers to tailor the flavour of a particular beer.

Given that alpha and beta acid content of hops has been linked to antibacterial activity (Acworth *et al*., 2012; Shen and Sofos, 2008) it is expected that the hop variants with the highest alpha and beta acid content would demonstrate the greatest antibacterial activity. In addition to being used in brewing, hops have a long history in herbal medicine and have been used to treat a variety of conditions such as stomach cramps, indigestion, insomnia and bacterial infections (Batchvarov and Marinova, 2001; Sumner, 2000). In this chapter, the medicinal properties of this plant, specifically its antibacterial activity against a range of medically important bacteria is investigated.

Of particular interest in the context of this study is the bacterium *Mycobacterium tuberculosis*. As previously described it is an acid fast organism with a thick waxy outer cell wall comprised of several mycolic acids, these contribute to the survival of the pathogen within human macrophages.

Due to the virulence and long incubation times of *M. tuberculosis*, a simulant organism, *Mycobacterium smegmatis* was employed to model the sensitivity of the pathogen to antibacterial compounds. *M. smegmatis* is well described as a model organism used within many screening assays. Unlike *M. tuberculosis*, *M smegmatis* is fast growing, requiring 72 hours incubation at 37 °C to reach 1 x 10^8 cfu/ml compared to *M. tuberculosis* which requires up to 6 weeks to achieve a similar level of growth (Chaturvedi *et al*., 2007b).

Mycobacterium smegmatis is non-pathogenic for healthy humans and as a consequence can be handled and manipulated in a Category II laboratory (Chaturvedi *et al*., 2007b; Tyagi and Sharma, 2002). Finally studies have shown that both bacteria demonstrate a similar level of sensitivity to antibacterial compounds and thus *M. smegmatis* has been used by a number of groups as a tool with which to identify compounds with the potential to be active against *M. tuberculosis* (Chaturvedi *et al*., 2007b).

A related organism, *M. bovis* is responsible for the animal form of the disease; bovine tuberculosis (bTB). As previously described, bovine infection with this pathogen is estimated to cost the UK economy over £100 million per year. *M. bovis* like *M. tuberculosis* is also classed as a Category III pathogen and replicates at a similar rate making it an impractical bacterium to use in high through put screening assays. In contrast, the vaccine strain of *M. bovis, Mycobacterium bovis* Bacillus Calmette-Guérin (BCG) rarely causes disease in healthy individuals (Category II organism) and replicates in the laboratory at a similar rate to *M. smegmatis* (Fitzgerald *et al*., 2010) In vitro studies have shown that BCG has a similar level of antibacterial sensitivity to anti-tuberculosis compounds making it a suiTable model organism with which to screen for antibacterial activity (Fitzgerald *et al*., 2010; Taneja and Tyagi, 2007).

Aside from *Mycobacteria* the sensitivity of other pathogenic bacteria to hop compounds was also determined:

Staphylococcus aureus is a Gram positive cocci and considered a commensal organism that is also an opportunistic pathogen within humans (Enright, 2003). One of the ways *M. smegmatis* differs from *S. aureus* is in the nature of its cell wall; mycobacteria have a thick waxy outer layer comprised of mycolic acids, which has been shown to increase their virulence (Murray *et al*., 2014), whereas Gram positive organisms have an outer layer of peptidoglycan. This difference in the cell wall has been shown to have an effect on the sensitivity of the organism to antibacterial compounds found in beer (Caballero *et al*., 2009).

Unfortunately the wide spread use of antibiotics has resulted in the emergence of variants of this pathogen which are resistant to a wide range of antibiotics. One such variant, Methicillin resistant *Staphylococcus aureus* (MRSA), has developed resistance to beta-lactam antibiotics such as penicillin and has become a major cause of healthcare acquired infection (Archer, 1998; Enright, 2003).

It is routinely used in clinical laboratories to determine the efficacy of antibiotics and was included in this study as a reference strain with which to assess the antibiotic activity of hop derived compounds. A second representative of Gram positive bacteria, *Bacillus subtilis* was also included in this study. In contrast to Staphylococci these bacteria form rods rather than cocci and rarely cause disease in healthy individuals (Reed, 2010). The bacteria has previously been used to screen plant extracts for the presence of antibacterial compounds (Rojas *et al*., 1992).

The final organism examined in this chapter, *Escherichia coli,* is considered a model bacterium for Gram negative bacteria which differ from Gram positive and acid fast organisms in that the cell wall contains an additional layer called the outer membrane (Jackson, 2014). This outer membrane contributes to antibiotic resistance by hindering the uptake and passage of compounds to the interior of the cell (Duffy *et al*., 2008).

This group of bacteria is made up of a large number of different serotypes the majority of which are harmless to healthy individuals, however some serotypes such as *E. coli* 0157, can lead to morbidity (Duffy *et al*., 2008). As with *B. subtilis*; *E. coli* is inexpensive to grow on routine media, grows relatively quickly compared to Mycobacteria and non-pathogenic variants are routinely used to determine antibiotic sensitivity (Duffy *et al*., 2008). The antibacterial compounds found in hops have been reported to show little, if any, antibacterial activity against Gram negative organisms (Beutler, 2009; Colegate and Molyneux, 2007; Dias *et al*., 2012).

This chapter describes the optimisation of an aqueous extraction and agar based assay that was used to determine the antibacterial activity of 50 hop variants against a range of bacterial species including Mycobacterium. The correlation of observed antibacterial activity and (supplier) stated alpha and beta acid content of each hop variety was investigated, assessing the hypothesis of (Caballero *et al*., 2009; Simpson and Smith, 1992) that the alpha and beta acid concentration make a major contribution to the antibacterial activity of beer.

3.2 Materials

3.2.1 Bacterial cultures

3.2.2 Mycobacterium smegmatis NCTC 8159 T

Mycobacterium smegmatis was purchased from the Health Protection Agency (HPA) culture collections service (Public Health England, Porton Down, UK). The strain was first identified by Trevisan, 1889 and isolated from smegma found in soil and water (Dostal *et al*., 2003). The bacterium has successfully been used as a safer, faster growing simulant of *M. tuberculosis* (Chaturvedi *et al*., 2007a; Tyagi and Sharma, 2002). Cultures were stored in 10 % glycerol on Microbank™ Cryo-protective beads (Pro-Lab Diagnostics Ltd, UK) at -80 °C until required.

3.2.3 Mycobacterium bovis Var. BCG NCTC 5692

A culture of *Mycobacterium bovis* BCG (Bacillus Calmette-Guerin*)* was purchased from the HPA culture collections service (Public Health England, Porton Down, UK). The original *M. bovis* BCG strain was developed by Calmette and Guerin between 1908 and 1919 by carrying out 231 passages of virulent *M. bovis* leading to spontaneous attenuation resulting in a strain of *M. bovis* that was no longer virulent in humans or animals. *M. bovis* BCG has been used as a surrogate organism for *M. bovis* and *M. tuberculosis* (Khan and Sarkar, 2008; Ritz *et al*., 2009). *M. bovis* BCG strains are all known to be resistant to pyrazinamide (Ritz 2009).

The culture was stored in 10 % glycerol on Microbank™ Cryo-protective beads (Pro-Lab Diagnostics Ltd, UK) at -80 °C until required.

3.2.4 *Escherichia coli* NCTC 1093

A culture of *Escherichia coli* NCTC 1093 was purchased from the HPA culture collections service (Public Health England, Porton Down, UK). *Escherichia coli* 1093 is a facultative anaerobic Gram negative bacterium which was originally isolated from human faeces by Dudgeon, 1921 at St. Thomas's hospital UK. The bacterium grows on nutrient broth (NB) and agar (NA) and forms smooth, cream colonies of 3 µm in length.

The bacterium has routinely been used in antibacterial assays (Khalil *et al*., 2013; Shami *et al*., 2013) and has no known resistance to antibiotics routinely used to treat Gram negative organisms. *E.coli* is used within this study as a comparison from acid fast (*Mycobacteria*) strains and Gram positive strains.

3.3.5 *Bacillus subtilis* NCTC 10315

A culture of *Bacillus subtilis* NCTC 10315 was purchased from the HPA culture collections service (Public Health England, Porton Down, UK). *Bacillus subtilis* NCTC 10315 is an aerobic Gram positive bacterium. The bacterium can be grown on NA and NB at 37 °C to form rough, cream, circular colonies of 2 µm in length. The strain used has been studied extensively and is a standard bacterial strain used to represent the Bacillus genre; indeed it has routinely been used to screen potential antibacterial plant extracts (Rojas *et al*., 1992)

3.3.6 *Staphylococcus aureus* (MSSA) NCTC 13277

Staphylococcus aureus NCTC 13277 is a gram positive facultative anaerobic organism that was originally isolated from a human nose (Davies, 1988). The bacterium is grown using NA and NB at 37 °C and forms smooth, cream, circular colonies of 1 µm in length. This organism is a well-established standard strain used as a comparison for other Gram positive organisms or comparing bactericidal susceptibility against the drug resistant strain, methicillin resistant *S. aureus* (MRSA).

3.3.7 Methicillin resistant *Staphylococcus aureus* (MRSA) NCTC 10442

A culture of methicillin resistant *Staphylococcus aureus* NCTC 10442 was purchased from the HPA culture collections service (Public Health England, Porton Down, UK). The strain was originally isolated from a human finger at St. Lukes hospital Guilford, UK. It is a facultative anaerobe which grows on NA and NB at 37 °C. It is well known healthcare associated infection (Enright, 2003), resistance to methicillin or indeed other penicillin class antibiotics is considered genetic and has been understood for over 50 years (Enright, 2003) and requires the resistance gene mecA that encodes a penicillin binding protein PBP2a (Enright, 2003).

MRSA was stored in 10 % glycerol on Microbank™ Cryo-protective beads (Pro-Lab Diagnostics Ltd, UK) at -80° C until required.

3.4 Hop varieties

The hop varieties used in this study are shown in Table 3.1. They were purchased from (themaltmiller.com) and were provided in vacuum sealed, non-transparent packages.

Hop Name/Type	Average estimated alpha acid content (% w/w)	Average estimated beta acid content (% w/w)	Year of Harvest	Country of Origin
Syrian Goldings	2.75	6.40	2010	Slovenia
Hallertauer	3.20	6.60	2010	Germany
Bobek	3.90	N/A	2010	New Zealand
Sonnet	4.10	6.60	2010	USA
Liberty	4.50	5.00	2009	USA
Tettnang	4.50	6.40	2009	Germany
Willamette	4.50	3.00	2011	USA
Fuggles	4.90	N/A	2010	UK
Fuggles Pellets	4.90	N/A	2010	UK
Cascade	5.50	6.70	2010	USA
Cascade Pellets	5.50	6.70	2010	USA
Progress	5.50	N/A	2009	UK
Goldings	5.80	3.80	2010	UK
Boadicea	6.00	3.80	2009	USA
Brambling Cross	6.00	N/A	2011	UK
Summer Pellets	6.00	N/A	2011	Australia
NZ Pacifica	6.10	8.80	2011	New Zealand
Delta	6.50	N/A	2010	USA
Glacier	6.70	3.50	2007	USA
Motueka Pellets	6.70	N/A	2011	New Zealand
Amarillo	6.90	3.40	2010	USA
Amarillo Pellets	6.90	2.50	2009	USA
Challenger	7.60	3.70	2011	UK
Aurora	7.80	2.30	2010	USA
First Gold	7.90	3.50	2011	UK
Northdown	8.00	N/A	2010	UK
Northern Brewer	8.00	N/A	2010	Germany
Centennial Pellets	8.60	N/A	2011	UK
Centennial	8.60	4.40	2010	USA
Perle	9.60	5.40	2010	Germany
New Zealand Rakau	10.80	N/A	2011	New Zealand
Chinook Pellets	11.00	N/A	2010	USA
Simcoe	12.20	N/A	2010	USA
Target	12.50	N/A	2009	UK
Chinnok	12.70	N/A	2011	USA
Nelson Sauvin	12.80	N/A	2012	New Zealand
Galena	13.00	N/A	2010	USA
Green Bullet	13.70	N/A	2010	New Zealand
Summit	14.30	N/A	2011	USA
Magnum	14.50	2.50	2010	Germany
Sorachi Ace	14.90	4.20	2010	Japan
Citra (whole) and Pellets	15.00	N/A	2011	USA
Galaxy	15.10	N/A	2012	Australia
Pacific Jade	15.10	N/A	2011	New Zealand
Colombus	16.20	N/A	2010	USA
Pacific Gem	17.00	N/A	2011	New Zealand
Warrier	18.20	N/A	2010	USA
Apollo	19.50	N/A	2010	USA
Savinjski Goldings	20.75	N/A	2010	Slovenia

Table 3. 1The hop varieties used in this study including country of origin, year of harvest and stated alpha and beta acid content (% w/w)

3.5 Methods

3.5.1 Maceration of hop material

Prior to the extraction of antibacterial compounds the hop material was macerated to increase surface area and maximise the extraction of compounds. For this, hop material was added to a processor (one touch power grater™, DKB Household, UK), fitted with a 4 inch, double blade attachment. The processor was used at full power (400 rpm) for 3 minutes at room temperature and pressure. After processing, the resulting hop material was passed through a 2 mm mesh.

Another method of hop maceration using a pestle and mortar was also employed. Hop material was ground using a pestle until no large fragments of hop material remained. These two methods were compared to identify the most effective maceration method.

3.5.2 Aqueous hop extraction method

To identify the optimum extraction conditions 5 g samples of macerated hop material were suspended in 200 mL of SDW and incubated at a range of temperatures (25, 50, 75 and 100 °C) in a water bath (Thermo Scientific, UK) for a range of times (30, 60, 120, 180 and 240 minutes). Following extraction the solutions were allowed to cool and were then filtered through a Whatman® grade 1 paper using vacuum filtration to remove any solids. The resulting extract was then used immediately in antibacterial assays.

3.6 Antibacterial Assay Methods

3.6.1 Agar Well Diffusion Assay

This is a well-established method and is commonly used to determine antibacterial activity against bacteria and fungi (Kim *et al.*, 1995; Ríos and Recio, 2005)

Firstly, a range of agars (typtone soya agar, nutrient agar, lysogeny agar and brain heart infusion agar) were assessed for their ability to support the growth of test organisms and to allow the diffusion of antibacterial compounds. Agars were prepared as per manufacturer's instructions and autoclaved at 121 °C for 15 minutes at 15 PSI (Prestige, 9 L classic bench top autoclave, LabShop, UK), after which agar was allowed to cool to 45 °C in a water bath (Princeton, Thermo-Scientific). The agar was then inverted gently and 20 mL aliquots were decanted into 45 x 90 mm petri dishes (Fisher Scientific, UK) and placed in a lamina flow cabinet where they were allowed to cool and set.

Plates were then inoculated with 150 µL of a bacterial suspension (Method 2.2.2) standardised to 1 x 10^6 cfu mL^{-1} (Method 2.2.3) and an even spread was made across the plate using a sterile flat sided spreader (Fisher Scientific, UK), plates were then incubated statically at 37 °C (Thermo Scientific, UK) for 3 hours.

A previously sterilised 6 mm borer (Fisher Scientific, UK) was then used to create 4 wells per plate. For each hop extract or test bacterium, newly sterilised equipment was used. In each well 80 µL of the aqueous hop extract was pipetted; in addition to this, on each plate 80 µL of H_2O was also pipetted into a well as a negative control and 80 µL of oleic acid (Sigma-Alrich, UK) at a concentration of 8 µg mL^{-1} in 50 % DMSO as a positive control. This was chosen as a control as it is a naturally derived unsaturated fatty acid which exhibits antibacterial properties (Zheng *et al.*, 2005). Plates were then carefully transferred to a static 37 °C incubator (Thermo Scientific, UK) for 24 or 72 hours depending on the test bacteria. Inhibition of bacterial growth was observed by a clear zone of inhibition around the test well. The size of the zone of inhibition was determined by measuring two diameters at right angles across the zone and subtracting the diameter of the well itself (6 mm). Each assay was performed on 3 separate occasions on each bacterial species with each hop extract unless otherwise specified.

3.6.2 Agar Incorporation Assay to determine the Minimum Inhibitory concentration of hop extracts

The agar well incorporation assay allows for the determination of antibacterial activity for different concentrations of hop extracts (v/v). In order to reduce the dilution effect of the hop extracts on the nutrient content of the medium each agar was made up at double its standard concentration (TSA 12 g 500 mL^{-1} diH_2O) and (Middlebrook 7H10 19 g 450 mL^{-1} diH_2O) and was autoclaved at 121 °C for 15 minutes at 15 PSI (Prestige, 9 L classic bench top autoclave, LabShop, UK) after which it was allowed to cool to 40 °C in a water bath (Thermo-Fischer, UK).

Aqueous hop extracts, sterile distilled water and molten agar where then added to a 24 well plate (Sterlin, UK) in amounts specified in Table 3.2 and mixed to give a final hop extract concentration as described in the table. The agar plates were then allowed to set within a laminar flow cabinet for 30 minutes.

Well Number	SDW Amount (µL)	Agar Amount (µL)	Hop Amount (µL)	% volume of hop extract
A1	0.00	2000	1000	33.00
B1	100	2000	900	30.00
C1	200	2000	800	26.00
D1	300	2000	700	23.00
A2	350	2000	650	21.60
B2	400	2000	600	20.00
C2	450	2000	550	18.30
D2	500	2000	500	16.00
A3	550	2000	450	15.00
B3	600	2000	400	13.00
C3	650	2000	350	11.60
D3	700	2000	300	10.00
A4	750	2000	250	8.00
B4	800	2000	200	6.60
C4	820	2000	180	6.00
D4	840	2000	160	5.30
A5	850	2000	150	5.00
B5	860	2000	140	4.60
C5	880	2000	120	4.00
D5	900	2000	100	3.30
A6	910	2000	90	3.00
B6	920	2000	80	2.66
C6	945	2000	55	0.83
D6	0.00	3000	0	0.00

Table 3. 2 The volumes of double concentrated agar, SDW, and aqueous hop extract used to generate a range of hop concentrations within the agar incorporation assay

After cooling, the plate was transferred to a static incubator (Thermo Scientific, UK) for 3 h at 37 °C. A previously prepared bacterial suspension (Method 2.2.2) was then standardised to 1 x10^6 cfu mL^{-1} (Method 2.2.3) and two 20 µL aliquots were pipetted onto the surface of each well. Plates were then incubated for 24 or 72 h depending on the test bacteria.

By using different concentrations of the hop extract against the test bacteria, the minimum inhibitory concentration (MIC) could be calculated. The MIC was determined by visually inspecting each well after incubation, the lowest concentration of hop extract which inhibited growth, was considered the MIC. To determine the minimum bactericidal concentration (MBC) the surface of each well was swabbed with a sterile wooden swab (Fisher Scientific, UK) and transferred to either 10 mL Middlebrook 7H9 broth in the case of *Mycobacteria* or 10 mL TSB in the case of other bacteria. The inoculated media was then incubated with shaking at 150 rpm at 37 °C for 24 or 72 h dependant on the test bacteria.

After incubation, the absorbance of each solution was read at 600 nm (UV -6010, Thermo Scientific, UK), using sterile broth as a negative control, the lowest concentration of hops that prevented the growth of bacteria was considered the minimum bactericidal concentration (MBC).

The method was repeated on 3 separate occasions for each test bacteria unless otherwise specified.

3.7 Results

Hops are employed in the brewing process to enhance the aroma and flavour of beer and to act as a natural preservative (Hornsey, 2003). It is known that the act of boiling hops with water, malt and barley releases antimicrobial compounds into the beer solution (Sakamoto and Konings, 2003b). This mixture, known as the wort, is heated for a specific length of time and temperature as determined by the brewer to achieve the desired aroma and flavour (Hornsey, 2003).

Hops which are used in brewing are provided in two forms; as un-macerated whole hop cones or as macerated, compressed pellets. The whole hop cones undergo minimal processing. In contrast, the pelleting process involves the hops cones being broken down into a fine power by hammer milling (maceration) after which they are compressed into 5 cm by 1 cm pellets (Hieronymus, 2012).

3.7.1 Optimisation assays for the extraction of antibacterial compounds from hops this is an intro not the results

The effect of hop cone processing along with the time and the temperature of the aqueous extraction of hop plants and the effect on antibacterial activity was determined by using the zone of inhibition assay against *M. smegmatis.*

3.7.2 The effect of maceration on the release of water soluble antibacterial compounds from hops

The degree of maceration and its effect on the release of water soluble antimicrobial compounds from hops was determined. The release of antimicrobial compounds into sterile distilled water from the following substrates was compared; hand macerated whole hop cones, mechanically macerated whole hop cones and mechanically disrupted pelleted hop cones. As a control, non-macerated hop cones were included. Following aqueous extraction (Method 3.5.2) the presence of compounds with antibacterial activity against *M. smegmatis* was determined using a zone of inhibition assay using Middlebrook 7H10 agar (Figure 3.1).

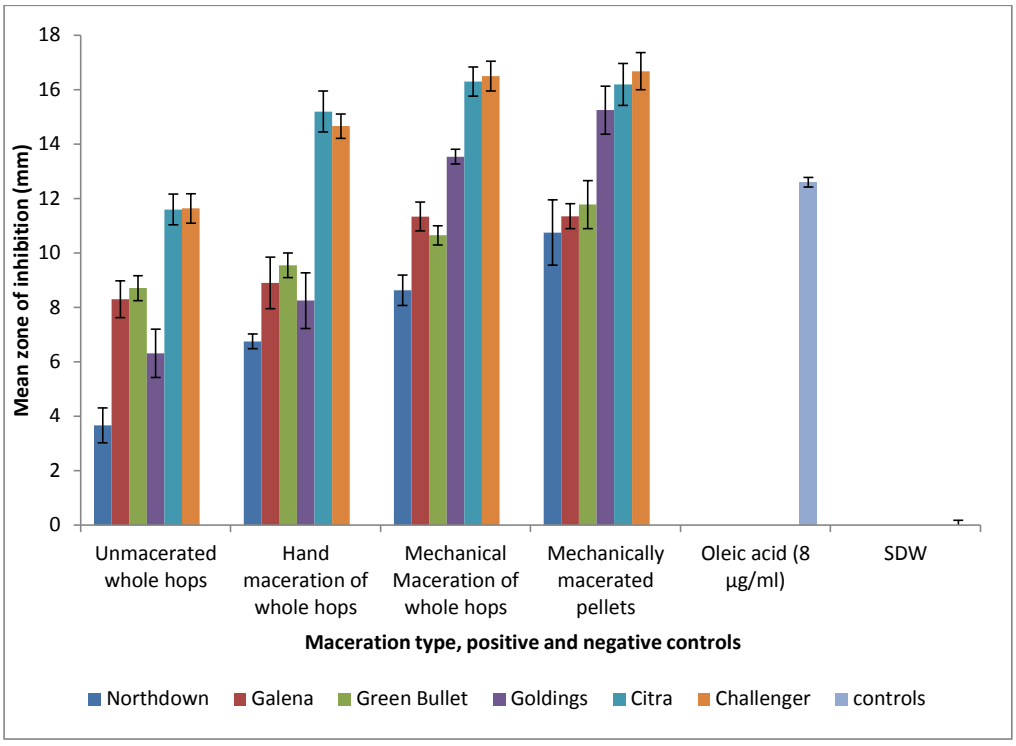

Figure 3. 1 The effectiveness of hand and mechanical maceration on the release of polar antimicrobial compounds from hops following aqueous extraction. Activity was determined using a zone of inhibition assay against *M. smegmatis*, positive controls and negative controls consisted of oleic acid (8 µg mL^{-1}) and SDW. Each assay was repeated 3 times; results presented are the mean, error bars represent standard deviation (±SD).

As can be seen from the data presented in Figure 3.1 physical disruption of the hop cone structure either by hand or using mechanical means resulted in an increase in the antibacterial activity of hop extracts following aqueous extraction when compared to undisrupted whole hops.

To determine if there were statistically significant differences between antibacterial activity and the level of maceration data was subjected to a one way ANOVA. A statistically significant difference ($p = 0.026$) between macerated and un-macerated hops in terms of antibacterial activity was observed. As a statistically significant difference was found a Tukey honest significant difference (HSD) post hoc analysis was used to determine if the type of maceration had an impact on antibacterial activity.

A statistically significant difference ($p = 0.027$) was observed between mechanically disrupted pelleted hop extracts (mean zone of inhibition (ZOI) 14.13 mm ±SD 1.203 mm) and untreated whole hops (mean ZOI = 12.82 mm ±SD 1.29 mm). In contrast no statistically significant difference was observed between hand ($p = >0.05$; mean ZOI 10.66 mm ±SD 1.22 mm) and mechanical ($p= > 0.05$); mean ZOI = 12.82 mm ±SD 1.29 mm) maceration of whole hops.

This result is not surprising given that during the production of hop pellets the whole hop cones are reduced to a powdered form prior to being compressed into pellets (Hieronymus, 2012). This form of processing offers two distinct advantages over the use of whole hops. The first is that the tight compression of the powdered hop material is thought to slow degradation by minimising exposure to oxygen (De Keukeleire, 2000). The second is that the powdering process increases the surface area of the hop material across which, compounds could be extracted.

Based on these results pelleted hops, when available, were used in all future antimicrobial assays. When pelleted forms of a particular variety were not available then whole hops were subjected to mechanical maceration prior to extraction.

3.7.3 The effect of temperature on the release of water soluble antibacterial compounds from hops

As previously mentioned hops are added to the wort and are heated at a temperature determined by the brewer. This heating process is essential as it leads to the thermal isomerisation of alpha and beta acids which enhances the taste of the beer and results in the generation of antibacterial isomers such isohumulone (Acworth *et al*., 2012; Jaskula *et al*., 2008; Stevens and Page, 2004).

To identify the optimum temperature for the maximum extraction of antibacterial compounds, pelleted hop varieties were suspended in SDW for a period of 60 minutes at 25 °C, 50 °C, 75 °C and 100 °C. Antibacterial activity was assessed against *M. smegmatis* in a zone of inhibition assay, with +ve oleic acid 8 µg/mL^{-1} and –ve SDW controls (Figure 3.2).

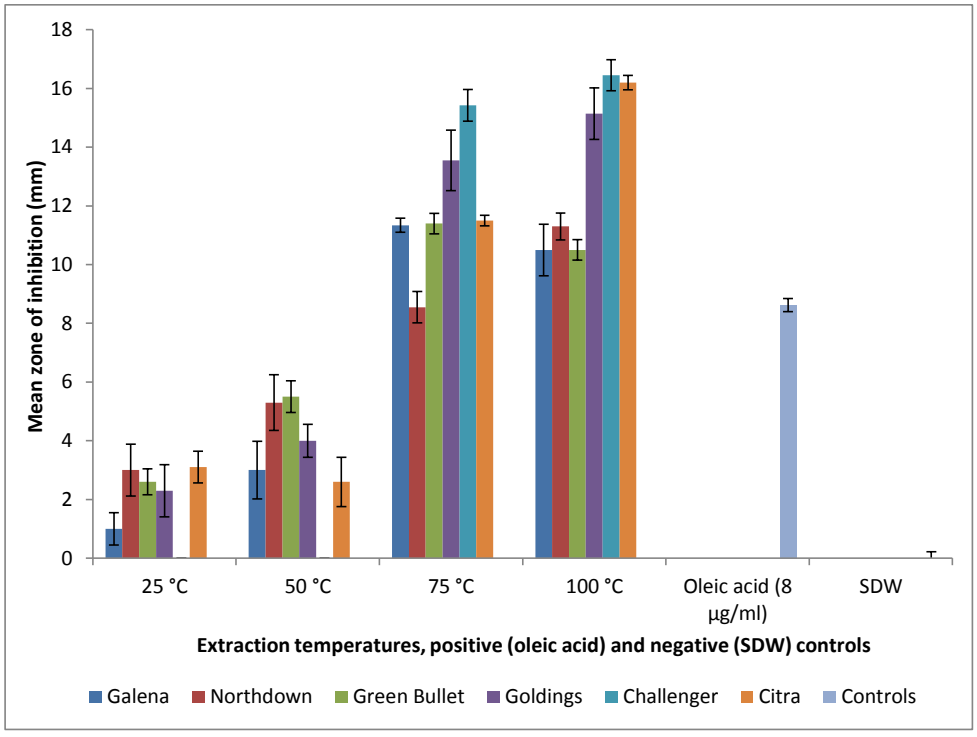

Figure 3. 2 The effect of extraction temperature on the release of antibacterial compounds from different varieties of hops. Pelleted hop variants were suspended in SDW at 25, 50 , 75 and 100 °C for 60 mins. Antibacterial activity was determined using a zone of inhibition assay against *M. smegmatis.* Each assay was repeated 3 times; results presented are the mean, error bars represent standard deviation (±SD).

It can be observed from Figure 3.2 that the antibacterial activity increased with the degree of heating for all hop variants tested.

Using a one way ANOVA analysis it was found that there was a statistically significant difference ($p = <0.05$) between heated (50 °C, 75 °C, 100 °C) and unheated (25 °C) hop extracts and the level of antibacterial activity. To determine the optimum extraction temperature data was subjected to a Tukey HSD analysis.

There was no statistically significant difference in activity between hops extracted at room temperature (25 °C) and 50 °C ($p = 0.693$); and between 75 and 100 °C ($p = 0.699$). There was however a statistically significant difference between hops extracted at 25 °C and 75 °C ($p = <0.05$), between 25 °C and 100 °C ($p = <0.05$) and between 50 °C and 100 °C ($p = < 0.05$).

It has long been understood that heating hops during the wort boiling process (approx. 100 °C) leads to the release of the alpha and beta acids which are responsible for aroma and bitterness (Arnold, 2005). It is these compounds which are isomerised to form *cis* and *trans* isomers which are also thought to be responsible for the antibacterial activity of beer (Caballero *et al*., 2009). Therefore it is possible that the heating during extraction could be directly affecting the isomerisation of the alpha and beta acids and thus the antibacterial activity.

It can be concluded from this data that an extraction temperature of 100 °C does not have a detrimental effect on antibacterial activity; moreover heating leads to antibacterial activity. Based on these results it was decided to adopt an extraction temperature of 100 °C for all future studies.

3.7.4 The effect of extraction time on the release of polar antibacterial factors from hops

In the previous assays the effect of maceration and temperature on the extraction of antibacterial compounds from hops was analysed. To determine if the time that hops were extracted influenced the recovery of antibacterial compounds pelleted hop material was extracted as previously described (Method 3.5.2) at a temperature of 100 °C for 30, 60, 120 and 180 minutes and a zone of inhibition assay was used to determine antibacterial activity against *M. smegmatis*. With +ve oleic acid 8 µg/mL^{-1} and –ve SDW controls

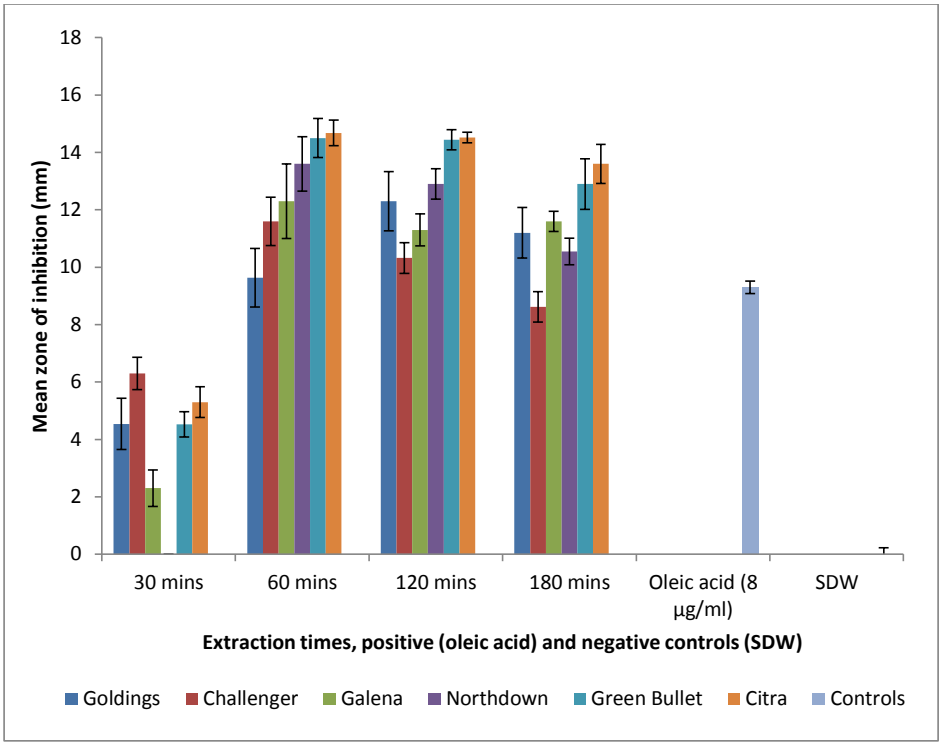

Figure 3. 3 The effect of extraction time of hop extracts at 100 °C and the level of antibacterial activity. Macerated hop material was heated at 100 °C for 30, 60, 120 and 180 minutes and after cooling to room temperature, were tested in a zone of inhibition assay against *M. smegmatis* on three separate occasions. Results represent the mean ZOI, error bars ± SD, positive control (oleic acid) and negative (SDW).

As can be seen from the results presented in Figure 3.3 the time that hops are exposed to 100 °C affects the magnitude of the resulting antibacterial activity and shows that some hop variants are more sensitive to the effects of time than others. The hop variant, Northdown failed to display any antibacterial activity following heating at 100 °C for 30 minutes while all other hop variants (n=5) displayed some level of antibacterial activity.

A one way ANOVA of the data compared the extraction time at 30, 60, 120 and 180 minutes and the level of antibacterial activity; a statistically significant difference ($p = <0.05$) was observed. Therefore a Tukey HSD post hoc test was used to evaluate the statistical differences between each extraction time.

Firstly the mean zone of inhibition of hop variants against *M. smegmatis* after heating at 100 °C for 30 minutes (mean 3.83 mm ± SD 0.935 mm) was compared to heating at 60, 120 and 180 minutes; and a statistically significant difference was observed ($p = < 0.05$).

However, comparing heating at 100 °C for 60 minutes (Mean ZOI 12.71 mm ± SD 0.790 mm) to 120 mins (mean ZOI 12.63 mm ± SD 0.686 mm) and 180 mins (mean ZOI was 11.47 ± SD 0.895 mm); it was found that there was no significant difference in activity ($p = 1.00$; 0.652 respectively).

Even though an increase in the overall antibacterial activity was observed after heating for 60 and 120 minutes, at 180 minutes there was a decrease in the mean zone of inhibition. Previous research (Caballero *et al*., 2009) has highlighted that hop compounds are heat sensitive; therefore prolonged heating further than 180 minutes could lead to thermal decomposition of the antibacterial compounds.

Based on these results all future hop extractions were performed at 100 °C for 60 minutes prior to antibacterial activity testing.

3.7.5 Identification of a common commercial agar based culture media with which to determine the sensitivity of MSSA, MRSA, *E. coli* and *B. subtilis* against aqueous hop extracts

To determine if the antibacterial activity demonstrated by the hop extracts was specific to a particular bacterial species, the hop variant Green Bullet was assessed for its ability to inhibit the growth of the following bacteria. Gram positive organisms: Methicillin sensitive (NCTC 13277) and resistant (NCTC 10442) isolates of *Staphylococcus aureus* (MSSA; MRSA) and *Bacillus subtilis* (NCTC 10315) and Gram negative bacteria *Escherichia coli* (NCTC 1093).

Due to the fact that these organisms grew poorly on Middlebrook 7H10 agar which was used to determine the sensitivity of *Mycobacteria*; a range of commercially available agars were compared for their ability to support growth and demonstrate antibacterial activity.

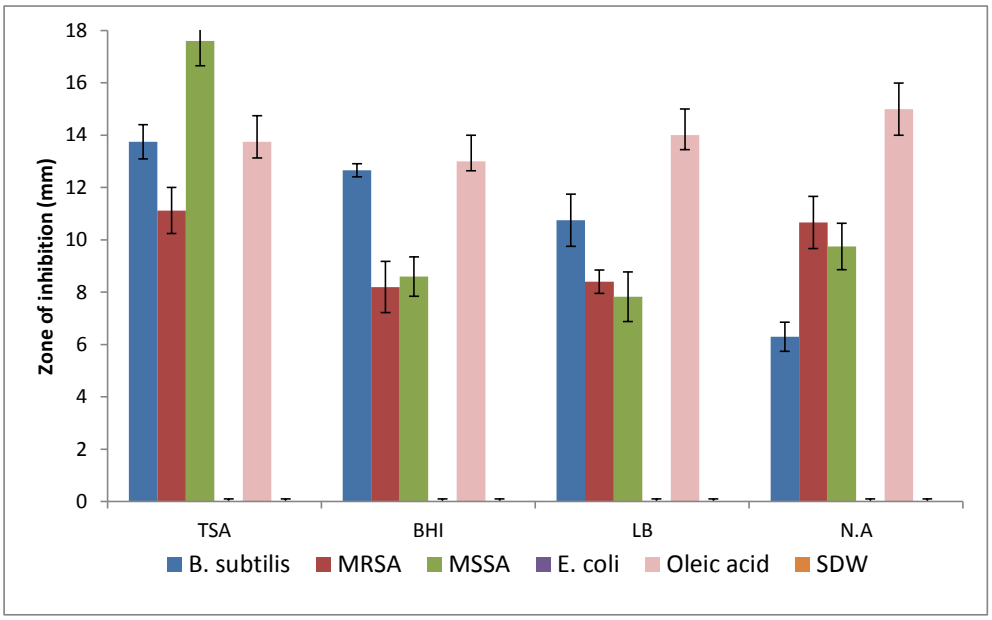

Figure 3. 4 A comparision of the antibacterial sensitivity of MRSA, MSSA, *E. coli* and *B. subtilis* to an aqueous extract of Green bullet using an aqueous extract of Green Bullet hops using different culture media. Antibacterial activity was determined using a zone of inhibition assay. Each assay was repeated 3 times; results presented are the mean, error bars represent standard deviation (± SD).

All of the culture media supported the growth of the test bacteria. While the growth of the Gram positive bacteria was inhibited by the Green Bullet hop extract the growth of the Gram negative representative *E. coli* was not (Figure 3.4). This finding was not suprising given that antibacterial compounds found in hops have been reported to show little, if any, antibacterial activity against Gram negative organisms (Beutler, 2009; Colegate and Molyneux, 2007; Dias *et al*., 2012).

To determine if there was a statistically significant difference between the zone of inhibition against all test bacteria and the agar used, data was subjected to a one way ANOVA. No significant difference was observed between TSA and BHI ($p=0.276$); TSA and LB ($p=0.142$) and TSA and NA ($p= 0.132$).

Due to the fact that TSA supported the growth of all test organisms and is regularly employed in antibacterial assays it was selected to support all future studies involving this selection of bacteria.

3.8 Comparison of the antibacterial activity of aqueous hop extracts with their average alpha and beta acid concentration

The alpha and beta acid content of hops has been linked to the antibacterial properties of beer (Dorman and Deans, 2000; Hornsey, 2003). Therefore to determine if the stated alpha and beta acid content of a particular hop variant correlated with observable antibacterial activity two agar based screening assays were used. These assays consisted of a zone of inhibition assay and an agar incorporation assay which were used to determine both a minimum inhibitory and bactericidal concentration of each hop variant.

3.8.1 A comparison of the antibacterial activity of hop extracts against M. smegmatis and their average alpha acid concentration using a zone of inhibition assay

To determine if the stated alpha acid concentration of an individual hop extract correlated with its observed antibacterial activity against *M. smegmatis* the mean zone of inhibition (n =3) of each aqueous hop extract (n=50) was plotted against the stated alpha acid content of each hop variant (Figure 3.5). To determine if a relationship existed between these two variables a linear regression analysis was undertaken.

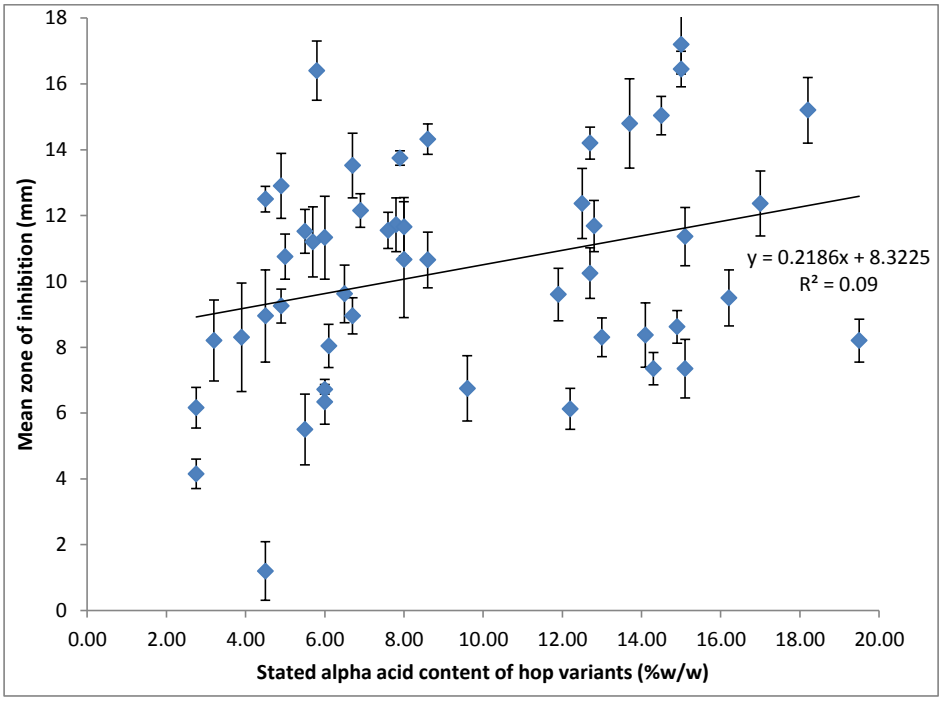

Figure 3. 5 Correlation of the stated alpha acid content of hop variants with the zone of inhibition against *M. smegmatis* using a zone of inhibition assay. Each assay was repeated 3 times; results presented are the mean, error bars represent standard deviation (± SD).

The R^2 value (0.09) generated by the regression analysis of the data presented in Figure 3.5 indicates that there is no significant correlation between alpha acid content and antibacterial activity against *M. smegmatis*. The R^2 value or coefficient of determination demonstrates how well data fits to a statistical model; the better the linear regression fits the data in comparison to the simple average the closer the R^2 value will be to 1. Therefore an R^2 value of 0.09 demonstrates that there is no correlation. To further determine if a statistical correlation existed the data was subjected to a Pearson's two tailed correlation test. This test determines the relationship between two variables (0.5 - 1 = strong relationship; 0.1 - 0.5 weak relationship) and determines the statistical significance of the correlation in the form of a p value (< 0.05 not statistically significant; > 0.5 statistically significant. A Pearson's R value of 0.104 was determined, showing a very weak relationship between alpha acid content and antibacterial activity.

3.8.2 A comparison of the antibacterial activity of aqueous hop extracts against *M. bovis BCG* and other non-*Mycobacterial* species with their stated alpha acid content using a zone of inhibition assay

In order to determine if there was a similar lack of correlation between the antibacterial activity against other bacteria and alpha acid content, the same analysis was performed using *M. bovis* BCG (NCTC 5692), the Gram positive bacteria; MRSA (NCTC 10442), MSSA (NCTC 13277), and *B. subtilis* (10315) and the Gram negative bacteria *E. coli* (NCTC 1093). The antibacterial activity of aqueous hop extracts was assayed against each organism using a zone of inhibition assay as previously described (Method 3.6.1). The mean zone of inhibition (n=3) was correlated against the stated alpha acid concentration of each hop variant.

A summary of the results are presented in Table 3.4 which shows the calculated R^2 values along with Pearson's two tail R values for each bacterial species.

Bacterial Species	Linear Regression Analysis R^2 value	Pearson's Two tailed Correlation R value
MRSA NCTC 10442	0.0030	0.1260
MSSA NCTC 13227	0.0014	0.1040
B. subtilis NCTC 10315	0.0746	0.1030
E. coli NCTC 1093	No inhibition	No inhibition
M. bovis BCG NCTC 5692	0.0040	0.0960

Table 3. 3 Statistical analysis of the correlation of the stated alpha acid content of hop variants (n=50) and the mean zone of inhibition against MRSA, MSSA, *E. coli*, *B. subtilis* and *M. bovis* BCG using a zone of inhibition assay (n=3). Table shows the linear regression analysis (R^2 value) as well as Pearon's R values.

From the results presented in Table 3.3, no obvious correlation between the alpha acid content of each hop variant and antibacterial was observed. As was previously observed, no antibacterial activity was demonstrated against *E. coli*.

These results are surprising given that alpha acids are widely recognised as possessing antibacterial activity and as such it would be expected that they would contribute to the antibacterial activity of the aqueous hop extracts (Caballero *et al*., 2009; Cragg and Newman, 2013; Natarajan *et al*., 2008). The lack of correlation could be due to a problem with how the alpha acid concentrations are determined, or the fact that the alpha acids and their heat derived isomers only make a small contribution to the overall antibacterial activity observed from the hop extracts.

3.8.2. A comparison of the antibacterial activity of aqueous hop extracts against *M. smegmatis* with their stated beta acid content using a zone of inhibition assay

To define if the stated beta acid concentration of an individual hop extract correlated with the antibacterial activity against *M. smegmatis*, the mean zone of inhibition (n=3) of each aqueous hop extract (n=50) was plotted against the stated beta acid content of each hop variant (Figure 3.6). To determine if a relationship existed, a linear regression analysis was undertaken on the data.

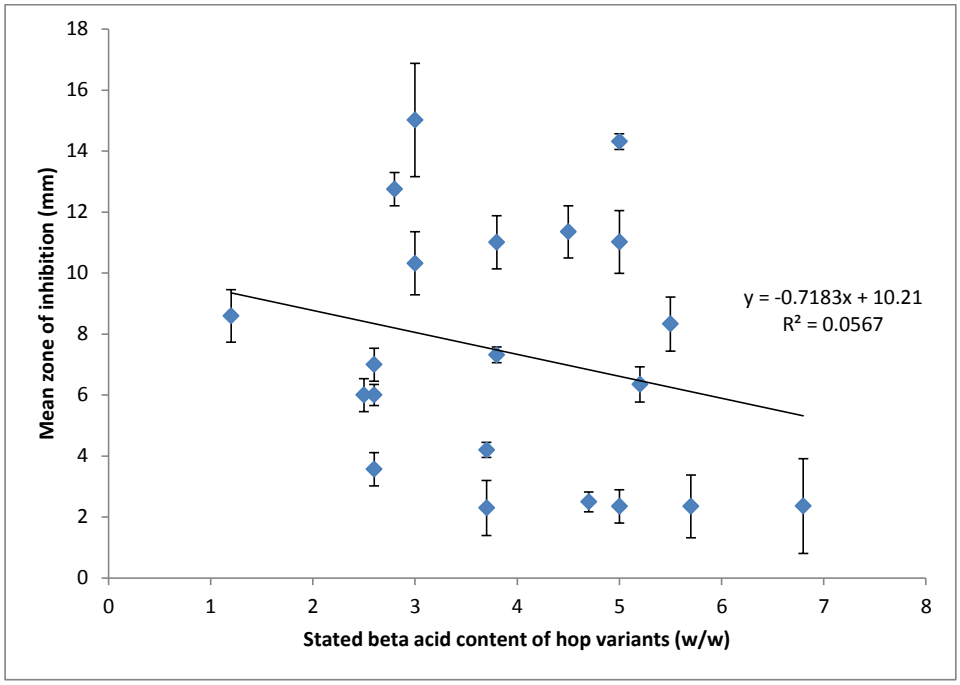

Figure 3. 6 The correlation of the stated beta acid content of hop variants (n=21) and the mean zone of inhibition against *M. smegmatis* using a zone of inhibition assay. Each assay was repeated 3 times; results presented are the mean, error bars represent standard deviation (± SD).

An initial observation of Figure 3.6 and the linear regression analysis (R^2 value 0.0567) suggests that there is no direct correlation between the beta acid content of hops and antibacterial activity against *M. smegmatis*. To confirm this observation the data was subjected to a Pearson's two tailed correlation which generated a Pearson's R value of 0.09 further suggesting no correlation.

3.8.3 A comparison of the antibacterial activity of aqueous hop extracts against *M. bovis* BCG and other non-Mycobacterial species with their stated beta acid content using a zone of inhibition assay

In order to determine if there was a similar lack of correlation between antibacterial activity and beta acid content with other bacteria the same analysis was performed using *M. bovis* BCG (NCTC 5692), the Gram positive bacteria; MRSA (NCTC 10442), MSSA (NCTC 13277), and *B. subtilis* (10315) and the Gram negative bacteria *E. coli* (NCTC 1093). The antibacterial activity of aqueous hop extracts was assayed against each organism using a zone of inhibition assay as previously described (Method 3.5.1). The mean zone of inhibition (n=3) was correlated against the stated alpha acid concentration of each hop variant.

A summary of the results are presented in Table 3.4 which shows the results of the linear regression analysis (R^2 values) along with Pearson's two tail R values for each bacterial species.

Bacterial Species	Linear Regression Analysis R^2 value	Pearson's Two tailed Correlation R value
MRSA NCTC 10442	0.0540	0.2150
MSSA NCTC 13227	0.0750	0.1470
B. subtilis NCTC 10315	0.0660	0.1100
E. coli NCTC 1093	No inhibition	No inhibition
M. bovis BCG NCTC 5691	0.0550	0.1320

Table 3. 4 Statistical analysis of the correlation of the stated beta acid content of hop variants (n=21) and the mean zone of inhibition against MRSA, MSSA, *E. coli*, *B. subtilis* and *M. bovis* BCG using a zone of inhibition assay (N=3). Table shows the linear regression analysis (R^2 value) as well as Pearson's R values.

The results from all of the hop variants found no obvious correlation between the stated beta acid content and antibacterial activity. As was previously observed no antibacterial activity was seen against *E. coli*.

Again, these results are surprising given that lupulone, a beta acid found in hops and beer is known to be antibacterial (Erdmann, 1951; Siragusa *et al*., 2008). The beta acids comprise of 3 compounds, lupulone, adlupulone and colupulone (Van Cleemput *et al*., 2009a) and the stated beta acid content of a hop refers to the total concentration (w/w) of all three compounds. Thus, it may be the case that lupulone is present in much smaller amounts than the other beta acids, this may explain the lack of correlation. It may also be the case, given that beta acids have been described as being weakly non-polar (Shen and Sofos, 2008), that the aqueous extraction process used may not have efficiently extracted all of the available beta acids.

3.8.4 Comparison of the antibacterial activity of aqueous hop extracts with their average alpha and beta acid concentration against *M. smegmatis* using an agar well incorporation assay

The zone of inhibition assay is a useful tool with which to rapidly screen the antibacterial activity of large numbers of hop extracts against all test bacteria. However, the information which can be obtained from this assay is limited when compared to a standardised agar incorporation approach which allows both the minimum inhibitory (MIC) and bactericidal (MBC) concentrations of the test agent to be determined.

For this reason, an agar incorporation assay (adapted from previous research (Varela *et al*., 2008)) was employed. A comparison of the antibacterial activity of the various aqueous hop extracts using the agar diffusion and agar incorporation assays against *M.smegmatis* is shown in the following table (Table 3.5).

	Antibacterial activity of hop extract		
Hop variant	Mean Zone of inhibition (mm)	Agar incorporation	
		MIC (% v/v hop extract)	MBC (As % v/v hop extract)
Galena	2.60	33.00	33.00
Styrian Goldings	4.15	33.00	33.00
Cascade	5.50	13.00	15.00
Simcoe	6.12	13.00	17.00
Savinjski Goldings	6.16	6.60	8.00
Summer Pellets	6.34	6.60	10.00
Boadicea	6.72	5.30	5.30
Perle	6.75	16.00	16.00
Galaxy	7.35	5.30	6.00
Summit	7.35	13.00	20.00
NZ Pacifica	8.04	10.00	10.00
Apollo	8.20	7.00	8.00
Hallertauer	8.20	10.00	10.00
Bobek	8.30	10.00	10.00
Liberty	8.30	33.00	33.00
Sonnet	8.37	4.60	5.00
Sorachi Ace	8.62	15.00	16.00
Motueka Pellets	8.95	13.00	15.00
Tettnang	8.95	10.00	15.00
Fuggles	9.25	13.00	15.00
Colombus	9.50	10.00	10.00
Delta	9.62	5.30	6.00
Chinnok	10.25	10.00	10.00
Centennial Pellets	10.65	11.60	13.00
Northdown	10.66	13.00	15.00
Amarillo	10.75	5.00	5.00
Cascade Pellets	11.20	11.60	11.60
Brambling Cross	11.33	7.00	7.00
Pacific Jade	11.36	6.60	8.00
Progress	11.52	10.00	10.00
Challenger	11.55	10.00	10.00
Northern Brewer	11.66	3.00	4.00
Nelson Sauvin	11.68	4.00	4.60
Aurora	11.72	5.30	6.00
Amarillo Pellets	12.15	4.00	4.60
Pacific Gem	12.37	16.00	20.00
Target	12.37	18.30	18.30
Williamette	12.50	3.00	5.50
Fuggles Pellets	12.90	11.60	11.60
Glacier	13.52	3.30	3.30
First Gold	13.75	3.30	4.00
Chinook Pellets	14.20	8.00	10.00
Centennial	14.32	4.60	5.00
Rakau	14.62	16.00	18.30
Green Bullet	14.80	10.00	10.00
Magnum	15.04	5.30	5.30
Warrier	15.20	10.00	10.00
Goldings	16.40	10.00	10.00
Citra	16.45	2.66	2.66

Table 3. 5 The antibacterial activity (ZOI, MIC and MBC) of aqueous hop extracts against *M. smegmatis* determined using a zone of inhibition and agar incorporation assay. (Results represent n=3)

Analysis of the results presented in Table 3.5 reveal that for several hop variants there was a marked difference in the results obtained using the zone diffusion and agar incorporation assays.

For example the hop variants Goldings and NZ Pacifica had identical MIC and MBC values (10 % v/v) yet there zones of inhibition differed markedly with diameters of 16.4mm and 8.4mm respectively.

A similar disconnection was observed between the Goldings and Citra variants which demonstrated similar mean zones of inhibition values (16.4 mm ±SD 0.13 and 16.45 mm ±SD 0.11) but differed markedly with regards MIC and MBC levels (Goldings MIC/MBC 10 % v/v and Citra MIC/MBC 2.66 % v/v).

To determine if the differences between the ZOI, MIC and MBC were statistically significant the mean zone of inhibition results were compared to the MIC and MBC values using a paired samples t test. A significant difference was seen between the mean zone of inhibition and the MIC ($p=0.001$) and the MBC ($p=0.001$) respectively.

From these results, it is possible to conclude that although the zone of inhibition assay is a useful method with which to detect antibacterial activity, the agar incorporation assay gives a more quantitative measure of the antibacterial activity of a hop extract.

3.8.5 A comparison of the antibacterial activity (MIC) of hops extracts against *M. smegmatis* and average alpha acid concentration determined using an agar incorporation assay

To determine if the stated alpha acid concentration of an individual hop extract correlated with its observed antibacterial activity against *M. smegmatis* the MIC (n=3) of each aqueous hop extract (n=50) was plotted against the stated alpha acid content of each hop variant (Figure 3.7). To determine if a relationship existed between these two variables data was subjected to a linear regression analysis.

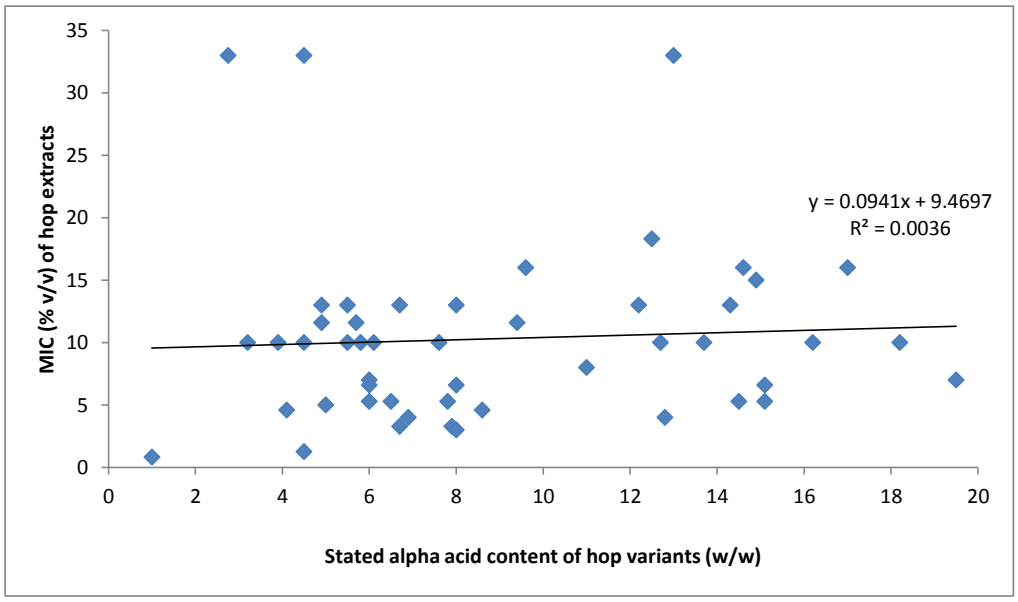

Figure 3. 7 A comparison of the antibacterial activity (MIC) of aqueous hop extracts with their stated alpha acid content. Antibacterial activity was determined using an agar incorporation assay against *M. smegmatis* grown on Middlebrook 7H10 agar Each assay was repeated 3 times; results presented are the mean.

The linear regression analysis suggests that there is no correlation between the stated alpha acid content and MIC. To confirm this observation the data was subjected to a Pearson's two tailed correlation which generated an R value of 0.681 suggesting no correlation.

3.8.6 A comparison of the antibacterial activity (MBC) of hops extracts against M. smegmatis and average alpha acid concentration determined using an agar incorporation assay

To determine if the stated alpha acid concentration of an individual hop extract correlated with its observed antibacterial activity against *M. smegmatis* the MBC (n=3) of each aqueous hop extract (n=50) was plotted against the stated alpha acid content of each hop variant (Figure 3.8). To determine if a relationship existed between these two variables linear regression analysis of the data was performed.

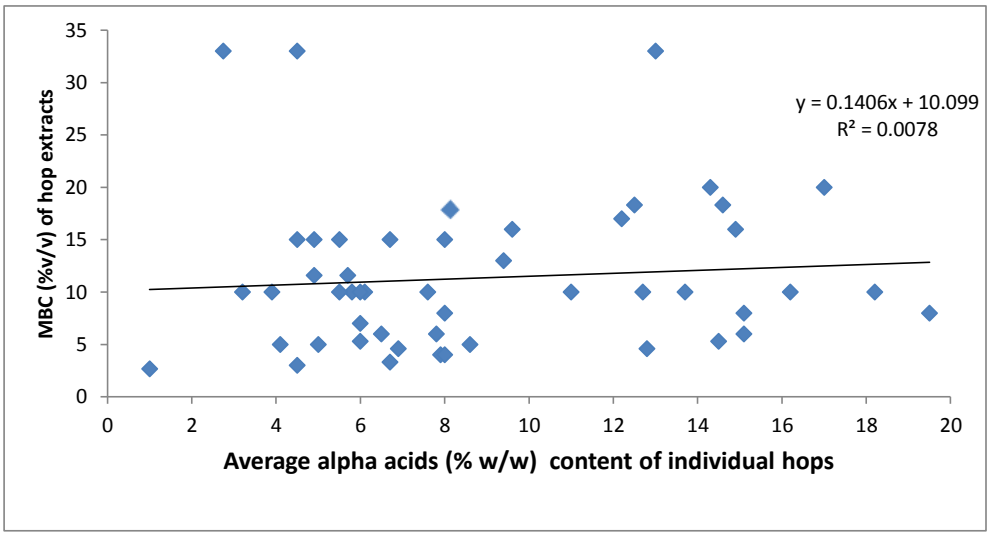

Figure 3. 8 A comparison of the antibacterial activity (MBC) of aqueous hop extracts with their alpha acid content. Antibacterial activity was determined using an agar incorporation assay against *M. smegmatis* grown on Middlebrook 7H10 agar Each assay was repeated 3 times; results presented are the mean

The linear regression analysis suggests that there is no correlation between the stated alpha acid content and MBC. To confirm this observation the data was subjected to a Pearson's two tailed correlation which generated an R value of 0.546 suggesting no correlation.

3.8.7 A comparison of the antibacterial activity (MIC) of hop extracts against *M. smegmatis* and the average beta acid concentration determined using an agar incorporation assay

To determine if the stated beta acid concentration of an individual hop extract correlated with its observed antibacterial activity against *M. smegmatis* the MIC (n=3) of each aqueous hop extract (n=50) was plotted against the stated alpha acid content of each hop variant (Figure 3.9). To determine if a relationship existed between these two variables a linear regression analysis of the data was performed.

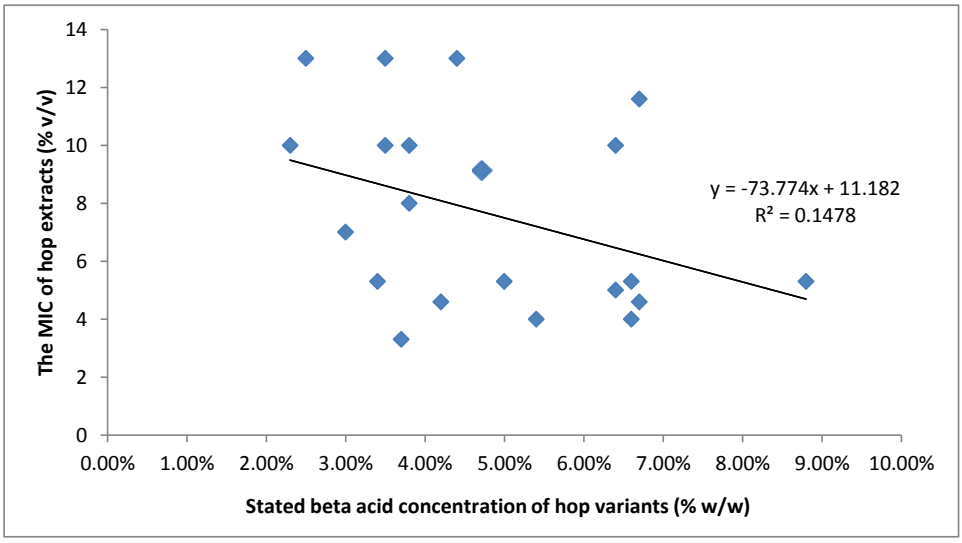

Figure 3. 9 A comparison of the antibacterial activity (MIC) of aqueous hop extracts with their beta acid content. Antibacterial activity was determined using an agar incorporation assay against *M. smegmatis* grown on Middlebrook 7H10 agar Each assay was repeated 3 times; results presented are the mean.

The linear regression analysis suggests that there is no correlation between the stated Beta acid content and MIC. To confirm this observation the data was subjected to a Pearson's two tailed correlation which generated a P value of 0.254 suggesting no correlation.

3.8.8 A comparison of the antibacterial activity (MBC) of hop extracts against *M. smegmatis* and the average beta acid concentration determined using an agar incorporation assay

To determine if the stated beta acid concentration of an individual hop extract correlated with its observed antibacterial activity against *M. smegmatis* the MBC (n=3) of each aqueous hop extract (n=50) was plotted against the stated alpha acid content of each hop variant (Figure 3.10). To determine if a relationship existed between these two variables a linear regression analysis of the data was performed.

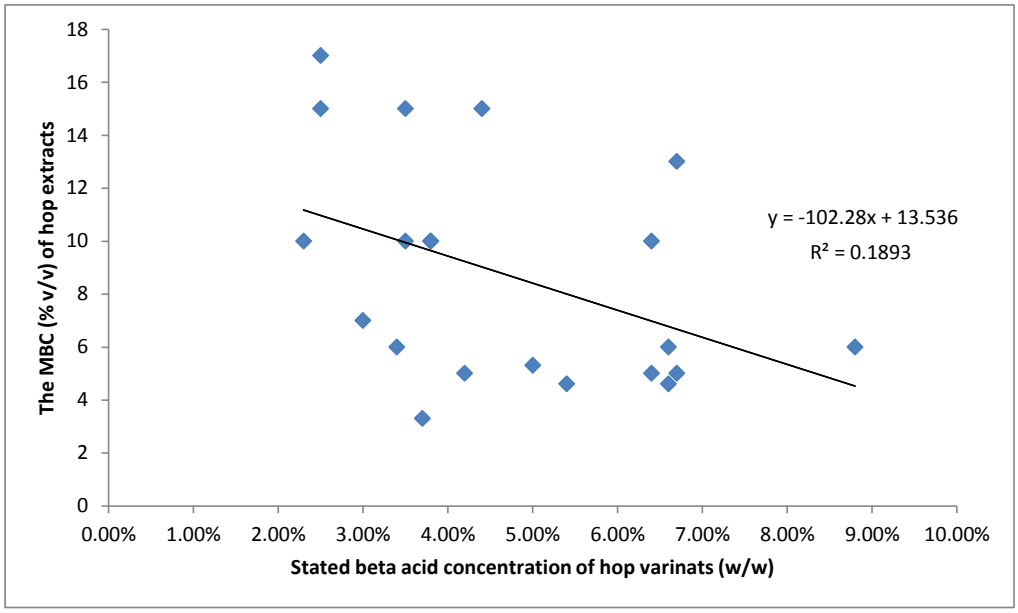

Figure 3. 10 A comparison of the antibacterial activity (MBC) of aqueous hop extracts with their beta acid content. Antibacterial activity was determined using an agar incorporation assay against *M. smegmatis* grown on Middlebrook 7H10 agar. Each assay was repeated 3 times; results presented are the mean.

The linear regression analysis suggests that there is no correlation between the stated Beta acid content and MBC. To confirm this observation the data was subjected to a Pearson's two tailed correlation which generated a Pearson's R value of 0.884 suggesting no correlation.

3.8.9 A comparison of the antibacterial activity (MIC and MBC) of aqueous hop extracts against *M. bovis* BCG and non-Mycobacterial species with their stated alpha acid content using an agar incorporation assay

In order to determine if there was a similar lack of correlation between MIC and MBC and alpha acid content with other bacteria the same analysis was performed using *M. bovis* BCG (NCTC 5692), the Gram positive bacteria; MRSA (NCTC 10442), MSSA (NCTC 13277), and *B. subtilis* (10315) and the Gram negative bacteria *E. coli* (NCTC 1093). The MIC and MBC of each organism was plotted against the stated alpha acid concentration of 50 hop variants and a linear regression analysis was performed. The data was then subjected to a Pearson's two tailed correlation. A summary of the results are presented in Table 3.6 which shows the results of the linear regression analysis (R^2 values) along with Pearson's two tail R values for each bacterial species.

Bacterial species	Linear regression analysis of MIC and alpha acid (R^2 value)	Linear regression analysis of MIC and alpha acid (R^2 value)
MSSA NCTC 13227	0.0005	0.0635
MRSA NCTC 10442	0.0098	0.0012
M. bovis BCG NCTC 5691	0.0016	0.0014
B. subtilis NCTC 10315	0.0450	0.0016

Table 3. 6 Statistical analysis (linear regression and Pearson's two tailed correlation) of the MIC and MBC of aqueous hop extracts against MSSA, MRSA, *M. bovis* BCG and *B. subtilis* with stated alpha acid content.

As displayed in Table 3.6, no correlation was found between the MIC and MBC of any organism and the stated alpha acid concentration of the hop variants.

3.8.10 Comparison of the antibacterial activity (MIC and MBC) of aqueous hop extracts against *M. bovis* BCG and non-Mycobacterial species with their stated beta acid content using an agar incorporation assay

The MIC and MBC of each organism was plotted against the average beta acid concentration of 21 hop variants and a linear regression analysis was performed. The data was then subjected to a Pearson's two tailed correlation. A summary of the results are presented in Table 3.7 which shows the results of the linear regression analysis (R^2 values) along with Pearson's two tail R values for each bacterial species.

Bacterial species	Linear regression analysis of MBC and beta acid (R^2 value)	Pearson's two tailed correlation of MIC and beta acid (R value)
MSSA NCTC 13227	0.0038	0.142
MRSA NCTC 10442	0.0035	0.147
M. bovis BCG NCTC 5691	0.0016	0.284
B. subtilis NCTC 10315	0.0001	0.514

Table 3. 7 The MIC and MBC of organisms MSSA, MRSA, *M. bovis* BCG and *B. subtilis* against aqueous hop extracts were correlated with the average beta acid concentration and the calculated R^2 values are displayed.

No correlation between the MIC and MBC of any organism and the stated beta acid concentration was observed.

3.9 Discussion

It is clear from this study that the level of maceration, extraction temperature and the time that the hop material is exposed to heat has a marked effect on antibacterial activity. Pelleted macerated hops demonstrated the greatest activity presumably due to increased surface area over which antibacterial compounds could be extracted and the fact that the pelleting process reduces the oxidation and thus the degradation of compounds.

The effect of heat on the antibacterial activity of the hop extracts is likely to have been the consequence of two processes, energy driven release of compounds from plant material into the aqueous solution and the isomerisation of released compounds such as the alpha and beta acids.

Heating of the hop extracts leads to the isomerisation of the alpha acids (humulone, cohumulone and adhumulone) into iso-α-acids via an acyloin type ring contraction. This isomerisation reaction has been understood since 1971 (De Keukeleire and Verzele, 1971), it has been shown that the isomerisation of hop acids leads to increased antibacterial activity in the beer (Caballero *et al*., 2009). The recovery of the *cis* and *trans* isomers of the alpha acids is comparatively low (utilising a maximum of 60 % of starting concentration of hop alpha acids) when using traditional hopping techniques such as simply adding hops to boiling wort when compared to using prepared hop extracts (Jaskula-Goiris, 2010). Furthermore, the isomerisation of hops and thus their utilisation within the brew, can vary significantly between brewers and in some cases between brew to brew, this can be due to many factors including pH, temperature and the degree of maceration (Jaskula-Goiris, 2010).

Indeed given that the hop plant is known to contain over 800 separate compounds it is not surprising that substances such as proteins and tannins may well interact with the hop acids resulting in a reduction of isomerisation (Acworth *et al*., 2012).

In brewing, the isomerisation of the hop alpha and beta acids during the boiling of the wort is used to increase the aroma and bitterness properties of the beer. The main alpha and beta acids humulone and lupulone, are relatively hydrophobic and thus aqueous extraction is a relatively inefficient process. Heat induced isomerisation of these compounds into their respective *cis* and *trans* isomers increases there solubility in water and thus promotes the extraction process (Acworth *et al*., 2012).

While heating increased the antibacterial activity of hop extracts, prolonged heating (> 60 mins) resulted in a loss of activity. Malowicki and colleagues observed that while isomerisation of alpha acids increased with time there was also increased thermal degradation. Suggesting that prolonged boiling at 100 °C could have an adverse effect on antibacterial activity (Malowicki and Shellhammer, 2005b).

In this study, two agar based screening methods were used to determine the antibacterial activity of heat treated hop extracts. The broth based micro dilution method was considered. However, due to the inherent turbidity of the hop extracts following heating it was not practical (Zgoda and Porter, 2001).

While the zone of inhibition assay proved a useful tool with which to rapidly determine the antibacterial of multiple hop extracts it does have limitations. The size of zone of inhibition is affected by the concentration of antibacterial agent in the sample, the thickness and nature of the supporting agar and also the density of the bacterial inoculum. Thus while rapid, the assay is a relatively insensitive tool with which to identify differences in the antibacterial activity of different hop variants (Ashworth, 2012, Brandon, 2014).

For this reason, an agar incorporation assay was developed and employed which allowed for the determination of the minimum inhibitory concentration and minimum bactericidal concentration of each hop variant. Agar incorporation methods have the advantaged of providing a more quantitative result than the zone of inhibition assay (Roberts 1998).

Using this assay it was observed that all hop variants demonstrated some level of antibacterial against the Mycobacterial isolates examined in this study. The Citra variety demonstrated the greatest inhibitory activity against *M. smegmatis* and *M. bovis* BCG with the Galena variety showing the least. No correlation between the antibacterial activity of the hop varieties examined in this study and their stated alpha and beta acid content was observed. To determine if this lack of correlation was specific to mycobacteria, the activity of the hop varieties against selected Gram positive and Gram negative bacteria was determined.

These organisms, unlike *Mycobacterium* require a more complex growth medium and for this reason I determined the ability of different commercial media, TSA, BHI, LB and NA, to support the growth of these organisms and to assess the antibacterial activity of the hop extracts.

The use of a zone of inhibition assay and a Green Bullet hop extract showed that all four media were equally effective at supporting growth and demonstrating antibacterial activity. Due to availability, TSA was used to determine the relative antibacterial activity of all of the hop variants. The results for the Gram positive bacteria mirrored those seen for mycobacteria with no correlation observed between antibacterial activity and the stated alpha and beta acid content.

As expected no antibacterial activity against *E.coli* was observed. As the only Gram negative bacteria the lack of activity is probably due to the nature of its cell wall (Cowan, 1999). In Gram negative bacteria, the outer cell membrane differs from the Gram positive by having a thick layer of lipopolysaccharides and proteins (Grayson, 2009). There are essentially two distinct pathways that an antibiotic can take: a lipid-mediated pathway for hydrophobic compounds and general diffusion porins for hydrophilic compounds. The lipid and protein compositions of the outer membrane of the Gram negative organism have a strong impact on the sensitivity of bacteria to antibiotics; rendering them less susceptible than Gram positive organisms (Delcour, 2009).

Overall the lack of correlation between the hop acid content and antibacterial activity was surprising given that the hop alpha and beta acids have been described as antibacterial (In Acworth *et al*., 2012). It is hypothesised that other compounds in the hop extract contribute for the observed activity. Hops have been shown to contain over 800 compounds some of which may be directly antibacterial or act in a synergistic manner to enhance the activity of other antibacterial compounds. Once such compound is xanthohumol, a bioactive prenylated chalcone which to date has only be found in hops and is known to be antibacterial (Acworth *et al*., 2012; Ceh *et al*., 2007; Stevens and Page, 2004). The compound is also isomerised in the wort during the boiling stage to form iso-xanthohumol which is also antibacterial (Acworth *et al*., 2012). The results presented in this chapter supports earlier work which suggests that other compounds in addition to hops acids, contribute to the antibacterial activity of beer (Acworth *et al*., 2012; Everard *et al*., 2012; Van Cleemput *et al*., 2009b).

Chapter 4

Quantification of the antimicrobial components of hops

4.0 Quantification of the antimicrobial compounds of hops

4.1 Chapter introduction

Natural products and their derivatives have been identified for their medical importance for thousands of years. A search of the NAPRALAERT database (an alert network for published literature on novel natural products) lists 58,725 plant species, of which, 6350 have been shown to have antimicrobial activity. Contrarily, even though natural products continue to be a primary source of new antimicrobials (Bérdy, 2012) only 20 new antibacterial compounds from natural products have been brought to the market since the year 2000 (Butler and Cooper, 2011; Wright, 2012).

Several reasons have been proposed for this decline in clinical development including; time of research and difficulty in extraction of the novel metabolite, the cost of taking the drug through clinical trials and the rise of bacterial resistance which can render the antimicrobial redundant before reimbursement of the initial investment (Abreu *et al*., 2012).

The importance of discovering novel antimicrobials has never been so significant. For example, the rise of multidrug resistant strains of *M. tuberculosis* (MDR-TB) has been linked to the acquired immunodeficiency syndrome (AIDS) epidemic, poor socioeconomic conditions, immigration and the lack of inhibitory antimicrobials. Due to a lack of novel antimicrobials, curing patients of MDR-TB is extremely low with 50% making a full recovery compared to more than 95 % of patients with antibiotic sensitive TB (Ahmad and Aqil, 2009; Mahady, 2005)

For these reasons, conventional medicine is increasingly receptive to plant-based medicines. This is due to not only the rise of bacterial resistance to traditional antibacterials, but also the rapid rate of botanical species extinction (Cowan, 1999). Furthermore, the general public have been made increasingly aware of the overuse and misuse of antibiotics, and are now becoming interested in alternative medicines such as ‘medicinal plants’. It has been estimated that up to 80 % of the population of developed countries worldwide use botanical products and consider them to be safe and effective (Mahady, 2001)

While thousands of plant species have been tested against many different strains of bacteria, the most common are *E. coli, B. subtilis, C. pneumonia* and Methicillin Resistant *S. aureus* (MRSA). However, little research has been paid to species of mycobacterium even though many strains such as *M. tuberculosis* and *M. abscessus* have developed increased resistance to front line antibiotics such as rifampicin (Jeon *et al*., 2009; Petrini, 2006).

In this chapter, the compounds which are directly responsible for the antibacterial activity against *M. smegmatis* was further investigated. Whilst it has been shown that several plant species are useful therapeutics (including; opium, belladonna and aloe) when used in their natural state, second generation natural products from the processing of the plant, to purified chemical compounds with an identified structure are now becoming more commonplace. Drugs such as taxol from *Taxus brevifolia* for the treatment of ovarian cancer followed the same development and evaluation as other pharmaceutical drugs (Ahmad and Aqil, 2009; Iwu, *et al*., 1999)

In Chapter 3, the antibacterial activity of crude hop extracts against a range of clinically relevant bacteria was investigated and data correlated with the alpha and beta acid concentrations. No statistically significant correlation was observed so therefore this chapter describes the extraction and bioassay guided fractionation of the antibacterial component molecules within hops.

In the production of beer, hops are primarily extracted in water during the wort boiling process and it has been shown that both the alpha- and beta-acids, as well as potential antibacterial compounds, are transferred to the brew during this stage (Caballero *et al*., 2009; Cornelison, 2008; Neve, 1991; Stevens and Page, 2004). For this reason, several volatile polar organic solvents such as methanol have been used in this project during the hop extraction process to mimic the polarity of water, while being simpler to manipulate in the laboratory. The use of solvents in plant extraction is well documented (Eloff, 1998; Parekh *et al*., 2005; Sharifa *et al*., 2008) and research by Wang *et al.* (2006) suggests that traditional solvent extraction is as efficient and reliable as other processes, such as supercritical CO_2 extraction (Wang and Weller, 2006).

Following treatment with a solvent, the crude hop extract is a complex mixture of over 800 compounds (Neve, 1991). The difficulties of separating this mixture further, not just in hops but many natural products, has been associated with the decline in drug discovery from nature (Vu *et al*., 2008). The key separation technique in natural product chemistry is chromatography (Colegate and Molyneux, 2007). Its most basic form, thin layer chromatography (TLC) has been used to separate crude hop extracts for over 50 years (Bhandari, 1964).

This method is used to separate non-volatile mixtures, usually on a thin layer of adsorbent material (the stationary phase) such as silica gel coated on an aluminium or glass plate. The sample is added to the plate and a suitable solvent (mobile phase) draws the analyte up the plate by capillary action. Different analytes will elute up the TLC plate at different rates depending on their polarity, producing separation (Reed, 2007). In this study, the use of TLC was combined with a bacterial assay to develop a bioassay-guided, bio autographic TLC technique originally described, and adapted from (Fleming, 1942). In summary, TLC methodology produces a separation of a complex mixture over which a thin layer of *Mycobacterium smegmatis* can be overlaid onto the surface of the TLC plate. Inhibition of growth can then be observed after bacterial exposure to a stain. Although not a quantitative method, it can locate potential antibacterial compounds (and eliminate inactive compounds) within the mixture (Rahalison *et al*., 1991).

Further separation methods, such as preparative TLC and column chromatography, can then be used for high volume recovery of a specific compound as they are relatively time consuming, laborious and can be of low resolution (Andersen, 1969; Bhandari, 1964; Hostettmann *et al*., 1998; Reed, 2007; Stavri *et al*., 2004; Zeković *et al*., 1994).

The primary method for analyte identification is high or ultra-pressure liquid chromatography (HPLC/UPLC) coupled to mass spectrometry (Acworth *et al*., 2012; Baker *et al*., 2008). HPLC utilises the development of high pressure pumps with reproducible, reliable flow rates to give rapid separation with reduced band broadening (Reed, 2007). To aid in the identification of separated compounds, the HPLC system is usually coupled to a mass spectrometer (MS). This method relies on splitting the eluent from the column so that only a small volume reaches the MS. Chromatograms show peaks which correspond to the elution of particular components. It is then possible to analyse individual peaks and obtain a mass for that component (Colegate and Molyneux, 2007; Koehn and Carter, 2005).

In this chapter, the validity of the methods used have not only been confirmed, but antimycobacterial analytes from methanol hop extracts have been tested, purified and identified. Differences between the most antimicrobial and least antimicrobial hop extracts have been observed and reasons for the differences in antibacterial activity discussed. This chapter also highlights the difficulties in obtaining pure bioactive compounds from complex natural products.

4.2 Materials

4.2.1 Sources of chemicals and reagents

All chemicals and reagents were purchased from Sigma Aldrich Ltd, UK, unless otherwise stated in the text.

All solvents were from purchased from Thermo Fischer Scientific Ltd, UK, unless otherwise stated in the text.

4.2.2 Diluents

Deionised water (diH_2O) was obtained from an ELGA purelab option BP15 dispenser (ELGA labwater, UK). The inorganic content of the water at 25 °C was between 1-15 MΩ-cm with a total organic carbon content of <30 ppb. It was sterilised at 121 °C for 15 minutes at 15 psi (Prestige, 9L classic bench top autoclave, LabShop, UK) to form sterile distilled water (SDW).

4.2.3 Bacterial cultures

Mycobacterium smegmatis 8159T was used as a simulant organism for *M. tuberculosis* and was grown and standardised as described in Section 2.2.2 and 2.2.3

All growth media was purchased and produced as described previously in Chapter 2, unless otherwise stated.

4.3 Methods

4.3.1 Organic solvent extraction of hop material

Fresh hop material was machine macerated as previously described and 20 g transferred to a flat bottom 1500 mL conical flask, which was suspended in 1000 mL of methanol. Other organic solvents (ethyl acetate, acetone) were also trialled to determine the greatest yield.

The solution was mixed continuously for 48 hours at 100 rpm at room temperature and pressure. After this time stirring was halted and solid hop material filtered through a Whatman® size 1 under vacuum pressure to remove any solid hop material. Resulting solvent was retained and solid hop material was re-suspended in 1000 mL of fresh methanol for a further 24 hours at room temperature and pressure with mixing at 100 rpm. Following this, hop material was filtered as previously described and weighed; hop extract solution was stored at room temperature in the dark until required.

4.3.2 Hop solution evaporation

Following extraction, both the 24 and 48 hour solutions were combined and re-filtered through a Whatman® size 1 filter to remove any remaining solids. The resulting solution was decanted into a 1000 mL round bottom flask. This was then attached to a Buchi Rotavapor 200 (Buchi, Switzerland) under vacuum pressure (15 bar) and rotated at 50 revolutions per minute (rpm) at 30 °C.

Waste solvent was firstly checked for compounds by using thin layer chromatography (Method 4.4.3) and if no compounds were found it was discarded. The resulting dry hop extract (crude hop extract) was removed from the flask, weighed and stored at 3 °C in the dark until required.

4.3.3 Thin Layer chromatography separation of hop extracts

Hops (n=50) detailed in (Section 3.1) were extracted using (Method 4.3.1) and dried by solvent evaporation (Method 4.3.2). Dry crude hop extracts (10 mg) were then re-suspended in 1 mL of methanol to give a final concentration of 10 mg/mL^{-1}; a 1:10 dilution series was then prepared in methanol to give final dilutions of 10, 1, 0.1, 0.01, 0.001 mg/mL^{-1} and each dilution was mixed gently using a desktop vortex (Vortex Genie 2T shaker, Fisher Scientific, UK) for 15 seconds.

Using a fresh capillary pipette 25 µL of each dilution was removed and spotted onto the surface of a thin layer chromatography (TLC) plate (50 mm x 75 mm, 0.20 mm Silica). The TLC plate was then placed horizontally within a fume cupboard at room temperature and pressure for 10 minutes to allow any excess solvent on the TLC plate to evaporate. TLC separation of the hop extract was dependant on the solvent system (mobile phase) and several ratios of the organic solvents hexane, ethyl acetate, methanol and ethanol were optimised to allow for optimum analyte separation. The solvent system methanol : ethyl acetate (ratio 1:1) was chosen for all future separations.

50 mL of freshly prepared solvent system was decanted into a TLC developing tank (100 mm^2 x 100 mm^2) and the TLC plate loaded with extract was placed into the solvent system allowing 1 cm between the surface of the solvent and the extract. The tank was then covered and the solvent allowed to run for 45 minutes or until the solvent covered 18 cm of the plate. The distance that the solvent travelled on the plate was recorded and the plate was examined under visible and UV light (365 nm, UVitech, UK). Each visible separated analyte was then measured for its total distance travelled from the baseline.

The Rf values of analytes could then be determined by using the following equation:

$$Rf = \frac{\text{Total distance travelled of selected spot (mm)}}{\text{Total Distance travelled of solvent (mm)}}$$

This method was repeated on three occasions under the same conditions and the Rf values recorded.

4.3.4 Bacterial overlay assay

The bacterial overlay assay utilises the TLC separation as described previously (4.3.3) but includes the addition of bacteria to allow visualisation of bioactive analytes as originally described by Fleming, (1942) and adapted from Rahalison, (1991).

Each hop variant (n=50) was extracted and a 0.1 mg/mL^{-1} solution of crude hop extract in methanol was prepared. As a control, *Streptomycin* solution (stock concentration 1 mg/mL^{-1} in 1 mM EDTA, Sigma-Aldrich, UK) was also diluted in methanol to give a final concentration of 6 µg/mL^{-1}.

All samples were then spotted onto the surface of a TLC plate as described earlier (Method 4.4.3) and were separated using the previously described solvent system. Once separated, each plate was dried within a fume cupboard at room temperature and pressure to ensure that no solvent remained on the plate. As a control, on each occasion a TLC plate without samples was also run and dried for the same amount of time, this was to ensure solvent evaporation.

A 72 hour *Mycobacterium smegmatis* suspension was prepared as described (Section 2.2.2), and was centrifuged at 5000 x g for 15 minutes (Avanti J-20 XP, Beckman Coulter Inc. UK) at which time a bacterial pellet was formed. Supernatant was removed and discarded; the remaining pellet was washed twice with diH_2O. The pellet was then re-suspended in 25 ml of sterile LB broth, mixed using a desktop vortex (Vortex Genie 2T) for 30 seconds at full power (3000 rpm) and standardised to 1 × 10^5 cfu /mL^{-1} as described in Section 2.2.3.

The inoculated broth was then spread evenly over the TLC plate which was then rested horizontally within a laminar flow cabinet for 5 minutes to allow evaporation of excess solution. The inoculated TLC plate was transferred to a sterile container and stored vertically in an incubator at 37 °C in high humidity for 72 hours.

The TLC plate was then removed from the incubator and placed inside a laminar flow cabinet. The tetrazolium salt, 2,3,5-triphenyltetrazolium chloride (20 µg/mL^{-1}), was prepared by diluting to 3 µg/mL^{-1} in SDW and then transferring to a spray equipped bottle. The solution was then sprayed evenly onto the surface of the plate, which was then re-incubated at 37 °C for 4 h in high humidity.

Upon visualisation, metabolically active bacteria convert the tetrazolium salt into an intensely coloured formazan; inhibitory compounds appear as clear spots against a red background. The Rf values of antibacterial analytes were recorded on three separate occasions. This method gives a qualitative indication of inhibitory activity from separated analytes and has been successfully used in antibacterial bio-autographic assays in other studies (Rahalison, 1991; Valgas, 2007; Waksmundzka-Hajnos, 2008). This method is used as a high-throughput screen to further guide separation of potentially inhibitory compounds.

4.3.5 Column chromatography

Column chromatography is another useful method of separation based on the TLC method and utilises the same solvent system. Separated analytes elute through a column with a stationary phase which can be made of either silica or Sephadex® (Reid and Sarker, 2012). Column chromatography has several advantages over the preparative TLC method. Perhaps most importantly is that a larger sample volume can be eluted through the column and larger amounts of the sample collected (Hostettmann *et al.*, 1998; Reid and Sarker, 2012).

4.3.6 Silica gel column chromatography

Silica gel is the most commonly used stationary phase in chromatography and separates compounds based on their polarity (Reid and Sarker, 2012). For this method, 15 g of silica (60Å 45 µm) was packed into a glass column (60 cm x 15 cm) so that it was 2/3 full. The column was then flushed with 1 L methanol : ethyl acetate (1:1) and 600 mL was then eluted and discarded.

Hops were extracted as previously described in Section 4.4.1, the solvent evaporated (Section 4.4.2) and 5 g of resulting crude hop extract was re-suspended in 10 mL of methanol.

The solution was mixed using a desktop vortex and the hop suspension was added drop-wise to the surface of the silica gel using a Pasteur pippete. Once loaded, the column was eluted under gravity and 20 mL fractions were collected until all visible bands were collected.

Each fraction was then concentrated to 5 mL and analysed using the bacterial overlay assay (Section 4.3.4), Rf values of antibacterial analytes were recorded and the relevant fractions of identical Rf values combined, concentrated and weighed. They were then stored at 3 °C in the dark until required. This method was repeated on 2 separate occasions.

4.3.7 Sephadex® LH-20 Column chromatography

Although silica gel is a useful method of separating compounds, it also has several problems. Yield/recovery of extracts can be low as compounds can bind to the silica, thus not eluting from the column. Since silica chromatography separates compounds based on polarity, the methanol extraction method extracts polar compounds and thus further polarity based separations can be problematic.

Another stationary phase which can be used is Sephadex®, a cross linked dextran gel that separates compounds based on their molecular size and is mostly used for high molecular weight molecules such as proteins, but Sephadex® LH-20 is commonly used in natural product separation (GE-Healthcare, 2011). Sephadex® LH-20 is a cross-linked dextran gel composed of macroscopic beads synthetically derived from a dextran polysaccharide.

To prepare a column, 15 g Sephadex® LH-20 was mixed with 200 mL of the solvent system (methanol : ethyl acetate (1:1)) to allow the dry powder to become swollen and form a slurry. The slurry was then transferred to a glass column (60 cm x 1.5 cm), suspended in 1000 mL of the solvent system and allowed to stand for 12 hours.

After resting, 600 mL of the solvent system was eluted under gravity through the column twice and 5 mL of the previously re-suspended crude hop extract (0.5 g/mL^{-1} in methanol) was added drop-wise to the surface using a Pasteur pippete.

To load the hop extract into the column, 25 mL of the solvent system was eluted under gravity so that the extract visually entered the Sephadex®; the column was then re-filled with 1000 mL of the solvent system and elution begun under gravity. Fractions of 20 mL were collected at a flow rate of 5-10 cm/hr^{-1}, until 250 mL of eluent had passed after the last visible band had eluted from the column.

Each fraction was then concentrated to 5 mL re-examined for antibacterial activity using the bacterial overlay assay and the Rf values of antibacterial analytes were recorded. Fractions which demonstrated antibacterial activity and had identical Rf values were combined, concentrated and stored at 3 °C in the dark until required.

4.3.8 Mass spectrometry

Antibacterial analytes separated and isolated from the crude hop mixture were primarily checked for purity and identification using liquid chromatography mass spectrometry (LC-MS). LC-MS is commonly used for the identification of hop compounds (Taylor *et al*., 2003; Vu *et al*., 2008), it is useful tool for detecting analytes of a low concentration and limited purity (Reusch, 2013; Vu *et al*., 2008) such as those extracted from natural products (Imperato, 2006).

4.3.8.1 Sample preparation for mass spectrometry

The sample was firstly prepared by identifying an antimicrobial spot on the TLC plate following overnight incubation (section 4.3.4) and separating the appropriate 'spot' on using column chromatography from the overall mixture. The Rf value of the compound was checked against the original inhibitory spot using TLC.

The solvent used in the extraction process was then evaporated using Method 4.3.2 and the remaining solid material weighed. This material was then re-suspended in acetonitrile to give a concentration of 1 mg/mL^{-1} and were finally filtered (0.45 µm Whatman) prior to analysis.

4.3.8.2 Sample analysis using mass spectrometry

The analysis was carried out using an Agilent 1100 series (Agilent Technologies, Polo Alto, CA, USA) chromatographic system equipped with a series binary pump, microvacuum degasser, series thermostatted column compartment and variable wavelength UV–vis detector. Sample injections were made through an autosampler. Data collection and processing were performed using an Agilent ChemStation software, version A.10.01 (Agilent Technologies).

The columns Agilent Zorbax SB-C18 (5 µm particle size, 250 mm × 4.6 mm) (Agilent Technologies, Polo Alto, CA, USA) and Waters XTerra® with hybrid particles containing both inorganic (silica) and organic (organosiloxane) components (5 µm particle size, 150 mm × 4.6 mm) (Waters, Milford, MA, USA) were used. Water for chromatography was obtained applying the System Simplicity 185 (Millipore, Billerica, MA, USA) purification system. The mobile phase and the solution to be injected were degassed and vacuum filtered prior to use through a Whatman 0.45 µm nylon membrane filter (Whatman International, Maldstone, UK) (Zivanovic *et al*., 2006).

4.3.9 High resolution mass spectrometry (HRMS)

Whilst LC-MS was used to identify dominant ions within hop extracts, high resolution mass spectrometry (HRMS) can be employed to precisely identify compounds by their high resolution mass values. HRMS is a double focusing technique meaning peaks of compounds can be differentiated by their molecular mass determined accurately to at least three decimal places (Reusch 2013).

On this basis it is possible for HRMS to distinguish precisely between empirical formulas with similar nominal masses which would only be determined to 1 decimal place in MS as demonstrated in Table 4.1.

Formula	C_6H_{12}	C_5H_8O	$C_4H_8N_2$
Mass	84.0939	84.0575	84.0688

Table 4. 1 An example of different empirical formulas of compounds separated using HRMS with similar molecular masses Adapted from (Reusch, 2013)

This makes HRMS a useful tool in natural product compound identification initially as it can distinguish between compounds of a similar nominal mass, but also using attached software it is able to estimate the elemental composition of potential compounds.

Sample analysis was undertaken by the EPSRC National Mass Spectrometry Service at Swansea University, using an LTQ Orbitrap XL.

4.3.10 HPLC

A Waters 2695 liquid chromatograph system comprising of a vacuum degasser, quaternary pump, auto- sampler, thermostatic column compartment and photodiode array detector was used. The column used was a C18 reversed phase Kingsorb 5 mm (150 x 4.6 mm) (Phenomenex Ltd., UK) with a Kingsorb 5 mm C18 (30 x 4.6 mm) guard column. Mobile phases consisted of 0.1 % ortho-phosphoric acid in ultra-purified water (v/v) (mobile phase A) and 0.1 % (v/v) ortho-phosphoric acid in methanol (mobile phase B). The gradient elution was performed as shown in Table 4.2.

	Mobile Phase (%)	
Time (min)	A	B
0 - 5	80	20
5 - 7	76	24
7 - 10	76	24
10 - 20	60	40
20 - 25	50	50
25 - 25.5	80	20
25.5 - 30	80	20

Table 4. 2 Composition of the mobile phase in the gradient elution system between 0 and 30 min. Mobile phase A consists of 0.1% ortho-phosphoric acid in ultra-purified water and mobile phase B 0.1 %(v/v) ortho-phosphoric acid in methanol (eluent B). All gradients were linear.

Post-run time was 5 min. Elution was performed at a solvent flow rate of 1 mL/min^{-1}. Detection was accomplished with a diode array detector and chromatograms were recorded at 210 and 280 nm. The column was maintained at 30 °C. The sample injection volume was 15 µL.

4.4 Results

4.4.1 Identification of optimum polar organic solvent for the extraction of hop components.

The organic solvents (methanol, ethanol and ethyl acetate) were used in the organic extraction method as described in (Method 4.3.1). The total yield from each hop following extraction in each solvent is displayed in Figure 4.1.

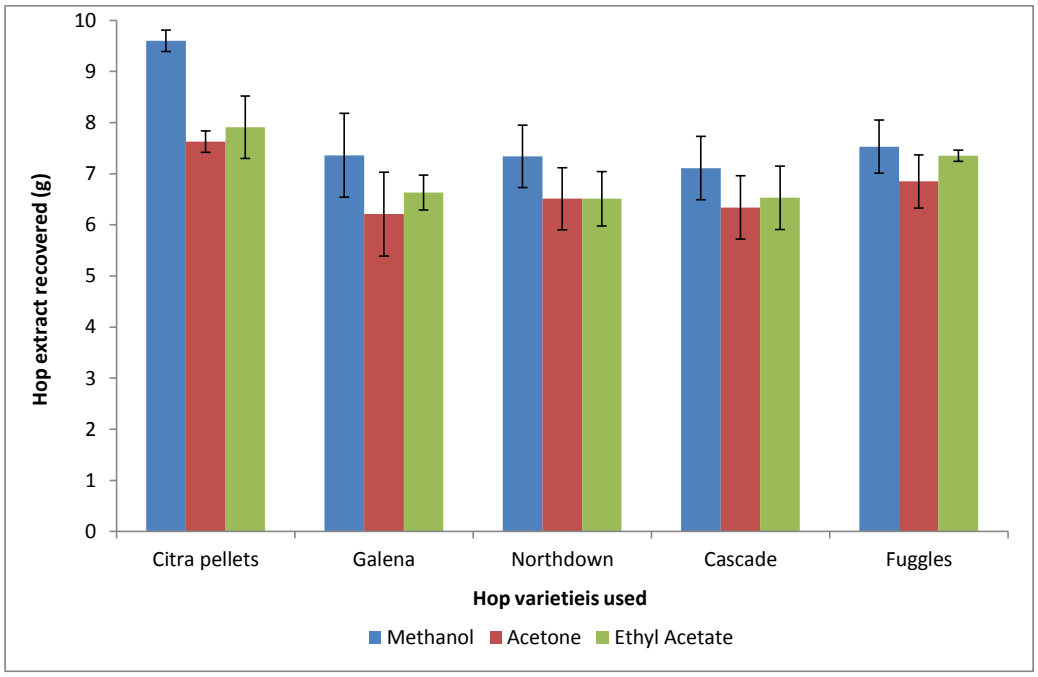

Figure 4. 1 Comparison of the total yield (g) of hop varieties Citra, Galena, Northdown, Cascade and Fuggles following organic solvent extraction (72h) of 20 g of hop material in 1 litre (L) of methanol, acetone and ethyl acetate (n=3; ±SD)

The recoverable yield of hop extracts following extraction was assayed by using five hop varieties (Citra (pellets); Galena; Northdown; Cascade and Fuggles) and extracting in three solvents (methanol, acetone and ethyl acetate) as described in Method 4.3.1.

Upon initial observation of Figure 4.1, it is apparent that the recoverable yield of the Citra pellets is increased when compared to the other hop variants. This, as explained in Chapter 3, may be due to the relative stability of the hop compounds after the pelleting process or the fact that maceration prior to pelleting of the hop materials increases the available surface area of the Citra hop variant.

To determine if there was a statistically significant difference between the total yield of extracted hop material after exposure to the organic solvents A one-way ANOVA was carried out on the data using SPSS software (Method 2.3). Results indicated a P value > 0.05 indicating no statistically significant difference between the extraction solvents and total recoverable yield.

On inspection of the Tukey Post Hoc analysis (Method 2.3) the methanol extraction produced an average 7.788 g ± SD 0.556 g yield per 20 g of dry hop material (38.9%) compared to acetone (6.708 g ± SD 0.478 (33.4%)) and ethyl acetate (6.986 g ±SD 0.442 g (34.93%)). It was on this basis that methanol was used in all future extractions.

4.4.2 Optimisation of a solvent system for the separation of organic solvent extracts of hops

Thin layer chromatography relies on the separation of the crude hop extracts using a mobile phase consisting of a solvent system of one or more solvents. Previous methods for hop natural product isolation reported several solvent systems (Bhandari, 1964; Hamburger and Cordell, 1987; Hostettmann *et al*., 1998) utilising ratios of hexane, ethyl acetate, methanol and acetone. In Table 4.3 the ratios of several solvent systems used in typical natural product separations are listed; and were trialled for separation of crude hop extracts.

Ratio number	Hexane	Ethyl Acetate	Methanol	Acetone
Ratio 1	1	1	1	1
Ratio 2	8	2	0	0
Ratio 3	7	3	0	0
Ratio 4	2	8	0	0
Ratio 5	3	7	0	0
Ratio 6	4	6	0	0
Ratio 7	5	5	0	0
Ratio 8	0	0	8	2
Ratio 9	2	4	4	0
Ratio 10	2	4	0	4
Ratio 11	6	4	0	0
Ratio 12	0	1	1	0
Ratio 13	0	0	1	1
Ratio 14	1	1	0	0

Table 4. 3 The ratios of organic solvents used for the determination of an optimum solvent system for hop extract separation using thin layer chromatography. Each ratio is defined as a number (e.g. 1:1:1:1 where each solvent would be in an equal volume) Adapted from (Bhandari, 1964; Hamburger and Cordell, 1987; Hostettmann *et al*., 1998)

Optimum separation was determined by ultraviolet (UV) light at 365 nm (UVitec, Cambridge, UK) and visible light. In the following Figure (4.2) separation of the Citra crude hop extract using methanol : ethyl acetate (1:1) (ratio 14) and using ratio 2, hexane : ethyl acetate (8:2) are shown.

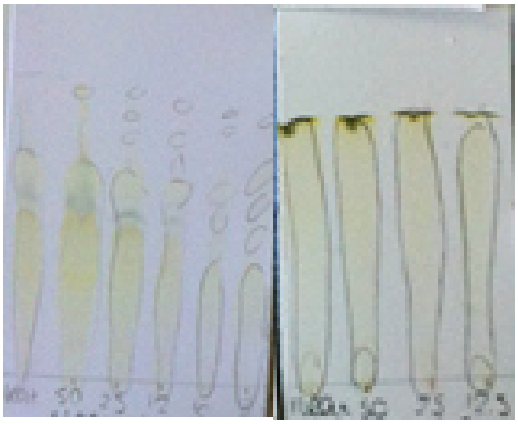

Figure 4. 2 Comparison of Citra crude hop extract (0.1 mg/ml) after separation using TLC and the solvent system methanol : ethyl acetate (1:1) (left) and using hexane : ethyl acetate (8:2) (right)

Solvent system optimisation relied on visual inspection of separated spots on the TLC plate. This was evident in Figure 4.2 where ratios 2 and 14 are compared; as it can be observed that ratio 14 (methanol : ethyl acetate (1:1) (left) has a much better compound resolution than ratio 2 (hexane : ethyl acetate (8:2) on the right.

The reason for the separation in different solvents is due to the polarity of the compounds present. The crude hop extracts used were extracted using the polar solvent methanol. It was hypothesised that the majority of compounds present will be polar and separation can be enhanced by using a more polar solvent system.

Based on these results the solvent system methanol : ethyl acetate (1:1) was used for all future separations.

4.4.3 Thin layer chromatography separation of hop extracts

TLC analysis of each hop variety (n=50) was completed and the Rf values of isolated hop analytes were recorded. In Figure 4.3 hops 1-14, were extracted in methanol and separated using TLC. The Rf values of separated spots were then calculated.

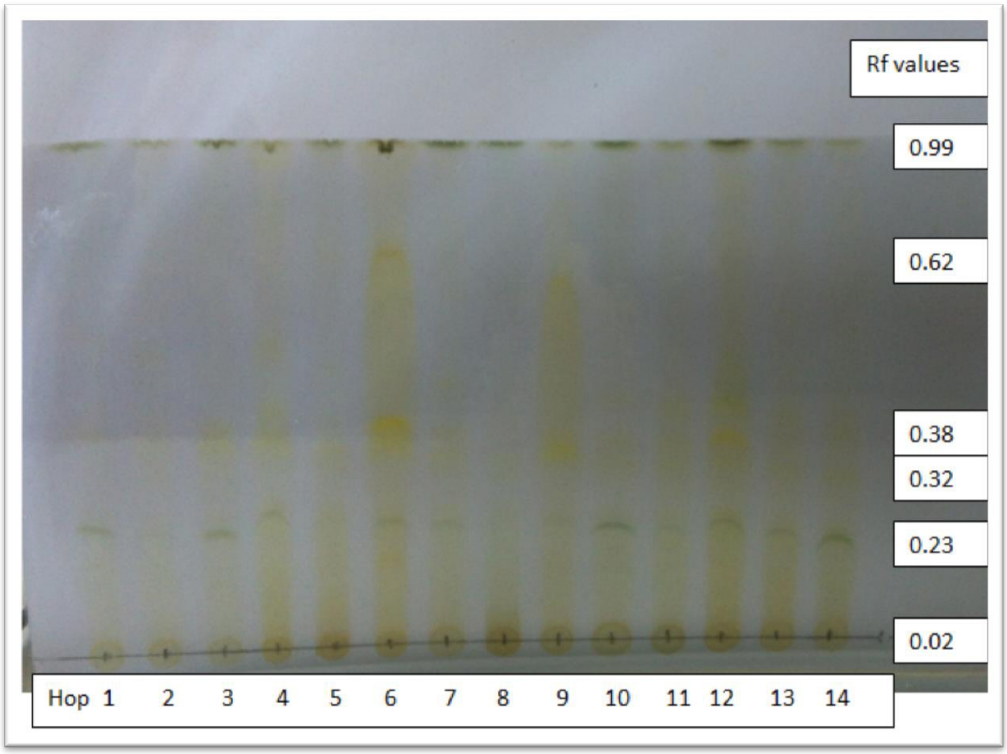

Figure 4. 3 The separation of hops 1 -14 using the TLC assay and calculation of Rf values of separated hop analytes (representative of 3 separate repeats)

After separation, six individual spots could be isolated and the Rf values were recorded and shown in the following Table (4.4). In several hop samples including sample 6 separation of the complete extract was challenging. This method however, was a precursor to antibacterial testing. Therefore, before further separation techniques were used, the antibacterial overlay assay was employed to guide further component purification.

Hop variety	Rf value 1 ± SD 0.001	Rf value 2 ± SD 0.003	Rf value 3 ±SD 0.002	Rf value 4 ±SD 0.003	Rf value 5 ±SD 0.004	Rf value 6 ±SD 0.011
Amarillo	0.02	0.23		0.38	0.62	1.00
Amarillo Pellets	0.02	0.23		0.38	0.62	1.00
Apollo	0.02	0.24	0.32	0.38	0.62	1.00
Aurora	0.02	0.24	0.32	0.38	0.62	0.99
Boadicea	0.02	0.23	0.32	0.38	0.62	1.00
Bobek	0.02	0.23	0.32	0.38	0.62	0.99
Brambling Cross	0.02	0.23	0.32		0.62	1.00
Cascade	0.02	0.23	0.32	0.38	0.62	1.00
Cascade Pellets	0.02	0.23	0.32	0.38	0.62	1.00
Centennial	0.02	0.23	0.32	0.38	0.62	0.99
Centennial Pellets	0.02	0.24	0.32	0.38	0.62	1.00
Challenger	0.02	0.23	0.32	0.38	0.62	0.99
Chinnok	0.02	0.24	0.32	0.38	0.62	1.00
Chinook Pellets	0.02	0.23	0.32	0.38	0.62	1.00
Citra	0.02	0.23	0.32	0.38	0.62	0.99
Citra Pellets	0.02	0.23	0.32	0.38	0.62	0.99
Colombus	0.02			0.38	0.62	
Delta	0.02		0.32	0.38	0.62	
First Gold	0.02		0.32	0.38	0.62	1.00
Fuggles	0.02	0.23	0.32	0.38	0.62	1.00
Fuggles Pellets	0.02	0.23	0.32		0.62	1.00
Galaxy	0.02	0.23	0.32		0.62	0.99
Galena	0.02	0.23	0.32	0.38	0.62	0.99
Glacier	0.02	0.23	0.32	0.38	0.62	0.99
Goldings	0.02	0.23	0.32	0.38	0.62	
Green Bullet	0.02	0.23	0.32	0.38	0.62	1.00
Hallertauer	0.02		0.32	0.38	0.62	1.00
Liberty	0.02		0.32	0.38	0.62	1.00
Magnum	0.02	0.23		0.38	0.62	0.99
Motueka Pellets	0.02			0.38	0.62	
Nelson Sauvin	0.02		0.32	0.38	0.62	1.00
Northdown	0.02		0.32	0.38	0.62	0.99
Northern Brewer	0.02		0.32	0.38	0.62	
NZ Pacifica	0.02		0.32	0.38	0.62	0.99
NZ Rakau	0.02	0.24	0.33	0.38	0.62	
Pacific Gem	0.02	0.23		0.38	0.62	0.99
Pacific Jade	0.02		0.32	0.38	0.62	0.99
Perle	0.02		0.32	0.38	0.62	
Progress	0.02	0.23		0.38	0.62	1.00
Savinjski Goldings	0.02		0.32	0.38	0.62	1.00
Simcoe	0.02		0.32	0.38	0.62	
Sonnet	0.02		0.32	0.38	0.62	
Sorachi Ace	0.02		0.32	0.38	0.62	0.99
Styrian Goldings	0.02		0.32	0.38	0.62	1.00
Summer Pellets	0.02		0.32	0.38	0.62	
Summit	0.02	0.23	0.32	0.38	0.62	
Target	0.21	0.23	0.32	0.38	0.62	0.99
Tettnang	0.21	0.23	0.32	0.38	0.62	
Warrier	0.02	0.23	0.32	0.38	0.62	
Williamette		0.23	0.32	0.38	0.62	0.99

Table 4. 4 Hop variants (n=50) were separated using the TLC assay and the Rf values separated spots (analytes) were recorded. Values are representative of 3 repeats; ± SD as described

4.4.4 The bacterial overlay assay

Whilst the TLC method is a useful assay for separation, this study was bioassay guided. The bacterial overlay assay is a useful means of determining the inhibitory activity of TLC separated hop analytes. Each hop (N=50) was overlaid with *M. smegmatis* using (Method 4.3.4). Inhibitory activity was recorded as clear spots and the corresponding Rf value was recorded. Figure 4.4 is a representative example of the bacterial overlay assay showing 6 clear areas of inhibition.

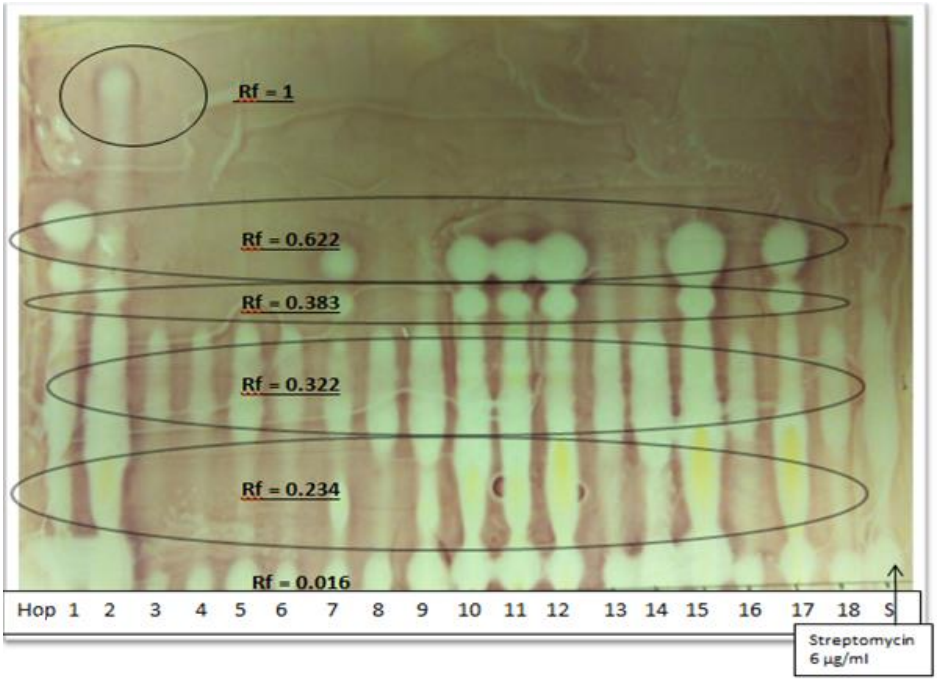

Figure 4. 4 The bacterial overlay assay using *M. smegmatis* against methanol hop extracts of hops 1-18 (Table 2.2). Inhibition is highlighted by unstained white spots in contrast to the stained (red) background indicating growth of the bacteria. Rf values refer to the Rf of separated spots. Figure representative of 3 separate repeats. Positive control Streptomycin (6 µg/ml^{-1})

Using the bacterial overlay assay, six separate Rf values proved to be inhibitory towards the growth of *M. smegmatis*. Each hop variety was assessed for the presence and antibacterial activity of these analytes, and corresponding Rf values of inhibitory analytes, are detailed in the following Table (Table 4.5).

Hop variety	Rf value 1 ±SD 0.001	Rf value 2 ±SD 0.001	Rf value 3 ±SD 0.000	Rf value 4 ±SD 0.000	Rf value 5 ±SD 0.000	Rf value 6 ±SD 0.001
Amarillo	0.02	0.23		0.38	0.62	
Amarillo Pellets	0.02	0.23		0.38	0.62	
Apollo	0.02	0.24	0.32	0.38		
Aurora	0.02		0.32	0.38		
Boadicea	0.02	0.23	0.32	0.38		
Bobek	0.02		0.32	0.38		
Brambling Cross	0.02	0.23	0.32			
Cascade	0.02	0.23	0.32	0.38		
Cascade Pellets	0.02	0.23	0.32	0.38		
Centennial	0.02	0.23	0.32	0.38		
Centennial Pellets	0.02	0.24	0.32	0.38		
Challenger	0.02	0.23	0.32			
Chinnok	0.02	0.24	0.32		0.62	
Chinook Pellets	0.02		0.32		0.62	
Citra	0.02	0.23	0.32	0.38	0.62	0.99
Citra Pellets	0.02	0.23	0.32	0.38	0.62	0.99
Colombus	0.02			0.38	0.62	
Delta	0.02		0.32		0.62	
First Gold	0.02		0.32	0.38	0.62	1.00
Fuggles	0.02	0.23	0.32		0.62	1.00
Fuggles Pellets	0.02	0.23	0.32		0.62	1.00
Galaxy	0.02	0.23	0.32		0.62	0.99
Galena			0.32		0.62	
Glacier	0.02			0.38	0.62	
Goldings	0.02	0.23	0.32	0.38	0.62	
Green Bullet	0.02	0.23			0.62	1.00
Hallertauer	0.02		0.32	0.38	0.62	
Liberty	0.02		0.32	0.38	0.62	
Magnum	0.02	0.23		0.38	0.62	1.00
Motueka Pellets	0.02			0.38	0.62	
Nelson Sauvin	0.02		0.32	0.38	0.62	
New Zealand Rakau	0.02	0.24	0.33	0.38	0.62	
Northdown	0.02		0.32	0.38	0.62	
Northern Brewer	0.02		0.32		0.62	
NZ Pacifica	0.02		0.32		0.62	
Pacific Gem	0.02	0.23		0.38		
Pacific Jade	0.02		0.32			
Perle	0.02		0.32	0.38		
Progress	0.02	0.23		0.38	0.62	
Savinjski Goldings	0.02		0.32			
Simcoe	0.02		0.32			
Sonnet	0.02		0.32			
Sorachi Ace	0.02		0.32	0.38		0.99
Styrian Goldings	0.02					1.00
Summer Pellets	0.02		0.32	0.38		
Summit	0.02	0.23		0.38		
Target	0.21		0.32			0.99
Tettnang	0.21		0.32		0.62	
Warrier	0.02			0.38	0.62	
Williamette		0.23	0.32	0.38		0.99

Table 4. 5 The Rf values of separated hop components in which inhibitory activity in the bacterial overlay assay against *M. smegmatis* 8159 was observed. Rf values are a result of 3 separate repeats (± SD)

Based on the results of the bacterial overlay assay (Table 4.5), it was observed that antibacterial activity resided in the six separated spots. However, each antimicrobial spot was not present in each hop variant.

As an example, the hop variant Citra has six separated compounds from the TLC analysis and when the bacterial overlay assay is used, each separated component has inhibitory activity against *M. smegmatis*. Compared to the hop variant Galena, which upon TLC separation also had six compounds, but only two (Rf 0.32; 0.62) were observed to be inhibitory against *M. smegmatis*.

In Table 4.6 each separated Rf value is logged alongside the number of instances where it was recognised using the TLC method and from the total number of hops (n=50). By using the bacterial overlay assay the number of hop extracts that this component was shown to be inhibitory against *M. smegmatis* is also displayed.

Rf Value	The total number of hop variants which contain the component (Out of a total of 50)	The total number of hop variants where the component is inhibitory against *M. smegmatis*	The % of hops in which component is present and has inhibitory activity
0.02	49	48	98 %
0.23	32	24	75 %
0.32	43	38	88.4 %
0.38	47	31	65.9 %
0.62	50	28	56 %
0.99	37	11	29.7 %

Table 4. 6 A summary of the total number of hop varieties that analytes are present and antibacterial against *M. smegmatis* as identified using the bacterial overlay assay (n=3)

It is reasonable to assume, from the data presented in Table 4.6 that each hop variant may have several different components leading to differences in the antibacterial activity observed.

For example, Rf value (0.02) is present in 49 out of 50 hops and 48 varieties, (98 %), of these have inhibitory activity against *M. smegmatis*. However, Rf value 6 (0.99) is present in 37 hop varieties but only antibacterial in 11, (29.7 %), of these.

As only six antimicrobial spots were separated from the crude hop extracts, it is possible that each spot contains a mixture of compounds or similar polarity which have not been fully separated by the TLC assay. However, to determine if there was any link between the Rf values and that of known antimicrobial compounds from hops, a literature search was undertaken though no related compounds were identified on this basis.

This was not surprising as TLC separation can be highly affected by the use of different solvent systems and local conditions (Reed, 2007). There are several reasons why the TLC assay is limited. The first is that only a low sample weight can be used. In each bacterial overlay assay, each hop variety was assayed at the same weight. In varieties which had lower concentrations of antibacterial analytes, the activity could be missed leading to negative results and the possibility of missing potential bioactive compounds. By using a higher initial sample weight separation of the extract would be reduced as the TLC plate would become overloaded.

However, even though separation is restricted; the bacterial overlay and TLC assay are used here solely for primary separation of hop extracts and to ascertain the antibacterial activity, so that further separation and purification methods can be guided.

A conclusion gained from using this assay was that several hop compounds may contribute to the overall biological activity and these may have similar polarities, leading to restricted separation in the TLC assay. For this reason, a further separation technique was used: column chromatography. Due to the laborious nature of the method and the use of high amounts of solvent, two hop variants were chosen for further investigation. The first hop variety, Citra, was chosen because all of the components separated using the TLC assay had inhibitory activity against *M. smegmatis,* the highest of all hop extracts tested. The other, Galena, had only two inhibitory components. This mirrors the result found in the previous chapter and may go some way to explain the different levels of activity.

4.4.5 Separation of antimicrobial hop components using silica gel column chromatography

Although the TLC assay is a useful method to separate an initial mixture of compounds, it is limited by the amount of sample that can be loaded and therefore collected. For this reason column chromatography was employed.

Once all fractions had been collected, *Citra* (n=126) *Galena* (n=130) solvent was removed by evaporation and each resulting extract was re-suspended in 1 mL of methanol. Each fraction was then assayed using the bacterial overlay assay against *M. smegmatis*; fractions with the same Rf values and antibacterial activity were combined, the solvent evaporated and extract weighed (Table 4.7).

	Hop variety	
	Galena 5 g of extract	**Citra 5 g of extract**
Total weight of recovered sample (g) including (1-6)	2.3 g (total loss of 2.7 g)	2.4 g (total loss of 2.6 g)
1) Recovered Weight of Rf 0.02	Recovered (0.015 g) not inhibitory	**Recovered (0.035 g) Inhibitory**
2) Recovered weight of Rf 0.23	Not recovered	Not recovered
3) Recovered weight of Rf 0.32	**Recovered (0.024 g) Inhibitory**	**Recovered (0.037 g) Inhibitory**
4) Recovered weight of Rf 0.38	Recovered (0.023 g) not inhibitory	**Recovered (0.048 g) Inhibitory**
5) Recovered weight of Rf 0.62	Not recovered	Not recovered
6) Recovered weight of Rf 0.99	Recovered (0.035 g) not inhibitory	**Recovered (0.094 g) Inhibitory**
Total weight of recovered antibacterial analytes	**0.024 g**	**0.214 g**

Table 4. 7 The recovered weight (g) of hop components from the Galena and Citra hop variants separated using silica gel column chromatography. Bold text highlights recovered antibacterial components as a result of 3 separate repeats

Several differences between the two hop variants and the recovered antimicrobial components were observed in Table 4.8. Firstly component 1 (Rf 0.02) was shown to have antibacterial activity against *M. smegmatis* using the bacterial overlay assay in the Citra variant only. This was also observed using the TLC overlay assay previously. Another difference was observed in that the recovered weight of component 1 differed between variant; with 0.035 g recovered from Citra and 0.015 g recovered from Galena.

In neither case was component two or five recovered. This was perhaps surprising as it was shown to be inhibitory in the Citra variant and as the solvent system was the same as previously used, suggests that the component had either become 'stuck' within the column or had eluted with other components.

Component three was recovered from both varieties and was observed to also still be inhibitory towards the growth of *M. smegmatis*. Again, a difference in the recovered weight was noticed between the two variants with Galena (0.024 g) having a considerably less yield than Citra (0.037 g).

As in component one, components four and six were also recovered, but again observed inhibitory activity in the Citra variant only, matching that of the TLC overlay assay, but also observed a much lower yield of the components in the Galena variant.

The differences in the recovered weight of the components highlights that there may be a difference in compounds separated between the two variants. However, it may indicate a problem with the separation and recovery method as Table 4.6 also details the total amount of hop extract that was recovered following column chromatography separation. Significant loss of extract was experienced in both Citra (-2.6 g) and Galena (-2.7 g) extracts as can be expected in column chromatography. This is due to the use of silica as a stationary phase (Moldoveanu and David, 2002), non-polar compounds which are absorbed into the silica should elute first followed by the more polar compounds. Depending on the solvent system in use, compounds adsorbed can bind to the silica reducing elution and the degree of separation.

This may be true of analytes 2 and 5, both of which were present in the original TLC analysis but where not recovered from the column.

Overall this method highlights that a high yield of analytes from the TLC analysis can be separated using column chromatography, isolated and assayed against bacteria for their biological activity. The method also highlights that there is a significant loss of yield due to the potential polarity of compounds within the hop extract. It was also noticed that no more separation of compounds was observed using this method.

4.4.6 Sephadex® LH-20 Column chromatography

Due to the ability of column chromatography to separate and recover a high yield of components from the extract, another form of stationary phase was used: sephadex® LH-20. As in the silica column chromatography method the hop variants Citra and Galena were again chosen for further separation and identification.

Once all fractions had been collected Citra (n=103) Galena (n=93). Each fraction was assayed for biological activity against *M. smegmatis*; fractions from the same variant with the same Rf values and antibacterial activity were combined, the solvent evaporated and extract weighed (Table 4.8).

	Extract weight and Hop variety	
	Galena 5 g of extract	**Citra 5 g of extract**
Yield of recovered sample (g) **Loss of sample (g)**	4.99 g Total loss – 0.01 g	4.98 g Total loss – 0.02 g
1) Recovered Weight of Rf 0.02	Recovered (0.141 g) not active	**Recovered (0.128 g) Active**
2) Recovered weight of Rf 0.23	Recovered (0.103 g) Not active	**Recovered (0.116 g) Active**
3) Recovered weight of Rf 0.32	**Recovered (0.086 g) Active**	**Recovered (0.095 g) Active**
4) Recovered weight of Rf 0.38	Recovered (0.112 g) not active	**Recovered (0.134 g) Active**
5) Recovered weight of Rf 0.62	**Recovered (0.103 g) Active**	**Recovered (0.126 g) Active**
6) Recovered weight of Rf 0.99	Recovered (0.995 g) not active	**Recovered (0.136 g) Active**
Total weight of Recovered antibacterial analytes	**0.189 g**	**0.735 g**

Table 4. 8 The total weight of recovered hop extracts and the weight of antibacterial hop components as determined by the bacterial overlay assay against *M. smegmatis* following separation using Sephadex® LH-20 column chromatography

By comparing this method to the silica column chromatography, the first difference is the overall yield of the hop extracts. In this method 4.99 g of the Galena extract and 4.98 g of the Citra extract was recovered, compared to 2.3 g and 2.4 g respectively using silica gel. The second difference is that all components identified in the TLC method were isolated from the column. In comparison using silica gel, analytes 2 and 5 were not recovered which shows the difference in the nature of the stationary phase.

4.5 Mass spectrometry

Mass spectrometry is a commonly used tool for the identification of natural products and has been used for the characterisation of hop extracts (Taylor *et al*., 2003; Vu *et al*., 2008).

Each component separated using Sephadex® column chromatography was assayed using mass spectrometry to determine the mass of dominant compounds. The masses of the compounds detected using mass spectrometry were compared to known antimicrobials previously isolated from hops (Table 4.9 below). Known antimicrobial compounds (Table 4.9) were discounted from further investigation.

Compound	M (molecular Weight)	$M + H^+$	$M + Na^+$	$2M + Na^+$	Formula
Adhumulone	579.003	580.003	602.003	1181.006	$C_{21}H_{30}O_5$
Adlupulone	428.290	429.290	451.290	879.580	$C_{27}H_{40}O_4$
Co-humulone	348.43	349.433	371.433	719.866	$C_{20}H_{28}O_5$
Co-lupulone	400.550	401.550	423.550	824.101	$C_{25}H_3O_4$
Farnesol	222.366	223.366	245.366	467.732	$C_{25}H_{26}O$
Geraniol	154.249	155.249	177.249	331.498	$C_{10}H_{18}O$
Geranyl acetate	196.286	197.286	219.286	415.572	$C_{12}H_{20}O$
Humulone	362.459	363.459	385.459	747.919	$C_{21}H_{30}O_5$
Isocohumulone	332.433	333.433	355.433	687.867	$C_{20}H_{28}O_4$
Limonene	136.234	137.234	159.234	295.468	$C_{10}H_{16}$
Linalool	154.249	155.249	177.249	331.498	$C_{10}H_{18}O$
Lupulone	414.577	415.577	437.577	852.155	$C_{26}H_{38}O_4$
Myrcene	136.234	137.234	159.234	295.468	$C_{10}H_{16}$
Trans-2-nonenal	140.222	141.222	163.222	303.445	$C_9H_{16}O$
Xanthohumol	354.396	355.396	377.396	731.792	$C_{21}H_{22}O_5$
α-humulene	204.351	205.351	227.351	431.702	$C_{15}H_{24}$
β-carotene	536.872	537.872	559.872	1096.745	$C_{40}H_{56}$

Table 4. 9 molecular weight (MW; MW+H_2; MW+Na; 2MW+Na) and chemical formula of known compounds from hops

Chemical formula search was conducted using the NCBI chemical search engine (NCBI, 2014).

4.5.1 Mass spectrum analysis of components isolated from Rf 6

The Citra hop extract was separated using Sephadex®® column chromatography and the component identified as Rf 6 was isolated, the mass spectrum of this the component is displayed in Figure 4.5.

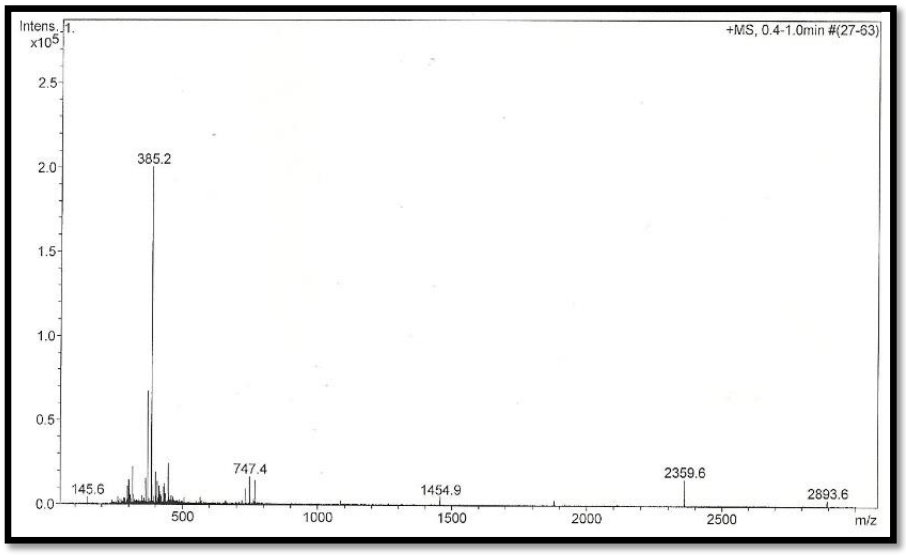

Figure 4. 5 Mass spectrum of component 6 extracted from Citra hop variant using column chromatography

The mass spectra of component presented a baseline peak with an *m/z* of 385.2, with a second lower intensity peak with an *m/z* of 747.2. Using the information gained from the literature search, it became apparent that this molecule was humulone with the peak at *m/z* 385.2 matching the MW+ Na from Table 4.10, the second peak with an *m/z* of 747.2 was determined as the 2M+Na species of this compound.

The Galena hop extract was also extracted and separated using Sephadex®® column chromatography and the MS of the component isolated is displayed in the following Figure (4.6)

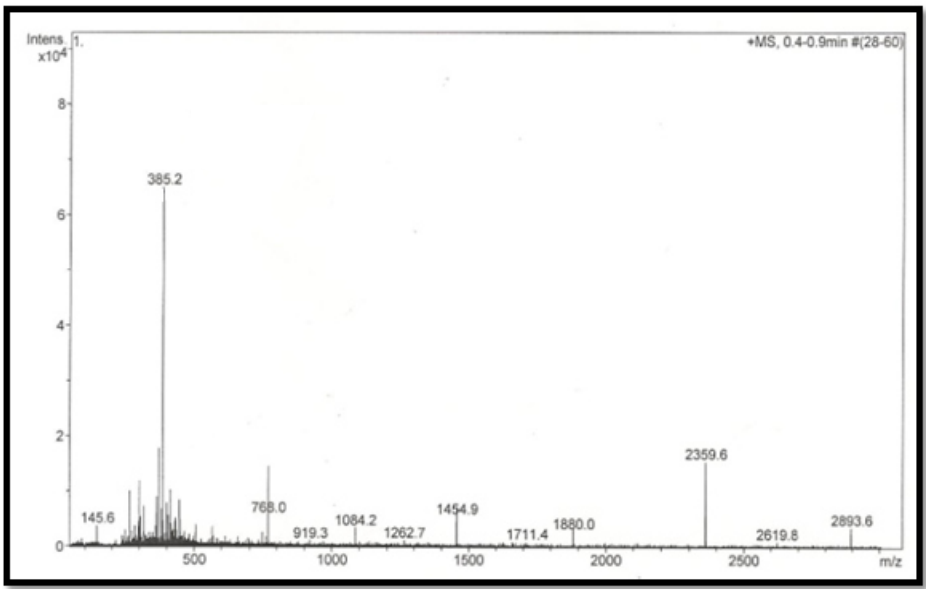

Figure 4. 6 Mass spectrum of Rf 6 extracted from Galena hop variant using column chromatography

From the mass spectra observed in Figure 4.6, it is possible to note the presence of a peak with an *m/z* 385.2, which was previously identified from the Citra extract as humulone. It is also noticeable that there are multiple secondary peaks within the fraction (*m/z* 273.2; 283.3; 768.0; 1454 and 2359.6).

It is hypothesised that the presence of these compounds has an antagonistic effect on the antibacterial activity of humulone. As the dominant compound humulone has been previously shown to be antibacterial and is well described in literature no further separation and purification was undertaken on this component (Erdmann, 1951; Stavri *et al*., 2004)

4.5.2 Mass spectrum analysis of components isolated from Rf 5

Component Rf 5 was separated from both the Citra and Galena extracts using Sephadex® column chromatography, and each extract was weighed to 1 mg/mL^{-1} and suspended in HPLC grade methanol. In Figure 4.7 the resulting spectra is displayed.

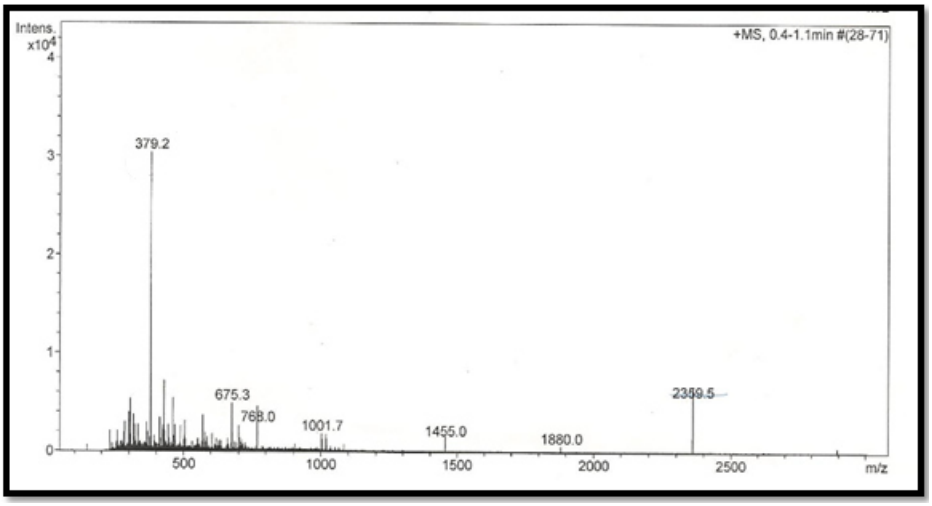

Figure 4. 7 Mass spectrum of Rf value 0.62 extracted from Citra hop variant using column chromatography and analysed using mass spectrometry (representative of 2 repeats)

The mass spectra of Citra has one baseline peak at *m/z* 379.2 Comparing the mass to published literature, this peak corresponds with the research carried out by (García-Villalba *et al*., 2006) who identified this mass as an oxidised derivative of humulone using a similar technique. Humulone and its oxidised derivatives have been shown to be inhibitory towards the growth of bacteria (Cornelison, 2008). The analysis was also carried out using the Galena variety as shown in the following Figure (Figure. 4.8).

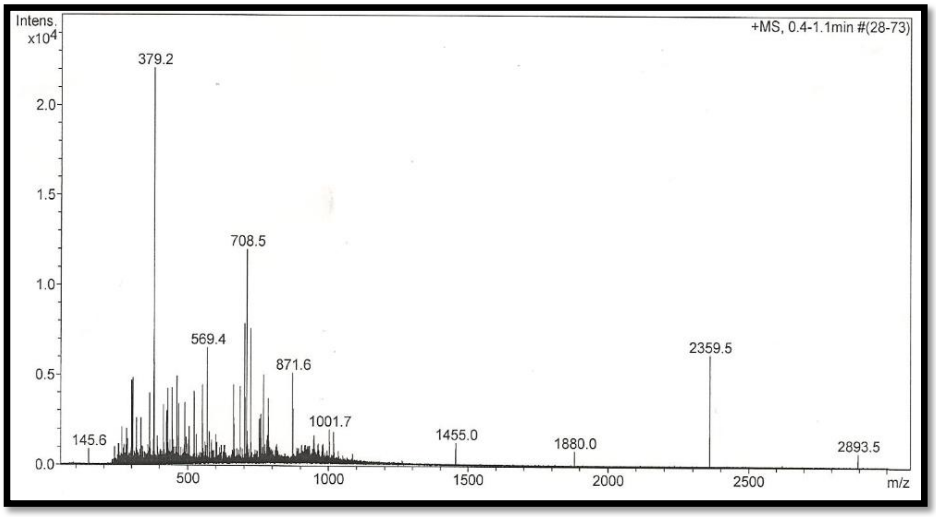

Figure 4. 8. Mass spectrum of Rf 0.62 extracted from galena hop variant using column chromatography and analysed using mass spectrometry (representative of 2 repeats)

After initial observation, it is possible to notice that the Galena extract, like the Citra extract has the oxidised humulone derivative at *m/z* 379.2. Several other peaks were also observed at *m/z* 569.4; 706.5; 708.5 and 871.

The bacterial overlay assay indicated that this component was inhibitory towards the growth of *M. smegmatis* in extracts from both the Citra and Galena varieties. Further investigation using mass spectrometry on this component reveals a dominant compound which can be identified using current literature as an oxidised humulone derivative which has been previously identified as antibacterial (Cornelison, 2008). Therefore, the inhibitory activity can be linked to this compound and no further separation or isolation procedures were undertaken.

4.5.3 Mass spectrum analysis of components isolated from Rf 4

Component four (Rf 0.38) was also separated from the both the Citra and Galena hop extracts using Sephadex® column chromatography and by using the bacterial overlay assay was shown to be inhibitory towards the growth of *M. smegmatis* in the Citra variant only. In Figure 4.9 the mass spectra of component three from the Citra variant is displayed.

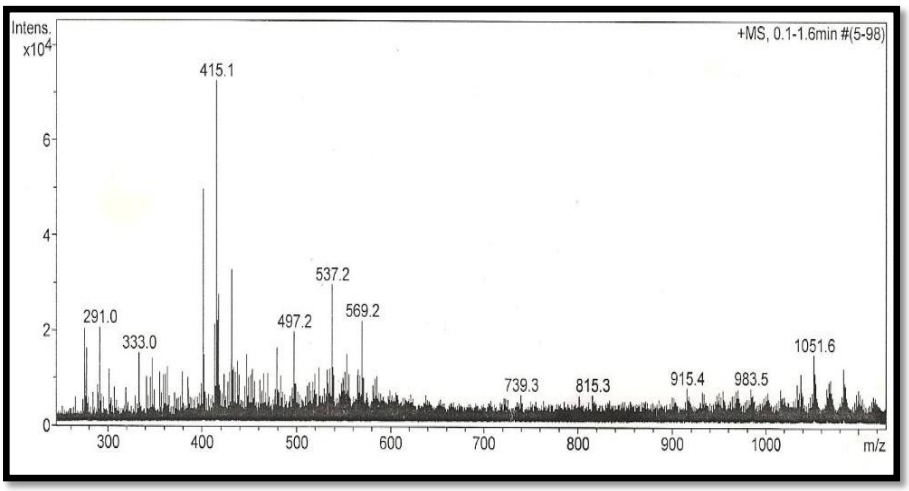

Figure 4. 9 Mass spectrum of Rf value 0.38 extracted from Citra hop variant using column chromatography

From the initial mass spectra several peaks were observed; the first with an *m/z* of 401; corresponds using Table 4.10 with the compound co-lupulone. Co-lupulone ($C_{25}H_{36}O_4$) is another example of a bitter acid related to lupulone, and differs in the number of carbonyls; co-lupulone is a well-researched bioactive compound commonly isolated from hops (Van Cleemput *et al*., 2009a)

Further investigation reveals that lupulone (*m/z* 415.1) can also be identified from the Citra hop extract. Based on the fact that both the lupulone and co-lupulone are the most dominant compounds, it can be assumed that the majority of antimicrobial activity can be associated with these compounds. Another peak identified (*m/z* 537.2) is associated with the pigment β-carotene and is a common pigment found within the majority of plant products (Vu *et al*., 2008) and has not been reported as antimicrobial.

The component 3 (Rf 0.38) was extracted from the Galena variety was also assayed using mass spectrometry and the mass spectrum is displayed in Figure 4.10.

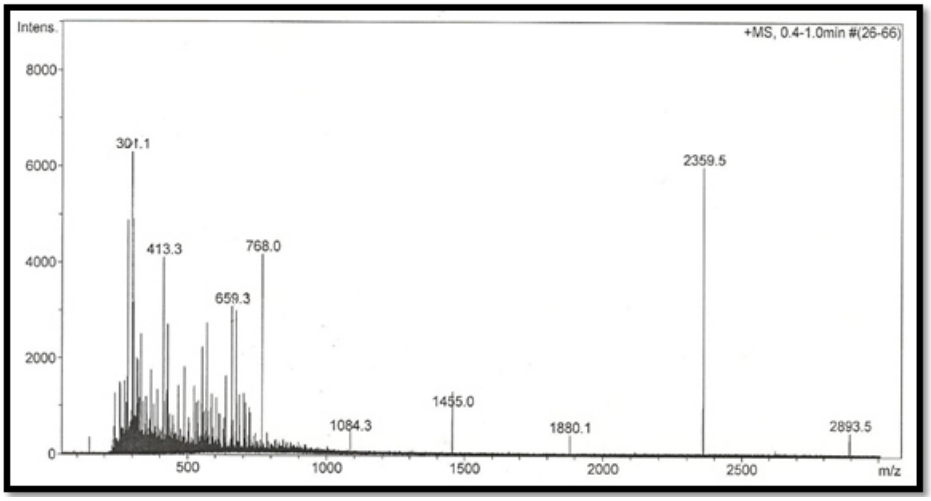

Figure 4. 10 Mass spectrum of Rf value 0.38 extracted from Galena hop variant using column chromatography

As can be seen from the mass spectra of component 0.383, there is a peak at 414.3 which was also previously identified in the Citra extract. A peak with an *m/z* of 301.1 was also observed but after investigation was shown to be a common contaminant within the mass spectrum. Peaks with an *m/z* of 659.3 and *m/z* 768.0 were also observed in the mass spectrum, although due to the lack of inhibitory activity against *M. smegmatis* no further investigation was undertaken. In conclusion, the inhibitory activity observed in the bacterial overlay assay by the Citra extract with an Rf of 0.383 can be associated with the presence of both lupulone and co-lupulone, this activity can be further associated with previous research (Erdmann, 1951; Siragusa *et al*., 2008).

4.5.4 Mass spectrum analysis of components isolated from Rf 3

Both Galena and Citra hop extracts were separated using Sephadex®® column chromatography and the analytes at Rf 0.32 present in both the Citra and Galena variants were observed to be inhibitory to the growth of *M. smegmatis*. In Figure 4.11 the mass spectrum of the Citra extract is displayed.

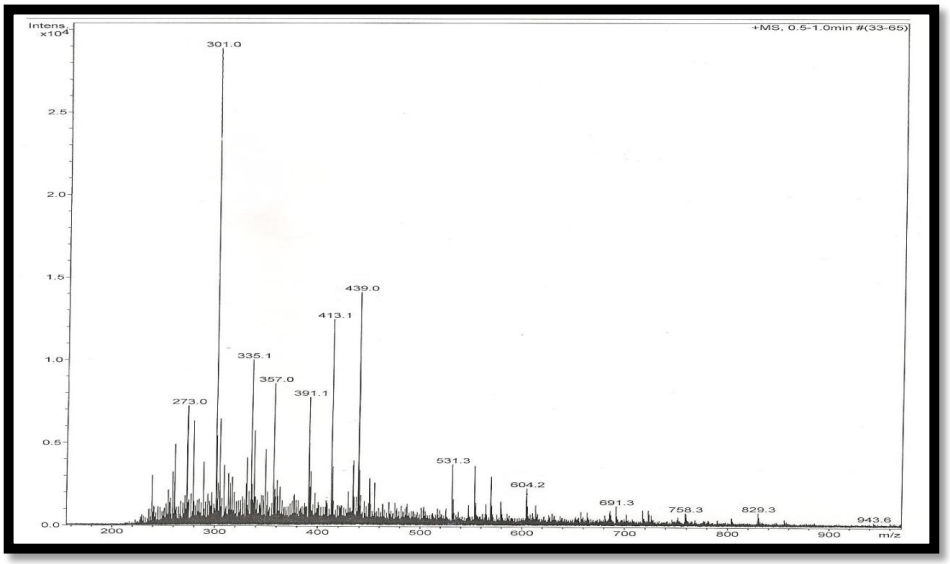

Figure 4. 11 Mass spectrum of Rf value 0.32 extracted from the Citra hop variant using column chromatography

Several peaks were observed in the mass spectrum analysis, the first (*m/z* 301.0) can be identified as a contaminant which is commonly observed in mass spectrometry and is not a concern.

The baseline peaks (*m/z* 439.0 and *m/z* 413.1) were observed and after a literature search compound *m/z* 413.1 was identified as the oxidised lupulone derivative, adlupulone as previously described by (Ceslova *et al*., 2009) . The second component with an *m/z* 439 can be identified as a fragment of the *m/z* 416.1, which identified by (Ceslova *et al*., 2009) is also an oxidised derivative of lupulone. Another peak at *m/z* 273.0 has also been previously identified as an epicatechin a catechin isomer (Shui and Leong, 2004).

The Galena extract which also showed inhibitory activity at Rf value 0.32 was also assayed using mass spectrometry and the mass spectrum is displayed in Figure 4.12.

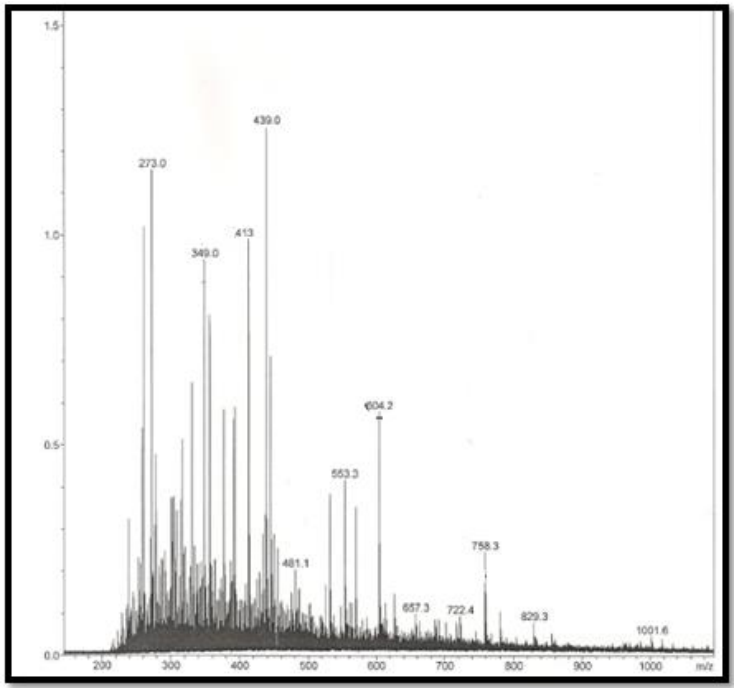

Figure 4. 12 Mass spectrum of Rf value 0.32 extracted from the galena hop variant using column chromatography

Again fragments in the instrument are observed at *m/z* 301.0 and are ignored from the investigation. Several peaks are observed from the Galena extract which are similar to the Citra extract namely *m/z* 273.0; previously identified as catechin; *m/z* 413.1; 439.0 which were also previously identified as the lupulone derivative adlupulone.

In conclusion, component 3 (Rf 0.32) separated from both the Citra and Galena hop extracts exhibited inhibitory activity against *M. smegmatis* in the bacterial overlay assay. Mass spectrum analysis revealed 3 dominant compounds with a *m/z* of 439.0; 413.1 and 273.0, relevant literature was searched and it was found that these *m/z* corresponded to compounds lupulone, adlupulone and epicatechin; all of which have previously been shown as antibacterial. Therefore no further identification was undertaken on this extract.

4.5.5 Mass spectrum analysis of components isolated from Rf 2

Component two (Rf 0.23) was also separated from the both the Citra and Galena hop extracts using Sephadex® column chromatography and by using the bacterial overlay assay the extract was shown to be inhibitory towards the growth of *M. smegmatis* in the Citra variety only. In Figure 4.13 the mass spectra of the Citra extract is displayed.

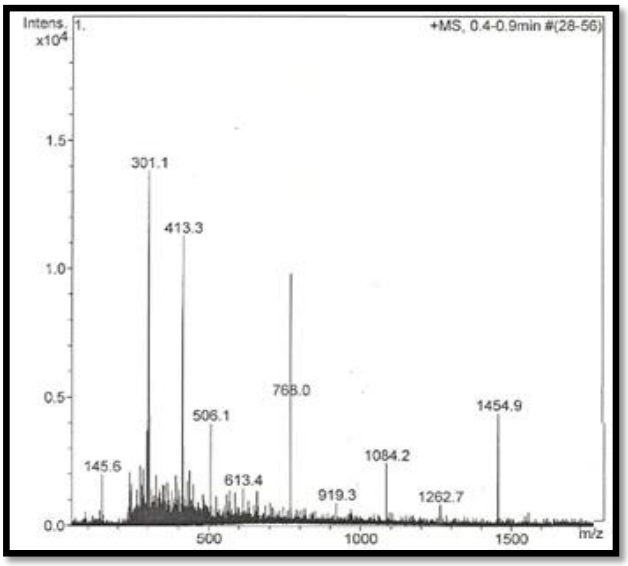

Figure 4. 13 Mass spectrum of Rf value 0.23 extracted from the Citra hop variant using column chromatography

From the mass spectra two peaks were identified (m/z 413.3 and m/z 768.0). Previous identification showed that peak m/z 413.3 was lupulone and whilst being present in the previous fraction, the compound has eluted from the column within this fraction also. Another peak at m/z 768.0 and 1454.9 was discounted from further studies as it was shown to be a common plasticiser.

The component from the Galena variety, although not inhibitory towards the growth of *M. smegmatis* was also assayed using mass spectrometry and the mass spectrum is displayed below in Figure 4.14.

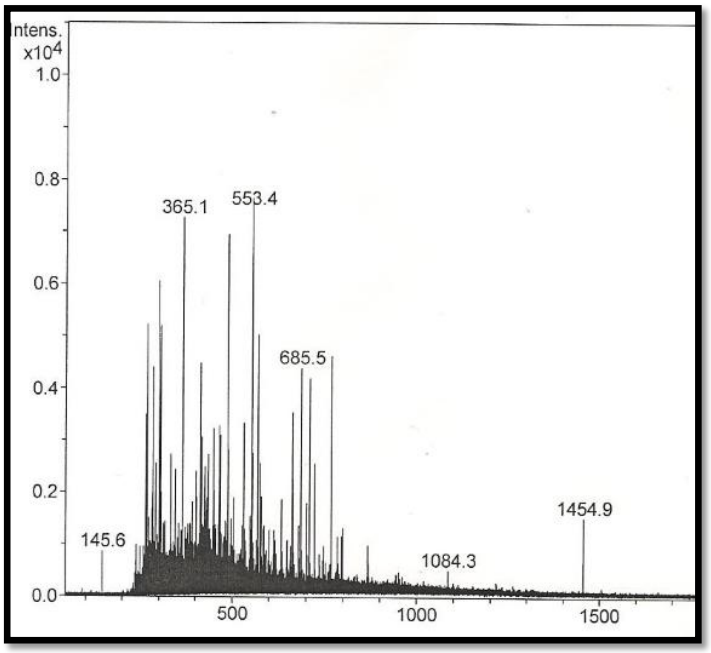

Figure 4. 14 Mass spectrum of Rf value 0.23 extracted from the Galena hop variant using column chromatography

The mass spectra of the Galena variety showed that there was an absence of m/z 413.3. Due to the lack of antibacterial activity from the fraction no further separation of the extract was undertaken.

4.5.6 Mass spectrum analysis of components isolated from Rf 1

Component one (Rf 0.02) was also separated from the both the Citra and Galena hop extracts using Sephadex® column chromatography and by using the bacterial overlay assay was shown to be inhibitory towards the growth of *M. smegmatis* in the Citra variety only. In Figure 4.15 the mass spectrum of the Citra extract is displayed.

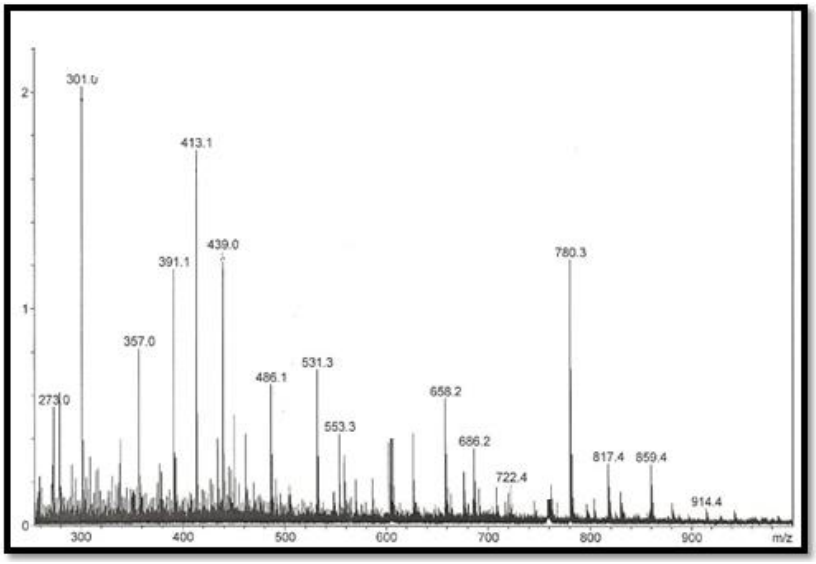

Figure 4. 15 Mass spectrum of Rf value 0.02 extracted from the Citra hop variant using column chromatography

This component was inhibitory towards the growth of *M. smegmatis* on the baseline of the bacterial overlay assay. This suggests that this component may contain compounds which are weakly non-polar and thus would not elute through the TLC plate.

Mass spectrometry analysis shown in Figure 4.15 above reveals the presence of lupulone (*m/z* 413.1),

The second peak of interest m/z 439.0 has been previously identified as adlupulone and was therefore not subject to any further investigation.

It was observed that there was a high amount of corresponding peaks within the mixture. It may be that these components are of a low polarity, meaning that separation using the current solvent system is not possible. Whilst it may be possible to further investigate this sample for any other antimicrobial compounds, it was hypothesised that the previously identified compounds were responsible for any antimicrobial activity.

Although component one isolated from the Galena extract did not exhibit any inhibitory activity, it was also analysed using mass spectrometry and the mass spectrum is displayed in the following Figure (4.16).

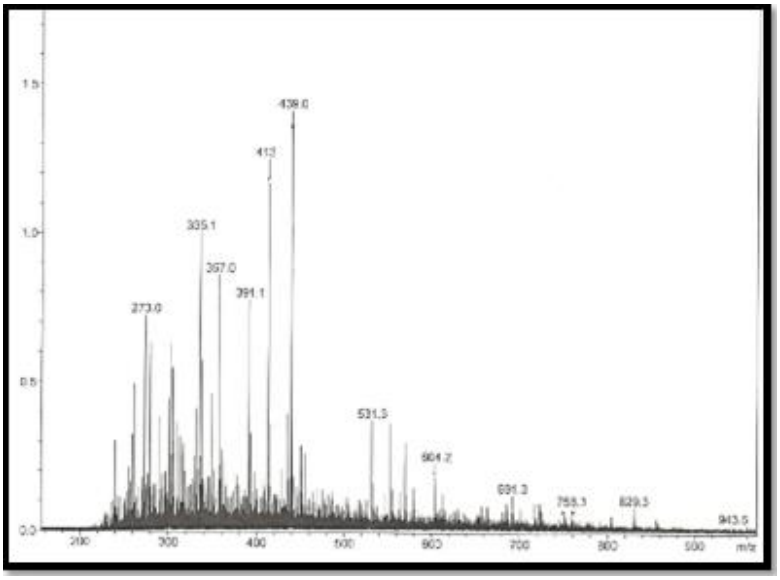

Figure 4. 16 Mass spectrum of Rf value 0.02 extracted from the galena hop variant using column chromatography

The mass spectrum of Rf 0.02 from the Galena extract also has a mass with an m/z of 413). The component also does not have a peak at m/z 439.0 which corresponded to adlupulone. These differences may allude to the variation in activity between the two varieties. It was also observed as in the Citra variant that that there were also a number of peaks. However, as this component did not produce any inhibitory activity towards the growth of *M. smegmatis* no further isolation techniques were undertaken. These compounds were also identified in the Citra component this was also another reason why no further separation was undertaken.

4.6 High Resolution Mass Spectrometry (HRMS)

High resolution mass spectrometry was used to determine more precisely the structure of any identified compounds and to help locate the predominant antimicrobial polyphenol xanthohumol (XN). It is known that the compound has a molecular weight of 354.39 and a chemical formula of $C_{21}H_{22}O_5$. By combining separated active extracts of both the Galena and Citra extracts and then analysing at molecular weights between *m/z* 200 and 400 it was hypothesised that XN could be identified.

In the following Figure 4.17 the HR-MS of the crude Citra hop extract is displayed with emphasis on the *m/z* between 200 and 500.

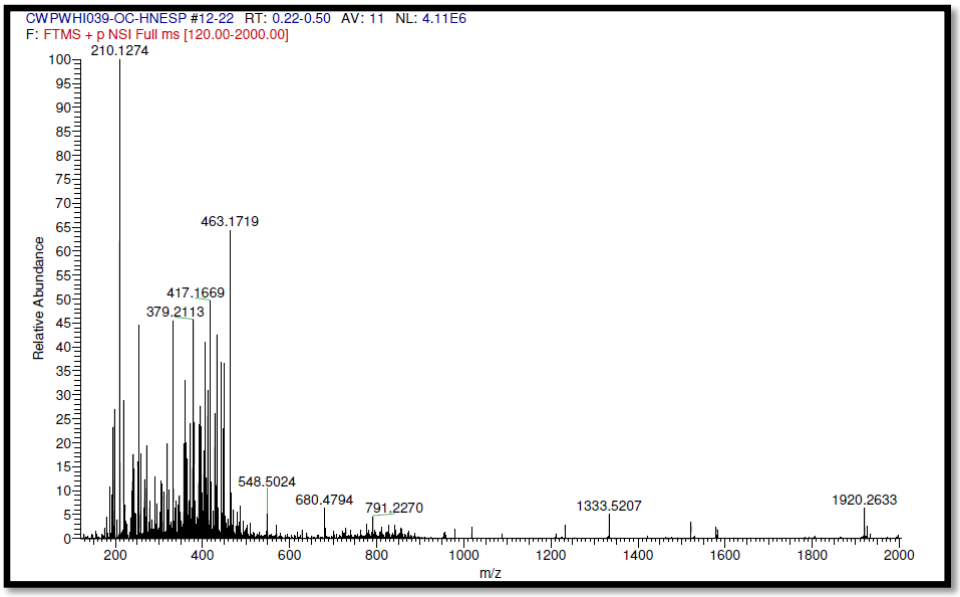

Figure 4. 17 High resolution mass spectra of the crude Citra hop extract

It can be initially observed from Figure 4.17 that the baseline compound has an *m/z* at 210.1274. Upon investigation this was reported to be a contaminant within the mass spectra and was therefore discounted from any further investigation.

The second compound of interest (*m/z* 463.1719) had not been previously observed in any of the previous mass spectras'. To further identify the compound, a more intense mass spectra is displayed in the following Figure 4.18.

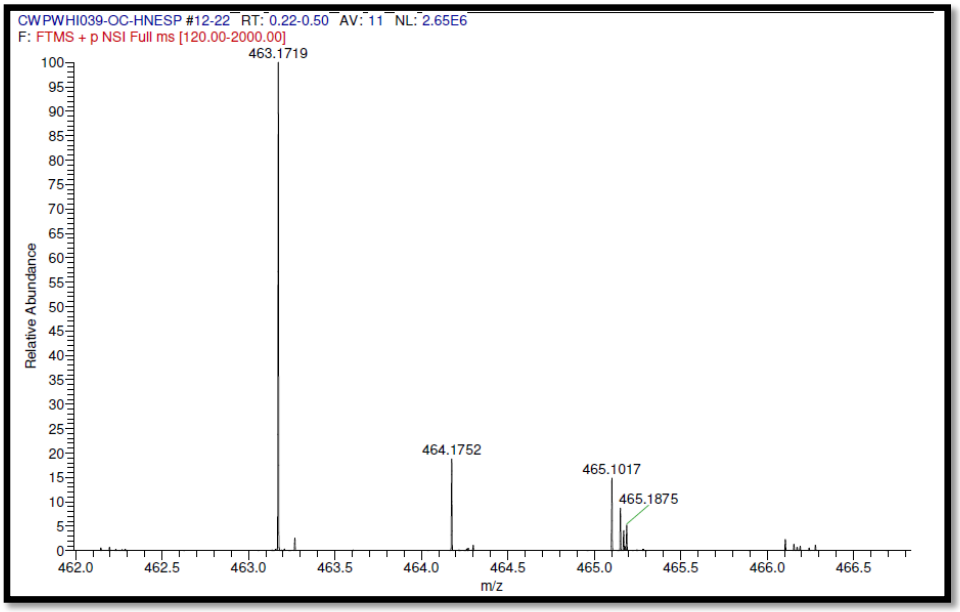

Figure 4. 18 High Resolution Mass Spectra of compound *m/z* 463.1719 isolated from the antibacterial Citra crude hop extract

By using high resolution mass spectrometry it is possible to use the instrument to estimate the theoretical mass and potential elemental composition of compounds isolated to an accuracy of ± 3 ppm. By estimating the chemical composition of the compounds, further investigations would be performed using databases such as SciFinder to try and match the compound with those previously identified.

In Figure 4.19 the estimations of the chemical formula of this compound are displayed

Isotope:	Min. .. Max.			
12 C	0....80			
1 H	0....100			
14 N	0....16			
16 O	0....16			
23 Na	0....1			
Tolerance Window:	+- 5.00 ppm			
Db/Ring Equiv:	-3.. 100	N-Rule:	Do not use	
Fits:	200	Charge:	1	

Mass	Theoretical Mass	Delta [ppm]	RDB	Composition
463.1719	463.1719	0.0	-0.5	$C_{9}H_{28}O_{12}N_{8}Na_{1}$
	463.1719	0.0	5.0	$C_{8}H_{22}O_{7}N_{15}Na_{1}$
	463.1716	0.6	-2.0	$C_{8}H_{24}O_{15}N_{7}$
	463.1716	0.6	3.5	$C_{7}H_{23}O_{10}N_{14}$
	463.1714	1.1	12.0	$C_{23}H_{26}C_{6}N_{4}Na_{1}$
	463.1714	1.1	17.5	$C_{22}H_{20}C_{1}N_{10}Na_{1}$
	463.1724	-1.2	21.0	$C_{22}H_{17}N_{13}$
	463.1724	-1.2	15.5	$C_{23}H_{23}C_{5}N_{6}$
	463.1711	1.7	10.5	$C_{22}H_{27}C_{9}N_{2}$
	463.1711	1.7	16.0	$C_{21}H_{21}C_{4}N_{9}$
	463.1727	-1.8	17.0	$C_{24}H_{22}C_{2}N_{7}Na_{1}$
	463.1727	-1.8	11.5	$C_{25}H_{28}C_{7}Na_{11}$
→	463.1730	-2.3	3.0	$C_{9}H_{25}O_{11}N_{11}$
	463.1730	-2.3	-2.5	$C_{10}H_{31}C_{16}N_{4}$
	463.1732	-2.9	4.5	$C_{10}H_{24}C_{8}N_{12}Na_{1}$
	463.1732	-2.9	-1.0	$C_{11}H_{30}C_{13}N_{5}Na_{1}$
	463.1705	2.9	0.0	$C_{7}H_{26}O_{11}N_{11}Na_{1}$
	463.1703	3.5	-1.5	$C_{6}H_{27}O_{14}N_{10}$
	463.1700	4.0	12.5	$C_{21}H_{24}C_{5}N_{6}Na_{1}$
	463.1700	4.0	18.0	$C_{20}H_{18}N_{13}Na_{1}$
	463.1738	-4.1	20.5	$C_{24}H_{19}C_{1}N_{10}$
	463.1738	-4.1	15.0	$C_{25}H_{25}C_{6}N_{3}$
	463.1698	4.6	11.0	$C_{20}H_{25}C_{8}N_{5}$
	463.1698	4.6	16.5	$C_{19}H_{19}C_{3}N_{12}$
	463.1741	-4.7	16.5	$C_{26}H_{24}C_{3}N_{4}Na_{1}$

Figure 4. 19 Estimated elemental composition report (± 3 ppm) of m/z 463.1719 isolated from the Citra crude hop extract provided by the high resolution mass spectrum. Red arrow indicates most likely compound composition

The elemental composition report of *m/z* 463.1719 highlights 25 possible elemental compositions for *m/z* 463, upon observation it was apparent that all but one of these included a nitrogen compound. The presence of nitrogen indicates alkaloid compounds, of which according to current literature, none have been isolated from hops. After searching relevant literature and an advanced search on SciFinder® (www.scifinder.cas.org) no matching chemical entities were found. Therefore on this basis, a novel chemical entity isolated from the antibacterial Citra crude hop extract with the chemical formula $C_{25}H_{28}O_{7}$ *m/z* 440 is presented.

4.7 UPLC-HR-MS determination of compounds isolated from the Citra crude hop extract

4.7.1 UPLC separation of crude Citra hop extract

Ultra pressure liquid chromatography (UPLC) coupled to HR-MS was used to further prove the methodology. In the following Figure (4.20) the chromatogram of the Citra crude hop extract is displayed.

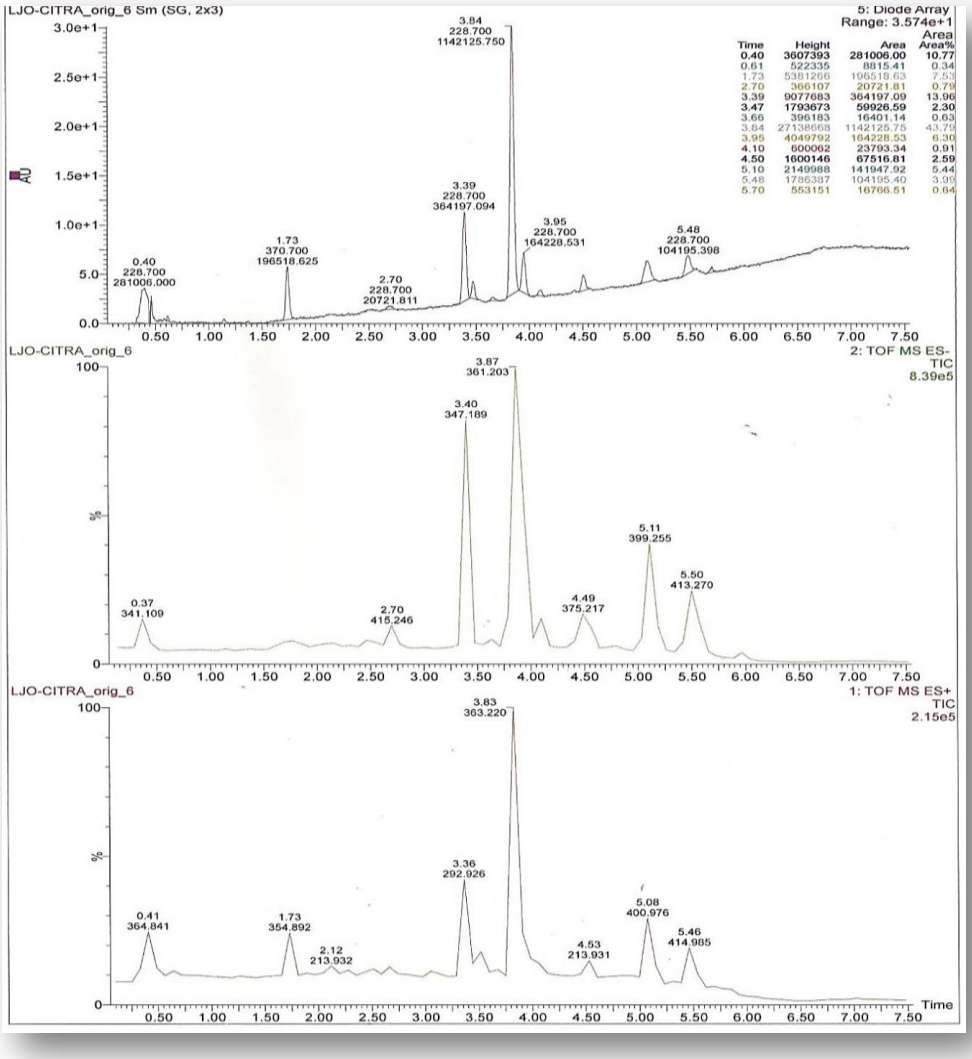

Figure 4. 20 UPLC chromatogram of Citra crude hop extract with selected peaks circled in red; image is representative of two separate repeats

Reviewing the chromatogram and comparing the peak molecular weight to that reviewed in Table (4.10) three peaks were apparent (*m/z* 353; 361 and 414).

4.7.2 Elemental composition of *m/z* 361

Firstlty the peak (*m/z* 361.200) was reviewed by using the attached high resolution mass spectrometer and was able to identify the chemical composition (± 3 ppm) in negative ion absorption as shown in the following Figure 4.25.

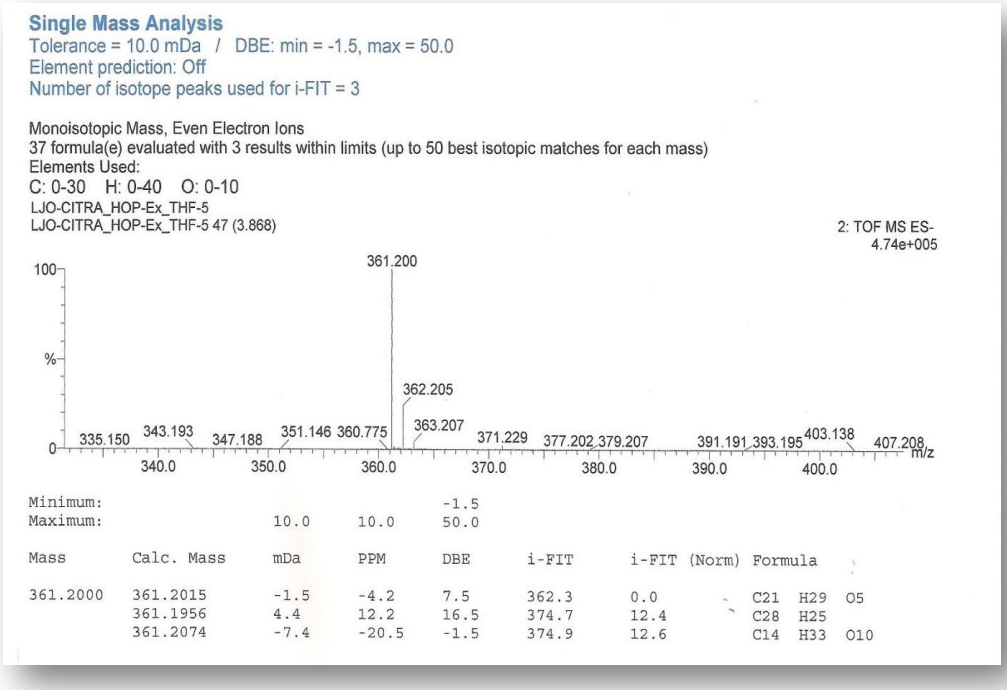

Figure 4. 21 UPLC HR-MS chromatogram and elemental composition report of *m/z* 361.200 isolated from the crude Citra hop extract

Figure 4.21 details 3 possible chemical compositions for the compound isolated within UPLC. The first with the lowest variation had a calculated *m/z* 361.2015 and a formula of C_{21} H_{29} O_5. This compound can therefore be positively identified as humulone (hop alpha acid) which has an *m/z* of 362 and a formula of C_{21} H_{30} O_5. One hydrogen is missing from the isolated compound due to the negative ion absorption.

4.7.3 Elemental composition of *m/z* 414

The next peak isolated had a m/z of 414.981 (ES+), after observing the data collected in Table 4.10 this mass matched that of lupulone. In the following Figure 4.22 the elemental composition report of the isolated compound.

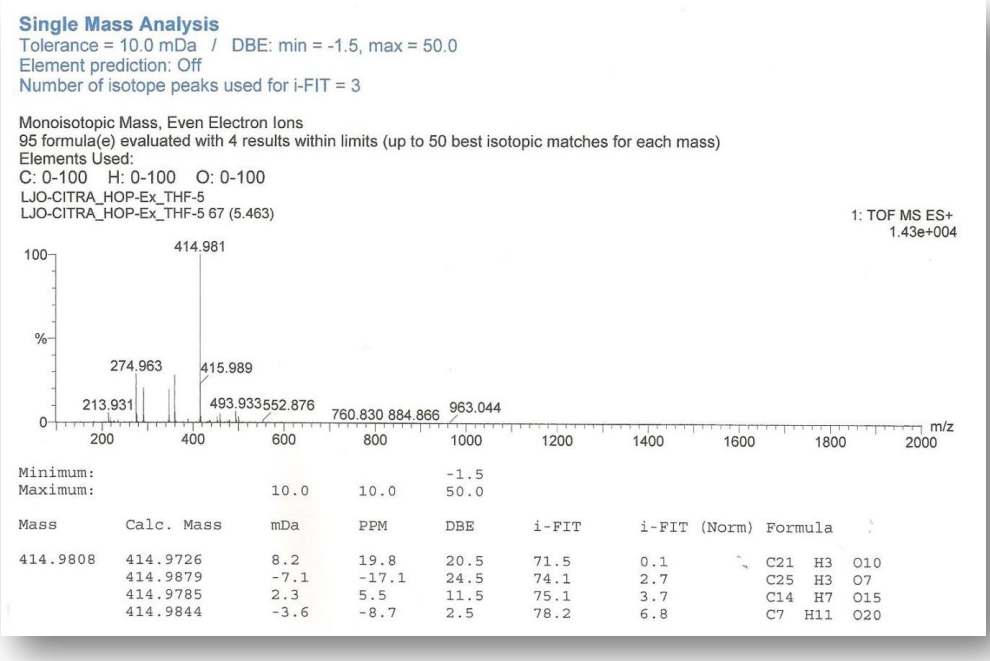

Figure 4. 22 UPLC HR-MS chromatogram and elemental composition report of *m/z* 414.981 isolated from the crude Citra hop extract

The elemental composition report of *m/z* 414.9809 identified four possible elemental compositions. The most probable match was hypothesised to be *m/z* 414.9879 with the elemental composition of C_{25} H_3 O_7 which can be positively identified as lupulone (hop beta acid).

Finally a third peak was also identified with an *m/z* 353.134. This peak was of particular interest as the mass matched that of xanthohumol as previously identified in Table 4.10. The chromatogram and elemental composition report of this component is displayed in the following Figure (4.23).

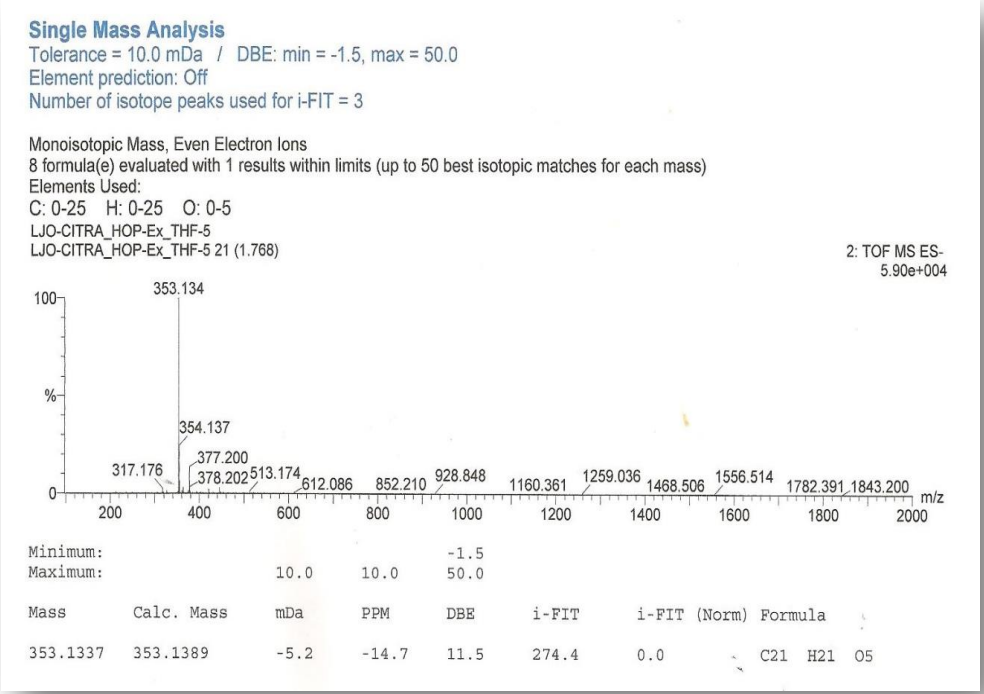

Figure 4. 23 UPLC HR-MS chromatogram and elemental composition report of *m/z* 353.1389 isolated from the crude Citra hop extract

The elemental composition report of *m/z* 353.134 gives one possible formula: $C_{21}H_{21}O_5$ with a calculated mass of *m/z* 353.1389 (ES-). This matches the chemical formula for xanthohumol as previously described.

Previous analyses did not locate the polyphenol xanthohumol. It is hypothesised that this compound did not elute up the TLC plate remaining in the mixture on the baseline and was masked by other compounds. Another possibility is that the compound concentration when separated using TLC is diluted therefore preventing its ability to inhibit the growth of the bacteria. It may also be the case that when separated based on the polarity an antagonistic effect is seen preventing any antimicrobial activity being observed from the compound.

4.8 Discussion

The use of plants in their crude form or purified extracts for the exploitation of their healing powers is an ancient theory (Cowan, 1999). It is therefore unsurprising that in the present day nearly 50 % of all pharmaceuticals worldwide have originated from plant derived sources (Beutler, 2009; Cragg and Newman, 2013).

However, since the development of antibacterials in the 1950's the use of plant derived natural products to treat bacterial infections have declined. Ever since the development of antimicrobials there have been incidences with bacterial resistance as a consequence there is a lack of new antibiotics and multidrug and extensively drug resistant organisms are becoming more apparent.

The main aim of this chapter was to build on the information gained in Chapter 4 and determine the chemical constituents of the antimicrobial hop extracts. A suitable volatile organic solvent was firstly identified. It is true that some antimicrobial constituents may be present in the non-polar extract. However, as antimicrobial activity has been identified in beer, a polar extraction method was employed.

Three solvents were trialled and the total yield of hop extract of five hop variants following 72 hour extraction was investigated. No statistically significant difference between the level of total yield (g) of hop material between the solvents used was observed. Previous research by (Verzele and Keukeleire, 2013) identified that methanol has been traditionally used for the extraction of hop alpha and beta acids, so therefore it was on this basis that methanol was used in all future extractions.

Upon initial observation of Figure 4.1 it appeared that the Citra hop pellet had a higher yield than the other hop variants when extracted in methanol, This, as previously explained in Chapter 3, may be due to the relative stability of the hop compounds after the pelleting process or the fact that maceration prior to pelleting of the hop materials increases the available surface area of the Citra hop variant.

The next stage of the extraction process was to separate the hop extracts on a TLC plate, several solvent systems were trialled to give an optimum separation. The solvent systems generated were as a result of a literature search concerning natural product separation and isolation. A solvent system of a 1:1 ratio of methanol : ethyl acetate was finally used. This predominantly polar solvent mix was based on the principle that the majority of the extract was from the polar fraction and therefore produced the most optimum separation.

Each hop variant (n=50) was then extracted in methanol and separated using TLC. It was observed from this method that 6 individual spots could be visualised. Several methods of visualisation (UV, Vanillin staining, etc) were also trialled but it was found that due to the pigments within the hops each spot could be observed by eye (data not shown). By calculating the Rf values of each separated spot it was possible to locate the spots at a later date if further analysed.

Upon analysis there were a total of 6 clear spots separated. However, in each hop variant there was a mixture of present and absent spots. To determine if the presence or absence of these spots had a positive effect on the antimicrobial activity employed a bacterial overlay assay.

The bacterial overlay assay employed was originally described by Fleming, 1942, and was adapted for use within this report. Briefly, a bacterial film suspended in growth media is applied to the surface of a solvent free TLC plate and is then incubated under high humidity at 37 °C. Bacteria can then be stained and metabolising cells visualised as a red colour against areas of no growth (clear). As a control, in each case a TLC plate which had not been used for separation of hop components was also inoculated and examined. A TLC plate which had been exposed to the solvent system and dried in the same way as the test plates was also used. In each case bacterial growth was observed.

It was interesting to observe that bacterial inhibition resided in the six spots separated in the initial TLC. However, this was not the same for every spot of equal Rf value, indeed comparing the most inhibitory hop variant Citra (6 antimicrobial spots) to the least active hop variant Galena (2 antibacterial spots) several differences could be noticed.

The most common (98% of variants) inhibitory spot was found to be close to the baseline of the TLC plate (Rf 0.02) and was hypothesised to contain a mixture of hop components of mixed polarity so therefore would not elute along the TLC plate using the mobile phase employed. It may also be the case that due to the number of components which may have been extracted, optimum separation was not possible using this method. In comparison, the least common (11 %) inhibitory spot was found to be at the solvent front of the TLC separation (Rf 0.99) and it was hypothesised that these compounds were the most polar and so therefore were readily dissolved into the solvent system.

To try and identify potential components of the antibacterial spots and to ascertain if there were differences in the chemical composition of the Galena and Citra hop variants, both variants were further separated using column chromatography. It was found that by using a stationary phase of silica gel a high amount of extract was lost within the column, possibly due to the compounds becoming 'stuck'. Therefore another form of stationary phase, Sephadex®, which separates compounds based on size was used and it was found that a much higher yield was achieved.

Upon fractionation of the hop extract, each fraction was further analysed using TLC and the solvent system previously identified. Post further TLC analysis each fraction was again assessed for inhibitory activity against *M. smegmatis* and the Rf values of inhibitory spots calculated. On no occasion was a new spot identified so therefore fractions with the same antimicrobial Rf value spots were combined, dried and weighed.

There were considerable differences in the weights of recovered components between the hop variants. This was the same for both non-inhibitory and inhibitory components. Reviewing the total yield from the extraction it is clear that these components were not lost within the column. It is hypothesised that the differences could be due to the differences in the chemical constituents of each component.

To help identify any known components of each fraction, mass spectrometry was used, as Citra had so far been the most inhibitory hop variant and Galena the least, these were chosen for further investigation. A literature search was conducted to identify the molecular weights and empirical formulas of known antimicrobial compounds from hops and was adjusted to show the common species detected in the mass spectrometer M, M+H, M+Na and 2M+Na..

Each separated spot from the hop variants Citra and Galena was isolated using column chromatography and analysed using mass spectrometry.

Component 6 (Rf 0.99) of the Citra hop variant was first investigated, and it was possible to hypothesise based on the molecular weight, that the compound humulone was present. This was also the case for the Galena extract. This compound was thought to be present in both hop variants antibacterial activity was only observed in the Citra variant. Upon further investigation the spectra from the Galena extract recognised a range of other peaks, it was hypothesised that the presence of these peaks, ultimately other compounds, may have an antagonistic effect on the antibacterial activity of humulone. It may also be the case that the concentration of this compound in the Galena variety may be lower than the Citra variant.

The next component investigated, 5, had an Rf value of 0.62 and it was found based on the mass of 379.2 that an oxidised derivative of humulone was present in the Citra variant. Humulone and its oxidised derivatives are known to be antimicrobial (Cornelison, 2008; García-Villalba *et al*., 2006). Component 5 was antimicrobial when isolated from the Galena variant and the same molecular weight peak was also identified from this extract. It may be that as this was the most dominant peak that the presence of this compound was responsible for the antibacterial activity of this spot.

Component 4 (Rf 0.38) was also investigated and several peaks were observed, notably from the Citra extract the peaks matched the molecular weights of the known antimicrobial compounds; lupulone and co-lupulone (Van Cleemput *et al*., 2009a). Based on the fact that both lupulone and co-lupulone are the most predominant compounds it can be assumed that, at least, the majority of antimicrobial activity can be associated with the presence of these compounds.

Interestingly as was the case with humulone, a compound with a mass matching lupulone was also detected in the Galena hop extract but no inhibitory activity was observed against *M. smegmatis*. Other peaks were observed with a molecular weight of *m/z* 659 and 768, but as there was no inhibitory activity, no further identification or separation procedures were undertaken. In conclusion the inhibitory activity observed in the bacterial overlay assay by the Citra extract with an Rf of 0.38 can be associated with the presence of both lupulone and co-lupulone, this activity can be further associated with previous research (Erdmann, 1951; Siragusa *et al*., 2008)

Component 3 (Rf 0.32) was also analysed and from the Citra hop variant and the relevant masses were matched to a hop beta acid adlupulone which has been previously described as antibacterial (Ceslova *et al*., 2009); Reed, 2007). Another peak at *m/z* 273.0 was matched to the mass of epicatechin. Catechin is a secondary plant metabolite belonging to the group flavan-3-ol, part of the natural family of flavonoids, found in many plant species, it has been described as an antioxidant and has been shown to be antibacterial (Bhandari, 1964; Ceslova *et al*., 2009; Stevens and Page, 2004). Catechin typically has two benzene rings and a dihydropyran heterocycle ring with an hydroxyl group (Figure 4.24). This configuration allows for two chiral centres on the molecule which can produce four diasteroisomers; two in t*rans* configuration (catechin) and two in *cis* configuration (epicatechin) (Ceslova *et al*., 2009).

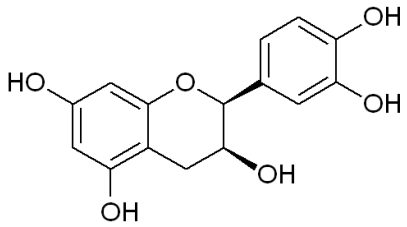

Figure 4. 24 the structure of epicatechin isolated from component 3 present in both Citra and Galena hop extracts

Several peaks are observed from the Galena extract which are similar to the Citra extract namely *m/z* 273.0: previously identified as catechin; *m/z* 413.1; 439.0 which were also previously identified as the lupulone derivative adlupulone. In conclusion, component 3 (Rf 0.32) separated from both the Citra and Galena hop extracts exhibited inhibitory activity against *M. smegmatis* in the bacterial overlay assay. Mass spectrum analysis revealed 3 dominant compounds with a *m/z* of 439.0; 413.1 and 273.0, relevant literature was searched and it was found that these *m/z* corresponded to compounds lupulone, adlupulone and epicatechin; all of which have previously been shown as antibacterial. Therefore no further identification was undertaken on this extract.

Component 2 (Rf 0.23) was also separated from the both the Citra and Galena hop extracts using Sephadex® column chromatography and by using the bacterial overlay assay the extract was shown to be inhibitory towards the growth of *M. smegmatis* in the Citra variety only. From the mass spectra 2 peaks were identified (m/z 413.3 and m/z 768.0). Previous identification showed that peak m/z 413.3 was lupulone and whilst being present in the previous fraction, the compound has eluted from the column within this fraction also. This is a hazard of TLC separation as a high starting weight of compound will elute throughout the whole plate possibly masking other compounds present (Reed, 2007).

Mass spectrum analysis was also carried out on the Galena extract, although as no inhibitory activity was observed, no further investigation was carried out. It was also noticed that this fraction did not contain any peaks with a molecular weight which matched that of previously identified antimicrobial hop compounds.

The final component, component 1, was isolated from the baseline of the TLC separation (Rf 0.02) and was hypothesised to contain the largest amount of compounds due to the lack of separation of non-polar compounds. Analysis revealed peaks with matching molecular weights to lupulone. Lupulone has been reported as weakly non-polar (Van Cleemput *et al.,*2009a) and therefore would be able to elute in mixtures with other compounds of mixed polarity (Hamburger and Cordell, 1987) which may explain why lupulone and related compounds are found at multiple points on the plate.

It was observed that there was a high amount of corresponding peaks within the mixture. It may be that these components are of a low polarity, meaning that separation using the current solvent system is not possible. Whilst it may be possible to further investigate this sample for any other antimicrobial compounds, it was hypothesised that the previously identified compounds were responsible for any antimicrobial activity.

The mass spectrum of Rf 0.016 from the *Galena* extract also has a mass with an m/z of 413 which matched that of lupulone. The component also does not have a peak at m/z 439.0 which corresponded to adlupulone. These differences may allude to the variation in activity between the two varieties. It was also observed as in the Citra variant that that there were also a number of peaks. However as this component did not produce any inhibitory activity towards the growth of *M. smegmatis* no further isolation techniques were undertaken.

It may be the case that several other compounds in each component are also inhibitory towards the growth of the bacteria but are outside the limits of detection using the relatively crude isolation technique. It is hypothesised that several compounds within each component are antibacterial and potentially act in a synergistic manner. When these compounds are separated using the TLC method and or column chromatography the synergistic activity is reduced which may lead to antagonistic combinations; or, that upon separation the relative concentration of these compounds is reduced so that no inhibitory activity is observed.

This may be the case for xanthohumol (XN). Xanthohumol is widely regarded as the most bioactive compound found within hops (Acworth *et al*., 2012; Ceh *et al*., 2007; Narvaez *et al*., 2013; Stevens and Page, 2004). However using the developed extraction method it was not apparently separated from the crude mixture. To try and identify this component and any other potential compounds, the entire bioactive Citra hop extract was isolated and analysed using high resolution mass spectrometry. As mentioned in the introduction, HR-MS is able to estimate to ± 3 ppm the elemental composition of separate peaks within a mixture. Interestingly, xanthohumol was not isolated using this method. However, a peak was observed at a mass of *m/z* 440 with an estimated elemental composition of C_{25} H_{28} C_{7}.

This peak was analysed further by searching SciFinder for corresponding compounds. In total 458 compounds with the same mass and formula were found from the database, which contains over 60 million (Ruddigkeit *et al*., 2012). Further refining of the results by search for "hops" revealed no corresponding literature. By searching "*Humulus lupulus",* 2 corresponding articles were identified (Hideo, 2007; Schaefer *et al*., 2005), but were not related to this compound in any way. On this basis the novel compound C_{25} H_{28} C_7 is presented as part of the antimycobacterial fraction of the Citra hop extract. This is an area that will require more investigation to secure a precise characterisation, further research would be required.

To further confirm the presence of xanthohumol ultra-high pressure liquid chromatography coupled to high resolution mass spectrometry was used. The initial separation revealed three peaks of interest (*m/z* 353; 361 and 414) which by using HR-MS were hypothesised to be humulone, lupulone and xanthohumol. This result shows that the initial biological assay and subsequent determination was able to successfully isolate both the alpha and beta acids; but was unable to separate xanthohumol.

This may be due to several reasons: the concentration of XN is reduced upon separation and therefore is not antibacterial when tested; extraction of the compound in methanol or other volatile solvents leads to an oxidation reaction of the compound, this would potentially lead to compound to form a polymer effectively taking it outside the limits of the chosen mass spectra limits. It may also be the case as previously mentioned that when separated the compound is in contact with antagonistic compounds preventing antibacterial activity.

This chapter therefore highlights the main difficulties of natural product separation and identification. An array of compounds can make separation difficult even when separation is based on polarity or molecular size. If separation is achieved, another problem that can arise is achieving a sufficient compound weight for identification purposes.

This chapter has successfully demonstrated that it is possible to separate each of the 50 hop variants and further confirmed that the Citra hop extract is the inhibitoriest whilst the Galena variant is the least inhibitory. Furthermore differences between these two variants have been identified and this has preluded to a hypothesis on the differences in antibacterial activity.

A novel compound which was associated with the antibacterial fraction of the crude Citra hop extract was isolated. This is a promising result and will need future investigation to purify and further characterise. It may be possible that alone this compound does not have any inhibitory activity but when combined with other separated hop compounds it may exhibit synergism.

Finally the principle of the methods used has been proven by isolating the most common antimicrobial compounds; humulone, lupulone and xanthohumol from the crude Citra hop extract using ultra pressure liquid chromatography.

Chapter 5

Synergistic Activity between Hop Extracts and Antibiotics and their Mode of Action

5.0 Synergistic activity between hop extracts and antibiotics and their mode of action

5.1 Introduction

The first antibiotic that was discovered to be inhibitory towards the growth of *Mycobacterium tuberculosis* was streptomycin. It was discovered in 1943 by Dr. Albert Schatz and is considered one of the top 20 discoveries of the 20th century (Pringle, 2013). Its mode of action is to inhibit protein synthesis. It functions by binding to the 16s rRNA of the 30S subunit of the bacterial ribosome thus preventing the binding of formyl-methionyl tRNA to the 30S subunit (Tanoira *et al*., 2014). Although a successful inhibitor of the pathogen the, toxicity to the antibiotic is well described and has been acknowledged since 1947, as has the emergence of bacterial resistance (McDermott, 1947; Avent *et al*., 2011; Siddiqui and Qureshi, 2014; Tanoira *et al*., 2014).

Antibiotic resistance can be defined as the ability of an organism to survive following exposure to one or more antibiotics. Organisms resistant to multiple antibiotics are considered multidrug resistant (MDR) (Conly, 1998; O'Brien *et al*., 2014).

The World Health Organisation (WHO) periodically completes global surveillance surveys on the level of *M. tuberculosis* to antibiotics and it is estimated that out of 100 clinical isolates of *M. tuberculosis* at least 23.8 % will be resistant to streptomycin (Cohn *et al*., 1997; Dooley *et al*., 2013; Gandhi *et al*., 2010); For these reasons streptomycin is infrequently used for the treatment of tuberculosis.

Resistance of *M. tuberculosis* to antimycobacterial drugs is not a new phenomenon as shown by streptomycin. However, resistance of TB to other mycobacterial drugs is increasing exponentially leading to the emergence of multidrug (MDR-TB) and extensively drug resistant (XDR-TB) strains (Murray *et al*., 2014). One of the most extensive studies undertaken to report the incidences of MDR-TB and XDR-TB worldwide analysed 17,690 clinical isolates and found that 20 % were MDR and were 2 % XDR (Centers for Disease Control and Prevention (CDC), 2006).

One of the ways to combat the development of resistance is to employ a combination therapy approach consisting of two or more antibiotics (Acar, 2000). This offers a number of potential advantages: (1) broad-spectrum coverage for the initial treatment of infected patients; (2) the ability to treat polymicrobial infections; (3) avert the selection of resistant microorganisms; (4) reduce dose-related toxicity; and (5) increase the potential for synergistic activity (Lienhardt *et al*., 2010).

The current treatment regime for pulmonary TB utilises a combination of antibiotics comprising of isoniazid and rifampicin (every day for 6 months) which provides broad spectrum coverage of the initial infection. This is supplemented by pyrazinamide and ethambutol which are taken every day for the first 2 months to avert the selection of resistant microorganisms. In the case of extra-pulmonary TB, the same combination therapy is used but treatment is extended to 12 months (Centers for Disease Control and Prevention (CDC), 2006).

There are two main reasons for using a combination therapy approach to treat *M. tuberculosis* infection. The first is to reduce the possibility of resistant organisms by using antibiotics with several modes of action. For example isoniazid (INH) inhibits the formation of mycolic acids on the cell surface effectively inhibiting the growth of the bacterium. As previously mentioned, INH is used in combination with rifampicin. Rifampicin inhibits RNA polymerase which inhibits DNA transcription, therefore reducing the ability of the bacterium to gain antibacterial resistance.

In this chapter the antibacterial activity of several antibiotics was assayed against antibiotic resistant clinical isolates of *M. abscessus* a fast growing organism related to *M. tuberculosis*. It is a poorly understood multidrug resistant organism which has been shown to cause abscesses in the lungs of patients with cystic fibrosis (Henry, 2014).

The antibiotics assayed in this chapter are commonly used to treat both *M. tuberculosis* and *M. abscessus* and as a consequence drug resistance is often encountered (Meziane-Cherif and Courvalin, 2014).

One of the antibiotics commonly used to treat mycobacterial infection is isoniazid (INH) which is a pro-drug activated by a bacterial peroxidase. The mode of action is to inhibit the synthesis of mycolic acids which are key components of the mycobacterial cell wall (Simons *et al.,* 2010) and thus prevent the growth of the bacteria (bactericidal activity). Although commonly used, the drug has several severe side effects such as liver damage which can lead to morbidity (Murray *et al*., 2014; Zhang *et al*., 1992).

Another commonly used antibiotic, Ethambutol (EM), also works by disrupting the formation of mycolic acids which increasing the permeability of the bacterial cell wall to antibiotics. Ethambutol, unlike INH is bacteriostatic so is commonly used in combination therapy with INH, rifampicin and pyrazinamide which have bactericidal effects. EM has several severe side effects including decreased vision and chronic liver failure (Coban *et al*., 2014).

Rifampicin (RIF) was introduced in 1967 and is used in combination therapy to treat tuberculosis and other Mycobacterial infections. Its mode of action is to prevent bacterial DNA-dependent RNA synthesis by inhibiting the action of RNA polymerase (Calvori, 2005). Bacterial resistance to RIF is common and occurs as a result of mutations in the *rpoB* gene which alters the rifampicin binding site on RNA polymerase and thus prevents the antibiotic from binding. For this reason RIF is always used in combination with other antibiotics (BNF.org). A further complication of using the drug is its hepatotoxicity which requires regular liver function tests to ensure patient health, (Coban, *et al*., 2014).

Pyrazinamide (PYR) is a prodrug which is commonly used in combination with INH and RIF to treat tuberculosis. Uniquely, PYR diffuses across the cell wall of the bacterium where the enzyme pyrazinamidase converts the drug into the active form pyrazinoic acid which increases internal pH (Cabellero, 2009). There are several theories as to the mode of action of the drug but it is generally assumed that pyrazinoic acid acts by disrupting membrane potential which prevents energy production (Zhang, *et al*., 2008). The drug is also thought to inhibit the action of fatty acid synthase required by the bacterium to synthesise fatty acids and binds to ribosomal protein S1 (RpsA) inhibiting trans-translation of tRNA (Coates *et al*., 2011; Shi *et al*., 2011). Pyrazinamide is also hepatotoxic and is the most common cause of drug induced hepatitis.

Pyrazinamide has no activity against actively growing TB bacilli at neutral pH but instead targets non-replicating bacteria at acid pH such as those found inside macrophages (McDermott and Tompsett, 1954; Shi *et al*., 2011; Tarshis and Weed, 1953).

Mycobacterium tuberculosis is just one bacteria within the mycobacterial species, other related organisms such as *M. avium* and *M. abscessus* can also cause serious healthcare associated infections (Petrini, 2006). To treat these pathogens and to counter the rise of multidrug resistant isolates two additional antibiotics, imipenem and ciprofloxacin, which have been shown to have inhibitory activity against these pathogens, have been employed to treat infected individuals (Inderlied *et al*., 1989; LaBombardi and Cataldo-Caputzal, 1993).

Imipenem (IM) is an intravenous β-lactam antibiotic and a member of the carbapenem class of antibiotics which is generally used to treat Gram negative bacteria such as *Escherichia coli* and *Pseudomonas aeruginosa*; it has also been shown to be active against several species of *Mycobacterium* such as *M. abscessus* (Dooley *et al*., 2013; Singh *et al*., 2014).

Beta lactam antibiotics act by binding to penicillin binding proteins (PBP's). In Gram negative organisms the PBP's which imipenem has the most affinity for are, PBP-1a, 1b and PBP-2. The binding of imipenem to PBP's results in inhibition of cell wall synthesis; in addition, direct binding PSP-2 and PBP-1b results in rapid cell lysis and death without filament formation (Inderlied *et al*., 1989; Rodloff *et al*., 2006; Carvela *et al*., 1984).

Commonly mycobacteria are recognised as being intrinsically resistant to beta lactam antibiotics. This is due, in part, to the presence of β-lactamase (an enzyme which inhibits the binding to PBP's). In addition to this, the lipid rich mycobacterial cell wall is thought to be a virtually impenetrable barrier to polar molecules such as beta-lactam antibiotics (Chambers *et al*., 1995). In a recent study however by Singh *et al*, (2014) the susceptibility of 67 clinical isolates of *Mycobacterium abscessus* to imipenem was investigated and it was found that only 14% were resistant; (MIC^{50} 5 µg) (Singh *et al*., 2014).

Imipenem has also been shown to be synergistic *in vitro*, with other anti-mycobacterial drugs (Singh *et al*., 2014) as well as being inhibitory towards the *M. tuberculosis* strain H37Ra (Chambers *et al*., 1995). Side effects are uncommon although it has been reported that when used in high concentrations IM can cause seizures (BNF.org).

Ciprofloxacin (CIP) is a broad spectrum, second generation, fluoroquinolone (BNF.org) and is commonly used in the treatment of a wide range of Gram positive and negative bacterial infections. Ciprofloxacin is also commonly used alone or in combination with other antibacterial drugs in the treatment of infections for which the bacterial pathogen has not been identified, including urinary tract infections and abdominal infections (Naber *et al*., 2006; Solomkin *et al*., 2010). CIP prevents cell division by inhibiting DNA gyrase, necessary to separate bacterial DNA (Drlica and Zhao, 1997; Inderlied *et al*., 1989; LaBombardi and Cataldo-Caputzal, 1993).

Given the ability of bacteria to evolve it is perhaps not surprising that they have developed resistance to the antibiotics which are currently in use. The situation is not helped by the fact that the cost of developing new antimicrobials far outweighs any financial gain to the pharmaceutical companies and as a consequence there are no new classes of antibiotic in the developmental pipeline, indeed no new classes of antibiotics have been brought to the market since 1980 (Phillips, 2013).

An alternative to discovering new antibiotics would be to identify natural compounds which restore the activity of redundant antibiotics or enhance their current level of antibacterial activity (Coates *et al*., 2011; Cragg and Newman, 2013). One such combination was identified by Cooper, *et al*., where synergistic activity was observed between Manuka honey and beta lactam antibiotics against an oxacillin resistant strain of *S. aureus* (Jenkins and Cooper, 2012). In another study Mossa (2004) and colleagues observed that plant derived compounds such as diterpenes, totarol, ferulenol and plumbagin increased the potency of isonicotonic acid hydrozide against *M. smegmatis*, *M. chelone, M. xenopei* and *M. intracellulare* (Mossa *et al*., 2004).

Another example of a natural product derived antibacterial is clavulanic acid which is derived from *Streptomyces clavuligerus.* Clavulanic acid or augmentin, is a Beta-lactam antibiotic which when used alone is not itself inhibitory but when used in combination with the penicillin type antibiotics such as amoxicillin, it can overcome antibiotic resistance in bacteria that secrete β-lactamase enzymes (Reading and Cole, 1977).

When reporting the activity of combinations of antibiotics there has been confusion regarding the classification of synergy, additive, co-active and indifferent. In this report I have used several methods to determine the interaction of the test compounds and therefore it is important to define the terminology used. In this report I class the activity of hop-antibiotic combinations as antagonistic (one will reduce the activity of the other); indifferent (the combination does not enhance or reduce the activity of each drug); co-active (appearance of increased activity in one, or both drugs); additive (statistically significant (FIC = >1 : <2) decrease in MIC of one compound when in combination); synergy (statistically significant (FIC < 0.5) decrease in MIC of both test compounds when in combination.

In this chapter the antibacterial activity of hop derived extracts against clinical isolates of *M. abscessus* including those resistant to multiple antibiotics was investigated. The ability of these extracts to potentiate the activity of the following antimicrobials: isoniazid, ethambutol, ciprofloxacin, pyrazinamide, imipenem and rifampicin were also studied. Finally the effect of exposure to hop extracts and antibiotics, alone and in combination on *M. abscessus* structure using scanning electron microscopy (SEM) was also investigated.

5.2 Materials and Methods

5.2.1 Clinical isolates of *Mycobacterium abscessus*

Clinical isolates were kindly provided by Dr. Joachim Burget and Dr. Mike Ruddy, University Hospital of Wales, Cardiff (UHW) and were collected from patients during July 2012 (Table 5.1); each isolate was characterised by a unique reference number (assigned by UHW) which is displayed in Table 5.1 alongside the known antibiotic resistance profiles.

Unique UHW strain number	Known antibiotic resistance (MIC > 128 µg/mL^{-1})	Collection date
8899	Imipenem, Rifampicin	11/07/2012
9495	Rifampicin Imipenem	11/07/2012
9568	Rifampicin	12/07/2012
9723	Rifampicin, Ciprofloxacin,	12/07/2012
10006	Rifampicin, Ciprofloxacin, Imipenem	21/07/2012
10332	Rifampicin	22/07/2012
11365	Rifampicin Ciprofloxacin, Imipenem	23/07/2012
11490	Rifampicin	24/07/2012

Table 5. 1 The UHW unique clinical isolate strain number, known antibiotic resistance (determined by an MIC > 128 µg/mL^{-1}), and isolation date of *Mycobacterium abscessus* strains

5.2.2 Growth media

Clinical isolates of *M. abscessus* were isolated using lysogeny agar (LA) and were supplied as actively growing cultures in lysogeny broth (LB). All subsequent antibacterial assays utilising *M. abscessus* were undertaken using LA and LB and were prepared according to manufacturer's instructions (10 g (LA/LB) / 500 mL of SDW) and were autoclaved at 121 °C for 15 minutes and 15 PSI before use (Prestige, 9 L classic bench top autoclave, LabShop, UK).

5.2.3 Antibiotics

The antibiotics used in this study were; isoniazid, ciprofloxacin, rifampicin, imipenem, ethambutol and pyrazinamide. For the agar incorporation assay; Etest strips which had a concentration gradient (0 µg to 256 µg) of each antibiotic were used. For the zone of inhibition assay each antibiotic was supplied as a sensitivity disc (Fisher Scientific, UK) and concentrations of 5, 10, 25, 50 and 100 µg were used. All antibiotic containing reagents were stored at -20 °C prior to use.

5.2.4 Crude hop extract

Based on the results presented in Chapter 3 the hop variant Citra demonstrated the highest level of antibacterial activity and was used to generate the extracts which were used in these studies. The method for obtaining aqueous hop extract was described in detail in Chapter 3.; briefly, 5 g of pelleted Citra hop variant was machine macerated into a powder with a diameter less than 2 mm. Macerated hop material was then suspended in 200 mL of SDW at 100 °C for 60 minutes and was then allowed to cool to room temperature. Solid hop material was removed by filtration (Whatman® size 2 filter), under gravity and resulting supernatant was used neat and termed crude hop extract; the crude hop extract was made fresh prior to each assay.

5.2.5 Purified hop extracts

The compounds in the Citra hop variant were separated as described in Chapter 4; briefly 1 mL of a 1 g/mL^{-1} Citra aqueous hop extract was separated using preparative thin layer chromatography and was assayed for antibacterial activity using the bacterial overlay assay (Method 4.3.4). Separated spots which displayed antibacterial activity towards *M. abscessus* 9495 were isolated, weighed and identified UPLC, as previously described in Chapter 4. The hop compounds isolated by this process are presented in Table 5.2.

Extract Number	Predominant compound as identified by mass spectrometry	Compound weight as tested (µg)
2737 G	Humulone	5
2737 Y	Lupulone	5
1426 G	Adlupulone	5

Table 5. 2 Extracted hop compounds used in the synergy assays. Compounds were separated from preparative thin layer chromatography based on their antibacterial activity and the predominant compound identified using mass spectrometry.

5.2.6 Characterisation of bacterial structure following exposure to antibacterial compounds using scanning electron microscopy

Fresh cultures of *M. abscessus* and *M. smegmatis* were prepared as previously described in section 2.2.2 and were standardised to 1 x 10^6 cfu/mL^{-1}.

Prior to fixing, 1 mL of bacterial culture was suspended in 1 ml of ciprofloxacin (0.12 µg/mL^{-1}), imipenem (8 µg/mL^{-1}) and Citra crude hop extract (0.125 % v/v). For synergy studies, antibiotics were prepared in 0.125 % v/v Citra crude hop extract at the concentrations previously mentioned. Controls consisted of *M. abscessus* and *M. smegmatis* suspended in SDW. Each sample (test and control) was incubated statically at 37 °C for 1, 6 10, 12, 18 and 24 hours on three separate occasions. At each time point the appropriate sample was prepared for imaging as descried below.

5.2.6.1 Sample preparation and imaging

Treated bacterial cultures were pelleted by centrifuging at 2600 x g for 10 min and the supernatant removed. Cultures were then fixed at room temperature for 1 h by suspending in 500 µL of 2 % glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4). The samples were then centrifuged at 2600 xg for 10 min and the supernatant was removed. To each sample, 500 µL of a solution containing a ratio of 2:2:1 of 2 % osmium tetroxide: 0.1 M sodium cacodylate (pH 7.4): 25 % glutaraldehyde and incubated for 1 h at room temperature.

Samples were centrifuged again and the supernatant was removed. The samples were re-suspended in 50 % ethanol in diH_2O for 1 minute and then centrifuged at 5000 x g. After removing the supernatant this process was repeated with 70, 80 and 95 % ethanol in diH_2O followed by three washes with absolute ethanol. The samples were stored in 1 mL of absolute ethanol at room temperature before dehydration under vacuum on carbon grids, sputter coating with platinum and imagining on a scanning electron microscope.

For each sample, 25 different fields of view were examined at 10,000 x and 50,000 x magnification using a Zeiss SIGMA FEG-SEM scanning electron microscope.

5.3 Methods to characterise the combined antibacterial activity of hops and antibiotics

5.3.1 Modified zone of inhibition assay

This method is a variation of the agar well diffusion assay (Section 3.6.1. In this assay LA was prepared as per manufacturer's instructions (10 g per 500 mL^{-1} SDW) and 20 mL of sterile, molten LA was transferred to a petri dish and allowed to set. A 1 x 10^6 cfu/mL^{-1} suspension of each test strain of *M. abscessus* (Section 2.2.2 and 2.2.3) was evenly spread onto the surface of the agar using a sterile spreader (Fischer Scientific, UK) and incubated statically at 37 °C for 3 hours.

Two agar based radial assays were employed to assess interaction. In the first, 80 µl of crude hop extract was added to a 6 mm diameter well which had been bored into the agar. An antibiotic impregnated disc was then placed 5 cm from the centre of the well after which the plate was incubated at 37 °C for 24 hours. In the second assay, sterile paper sensitivity test discs were loaded with hops compounds at a concentration of 5 $\mu g/mL^{-1}$ and left to dry. Discs containing antibiotics and hop compounds were then placed 5 cm apart on the surface of an inoculated agar and incubated at 37° C for 24 hours. Following incubation the agar plates were inspected for the presence of zones of inhibition. The shape of the interaction between the zones of inhibition generated by each disc was used to indicate the type of interaction between the two agents (Figure 5.1).

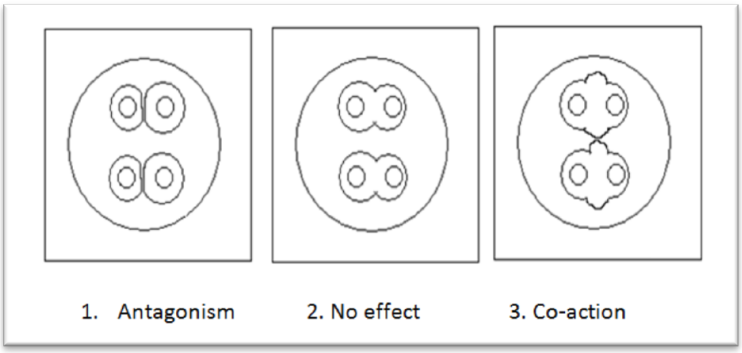

Figure 5. 1 The possible types of interaction between two antibacterial agents. Depending on the appearance of the interaction it is classified antagonistic, no effect or Co-active. Adapted from Piddock, (1990).

5.3.2 Modified agar incorporation assay

To further characterise the interaction of hop derived compounds and antibiotics, the hop agar incorporation assay was employed. This allowed the minimum inhibitory concentration (MIC) of each hop extract to be determined against each test organism.

To determine the interaction of hop extracts and antibiotics the following method was employed. A sub MIC concentration of hop compound was incorporated into agar using a variant of the previously described method (Method 3.6.2). Following inoculation of the surface of the agar plate with the test organism the plate was incubated at 37 °C for 3 hours.

To determine the sensitivity of the organism to a chosen antibiotic the E-test or episolmeter test was employed. The Etest consists of a predefined gradient (usually between 256 µg – 0 µg) of antibiotic concentrations on a plastic strip, allowing the MIC value of antibiotics, antifungal and antimycobacterial agents. The addition of a concentration gradient allows for the results to be less subjective, in the zone of inhibition assay the pattern of growth is used to describe positive co-action whereas using the E-test a reduction in the MIC value is used (Reed, 2007; Wanger *et al*., 1995)

An Etest strip containing the chosen antibiotic was then positioned onto the surface of the agar and the plate was returned to the incubator at 37 °C for 24 hours. As a control the assay was performed using agar from which the hop compound had been omitted.

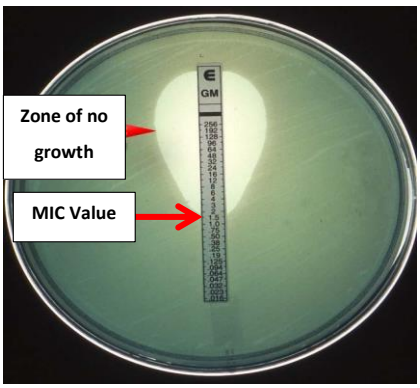

Figure 5. 2 Determination of the MIC value of Gentamicin against *E.coli* using an Etest strip. The clear zone represents the area of inhibition and the point at which the edge of the zone meets the scale on the strip represents the MIC.

An example of an E-test strip test plate following incubation can be seen in Figure 5.2. A clear area of no growth is observed which leads to a narrowing point indicating the minimum inhibitory concentration. When crude hop extract is incorporated into the agar the zone is again read, and depending on the result the combination was classed as co-active (decrease in MIC), antagonistic (increase in MIC) or indifferent (no change to MIC). All assays were repeated on no less than 3 separate occasions unless otherwise stated.

To identify a change in the MIC rather than experimental error a 3 dilution decrease in the MIC value was used. Any changes below this value were taken to be experimental error or 'no effect'. The 3 dilution rule has been previously used to determine additive effects using this assay (Reed, *et al*., 2007).

5.3.3 Identification of synergy using a checkerboard method

To test for synergy, indifference or antagonism the checkerboard method was used and was adapted from White *et al*., 1996. The combinations tested against each clinical isolate of *M. abscessus* were ciprofloxacin plus Citra crude hop extract and imipenem plus Citra hop extract. The concentration range for each antimicrobial was 0.015 – 32 $\mu g/mL^{-1}$ and was prepared from a stock solution prepared in diH_2O. For the Citra crude hop extract concentration ranged from 0.05 % - 0.8 % v/v and was prepared as previously described.

Each inoculum of *M. abscessus* was prepared as previously described and standardised to a concentration of 1 x 10^6 cfu/mL^{-1} in double concentrated lysogeny broth (20 g/l). Within an MIC plate (Sterlin, UK) 80 µL of standardised bacterial suspension along with 50 µL of fresh double concentrated broth was added. To each well a 100 µL combination of ciprofloxacin or imipenem (Range 0.015 $\mu g/mL^{-1}$ – 32 µg $/mL^{1}$) prepared in Citra hop extract (Range 0.05 – 0.8 % v/v) was added to give a range of concentrations of each test antibacterial. Plates were then incubated at 37 °C for 24 hours with shaking at 50 rpm.

Controls consisted of imipenem and ciprofloxacin and Citra hop extract only at the concentrations listed above; sterile distilled water; and 80 µL of *M. abscessus* suspended in 50 µL of double concentrated broth with 100 µL of SDW. Growth was observed by eye as either turbid (growth) or clear (no growth) in each well.

To evaluate the effect of different combinations of antibiotics and hops the fractional inhibitory concentration (FIC) was calculated for each antibiotic in each combination. The following formula was used to calculate the FIC:

Definitions:

- MIC_A - The MIC of ciprofloxacin/ imipenem (Drug A) alone
- MIC_B – The MIC of Citra hop extract (Drug B) alone
- MIC_{AB} – The MIC of drug A in combination with B
- MIC_{BA} – The MIC of drug A in combination with A

FIC (fractional inhibitory concentration) index calculations:

FIC Index = MIC_{AB} / MIC_A + MIC_{BA} / MIC_B

MIC_{AB} = MIC of A in the presence of B; MIC_{BA} = MIC of B in the presence of A

Results were then interpreted as follows:

Interpretation	FIC
Synergy	< 0.5
Additive	> 0.5 and < 1.0
Indifference	> 1 and < 4.0
Antagonism	> 4.0

Table 5. 3 Interpretation of the FIC to identify synergy, additive, indifference or antagonism between antibiotics and crude hop extract

Adapted from Acar, (2000); White *et al.*, (1996)

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5.4 Results

5.4.1. Determination of the antibiotic sensitivity of clinical isolates of *M. abscessus*

To confirm the antibiotic sensitivity of the clinical isolates of *M. abscessus* provided by the UHW a disc diffusion assay was employed (Method 5.3.1). The following range of antibiotics and disc concentrations were used; rifampicin, imipenem, ciprofloxacin and pyrazinamide at concentrations of 5 and 10 µg. The resulting zone of inhibition following overnight incubation is shown in Table 5.4; no inhibition is indicated by (NI).

Strain number	Imipenem		Ciprofloxacin		Pyrazinamide		Rifampicin		Ethambutol	
	5 µg	**10 µg**	**5 µg**	**10 µg**	**5 µg**	**10 µg**	**5 µg**	**10 µg**	**5 µg**	**10 µg**
	The mean zone of inhibition (mm)									
8899	NI	NI	22.8	46.2	NI	NI	NI	NI	NI	1.2
9495	22.3	48.3	22.6	44.3	NI	NI	NI	NI	NI	1.4
9568	25.3	49.6	28.6	52.6	1.4	3.7	NI	NI	NI	1.1
9723	26.7	51.6	NI	NI	NI	1.4	NI	NI	NI	NI
10006	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI
10332	22.6	46.2	25.3	49.7	1.6	3.5	NI	1.1	NI	1.2
11365	25.8	50.2	NI	NI	NI	NI	NI	NI	NI	NI
11490	26.4	51.3	29.4	50.5	NI	NI	NI	NI	NI	NI

Table 5. 4 The zone of inhibition (mm) of 5 and 10 µg antibiotic sensitivity discs (imipenem; ciprofloxacin; pyrazinamide; rifampicin and ethambutol) against clinical isolates of *M. abscessus* as a result of 3 separate repeats (NI= no inhibition). Controls consisted of +ve Gentamicin at 10 µg/mL^{-1} and –ve an inoculated filter disc with 50 µL of SDW

Imipenem was found to be the most effective antibiotic at the concentrations tested for these isolates while rifampicin was the least.

Comparing the results obtained from Table 5.1 the bacterial sensitivities to the antibiotics appears to match those expected. This is especially the case for strain number 10332 which appears sensitive to all antibiotics assayed at both 10 µg/mL^{-1}, in comparison; strain 11365 is the most resistant.

5.4.2. Determination of the sensitivity of clinical isolates of *M. abscessus* to Citra hop extract

The antibacterial activity of the Citra crude hop extract was next determined by the using the previously developed zone of inhibition assay (method 3.6.1). The average zone of inhibition was calculated on 3 separate occasions against all of the clinical isolates of *M. abscessus* and the results displayed in Figure 5.3.

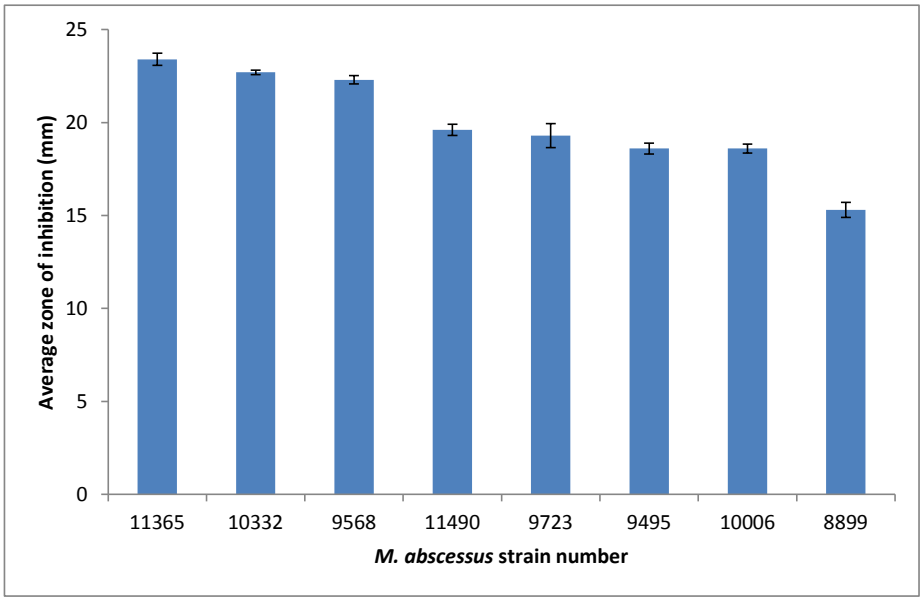

Figure 5. 3 The average zone of inhibition (mm) of Citra crude hop extracts against clinical isolates of *M. abscessus*. Results shown are the mean of 3 separate repeats, error bars represent ± Standard deviation (SD)

All of the clinical isolates of *M. abscessus* were susceptible in varying degrees to the hop extract. To determine if there was a statistically significant difference in the level of antibacterial activity observed against theses isolates, a one way ANOVA and Tukey post hoc analysis of the zones of inhibition generated by strains 11365 and 8899 was performed. A p value (>0.05) indicated that there was no significant difference in the zones of inhibition suggesting that all of the isolates demonstrated a similar level of sensitivity to the hop extract. The fact that antibiotic resistant isolates were as susceptible as there antibiotic susceptible counterparts suggests that the extract contains compounds that may act in a manner which differs from that of the particular antibiotics and that they may have utility as antimicrobial agents.

5.4.3 Characterisation of the combined antibacterial activity of hops and antibiotics against clinical isolates of *M. abscessus*

To characterise the interaction of individual antibiotics and the hop extract, an agar diffusion assay was used (section 3.6.1). An antibiotic sensitivity disc containing 10 µg of the antibacterial agent was placed 5 cm from a well containing Citra hop extract and was incubated overnight. The resulting growth was examined for evidence of synergism, antagonism or no effect.

Figure 5.4 shows an example of an antibiotic/ hop interaction which demonstrated coactivity against *M. abscessus* 9495.

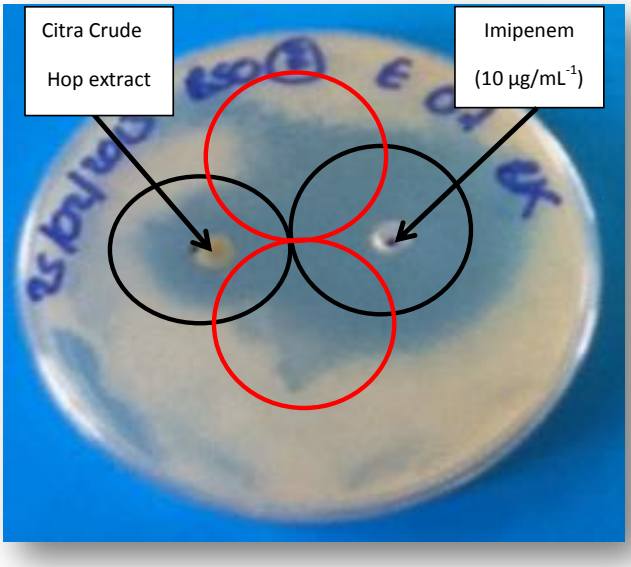

Figure 5. 4 Synergistic interaction between the antibiotic imipenem (10 µg/mL^{-1}) and crude Citra hop extract against the clinical isolate *Mycobacterium abscessus* 9495. Zones of inhibition shown in black indicate normal inhibition whilst zones in red indicate synergism. Figure shown is representative of 3 separate repeats.

Figure 5.5 is an example of the zone of inhibition assay between the Citra crude hop extract and the antibiotic ethambutol (10 µg) against the clinical isolate *M. abscessus* 9568. The figure details 'no effect' (Figure 5.1), meaning that no coactivity or antagonistic activity was observed.

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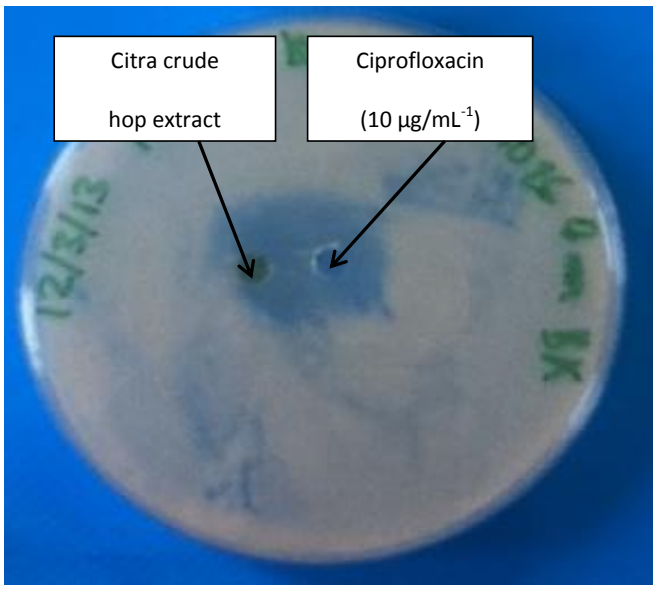

Figure 5. 5 An example of the zone of inhibition assay detailing no effect between the antibiotic ciprofloxacin (10 µg/mL^{-1}) and the crude Citra hop extract against the clinical isolate *Mycobacterium abscessus* 9568. Figure shown is representative of 3 separate repeats

For ease of analysis the interaction for each clinical isolate has been collated and is presented in Table 5.5

M. abscessus strain numbers	Imipenem 10 µg	Ciprofloxacin 10 µg	Pyrazinamide 10 µg	Rifampicin 10 µg	Ethambutol 10 µg
8899	NE	NE	NE	NE	NE
9495	CO	NE	NE	NE	NE
9568	CO	CO	NE	NE	NE
9723	CO	NE	NE	NE	NE
10006	NE	NE	NE	NE	NE
10332	CO	CO	NE	NE	NE
11365	NE	NE	NE	NE	NE
11490	CO	CO	NE	NE	NE

Table 5. 5 A summary of the activity of crude hop extract and antibiotics against clinical isolates of *M. abscessus* determined using an agar diffusion assay. (Key – CO = Co-active; NE = No effect; A = antagonism. Results determined on three separate occasions

As can be seen from Table 5.5 the degree of antibacterial interaction varied depending on the type of antibiotic and the test organism. Antimicrobial co-action was observed between hop extracts and imipenem (n=5) followed by Ciprofloxacin (n=3). In no case was antagonism observed.

While the Citra hop extract demonstrated antibacterial activity against all strains of *M. abscessus* tested, the isolates varied in their response to particular combinations of hop extract and antibiotics. Isolates 9568, 10332 and 11490 demonstrated coactivity with two of the antibiotics tested.

Strain numbers 10006, 8899 and 11365 showed no effect with any of the antibiotics which suggests fundamental differences between the sensitivity of these bacteria. It is interesting to note that 10332 demonstrated the greatest range of antibiotic sensitivities while 10006 was the most resistant.

5.4.4. Determination of the sensitivity of clinical isolates of *M. abscessus* to individual compounds isolated from the Citra hop extract

To determine the degree of antibacterial activity of the Citra hop purified components, isolated in Chapter 4, 5 µg of test compound was transferred to a paper sensitivity disc and employed in a zone of inhibition assay (Method 3.6.1). The assay was repeated on 3 separate occasions and the results are presented in Table 5.6.

M. abscessus strain numbers	Extract 2737 G Mean ZOI ±SD (mm)	Extract 2737 Y Mean ZOI ±SD (mm)	Extract 1426 G Mean ZOI ±SD (mm)
8899	18.3 ± 0.45	14.2 ± 0.10	16.5 ± 0.33
9495	12.3 ± 0.11	15.3 ± 0.11	17.3 ± 0.42
9568	12.3 ± 0.21	16.6 ± 0.32	15.4 ± 0.18
9723	11.6 ± 0.44	14.8 ± 0.88	18.2 ± 0.14
10006	11.2 ± 0.52	14.1 ± 0.23	15.6 ± 0.77
10332	10.9 ± 0.19	12.4 ± 0.31	14.7 ± 0.11
11365	12.3 ± 0.18	13.6 ± 0.54	15.2 ± 0.46
11490	12.2 ± 0.16	13.4 ± 0.44	14.9 ± 0.14

Table 5. 6 The antibacterial activity of Citra hop sub-fractions (2737G; 2737Y and 1426G) against clinical isolates of *M. abscessus* determined using a zone of inhibition assay. Each value is a mean ZOI ± SD (mm) based on three separate repeats

As can be observed in Table 5.6 all three extracts demonstrated varying degrees of inhibitory activity against all of the clinical isolates of *M. abscessus*. The data was subjected to Tukey HSD Post Hoc analysis to determine if there was a statistically significant difference in the sensitivity of the strains. A p value of > 0.05 indicated that there was no difference in strain sensitivity to any of the hop extracts tested.

Comparing the sensitivity data from Table 5.3 and Table 5.6, initial observations indicate that there is a decrease in the average zone of inhibition (mm) of the purified extracts when compared to the crude hop extract. A one way ANOVA compared the average ZOI (mm) of the crude hop extract against all strains with the average ZOI of the purified hop extracts against all strains. It was found that there was a statistically significant difference ($p=<0.05$) in the level of antibacterial activity, therefore a reduction in the overall activity.

This is perhaps not surprising as hops are known to contain more than 800 separate compounds of which more than 20 have been reported to be antibacterial (Sumner, 2010). It is possible that individually these compounds do not possess a measurable level of antibacterial activity but when in combination, as in the crude extract, an antibacterial affect can be observed. It may be the case that the separation and purification of these fractions actually increases the availability of these compounds compared to the original extract. It may be the case that other compounds can act antagonistically preventing their antibacterial activity.

5.4.5. Determination of synergy between hop derived compounds and antibiotics using an agar based zone of inhibition assay

The interaction between the antibacterial hop sub-fractions with antibiotics was assayed using an agar based method. No effect between any of the hop extracts and antibiotics assayed was observed.

Given that coactivity was observed using the crude hop extract these results suggest that either the hop compounds necessary for synergy were missing from the purified fractions or that synergy is the consequence of the combined activity of a number of compounds found in the crude hop extract.

5.4.6 Determination of the MIC of Citra hop extract against clinical isolates of *M. abscessus* using an agar incorporation assay

To further determine if there was a positive co-action between the crude hop extract and the antibiotics a modified agar incorporation assay utilising E-test strips was used (section 5.3.2). By measuring changes in the MIC I hypothesised that the any changes in antimicrobial activity could be more accurately determined.

The first stage of this process was to determine the level of sensitivity of the bacterial isolates to the crude hop extract so that the level of hop extract to incorporate into the agar for the E-strip assay could be determined. As can be seen in Table 5.7 all isolates were sensitive to the Citra hop extract. A p value of > 0.05 using a one way ANOVA indicated that there was no statistically significant difference between the MIC of the crude hop extracts and the bacterial isolates

M. abscessus strain number	The MIC of crude Citra hop extract (% concentration of hop extract v/v)
8899	1.25
9495	0.625
9568	1.25
9723	1.25
10006	1.25
10332	0.625
11365	0.625
11490	0.625

Table 5. 7 The MIC of crude Citra hop extract against clinical isolates of *M. abscessus* determined using an agar incorporation assay. Results represent the mean of three separate assays

It was interesting to observe in Table 5.7 that even though the organisms were resistant or sensitive to different antibiotics there was no variation in their sensitivity to the hop extract. This highlights fundamental differences in the mode of action(s) of the hop extract and the antibiotics previously assayed.

This assay also supported the findings of previous research (Table 5.3) which detailed the zone of inhibition and showed no variation in the sensitivity of the isolates to the hop extract.

5.4.7 Determination of the MIC of antibiotics against clinical isolates of *M. abscessus* using the E-strip test.

The method employed was a variant of the agar incorporation method developed in Chapter. A sub-inhibitory concentration of Citra hop extract was incorporated into the agar prior to the addition of the E-strip. As a control the hop extract was omitted. Typical examples of the results generated with this method are presented in the following figures (5.6 and 5.7).

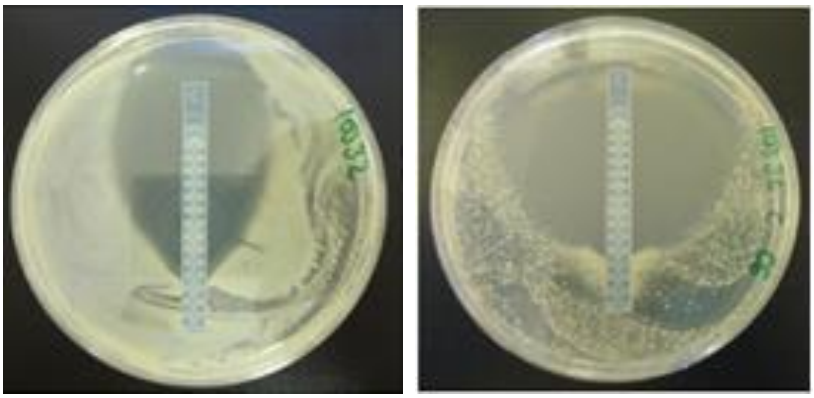

Figure 5. 6 The effect of imipenem 0-256 µg on the left and with the incorporation of a sub-inhibitory concentration (0.3125 % (v/v)) of Citra crude hop extract on the right. Figure representative of 3 separate repeats.

Figure 5.6 shows the effect of hop extract at a concentration of 0.3125 % v/v on the sensitivity of *M. abscessus* 10332 to imipenem. In the presence of the hop extract, the zone of inhibition was larger and the MIC value reduced from 6.0 µg to 0.03 µg suggesting a synergistic effect.

As can be observed in Figure 5.7 the addition of a ciprofloxacin Etest had an inhibitory effect on the growth of *M. abscessus* 10332 (MIC – 0.12 µg). When compared to the image on the right, in which 0.3125 % (v/v) Citra hop extract was incorporated into the agar. The zone of inhibition surrounding the Etest strip was much larger, the MIC value had also decreased to 0.06 µg from 0.12 µg. Although not considered a significant decrease in MIC to be synergistic, a positive co-action was assumed due to the increase in the zone of inhibition.

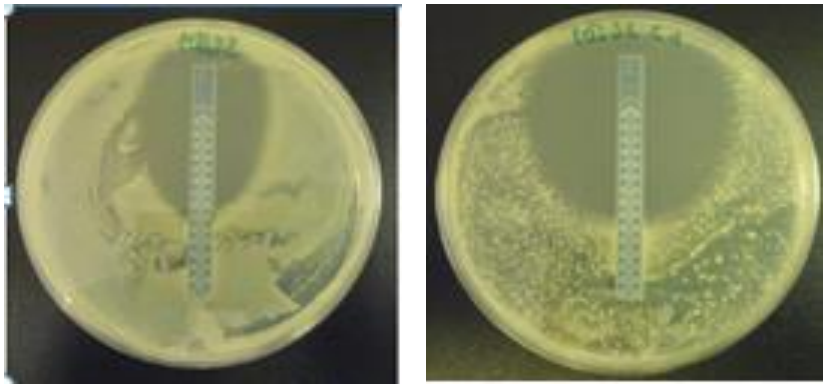

Figure 5. 7 The Etest of ciprofloxacin (256 – 0 µg) against *M. abscessus* 10332 without the incorporation of Citra hop extract (0.0625 % v/v) (left) and with the incorporation of extract.

A summary of the results for all of the isolates of *M. abscessus* examined in this study to determine the interaction between hop extract and antibiotics is shown in the following Table (Table 5.8).

	Imipenem		Ciprofloxacin		Pyrazinamide		Rifampicin		Ethambutol	
Strain numbers	MIC (µg)	MIC with Hop Ext. (0.312%v/v) (µg)	MIC (µg)	MIC with Hop Ext. (0.312%v/v) (µg)	MIC (µg)	MIC with Hop Ext. (0.312%v/v) (µg)	MIC (µg)	MIC with Hop Ext. (0.312%v/v) (µg)	MIC (µg)	MIC with Hop Ext. (0.312%v/v) (µg)
8899	>32	>32	6	6	>32	>32	>32	>32	>32	>32
9495	8	2	6	6	>32	>32	>32	>32	>32	>32
9568	8	2	0.12	0.06	>32	>32	>32	>32	>32	>32
9723	8	2	>32	>32	>32	>32	>32	>32	>32	>32
10006	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32
10332	6	0.03	0.12	0.06	>32	>32	>32	>32	>32	>32
11365	6	0.12	>32	>32	>32	>32	>32	>32	>32	>32
11490	8	2	1	0.5	>32	>32	>32	>32	>32	>32

Table 5. 8 The effect of Citra hop extract on the antibiotic sensitivity of clinical isolates of *M. abscessus*. The MIC of imipenem; ciprofloxacin; pyrazinamide; rifampicin and ethambutol was determined using the Etest before and after the incorporation of Citra hop extract (0.312 % v/v). The numerals in red denote a reduction in the MIC, suggesting coactivity. Results are representative of 3 separate repeats.

In this assay the concentration of the hop extract did not change, instead a variation of the MIC of the antibiotic was used to determine an antagonistic (> MIC); indifferent (no change in MIC); co-active (< 3 fold decrease in MIC).

Therefore on this basis, the antibiotic which demonstrated the highest level of Co-activity, was imipenem, specifically in strains 10332 and 11365.

It has been proposed that hops contain compounds which induce the release of bacterial cell wall degrading enzymes which contribute to their antibacterial activity (Carder, 1987). Given that imipenem also targets the bacterial cell wall it is interesting to speculate that the effect seen in these studies is a consequence of an interaction which enhances the access of either drug to its target. The fact that the purified antibacterial hop compounds failed to interact with imipenem suggests that their mode of action does not involve any form of direct cell wall damage.

The interaction between hops and ciprofloxacin although detectable was not as dramatic as that seen with imipenem. Whilst no evidence of co-activity was observed, an increase in the size of the zone of inhibition was seen suggesting a form of positive interaction.

Ciprofloxacin is a broad spectrum antibiotic used against both Gram positive and Gram negative organisms and works by inhibiting DNA gyrase enzymes inhibiting cell division. Resistance to ciprofloxacin has been linked to a lack of cell wall permeability which is a classical defence mechanism in mycobacteria (Chatterje., 1997; Jarlier., 1994)

As previously discussed in this chapter, each hop extract had observable antibacterial activity against the *M. abscessus* isolates. Therefore it may be that the hop extract is degrading the bacterial cell wall, leading to intracellular exposure to ciprofloxacin. In each case between the hop extract and pyrazinamide; rifampicin and ethambutol an indifferent result was given. Interestingly all isolates had an MIC value of more than 32 $\mu g/mL^{-1}$ to the anti-tuberculosis drugs ethambutol, pyrazinamide and rifampicin. This highlights previous research which suggests that *Mycobacterium abscessus* is intrinsically resistant to these antibiotics (Brown-Elliott *et al*., 2012; Griffith *et al*., 2007; Medjahed *et al*., 2010).

5.4.8 Checkerboard method for determining synergy between imipenem, ciprofloxacin and hop extracts against *M. abscessus* 9568, 10332 and 11490

To further determine and quantify if the combinations of hop extract and antibiotic were synergistic, additive, indifferent or antagonistic. The checkerboard method was used.

Based on the results gained from Table 5.8, three strains of *Mycobacterium abscessus* were employed: 9568, 10332 and 11490. These strains were chosen due to the changes in MIC values of the antibiotics (imipenem and ciprofloxacin) seen in the previous assay 5.4.7. Results are presented in Tables 5.9 and 5.10 and represent 3 separate repeats.

Definitions:

- MIC_A - The MIC of ciprofloxacin/ imipenem (Drug A) alone
- MIC_B – The MIC of Citra crude hop extract (Drug B) alone
- MIC_{AB} – The MIC of drug A in combination with B
- MIC_{BA} – The MIC of drug A in combination with A

M. abscesuss	**Single Drug Imipenem Hop ext.**		**Combination**		**FIC Index**	**Interpretation**
	MIC_A	MIC_B	MIC_{AB}	MIC_{BA}		
9568	8	1.25	2	0.625	0.77	Additive
10332	6	0.625	0.03	0.156	0.255	Synergistic
11490	8	0.625	2	0.625	1.25	Indifference

Table 5. 9 Results of the checkerboard assay determining the MIC of imipenem and Citra crude hop extract alone and in combination with subsequent interpretation of the FIC index. Values of 3 separate repeats ±SD 0.00

M. abscessus	**Single Drug CIP Hop ext.**		**Combination**		**FIC Index**	**Interpretation**
	MIC_A	MIC_B	MIC_{AB}	MIC_{BA}		
9568	0.12	1.25	0.06	0.625	1.0	Indifference
10332	0.12	0.625	0.06	0.312	0.99	Additive
11490	1	0.625	0.5	0.625	1.5	Indifference

Table 5. 10 Results of the checkerboard assay determining the MIC of ciprofloxacin (CIP) and Citra crude hop extract alone and in combination with subsequent interpretation of the FIC index. Values of 3 separate repeats ±SD 0.00

The checkerboard method represents the gold standard in synergy testing and its effectiveness has been evaluated previously by researchers in the field of antibiotic synergy (Petersen *et al*., 2006; Harada *et al*., 2014; Zuo *et al*., 2014).

The advantages of this method allow the subsequent MIC's of both test agents, in this case the antibiotics imipenem and ciprofloxacin and Citra crude hop extract, to be determined alone and in combination. Furthermore the method allows for the fractional inhibitory concentration (FIC) to be defined and a synergistic, additive, indifferent or antagonistic result being given for each combination. The FIC was first described by Elion (1954) and determines the statistical significance that two separate drugs will act in a synergistic or antagonistic manner based on a number determined between 0 and > 4 (Elion *et al*., 1954; Hall *et al*., 1983b).

In the case of imipenem, 3 different interpretations of interaction were determined. Interestingly the MIC of imipenem in the checkerboard method matched that as determined in the modified agar incorporation method, however, several concentrations of hop extract were used in combination with the antibiotics. An observed synergistic effect (FIC = 0.255) between imipenem (MIC 0.03 $\mu g/mL^{-1}$ in combination with Citra crude hop extract 0.156 % v/v) against the strain 10332 was seen. This is a reduction from 6 $\mu g/mL^{-1}$ of imipenem and 1.25 % v/v of Citra hop extract when used alone.

An additive effect (FIC = 0.77) between imipenem (2 $\mu g/mL^{-1}$) and Citra crude hop extract (0.625 % v/v) from an original concentration of 8 $\mu g/mL^{-1}$ and 1.25 % v/v against strain number 9568 was also observed.

In the third bacterium trialled 11490, although the MIC of imipenem dropped from 8 $\mu g/mL^{-1}$ to 2 $\mu g/mL^{-1}$ when in combination with a 0.625 % v/v hop extract. The FIC (1.25) indicated that this was an indifferent effect.

When compared to results of ciprofloxacin alone and in combination with the hop extract similar activity to the results obtained in the modified agar incorporation assay against the test bacterium was observed.

Whilst the FIC in each case indicated that there was no synergistic effect, an additive interaction was seen against *M. abscessus* 10332. This is identified by the MIC of ciprofloxacin reducing to 0.06 µg/mL^{-1} from 0.12 µg/mL^{-1} and the Hop extract MIC being reduced to 0.312 % v/v from 0.625 % v/v which resulted in an FIC of 0.99 which was just below the threshold of indifferent (FIC >1 and <4).

Whilst an indifferent effect was seen between the test agents against strains 9568 (FIC = 1.0) and 11490 (FIC = 1.5) a decrease in both the MIC of the ciprofloxacin and hop extract in both cases was observed. This is considered statistically insignificant 'indifferent' according to the FIC.

5.5 Scanning Electron Microscopy (SEM) imaging of antibiotic and hop combinations

To characterize the interaction of hop derived compounds and antibiotics with the bacteria used in this study scanning electron microscopy was employed. The structures of two species of mycobacteria: *M. abscessus* and *M. smegmatis* was firstly compared.

Figure 5.8 below a scanning electron microscopy image at 100k magnification of *M. smegmatis* 8159 is presented.

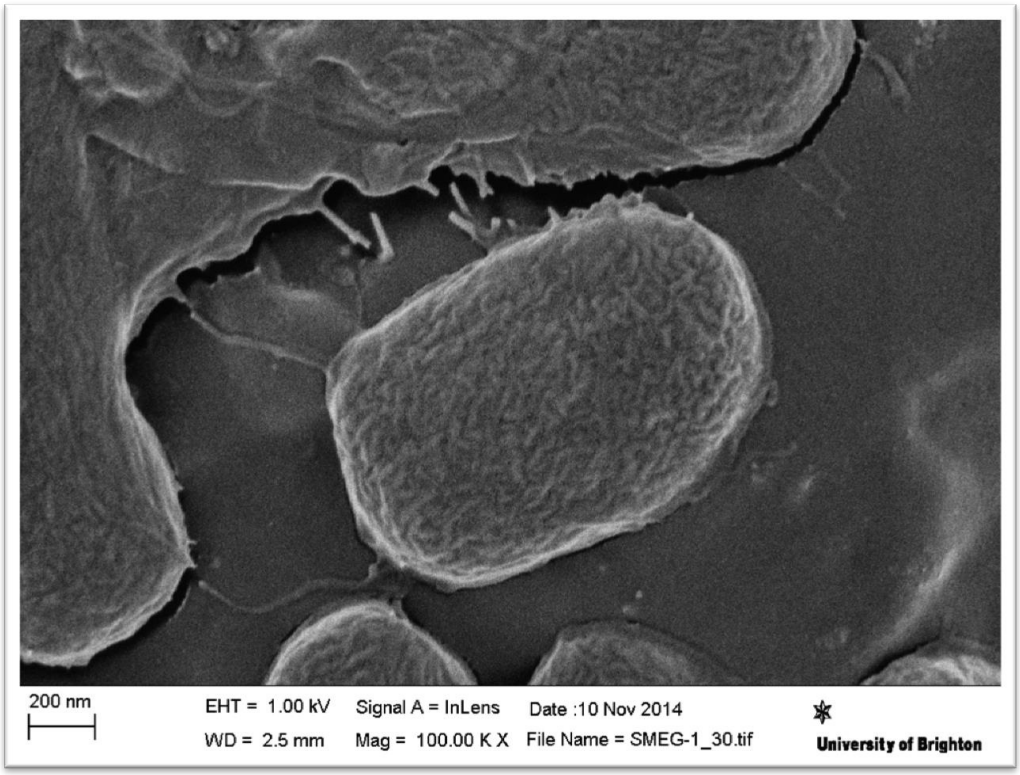

Figure 5. 8 SEM image of *Mycobacterium smegmatis* 8159 at 100 k magnification.

As can be seen from Figures 5.8 and 5.9 both bacteria are covered in what appears to be a rough, variegated surface which probably corresponds to the waxy outer layer which is known to coat mycobacteria.

A comparison of the size of *M. smegmatis* to *M. abscessus* revealed that these bacteria varied in size with *M. smegmatis* (approx. 1 µm) being shorter in length than *M. abscessus* (approx. 2 µm).

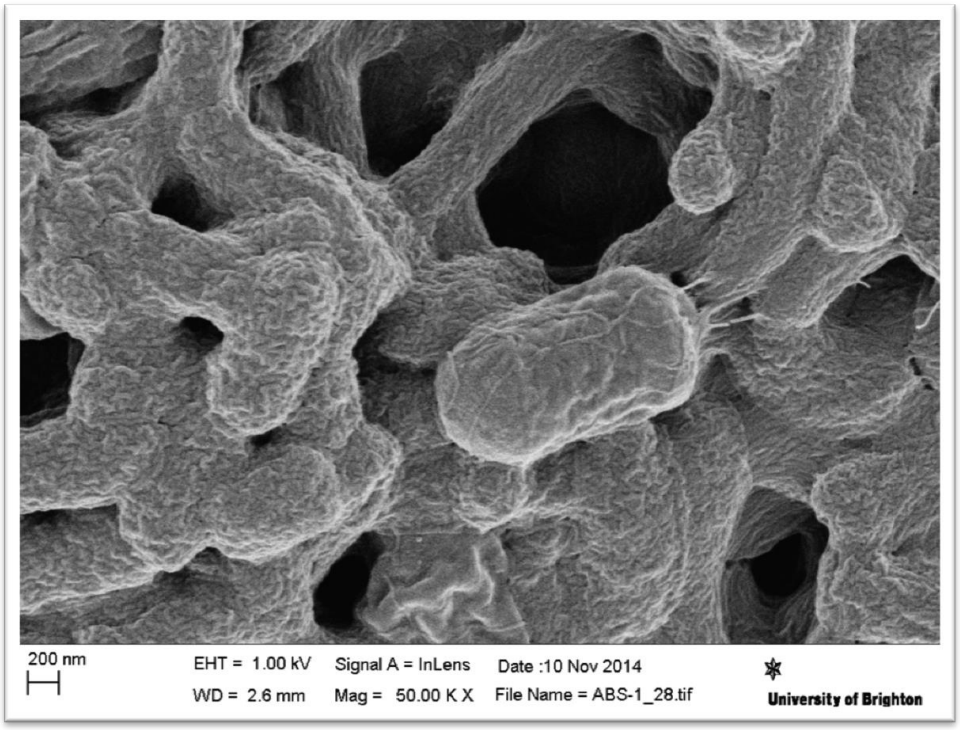

Figure 5. 9 SEM image of *Mycobacterium abscessus* 10332 at 50, 000 x magnification representative of 25 separate images.

Figure 5.9 represents 25 separate images of the bacteria in situ. Several observations can be made; notably there is a lack of single cells. This may be due in part to the waxy surface of the bacteria comprised of hydrophobic lipids and glycoproteins which allow the bacteria to adhere (Brennan, 2003; Ryan *et al*., 2004; Tyagi and Sharma, 2002). The lipid rich cell wall is a well-known virulence factor in mycobacteria and aids in drug resistance by acting as a permeability barrier (Ryan *et al*., 2004).

5.5.1 Characterisation of bacterial structure following exposure to Citra hop extract using SEM

To determine if exposure to Citra hop extract had any effect on cellular morphology, cells of *Mycobacterium abscessus* 10332 were incubated for 10 hours with (0.3125 % v/v) of hop extract. After which, they were examined using SEM (Figure 5.10).

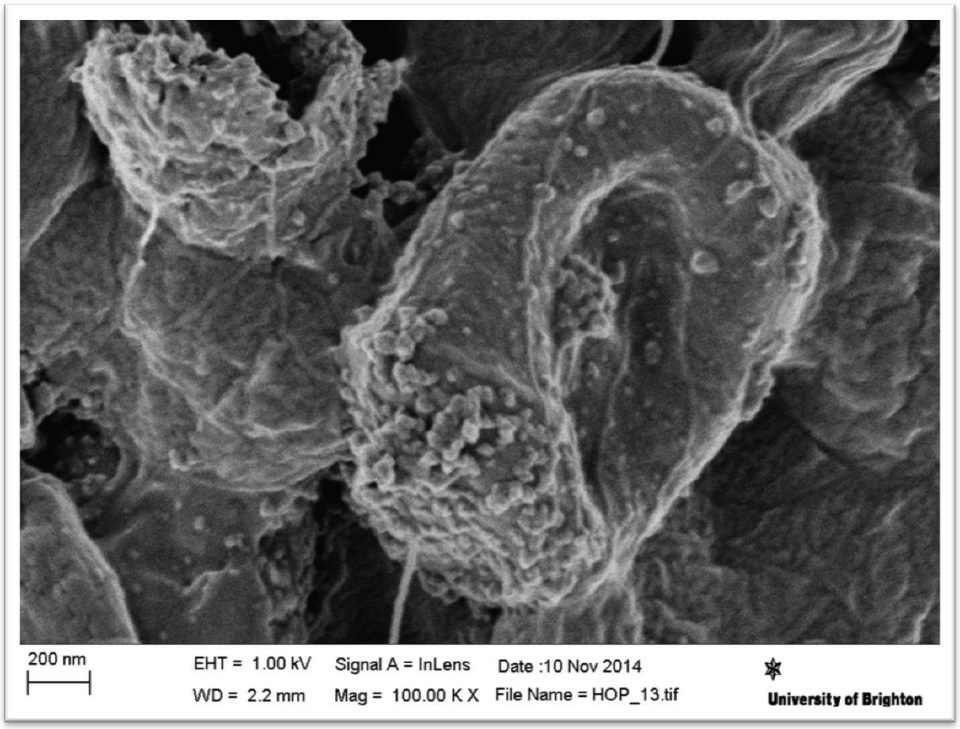

Figure 5. 10 SEM image of *M. abscessus* 10332 (50k magnification) after 10 h incubation with 0.3125 % (v/v) Citra crude hop extract- representative of 25 separate images

In comparison with the control *M. abscessus* (Figure 5.9), the hop extract is having a demonstrable effect on the cell structure, whilst size does not seem to be affected, pitting of the cells forming crevices is clearly visible.

5.5.2 Characterisation of bacterial structure following exposure to Ciprofloxacin using SEM

To determine if exposure to ciprofloxacin had any effect on cellular morphology, cells of *Mycobacterium abscessus* 10332 were incubated for 10 hours with 6 µg/mL^{-1} ciprofloxacin after which they were examined using SEM (Figure 5.11).

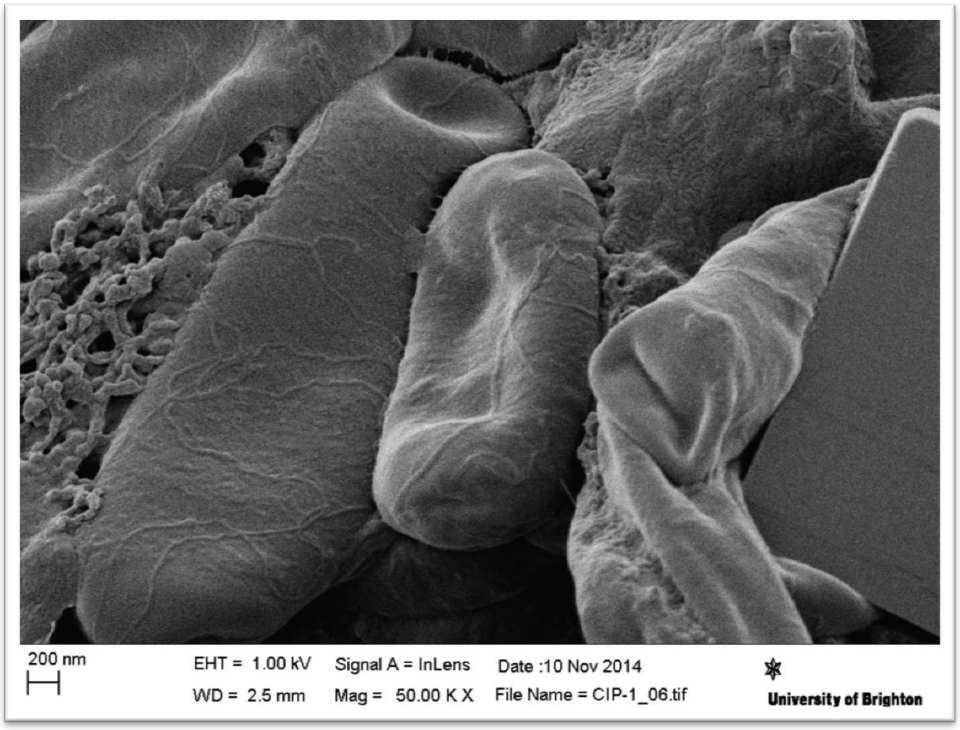

Figure 5. 11 SEM image of *M. abscessus* 10332 at 50k magnification after incubation with ciprofloxacin (6 µg/mL^{-1}) for 10 hours. representative of 25 separate images

Comparison of these images with those of untreated bacteria (Figure 5.9) revealed obvious differences in the appearance of the cell. The waxy cell surface has been lost and the cells are noticeably larger (approx. 3-4 µm) and are visibly pitted. These differences may be due to the antibiotic inhibiting the action of DNA gyrase and thereby preventing cell division and cell surface differentiation.

5.5.3 Characterisation of bacterial structure following exposure to imipenem using SEM

Next investigated was the effect of exposure to the bet-lactam antibiotic, imipenem, on the cellular morphology of *M. abscessus* following incubation with imipenem (6 µg/mL^{1}) for 10 hours. As was the case for ciprofloxacin treatment the bacteria exposed to imipenem had smoother cell surfaces than those of the untreated control. Imipenem is known to interact with penicillin binding proteins in the cell wall which may explain the effect on cell surface morphology (Chambers *et al*., 1995).

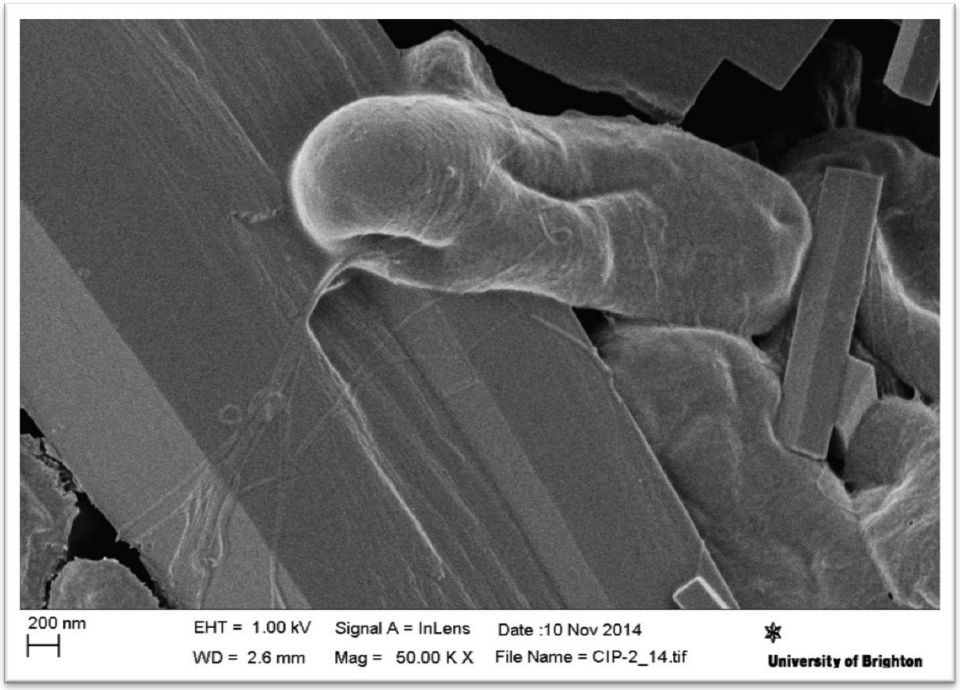

Figure 5. 12 SEM image of *M. abscessus* 10332 at 50 k magnification after incubation with imipenem (6 µg/mL^{-1}) for 10 hours. Figure representative of 25 separate images

5.5.4 Characterisation of bacterial structure following exposure to Citra hop extract and ciprofloxacin using SEM

To determine the effect of a combination of Citra hop extract and ciprofloxacin, *M. abscessus* 10332 was incubated with 0.312 % v/v hop extract and 0.06 µg/mL^{-1} ciprofloxacin for 10 hours (Figure 5.13).

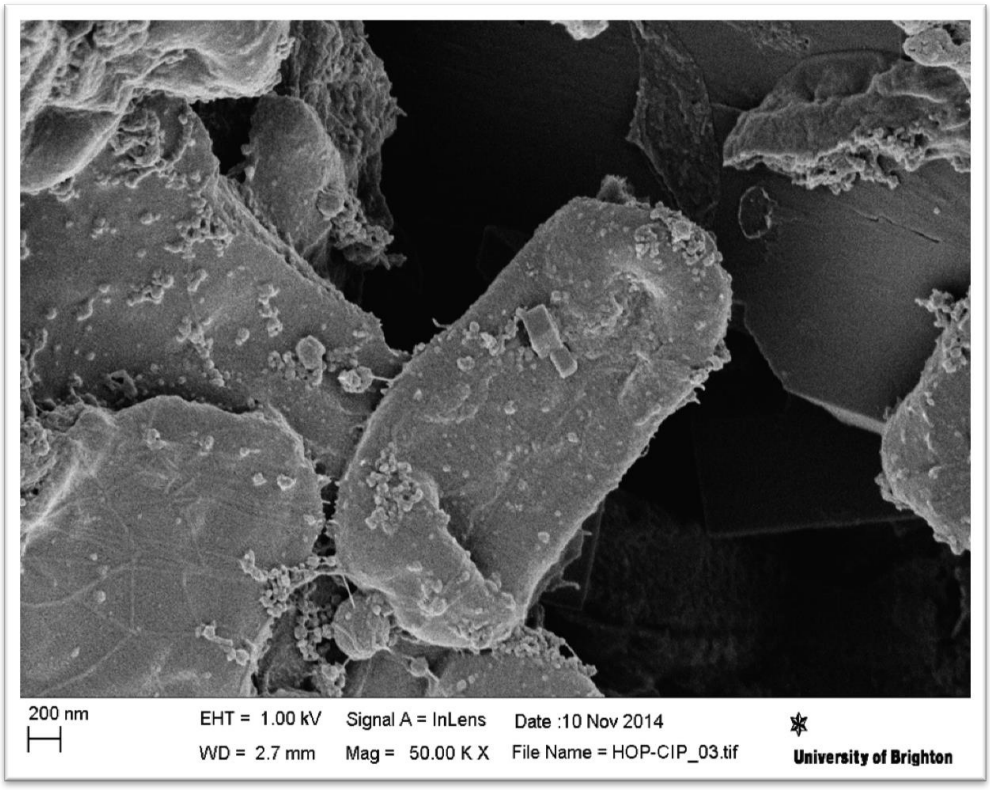

Figure 5. 13 SEM image of *M. abscessus* 10332 after incubation with ciprofloxacin (0.06 µg/mL^{-1}) and Citra crude hop extract (0.312 % v/v) for 10 h. Representative of 25 separate images

A large decrease in the number of intact cells was observed. This suggested that the combination of agents had resulted in the destruction of the cell wall of the bacterium. Those cells which had not been destroyed displayed a similar appearance to the ciprofloxacin treated bacteria in Figure 5. 11.

5.55 Characterisation of bacterial structure following exposure to Citra hop extract and imipenem using SEM

The effect of imipenem and the Citra crude hop extract on the structure of *M. abscessus* 10332 was next investigated. The bacterium was incubated for 10 hours with a sub-inhibitory concentration of hop extract (0.156 % (v/v) and 0.03 µg/mL^{-1} of imipenem and then visualised (Figure 5.14).

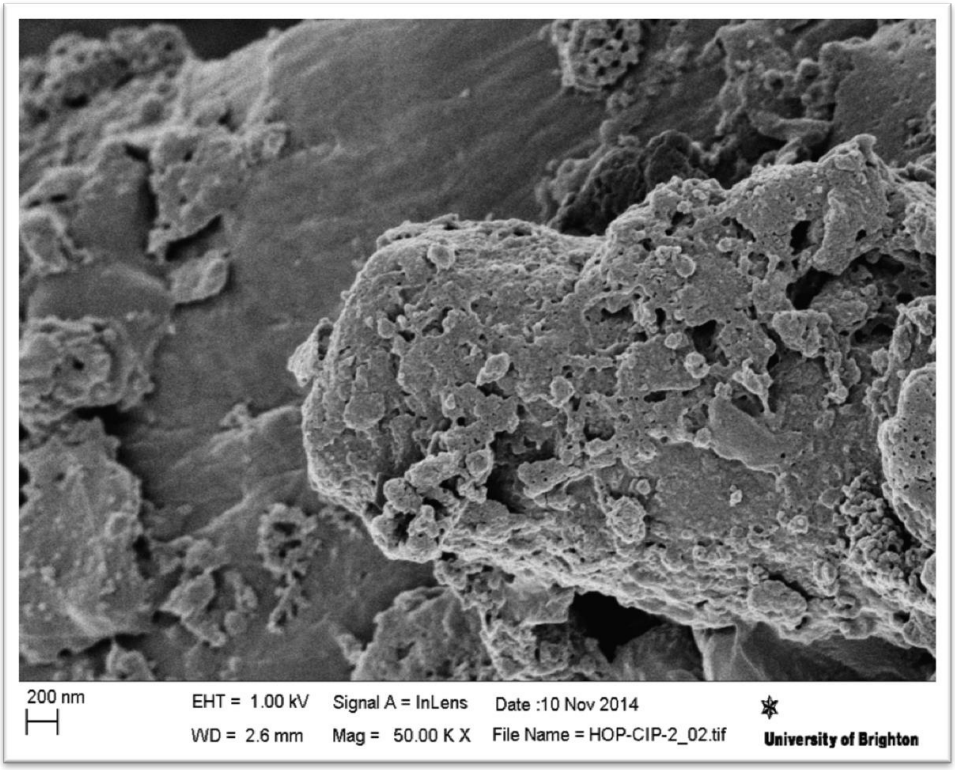

Figure 5. 14 SEM image of *M. abscessus* 10332 after incubation with imipenem (0.03 µg/mL^{-1}) and Citra crude hop extract (0.156 % v/v) for 10 h. Representative of 25 separate images

A large decrease in the number of intact cells was observed, suggesting that the combination of agents had resulted in the destruction of the bacterial cell wall.

To confirm that this cellular disruption was not a localised artifact, additional images were captured at a lower magnification (10,000 versus 50,000) which also showed extensive cellular disruption suggesting that the combination of hops and imipenem was extremely effective at destroying cells of *M. abscessus* 10332 (Figure 5.15)

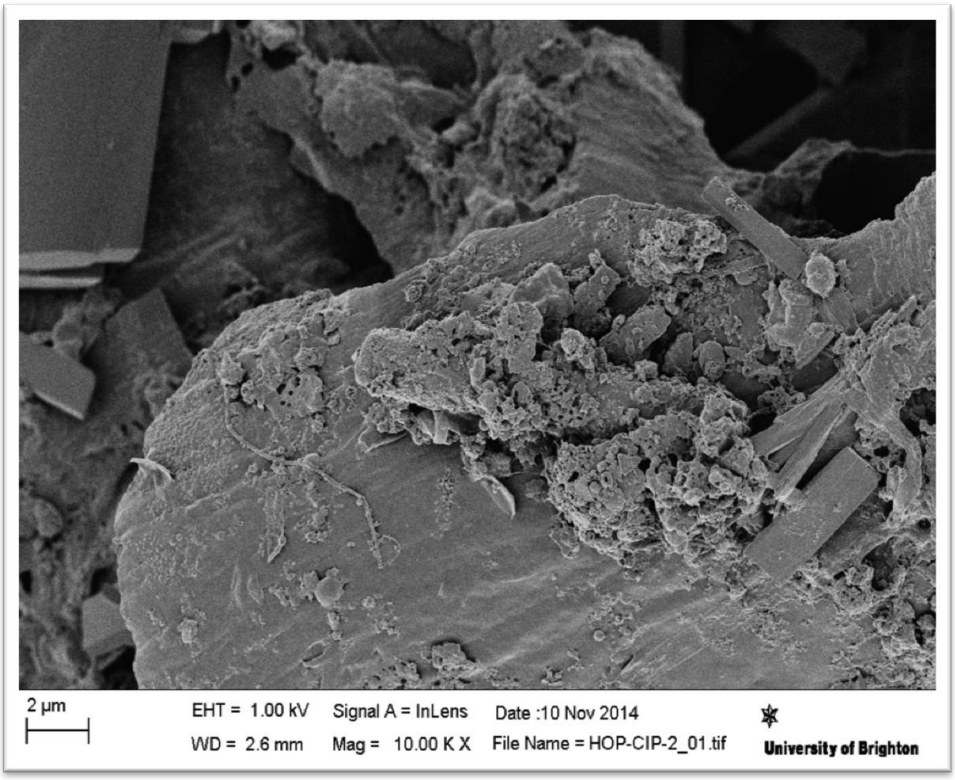

Figure 5. 15 SEM image of *M. abscessus* 10332 at a magnification of 10 k after incubation with Imipenem (0.03 µg/ml) and Citra crude hop extract (0.156 % v/v) for 10 h detailing the lack of visible cells representative of 25 separate images

5.6 Discussion

The organism *Mycobacterium abscessus* has been described as a new antibiotic nightmare (Nessar *et al*., 2012). While the bacterium is intrinsically resistant to some classes of antibiotics, its ability to acquire resistance to other front line drugs makes it a difficult pathogen to treat, particularly when encountered in patients with cystic fibrosis (CF) (Cullen *et al*., 2000; Petrini, 2006; Seddon *et al*., 2013).

Distinguishing *M. abscessus* from other chronic bacterial pulmonary infections found in the advanced stages of CF is extremely challenging, as such, there is a concern that the use of broad spectrum antibiotics may simply select for the emergence of antibiotic resistant isolates of *M. abscessus* (Cullen *et al*., 2000).

Incidences of *M. abscessus* infections are increasing: the CDC assessed 3,805 adults and 3,317 children with cystic fibrosis and found that 5.0 % and 3.3 % of these patients respectively had been diagnosed with an mycobacterial infection, of those, only 44 % of adults and 47 % of children were receiving or had received treatment (Seddon *et al*., 2013).

M. abscessus is often a neglected infection and as a consequence is becoming a major healthcare concern. As was shown in Chapter 3 hop extracts contain compounds which inhibit the growth of *Mycobacterium smegmatis* and for this reason, the antibacterial activity of Citra hop extract and compounds isolated from this extract against clinical isolates of *Mycobacterium abscessus* was determined.

Clinical isolates were obtained from patients treated at the University Hospital of Wales, Cardiff during July 2012. In total eight clinical isolates which varied in their susceptibility to commonly used antibiotics were assayed for their susceptibility to 5 and 10 µg antibiotic discs of imipenem, ciprofloxacin, pyrazinamide, rifampicin and ethambutol. From this it was observed that the most sensitive organism was *M. abscessus* 10332 whilst in comparison strain 11365 was the most resistant.

As had previously been shown in Chapter 3, the Citra hop extract is effective at inhibiting the growth of *Mycobacterium smegmatis* for this reason this extract was also trialled against clinical isolates of *Mycobacterium abscessus*. This was achieved by determining antibacterial activity using a previously developed zone of inhibition assay. No significant difference in the sensitivity of the isolates to the crude Citra hop extract was observed. This was an interesting result due to the fact that several of the isolates were multidrug resistant but were as susceptible as their antibiotic susceptible counterparts, suggesting that the extract contains compounds that exerted inhibitory activity in a manner which differs from that of the antibiotics.

Natural products offer a great resource of novel antimicrobials due to the presence of compounds which have multiple modes of action. For example, the bioactive compound in garlic, allicin, has been shown to stimulate the immune system, attracting macrophages and lymphocytes to its site of injection (Cseke *et al*., 2010). In the case of hops, the constituents humulone and lupulone have been shown inhibit the efflux pumps responsible for nutrient supply to the cell (Teuber and Schmalreck, 1973).

In addition to direct antimicrobial activity it has also been shown that natural products can potentiate the activity of antibiotics (Obiang-Obounou and Jang, 2011; Shami *et al*., 2013). To identify if there was any potential co-action between the hop extract and the antibiotics in use, three commonly used agar and broth based methods were compared for their suitability. Previous research has shown that methods differ in their predictive efficacy (Orhan *et al*., 2005a; White *et al*., 1996).

The first method used a modified zone of inhibition assay. This relied on observing the shape of the zones of inhibition generated around the test compounds. Using this approach co-action between the crude hop extract and the following antibiotics: imipenem (5 isolates) and ciprofloxacin (3 isolates) was observed. No effect was observed when using pyrazinamide, ethambutol and rifampicin.

It was also observed that strains 10006 and 11365 showed no increased sensitivity to any of the antibiotics tested and that strain 10332 demonstrated the greatest range of antibiotic sensitivities whilst 10006 was the most resistant.

To further investigate antibacterial coaction, the antibacterial compounds: humulone, lupulone and adlupulone were also trialled using the same method. Unfortunately in each case, no effect was observed between the combinations. This suggested that synergistic activity is a consequence of the combined activity of a number of compounds present in the crude hop extract.

As previously mentioned in Chapter 4, compound extraction relies on the demonstration of antibacterial activity. Thus, it may be the case that the compounds responsible for the co-active effect with antibiotics lack any direct antibacterial activity and therefore would not have been selected for extraction.

To further identify if there was a coactive effect, the agar incorporation assay previously described in Chapter 3 was employed. Determination of the MIC of the crude Citra hop extract against each clinical isolate of *M. abscessus* revealed no statistically significant difference in the level of sensitivity to the extract.

Based on these results, a sub MIC concentration (0.312 % v/v) was chosen for all bacterial isolates and incorporated into the agar and an Estrip of each antibiotic placed on the surface of the inoculated agar. The results obtained were compared to those generated in the absence of the hop extract. In the context of this project coactivity was determined by a 3 fold decrease in the antibiotic MIC, indifference as no change in MIC and antagonism as an increase in MIC. In previous studies a single dilution change in MIC was considered coactive (White *et al*., 1996).

The advantage of utilising agar incorporation over that of the zone of inhibition assay is that it allows for the measure of different levels of antibiotic sensitivity and thus generates a semi quantitative result. By using this technique it was found that this was most apparent in strains 10332 and 11365 when in conjunction with imipenem and 9568 and 10332 when subjected to ciprofloxacin. In each case between the hop extract and pyrazinamide, rifampicin and ethambutol an indifferent result was given.

There are several possible reasons for the different results observed between the two assays:

1. The zone of inhibition is observed by eye and therefore the observed 'pattern' of growth is subjective. A pattern which is suggestive of coactive may indeed represent indifferent.

2. The Estrip gives a much more quantifiable result which enables a determination to be made of any changes in relative antibiotic sensitivity. However, the strip is not designed for the purposes of synergy and therefore small changes in MIC below the limits of detection on the E-strip may go unnoticed.

In the context of this study, these two agar based screening assays were used as a means of selecting hop and antibiotic combinations worthy of a more detailed study such as checkerboard method. This method represents the "gold standard" assay with which to determine potential synergistic interactions (Petersen *et al*., 2006; Harada *et al*., 2014; Zuo *et al*., 2014).

Using this method a synergistic effect for the combination of imipenem and hop extract against strain 10332, an isolate which was sensitive to all of the antibiotics examined in this study was observed. An additive effect was seen against strain 9568 while 11490 demonstrated indifference. This differs from the results obtained using the agar assays which found a coactive effect highlighting the need to appreciate the limitations of each assay.

For example, while the zone of inhibition assay is a classical method for determining synergy, the interpretation of the results can be subjective and is dependent on several factors including; the confluence of bacterial growth on the surface of the agar and the chance of human error when spreading organisms. The E-test method suffers from the same drawbacks and is considerably more expensive than the zone of inhibition assay. Also whilst the effects of different concentrations of antibiotic could be assessed, only one fixed concentration of hop extract is employed.

While the checkerboard method is considerably more labour intensive than the other assays, it does overcome many of these issues and has the added advantage that several concentrations of the test agents can be analysed at the same time. This allows for any changes in the MIC of both test agents to be determined (Orhan *et al*., 2005a; White *et al*., 1996). The checkerboard assay is also more reproducible than the E-test in that White (1996) reports a 25% discrepancy between the two assays when used to assess the coactivity of the same antibiotic combination (White *et al*., 1996)

In conclusion, the separate assays presented different results. In using the checkerboard assay, synergy was observed between imipenem and the hop extract against 10332 and an additive effect against strain 9568. When compared to the Etest, an additive effect was seen against strain 10332 when exposed to ciprofloxacin and hop extract, and an indifferent effect against 9568 using the Etest method.

While *M. abscessus* 10332 appeared to be sensitive to clinically relevant levels of imipenem, the occurrence of isolates of *M. abscessus* which are resistant to the drug is becoming increasingly common (Medjahed *et al*., 2010). This is due to the expression of β-lactamase enzymes that degrade the activity of β-lactam antibiotics such as imipenem. A number of the isolates examined in this study demonstrated a level of drug resistance indicative of the production of β-lactamase enzymes. Unfortunately, none of the resistant isolates examined in this study demonstrated any evidence of co-activity with hop extracts

To determine the potential mode of action of the hop extract and the antibiotics alone and in combination, scanning electron microscopy was used. One of the limiting factors of SEM is the fixing and dehydration steps. This severe process can adversely affect the structure of the bacterium and even destroy intact cells. This was a major concern and for this reason the method utilised a short (30 seconds) contact time with 100 % alcohol to avoid dehydrating the cells.

Another problem that can be encountered when using SEM is to ensure that enough field of view are examined to ensure that the images seen are representative of the entire sample. Each image presented in this project is representative of a minimum of 25 separate images (all images are available in the appendix in CD form).

Firstly the structures of *M. smegmatis* 8159, the surrogate organism of *M. tuberculosis* and the clinical isolate *M. abscessus* 10332 (chosen because it showed the most promising interactions between hop extracts and antibiotics) were compared. No obvious evidence of any cell damage (cell destruction, pitting) due to the fixing process was observed in any of the bacteria visualised.

The two bacteria appeared to differ in size in that the mean length of *M. smegmatis* was approximately 1 µm while *M. abscessus* was approximately 2 µm. Examination of the bacterial surface revealed a rough, crenulated surface which presumably is evidence of the waxy coat which is known to surround these bacteria (Ryan *et al*., 2004)

Exposure to a sub-inhibitory concentration of Citra hop extract (0.3125 % v/v) had a noticeable effect on the surface of the bacteria in that it became much smoother and the cells were visibly pitted. Previous studies to identify the mechanism of antimicrobial action of hop extracts have found that it was due to changes in the permeability of the cell wall or membrane (Simpson and Smith, 1992; Teuber and Schmalreck, 1973) with subsequent inhibition of respiration and of DNA and RNA synthesis (Behr and Vogel, 2009). It may be the case that the hop extract exerts both of these properties; firstly by breaking down the waxy outer layer of the cell wall which secondly allows the compounds to access the cell and disrupt cellular mechanisms.

Indeed the intrinsic resistance of mycobacteria to antibiotics has been linked to the impermeability of the mycolic acid containing cell wall which is responsible for the bacterium's natural resistance to antibiotics such as tetracycline, fluoroquinolones and aminoglycosides.

Exposure of *M. abscessus* to ciprofloxacin and imipenem and its effect on cell wall structure was next investigated. In the case of ciprofloxacin, the apparent waxy cell surface was lost and the cells were noticeably larger (approx. 3-4 μm) and as was seen following exposure to hop extract, visibly pitted. These differences may be due to the antibiotic inhibiting the action of DNA gyrase and thereby preventing cell division and cell surface differentiation.

A similar effect was noticed after the bacteria had been exposed to imipenem. The bacteria had a much smoother surface than the untreated control. Imipenem is known to interact with penicillin binding proteins involved in cell wall biosynthesis which may explain the effect on cell surface morphology (Chambers *et al*., 1995).

Exposure of the bacterium to a combination of hop extract and ciprofloxacin at the concentrations identified as being synergistic in the checkerboard assay resulted in a marked reduction in whole bacterial cells. This was surprising given that the mode of action of ciprofloxacin is to inhibit bacterial DNA synthesis. At this moment in time there is no reasonable explanation of this phenomenon. Exposure of the bacterium to a combination of hop extract and imipenem, at the concentrations identified as being synergistic, had a similar effect on cellular morphology, in that the majority of bacteria were lysed.

It has been reported that several hop compounds, namely the alpha and beta acids (Orhan *et al*., 2005a) and xanthohumol (Stevens and Page, 2004), have antimicrobial effects and demonstrate coactivity with several antimicrobials (Natarajan *et al*., 2008). In the case of imipenem, both the hop extracts and the antibiotic have been shown to act on the cell wall of the bacterium by inhibiting efflux pump mechanisms.

In conclusion, this chapter demonstrates that the Citra hop extract contains compounds capable of inactivating multidrug resistant clinical isolates of *M. abscessus*. It has also been shown that the extract contains compounds which enhance the activity of the antibiotics imipenem and ciprofloxacin; the mechanisms by which this enhancement is achieved and the identity of the compounds responsible has yet to be determined. Electron microscopy studies suggest that these compounds affect the cell wall of the bacterium in a manner which when give together result in the destruction of the bacterium.

These encouraging results suggest that further research is merited to identify the hop derived compounds responsible for these effects with a view to developing next generation therapeutics for the treatment of drug resistant mycobacterium such as *M. tuberculosis.*

Chapter 6

General Discussion, Conclusions and Future work

6.0 General Discussion

Bacterial resistance to antibiotics is a worldwide problem; indeed It is estimated that almost one third of the world's population is infected with *M. tuberculosis* of these approximately 10% of cases are resistant to first line antibiotics (Murray *et al*., 2014)

The current cost of $1.5 billion required to bring a new antibiotic from the benchtop to the market, coupled with the velocity of bacterial resistance has led to a lack of novel antibiotics over the last 20 years. Due to this, current research into plant derived drug discovery has seen resurgence. Indeed, over 50% of the drugs used today are derived from natural products, and recent evidence suggests that as the general public are being made aware of the decline of antibiotics, natural therapeutics are becoming more accepted.

One such natural product which has been utilised for its antibacterial activity for hundreds of years is the hop plant. Beer was originally produced without the addition of hops. However, it was observed that during summer months 'unhopped' beer did not last as long as its hopped counterpart due to bacterial contamination. As a consequence the popularity of hopped beer increased (Sumner 1988). During the mid-19th century, drinking water was heavily contaminated by faecal pathogens due to poor sanitary arrangements. Beer was considered a safer drink due to the fact that bacteria present were reduced due to a combination of boiling and the presence of hop derived antimicrobial compounds (Sumner 1988).

The majority of research to date has been focused on characterising the antimicrobial activity of hops against Gram positive bacteria such as lactobacillus which represent the principal spoilage agents of beer (Ahn *et al*., 2007; Sagdic *et al*., 2003). Surprisingly little attention has been paid to determine if these plants contain compounds that have inhibitory activity against clinically important bacteria such as *Mycobacterium*. This thesis sought to characterise the antibacterial activity of hops and their extracts against a range of medically important Gram negative and positive bacteria; *Escherichia coli, Staphylococcus aureus* and *Mycobacteria.*

Due to the pathogenicity of *M. tuberculosis* and its slow rate of growth on laboratory media two simulant organisms: *M.smegmatis* and *M. bovis* BCG were used. Both organisms have been shown to have similar sensitivities to antibiotics compared to *M. tuberculosis* (Chaturvedi *et al*., 2007b; Tyagi and Sharma, 2002). *M. smegmatis* and *M. bovis* both grow at a higher rate than *M. tuberculosis* and due to their lack of virulence can be handled within a category II laboratory.

To determine the antibacterial activity of individual hop varieties an extraction process was developed which mirrored that used during the brewing process. It was found that the degree of maceration, extraction temperature and time influenced the inhibitory activity of the crude extract. Furthermore, by increasing the time that machine macerated hop material was exposed to 100 °C, the antibacterial activity was increased.

The effect of heat on the antibacterial activity of the hop extracts is likely to have been the consequence of two processes: energy drive release of compounds from plant material into the aqueous solution and the isomerisation of released compounds such as the alpha and beta acids. Isomerisation of these compounds due to heating has been shown to increase the antibacterial activity of hop extracts. This was further shown by the fact that when heated at room temperature (25 °C) there was little or no antimicrobial effect. Prolonged heating of the extracts resulted in a loss of activity. Increasing the time of heating increases isomerisation, and concurrently causes thermal degradation. It has been suggested that prolonged heating could have an adverse effect on the antibacterial activity (Jaskula-Goiris, 2010)

In this study two agar based screening methods were used to determine the antibacterial activity of 50 hop variants. A broth based micro dilution method had been considered. However, progress using this approach was inadequate due to the inherent turbidity of the hop extracts following heating (Zgoda and Porter, 2001)

While the zone of inhibition assay proved to be a useful tool with which to screen the activity of multiple hop extracts it lacked sensitivity. For this reason, an agar incorporation assay was developed which allowed for the determination of the minimum inhibitory and bactericidal concentrations (Roberts 1998). Using this assay it was found that all of the hop variants demonstrated an inhibitory effect against all of the test bacteria with the exception of *Escherichia coli*. Gram negative organisms such as *E.coli* are often resistant to antibiotic substances due to the nature of their cell wall, as such natural products rarely have an inhibitory effect against these organisms (Yadav *et al*., 2012).

As has been hypothesised previously, an investigation into the correlation between alpha and beta acid content of a particular hop variant and its antibacterial activity was undertaken. No statistically significant correlation was observed. This was surprising given that the hop alpha and beta acids have been reported to be antibacterial (In Acworth *et al*., 2012). I hypothesise that other compounds in the hop extract are responsible for the observed antibacterial activity. Hops have been shown to contain over 800 compounds some of which may be directly antibacterial or act in a synergistic manner to enhance the activity of other antibacterial compounds (Barbara Jaskula-Goiris, 2010; Cornelison, J.M., 2008).

The results suggest that the antimicrobial activity demonstrated by the hop variants was not due to a single component but was more likely due to a mixture of components acting in a synergistic manner; the individual components were next investigated.

To identify the antibacterial compounds present in the hop extracts an extraction and identification system based on a combination of solvent extract and thin layer chromatography (TLC) was developed. The technique was modified by overlaying the separated compounds on the TLC plate with a layer of *M. smegmatis*. Following incubation the plate was stained and for the presence of viable bacteria and any spots of non-staining were taken to indicate the presence of antibacterial compounds.

Using this approach it was observed that the Citra hop variant (the variant with the greatest antibacterial activity: (Chapter 3) had the greatest number of antimicrobial spots, whilst the Galena variant (the least antimicrobial: Chapter 3) had the least number of antimicrobial spots. These results support the methodology used in Chapter 3 which ranked the hops based on their antimicrobial activity and shows that the TLC overlay method was a reliable means of determining antimicrobial activity.

A limitation of this approach is that it separates compounds on the basis of their polarity, and as a consequence separated spots may contain a mixture of antimicrobial and non -antimicrobial components of similar polarity. Based on the information gained from Chapter 3 the Citra and Galena hop variants were selected for further analysis. To further enhance the separation of compounds, column chromatography was used.

The advantage of column chromatography in comparison to TLC was that a greater quantity hop extract could be separated and as a consequence higher yields of components of interest could be achieved. It is recognised that it's possible for more than one compound to be present of equal polarity in a single spot and that conventional techniques could not separate them.

To identify the active components of the Citra and Galena spots selected based on antimicrobial activity; both were analysed using mass spectrometry to elucidate the compounds present. The compounds: adhumulone, lupulone, colupulone, adlupulone and epicatechin were found to have similar masses to those extracted from the hop extracts. There were differences in the isolation of these compounds from both variants which suggested that their presence has an effect on the antimicrobial activity.

The bioassay guided extraction technique failed to identify all of the antibacterial compounds that are known to be present in hops. The most notable missing compound was the antibacterial polyphenol, Xanthohumol which has been isolated by others from this plant and is known to be active against mycobacteria (Acworth *et al*., 2012; Ceh *et al*., 2007; Stevens and Page, 2004).

Further analysis was conducted to screen for the presence of XN via ultra-pressure liquid chromatography which is an approach that has been used for a number of years to identify natural product derived compounds (Acworth *et al*., 2012; Ahuja and Rasmussen, 2011; Baker *et al*., 2008). Comparison of the crude extracts of the Citra and Galena hop variants confirmed the presence of known antibacterial compounds humulone and lupulone. Xanthohumol was also identified, it may have been the case that this polyphenol was at a low concentration when separated from the hop extract and therefore was missed during the initial screening process.

The presence of a new compound was detected and isolated from the antibacterial fraction of the Citra hop variant; however it was found that this compound was not itself antimicrobial. Although not singularly antimicrobial, as with many plant extracts, compounds within an antimicrobial mixture are often synergised or potentiated by each other. It is hypothesised that this is the case. However, more research is needed to investigate the synergies between this compound and others from the extract.

By using several different methods of characterisation it was possible to gain an insight into the constituents responsible for the antimicrobial activity of the extracts.

Natural product derived compounds have been successfully used to potentiate the activity of antibiotics. As bacterial resistance increases, antibiotics are used at ever increasing concentrations to the point where they are no longer efficacious and could produce harmful side effects. A synergistic combination of antibiotics and a natural product not only decreased the efficacious concentration but due to multiple mechanisms of action, substantially decreases the likelihood of bacterial resistance (Ahn *et al*., 2007; Jenkins and Cooper, 2012; Mossa *et al*., 2004; Shami *et al*., 2013).

Mycobacterium abscessus is a clinically relevant multidrug resistant organism which has been shown to be intrinsically resistant to many first line antibiotics (Nessar *et al*., 2012). To determine if hop extract contained compounds which potentiated the activity of antibiotics the ability of the extract alone and in combination with antibiotics to target clinical isolates of *M. abscessus* was investigated. In recent years this fast growing non-tuberculosis mycobacterium and opportunistic pathogen has emerged as a major burden to patients with cystic fibrosis (Cullen *et al*., 2000). Variants have developed high level resistance to commonly prescribed antibiotics which has resulted in the pathogen being described as an emerging antibiotic nightmare (Nessar *et al*., 2012).

To investigate the potential synergistic activity between antibiotics and hops the previously isolated hop compounds; (humulone; lupulone and xanthohumol) were assayed against 8 clinical isolated of *M. abscessus*. Surprisingly, no synergistic effect was observed.

As previously stated, antibacterial activity of hops is often as a result of a combination of separate compounds. Therefore, the interaction between the whole crude extract and antibiotics against clinical isolates of *M. abscessus* was characterised. To determine the nature of these interactions three methods were employed.

Using the previously developed zone of inhibition assay, a positive coaction between the Citra crude hop extract and the following antibiotics; imipenem and ciprofloxacin, was identified. In no case was antagonism observed. To confirm these results, an agar incorporation assay was used. For this, a sub MIC concentration of hop extract was incorporated into the agar and an E-test strip of the antibiotic was placed on the surface of an *M. abscessus* inoculated agar plate. As there is no standard regimen for identifying a synergistic effect using this method, a threefold decrease in the MIC when in combination with a sub-inhibitory concentration of hop extract indicated a positive co action.

This method gave a quantifiable result as decreases in the MIC value could be measured and thus were less subjective than relying on visualising the shape of the interaction of zones of inhibition as in the previous method. While the two methods agreed with regards the co-action between the hop extract and imipenem and ciprofloxacin, no change in the MIC against any organism when the hop extract was assayed alongside pyrazinamide, rifampicin and ethambutol.

As no co-activity had been observed from pyrazinamide, rifampicin and ethambutol, the antibiotics ciprofloxacin and imipenem were assayed using the checkerboard method. The checkerboard method is recognised as the old standard in characterising antibiotic synergy and allows the classification of the interaction between the antibiotics: imipenem and ciprofloxacin and the Citra crude hop extracts into the following groups; synergistic, additive, indifferent or antagonistic (Hall *et al*., 1983a; Orhan *et al*., 2005b).

For imipenem a synergistic interaction with the Citra hop extract against strain 10332; an additive effect against strain 9568 and an indifferent effect against 11490 was observed. These results differed from those seen with the other methods which reported coactivity in each case. The results obtained for the interaction between ciprofloxacin and hop extract also differed from the earlier assays in that an additive effect against strain 10332 was observed. Whilst against strains 9568 and 11490 the interaction showed an indifferent effect.

Taken together these results suggest that the hop extract contains compounds which are capable of inhibiting the growth of mycobacteria and of enhancing the activity of the cell wall damaging antibiotic imipenem.

The activity against antibiotic resistant strains suggested that the hop extract contained compounds whose antimicrobial mode or modes of action differed from that of the antibiotics assayed in this study. It is known that certain hop constituents such as humulone can degrade the cell wall of Gram positive organisms (Cornelison, 2008) and therefore it was hypothesised that this compound could act synergistically with other cell wall damaging antibiotics such as imipenem.

To determine if the hop extract damaged the architecture of the cell wall of the Mycobacteria, selected bacteria were exposed to hop extract alone, and in combination with antibiotics and examined the effect on the structure of *M. abscessus* 10332 by using scanning electron microscopy.

Exposure of *M. abscessus* 10322 to a sub-inhibitory concentration of hop extract changed the topography of the cell wall from a rough to a smooth surface. It also caused the cells to pit. One of the mechanisms of antimicrobial action of hop extracts is thought to be due to changes in the permeability of the cell wall and membrane (Simpson and Smith, 1992; Teuber and Schmalreck, 1973) which leads to the subsequent inhibition of respiration and of DNA and RNA synthesis (Behr and Vogel, 2009). These images support the idea that exposure to hop derived compounds can alter cell wall structure.

Exposure of the test bacteria to imipenem and ciprofloxacin also affected the cell surface in a similar manner. In the case of ciprofloxacin this result was surprising in that this antibiotic does not target the cell wall but rather interferes with the activity of DNA gyrase which in turn inhibits the uncoiling of DNA gene expression. Indeed following exposure to the ciprofloxacin the individual bacterial cells were noticeably larger (approx. 3-4 μm); as was also the case with the imipenem exposed cells.

The exposure of the bacteria to both hop extract and ciprofloxacin, at the synergistic concentration identified in the checkerboard assay, appeared to cause no additional morphological changes to those seen when the bacteria were treated with ciprofloxacin alone. Interestingly, a marked reduction in the number of intact bacterial cells suggested that the combination of treatment had breached the cell wall.

A similar effect was also observed between the hop extract and imipenem. This destructive interaction is perhaps not surprising given that both agents are known to act on the cell wall of the bacterium (Vaughan *et al*., 2005).

6.1 Conclusions

The overall aim of this research was to identify hop derived compounds which had the potential to be developed as drugs capable of treating antibiotic resistant strains of mycobacteria. Using *M. smegmatis*, a fast growing simulant for *M. tuberculosis*, as a screening tool it was found that hop varieties differed markedly in their ability to inhibit bacterial growth. Subsequent studies showed that the most active hop variant, Citra, was also able to inhibit the growth of clinical isolates of *M. abscessus*, a known pathogen of humans. Of particular interest in the context of multi-drug resistant mycobacteria was the fact that the Citra hop extract was equally effective against sensitive and drug resistant isolates of *M. abscessus* suggesting that its mode of action differed from that of the ciprofloxacin and imipenem.

Whilst the study identified the presence of previously described antibacterial compounds with activity against mycobacteria it failed to identify any new antimicrobial compounds. This could be due to the fact that there are no new compounds to find or that the methods lacked the required sensitivity and specificity to detect their presence.

Combined, the developed bioassay guided methods were able to detect the presence of known antimicrobial compounds; whilst the detection of these compounds was limited by the concentration of the compound and the concentration of bacteria; the methodology was found to be successful in identifying differences between the hop variants.

Whilst novel pure compounds were found to have no synergistic activity with antibiotics, synergistic activity of the crude hop extract with imipenem against *M. abscessus* raises the possibility that once identified; mixtures of pure compounds could be used to potentiate the activity of antibiotics against drug resistant isolates of mycobacterium.

6.2 Future work

This research highlights the possibility of the hop plant to successfully be used as an antibacterial agent and as such raises several possibilities for further research.

Firstly, the hop variants should be analysed against more clinical isolates of drug resistant *M. abscessus* and *M. tuberculosis*. Tubercculosis is going to become a major healthcare concern worldwide over the next decade and as such the development of novel antibiotics with differing modes of action or the ability to potentiate the activity of redundant antibiotics should be of prime concern.

It would be interesting to separate as many compounds as possible from the hop extract and testing different combinations of these to try and identify synergistic combinations. By using HPLC or associated techniques to identify individual components it may be the case that a new class of antimicrobials could be identified which may not be specific to hops but may be wide ranging in nature.

Of course *M. tuberculosis* is not the only drug resistant organism, species such as MRSA and *C. difficle* are a common hospital acquired infection and it would be interesting to repeat the synergy assays with antibiotics such as methicillin or metronidazole to identify other synergistic combinations.

Following the results of this research, it would be interesting to examine the potentiation of intravenous antibiotics used for the treatment of *M. abscessus* infection by hop extracts or hop compounds.

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8.0 Appendices

Attached CD with Scanning Electron microscopy images