Targeting Natural Products to counter the challenge of MRSA

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Abstract

As the global threat of antibiotic resistance grows the approval rate of new antibiotics falls. Pharma companies are dropping their antibiotic research programmes at the time when new antibiotics are needed the most due to antibiotic resistance. Paradoxically antibiotic resistance is one of the many reasons that the discovery of new antibiotics is not lucrative enough for it to be a financially viable pursuit. Promising high-throughput technologies have been unsuccessfully employed to streamline drug discovery due to the lack of novel chemicals within libraries. Natural products have contributed massively to drug discovery in the past although its contribution to the declining number of antibiotics discovered recently has also diminished. To revive the drug discovery pipeline invasive weeds were targeted as a potential source of novel chemical compounds. Three species; *Fallopia japonica*, *Impatiens glandulifera* and *Rhododendron ponticum* were collected. Each species was extracted and fractioned to discover any potential antimicrobial compounds. A particularly active compound 2-methoxy-1,4naphthoquinone (MNQ) was discovered. This compound was found to have a broad range of activity against clinically relevant bacteria. The methoxy group was found to be crucial for the potent antimicrobial activity of this compound. It was also a potent inhibitor of *Schistosoma mansoni*. Cytotoxicity of this compound was found to be a potential issue with mixed results. High throughput metabolomic methodologies were developed to understand the mechanism by which MNQ inhibits the growth of MRSA. The metabolomic effect of MNQ was compared to other antibiotics, it was found that MNQ had a distinctive metabolomic effect. This unique effect was further investigated with in-depth metabolic pathway analysis using statistical methods twinned with KEGG metabolomics pathway database. Once a tentative mode of action was identified transmission electron microscopy and specific antimicrobial assays were used to support this theory.

This research project has discovered a promising antimicrobial and anthelminthic compound and the developed metabolomic methodology yielded a large amount of useful data regarding the mode of action of MNQ and other antibiotics.

Contents

Chapter 1 - Natural Products; Invasive weed species as a potential source of antimicrobials

1.1 – Introduction – Natural Products

1.1.1 - Chapter Aims

Natural products have been used to treat disease for thousands of years yet traditional approaches to drug discovery have been declining with the modernisation of the drug discovery pipeline. With an emphasis on synthetic methods and high throughput screening in recent research there has been a decline in the number of novel drugs being discovered. The effectiveness of drug discovery is critical especially in the face of antibiotic resistance. The contribution of natural products and alternative methods to drug discovery will be discussed as well as the issue of antibiotic resistance. Invasive weeds are a problematic yet could potentially be a plentiful source of novel antimicrobial compounds. Three particularly plentiful and aggressive invasive weed species were chosen for analysis. A bioactivity led purification process will be carried out to identify compounds which have the potential to treat MRSA infections.

1.1.2 - History of natural products

Humans have always been dependant on nature to cater for their basic needs such as food and shelter but also medicines. Initially medicines were in the form of crude treatments such as tinctures, teas, poultices, powders and other herbal formulations. There is evidence that Neanderthals living 60,000 years ago used plants from the Alcea genus to treat infections (Stockwell, 1988), this plant genus is widely used in ethnomedicine and there are current research efforts attempting to identify active compounds within this genus (Seyyednejad et al., 2010; Ghasemi and Atakishiyeva, 2016; Azab, 2017). More recent approaches involved the isolation of a single active compound (Balunas and Kinghorn, 2005). The first active compound to be isolated in this way was

morphine from opium by Friedrich Setürner in 1804 (Schmitz, 1885). Morphine was used as a potent analgesic at this time and became the first of the opioid drug class. Current semi-synthetic opioid drugs, such as oxycodone, are all derived from the initial research on *Papaver somniferum* commonly known as the breadseed or opium poppy. Much natural product research at this time was based on plants linked to known medicinal properties and this led to discoveries such as aspirin, quinine, and pilocarpine (Butler, 2004). The analgesic aspirin is synonymous with the willow tree because in the 18th century willow extracts were used to relieve pain. The active components of the willow tree were identified as salicilin and salicylic acid (Mahdi, Mahdi and Bowen, 2006). The salicylic acid synthetic derivative acetylsalicylic acid (aspirin) is a Nonsteroidal anti-inflammatory drug (NSAID) which is a drug class which reduces pain, fever and inflammation. It also reported to have a range of other beneficial health benefits being said to have, antiproliferative, anticancer properties (Ridker et al., 1997; Mahdi, Mahdi and Bowen, 2006). Pilocarpine, a treatment for intraocular pressure and xerostomia, is another example of an effective natural product isolated from Pilocarpus jaborandi in 1874 which is still in use today (Sneader, 2005). Quinine has origins as far back as the 1600s when the bark of the cinchona tree was used to treat malaria. Later the active component was identified as quinine and the use of which to treat malaria marked the first successful treatment of an infectious disease. It was replaced as the frontline treatment for malaria in 2006 yet it remains as an alternative if artemisinin fails and is still the favoured antimalarial drug in pregnant patients (Achan et al., 2011). The replacement frontline treatment for malaria is now artemisinin which is a natural product derived from *Artemisia annua*. It was discovered in 1972 although the medicinal properties of the herb have been known for over 2000 years. The herb immersed in 2 L of water was prescribed between 284-346 CE and the handbook remains (Tu, 2011). From these examples isolated natural products alone or with some synthetic variation have made major contributions to medicine even before the causes of diseases were understood.

It was not until the late 19th century that microorganisms were known to causes disease, despite microorganisms being discovered by Robert Hooke and Antoni Van Leeuwenhoek in 1665.

The link between microorganisms and disease was not made until Louis Pasteur between 1860-1864. With the definition of Koch's postulates (basic criteria for demonstrating a disease was caused by an organism) the discovery of the causative bacterium enabled drugs to be disease/bacteria specific. However, up to this point toxic substances were used to treat such diseases where the "drugs" being used were just as poisonous to human as to the bacteria causing the disease. For example, mercury was used to treat syphilis. Using the insights gained from discovery of microorganisms Paul Ehrlich developed approaches to detect specific substances which would kill microbes without harming human cells using dyes. He also coined the term 'chemotherapy' and 'magic bullet', which is the best description for modern day medicines. The first successful 'magic bullet' was arsphenamine which was able to treat syphilis, sold under the trade name salvarsan in 1910. This was synthesised, opposed to the traditional method of searching for the active compound from plant extracts (Ehrlich and Hata, 1910; Williams, 2009). This systematic screening approach developed by Ehrlich became the cornerstone for drug research in pharmaceutical industry leading to the identification of thousands of drugs, not only antimicrobials.

Around the same time in 1928 was possibly the most famous discovery made in the antibiotic era, the discovery of penicillin. Its discovery was serendipitous and contrasted with the methodical systematic approaches laid down by Ehrlich a few years earlier. Its discovery was unplanned, untargeted and it was a purely natural origin rather than being (semi) synthesised.

The discovery of a potent antimicrobial compound was desperately needed at the time of World War II and led to the discovery of many more antimicrobial agents. Given the example of penicillin there is no absolute correct way to carry out drug discovery. It should use of historical knowledge and the technological advances available at the time, while also taking advantage of unforeseen opportunities which may arise at the time.

Research into natural products has diminished in the past two decades due to a lack of interest by major pharmaceutical companies. Caused by the laborious nature of natural product

discovery and its incompatibility with high throughput screening (HTS) directed at molecular targets (Harvey, Edrada-Ebel and Quinn, 2015). HTS have many benefits; they provide large amounts of high-quality data in relation to standard techniques, it is high throughput (as the name suggests) therefore large volumes of samples can be tested in small duration of time, increased cost-effectiveness. The increase popularity of HTS has also led to the development of underlying technologies such as automation, miniaturisation, data capture, data analysis, and new bioassay formats. However, the results of HTS large chemical libraries have been disappointing in practice (these libraries containing a range of compounds from many diverse sources) and so natural products have emerged with a significantly higher hit rate compared to fully synthetic and combinatorial libraries (Sukuru et al., 2009). Furthermore, it has been shown that 83% of core ring scaffolds present in natural products are not represented in commercially available screening libraries leading to fewer drug leads (Hert et al., 2009). This stated HTS should be considered as a tool to be used alongside other drug discovery methods and should not be the only means for drug discovery (Macarron et al., 2011).

Even with the recent neglect of natural products as a source of drugs, plants have been the single most productive source of leads, particularly as anti-cancer agents and anti-infectives (Harvey, 2008). Using the most recent Newman and Cragg paper results tracking the number of new antimicrobial agents approved between 1981 and 2002 and then in 4-year intervals up to 2014 (Figure 1). There are three main trends in approved antimicrobials between 2002-2014: i) the new biological compound, ii) the sharp rise in the number of vaccines iii) and the consistent performance of natural and synthetics. (Newman, Cragg and Snader, 2003; Newman and Cragg, 2007, 2012, 2016).

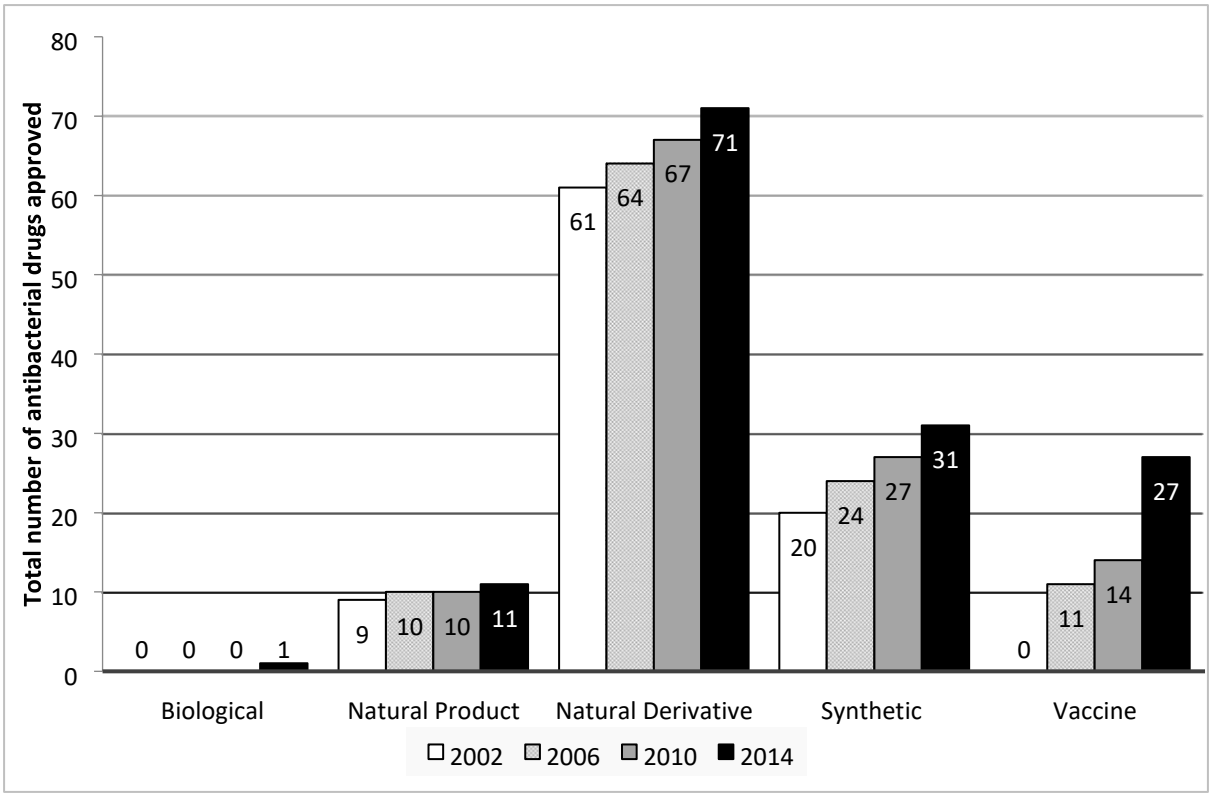


Figure 1: Total number of antibacterial drugs approved in different ways from 01/01/1981 to Recent Years (data adapted from Newman, Cragg and Snader, 2003; Newman and Cragg, 2007, 2012, 2016)

Biologicals as defined by Newman & Cragg are "usually large (>50 residues) peptides or proteins either isolated from an organism/cell line or produced by biotechnological means in a surrogate". The example given is Raxibacumab; a recombinant human monoclonal antibody developed against inhalation of *Bacillus anthracis*. A part of *B. anthracis* pathologic process it produces the anthrax toxin, which is composed of two binary combinations, each containing a protective antigen (PA) responsible for binding, which can combine with either an oedema factor or a lethal factor to form the respective toxin. Raxibacumab binds the PA preventing the formation of either toxin. Raxibacumab therefore does not have any antibacterial activity but works by preventing the internalisation of toxins and the progression of infection. As such it should be used alongside other antibiotic treatments (Kummerfeldt, 2014). The treatment of bacteria with non-lethal drugs such as raxibacumab, quorum sensing inhibitors which reduce virulence of the

pathogen could potentially have a lower selective pressure compared to targeting critical molecular targets that result in death and lead to a slower onset of resistance (Hentzer and Givskov, 2003; Rasmussen and Givskov, 2006).

Similarly, there has been a sudden increase in the number of vaccines between 2010-2014, almost doubling from 14 to 27. Most of the new vaccines treat meningococcal vaccines, but also include most notably the first vaccine for typhoid fever (Newman and Cragg, 2016).

One new natural compound, fidaxomicin was the first in a new class of narrow spectrum macrocyclic antibiotics used to treat *Clostridium difficile*. The original discovery of this compound from *Actinoplanes deccanensis* ATCC 21983 dates to 1975, with the structure elucidated in 1988, approval for use as a drug in 2011 and the mode of action revealed in 2012. This compound inhibits RNA polymerase, but unlike other antibiotics such as rifampicin, fidaxomicin binds to and prevents the movement of the switch regions of RNA polymerase (Parenti, Pagani and Beretta, 1976; Cavalleri et al., 1988; Artsimovitch, Seddon and Sears, 2012). Other new natural derivatives and synthetic compounds have also been approved. For example, natural derivatives have included ceftaroline (cephalosporin), cetolozane (cephalosporin with tazobactam β-lactams inhibitor), dalvabancin (glycopeptide), and oritavacin (glycopeptide). There were no new classes of antibacterial compounds. One synthetic drug of interest, bedaquiline is a quinolone-based antibacterial. The first tuberculosis drug in 40 years with a unique activity which inhibits proton pumps of mycobacterial ATP synthetase enabling efficacy against multidrug resistant TB (Matteelli et al., 2010).

The pharmaceutical industry has been deemphasising its natural products since before 2002 even though they also provide a platform for synthetic derivatisation to take place and enhance the pharmacophoric spaces available in HTS libraries. In 2013 there were 14 classes of antibiotics, of these 4 had a synthetic origin and the remaining 10 were all from natural sources (Lewis, 2013). Therefore, due to the vast diversity of natural products and wide possible applications means that their isolation and characterisations for medicinal purposes remains of importance today.

1.1.3 - Antimicrobial Resistance

Antimicrobial efficacy is highlighted following their introduction; circa 1930 pre-antibiotic hospitals were populated with patients suffering from pneumonia, meningitis, bacteraemia, typhoid fever, endocarditis, mastoiditis, syphilis, tuberculosis, and rheumatic fever. While in the post-antibiotic era wards of the 1980s were filled with patients with cancer, heart disease, or complications from diabetes or hypertension (McDermott and Rogers, 1982; Cohen, 1992). Therefore, antibiotic resistance will have a profound effect and was forewarned when penicillin was first used to treat disease:

> *"The time may come when penicillin can be bought by anyone in the shops. Then there is the danger that the ignorant man may easily underdose himself and by exposing his microbes to non-lethal quantities of the drug make them resistant. Here is a hypothetical illustration. Mr. X. has a sore throat. He buys some penicillin and gives himself, not enough to kill the streptococci but enough to educate them to resist penicillin. He then infects his wife. Mrs. X gets pneumonia and is treated with penicillin. As the streptococci are now resistant to penicillin the treatment fails. Mrs. X dies. Who is primarily responsible for Mrs. X's death? Why Mr. X whose negligent use of penicillin changed the nature of the microbe. Moral: If you use penicillin, use enough"*
>
> *(Fleming, Chain and Florey, 1945).*

Even with this forewarning we have been unable to prevent the global propagation of antibiotic resistance, due to a range of factors. The inappropriate prescription of antibiotics is a completely avoidable contributing factor to antimicrobial resistance. Antibiotics are not recommended for coughs, colds, and viral sore throats however, half of these conditions were incorrectly provided with an antibiotic prescription in the UK between 1995-2011 (Hawker et al.,

2014). Antibiotics are provided in these situations in fear of a suppurative complication or to ease the severity or duration of the illness, but these concerns are not supported by data. For example, it has been shown that the risk of serious complications following a sore throat, otitis media, and upper respiratory tract infections is low and the number of antibiotics needed to prevent one serious complication is over 4,000 prescriptions (Petersen et al., 2007). Furthermore, there is little evidence that antibiotics significantly reduce duration or severity of a sore throat (Little et al., 2013) and the inappropriate use of antibiotics has no clear benefit to the patient and will only act to further propagate antibiotic resistance. This has been evidenced by randomised double-blind placebo-controlled study for the macrolides, azithromycin and clarithromycin, which showed a significant increase in the proportion of macrolide-resistant Streptococci in healthy patients (Malhotra-Kumar et al., 2007). A future consequence of this increased carriage of resistant bacteria may include the spread of bacteria which could also cause a resistant infection to develop. The inappropriate prescription of antibiotics is widespread, but the variations can vary significantly in relatively small area. There is a marked variation in antibiotic prescriptions between clinical commissioning groups in England, with, 8.4% of patients provided antibiotics in Newcastle West compared to only 4% in Camden with a national average of 6.4% (Shallcross and Davies, 2014).

When compared to rest of the European Union, UK consumption was slightly lower with 18.8 defined daily doses per 1000 habitants (DID) compared to the EU average of 14.9 DID. France reported the highest antibiotic consumption (32.2 DID) and the Netherlands with the lowest (10.0 DID) (Goossens et al., 2005). Most countries are now taking further measures against antibiotic resistant bacteria such as isolating MRSA and multi-drug resistant (MDR) Staphylococci colonised patients in specialised and have been key in reducing MRSA (Cooper et al., 2004). Similar approaches are taking for other serious drug resistant bacterial infections such as *C. difficile* (Hsu et al., 2010), *Klebsiella pneumoniae*, *E. coli*, Enterobacter cloacae (Gupta et al., 2011) infections. However, no matter how well individual countries perform in prevention, prescriptions and isolation, resistant

bacteria do not respect international boundaries with active infections and colonising drug-resistant bacteria being spread throughout the globe by population movement.

Therefore, this is a global issue which needs coordinated antibiotic controls between all countries however, this can be challenging due to limited coordinated action at political, governmental and international levels (Laxminarayan et al., 2013). Self-medication with antibiotics is common in many parts of the world (Väänänen, Pietilä and Airaksinen, 2006; Plachouras et al., 2010; Morgan et al., 2011). This is unacceptable when the repercussions of not completing a course of antibiotics have been known since they were first discovered.

Another source of antibiotic resistance is the millions of kilograms of antibiotics used for agricultural. Whereby, approximately 80% of antibiotics sold in the US are used animal agriculture and 60% of which are medically important for human disease. These antibiotics are administered through animal feed to marginally improve growth rates and prevent infection and are predicted according to the FDA to increase overall in the next 15 years (Martin, Thottathil and Newman, 2015). Tracking the recent total use antibiotics over the past few years from 2009-2016 based on US Food and Drug Administration (FDA) figures submitted over consecutive years (Figure 2) there has been steady increase in the antibiotic use in the agricultural industry up to 2015, with a significant drop in 2016.

The impact of non-action or lack of action over the misuse of antibiotics could result in a return to pre-antibiotics conditions in hospitals. Where the contraction of a relatively harmless bacterial infection by modern standards this could result in a death sentence. Although currently unimaginable, if resistance spreads globally current antibiotics will become obsolete. If effective controls are not placed on antibiotic use, the fate of future antibiotic efficacy will depend on new antibiotics being developed.

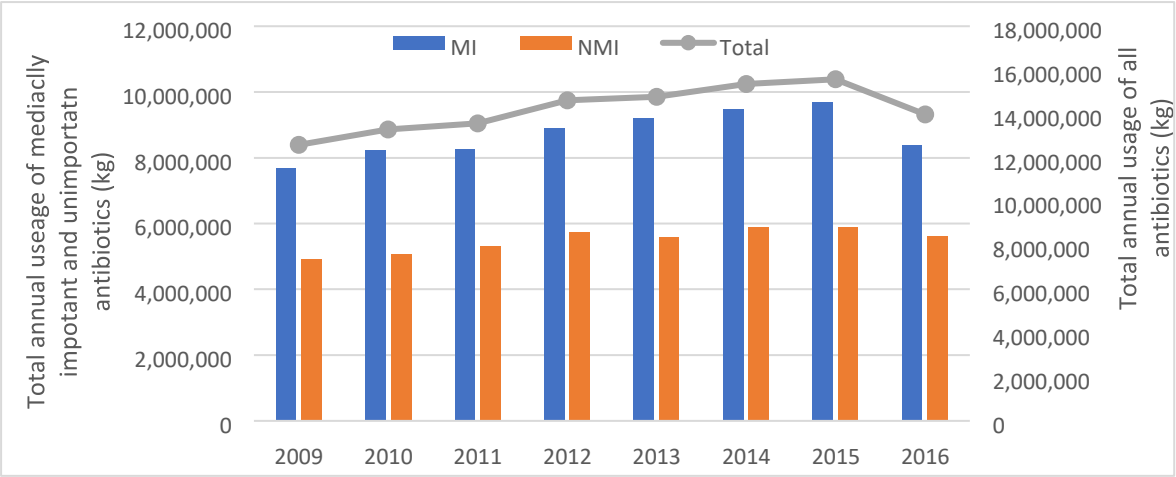


Figure 2: Bar chart showing percentage of medically important and unimportant antibiotics used for agricultural use over time and line showing the total mass of antibiotics used in the agricultural industry annually (Cvm, 2017)

The rise in antimicrobial resistance in bacterial is compounded by a decrease in the number of new classes of antibiotics being developed, with only two new classes: fidaxomicin and bedaquiline introduced in 2011 and 2012, respectively. This slight upward trend in novel antimicrobial discovery, although encouraging, is unlikely to solve the problems of resistance. Figure 3 is a timeline of antibiotic discovery from 1936 to 2012, during this time 14 classes antibiotics were discovered. Five of which; tetracyclines, streptogramins, lipopeptides, fidaxomicin, and bedaquiline (diarylquinolines class), resistance was detected before they were approved for use as an antimicrobial agent. A combination of a slow discovery rate and lengthy approval processes the antibiotic discovery pipeline has run dry. The market is crowded with many versions of the same class, neglecting to risk the potential benefits of discovering new modes of action. The development of new antibiotics is high risk and expense, the development process can be separated into 5 sections: Lead compound identification, Lead compound optimisation, preclinical testing, phase I clinical trials and phase II clinical trials. The associated cost of each stage can vary massively; lead compound identification can range for € 100,000 to more than € 1 million and can take between 6 months and 4 years. Optimisation is far more costly, estimated to cost between € 1-5 million and expected to take between 6 month and 4 years. Preclinical testing would be expected to take one to two years and cost € 1-5 million but can be as high as € 10 million. The cost of phase I clinical

trials ranges from less than € 1 million to as much as € 15 million, taking around 1 year to complete. Phase II costs are likely to be higher ranging from less than € 1 million to over € 20 million (Årdal et al., 2018). Additionally, obtaining regulatory approval is often a significant obstacle for drugs attempting to make it to market (Gould and Bal, 2013; Ventola, 2015).

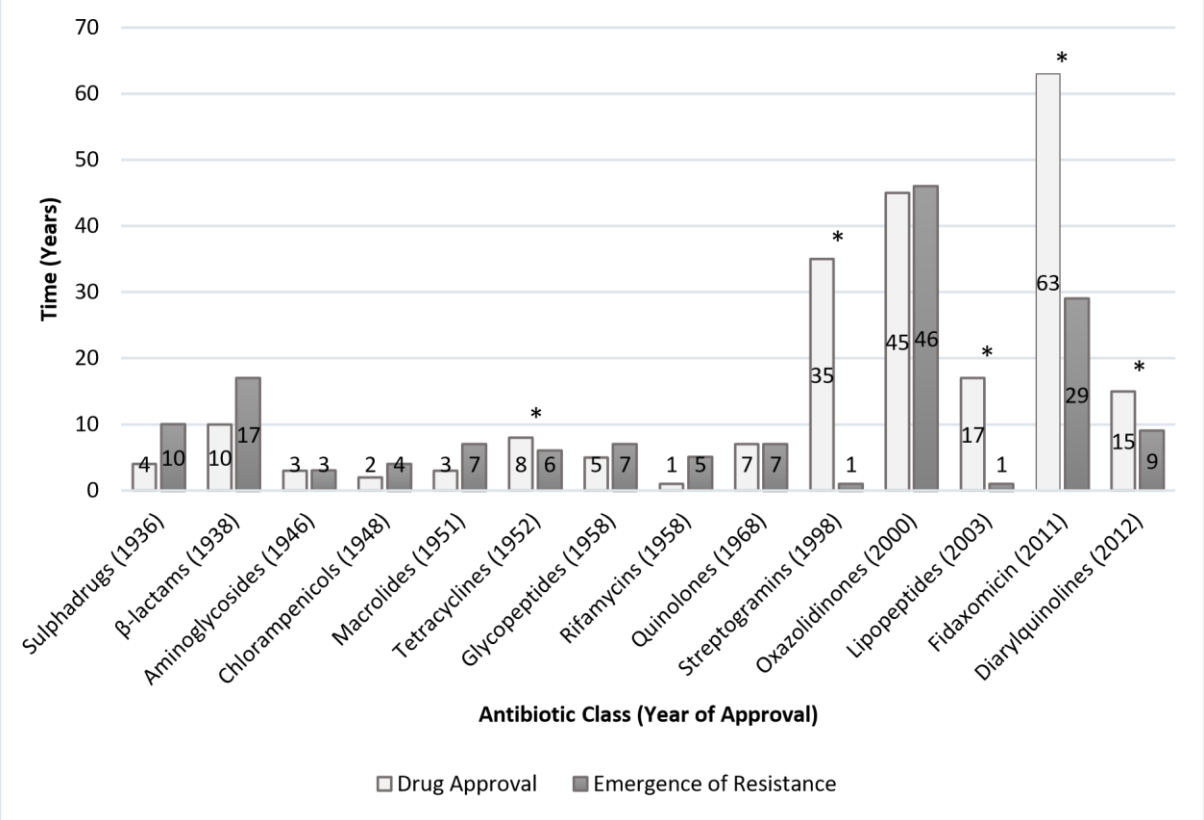


*Figure 3: Column cluster graph showing the number of years taken to approve antimicrobial class for use in humans from the first point of discovery compared to the time taken for resistance to develop. With the first approved antimicrobials on the left to the newest approved on the right. *- indicate instances where resistance developed before approval. (Graph made using data from Lewis, 2013).*

The nature of antibiotics is that they are "singe use", only required when there is an infection and once the course is complete, they are not needed. This reduces their profitability compared to other drugs for chronic conditions which are required to be taken over a long period of time or drugs can be taken sporadically as and when needed. The single use nature of antibiotics, the significant expenditure required to take a new drug to market, the inevitability of resistance, and a global lack initiative to prevent misuse of antibiotics are all contributors which have led to big

pharmaceutical companies reducing their interest in antibiotic discovery. This is especially true for early phase interest which are vital for discovering novel compounds yet are extremely unlikely to make it to market. Of tens of thousands of drug candidates which have found to be active can be whittled down to just one approved medicine afters years of investment.

1.1.4 - Invasive Weeds

Invasive non-native species (INNS) are organisms that have been introduced into a new environment once established are able to outcompete the native flora/fauna. These species have generally been introduced by humans into areas outside their natural range and can have drastic effects on the ecosystem. These INNS can also incur economic cost due to control and eradication, structural damage to infrastructure and loss of crop production with impact to the UK estimated to be between £200-300 million (Williamson, 2002). However, it can be proposed that INNS may provide an alternative source in the discovery of new pharmaceuticals. (Williams et al., 2010).

When INNS are introduced into a new environment, they may be eaten by native insects which can result in a reduce their likelihood of survival (Singer, Thomas and Parmesan, 1993). The INNS could be phytochemically like the native species herbivores often switch to novel hosts compared to their traditional host (Strong, Lawton and Southwood, 1984). Conversely, if the INNS have a different phytochemical make up this could deter insect feeding; referred to as the enemy release hypothesis (ERH). Reduced feeding by herbivores and other natural enemies can result in a rapid increase in distribution and abundance (Keane and Crawley, 2002). However, it is not only herbivory which could influence the success of the INNS but the outcome of interaction with soil microbiological communities (Kourtev, Ehrenfeld and Häggblom, 2002) and other plant species through allelopathy (Callaway and Ridenour, 2004). For example, 57.5% of phytochemicals from invasive plants have shown activity against more than one type of microorganism and several had general biocidal effects (Cappuccino and Arnason, 2006). The increases in novel phytochemistry and bioactivity were linked to invasive plants which benefit from the ERH. These plants can produce a

plethora of novel compounds in their new environment as the herbivorous, bacterial and allelopathic pressures are avoided and may provide a continually evolving source of novel antimicrobial compounds. For this study three invasive weeds were used and explained in detail below. There were chosen since they are currently widespread throughout the UK as a result would be a plentiful source of biomass for extraction of bioactive compounds. They are also problematic therefore their removal in large amounts will be beneficial to the environment.

1.1.4.1 – *Impatiens glandulifera*

Impatiens glandulifera or Himalayan balsam is a tall annual herb with explosive seed heads native to west and central Himalayas. It has pink-purple flowers, fleshy stem and finely serrated leaves. It was first introduced in the early 19th century as an ornamental and nectar producing plant and later recoded in the growing wild in 1885 becoming widespread in the UK and throughout the norther hemisphere (Beerling and Perrins, 1993). It is currently widespread and common across the UK, primarily on riverbanks and in other damp areas (NNSS, 2018). Due to the height of *I. glandulifera* it casts shade reducing the germination and establishment of other species beneath its canopy, resulting in a 25% reduction in the richness of diversity (Hulme and Bremner, 2006). *I. glandulifera* has an estimated annual cost of £1,000,000 on the UK economy (Williams et al., 2010). *I. glandulifera* is native to high altitude meadows and fringe woodland and have been found to grow taller in non-native sites. The invasive plants within the UK show clear signs of the EHS, where generalist natural enemies are not inflicting as much damage as the specialists within the native environment (Tanner et al., 2014).

1.1.4.2 – *Fallopia japonica*

Fallopia japonica or Japanese knotweed is a tall herbaceous perennial with bamboo-like stem which grows into dense thickets. It was introduced into the UK from Japan as an ornamental garden plant in the 19th century and has now become widespread in a range of habitats particularly on roadsides, riverbanks and derelict land displacing native flora. It causes serious structural damage

to buildings and infrastructure due to its rapid growth rate and ability to push through tarmac, concrete and drains. The presence of Japanese knotweed can devalue housing, damage roads and rail networks, displace of local flora to impact on leisure and tourism. The costs linked to its presence and removal can be considerable, estimated to be £165, 609,000 within the UK in 2010 (Williams et al., 2010).

1.1.4.3 – *Rhododendron ponticum*

Rhododendron ponticum is a large evergreen shrub with attractive purple to pink flowers, leathery leaves and solid stems. *R. ponticum* is extensively naturalised throughout the British Isles with very few natural enemies facilitating its widespread growth (Judd and Rotherham, 1992) and adverse impact on forestry (Cross, 1981). It is unclear from which area *R. ponticum* originated prior to introduction into the UK, as well as the extent of introgression which has altered British species (Cross, 1975). The total cost of *R. ponticum* to the UK in 2011, which includes public, charities, private and forestry related cost, was calculated to be £670,924 (Dehnen-Schmutz, Perrings and Williamson, 2004) yet the annual cost has also been estimated to be £8,621,000 on the UK economy by Williams et al., 2010.

1.1.5 – Liquid Chromatography

1.1.5.1 – Overview

The term chromatography was first coined in 1906, when coloured constituents of leaves were separated using a column of calcium, alumina, and sucrose. The International Union of Pure and Applied Chemistry's (IUPAC) definition of chromatography is:

> *"Chromatography is a physical method of separation in which the components to be separated are distributed between two phases, one of which is stationary (stationary phase), while the other (the mobile phase) moves in a definite direction."*

The two principle types of chromatography are gas chromatography and liquid chromatography. Gas chromatography separates gaseous substances based on adsorption on or partitioning in a stationary phase. Liquid chromatography includes a range of techniques based on molecular size, charge, adsorption or partitioning from a liquid phase. All chromatography is based upon the establishment of an equilibrium between a stationary phase and a mobile phase. The distribution of this equilibrium is described by the distribution constant (Equation 1).

$$Kc = \frac{[X]s}{[X]m}$$

Equation 1: The distribution constant (Kc). Where [X]s is the concentration of component X in the stationary phase at equilibrium and [X]m is the concentration in the mobile phase.

This is dependent upon temperature, type of compound, and the stationary and mobile phases. A larger Kc value will result in a stronger affinity to the stationary phase moving slowly along the column and those with lower values have a stronger affinity to the mobile phase and move quickly along the column. (Christian, 2003). Pre-1970s liquid chromatography was based on the use of large columns with large particles under gravity feed and fractions collected manually for measurement in a spectrophotometer. With the advancements of increased flow pressure and small silanized silica particles resulting in improved peak dispersion becoming known as high powered liquid chromatography (HPLC). In addition to conventional HPLC columns, Ultra High-Pressure Liquid Chromatography (UHPLC) is also an option, which as the name suggests, operates at a higher pressure then HPLC. This higher pressure results in more rapid chromatography (typically <10 min) with better resolution and efficiency due to smaller particle size (<3 µm). The conventional maximum operating pressure of HPLC is around 400 bar (6,000 psi), whereas UHPLC operates at pressures as high as 1300 bar (19,000 psi) (Fekete et al., 2014). As well as smaller particle sizes Superficially Porous Particles (SPP) are an alternative to smaller particles sizes, comprised of a solid core coated with porous silica layer, offering increased efficiency and speed comparable to HPLC. The most important measure of successful chromatography is the resolution of the peaks, this is

vital whether chromatography is being used for purification of analysis. In order to obtain good resolution three key factors, retention, selectivity and efficiency need to be considered. There is a visual summary of these in Figure 4.

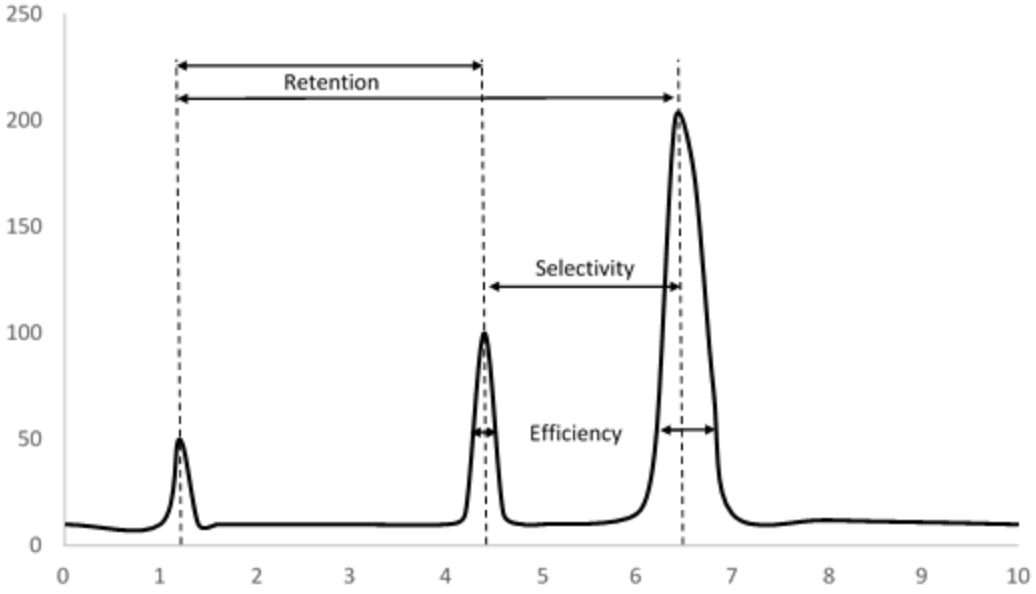


Figure 4: Typical chromatography highlighting the major measurable factors of chromatography.

Retention is the time an analyte spends on the column compared to unretained compounds which pass through the column unaffected by the stationary phase. Analytes are retained on a column based in their affinity with the stationary/mobile phase. There are many different stationary phases available which retain analytes based on their polarity in the case of normal and reversed phase but also charge in the case of ion-exchange chromatography and size-exclusion chromatography. In order to obtain desired retention, the correct stationary phase is the most important first step. Once a relevant column is selected then mobile phase selection and composition need to be considered carefully. Each type of column is only suitable with certain solvents and the most effective way to alter retention is to change mobile phase composition.

The retention of peaks needs to be controlled so analytes are clearly defined from one another, this is selectivity. The selectivity factor is the ability of a chromatographic system to distinguish between different components. This can be visualized as the distance between two peak

and measured as a ratio of the retention time of two peaks. Selectivity factor is calculated by dividing the latest retaining peak by the earlier retaining peak, the larger the selectivity factor the better the separation between peaks. The space between peaks is dependent on the retention, if too strong mobile phase is used peaks will elute quickly and there will be poor separation between the peak causing them to coelute. The pH of the mobile phase effects the retention and particularly the selectivity of certain analytes which have ionisable groups. The pH has a significant effect on the ionization state of the analyte, for successful chromatography. For example, when pH = pKa the analyte can be in both ionized and neutral states, these two forms will have different retention properties and lead to broad, tailing or splitting peaks. If an acidic analyte is present within acidic mobile phase which has a pH below the pKa there will be sufficient protons to maintain a neutral analyte which will retain for longer on the column. If this acidic analyte is in a basic environment the analyte dissociates into its ionized conjugate base, resulting in less retention. However, if an analyte is particularly polar or ionic then ion pairing agents can be added to the mobile phase which will bind to ionized molecules increasing their retention (Berg et al., 2009).

Efficiency is the ability to obtain the correct peak shape, which is as thin as possible. This is vital for good chromatography and the efficiency of a column is measured using a plate count equation (Equation 2)

$$N = 5.54\ (tr/\mathrm{W}_{0.5})$$

Equation 2: Where tr is the retention time and W0.5 is the peak width at half height. There are other plate count calculations, but this is the most widely used.

The larger the number of plates the more efficient the column. The length of the column as well as the particle size and pore size have a significant effect on efficiency of a column. Smaller particles and pores result in higher number of plates due to reduced eddy diffusion, which is the lateral movement through column packing. Longer columns result in poor peak shape due to longitudinal

diffusion. Both lateral and longitudinal diffusion are key factors within the Van Deemter equation (Equation 3):

$$H = A + \frac{B}{u} + Cu$$

Equation 3: Van Deemter equation is a measure of the resolving power of a column (H). Where A is eddy diffusion, B is longitudinal diffusion, C is equilibration time and u is linear flow rate.

Each of these factors have an effect in the efficiency of the column and can be used to calculate the flow rate required to obtain the ideal chromatography (Van Deemter, Zuiderweg and Klinkenberg, 1956).

1.1.5.2 – Normal Phase

Normal phase as the name implies is the original form of chromatography. The columns are made from silica, which is an ideal material for chromatography. These rigid particles resist compaction due to flow and have a large surface area. This has been the main packing material for HPLC columns for decades. The surface of the silica is covered with strongly polar silanol groups (Si-OH) which form hydrogen bonds, π- π bonds and dipole-dipole interactions with analytes in a nonpolar mobile phase. Typical normal phase stationary phases include bare silica, cyano, diol and amino bonded phases. Cyano bonded silica is the most robust of all normal phase options with the ability to retain dipolar compounds such as chloro, nitro and nitrile substituents more strongly than other normal phases. Diol is the most polar and retains amines, ethers, ester and ketones more strongly. Amino being the least robust of the boned phases and have similar properties to that of diol but have the ability to separate vitamins A and D. Silica alone is the least convenient for stability and reproducibility reasons but it does have the ability to separate isomers and particularly useful for large scale applications due to its simplicity. Normal phase works well with analytes which are insoluble in water and can even separate isomers, due to separation selectivity being greatly influenced by altering the mobile phase constituents and solvent ratios. Another advantage of normal phase is that organic compounds are highly soluble in normal phase solvents and these

solvents have a low viscosity therefore allowing higher flow rates increasing sample throughput (Jandera, 2011)

1.1.5.3 – Reversed Phase

Reversed phase chromatography was chronologically the second mode of chromatography after normal phase. It is named reversed because the mobile phase used is more polar than the stationary phase, effectively the reverse of normal phase. Reversed phase stationary phases are silica at their core but with the addition of hydrophobic groups. Typically, these groups include differing lengths of carbon chains, C4, C8 or C18. Reversed-phase columns do not suffer for poor reproducibility from solvent usage resulting in reversed-phase becoming the main HPLC technique. The retention of an analyte on a column is primarily dependent on its hydrophobicity. Linear carbon chains of increasing length result in higher retention.

1.1.5.4 – Other Liquid chromatography and their uses

Ion exchange chromatography is particularly useful for proteins, nucleotides and other macro biomolecules. It is based on the attractive forces between the charged solute molecules and immobilized groups of the opposite charge. This method is not compatible with MS and other analytical equipment and is solely used as a purification technique (Kopaciewicz et al., 1983). Similarly, size exclusion chromatography is also a technique used for protein analysis rather than small molecules. This chromatography is based on the observation that zeolites could act as a molecular sieve to exclude molecules based on their size (Barrer, 1944).

Hydrophilic interaction liquid chromatography (HILIC) provides a chromatography approach to separate small polar compounds which combine characteristics from normal, reversed phase and ion exchange chromatography. HILIC uses polar stationary phases like those of normal phase but utilizes mobile phases like those used in reversed phase. HILIC overcomes reversed phase solid phases which are unable to retain particularly polar molecules and overcomes the polarity limits of normal phase mobile phases which are unable to solubilize very polar molecules. HILIC is also able

to separate ionic analytes without the addition of ion paring agents required by ion exchange chromatography. In contrast to reversed phase, HILIC gradients begin with organic solvents such as acetone and increase in polarity up to high aqueous content mixtures to elute increasingly polar analytes (Guo and Gaiki, 2005).

Solid phase extraction (SPE) is a sample preparation technique which allows samples to be extracted, concentrated and cleaned prior to other purification techniques or analysis. SPE cartridges are available in a range of sizes from small scale < 1 mL to over 1 L and a range of phases: normal, reversed, ion exchange and adsorption (Thurman and Mills, 1998). Flash purification is a cost-effective high-throughput alternative to chromatography purification. It requires less optimization and offers a range of columns options and sizes depending on the scale of the sample. Like HPLC solvent is forced through a column under pressure resulting in fast flow rates in comparison to HPLC but at far lower pressure. The columns used for flash purification tend to be much larger and wider than HPLC allowing purification of several grams of sample in a short time with moderate resolution (Andrews, 1986).

1.1.6 – Mass Spectrometry

Mass spectrometry is an analytical technique which measures the mass-to-charge ratio of compounds and fragments. The invention of mass spectrometry began in 1858 when Julius Plucker attempted to pass electricity through a vacuum and discovered cathode rays (Plucker, 1858) and later the discovery of electrons by J.J. Thomson. Thomson went on to construct a parabola mass spectrograph which is was the forerunner to modern mass spectrometer (MS). The original MS released a beam of positive ions through parallel magnetic and electrical fields which would deflect the beam onto a fluorescent screen or photographic plate. It was observed that the extent of deflection was related to the mass-to-charge ration of the positive ion (Thomson, 1913). In 1918 Dempster constructed the first true MS which ionized a salt by electron bombardment and accelerated the ions through a magnetic field into a mass analyser (Dempster, 1918). MS

instrumentation can be broken down into three parts: ionization source, mass analyser and the detector. Each of these underwent rapid development following 1918 to obtain the various ionization sources, mass analysers and detectors available for modern MS.

There are a wide variety of ionization sources available, Electrospray Ionization (ESI) being one of the most popular, as well many others; Electron Ionization (EI), Chemical Ionization (CI), Field Ionization (FI), Fast Atom Bombardment (FAB), Field Desorption (FD), Plasma Desorption (PD), Laser Desorption (LD), Matrix-Assisted Desorption Ionization (MALDI), Thermospray, Atmospheric Pressure Ionization, Atmospheric Pressure Chemical Ionization, Atmospheric Pressure Photoionization, and Atmospheric Pressure Secondary Ion MS which is Direct analysis on real time (DART). Each with their own advantages and disadvantages depending on the samples to be analysed. The chosen ionization sources must be combined with a mass analyser, of which there are also many to choose from each with different capabilities; electric sector, magnetic sector, quadrupole, ion trap, time-of-flight, Fourier transformation ion cyclotron resonance, Fourier transform orbitrap and hybrids of these different analysers. The final constituent of a MS instrument is the detector which can be a: Photographic Plate, Faraday Cup, Electron Multiplier or an Electro-optical Ion Detector.

1.1.6.1 – Ionisation

EI ionization required the sample to be in gas-phase through which a beam of electrons is passed, electrons collide with the neutral analytes to produce a charged ion. The common term “collide” and “impact” to describe how electrons impart charge to molecules is a misconception, an electron has a certain wavelength and kinetic energy value which are correlated. A 2.7 Å wavelength has an energy of 20 eV and 1.4 Å is equal to 70 eV. When this energy corresponds to a transition within the molecule it leads to various electronic excitement, where there is enough energy an electron can be expelled resulting in a changed analyte (Bentley and Johnstone, 1970). EI is a hard ionization technique which induces extensive fragmentation which provides useful information for

elucidating the structure of unknown analytes, however the molecular ion is not always observed. CI is a softer ionization technique which yields spectra with less fragmentation and molecular ion is clearly recognized. Like EI, CI also uses electron “impact”, but it is used to ionize a reagent gas which then reacts with the analyte to produce an ion. This is a softer ionization technique that EI which was particularly useful for larger molecules and polyfunctional compounds (Munson and Field, 1966). FAB ionization is like CI and is a relatively low fragmentation technique. It is carried out by colliding electrons with slow moving atoms such as Ar, Xe, or Cs to ionize them. These ionized atoms are then accelerated to a beam which is directed at a sample which is either solid or within a matrix such as glycerol, which in turn ionizes the analyte. FAB is particularly useful for large, non-volatile, thermally unstable compounds as samples are not required to be in gas-phase for analysis (Barber et al., 1981). FI uses very strong electric fields to produce ions from gas-phase molecules. The energy transferred during FI is a fraction of 1 eV, therefore generates ions with an extremely low excess of internal energy thus exhibiting no fragmentation. FI is one of the softest ionization methods, however it does require the samples to be volatile and thermally stable as breakdown of the analyte can occur during the evaporation process (Beckey et al., 1969).

There are several desorption ionization techniques, the first of which was field desorption. Desorption based methods overcomes the need for samples to be in gas phase, allowing non-volatile samples to be analysed using MS. FD has be mostly replaced by other desorption methods yet it remains particularly useful for the analysis of high molecular mass non-polar compounds such as polymers (Beckey, 1977). The most widespread and powerful desorption ionization technique is MALDI, which is ideal for the analysis large, non-volatile, and thermally liable compounds such as proteins, oligonucleotides, synthetic polymers and large inorganic compounds. MALDI involves the analyte is dissolved in a matrix. This matrix is a solvent containing small organic molecules which have strong absorption at the laser wavelength. The analyte and matrix are dried prior to placing under vacuum and then ablated by intense laser pulses over a short duration. Although not fully understood, the laser irradiation is believed to induce rapid heating which causes sublimation of the

matrix crystals which also removes analyte for the surface. The charged matrix then imparts charge to the analyte via proton transfer. MALDI is more sensitive than other laser ionization techniques due to the matrix minimizing damage to the analyte from the laser pulse and increasing energy transfer to the analyte (Zenobi & Knochenmuss, 1998). MALDI can detect femtomoles of proteins with a molar mass up to 300,000 Da (Spengler & Cotter, 1990).

Although there is are plentiful ions sources available to most relevant for the use of identifying a wide range of unknown plant compounds and bacterial metabolites is ESI. The success of ESI is due to its versatility, although was originally considered to be an ionization technique for protein analysis. ESI its use extended to polymers, biopolymers and small polar molecules. ESI allows very high sensitivity to be reached and is easily coupled with HPLC. ESI is based on the generation of gas-phase ions from a solution flowing through a small outlet, when voltage is applied. Once enough voltage is supplied between the solution and MS inlet, the solution is dispersed in fine droplets with the aid of a sheath gas which is usually N2 which undergo a succession of solvent evaporation and coulombic fission, lastly leading to multi-charged molecular ions with intact analytes (Rohner, Lion & Girault, 2004). Electrospray can used with either positive or negative applied voltage leading to the formation of cations or anions, respectively. A power supply is connected the solution and a counter electrode, when there is no voltage applied the only force acting on the liquid is surface tension causing the surface to be hemispherical. Once electrostatic force is applied and is balanced with the surface tension, the surface forms a conic shape called the Taylor cone (Taylor, 1964). With a slight increase in applied potential, the solution/air interface at the apex of the cone becomes unstable dispersing the solution into charged droplets. The droplet evaporates and as the coulombic repulsion exceeds the surface tension, each drop explodes to produce further smaller droplets. Continuous generation of smaller and smaller droplets leads to the production of gas-phase ions (Rosell-Llompart & De La Mora, 1994). The sensitivity of ESI is based on the concentration of the analyte rather than the quantity of samples injected into the source, in fact sensitivity is improved the lower the flow rate of sample down to tens of nanolitres per minute.

ESI along with all other ionization methods suffer from ion suppression, which is a phenomenon occurring when there is a high concentration of non-volatile compounds inhibiting droplet formation, release into gas phase and ultimately the detector. Highly concentrated samples compete for limited charges and space on the droplet. This results in the molecules with higher mass suppressing smaller molecules, and more polar compounds are more susceptible to suppression. (Sterner et al., 2000, Bonfiglio et al., 1998, and Annesley, 2003). Ion suppression can be affected several variables, system variable, compound variables and method variables. System variables are electric field, ES-capillary diameter, ES-capillary voltage, distance to counter electrode, heat capillary of ambient gas, solvent saturation level of ambient gas. Compounds variables are surface activity, proton affinity, pKa and solvation energy. Method variables are flowrate, electrolyte concentration, pH and surface properties such as boiling point and surface tension (King et al., 2000). The presence of electrolytes is vital to observe a stable spray, however normal solvents contain enough electrolytes for this purpose. As the maximum tolerable concentration of electrolytes to have good sensitivity is 10-3 M, it is more important to remove electrolytes for the samples to obtain good ionization (Hoffmann & Stroobant, 2007). Therefore, ion suppression can be reduced by implementing several strategies. Sample matrix, coeluting compounds and crosstalk can contribute to ion suppression, therefore sample purification using SPE (or other means of purification) and suitable chromatographic separation can reduce this effect. With respect to sample matrix impacting on ion suppression TFA is a known to be one of the best liquid chromatography modifiers. TFA does not interfere with spectrophotometric detection but it does significantly cause problems for mass spectrometric analysis. TFA increases the surface tension of the mobile phase which causes the “Taylor cone” to be unstable causing the spray to often break down reducing the analyte ion signal, by using a weaker acid such as acetic or formic acid ion suppression can be reduced (Hayati et al., 1986).

ESI ion formation occurs at ambient pressure meaning that there are numerous collisions between ions and molecules which are not seen in a vacuum. Proton transfer, producing a cation or anion is

the most common ion-molecules reaction at ambient or high pressure. This reaction is based on proton affinity, the neutral or anionic acceptor of a proton must have a higher affinity that the neutral or cationic donor thus transferring the proton. This reaction also occurs when the molecule fragments, the proton can only be present on one of the fragments. An adduct is an ion formed by the combination of a neutral molecule and an "ionizing" ion other than a proton. Sodium (M+23)+ and ammonium (M+18)+ are common positive adducts and chlorine (M+35)- , (M+37)- and acetate (M+59)- are common negative adducts. Commonly acids are added to mobile phases and can for adducts such as formic acid forming formate (M+45)- negative ions. Dimers and other aggregates can also form (M+M+H)+ (Hoffmann & Stroobant, 2007).

1.1.6.2 – Mass Analyser

Once gas phase ions have been produced, they must be separated based in their masses and determined. The physical property which is measured is the mass to charge ratio rather than the mass alone, therefore multiply charged ions will be a fraction of the actual mass. There are 5 main characteristics of mass analysers: mass range limit, analysis speed, transmission, mass accuracy and resolution. Mass range is the limit of m/z over which a mass analyser can measure, and analysis speed is the rate at which the analyser is able to measure over that range. Transmission is a measure of the number of ions lost during mass analysis, it is the ratio between the ions reaching the detector and the ions entering the mass analyser. Mass accuracy is a measure of the difference between the measured m/z and the theoretical m/z, high mass accuracy has significant applications in elemental composition determination. Resolution concerns the ability of an analyser to distinguish between two ions with small m/z differences. Mass analysers ability to resolve m/z are categorized into low and high; the distinction being less than or greater than 10,000 FWHM (Hoffmann & Stroobant, 2007).

Quadrupole analysers separate ions based on the stability of their trajectory in oscillating electric fields. Quadrupoles are made up of a source, focusing lenses, and four perfectly parallel

rods. As the ions enter the focusing lenses, they are passed longitudinally through the centre of these charged parallel rods to the detector. Opposite rods are electrically connected with RF and/or DC voltages, meaning adjacent rods have opposite charges. The ions will be drawn laterally to the opposite charge. If the potential changes sign before it discharges itself on the rod, the ion will change direction. If the lateral pull to either charged rod remain stable for the length of the quadrupole the ion will be detected, electrical potentials are adjusted so only certain ions with specific m/z are selected to pass through the rods to the detector. RF only voltage results in a wide range of m/z traversing through the quadrupole, which is usually used for ion-focusing. When both DC and RF are applied ions of a specific m/z can be tuned, by increasing the DC and RF voltage and keeping the ratio constant the mass range can be scanned to transmit ions of increasing m/z to acquire a mass spectrum. In practice the highest detectable m/z of a quadrupole is about 4,000 Th and resolution of around 3,000 FWHM making them low resolution instruments. Quadrupoles are the cheapest analyser but are also very robust, they do not depend of kinetic energy of the ions, robust in terms of pressure changes and have high scan speed therefore are well suited to be coupled with chromatographic techniques. Multiple quadrupoles can be applied in tandem to obtain fragmentation analysis. If three quadrupoles were lined up a collision gas such as He would be introduced into the central quadrupole and then the first and last could either scan of a range of ions or select for specific m/z ranges.

Ion trap analysers use an oscillating RF quadrupolar electric fields to trap ions in two or three dimensions and store them. An ion trap analyser is made up of a circular electrode with two ellipsoid caps. They work in similar way to a quadrupole only in a loop instead of a rod. Instead of changing the potentials to allow ions through the rods, ions of different masses are stored within the ion trap and then expelled according to their masses to obtain a spectrum (Stafford et al., 1984). Ions can be produced by injecting electrons in the ion trap. The 3D ion trap also known as Paul ion trap is subject to spatial charge effects which effect the stability of the ions within the trap when there are too many ions present. This effect is lessened in 2D ion trap known as linear ion traps (LIT), which can

accommodate ten-fold more ions than 3D without suffering from poor resolution. Ion traps are often combined with other mass analysers in order to improve the efficiency of the MS instrument allowing more ions to be analysed (Douglas et al., 2005).

Time of flight (TOF) analysers separates ions based on the velocity of the ions within a flight tube after being accelerated by an electric field. TOF analysers work well with ion sources which produce pluses of ions such as laser-based techniques. The m/z is measured by the time ions takes to move from the source to detector. TOF analysers have a high analysis speed, broad m/z ranges and no upper limit therefore ideally suited to soft ionisation techniques and work with proteins. However, they detect a weak number of ions which is insufficient to produce high precision and resolution (Moniatte et al., 1996).

1.2 - Methods – Natural Products

1.2.1 – General Reagent

Table 1: List of all general reagents, suppliers and grades.

Reagent name	**Supplier**	**Grade**
Methanol (MeOH)	Fisher Scientific	HPLC grade 99.9 %
Methanol-d_4	Sigma-Aldrich	99.8 atom % D
Dimethyl Sulfoxide (DMSO)	Supelco –	LiChrosolv ≥99.7 %
Water	Milli-Q	18.2 Ω – Q-pod purifier
Formic acid (FA)	Supelco	LC-MS LiChropur 98-100%
2-methoxy-1,4-naphthoquinone (MNQ)	Merck	98%
Mueller Hinton agar	Merck	For microbiology
Mueller Hinton broth	Merck	For microbiology
Yeast malt agar	Merck	For microbiology
Yeast malt broth	Merck	For microbiology
Brucella Agar with 5% Sheep Blood, Hemin and Vitamin K	Millipore	For microbiology
Dulbecco's modified eagle media (DMEM)	Gibco	Suitable for cell culture
Hepes	Gibco	1 M, Suitable for cell culture
Foetal Calf Serum	Gibco	Suitable for cell culture
Kanamycin solution	Sigma-Aldrich	10 mg/mL in 0.9% NaCl, BioReagent, suitable for cell culture
Amphotericin B solution	Sigma-Aldrich	250 mg/L in deionized water, BioReagent, suitable for cell culture
Defibrinated sheep blood	Oxoid	NA
Phosphate buffered saline	Sigma-Aldrich	10x concentrate, suitable for cell culture
Triton-X 100	Sigma-Aldrich	Laboratory grade
Eagle modified essential media (EMEM)	Gibco	Suitable for cell culture
Basement membrane extract with phenol red	Gibco	Suitable for cell culture
Foetal Bovine Serum (FBS)	Gibco	Value FBS, suitable for cell culture
MEM Non-essential amino acid solution (100x)	Gibco	Without glutamine, BioReagent, suitable for cell culture
L-Glutamine	Gibco	200mM, suitable for cell culture
Ampicillin sodium salt	Sigma-Aldrich	Powder, BioReagent
Meropenem trihydrate	Sigma-Aldrich	Powder, BioReagent
Ciprofloxacin HCl	Supelco	Pharmaceutical secondary standard
Lithium Mupirocin	Sigma-Aldrich	Powder, >95%
Levofloxacin	Sigma-Aldrich	Powder, >98%
Chloramphenicol	Sigma-Aldrich	Powder, BioReagent
Rifampicin	Sigma-Aldrich	Powder, BioReagent
Tetracycline	Sigma-Aldrich	Powder, 98-102%
Cefotaxime sodium salt	Sigma-Aldrich	Powder, BioReagent

Nalidixic acid sodium salt	Sigma-Aldrich	Powder
Gentamycin sulphate	Sigma-Aldrich	Powder
Streptomycin sulphate salt	Sigma-Aldrich	Powder, BioReagent
2-hydroxy-1,4-naphthoquinone (HNQ or lawsone)	Sigma-Aldrich	97%
Urea	Supelco	8 M (after reconstitution with 16 mL high purity water)
DL-Dithiothreitol (DDT)	Sigma-Aldrich	>98%
Iodoacetamide	Sigma-Aldrich	BioUltra
TRIS Solution	Millipore	OmniPur, 1.0 M pH 7.5
Trypsin	Promega	Mass Spectrometry Grade
Chloroform	Sigma-Aldrich	HPLC Plus, ≥99.9%

1.2.2 – Extraction and purification

I. glandulifera was collected from a riverbed in Llanfarian, Ceredigion, Wales, UK (52.37667°N, -4.07833°W). *F. japonica* was collected from a riverbed in Penparcau, Aberystwyth, Ceredigion, SY23 3TL (52.403472°N, -4.068028°W). *Rhododendron ponticum* plant material was collected from Llanilar, Aberystwyth, Ceredigion, SY23 4SB (52.352833°N, -4.020306°W). Only the aerial parts of the plant were collected and stored in a -80 °C freezer within 1 hour of collection.

In order to fully extract all compounds contained within each plant all cellular structure needed to be disrupted therefore all plant material was ground into a powder using liquid nitrogen, pestle and mortar. Powdered plant material was extracted in 75% aqueous methanol stirring at room temperature for 24 hours. The plant material was separated from the solvent using a sieve, discarding the plant material and drying the solvent under vacuum.

Purification and analysis of samples will be carried out using C-18 solid phase, therefore samples need to be prepared as such. After conditioning a 10 g LC-18 SPE cartridges (Supelco, UK), dry sample extract was added. Water was added to the sample and pulled through the cartridge under vacuum. This fraction was collected and will be referred to as “unbound”. This is because analytes present within this fraction have not bound to the C-18 solid phase within the SPE cartridge. Further purification and analysis of this fraction would require chromatographic techniques which are suitable for the most polar of analytes such as HILIC. Due to the benefits and ubiquity of reversed

phase chromatography (see 1.1.5 – Liquid Chromatography) it was decided that analytes which are suitable for these separation technique would be the main focus. However, the unbound fraction was freeze dried under vacuum and stored at -80 ˚C, should we need to return to this sample. The cartridge was then washed with methanol and collected separately. This fraction will be referred to as the "bound" fraction, as any analytes which are passed through the column can be separated using reversed phase chromatography. This fraction was also freeze dried at stored at -80 ˚C. In addition to separating the analytes relevant for further purification based on which type of chromatography would be most relevant this also cleaned the samples for further purification. Any particulate matter or analytes which cannot pass through a C-18 column would be removed improving the quality of any further chromatography carried out.

Using the bound fraction, further separation was carried out using Isolera 4 (Biotage, Sweden) flash purification system into fractions using a gradient of ultrapure 18.2 Ω H2O and analytical grade MeOH from 0-100% MeOH (flow rate of 100 mL/min) using a 60 g Biotage SNAP Ultra C18 flash cartridge (Biotage, Sweden). This "SNAP Ultra" column allowed the bound fraction of sample to be purified quickly and with the highest purification performance offered by the Biotage flash purification system. This yielded a range of fractions for each plant which were assayed for a range of biological activities. A fraction was exhibiting potent biological activity and further purification using C18 semi-preparative HPLC system (Dionex, ThermoFisher Scientific, UK). A custom isocratic gradient using ultra-pure 18.2 Ω H2O and analytical grade MeOH were used to purify this fraction into two pure compounds only one of which was found to be a potent antimicrobial.

1.2.3 – HPLC-PDA-MS Analysis

1.2.3.1 – HPLC-PDA- Low resolution MS

The instrumentation and method described below was used to analyse crude plant extracts and fractions from flash purification. This is run a relatively long gradient to isolate peaks and

tentatively identify their structure. The aim was not full structural characterisation but to understand the purity and type or compounds present within these samples.

The bound and unbound plant extract (100 µL) were inserted into HPLC vials. The analysis used a Thermo-Finnigan HPLC/MSn system (Thermo Electron Corporation, USA) which comprised a Finnigan surveyor photodiode array (PDA) plus detector and a Finnigan LTQ (linear trap quadrupole) with an electron spray ionisation (ESI) source, the system was linked to an analytical workstation. The column used was a reversed phase Waters C18 Nova-Pak (4 µm, 3.9 mm × 100 mm). 10 µL of sample was injected for each run. The PDA detection wavelengths were set between 240 nm – 600 nm, with a flow rate of 1 mL min-1. The mobile phase consisted of water-formic acid (solvent A; 100:0.1, v/v) and HPLC grade methanol-formic acid (solvent B; 100:0.1, v/v). The starting condition was 95%A/5%B, with the percentage of solvent B increasing linearly to 100% over 30 minutes. The LC-MS interface used N2 as the sheath and auxiliary gas and He was used as the collision gas. For ionisation in negative mode, the interface and mass-selective detection parameters were as follows: sheath gas, 30 arbitrary units; auxiliary gas, 15 units; spray voltage, -4 kV; capillary temperature 320 °C; capillary voltage, −1 V; tube lens offset, −68 V. Xcalibur analytical software (Thermo Fisher Scientific, USA) was used to process the chromatographic data. Results were compared to various online libraries such as KEGG ligand (KEGG, Japan) and Mass bank (Horai et al., 2010) as well as scientific journals.

1.2.3.2 –HPLC-PDA- High resolution MS

The instrumentation and method described below was used once pure compounds were achieved. A relatively short gradient and run time was utilised as these compounds did not require separation. The aim of this method was to be able to aid in the full structural characterisation of compounds using the high-resolution MS and running standards for comparison.

LC-HRMS analysis was performed on an Orbitrap Fusion Tribrid mass spectrometer (Thermo Scientific) that was coupled to an UltiMate 3000 liquid chromatographic system (Dionex, Thermo

Scientific). A Hypersil Gold (Thermo Scientific) reversed phase C18 column (2.1 mm x 150 mm; particle size 5 µm) was used and maintained at a temperature of 60 °C at a flow rate of 0.6 mL/minute. 10 µL was injected and eluted using a gradient mobile phase system with eluent A containing ultra-pure water (18.2 Ω) with 0.1% formic acid (mass spectrometry grade, Fluka), and eluent B MeOH (HPLC grade, Fisher Scientific) with 0.1% formic acid. The initial conditions were sat at 95% A with a linear increase to 95% B over 7 minutes. 100% B was held for 3.5 minutes before equilibration at initial conditions for a further 2.5 minutes.

Ions were generated in a HESI-II source with a source voltage of 3500/2500 V for positive/negative mode. The sheath gas was set to 45 psi, aux gas flow set to 13 AU, with a vaporiser temperature of 358 °C and an ion transfer temperature of 342 °C. Ions were detected in profile mode over 100-2000 m/z in the orbitrap detector at a resolution of 240000 and an injection time of 100 milliseconds in both positive and negative mode.

1.2.4 – NMR Analysis

1H NMR spectra were recorded on a Bruker DRX500 instrument operating at 500 MHz using CD3OD as the solvent and SiMe4 as the internal standard. Chemical shifts (δ) are given in ppm relative to SiMe4. Post-acquisition processing was performed on MestReNova (Version 6.0.2) Mestrelab Research, Compostela). 1H spectra were processed using automatic peak picking and manual integration. Where applicable, coupling constants (J) were calculated using the MestReNova manual multiplet analysis function and are expressed in Hz.

1.2.5 – Antimicrobial susceptibility testing – 24-hour growth curve

Initial plant extracts and early purified products were subjected to a 24-hour growth curve antimicrobial assay. This method allows many samples to be tested on one plate against 4 different aerobic bacterial species: *Bacillus subtilis* ATCC 663, *Escherichia coli* – ATCC 25922,

Pseudomonas aeruginosa ATCC 27853, and *Staphylococcus aureus* ATCC 29213. All samples were tested in triplicate with no serial dilution at concentration of 1 mg/mL.

Bacterial suspensions were prepared by direct colony suspension in Mueller Hinton broth (Merck, Darmstadt) and incubated overnight at 37 °C in a temperature-controlled orbital shaker at 300 rotations per minute (rpm). The overnight cultures were standardized to a McFarland standard value of 0.5 which is equivalent to 1×10^8 cfu/mL. This is done by diluting the suspension to obtain an optical density (OD) of 0.1 at 600 nm using a spectrophotometer (Wiegand, Hilpert and Hancock, 2008; Hecht et al., 2007). A further 1 in 10 dilution of this bacterial suspension was carried out to make a standardised bacterial solution of 1×10^7 cfu/mL. Mueller Hinton broth (150 µL) was added to each well in the 96-well plate. Into rows A-E was added 40 µL of 5 mg/mL solution of sample was added and mixed, one sample per row. Rifampicin (61 µM, control) was added to row F. The wells in rows G and H were left untreated. Standardized overnight culture (10 µL; 1×10^7 cfu/mL) was added to rows A−G to provide a final in-well concentration of 5 × 105 cfu/mL. Row H left with no sample and no bacteria as a blank and a contamination control. Plates were incubated in a Hidex Sense Plate Reader (LabLogic, Sheffield UK) at 37 °C within an orbital shaker at 300 rpm for 24 hours. The OD was measured at 600 nm every 20 minutes for 24 hours this data was used to produce a growth curve. The high concentration is justified as the samples contain a mixture of many compounds therefore a high concentration is required to see any activity. By measuring growth over 24 hours any disturbances in growth can be analysed even if the sample did not affect the total amount of bacteria present after 24 hours. A percentage inhibition can be calculated by dividing the OD at 24 hours of a sample by the OD at 24 hours of the positive control and multiplying by 100.

1.2.6 – Antimicrobial susceptibility testing – Minimum inhibitory concentration

Calculating the minimum inhibitory concentration (MIC) of a compound was carried out once a pure compound has been obtained and involves testing a range of concentrations to show

the lowest amount of compound which can prevent the growth of different bacteria over the generation tome of the bacteria.

Bacterial suspensions were prepared by direct colony suspension in Mueller Hinton broth (Merck, Darmstadt) and incubated overnight at 37 °C in a temperature-controlled orbital shaker at 300 rpm. The overnight cultures were standardized to a McFarland standard value of 0.5 which is equivalent to 1×10^8 cfu/mL. This is done by diluting the suspension to obtain an OD of 0.1 at 600 nm using a spectrophotometer (Wiegand, Hilpert and Hancock, 2008; Hecht et al., 2007). Broth (190 µL) was added to each well in the 96-well plate. Further broth (160 µL) was added to wells A1− A12, followed by test solution (40 µL). The sample was mixed, and aliquots (200 µL each) were taken and added to each well through to row E. Rifampicin (61 µM, control) was added to row F. The wells in rows G and H were left untreated. Standardized overnight culture (10 µL; 1×10^7 cfu/mL) was added to rows A−G to provide a final in-well concentration of 5×10^5 cfu/mL. Plates were incubated in a Hidex Sense Plate Reader (LabLogic, Sheffield UK) at 37 °C within an orbital shaker at 300 rpm for 24 hours (72 hours in the case of *B. anthracis*). The OD was measured at 600 nm at initial time point and after 24 hours, this was used to calculate the percentage growth when compared to bacterial growing without the presence of an antimicrobial compound.

Samples and bacterial combinations were carried out in triplicate, the lowest concentration of MNQ which inhibited the growth of bacteria was deemed to be the MIC. If there is a discrepancy between the triplicate analyses an average of the three MIC values were taken.

Most of the bacteria used for this test were all aerobic; *Bacillus anthracis* NCTC 10340 (72-hour generation time), *Bacillus subtilis* ATCC 663, *Escherichia coli* – ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella enteritidis* wild type, *Staphylococcus aureus* ATCC 29213 and *Staphylococcus aureus* MRSA ST8: USA300.

Two isolates of *Clostridium difficile* were also assayed; 291 previously described by Calabi et al. 2001, and 630 previously described by Farrow, Lyras and Rood 2000. These are strictly anaerobic

bacteria, meaning they are very sensitive to the presence of oxygen. They were incubated in an anaerobic chamber with a gas mix of 5% CO2, 10% H2, 85% N2. Unfortunately, there was no option to shake while incubating so they were placed on an orbital shaker at 300 rpm for 10 mins prior to taking the initial OD reading then entering the anaerobic chamber. The same was shaking was carried out prior to the final reading taken at 72 hours.

1.2.7 – Antifungal susceptibility testing – 24h growth curve and minimum inhibitory concentration

Antifungal assays were carried out in the same format as antimicrobial assays only using Candida albicans (ATCC 10231) and yeast malt broth (Merck, Darmstadt) as a growth medium. The only fungal species used for this assay was Candida albicans NCTC 3255.

1.2.8 – *Schistosoma mansoni* schistosomula culture compound screening - Roboworm Platform

S. mansoni (Puerto Rican Strain, Naval Medical Research Institute- NMRI) cercariae were collected from infected Biomphalaria glabrata (NMRI) snails after exposure to 2 hours of light at 26°C and then mechanically transformed into schistosomula as described (Colley and Wikel, 1974). Newly transformed schistosomula were prepared for 72 hours high throughput screening (HTS) in 384-well black-sided microtiter plates (Perkin Elmer, MA, USA) as described in Nur-E-Alam et al., 2017, with a final DMSO concentration of 0.625%. The effect of compounds on 72 hours cultured schistosomula was deduced by analysing the effect on both motility and phenotype of treated schistosomula using the image analysis model described by Paveley et al., 2012.

1.3 - Results – Natural Products

The following summarises the strategy used to isolate anti-microbial compounds. All powdered plant material was extracted in 75% methanol, dried under vacuum and bound to C18 reversed phase silica prior to flash purification using gradient of ultra-pure 18.2 Ω H2O and analytical

grade MeOH from 0-100% MeOH (flow rate of 100 mL/min) using a 60 g Biotage SNAP Ultra C18 flash cartridge. To identify which fractions and compounds were of interest a range of biological assays were carried out. Following flash purification 24h bacterial growth assays were carried out at 1 mg/mL. Further purified fractions and compounds were tested for antimicrobial activity using MIC assay and other bioassays. Following flash purification fractions of interest were purified further using semi-preparative HPLC. HPLC-PDA-ESI-MS/MS was carried out to identify compounds. For compounds of interest which were purified fully NMR was also carried out.

1.3.1 – *Impatiens glandulifera*

1.3.1.1 – *Impatiens glandulifera* - Isolation of compounds – Flash Purification

In order to obtain antimicrobial compounds of interest from the plant extract a bioactivity led purification method was employed, this is summarised in Figure 5. The *I. glandulifera* plant extract was prepared for purification by passing through a C18 SPE cartridge firstly eluting with water and then methanol. The methanol fraction was purified further using C18 reversed phase flash purification. The mobile phases were water and methanol, a gradient from 0-100% methanol over 30 mins at a flow rate of 10 mL/min was used (Figure 6).

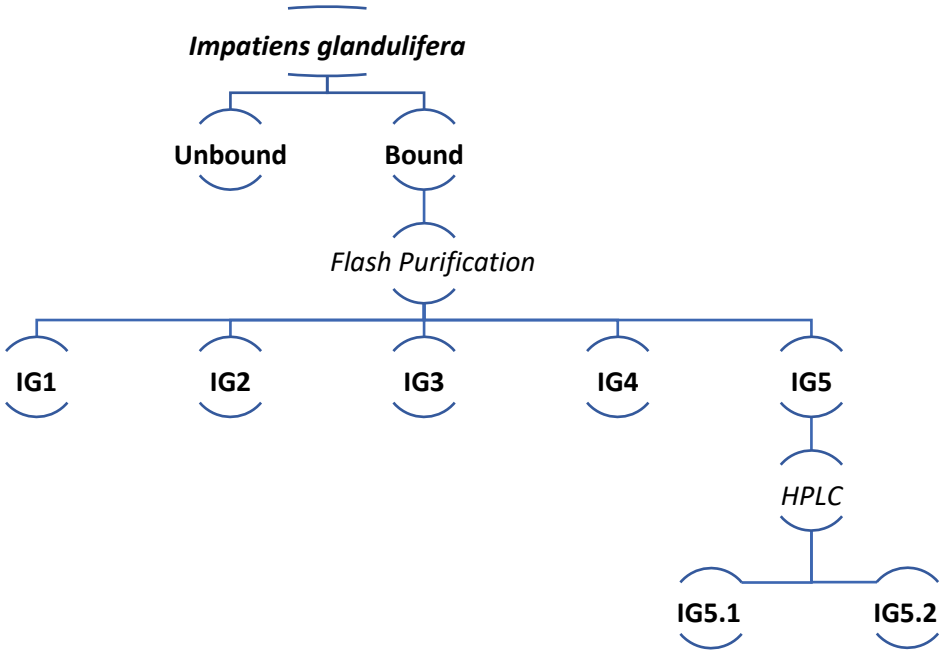


Figure 5: Summary of the separation pathway of I. glandulifera material

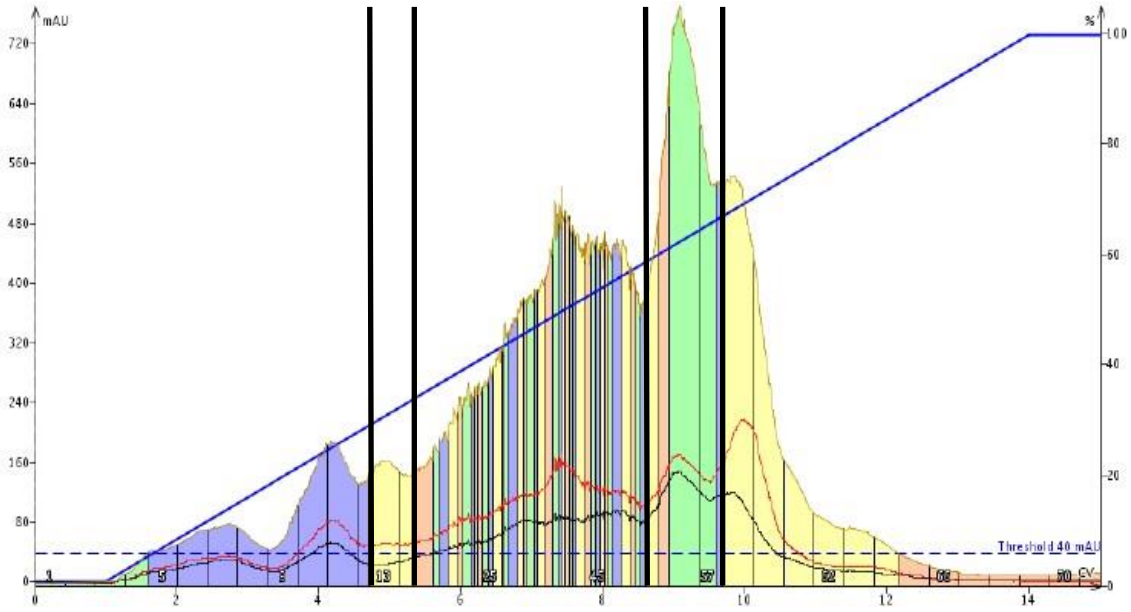


Figure 6: Flash purification of I. glandulifera material, which was separated into 5 fractions denoted by the vertical black lines. The coloured area of the graph represents the total UV absorption of the analytes leaving the column at that time. The red line represents UV absorption at 280 nm and black line represents UV absorption at 330 nm. Colour changes indicated that the flash purification system has detected a significant change in UV absorption which could indicated a different analyte being eluted. The blue line represents the increasing gradient of methanol over time.

1.3.1.2 – *Impatiens glandulifera* -Identification of compounds

Five fractions of *I. glandulifera* were obtained using flash purification each was analysed using HPLC-PDA-ESI-MS/MS (Appendix 1). Each major peak present in both PDA and negative mode mass chromatogram is shown in **Error! Reference source not found.**. The tentative identifications are based upon the use of online mass spectral database MZedDB and based on their fragmentation patterns.

Table 2: Summarised HPLC-PDA-ESI-MS/MS data of the 5 fractions obtained from *I. glandulifera*

Fraction No.	RT (min)	PDA λ max (nm)	MS1 $[M-H]^-$ ions (*m/z*)	MS2 $[M-H]^-$ ions (*m/z*)	Tentative identification
1	6.25	289, 312	400.62	302.89, 228.80, 200.80	Unknown
	9.39	251, 273, 326	316.92	229.79,228.77, 200.79, 184.80	Myricetin
2	2.53	261	286.73	240.74	Unknown
	5.20	281	480.87	258.73, 288.70, 212.72, 200.73	Unknown
	8.25	272, 355	332.82	272.79, 260.74, 104.54	Unknown
	9.35	251, 273, 326	316.92	229.79, 228.77, 200.79, 184.80	Myricetin
	10.64	285, 379	258.81	258.75, 231.88, 230.74	Unknown
	11.17	252, 282, 338, 379	634.90	616.82, 482.82, 472.77, 454.81	Trigalloyl glucose
3	8.92	274, 343	550.37	303.11	8-methylsulfinyl-*n*octylglucosinolate
	9.39	289, 340, 379	584.74	538.69, 376.60	Unknown
	11.22	252282,338, 379	634.90	616.82, 482.82, 472.77, 454.81	Trigalloyl glucose
	12.63	255, 353	548.62	504.8	Quercetin-3-*O*malonylglucoside
	14.06	265, 345, 379	532.50	488.79	Luteolin-7-*O*-6malonylglucoside
4	11.96	255, 354	462.95	301.88, 300.85, 299.87	Quercetin-3-*O*-glucoside
	12.16	255, 354	608.96	300.73	Rutin
	13.52	265, 347	593.00	286.00, 284.87	Tiliroside

5	12.09	249, 278, 339, 379	462.95	301.88, 300.85, 299.87	Quercetin-3-*O*-glucoside

1.3.1.3 – *Impatiens glandulifera* -Antimicrobial activity of flash purification fractions

In order to streamline the purification process, antimicrobial assays were carried out on the 5 flash purification fractions obtained from *I. glandulifera* prior to any further purification. A 24-hour antimicrobial growth curve method was carried out using fractions at 1 mg/mL (see 1.2.5). The total percentage inhibition of growth over 24 hours for each fraction was calculated and displayed in Table 3.

Table 3: Summary of all flash purification fractions (1 mg/mL) antimicrobial activity expressed as percentage inhibition after 24 hours of growth (n=3)

Fraction	*E. coli* (ATCC 25933)	*P. aeruginosa* (ATCC 700603)	*S. aureus* (ATCC 29213)	*B. subtilis* (ATCC 6633)
IG 1	9	0	20	17
IG 2	15	0	16	20
IG 3	3	0	47	19
IG 4	17	0	53	0
IG 5	55	0	95	48

Of the 5 fractions tested, IG5 clearly showed the most significant antimicrobial activity. IG5 had the highest % inhibition against all the bacterial species tested, although *P. aeruginosa* remained unaffected by all the fractions. IG5 showed the most significant activity against *S. aureus*, allowing only 5% growth over a 24-hour period.

1.3.1.4 – *Impatiens glandulifera* – Isolation of compounds – HPLC Purification of IG5

Of the five fractions IG5 was chosen for further purification due to its significant antimicrobial activity. To obtain pure compounds HPLC purification was carried out using a Nova-

Pak C18 Prep Column, 60 Å, 6 µm, 7.8 mm X 300 mm on a preparative HPLC system with a PDA detector (Dionex, ThermoFisher Scientific, UK). 100 µL of a 10 mg/mL solution was made up in 60% MeOH and injected using a custom gradient of 60% Methanol to 80% methanol over 10 minutes before washing at 100% and returning to starting conditions over a total of 25 minutes. The PDA detector was set to 240 nm and all eluent was collected into tubes. This method was developed to obtain two well-resolved peaks along with four small peaks. The two main peaks were named IG5.1 and 5.2 yielding 2 and 5 mg of dry product respectively, the other smaller peaks were also collected but their weights were negligible. The injection volume as increased to 200 µL and the resolution between peaks was maintained, and repeated a further 20 times, obtaining around 10 mg of IG5.1 and over 25 mg of IG5.2.

1.3.1.5 – *Impatiens glandulifera* – Antimicrobial activity of IG5.1 and IG5.2

Using the material gained from the HPLC purification along with the flash purification fractions an MIC assay was carried out against MRSA (Table 4). This clearly demonstrates the result of the purification, it has marginally improved the potency of IG5 in the case of IG5.2 by removing any inactive impurities, present in IG5.1 and other peak too small to carry out an assay. This compound has shown activity 20-fold better than any other fraction against MRSA.

Table 4: Minimum inhibitory concentration of IG fractions against MRSA ST8: USA300 (n=3)

Fraction	**MIC (mg/L)**
IG 1	1420
IG 2	1660
IG 3	490
IG 4	1130
IG 5	39
IG 5.1	100
IG 5.2	23

The Les Bailie group had a range of interesting pathogens which I was able to also able to use. This widened the scope of the anti-bacterial activity of the IG5.1 and 5.2. Their interest was in *Bacillus anthracis* along with a few other bacteria (Table 5). Yet again the IG5.2 fraction showed impressive antimicrobial activity against more species of bacteria, *C. difficile*. Although these results are not as impressive as the activity seen against *S. aureus*.

Table 5: Minimum inhibitory concentration of IG5.1 and IG5.2 against a further 5 strains of bacteria (mg/L) (n=3)

Fraction	*Bacillus anthracis*	*Bacillus subtilis*	*Salmonella enteritidis*	*Clostridium difficile (291)*	*Clostridium difficile (630)*
IG5.1	462	>1000	895	256	>1000
IG5.2	116	533	329	79.7	290

1.3.1.6 – *Impatiens glandulifera* –Identification of IG5.2

The fraction IG5.2 was identified as 2-methoxy-1,4-naphthoquinone (MNQ) using NMR (Figure 7 and Table 6) and HR-MS (Figure 8). 1H NMR revealed that IG5.2 only had 4 distinct proton environments, in combination with the HRMS data it was known that this was a small molecule with a molecular mass of 188 m/z (189.05472 [M+H]+). Using this information and literature (Yang et al., 2001; Mitchell et al., 2007) to support this compound being derived from *I. glandulifera* the structure was confirmed to be MNQ. For further proof of this structural elucidation commercially available MNQ was obtained (Merck, Darmstadt) and run using the same HR-MS methodologies and compared the chromatography obtained from IG5.2 (Figure 9). The retention time matched and the MS ion was the same, therefore the structure is confirmed as MNQ.

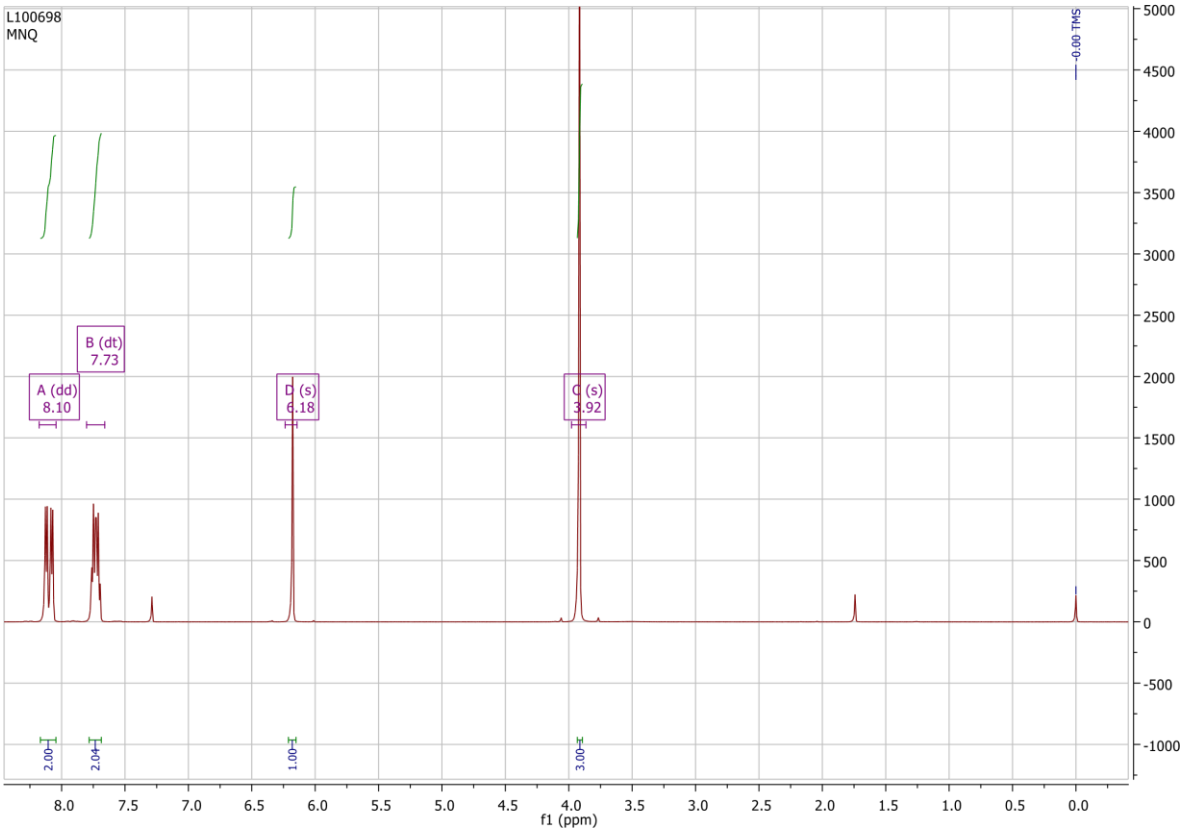
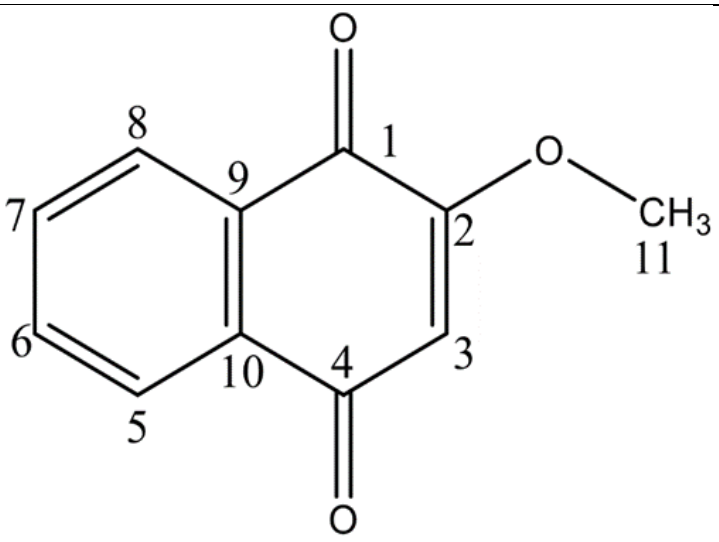


Figure 7: 1H NMR spectrum of IG5.2

Table 6: ^{1}H NMR chemical shifts and identification of IG5.2 as 2-methoxy-1,4-naphthoquinone (MNQ)

Carbon Number	Chemical shift ^{1}H
C-1	-
C-2	-
C-3	6.18 s
C-4	-
C-5	8.13 dd
C-6	7.67 dt
C-7	7.73 dt
C-8	8.08 dd
C-9	-
C-10	-
C-11	3.92 s



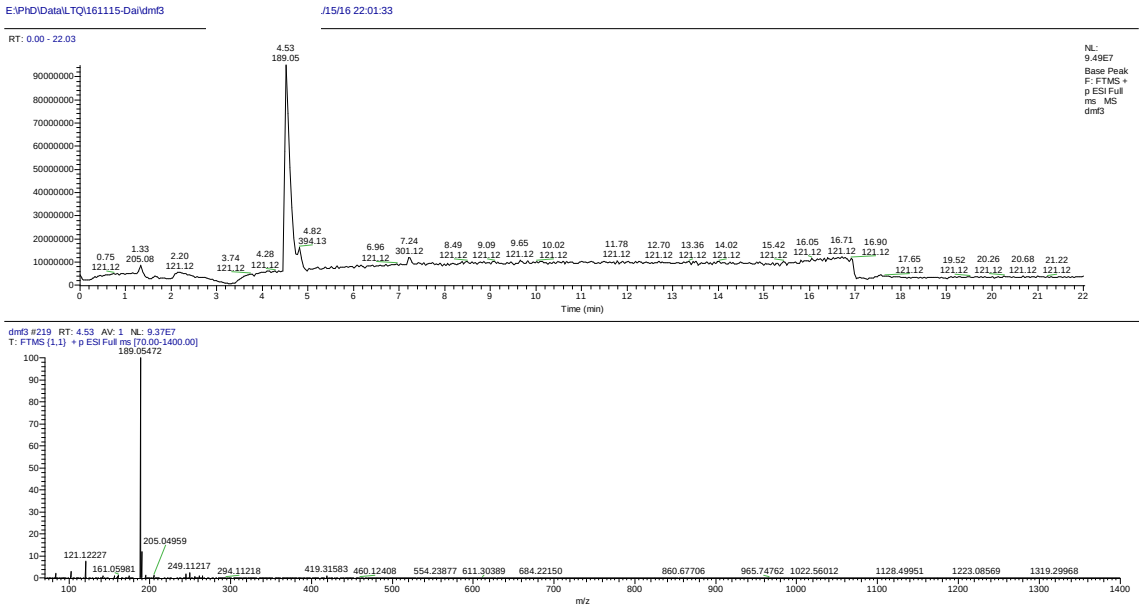


Figure 8: HPLC-HR-MS of IG5.2. The top graph is a mass chromatogram over the 7 min run time showing the relative abundance of ions being detected at certain retention times. The bottom graph is a mass chromatogram taken at the apex of the peak within the mass chromatogram.

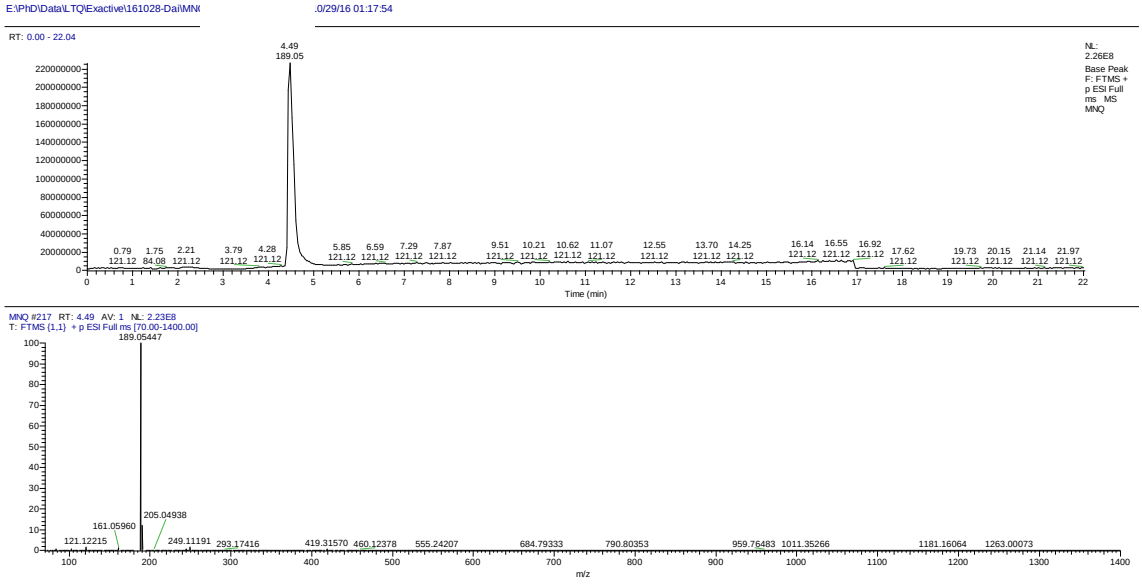


Figure 9: HPLC-HRMS of commercially available MNQ. The top graph is a mass chromatogram over the 7 min run time showing the relative abundance of ions being detected at certain retention times. The bottom graph is a mass chromatogram taken at the apex of the peak within the mass chromatogram.

1.3.2 – *Fallopia japonica*

1.3.2.1 – *Fallopia japonica* - Isolation of compounds

The same methodologies used for *I. glandulifera* were also applied to F. japonicum. A summary of the purification and assays carried out is depicted in Figure 10. The plant extract was prepared for purification by passing through a C18 SPE cartridge firstly eluting with water and then methanol. The methanol fraction was purified further using C18 reversed phase flash purification. The mobile phases were water and methanol, a gradient from 0-100% methanol over 30 mins at a flow rate of 10 mL/min was used (Figure 11).

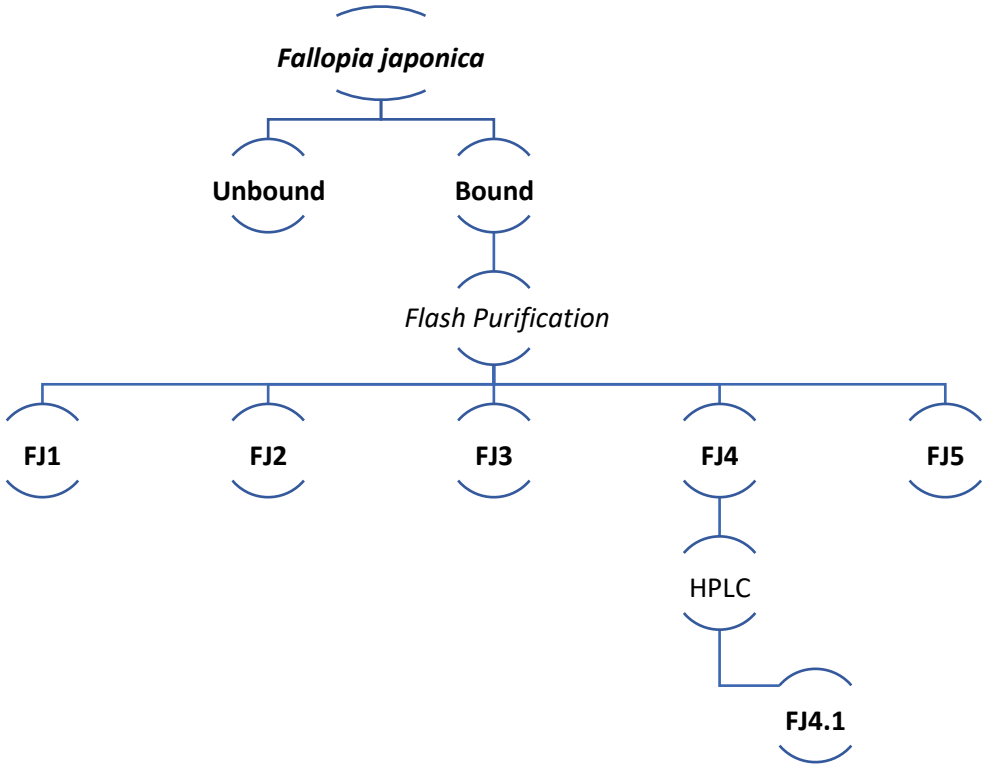


Figure 10: Summary of the purification of F. japonica

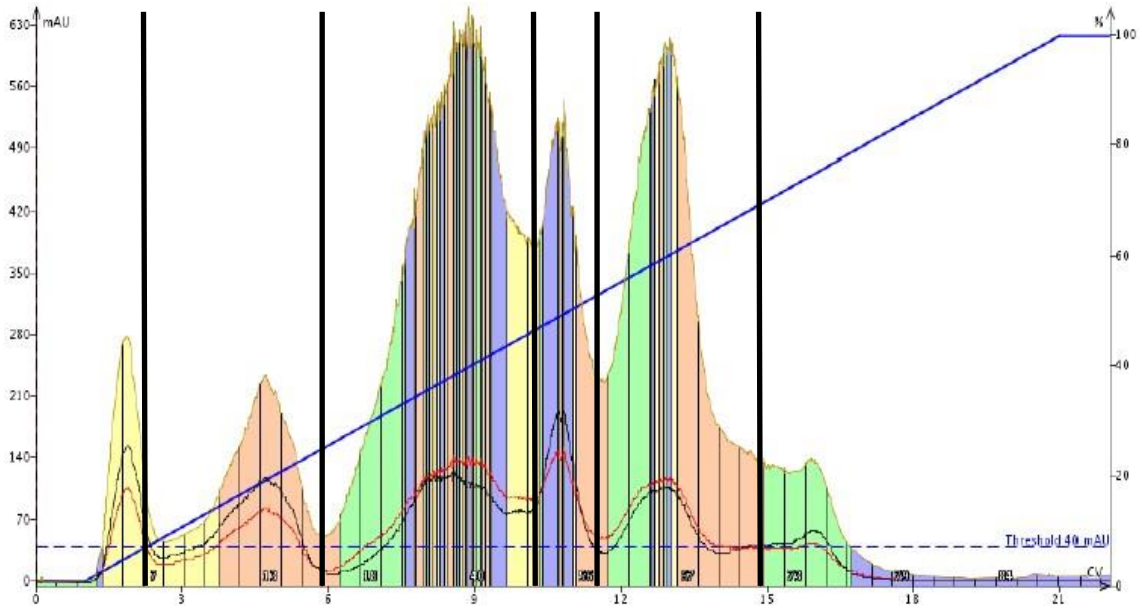


Figure 11: Flash purification of F. japonica material which was separated into 6 fractions denoted by the vertical black lines. The coloured area of the graph represents the total UV absorption of the analytes leaving the column at that time. The red line represents UV absorption at 280 nm and black line represents UV absorption at 330 nm. Colour changes indicated that the flash purification system has detected a significant change in UV absorption which could indicated a different analyte being eluted. The blue line represents the increasing gradient of methanol over time.

1.3.2.2 – *Fallopia japonica* – Identification of compounds

Five fractions of *F. japonica* were obtained using flash purification each was analysed using HPLC-PDA-ESI-MS/MS (Appendix 2). Each major peak present in both PDA and negative mode mass chromatogram were summarised in Table 7.

Table 7: Summarised HPLC_PDA_ESI_MS/MS data of the 5 fractions obtained from F. japonica

Fraction	RT (min)	PDA λ max (nm)	MS1 $[M-H]^-$ ions (*m/z*)	MS2 $[M-H]^-$ ions (*m/z*)	Tentative identification
FJ1	5.5	310	336.89	190.78, 162.76	*Trans*-5-*para*-coumaroylquinic acid
	6.46	323	366.99	192.80, 193.85, 172.81, 133.77	Feruloylquinic acid

	6.65	325	352.94	191.96, 190.82, 178.82	3-*O*-caffeoylquinic acid
	7.96	313	294.76	162.74, 148.70, 130.59	*Para*-coumaroyl-pentose
	8.88	327	324.86	192.83	Feruloyl tartaric acid
FJ2	8.29, 9.29	311	336.89	190.78, 162.76	*Trans*-5-*para*-coumaroylquinic acid
	7.9	279	578.69	301.03, 288.87, 245	Naringin
	10.5	314	308.87	276.81 162.77, 144.72	Unknown
FJ3	9.31	304	434.75	388.74, 227.06	Resveratrol glycoside
FJ4	10.08	306	435.75	388.74, 227.06	Resveratrol glycoside
FJ5	12.48	256, 354	609.06	300.86	Unknown
	13.34	256, 353	478.47	337.22, 460.14	Myricetin-*O*-hexoside
	13.6	256, 349	492.48	472.98, 447.07, 286.90	Patulitrin

1.3.2.3 – *Fallopia japonica* – Biological Assays

Antimicrobial assay was carried using the *F. japonica* fractions in the same way as for *I. glandulifera* for comparison (**Error! Reference source not found.**). Fractions 3,4 and 5 showed the

most significant activity against *S. aureus*, however this activity was far below that seen in *I. glandulifera*.

Table 8: Summary of all flash purification fractions (1 mg/mL) antimicrobial activity expressed as percentage inhibition after 24 hours of growth (n=3)

Fraction	*E. coli* (ATCC 25933)	*P. aeruginosa* (ATCC 700603)	*S. aureus* (ATCC 29213)	*B. subtilis* (ATCC 6633)
FJ 1	2	0	0	0
FJ 2	4	0	0	0
FJ 3	13	0	39	0
FJ 4	8	0	37	0
FJ 5	9	0	30	0

Due to the low level of antimicrobial activity seen in these fractions no further purification was carried out and these fractions were not assayed against other bacteria species. However, the Hoffmann Research Laboratory offered their services to Life Sciences Research Network Wales projects. They have a high-throughput screening platform called Roboworm, which tests the ability of compounds to inhibit the viability of a larval schistosomula. As these fractions were plentiful and not as important as the IG5.2 fraction, which was critical, these fractions were a good opportunity to trial this assay. As these fractions were not active against bacteria, there is no reason they would not be a potent inhibitor of *Schistosoma mansoni* schistosomulae. Figure 12 shows that JK5 manage to significantly affect the motility of the schistosomulae but unfortunately did not affect the phenotype enough to be considered a hit compound and this was not investigated further.

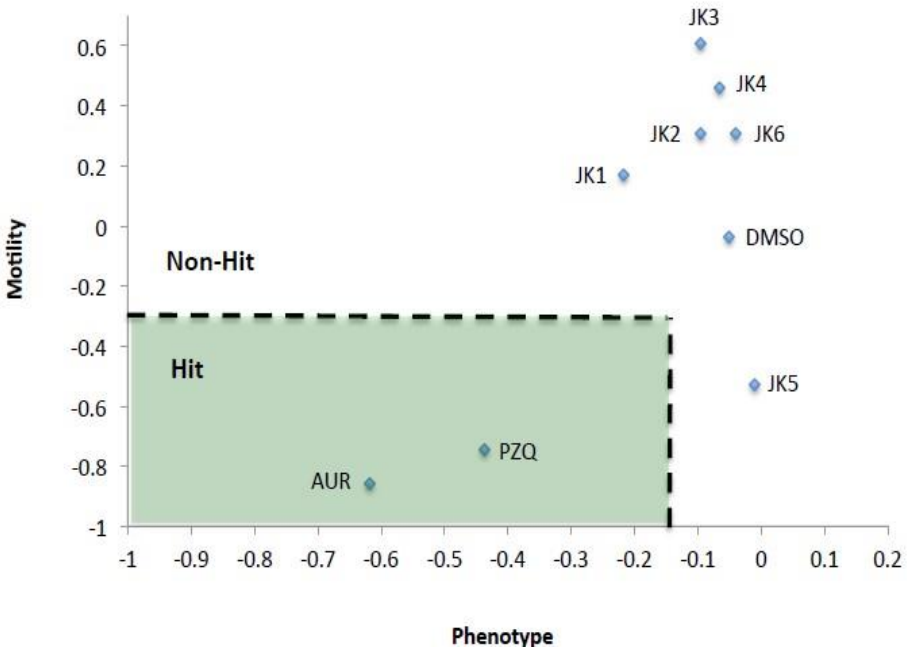


Figure 12: Anti-helminthic activity of F. japonica fractions against S. mansoni. There are two parameters measured, motility and phenotype. In order to regarded as an active compound (a hit within the green area) the compound must score below -0.15 for phenotype and below -0.3 for motility. Auranofin (AUR) and Praziquantel (PZQ) are proven anti-schistosomal drugs.

1.3.3 – *Rhododendron ponticum*

1.3.3.1 – *Rhododendron ponticum* - Isolation of compounds

As for the previous two plants a summary of the purification carried out is shown in Figure 13 and Figure 14 shows the flash purification of *R. ponticum* which was carried out in the same way as previous sections using a C18 column after SPE.

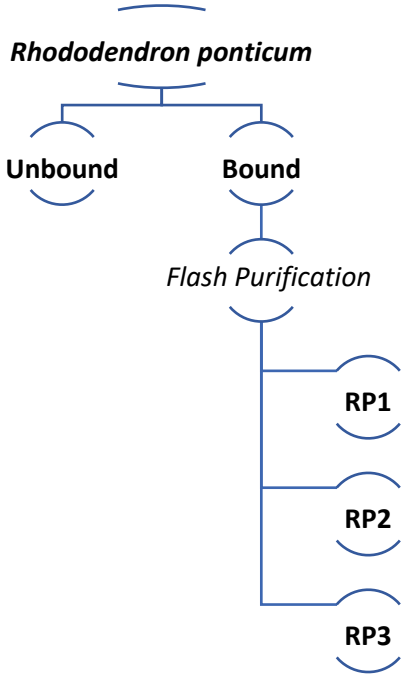


Figure 13: Summary of the purification of R. ponticum

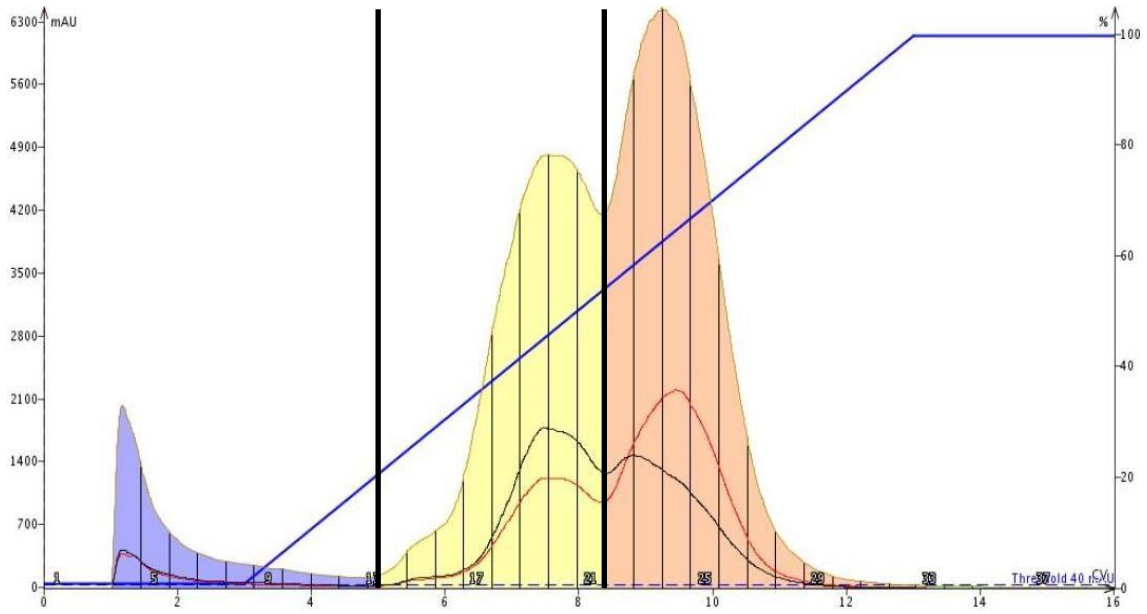


Figure 14: Flash purification of R. ponticum material which was separated into 3 fractions denoted by the vertical black lines. The coloured area of the graph represents the total UV absorption of the analytes leaving the column at that time. The red line represents UV absorption at 280 nm and black line represents UV absorption at 330 nm. Colour changes indicated that the flash purification system has detected a significant change in UV absorption which could indicated a different analyte being eluted. The blue line represents the increasing gradient of methanol over time.

1.3.3.2 – *Rhododendron ponticum* – Identification of compounds

Three fractions of *R. ponticum* were obtained using flash purification each was analysed using HPLC-PDA-ESI-MS/MS (Appendix 3). Each major peak present in both PDA and negative mode mass chromatogram were summarised in Table 9.

Table 9: Summarised HPLC_PDA_ESI_MS/MS data of the 3 fractions obtained from R. ponticum (-PDA data not available)*

Fraction No.	RT (min)	PDA λ max (nm)	MS1 [MH]- ions (*m/z*)	MS2 [MH]- ions (*m/z*)	Tentative identification
1	1.96	270	204, 338.76	168.99, 468.88	Unknown
	4.57	296, 323	353.07	190.92, 178.92	5-hydroxy-6-methoxy-3'-4'-methylenedioxyfuranol [2",3":7,8] flavanone
	5.433	287	370	324.93, 162.95	Unknown
	5.88	287, 298	337.06	163	2-O-acetyl-trans-coutaric acid
2	1.96	N/A*	168.99	124.88	Unknown
	3.27	N/A*	593	425	Quercetin 2-glucoronide sulphate
	4.74	N/A*	577.01	424.99	Kaempferol 3-glucoronide-7-sulphate
	5.03	N/A*	577.01	424.99	Kaempferol 3-glucoronide-7-sulphate
	5.89	N/A*	337.06	163	2-O-acetyl-trans-coutaric acid
	6.61	N/A*	483.08	207.98	Artonin P
	7.58	N/A*	502.65	456.94	Unknown
	8.12	N/A*	334.86	288.98	Unknown
	10.27	N/A*	373.04	327.04	Ovalitenone
	10.94	N/A*	479.05	315.95, 316.98	Myricetin 3-galactoside
	11.87	N/A*	463.07	315.97, 316.94	Catechin 3-O-(1-hydroxy-6-oxo-2-cyclohexane-1-carboxylate)
3	9.78	288	243.11, 259.05	N/A	Unknown
	10.28	251, 340	259.1	N/A	Dihydropyrans

	10.94	255, 357	243.14	N/A	Geranyl benzoquinone
	11.85	256, 352	258.97	N/A	Caffeic acid-3-sulphate
	12.38	255, 354	259.08	N/A	Dhelwagin
	13.53	256, 354	249.06	N/A	Lunularin

1.3.3.3 – *Rhododendron ponticum* – Biological Assays

As for the other two plants the same antimicrobial assay was carried out for *R. ponticum* (Table 10).

Table 10: Summary of all flash purification fractions (1 mg/mL) antimicrobial activity expressed as percentage inhibition after 24 hours of growth (n=3)

Fraction	*E. coli* (ATCC 25933)	*P. aeruginosa* (ATCC 700603)	*S. aureus* (ATCC 29213)	*B. subtilis* (ATCC 6633)
RP 1	25	46	20	37
RP 2	19	58	37	55
RP 3	8	22	13	11

R. ponticum fraction 1 and 3 showed barely any activity against any bacterial species when carrying out a 24-hour assay. Although RP2 showed moderate antimicrobial activity across all four bacterial species. An MIC assay was carried out using RP2, although the antimicrobial activity was not enough to kill the bacteria at a concentration tested. In order to display this data in a meaningful way the minimum concentration of RP2 required to limit the growth of the bacteria to 50% and 90%. This is referred to as the minimum limiting concentration (MLC) (Table 11). The concentration of RP2 required to inhibit the growth of all four bacteria is relatively poor compared to that of *I. glandulifera*. Although activity was relatively poor RP was consistently active against all four species, which was not seen in any other plant. The activity seen against *P. aeruginosa* was particularly good considering the other plant fractions were unable to affect the growth of this bacteria in the slightest.

Table 11: The minimum concentration of R. ponticum fraction 2 required to limit growth of four bacterial species by 50 and 90% over 24 hours (mg/L) (n=3)		
Bacterial Species	**MLC50**	**MLC90**
E. coli	740	1340
S. aureus	530	950
P. aeruginosa	470	840
B. subtilis	450	810

To increase the breadth of the biological testing carried out the *R. ponticum* fractions were also assayed for their ability to inhibit the growth of Candida albicans. This opportunistic pathogenic yeast is common in human mouths and throughout the gastrointestinal tract. These yeast cells are morphologically like bacteria and able to develop the antimicrobial susceptibility testing to be able to also assay C. albicans (Figure 15).

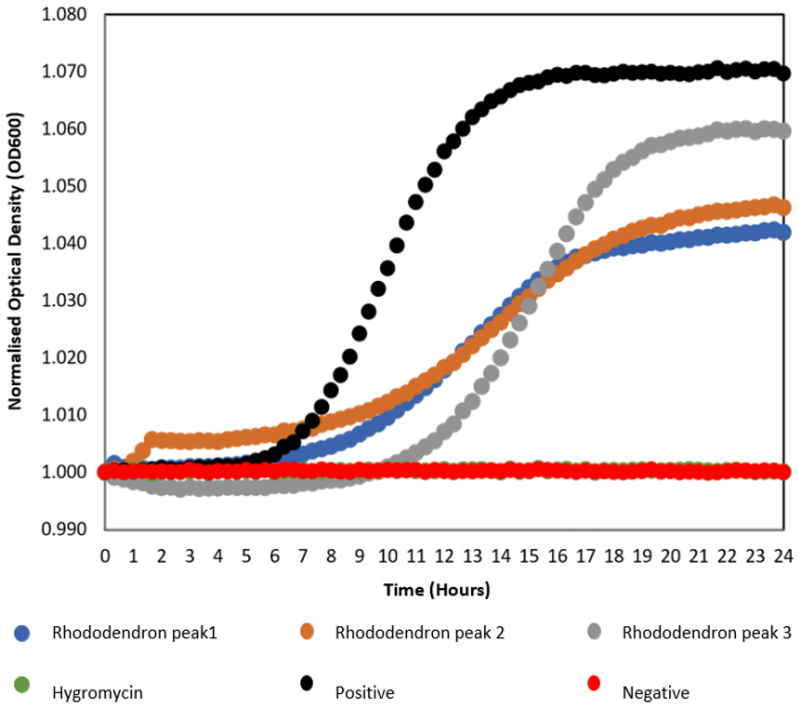


Figure 15: The 24-hour growth curve of C. albicans ATCC 102313 in the presence of three R. ponticum fractions (1 mg/L), hygromycin as an antifungal control, with no inhibiting compounds as a positive control and no fungi as a negative contamination control.

None of the fractions showed significant activity, with RP1 showing the best antifungal activity with an MLC50 of 1.4 mg/L, which was calculated based on the linear extrapolation of the single concentration tested.

1.4 - Discussion – Natural Products

1.4.1 – *Impatiens glandulifera*

I. glandulifera fractions were found to be effective against *E. coli*, *S. aureus* and *B. subtilis* at 1 mg/mL. Fraction IG5 was by far the most active fraction overall; 55%, 95% and 48% inhibition respectively yet it was unable to affect *P. aeruginosa* (Table 3). Further purification was carried out on IG5 which yielded two fractions, IG5.1 and IG5.2. Of these two fractions IG5.2 was found to be an extremely potent inhibitor of MRSA, *Bacillus anthracis*, *B. subtilis*, *Salmonella*, and *C. difficile* (Table 4 and Table 5). The best activity was seen against MRSA, with an MIC of 23 mg/L. A range of antibiotics can be used to treat MRSA infections each with ranging MIC values depending on the strain; clindamycin (0.125-0.5 mg/L), daptomycin (0.063-4 mg/L), Doxycycline (0.25-32 mg/L), Erythromycin (0.25-<64 mg/L), Linezolid (2-32 mg/L), trimethoprim-sulfamethoxazole (0.06-20 mg/L), and vancomycin (1-4 mg/L) (Fowler et al., 2006; Kalil et al., 2014; LaPlante et al., 2008). Comparing the MIC of MNQ to currently used antibiotics it is around 10-fold less potent. However, activity can be dependent on the strain being used. The above studies did not use USA300 so it cannot be directly compared. Further strains will need to be tested to fully understand the potential of MNQ. Only IG5.2 has been tested, which is a plant extract, commercially available MNQ has been purchased and further screens can be carried out using this. Looking at the mass chromatograms Figure 8 and Figure 9, the purchased MNQ has a cleaner chromatogram than the plant extract. This increase in purity could improve the potency to rival the efficacy of antibiotics such as vancomycin.

Using a combination of HPLC-PDA-HRMS and NMR (Table 6 and Figure 8) IG5.2 was identified as 2-methoxy-1, 4-naphthoquinone (MNQ). This compound has been isolated previously from *Impatiens* balsamina which is a close relative of *I. glandulifera*, and MNQ was also shown to inhibit the growth of bacterial species *Staphylococcus aureus*, *Bacillus cereus*, *B. megaterium, B. subtilis Aeromonas salmonicida*, *Aquaspirillum serpens* and fungal species *Candida albicans*, *Fusarium oxysporum*, *Aspergillus fumigarus*, *Macrospore gypseum*, *Trichophyton mentagrophytes* (Yang et al., 2001). The activity of MNQ against *S. aureus* in this paper was found to be 16 mg/L which is marginally better than this study. This could be due to the strain or the presence of impurities as discussed earlier.

1.4.2 – *Fallopia japonica*

The most active fractions FJ3 & FJ4 isolated from *F. japonica* showed moderate antimicrobial activity against *S. aureus* (Table 8). The major compounds present in these fractions were identified as resveratrol glycoside using HPLC-PDA-ESI-MS/MS (**Error! Reference source not found.**). According to the mass spectrometry data, FJ3 and 4 yielded a base ion of m/z 435 and secondary fragmentation ion of 389 [(M-H)-46]- which is consistent was a formate adduct formed due to there being formic acid in the mobile phase. Therefore, the molecular ion is 389 [M-H]-. There is further fragmentation from m/z 389 to 227 which is a loss of m/z 162 which is consistent with the loss of a hexose sugar in literature (Jerkovic et al., 2007; Vukics and Guttman, 2010). FJ3 and 4 have different retention times although they share the same mass spectra, resveratrol has two isomers in cis and trans conformation and these effects the retention time on C18 column chromatography (Lamuela-Raventos et al., 1995).

Resveratrol has been identified in over 70 plant genera and has been shown to have many beneficial biological activities; antimicrobial activity, anti-inflammatory, anticancer, and antioxidant activity (Frémont, 2000; Joe et al., 2002; Pervaiz, 2003). The presence of resveratrol in *F. japonica* is well documented as being considered as a commercially viable source of raw material for resveratrol

production as well as other anthraquinones such as emodin and physcion (Chen et al., 2013). Due to the fact resveratrol and *F. japonica* are so well documented no further research was carried out on *F. japonica*.

1.4.3 – *Rhododendron ponticum*

All three *R. ponticum* fractions were consistently active against *E. coli*, *P. aeruginosa*, *S. aureus*, *B. subtilis* and the fungi C. albicans. Although the level of activity was relatively low compared to other fractions, *R. ponticum* was the only plant which was able to inhibit the growth of *P. aeruginosa*. Although level of activity was not significant, to see any activity against the gram-negative bacterium *P. aeruginosa* is interesting as this suggests the compounds within this fraction of *R. ponticum* are showing a different pattern of antimicrobial activity to that found in the other plant extracts.

The compounds tentatively identified in RP2 using HPLC-PDA-ESI-MS/MS were mainly flavonoid, flavonoid glycosides and related compounds, because there was characteristic mass fragmentation which matches that see in the literature (Vukics and Guttman, 2010) (Table 9). Flavonoids and their glycosides have been previously reported and were shown to have anti-inflammatory, antinociceptive (Erdemoglu et al., 2008), antiapoptotic (Zhang et al., 2010). The antibacterial and antifungal activity of *Rhododendron* species have been previously studied (Ertürk et al., 2009). The activity seen previously was tested using plate diffusion assays and the activity seen in our study exceeds that seen by Ertürk et al. This difference could be accounted due to different methods. However, the increased activity in the UK variety could be due to the UK variety being invasive. As an invasive species this plant is not exposed to its natural predators and pests which would be present in Turkey. The predator-prey relationship would have evolved over time to keep both species in check, once the predator is removed by moving the plant to the UK this plant is able to grow faster and be more aggressive. Without the stress of the predator the UK variety may

be able to produce different bioactive, or in different quantities which cause a more antimicrobial extract. This could be an example of the ERH in action.

Unfortunately, the flash purification of *R. ponticum* did not yield as many fractions as the other two plants. Fortunately, in the case of the other two plants a relatively pure, biologically active fraction was yielded, identified and assayed. The issue with carrying out bioactivity led purification when all fractions show relatively low activity it is difficult to choose which fraction to purify further. Ideally a better first round of purification should have been carried undertaken, this could have been done by either utilising a longer, slowed gradient which would have separated peaks better or a customised step gradient. A step gradient approach would involve running a series of isocratic elutions only increasing the percentage of organic solvent when it looks like there are no more analytes being eluted. This could have easily been applied to purification using the Biotage flash purification systems PDA detector.

1.5 – Conclusion

The focus of this project is to discover novel drug candidates for the treatment of MRSA. The aim of this chapter is to screen a range of problematic invasive weed species for molecules which show antimicrobial activity. Antibiotic resistance is a particularly big issue facing society and MRSA is the most high-profile pathogen of all. If a compound can be found which is able to treat this infection, then this will be the highest priority of all.

The main candidate compound found in *F. japonica* was tentatively identified as a Resveratrol glycoside. This compound is well documented and has a broad range of bioactivities. However, the activity against *S. aureus* isn't particularly impressive and does not have any potential to be a future treatment for MRSA infections. Therefore, no further research was carried out on *F. japonica*.

R. ponticum showed a low level of activity against all bacteria and fungi. This is interesting as no other compounds had any impact on the growth of *P. aeruginosa*. The quality of the purification of the *R. ponticum* was not as effective as the other plants. No single compounds were isolated or identified. The low level of activity could be due to active compounds being diluted by the presence of other non-active compounds being present in the fractions. This plant has the potential to produce an active inhibitor of gram-negative bacterial growth. However, this is not the focus of this project and *R. ponticum* did not show significant activity against *S. aureus*.

A potential drug has been found within *I. glandulifera*, in the form of MNQ. This exciting molecule has been shown to have antimicrobial activity far beyond any other found within this project. It was found to have a broad range of activity against a range of pathogenic bacteria but showed particularly potency against *S. aureus* and MRSA. This molecule will be the focus of this project going forward.

Chapter 2 Lead Compound MNQ bioactivity and Drug suitability

2.1 Introduction – MNQ Bioactivity

2.1.1 – Chapter Aims

The major outcome of the first chapter was the isolation of MNQ from *I. glandulifera* which was shown to have potent antimicrobial activity, especially against MRSA which was comparable to that of current antibiotics. The known bioactivity of naphthoquinones will be discussed to understand the potential of this molecule as a future antibiotic. MNQ has been tested against a relatively small range of bacteria. To comprehensively understand the antibacterial profile, a broad range of clinically relevant bacterial species and strains must be tested. Although MRSA is the focus of this project it is important to understand the full bioactive ability of this molecule. *Schistosoma mansoni* is a pathogenic parasite which needs new treatment options, therefore MNQ will be tested against three stages of the *S. mansoni* life cycle. Cytotoxicity is vital factor for any potential drug, MNQ must show selective toxicity against bacteria so a range of tests must be carried out to assay the toxicity of MNQ.

2.1.2 Bioactivity of Naphthoquinones

Naphthoquinones (NQ) are a class of organic compounds derived from naphthalene, the simplest polycyclic aromatic hydrocarbon, with the addition of two ketones. These carbonyls can be in various positions; 1, 2-NQ and 1, 4-NQ:

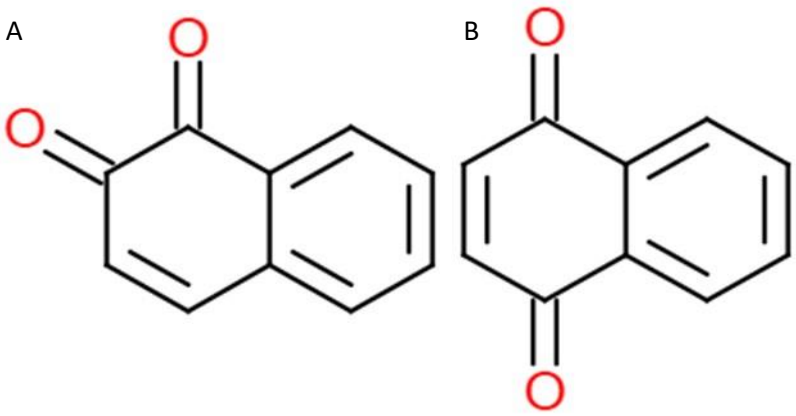


Figure 16: Chemical structure of 1,2-naphthoquinone (A) and 1,4 naphthoquinone (B)

2.1.2.1 - 1, 2-Naphthoquionines

A range of 1, 2-NQ derivatives have been found to show potent anti-diabetic activity based on their ability to inhibit protein tyrosine phosphate 1B (PTP1B). PTP1B pays a role in the negative regulation of insulin signalling and is involved in the insulin resistance associated with Type-2 diabetes (Kennedy and Ramachandran, 2000). 1, 2-NQ itself with no alteration was considered a hit with an IC50 of 1.64 µM. Thirty-one 1, 2-NQ derivatives were synthesised by Ahn et al. with alteration made to the 4 position (to increase stability by preventing Michael type nucleophilic addition) and subsequently evaluated for their in vitro inhibitory activity against recombinant human PTP1B. 5 of the 31 derivatives were completely inactive, 10 showed improved activity verses the original NQ and 17 were still active but no improvement on the original 1, 2-NQ core. The addition of aryl groups significantly improved antidiabetic activity of 1, 2-NQ derivatives and the addition of fluorine or hydroxide groups to the aryl further improved potency. Addition of a cyclohexyl group, proved to be the most potent (IC50 of 0.32 µM) but other alkyl substitutions only decreased activity (Table 12) (Ahn et al., 2002).

Table 12: Inhibitory activity of 1,2-naphthoquinone derivatives against PTP1B (Ahn et al.,2002)

Position 4 derivative	**IC_{50}**
Cyclohexyl	0.32
C_6H_4–4-OH	0.44
C_6H_3–2,5-F_2	0.5
C_6H_5	0.86
C_6H_4–4-OCH_2CO_2Et	1.07
3-Indole	1.13
C_6H_4-2-NO_2	1.17
Benzyl	1.42
$C_6H_4COOCH_3$	1.54
C_6H_4–2-OH	1.6
C_6H_4–2-OCH_2CO_2Et	2.15
1-Naphthyl	2.15
3-Indole-5-carboxylic acid	3
$(CH_2)5CH_3$	3.3
Cyclopentyl	4.2
3-Indole-6-carboxylic acid	4.56
C_6H_5–2,5-Cl_2	5.05
$C_6H_5OCH_3$	5.24
SO_3Na	5.29
Biphenyl	5.4
C_6H_2-3,5-di-t-butyl-4-OH	5.73
Isopropyl	10.13
NH_2	24.59
OCH_3	29.11
N(CH_3) C_6H_5	34.88
$OCH_2CH_2CH_2OH$	36.47
$N(CH_2)_4$	N/A
3-Indole-1,2–$(CH_3)_2$	N/A
–3-Indole-2–C_6H_5	N/A
Butyl	N/A
Decyl	N/A

1,2-NQs are also known to disrupt the effects of other Protein Tyrosine Phosphatases (PTP) which are involved in a wide range of biological functions. The presence of two neighbouring

carbonyls which is seen in all 1,2-NQs is essential for PTP inhibition which (Urbanek et al., 2001). For example, CD45 is involved in cellular growth, differentiation, mitotic cycle and oncogenic transformations, and is critical for T-cell receptor mediated T-cell activation. Derivatives of 1, 2-NQ with aryl alterations at the position 4 were found to be potent inhibitors of CD45 and T-cell proliferation in vitro. It is suggested that the 1, 2 NQs carbonyl of the dione attaches to the active site cysteine851 of CD45 rendering the enzyme catalytically inactive. However, removal of the vicinal carbonyl groups (replaced with Fluorine) yielded no activity and 1,4-NQs showed poor activity implicating additional mechanisms of action. Nocardione A (1,2-NQ with a hydroxyl group at position 7 and a pentose attached directly onto positions 3 and 4) inhibit three different type of PTPs; CDC25B (involved in cell proliferation), PTP1B (T-cell activation), and FAP-1 (linked to cell death and apoptosis) (Otani et al., 2000).

1,2-NQ have been shown to have anti-tumour activity. 1,2-NQ with thiosemicarbazone (TSC) group in the 2-position showed significant inhibitory activity against MCF-7 human breast cancer cells. Unaltered 1,2-NQ had an IC50 of 13.37 μM, the addition of TSC significantly improved the activity with an IC50 of 3.14 μM. Further improvement can be made by forming a NQ-TS Ni2+ complex which has an IC50 of 2.25 μM. Other metal complexes were formed with Cu and Pd, but these marginally decreased the activity (Chen et al., 2004).

2.1.2.2 - 1, 4-Naphthoquionines

1,4-Naphthoquinones have a wide variety of biological activities including antibacterial and anticancer. For example, shikonin is a major component of *Lithospermum erythrorhizon* which is a Chinese herbal medicine with various biological activities, one of which is the inhibition of human immunodeficiency virus (HIV) type 1-induced cytopathology (Chen et al., 2003). Four 1 ,4-NQ derivatives have been assessed for anti-fungal activity based around shikonin (5,8-dihydroxy-6-(1-hydroxy-4-methyl-3-pentenyl)-1,4-naphthalenedione). These derivatives at position 6; include the additions of either an acetyl group, a hydroxy-iso-valeryl group or removal of the hydroxy group.

Each derivative showed decreased potency against a range of fungal species, compared to shikonin which showed similar levels of activity to fluconazole, a first generation triazole (Sasaki, Abe and Yoshizaki, 2002). This illustrates that natural selection can produce the most bioactive derivative so any synthetic changes have the potential to reduce potency.

Several 1, 4-naphthoquinoe derivatives have also been tested for anti-cancer activity against Walker 256 carcinosarcoma implanted into rats. Two active derivatives were 2,3bis(phenylsulfanyl)-1,4-naphthoquinone and 5,8-dihydroxy-2-methyl-1,4-naphthoquinone (Tandon et al., 2004). While another 1,4-naphthoquine derivative, S-(1,4-naphthoquinone-2-yl)-mercaptoalkanoic acid amide, was active against Lymphoid leukaemia P 388 in rats (Tandon, Singh and Yadav, 2004).

For anti-viral activity two 1,4-NQs; S-(1,4-NQ-2-yl)-5,8-dihydroxymercaptoalkanoic acid with an additional hydroxyl group and 5,8-dihydroxy-2-arylthio-1,4-NQ showed in vitro anti-viral activity against influenza-A virus and Herpes simplex virus. (Tandon, Singh and Yadav, 2004).

NQs have been shown to be useful in areas other than biology; 1,4-NQ has been shown to inhibit the corrosion of aerated and de-aerated aluminium in sodium chloride solutions. Showing that the presence of this naphthoquinone reduced pitting potentials, decreased anodic currents, and surface polarisation resistances are increased. The most effective concentration of naphthoquinone was found to be 1x10-3 M (Sherif and Park, 2006). Combining the broad antibacterial activity and the anti-corrosion activity of 1,4-NQs means this molecule could have potential as a cleaning solution for medical devices.

2.1.3 Biosynthesis of naphthoquinones

NQs are a diverse group of natural products naturally occurring in many plant species, fungi and even bacteria. If MNQ is to be considered as potential antibiotic it is important to understand how this molecule is produced by plants and what purpose it serves.

The o-succinylbenzoate (OSB) pathway which begins with chorismate and after 9 reactions results in MNQ (Figure 17). The OBS pathway was first discovered in the 1960s when [14C]-shikimate fed to *E. coli* (Cox & Gibson, 1964) and maize shoots (Whistance, Threlfall & Goodwin, 1966) to investigate the biosynthesis of vitamin-K. Tracer studies carried out using I. balsamina and [U-14C]-α-ketoglutarate showed that this was incorporated into lawsone (Grotzinger & Campbell, 1972). Although this pathway has been studied for many years many reactions are not fully understood.

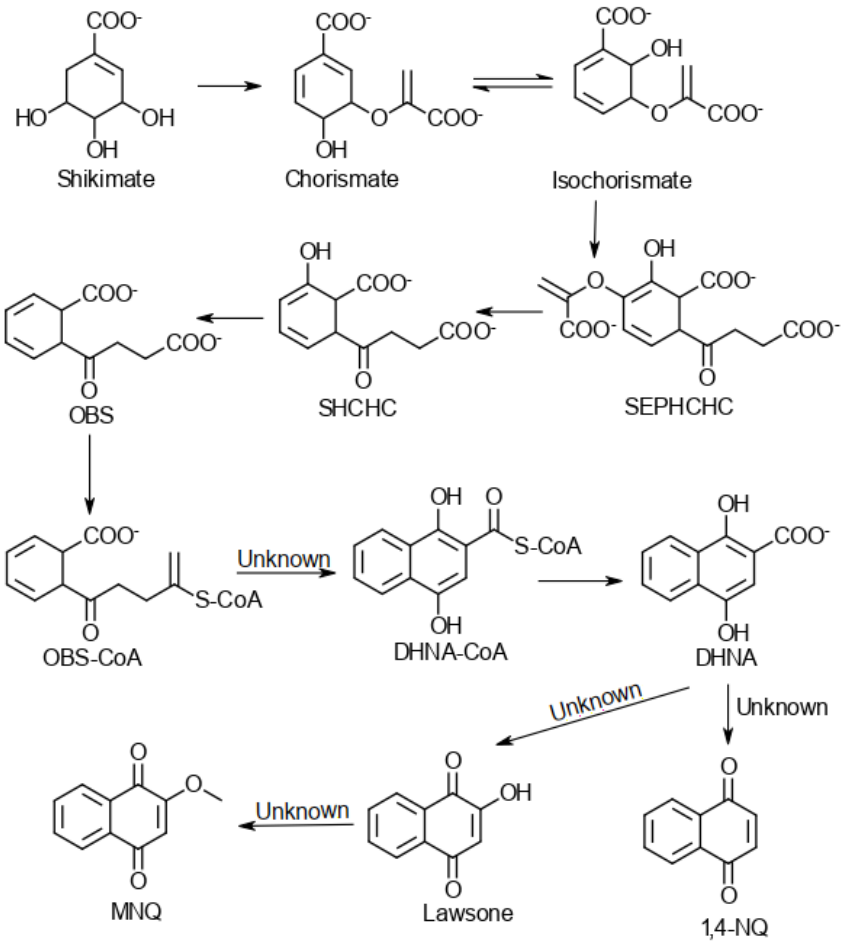


Figure 17: The o-succinylbenzoate (OSB) biosynthetic pathway producing 2-methoxy-1,4-naphthoquinione (MNQ) from shikimate via 10 reactions. The first 6 steps convert shikimate into chorismate, then isochorismate, then 2,2-succinyl-5-enolpyruvyl-6-hydroxy-3-cyclohexane-2-carboxylate (SEPHCHC), then 3, 2-succinyl-6-hydroxy-2,4-cyclohexadiene-2-carboxylate (SHCHC), and finally OBS into OBS-coenzyme A (CoA). These steps have genetic evidence to support these reactions. The reaction which converts OBS-CoA to dihydroxynaphthoyl (DHNA)-CoA has a lack of supporting genetic evidence to be sure this is the correct reaction. All further reactions from DHNA into other naphthoquinones have little supporting genetic information and the enzymes responsible for these reactions are unknown.

The pathway begins with chorismate which is derived from shikimate, an important metabolite for the biosynthesis of aromatic amino acids (Herrmann & Weaver, 1999). The first reaction is the isomerisation of chorismate to isochorismate by isochorismate synthase (Garcion et al., 2008). Followed by the addition of α-ketoglutarate which is catalysed along with the next two reactions by the trifunctional enzyme PHYLLO; containing 2-succinyl-5-enolpyruvyl6-hydroxy-3-cyclohexene-2-carboxylate (SEPHCHC) synthase, 2-succunyl-6-hydroxy-2,4-cyclohexadiene-2-carboxylate (SHCHC) synthase, and OBS synthase domains (Gross et al., 2006). This addition of α-ketoglutarate results in SEPHCHC (Grotzinger & Campbell, 1972), followed by the 2,5-elimination of pyruvyl side chains to form SHCHC (Jiang et al., 2008), and dehydration to form OSB (Meganathan & Bentley, 1983). The ring (now aromatic) will form one of the rings of the naphthalene. OSB-Coenzyme A (CoA) ligase attaches a CoA group to the succinyl side chain of OSB which is then cyclised by dihydroxynaphthoic acid (DHNA)-CoA synthase to form the second ring of the naphthalene core in DHNA-CoA (Kwon, Bhattacharyya & Meganathan, 1996). The removal of CoA by hydrolysis catalysed by thioesterases results in DHNA (Meganathan, 2001). At this point there is a significant fork in the pathway, where DHNA can be converted into phylloquinones (Lohmann et al.,2006), anthraquinones (Eichinger et al., 1999), juglone (Leistner & Zenk, 1968), and most importantly for this project lawsone which is a precursor of MNQ (Zenk & Leistner, 1967). Lawsone is formed into MNQ via an S-adenosylmethionine-dependent O-methyltransferase (Liscombe, Louie & Noel, 2012).

There are two other NQ producing metabolic pathways stemming from chorismate, the homogentisate (HGA)/mevalonic acid (MVA) pathway which produces chimaphillin (Bolkart & Zenk, 1969) and the 4-hydroxybenzoic acid/MVA pathway yields deoxyshikonin, shikonin, alkannin and other derivatives (Schmid & Zenk, 1971). One other metabolic pathway, the acetate-polymalonate pathway, can produce NQs. These are plumbagin, droserone, 5-O-methyldroserone and 7-methyljuglone although the reactions and enzymes responsible for these NQs are unknown (Durand & Zenk, 1971).

NQs are synthesized by organisms throughout all kingdoms of life involved in a range of metabolic processes. For example, within plants and algae 1,4-NQs are involved in photosynthesis although this is not linked to their bioactivity which is not relevant to this project (Brettel, Sétif & Mathis, 1986). NQs produced by certain plants have an allelopathic effect on the plants around them, an example of this would be the phytotoxic effect of juglone from *Juglans nigra*. By excreting this NQ into the surrounding area to prevent growth of other plants (Soderquist, 1973). This allelopathic activity has also been observed in *I. glandulifera* and the compounds responsible is thought to be MNQ, this is likely a contributing factor to this plant's successful invasion of most of the norther hemisphere (Ruckli et al., 2014). NQs produced by plants also interact with bacteria and fungi to the benefit on the plant. As has been clearly shown MNQ has significant antimicrobial activity and this will protect *I. glandulifera* from any bacterial of fungal infections. MNQ has been shown to be an effective inhibitor of Ecdysone 20-Monooxygenase, which is the insect cytochrome P450-dependent hydroxylase which plays a pivotal role in insect reproduction and development (Mitchell et al., 2007). Many species of fungi produce a wide range of different NQ which cause pigmentation of fruiting bodies for sexual reproduction as well as protection from UV light and insects (Studt et al., 2012). They are also produced in reaction to unfavourable factors in the environment, combining this with their broad range of biological activity it can assumed these compounds play a protective role (Medentsev & Akimenko, 1998; Darvill & Albersheim, 1984). Within the bacteria Actinomycetes numerous 1,4-NQs are produced which have a range of bioactivity seen previously but these are also used as precursors to produce polyketides which also have a broad range of bioactivities with protective properties (Moore & Hopke, 2001).

The natural purpose of NQs are for protections from other organisms, whether that be plant versus plant in the case of allelopathy see in *I. glandulifera*, or plant versus pest in the case of MNQ inhibiting insect development enzymes, or fungi producing NQs for a selective advantage over competitors in unfavourable environments. Many antibiotics, most famously penicillin, are produced by fungi in order to harm surrounding competitors to gain a selective advantage (Losada

et al., 2009). Antibiotic compounds such as penicillin are isolated and utilised by humans to destroy infectious pathogens. NQs seemingly serve the same protective purpose within plants, fungi and bacteria; therefore, it can be assumed these molecules can also be utilised to treat infections.

2.1.4 Bacterial Cell Membranes

Bacteria can be grouped generally into three groups based on their membranes; gram-negative, gram-positive and mycobacteria. Each offer different challenges for antibiotic compounds which need to be overcome to prevent the growth of bacteria. These structures can influence the efficacy of drugs with many antibiotics being selective for gram-positive, gram-negative, mycobacteria or in some cases broad spectrum covering a wide variety of bacteria. These factors are important to consider when determining the antimicrobial profile of MNQ.

A wide range of bacterial species and strains will be tested in this chapter tested for susceptibility against MNQ to evaluate its potential as an antimicrobial chemotherapeutic agent. Two factors which are important when selecting which bacteria to screen is variation in cell wall composition and possible resistance mechanisms. These can provide information on the suitability of the potential drug to a therapeutic use and any known resistance mechanism which can thwart its activity. Although MRSA is the focus of this project the bioactivity of MNQ is considerable, so to fully understand its potential a broad range of bacteria will be tested with different barriers to overcome.

Gram negative cell envelopes are made up of three principle layers; the outer membrane (OM), the peptidoglycan cell wall, and the inner membrane (IM). Between these two concentric membranes are areas called periplasmic spaces. The OM is a lipid bilayer composed of phospholipids on the inner leaflet with the outer composed of glycolipids, principally, lipopolysaccharides (LPS) (Kamio and Nikaido, 1976). The OM is crucial for the survival of *E. coli* and other gram-negative bacteria, yet its only known function is to serve as a protective barrier. It is known to contain enzymes such as phospholipase, protease, and enzymes which modify LPS, but these are not

essential as mutants lacking these shown no striking phenotype (Silhavy, Kahne and Walker, 2010). There are two classes of protein embedded within the OM; lipoproteins and β-barrel proteins which are involved in bacterial growth (Wu et al., 2006). There are about 100 lipoproteins encoded by in *E. coli* and the function of most are unknown (Miyadai et al., 2004). Most of the integral transmembrane proteins are assumed to be β-barrel proteins, they are formed of β-sheets wrapped into cylinders which cross the OM. These are also referred to as porins and they allow passive diffusion of small molecules, such as monosaccharides, disaccharides, and amino acids (Cowan et al., 1992). LPS play a crucial role in the barrier function of the OM and represent an effective barrier against hydrophobic molecules. Within this OM there is also a rigid peptidoglycan exoskeleton which determines bacterial cell shape. Peptidoglycan is made from disaccharide N-acetyl glucosamine-N-acetyl muramic acid repeating units crosslinked by pentapeptide chains. The peptidoglycan in gram negative bacteria is smaller than in gram positive bacteria but it still plays a significant role, interacting with proteins in the cell envelope and providing stability (Godlewska et al,. 2009). Between the OM and the IM is the periplasmic space, which is an aqueous compartment filled with proteins, that are more viscous than cytoplasm (Mullineaux et al., 2006). This compartment allows bacterial to sequester potentially harmful degradative enzymes but also beneficial proteins which serve a range of functions. Which include periplasmic binding proteins, involved in sugar and amino acid transport and chemotaxis as well as chaperone-like molecules are involved in envelope biosynthesis (Ehrmann, 2007).

Eukaryotic cells contain several membranous organelles such as mitochondria for energy production, smooth endoplasmic reticulum (ER) for lipid synthesis, rough ER for protein secretion, and cytoplasmic membrane sense the environment and transporting molecules. Bacteria do not have these organelles therefore these membrane-associated functions are performed by the IM which is a phospholipid bilayer. There are several structures embedded into this envelope such as flagella, secretion systems, and efflux pumps. Efflux pumps play important roles in pathogenicity

and antibiotic resistance, they export xenometabolites such as antibiotics from the cell across the envelope and into the surrounding media (Eswaran et al., 2004).

The gram-positive cell envelope differs in several ways, the major difference being the lack of an OM. As discussed earlier this outer membrane provides protection from toxic molecules and structural rigidity along with the peptidoglycan and periplasmic space. Gram-positive bacteria often live in harsh environments just like their negative counterparts yet are less protected and as a result, the peptidoglycan layer in gram positive bacteria is far thicker (30-100 nm versus a few nm in gram-negative) In addition to the increased thickness, there are teichoic acids (TA) threading though gram positive peptidoglycan. These are linear anionic polymers consisting of repeating units of ribitol and glycerol linked by phosphodiesters, covalently bonded to the peptidoglycan giving the wall and net negative charge. TA can form larger structures such as wall teichoic acids (WTA), by coupling to peptidoglycan, and lipoteichoic acids (LTA), anchored to the cell membrane. WTAs are mostly composed of disaccharide linkages of polyribitol phosphate (polyRboP) or polyglycerol phosphate (polyGroP) with as many as 60 repeating units. These extend perpendicularly through the peptidoglycan mesh to form a “fluffy” layer beyond the cell envelope (Neuhaus and Baddiley, 2003). While LTAs are composed of polyGroP polymers, often with D-alanine or a sugar moiety, and their glycerolphosphate repeating units have the opposite chirality to those in WTAs. They also typically contain fewer repeating units and are shorter resulting in the molecules extending from the cell membrane into the peptidoglycan, rather than through and beyond. These TA are not essential for viability, but biosynthetic mutants show morphological and growth defects demonstrating a structural importance (Morath, von Aulock and Hartung, 2005). TAs also have roles in cation homeostasis (Marquis, Mayzel and Carstensen, 1976), influencing the rigidity and porosity of the cell wall and its susceptibility to antibiotics (Peschel et al., 2000). Several functionally important proteins are also bound to the peptidoglycan surface of the cell, some analogous to those found in the periplasm of gram-negative bacteria (Lambert, 2002; Dramsi et al., 2008). The structure of peptidoglycan in gram positive bacteria also differs from gram negative with peptides cross-linking

between the glycan strands a connected through pentaglycine branches extended (instead of pentapeptide side chains) from the third amino acid of the stem peptide (Vollmer, 2008). These branches are assembled by nonribosomal peptidyl transferases which are crucial for the survival of the bacteria. The branched stem peptide plays a variety of roles; they serve as attachment sites for covalently associated proteins and implicated in β-lactam resistance (Rohrer and Berger-Bächi, 2003). Proteins present on the surface of the peptidoglycan layer play many pivotal roles; adhesins, fibronectin, fibrinogen and elastin are vital for colonisation of the host, modifications of the cell envelope for immune system evasion, internalisation, phage binding are carried out by sortases (Silhavy, Kahne and Walker, 2010).

Mycobacterial cell walls are more complex than those of typical gram-positive bacteria and this increased complexity contributes to their virulence. They possess a standard IM attached to a peptidoglycan layer, but this contains arabinogalactan which is covalently attached to long chain (up to C90) mycolic acids. In addition, modified peptidoglycan mycobacteria have a symmetrical OM which is dependent on mycolic acids, but their organisation is unclear (Hoffmann et al., 2008). This cryptic barrier provides an extremely permeable barrier to noxious compounds resulting the in the high intrinsic resistance of mycobacteria to many drugs (Brennan & Nikaido, 1995).

2.1.5 – Schistosomiasis

The levels of potency seen within MNQ and the breadth of its activity within the literature is such that it could be considered as a potential treatment for parasitic infections as well as bacterial. There is a clear need to fight antibiotic resistance, yet parasitic diseases are also devastating and are in desperate need to new treatments. In collaboration with Hoffmann Research Laboratory at Aberystwyth University a range of Schistosomal screens would be carried out using MNQ.

Schistosomiasis is the most devastating parasitic disease in humans behind malaria. It is an infection with blood fluke from the genus *Schistosoma* with 200 million people infected and a

further 800 million people at risk (Steinmann et al., 2006). There are 5 species of *Schistosoma* parasites in humans; *S. mansoni*, S. haematobium*, S. japonicum*, S. *intercalatum* and *S. mekongi*. *S. mansoni* which is the widest geographically distributed of all species is transmitted by Biomphalaria snails and causes intestinal and hepatic schistosomiasis in Africa, the Arab peninsula, and South America, Adult Schistosomes are white/greyish worms 7-20 mm in length with cylindrical bodies, two terminal suckers, a complex tegument, a blind digestive tract, and reproductive organs. Other trematodes have separate sexes which live independently and must meet for reproduction to take place. Both male and female *S. mansoni* worms exist together, the male body forming a groove in which the longer thinner female is held, the permanently embraced couples live within the mesenteric venous plexus feeding on blood and globulins. The females produce hundreds to thousands of eggs daily which migrate to the hosts intestine for excretion and can remaining viable for up to 7 days. Once out of the primary human host it begins the search for an intermediate host such as a freshwater snail, it does this by releasing a miracidium on contact with water guided by light and chemical stimuli. Once within the intermediate host asexual multiplication occurs producing multicellular sporocysts and later into cercarial larvae with embryonic suckers and characteristic bifurcated tail. After 4-6 weeks in the snail infectious cercariae are released to return to a human/mammalian host by penetrating the skin. This maturing larval form of the parasite is called the schistosomula and requires 5-7 weeks to become an egg producing adult worm, with the adult surviving 3-10 years on average (Colley et al., 2014). An adult worm pair can produce and disseminating thousands of eggs daily propagating the disease further. Evidence suggest that schistosome eggs are the cause of morbidity rather than the adult worm. Many eggs are not excreted and become permanently lodged in the intestines or liver. Trapped eggs cause an aggressive response from the host immune system along with many pathologies; anaemia, growth stunting, impaired cognition, decreased fitness, periportal fibrosis, portal hypertension, urogenital inflammation and scarring, hepatosplenism and other organ-specific effects (Burke et al., 2009).

Praziquantel (PZQ) in the current drug of choice for schistosomiasis, effective against all *Schistosoma* species but the mechanism of action is not fully understood. PZQ is also ineffective against certain stage of the life cycle of *S. mansoni*. Multiple people within a population are likely to be infected at once, however, not all will be showing symptoms and therefore do not take PZQ. This leads to the propagation of the disease within communities and makes it difficult to treat comprehensively (Pica-Mattoccia and Cioli, 2004). This leads to massive repeated use on large number of individuals accelerating the development of resistance. PZQ is currently a broad-spectrum anthelminthic yet certain parasites such as *Fasciola hepatica* and *F. giganica* are refractory and *S. mansoni* and *S. japonicum* are becoming resistance (Chai, 2013). It can be argued that new treatments for parasitic infections are needed as badly as bacterial infections. Due to this great need for anthelminthic drugs MNQ will also be assayed for its ability to inhibit the growth of *S. mansoni* at multiple stages of its life cycle to ensure it does not have the same issues as PZQ.

2.2 Methods – MNQ Bioactivity

2.2.1 - Antimicrobial susceptibility testing – All aerobic bacterial strains

This method is described in section 1.2.6 – Antimicrobial susceptibility testing – Minimum inhibitory concentration were carried out by the Specialist Antimicrobial Chemotherapy Unit at Public Health Wales which offer this antimicrobial evaluation service through the Life Sciences Research Network Wales who fund this PhD. A list of all bacteria used can be seen in Appendix 4 – All bacterial strains.

2.2.2 - Antimicrobial susceptibility testing – *Clostridium difficile* and *Bacteroides fragilis*

The Clinical and Laboratory Standards Institute (CLSI) agar dilution procedure was undertaken to assess the susceptibility of a range of *C. difficile* and *B. fragilis* strains. Brucella agar supplemented with lysed sheep blood, haemin and vitamin K (Hecht et al., 2012). These assays were carried out by the Specialist Antimicrobial Chemotherapy Unit at Public Health Wales which offer

this antimicrobial evaluation service through the Life Sciences Research Network Wales who fund this PhD.

2.2.3 - Antimicrobial synergistic assay

The antimicrobial synergistic checkerboard assay was performed as previously described by Orhan et al (2005) using MNQ in combination with a range of known antibiotic compounds and 2-hydroxy-1,4-naphthoquinone against MRSA ST8:USA300. As a measure of the synergistic activity the fractional inhibitory concentration (FIC) index (ΣFIC) (Equation 4):

$$\Sigma FIC = FIC\ A + FIC\ B$$

$$FIC\ A = \frac{MIC\ MNQ\ in\ combination\ with\ antibiotic}{MIC\ MNQ\ alone}$$

$$FIC\ B = \frac{MIC\ antibiotic\ in\ combination\ with\ MNQ}{MIC\ antibiotic\ alone}$$

Equation 4: Fractional inhibitory concentration (FIC) index (ΣFIC)

The combination of antimicrobial agents which result in an ΣFIC or ≤0.5 is considered synergistic, a value >0.5 and <2 is indifferent and ≥2 is antagonistic (Orhan et al., 2005) although the due to low reproducibility in other research the level for antagonism has also been set to ΣFIC>4 in other literature (Odds, 2003). Both values will be considered.

2.2.4 - *Schistosoma mansoni* schistosomula culture compound screening - Roboworm

This method was used in chapter 1 for *F. japonica* fractions to find some novel bioactivity within this plant extract, however none of the fractions met the "hit" criteria. The most bioactive

compound in the form of MNQ has been identified and will be assayed for its anthelmintic activity in the same way, see 1.2.8 – *Schistosoma mansoni* schistosomula culture compound screening - Roboworm Platform.

2.2.5 - *Schistosoma mansoni* adult worm culture and compound screening

MF-1 mice (Harlan, UK) were infected by percutaneous exposure to 200 cercariae. Mature adult parasites were recovered from hepatic portal veins by perfusion seven weeks post infection as described by Smithers and Terry. Three adult male and three adult female worms (i.e. three worm pairs) were cultured per well in a 48-well tissue culture plate (Fisher Scientific, Loughborough, UK) containing 1 mL of Dulbecco's modified eagle media (DMEM) (Gibco, Paisley, UK) media (containing 10% v/v Hepes (Sigma-Aldrich, Gillingham, UK), 10% v/v Foetal Calf Serum (Gibco, Paisley, UK), 0.7% v/v 200 mL-Gluta-mine (Gibco, Paisley, UK), 1% v/v Kanamycin/Amphotericin B (Gibco, Paisley, UK)). 6 worm pairs per treatment were incubated for 1 hour at 37°C in a humidified atmosphere containing 5% CO2 before being dosed with test compounds obtaining final concentrations of 50, 25, 12.5 and 6.25 mM (0.3% DMSO final concentration). While all worms were scored manually after 24, 48 and 72 hours using microscopic methods described in the literature (Ramirez et al., 2007), only motility metrics at 72 hours are reported. At 72 hours, eggs were also collected and counted from each well.

2.2.6 - Erythrocyte Lysis

To this point the toxicity of MNQ has been tested against a variety of bacteria which has produced positive results. All these positive antibacterial results can be undone if MNQ is found to be as toxic against humans. An antibiotic must show selective toxicity against bacteria. Toxic phenolic compounds have been shown to have detrimental effects on human erythrocytes, including haemolysis (Bukowska & Kowalska, 2004). Haemolysis is a relatively simple assay to carry out and will rule out the first possibility of cytotoxicity without the cost or complication of cell lines assays.

Defibrinated sheep blood (Oxoid Ltd Hampshire UK) was used to determine the ability of the isolated compounds to cause erythrocyte lysis. 1 mL of defibrinated sheep blood (RBC) was added to six sterile microcentrifuge tubes and centrifuged for 5 minutes at 1,000 x g (Heraeus Biofuge Pico). The supernatant was discarded, and pellet washed three times in phosphate buffered saline (PBS) with centrifugation at 1,000 x g between each wash. The RBC were diluted in PBS 50% v/v. A 180 µL volume, of PBS was added to wells A1-A12 in sterile, transparent, flat bottomed 96 well plate and 100 µL to all wells except row G to which 90 µL was added. A total of 20 µL of each sample being screened was added to row A in triplicate (four per plate) and serial diluted down the plate from A to F. 10 µL of 2% triton-X 100 (Sigma-Aldrich, Gillingham, UK) was added to wells G1-12 as a RBC lysis control. To each well was added 100 µL of the 50% v/v RBC solution. Plates were then covered and left at 37°C for 1 hour. The plates were then centrifuged at 1,000 x g (Heraeus Multifuge 3 S-R). The 100 µL of supernatant from each well was transferred to a new transparent, flat bottomed 96 well plate. The optical density (OD) of the supernatant was measured at 450 nm using a Hidex Sense Plate Reader expressed as percentage haemolysis compared to 0.1% triton-X (Blazyk et at, 2001; Oyama et al., 2017).

2.2.7 - HepG2 cell culture and MTT assay

Cells were grown to 80% confluency in culture media (basement membrane extract with phenol red for HepG2 cells, 10% v/v Foetal Bovine Serum, 1% v/v EMEM non-essential amino acid solution, 1% v/v 200 mM L-Glutamine, 1% v/v gentamycin/amphotericin B). Confluent cells were prepared for cytotoxicity assays in the same manner as stated by Nur-E-Alam et al., 2017. Briefly, 2.5 × 104 were seeded in a black walled 96-well microtiter plate (Fisher Scientific, Loughborough, UK) and incubated for 24 h at 37 °C in a humid atmosphere containing 5% CO2. Test compounds in triplicate were then added at final concentrations of 100, 75, 50, 25, 10, 1, 0.1, 0.01 µM (1.25 final % DMSO) in parallel to negative (DMSO; 1.25%) and positive (1% v/v Triton X-100) (Dayeh et al.,

2004) controls. Following a further incubation for 24 h, the MTT assay was performed as previously described Nur-E-Alam et al., 2017.

2.2.8 - *Galleria mellonella* cytotoxicity screen and infection model

Galleria mellonella (Live Foods, UK) were purchased and injected within one week of arrival. Only individuals between 225 and 275 mg were selected with an assumed average of 250 mg. Samples were diluted to working concentrations with a maximum of 10% DMSO and bacteria (MRSA USA300) were grown at 37°C overnight in Mueller-Hinton (MH) broth. 500 µL of overnight culture was used to inoculate 4.5 mL of MH broth which was incubated at 37 °C, 180 rpm for 3 hours. Cells were harvested and via centrifugation and resuspended to obtain an OD600 of 0.1. Larvae were inoculated with 10 µL in the front right proleg using 10 µL Hamilton syringe. Bacterial injection was used as a negative control showing survival rates when infected with bacteria versus compounds and for an infection model as previously described by Desbois and Coote, 2011.

2.3 – Results – MNQ Bioactivity

2.3.1 – Antimicrobial susceptibility testing – Aerobic bacteria

2.3.1.1 – Antimicrobial activity against Staphylococcal Species

After identifying that MNQ was the most active compound within the plants tested further antimicrobial assays were undertaken to assess the potential of MNQ as an antibacterial agent. As this project is aimed at discovering a compound which can counter the challenges of MRSA a broad range of Staphylococcal species and strains were tested. Initially, *S. aureus* ATCC 29213 was used to assess anti-staphylococcal activity of plant extracts, but in order to counter the challenges of MRSA multi resistant strains must be assayed (Table 13). MNQ was effective against all staphylococcal strains tested. The best activity was observed against S. epidermis NCTC 11047.

Table 13: Antimicrobial activity of 2-methoxy-1,4-naphthoquinone against a range of staphylococcal strains (n=3)

Bacterial strain	**MIC (mg/L)**
S. aureus- ATCC 29213 (MSSA)	19
S. aureus-ATCC 33591 (MRSA)	11
S. aureus-H-EMRSA-15 (MRSA)	13
S. aureus-ST8-USA300 (MRSA)	13
S. epidermis- NCTC 11047	4
S. saprophyticus- Wild strain	31

MNQ was shown to be active against the highly virulent strains of MRSA however to screen a larger number of strains further testing was undertaken at the Specialist Antimicrobial Chemotherapy Unit (SACU), University Hospital of Wales (Table 14). The same standard *S. aureus* strain was used by SACU, but the methods used by this laboratory suggested that MNQ had better antimicrobial activity than we described previously. Three different stock strains of MRSA were tested with either resistance to flucloxacillin (conferred by mecA), erythromycin/clindamycin (conferred by macrolidelincosamide-streptogramin B (MLSB) resistance), and vancomycin heteroresistance Mu3 strain. In addition to these three strains, *S. aureus* (11051), a tetracycline resistant clinical isolate from a patient in Cardiff hospital, was also tested. The exact mechanism of tetracycline resistance in 11051 is unknow.

Table 14: Antimicrobial activity of 2-Methoxy-1,4-naphthoquininone (MNQ) against a range of staphylococcal bacteria by the Specialist Antimicrobial Chemotherapy Unit at University Hospital of Wales (n=3)

			MIC (mg/L)	
Strain No.	Organism	Resistance	Ampicillin	MNQ
ATCC 29213	*S. aureus*	-	4	8
NCTC 12493	*S. aureus*	Flucloxacillin	>128	8
ATCC BAA-977	*S. aureus*	Erythromycin/Clindamycin	64	8
ATCC 700698	*S. aureus*	Vancomycin	>128	8
11051	*S. aureus*	Tetracycline	>128	8
25760	*S. epidermidis*	-	64	4
25495	*S. epidermidis*	-	8	4
194073	*S. saprophyticus*	-	4	8

2.3.1.2 – Antimicrobial activity against other clinically relevant Gram-positive bacteria

MNQ was assessed for broad spectrum efficacy against a range of clinically relevant gram-positive bacteria (Table 15). MNQ was able to consistently inhibit the growth of all gram-positive bacteria tested., However, *E. faecalis* which does not possess any antibiotic resistant mechanisms showed the highest level of resistance against MNQ with an MIC of 64 mg/L which is regarded as very poor activity. The best activity was seen against M. smegmatis, where MNQ far outperformed the antibiotic ampicillin with an MIC of 1 mg/L compared to 16 mg/L. A range of *S. pneumoniae* were tested with three antibiotic resistant strains, MNQ performed consistently against all with an MIC of 16 mg/L.

Table 15: Antimicrobial activity of 2-methoxy-1,4-naphthoquininone (MNQ) against a range of gram-positive bacterial species (n=3)

			MIC (mg/L)	
Strain No.	Organism	Resistance	Ampicillin	MNQ
ATCC 29212	*Enterococcus faecalis*	-	2	64
NCTC 12201	*E. faecalis*	Vancomycin	4	4
ATCC 51299	*E. faecalis*	Vancomycin	8	4
NCTC 333	*Mycobacterium smegmatis*	-	16	1
20456	*Streptococcus bovis*	-	1	32
21816	*S. bovis*	-	1	32
21818	*S. bovis*	-	1	32
22358	*S. Group A*	-	0.06	16
22362	*S. Group G*	-	0.25	16
ATCC 49619	*S. pneumoniae*	-	0.25	16
18778	*S. pneumoniae*	Erythromycin / Tetracycline	0.25	16
21394	*S. pneumoniae*	Penicillin	32	16
21395	*S. pneumoniae*	Erythromycin / Clindamycin	0.5	16
13121	*S. pneumoniae*	-	0.25	16
13122	*S. pneumoniae*	-	0.25	16

2.3.1.3 –Antimicrobial activity against clinically relevant Gram-Negative bacteria

MNQ has been shown to inhibit the growth of a wide range of gram-positive bacterial species, however, gram-negative cells possess a more complex barrier (outer membrane)that can

have a major effect on the potency of antimicrobial compounds (Table 16). Other than *A. baumannii* and *B. cepacia* with poor MIC of 32 and 64 mg/L respectively MNQ was unable to affect the growth of gram-negative bacteria (≥128 mg/L)

Table 16: Antimicrobial activity of 2-methoxy-1,4-naphthoquininone (MNQ) against a range of gram-negative bacterial species (n=3)

			MIC (mg/L)	
Strain No.	**Organism**	**Resistance**	**Ampicillin**	**MNQ**
572	*Acinetobacter baumannii*	-	128	32
NCTC 10661	*Burkholderia cepacia*	-	>128	64
ATCC 25922	*Escherichia coli*	-	8	128
ATCC 35218	*Escherichia coli*	Ampicillin	>128	128
NCTC 13353	*Escherichia coli*	3rd gen cephalosporins	>128	128
353	*Escherichia coli*	Nitrofurantoin/ trimethoprim	>128	>128
21856	*Klebsiella pneumoniae*	-	>128	>128
Controls	*Klebsiella pneumoniae*	Carbapenems	>128	128
ATCC 700603	*Klebsiella pneumoniae*	4th gen cephalosporins	>128	>128
NCTC 13442	*Klebsiella pneumoniae*	Carbapenems	>128	128
NCTC 10975	*Proteus mirabilis*	-	>128	128
ATCC 27853	*Pseudomonas aeruginosa*	-	>128	>128
8204	*Salmonella enteritidis*	-	4	128

2.3.2 - Testing antimicrobial activity against anaerobic bacteria

Two other bacteria *Bacteroides fragilis* and *Clostridium difficile* were also assayed for susceptibility to MNQ. These clinically relevant species are obligate anaerobes and required a different susceptibility testing methodology to aerobic species (Table 17). MNQ showed very consistent activity against all *C. difficile* strains, on par with the antibiotic in most cases (MIC 1-4 mg/L). The ability of MNQ to inhibit the growth of *B. fragilis* ranged from 2 to >128 mg/L. However, the >128 mg/L result was due to metronidazole resistance within one strain. This could indicate that MNQ has a similar mechanism of action to metronidazole.

Table 17: Antimicrobial activity of 2-methoxy-1,4-naphthoquininone (MNQ) against a range of Bacteroides fragilis and Clostridium difficile (N=3)

			MIC (mg/L)	
Strain No.	**Organism**	**Ribotype**	**Meropenem**	**MNQ**
ATCC 25285	*Bacteroides fragilis*	-	0.06	2
13350	*Bacteroides fragilis*	Metronidazole resistance	0.03	>128
1579	*Bacteroides fragilis*	-	0.125	16
1580	*Bacteroides fragilis*	-	0.125	16
1581	*Bacteroides fragilis*	-	0.5	16
1582	*Bacteroides fragilis*	-	0.125	16
1583	*Bacteroides fragilis*	-	0.25	8
1584	*Bacteroides fragilis*	-	4	8
1585	*Bacteroides fragilis*	-	1	8
1591	*Bacteroides fragilis*	-	128	16
1592	*Bacteroides fragilis*	-	1	16
1593	*Bacteroides fragilis*	-	1	16
1594	*Bacteroides fragilis*	-	4	16
1595	*Bacteroides fragilis*	-	32	4
1596	*Bacteroides fragilis*	-	0.5	8
1597	*Bacteroides fragilis*	-	1	16
1598	*Bacteroides fragilis*	-	2	16
1599	*Bacteroides fragilis*	-	1	16
1600	*Bacteroides fragilis*	-	0.125	32
1601	*Bacteroides fragilis*	-	0.25	32
1602	*Bacteroides fragilis*	-	0.25	16
R43812	*Clostridium difficile*	Ribotype 001	1	2
R43875	*Clostridium difficile*	Ribotype 001	1	4
R43935	*Clostridium difficile*	Ribotype 001	1	1
R43943	*Clostridium difficile*	Ribotype 001	1	1
R43968	*Clostridium difficile*	Ribotype 001	1	1
R43883	*Clostridium difficile*	Ribotype 014	1	2
R44000	*Clostridium difficile*	Ribotype 014	1	1
R44002	*Clostridium difficile*	Ribotype 014	0.5	1
R44003	*Clostridium difficile*	Ribotype 014	0.5	1
R44015	*Clostridium difficile*	Ribotype 014	1	2
R43874	*Clostridium difficile*	Ribotype 027	2	1
R43942	*Clostridium difficile*	Ribotype 078	1	1
R43997	*Clostridium difficile*	Ribotype 078	1	1
R43998	*Clostridium difficile*	Ribotype 078	1	1
R44004	*Clostridium difficile*	Ribotype 078	1	1
R44007	*Clostridium difficile*	Ribotype 078	1	2

2.3.3 - Synergistic assessment of MNQ interactions with other antimicrobials

Antimicrobial drugs as they are often used in combination to treat bacterial infections. Therefore, to understand how MNQ works in conjunction with other antibiotics is useful information for its development as a potential antimicrobial chemotherapy agent. MRSA USA300 was used as the test bacterial strain a model for this assay as MNQ has a good MIC against MRSA. MNQ was combined with 11 antibiotics to establish to test for augmented activity against MRSA (Table 18). MNQ was found to be synergistic with ciprofloxacin and rifampicin which shows these have complementary modes of action. MNQ shows poorer activity when combined with gentamycin, streptomycin and lawsone. However, some literature suggests this methodology has poor reproducibility and only ΣFIC values of ≥4 should be considered as true antagonism, therefore only lawsone showed antagonism with MNQ.

*Table 18: Synergistic activity of 2-methoxy-1,4-naphthoquinone (MNQ) with other antibiotics against Staphylococcus aureus MRSA USA 300 (n=3). * - high level of significance*

	MIC (mg/L)	**MIC MNQ (mg/L)**	**FIC A**	**FIC B**	**ΣFIC**	**Activity**
Ciprofloxacin	5	12.5	0.125	0.125	0.25	Synergistic
Mupirocin	1.25	12.5	0.25	0.5	0.75	Indifferent
Levofloxacin	1.25	12.5	1	1	2	Indifferent
Chloramphenicol	3.125	12.5	0.25	0.5	0.75	Indifferent
Rifampicin	0.07	12.5	0.125	0.0313	0.15	Synergistic
Tetracycline	6.25	12.5	0.5	0.5	1	Indifferent
Cefotaxime	25	12.5	1	1	2	Indifferent
Nalidixic acid	12.5	12.5	0.25	0.5	0.75	Indifferent
Gentamycin	3.125	12.5	2	1	3	Antagonistic
Streptomycin	50	12.5	1	2	3	Antagonistic
Ampicillin	100	12.5	1	1	2	Indifferent
Lawsone	100	12.5	2	2	4	Antagonistic*

2.3.4 – *Schistosoma mansoni* schistosomula compound screening - Roboworm Platform

MNQ was assessed for its ability to effect general health and viability of both *S. mansoni* adult worms and schistosomula. The effect of MNQ on the schistosomula stage of the life of *S.*

mansoni was assessed using the Roboworm platform. The Roboworm platform is an automated high-throughput, imaging analysis platform measures both phenotype and motility of treated larvae. To be considered a positive result on this screen the phenotype and motility score of the compound must fall below -0.15 and -0.35, respectively, in ≥70% of the larvae assayed. A positive control is present in the form of auranofin which is a potent inhibitor of *S. mansoni* at the schistosomula stage of the life cycle. A negative control is also run in the form of DMSO (0.625%) which is used to solubilise the samples. MNQ was tested at a concentration of 1.88 mg/L (10 µM) with an average phenotype score of -0.614 and average motility score of -0.945. These values are far below the activity threshold suggesting that MNQ was an effective anti-schistosomal agent (Figure 18).

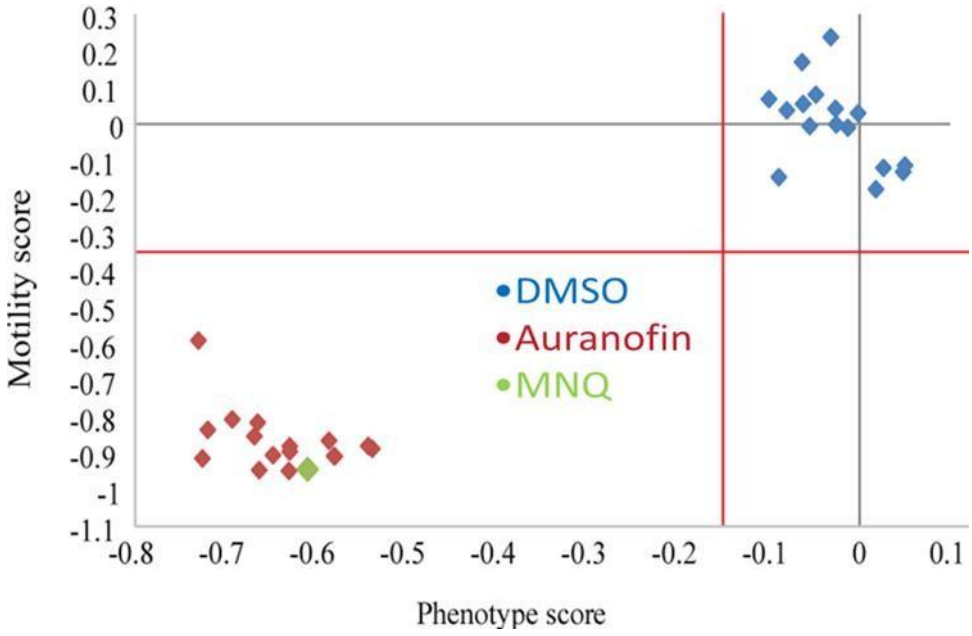


Figure 18: Anthelminthic activity of 2-methoxy-1, 4-naphthoquinone (MNQ), Auranofin (positive control) and dimethyl sulfoxide (DMSO) (negative control) against Schistosoma mansoni schistosomula based on mobility and phenotypic impact (n=2)

2.3.5 – *Schistosoma mansoni* adult worm compound screening

Further tests were carried out using the adult *S. mansoni* worm, measuring the effect of MNQ on the motility in both male and female worms and the total viable egg production. MNQ had a significant effect on egg production at all concentrations tested (50-6.25 µM), and significantly

inhibited motility at 4.7 mg/L (25 µM) with an IC50 of 4 mg/L (21.71 µM) (Figure 19). MNQ showed a preference against female worms with an IC50 3.69 mg/L (19.61 µM) and IC50 4.73 mg/L (25.11 µM) against male worms, with there being a significant difference between male and female motility at 50 µ (Figure 20). This contrasted with PZQ which is more active against male worms.

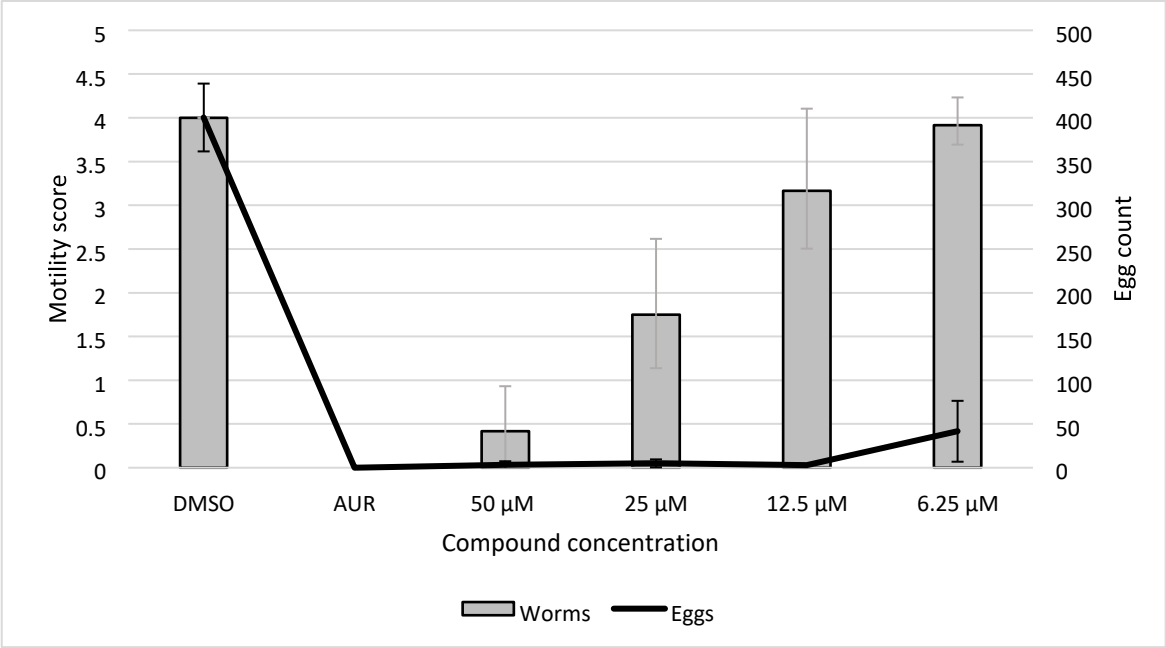


Figure 19: Anthelminthic activity of 2-methoxy-1, 4-naphthoquinone tested at 4 concentrations compared to a positive control auranofin (AUR) and negative control dimethyl sulfoxide (DMSO) in adult S. mansoni measured using motility score (bar) and counting the total number of viable eggs produced (line) after 72 hours (n=6).

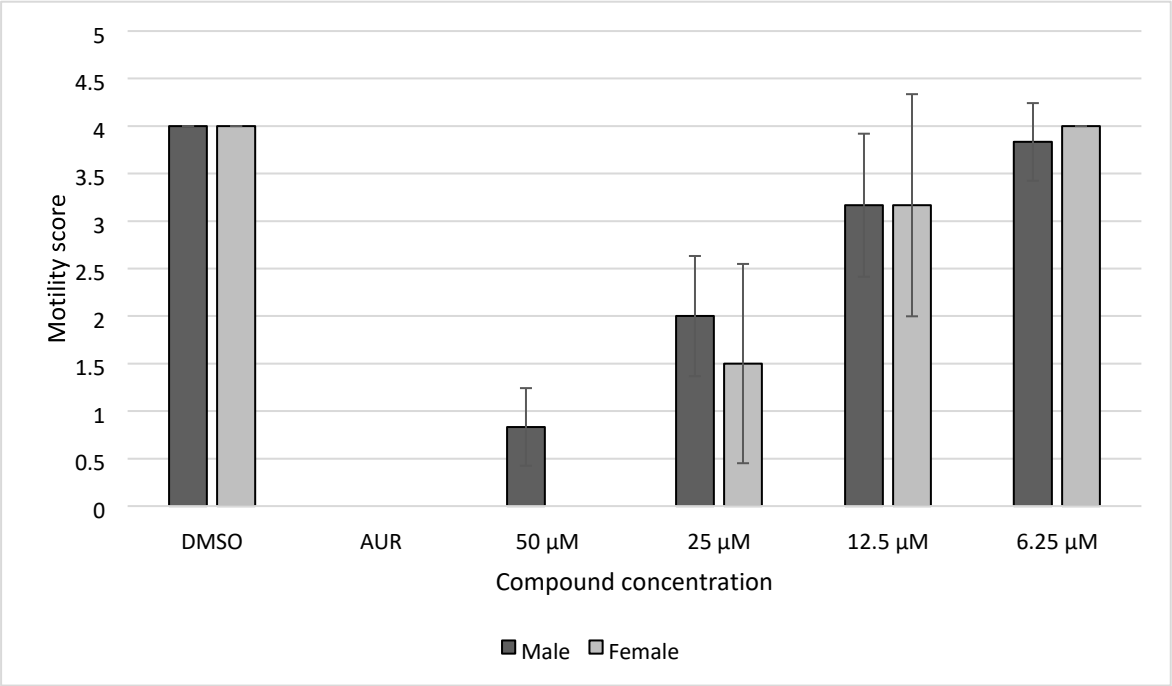


Figure 20: Anthelminthic activity of 2-methoxy-1, 4-naphthoquinone naphthoquinone tested at 4 concentrations compared to a positive control auranofin (AUR) and negative control dimethyl sulfoxide (DMSO) in adult male and female S. mansoni measured using motility score (n=6).

2.3.6 – Erythrocyte lysis

To consider MNQ as a potential chemotherapeutic agent, its human toxicity must be established. This was undertaken using a range of assays including; erythrocyte lysis, HepG2 cell lines, *Galleria mellonella* model organism toxicity assay.

A simple assay was carried out to determine the concentration of MNQ required to lyse RBC (Table 19). RBC lysis of > 10% was only seen at ≥ 50 mg/L which was considerably greater than most MIC of MNQ against susceptible strains ranges from 1-12 mg/L.

Table 19: Percentage haemolysis of erythrocytes in the presence of 2-methoxy-1, 4-naphthoquinone (n=3)	
Concentration (mg/L)	Percentage haemolysis
100	33.519
50	13.676
25	3.741
12.5	0.830
6.25	2.702

2.3.7 – HepG2 Cytotoxicity

HepG2 human liver hepatocellular carcinoma cells are routinely used as a reproducible cell line for the screening liver toxicity of compounds. MNQ was found to have an EC50 of 0.269 mg/L (1.431 µM). This was lower than the concentration needed to kill even the most susceptible bacteria (Figure 21). At log values below 0 (concentrations below 1 µM) the normalised absorbance values are around 100 indicating health cells, however at log 0 there is a large error bar this is because at this concentration the cells begin to die and there is a large amount of variability at this point. Beyond log 0 the normalised absorbance drops to around 0 because the cells have dies and all replicates agree with small error bars.

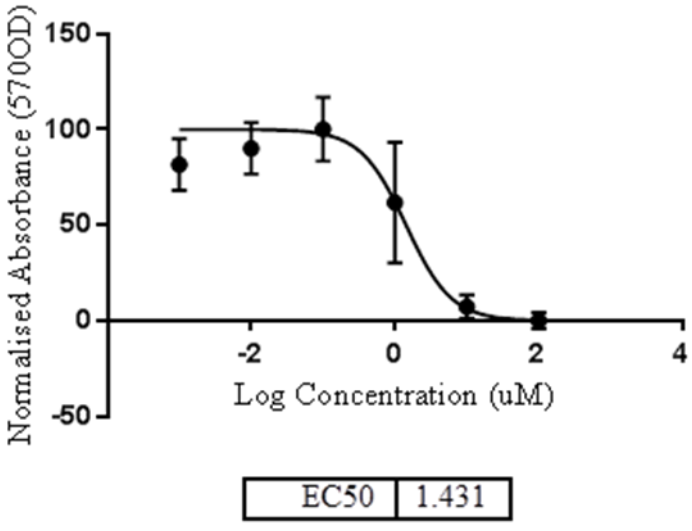


Figure 21: Line graph of log concentration against normalised absorbance, showing cytotoxicity of 2-methoxy-1, 4naphthoquinone against HepG2 cells

2.3.8 – *Galleria mellonella* toxicity

G. mellonella has been shown to be a simple and inexpensive in vivo infection model which can provide information based on the likely cytotoxicity and antibacterial efficacy (Desbois and Coote, 2011). This wax moth model has been shown to respond similarly to mice in bacterial infection models (Jander, Rahme and Ausubel, 2000) and has the added advantage of being less expensive and time consuming. This assay tests both the cytotoxicity of MNQ against a live organism and the ability to fight an infection within a living system.

The tolerance of *G. mellonella* to MNQ alone needed to be established prior to carrying out an infection model experiment to ensure MNQ was not toxic to *G. mellonella*. A range of MNQ concentrations were injected into healthy wax moth larvae and observed over 72 hours monitoring the survival rate (Figure 22). MNQ has an MIC of 8 mg/L and 10x and 100x MIC was tested to see if any toxicity was observed in the wax moth larvae. Of the 20 replicate larvae in each group, only one individual larva died over all MNQ treatment after 72 hours. There was no statistically significant difference between the highest MNQ concentration and no treatment (Log-rank (Mantel-Cox) test p=0.3173). Showing that MNQ has limited toxicity on wax moth larvae even at 800 mg/L.

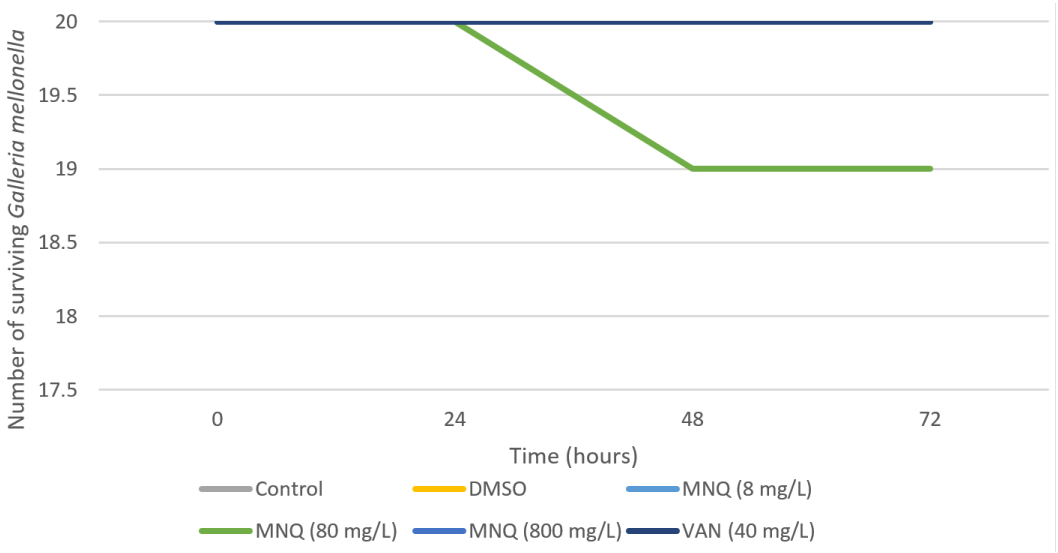


Figure 22: Line graph showing he number of G. mellonella surviving after 72 hours of treatment with 2-methoxy-1,4-naphthoquinone (MNQ) at a range of concentrations, vancomycin (VAN), dimethyl sulfoxide (DMSO) and untreated.

2.3.9 – *Galleria mellonella* infection model

Given the limited toxicity of MNQ it was possible to infect the larvae with MRSA and assess if MNQ could reduce infection development of MRSA. A lethal dose of MRSA was injected into the 100 larvae and separated into 6 groups of 20 for different treatments. Three MNQ treatments were applied to the infected larvae at x1 (8 mg/L), x10 (80 mg/L) and x100 (800 mg/L) MIC. Vancomycin is a proven treatment for MRSA and one group was injected with 40 mg/L of vancomycin as an antibiotic comparison. 20 other larvae were treated using the 10% DMSO solution used to solubilise the samples as a negative control. A further 20 larvae obtained from the same batch as the others were not infected and not treated as a positive control. This test will show the antimicrobial activity of MNQ within a living organism compared to a well-established antibiotic in the form of vancomycin.

MNQ seemed to show some protective activity at the highest concentration of 800 mg/L with 7 larvae surviving after 48 hours and 3 at 72 hours (Figure 23 and Table 20). This was more than DMSO alone, however the difference was not found to be significant (Table 21). Even with the vancomycin not all the larvae survived but after 72 hours 14 were surviving, significantly more than MNQ and DMSO and at a far lower concentration.

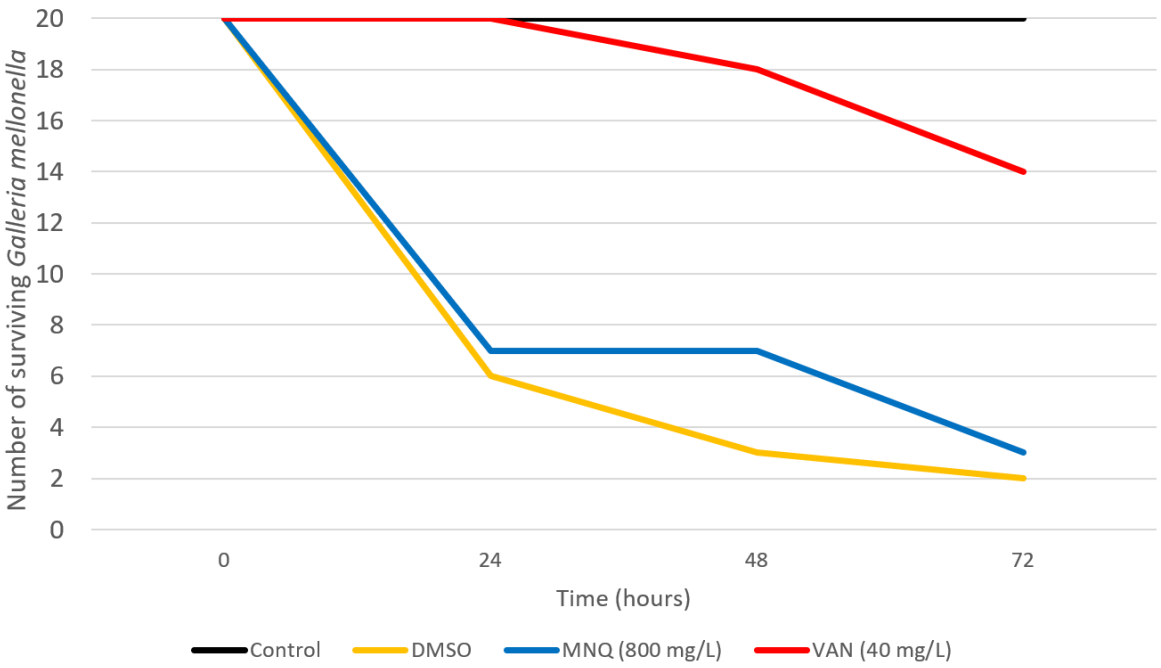


Figure 23: Line graph showing the number of surviving G. mellonella infected with MRSA treated with 2-methoxy-1,4-naphthoquinone (MNQ), vancomycin (VAN), dimethyl sulfoxide (DMSO) and untreated over 72 hours.

Table 20: The number of larvae surviving after each time point after injection of a lethal dose of MRSA and treatment with antibiotics (n=20)

Treatment	Alive 0h	Alive 24h	Alive 48h	Alive 72h
Control	20	20	20	20
DMSO	20	6	3	2
MNQ (8 mg/L)	20	5	3	1
MNQ (80 mg/L)	20	4	4	2
MNQ (800 mg/L)	20	7	7	3
VAN (40 mg/L)	20	20	18	14

Table 21: Statistical Comparison of Survival curves for 2-methoxy1, 4-naphthoquinone (800 mg/L) vs Untreated infection model	
Log-rank (Mantel-Cox) Test	
Chi square	0.7554
df	1
P value	0.3848
P value summary	ns
Are the survival curves sig different?	No
Gehan-Breslow-Wilcoxon Test	
Chi square	0.3444
df	1
P value	0.5573
P value summary	Ns
Are the survival curves sig different?	No
Median survival	
Treatment A	24.00
Treatment B	24.00
Ratio	1.000
95% CI of ratio	0.4846 to 1.515
Hazard Ratio	
Ratio	0.6265
95% CI of ratio	0.2182 to 1.798

2.4 – Discussion – MNQ Bioactivity

MNQ, which showed promising activity against *S. aureus* was isolated from *I. glandulifera* and subjected to a broad range of biological assays.

2.4.1 – Staphylococcal species

2.4.1.1 – *Staphylococcus aureus* and MRSA

Of the staphylococcal species tested (Table 13 and Table 14) MNQ was most effective against S. epidermis NCTC 11047 with an MIC of 4 mg/L. S. epidermis is of clinical interest as it is an opportunistic pathogen able to cause a range of diseases (Otto, 2009). The MRSA strains of most clinical relevance are ATCC 33591, EMRSA and USA300. These have different resistance profiles to antibiotics where ATCC 33591 possesses a type III staphylococcal cassette chromosome (SCC) mec

which conveys methicillin resistance. This mobile genetic element is widely disseminated through staphylococci and contains four commonly shared features (IWG-SCC, 2009):

They carry a mec gene complex:

mecA (can be A, B, C, D or E depending on type, A in this case because it is type III) – this is a gene encoding penicillin binding protein (PBP) 2A which has lower affinity for β-lactams than the usual PBP1,2,3 and 4. This provides an alternative PBP that is not affected by the presence of β-lactam antibiotics meaning transpeptidase remain active allowing cell wall synthesis to continue uninhibited.

Two regulatory genes. mecI gene produces a repressor protein (MecI), when bound to mecA transcription is repressed. mecR1 a signal transducer protein, when bound to β-lactam antibiotics, MecR1 releases a polypeptide with proteolytic activity to degrade MecI increasing transcription of mecA, and insertion sequence IS431.

They carry a ccr gene complex:

Composed of two site specific recombinase genes responsible for the motility of SCCmec, this makes the mec gene complex a mobile genetic element allowing this virulent complex to be transferred to other bacteria. ccrA3 and ccrB3 (number dependent on SCC type – in this case III) catalysing the precise excision, site- and orientation- specific integration of SSCmec elements.

They have characteristic directly repeated nucleotide sequences and inverted complementary sequences at both ends.

They can be accepted into integration site sequence for SSCmec (ISS), situated at the 3’ end of orfX.

The presence of SCCmec type III in ATCC 33951 provides resistance only to β-lactams. As MNQ does not possess a 4 membered lactam ring it is unlikely to have the same mechanism of action as β-lactams. The protection which SCCmec type III offers is ineffective as MNQ does not bind

to PBP therefore it is unsurprising that MNQ can effectively inhibit the growth of this strain of MRSA at the same concentration as MSSA.

The other two strains, EMRSA and USA300, are clinical isolates from hospitals and are considered the two most successful MRSA strain to disseminate globally and these both have SCCmec type IV (Sabirova et al., 2014). The first epidemic of MRSA, designated EMRSA-1 was recognised in 1981 and followed by many other outbreaks over the years with EMRSA-15 emerging in 1991, rapidly displacing most other EMRSA strains (O'neill et al., 2001). EMRSA-15 accounts for >95% of MRSA bacteraemia in UK hospitals (Moore and Lindsay, 2002). This strain has become the dominant strain in the UK and has also been identified in Australia, New Zealand, Germany, Sweden, and Finland. This strain is characterised by weak lysis with phage 75, production of staphylococcal enterotoxin-C (SE-C) and nonproduction of urease. The production of enterotoxins contributes to the pathogenicity of staphylococcal infections such as food poisoning and toxic shock syndrome (TSS). SE-C is one of the least common and is usually isolated form animals (Marr et al., 1993). SEs are classified as superantigens, which can be defined as a toxin of foreign substance which can stimulate large populations of T-cells leading to the production of a cytokines which are usually produced by pathogenic bacteria as a mechanism of defence against immune response. SEs bind Major Histocompatibility Complex (MHC) class two molecules on antigen presenting cells (APC), crosslinking APCs with T-cells eliciting the release of cytokines (Pinchuk, Beswick and Reyes, 2010).

USA300 was first reported in 2003, one of eight USA MRSA strains (USA100-800) (McDougal et al., 2003) but actually came to the attention of the CDC in 2000 during multiple community MRSA infection outbreaks in a Mississippi prison (CDC, 2001) and football players in Colorado, Indiana, Pennsylvania and Los Angeles (CDC, 2003). It was remarkable at the time that all these individuals were infected with not only the same strain, but these young, healthy individuals are not traditionally considered to be at risk of MRSA infection. Over time USA300 became the most isolated MRSA strain (other than USA100) and particularly in a community setting amongst otherwise

healthy individuals (Tenover and Goering, 2009). USA300 diverged form ST8-MSSA acquiring SCCmec type IV leading to strains such as EMRSA 2, 5 and USA500, and diverged further with the acquisition of genes encoding Panton-Valentine leukocidin (PVL), arginine catabolic mobile element (ACME), and msr(A) erythromycin resistance gene, therefore becoming far more virulent. PVL is a β-pore forming cytotoxin (leukotoxin, leukocidin, or more broadly invasin) which causes increased virulence of *S. aureus* infections and the specific production of PVL is preferentially linked to furuncles, cutaneous abscesses, and severe necrotic skin infections (Cribier et al., 1992; Couppie et al., 1994). Leukotoxins in general (others being γ-haemolysin, leukotoxins ED, and AB/HG) have two protein subunits classified 'S' and 'F', referring to slow and fast based on elution speed. The current understanding is that the S subunit serves as a homing component which binds specifically to host receptor, and then recruits the F subunit. Once localised on the cell surface, subunits assemble into an octameric prepore with four of each subunit alternating. The stem domains of the assembled subunits unfold and insert into the cell membrane to form β-barrel pores (Kaneko and Kamio, 2004). As the name suggests leucocidins form these pores and therefore lyse cells of the leukocytic lineage, yet different leukotoxins target different leukocytes and can be more effective against different host species. PVL mostly effects human neutrophils (no effect on RBCs unlike haemolysin) yet mouse and monkey cells are resistant to lysis by PVL (Löffler et al., 2010). Although this PVL is strongly associated with community acquired MRSA strains (~85%) the contribution of this leukotoxin to the pathogenesis of MRSA remains inconclusive (Yoong and Torres, 2013). ACME includes two main gene clusters; arc (arcA, B, C, D) and oligopeptide permease operon opp (opp-3A, B, C, D, E). The 4 arc genes encode a complete arginine deiminase pathway which converts L-arginine to CO2, adenosine triphosphate (ATP) and ammonia; the production of which is thought to enhance ability to grow and survive within a host. The opp genes encode ABC transporter systems. The current understanding is that ACME does not directly enhance virulence but improves general fitness and ability to colonise the host (Diep et al., 2008; Shore et al., 2011).

The three known strains tested in SACU had resistance to Flucloxacillin (conferred by mecA), Erythromycin/Clindamycin (conferred by macrolide-lincosamide-streptogramin B (MLSB) resistance), and vancomycin heteroresistance Mu3 strain. MLSB resistance is due to the ermC genes which encode a 23S rRNA methyltransferase, facilitating methylation of 23S rRNA at adenine. Rendering the ribosomes incapable of binding the MLS antibiotics becoming resistant (Jenssen et al., 1987). The first MRSA strain with heterogeneous intermediate resistance to vancomycin (hVISA) was reported in 1997 in Japan (Hiramatsu et al., 1997). The heterogeneous resistance to vancomycin indicates there are subpopulations of vancomycin-intermediate daughter cells that are susceptible to vancomycin. hVISA strains of *S. aureus* quickly gain resistance to vancomycin when exposed to the antibiotic with MIC ranging from 8- 24 mg/L (Saito et al., 2014). This strain is very closely related to a fully VISA strain Mu50 (Liu and Chambers, 2003). These resistance mechanisms had major effects on the efficacy of ampicillin yet no observable impact on MNQ's ability to inhibit the growth of these bacteria. The MICs for MNQ against all *S. aureus* strains were 8 mg/L. The breadth of this activity across so many resistant strains exceeds all current antibiotics. This suggests that MNQ does not work in the same way as many of the antibiotics currently used today, an antibiotic with a new mode of action is what is needed to counter the challenge of MRSA.

2.4.1.2 – Staphylococcus epidermis and *S. saprophyticus*

S. epidermis is a common skin coloniser and usually has a benign relationship with its host. However, it can become an opportunistic pathogen causing a range of infections. S. epidermis belongs to a group of bacteria called coagulase-negative staphylococci (CoNS), which distinguishes it from *S. aureus* which is coagulase-positive. CoNS bacteria commonly cause infections linked to medical devices such as catheters, prosthetics, pacemakers, shunts as well as grafts (Rogers, Fey & Rupp, 2009). *S. saprophyticus* is the second most frequent cause of urinary tract infections, it can also cause kidney infections, septicaemia and endocarditis (Hedman & Ringertz, 1991). Growth of the three other clinical isolates of S. epidermis and *S. saprophyticus* with unknown resistance mechanisms were also inhibited by MNQ with MICs of 4-8 mg/L, despite one of the S. epidermis

strains showing some resistance to ampicillin. MNQ has outperformed the antibiotic against other Staphylococcal species. Antibiotics are usually prescribed in advance of identifying the bacteria causing the infection, therefore it is important that MNQ can inhibit the growth of many bacterial species especially closely related species such as these.

2.4.2 - Testing antimicrobial activity against clinically relevant Gram-positive bacteria

2.4.2.1 – *Enterococcus faecalis*

MNQ was assayed for antimicrobial activity against a range of gram-positive bacteria (Table 15). *Enterococcus faecalis* is an opportunistic pathogenic commensal bacterium which inhabits the GI tract of humans and other mammals yet can cause diseases such as endocarditis, septicaemia, UTIs, meningitis etc (Murray, 1990). Three strains have been used; a standard susceptibility testing strain, and two strains which are resistant to vancomycin through two different mechanisms, vanA and vanB. Glycopeptide antibiotics (vancomycin, teicoplanin etc.) are often used to treat Enterococcus and Staphylococcus infections. These antibiotics bind the peptidyl-D-alanine-D-alanine termini of peptidoglycan precursors blocking their incorporation into the cell wall (Reynolds, 1989).

VanA resistance is characterised by high level resistance to vancomycin (MIC ≥64 mg/L) and teicoplanin (MIC ≥16 mg/L). This is mediated by Tn1546 type transposon containing vanA genes encoding a D-alanine-D-alanine ligase with results in a peptidoglycan precursor with reduced affinity for glycopeptide antibiotics (Bugg et al., 1991; Lester et al., 2006). VanB resistance results in lower level vancomycin resistance (MIC 8-64 mg/L), remaining susceptible to teicoplanin, with the vanB locus encoded by a conjugative transposon of the TN1549-/Tn5382-subtype (Bjørkeng et al., 2011). These plasmid mediated resistances are worrying as it can be spread quickly not only in Enterococci, but other species such as *Staphylococcus aureus* leading to VRSA. MNQ was relatively ineffective against the ATCC 29212 strain of *E. faecalis* with an MIC of 64 mg/L. This strain is considered as a control strain in food testing, susceptibility testing, quality control and does not possess any specific

resistance mechanisms. However, MNQ was found to be a particularly potent against vanA and vanB encoding strains of *E. faecalis* with an MIC of 4 mg/L. An MIC of 64 mg/L would be considered a very poor result for an antibiotic, however its very impressive MIC of 4 mg/L against vancomycin resistant strains is interesting. As vancomycin resistance is very common within *E. faecalis* finding a compound which can treat these resistant strains is extremely valuable (Murray, 2000). This is especially valuable as it has been found that vancomycin resistance within *E. faecalis* can transferred to *S. aureus* (Noble, Virani & Cree, 1992).

It is odd that the presence of one transposable element in the form of vanA and vanB would reduce the ability of *E. faecalis* to resist the antimicrobial activity of MNQ which seeming has an entirely different mechanism of action. However, the acquisition of resistance to an antibiotic does come at a fitness cost, these can be measured indirectly by the relative growth rate, survival and competition performance in vitro. In most investigations, a chromosomal mutation which yielded resistance did incur a fitness cost (Andersson and Levin, 1999). In the specific case of the vanA operon within *S. aureus*, the expression of resistance is costly to the host especially when challenged with vancomycin (to ensure the operon is induced) with minimal fitness cost without vancomycin (Foucault, Courvalin and Grillot-Courvalin, 2009). The results in Table 15 is a clear representation of this fitness cost, represented by the different MICs of MNQ versus vancomycin resistant and susceptible strains of *E. faecalis*.

Further to this point, PBP 2 is not capable of cross-linking peptidoglycan which has been modified due to van genes (Severin et al., 2004). This means that the van operon which is responsible for alteration of peptidoglycan resulting in vancomycin resistance in not compatible with mecA which is produces a modified PBP which renders β-lactam antibiotics ineffective. As these two mechanisms are conflicting and the fitness cost associated will be very high and there have been instances where there has been a deletion of mecA in the presence of vancomycin to compensate for this issue (Adhikari et al., 2004; Noto, Fox and Archer, 2008). The incompatibility of

different resistance mechanisms is very promising for the use of combination drug therapies, having two antibiotics with different modes of action can theoretically slow the onset of resistance (Mouton, 1999). Using combination therapy to treat tuberculosis (Chaisson, 2003) and *P. aeruginosa* (Lister & Wolter, 2005) has been found to be successful for slowing the occurrence of resistance. However, there are conflicting accounts in the literature which state that combination therapy has little to no impact on the development speed of resistance (Sanders Jr & Sanders, 1988l; Carmeli et al., 1999).

2.4.2.2 – Mycobacterium smegmatis

Tuberculosis (TB) remains a major global threat caused by the bacterium Mycobacterium tuberculosis. It typically affects the lungs but can also affect other such as the skin, lymph nodes, urinary tract, skeletal system, central nervous system and many other sites (Dye et al., 1999). Overall, only 5-15% of the 2-3 billion people infected with M. tuberculosis will develop TB disease during their lifetime, yet one developed without treatment the death rate is high. There were estimated to be 1.8 million TB deaths and 10.4 million new cases in 2015 (WHO, 2016). M. smegmatis was used as a fast-growing that is widely used as a surrogate for TB. This is the case, although M. smegmatis tends to be more robust and can miss active compounds which can effectively inhibit the growth of M. tuberculosis (Altaf et al., 2010). MNQ can effectively inhibit the growth of M. smegmatis with an MIC of 1 mg/L. This level of activity exceeds its MICs against Staphylococcal species suggesting MNQ could prove to be an antitubercular lead compound.

2.4.2.3 – *Streptococcus pneumoniae*

The *Streptococcus* genus contains over 50 species that are associated with a wide range of infections. *Streptococcus pneumoniae* typically colonises the respiratory tract and can be carried asymptomatically, however in individuals with weaker immune systems cause pneumonia, meningitis, sepsis and other diseases (Krzyściak et al., 2013). A wide range of clinical isolates with erythromycin and tetracycline, penicillin, and erythromycin and clindamycin with a non-resistant

reference strains were tested. All strains of *S. pneumoniae* tested were equally susceptible to MNQ with an MIC of 16 mg/L. MNQ had the same MIC against S. pyogenes, also known as group A *Streptococcus* (GAS) and S. dysgalactiae also known as group G *Streptococcus* (GGS). GAS causes mild human infections such as pharyngitis, impetigo, and more serious infections such as necrotizing fasciitis and streptococcal toxic shock syndrome. GGS typically causes chronic skin conditions among other infections. Penicillins are the recommended treatment for GAS and GGS infections and these bacteria have remained susceptible with no development of penicillin resistance anywhere in the world (Walker et al., 2014). *S. bovis* is commonly found as part of the bowel flora of humans and animals, responsible for such diseases as bacteraemia, endocarditis, neonatal infection, and meningitis. *S. bovis* was less susceptible to MNQ treatment than other *Streptococcus* species with an MIC of 32 mg/L. MNQ having again such consistent activity against another range clinically relevant species is a promising sign that MNQ has real potential as a broad-spectrum antibiotic.

2.4.3 – Testing Antimicrobial activity against clinically relevant Gram-Negative bacteria

2.4.3.1 – *Acinetobacter baumannii*

Acinetobacter baumannii has become increasingly significant as a pathogen over the past 20 years due to its ability to upregulate or acquire resistance determinants and survive for prolonged periods in hospital environments threatening the efficacy of antibiotics. There is a wide array of antimicrobial resistance mechanisms described in *A. baumannii* and a global emergence of β-lactam resistance *A. baumannii* (Peleg, Seifert and Paterson, 2008). Therefore, the traditional treatment of β-lactam antibiotics are ineffective and other drugs must be used such as colistin, however, this can cause a range of side effects and is only used as a last resort (Abbo et al., 2005). MNQ was shown to inhibit *A. baumannii* growth with an MIC of 32 mg/L. The strain used is a clinical isolate which is resistant to ampicillin which has an unknown resistance mechanism against β-lactam. As MNQ has been proven to be unaffected by the presence of β-lactam resistance mechanisms within all strains

of bacteria tested so far, this indicates that MNQ could hold a potential solution to the global emergence of β-lactam resistance.

2.4.3.2 – *Burkholderia cepacia*

Burkholderia cepacia, an opportunistic human pathogen typically colonizing the airways leading to the exacerbations of pulmonary infections. *B. cepacia* plays a role in cystic fibrosis patients' respiratory failure and can generally cause lung infections. Treatment for this pathogen is difficult as it is resistant to most antimicrobial agents (Coenye et al., 2001). Unfortunately, the MIC of MNQ for *B. cepacia* was MIC of 64 mg/L, which is not considered to be particularly good. However, this is better than ampicillin which has an MIC >128 mg/L.

2.4.3.3 – *Escherichia coli*

E. coli which is usually a commensal bacterium of humans and animals and is the most common cause of gram negative nosocomial and community-acquired infections causing diseases such as gastroenteritis, UTI, meningitis, peritonitis, and septicaemia (Kaper, Nataro & Mobley, 2004). In addition to causing a range of diseases, drug resistant *E. coli* has been reported worldwide and the occurrence of resistance in increasing (Von Baum and Marre, 2005). Four strains of *E. coli* were therefore tested for their susceptibility to MNQ, three of which had resistance mechanisms. MNQ had an MIC of 128 mg/L against *E. coli* with no resistance mechanism, ampicillin and cephalosporin resistant *E. coli*. MNQ has an MIC >128 mg/L against nitrofurantoin/trimethoprim resistant *E. coli*. These are very poor activity profiles against *E. coli*, likely due to the structure of the bacterial membranes of gram negative.

2.4.3.4 – *Klebsiella pneumoniae*

Klebsiella pneumoniae naturally occurs in the soil and the normal flora of the mouth, skin and intestines. Again, this bacterium is an opportunistic pathogen causing pneumonia and other infections usually in immunocompromised patients (Podschun and Ullmann, 1998). Four strains were tested for susceptibility to MNQ. Two strains resistant to carbapenems were susceptible to

MNQ at the highest concentration tested, 128 mg/L. MNQ was not able to inhibit the growth of the other two strains, one being non-resistance and the other resistant to cephalosporins. These results reflect that seen in *E. coli* further adding to the hypothesis that MNQ is ineffective against gram-negative bacteria. Also, that the presence of carbapenem resistance is reducing the fitness of *K. pneumoniae* resulting in higher susceptibility to MNQ.

2.4.3.5 – *Proteus mirabilis*

Proteus mirabilis is widely distributed in the environment, including polluted water, soil, and manure where it plays a role in decomposition. These bacteria are the causative agent of a variety of opportunistic nosocomial infections in the respiratory tract, eye, ear, nose, skin, burns, throat, wounds, also causing gastroenteritis and UTI (Jacobsen et al., 2008). Growth of *P. mirabilis* was not affected by MNQ in any major way, with a high MIC of 128 mg/L, although MNQ is more effective than ampicillin. This is promising as MNQ is consistently showing activity even if it is at a high concentration suggesting the killing mechanism of MNQ is difficult to resist.

2.4.3.6 – *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a versatile bacterium able to grow in soil, marches, habitats as well as plant and animal tissue. *P. aeruginosa* is proficient in the formation of biofilms which is a key factor in the cause of disease and resistance to antibiotics (Hardalo and Edberg, 1997; Costerton, Stewart and Greenberg, 1999). *P. aeruginosa* is one of the top three causes of opportunistic human infections which is resistant to antibiotics and disinfectants (Bodey et al., 1983) and MNQ was unable to inhibit the growth of *P. aeruginosa* at the highest concentration tested, 128 mg/L.

2.4.3.7 – *Salmonella enteritidis*

Salmonella enteritidis is a major pathogen associated with the consumption of eggs and the major cause of nontyphoidal salmonellosis (Rabsch, Tschäpe and Bäumler, 2001). MNQ again only inhibited the growth of this gram-negative pathogen at 128 mg/L.

The average MIC of MNQ against gram-positive bacteria is 15 mg/L and the MIC against gram-negative bacteria is 155 mg/L (assuming MNQ would be able to inhibit MNQ-resistant strains at 256 mg/L). This ten-fold reduction in efficacy suggests the presence of an outer membrane has had a significant effect on the ability of MNQ to inhibit the growth of bacteria. This extra barrier of protection plays a key role in preventing the mechanism of action of MNQ, either by limiting the internalisation of MNQ or preventing access to the membrane if this is the site at which MNQ effect the bacteria.

2.4.4 - Testing antimicrobial activity against anaerobic bacteria

2.4.4.1 – *Bacteroides fragilis*

Bacteroides species comprise nearly half of the faecal flora community with some critical to the host nutrition. *B. fragilis* account for <1-2% of cultured faecal flora but play a role in the host mucosal and systematic immunity. *B. fragilis* is also an opportunistic pathogen and a leading anaerobic isolate in clinical specimens, bloodstream infections, and abdominal abscesses (Sears, 2009). *B. fragilis* is inherently resistant to a range of antibiotics due to resistance being conveyed via a plasmid which can be quickly passed throughout bacterial population. This plasmid can resist the antibiotics clindamycin, erythromycin, streptogramins and tetracycline (Privitera, Dublanchet & Sebald, 1979). Therefore Metronidazole (Met) or a complex mix of antibiotics are used to treat infections (Wexler, 2007). MNQ exhibited an MIC against *B. fragilis* between 2-32 mg/L. However, there was one Met resistant strain of *B. fragilis* which was entirely resistant to MNQ with an MIC >128 mg/L.

Met is administered as a prodrug which is inactive and requires activation by the partial reduction of its nitro group, the active form is a toxic nitroso- radical which binds DNA causing double strand DNA breakage. Met resistance in *B. fragilis* can be caused by the nim genes which is carried on a novel conjugative transposon (Husain et al., 2013). This gene is thought to code a nitroimidazole reductase which reduces the nitro group of 4- or 5-nitroimidazole to an amino group

to make the inactive compound, 5-aminoinidazole preventing the toxic effects of nitroso radicals key for the antibacterial activity of Met (Müller, 1983; Carlier et al., 1997). The antibacterial activity of MNQ is dependent on the presence of the methoxy group as seen in the different MIC of MNQ and HMQ. If nitroimidazole reductase can reduce MNQ it will lose the methoxy group therefore lose its potency. However, Met resistance can persist within nim-negative strains due to increased efflux gene transcription levels, alterations in DNA repair systems, metabolic changes and lack of activation of the Met molecule. Similarly, naphthoquinones can produce adducts and reactive oxygen species (ROS) which can cause alkylation or oxidation of DNA and proteins (Bolton et al., 2000). Thus, resistance to Met could explain its poor efficacy of MNQ in this strain. However, as this is a clinical isolate it is unknown whether Met resistance is mediated through nim genes, yet we can tentatively deduce that the presence of nim genes within bacteria could confer resistance against MNQ. This is worrying as nim homologues are found in both gram-negative and positive genera or both aerobic and anaerobic bacteria and archaea, suggesting that the nim gene family is ancient and widespread (Husain et al., 2013). Although MNQ does not contain a nitro group which requires reduction for activation therefore it would not make sense for resistance to be conveyed through the presence of a nitroimidazole reductase, unless there are other factors at play as this mechanism of resistance is not fully understood.

2.4.4.2 – *Clostridium difficile*

C. difficile is a spore forming bacterium, which causes symptoms such as diarrhoea, fever, nausea and abdominal pain. It is involved in just under half of all infections occurring within hospital (CDC, 2012). *C. difficile* infection (CDI) is a major cause of morbidity and mortality from healthcare-associated infections in economically developed countries. MNQ was tested against 16 clinical isolates of *C. difficile* including 4 different ribotypes (RT). Certain RT have been linked to severe outbreaks. RT 78 has been described as “hypervirulent” which tends to affect a younger population and is more frequently community associated when compared to type 027 which is also responsible for severe CDI outbreaks and is said to be have increased virulence (Goorhuis, Bakker, et al., 2008).

RT 014 is a highly successful among the most common RTs causing CDI, predominantly in paediatric populations (Knight et al., 2017). RT 78 is most commonly isolated from swine and calves in the US, and associated with human community onset infection (Goorhuis, Debast, et al., 2008). RT 001 has been associated with increasing prevalence of CDI in human along with a range of antibiotic resistance in Germany (Borgmann et al., 2008).

Although RT is linked to virulence and severity of the infection this does not necessarily increase resistance to drugs although certain RT do tent to pick up resistance mechanisms more readily and are thought to cause epidemics. However, even the link between RT and severity of infection has been disputed; RT was found to be not significant as a predictor of severity of CDI. Other factors such as white blood cell count and albumin count are more clinically relevant factors which effect the severity of CDI (Walk et al., 2012). The MIC of MNQ against *C. difficile* ranged between 1-4 mg/L and did not correlate with RT, this is lowest MIC of MNQ seen against any bacteria. The level of activity seen in MNQ was as good as meropenem in most cases which is impressive activity increasing the range of bacteria MNQ is known to be effective against. This activity against all RT of *C. difficile* is an indication MNQ possesses the level of potency required to be a future treatment for *C. difficile* infections.

2.4.5 - Synergistic assessment of MNQ interactions with other antimicrobials

With 7 of the 11 antibiotics the presence of MNQ had no effect ("indifferent"). With gentamycin and streptomycin their ability to inhibit the growth of MRSA was antagonised by the presence of MNQ. Both gentamycin and streptomycin are aminoglycosides which irreversibly bind the 30S subunit proteins and 16S rRNA preventing protein synthesis, which presumably for MNQ was affecting via an unknown mechanism. MNQ showed synergistic activity when combined with ciprofloxacin and rifampicin. Ciprofloxacin is a fluoroquinolone which inhibits enzymes topoisomerase II (DNA gyrase) and topoisomerase IV, which are required for bacterial DNA replication, transcription, repair, strand supercoiling and recombination (Drlica and Zhao, 1997).

Rifampicin specifically inhibits bacterial DNA-dependent RNA polymerase leading to suppression of RNA synthesis and cell death (Campbell et al., 2001). Although these two antibiotics have different mechanisms of actions, they both have a quinones at their core. This could be a contributing factor to their synergistic relationship with MNQ which is also has a quinone core. Ciprofloxacin has been shown to stimulate the production of reactive oxygen species (ROS) which have a toxic effect, it has been suggested this generation of ROS contributes to the antimicrobial activity of ciprofloxacin (Goswami, Mangoli & Jawali, 2006). If a quinolone core is responsible for the generation then MNQ is likely also able to form ROS. The presence of two agents generating these toxic molecules will have a synergistic affect, therefore explaining these results. However, levofloxacin like ciprofloxacin is a second-generation fluoroquinolone and would therefore expect synergism, but it has an ΣFIC value of 2 which is perfect indifference.

In addition to the 11 antibiotics tested for synergistic activity with MNQ, 2-hydroxy-1,4naphthoquinone (HNQ) also known as lawsone was also assayed for its activity against MRSA and in combination with MNQ. HNQ showed 100-fold higher MIC compared to MNQ, therefore, the methoxy group of MNQ is required for the highly potent antimicrobial activity against MRSA. HNQ was also found to be the most antagonistic of all the tested compounds. HNQ has been shown to inhibit the formation of ROS (Saeed et al., 2013), which was found to be the cause of the synergism seen in other antibiotics combined with MNQ. This would explain the significant antagonism seen when MNQ and HNQ are combined.

2.4.6 – Screening for anthelminthic activity

The current treatment for *S. mansoni* infection is praziquantel (PZQ) which is ineffective against schistosomula. Its mechanism of action is thought to target the schistosome calcium ion channels, causing rapid calcium ion uptake along with vacuolation a blebbing near the surface. In addition, PZQ is also thought to increase the exposure of antigens on the worm's surface increasing susceptibility to host antibodies (Doenhoff, Cioli and Utzinger, 2008). PZQ has an IC50 5.44 mg/L

(17.4 µM) against the adult worm (Kasinathan, et al., 2010), although the efficacy is dependent on the age of the infection, the sex of the worm and whether they are paired or unpaired. Immature 28-day schistosomes are 30 times more resistant to PZQ than the 7-week-old adults. Being female and unpaired also decreased the efficacy of PZQ. The inconsistency against different forms of the same organism is a problem for the clinical use of PZQ (Pica-Mattoccia and Cioli, 2004), therefore it is pivotal to develop new anti-schistosomiasis drugs which are effective against all stages of the parasite. MNQ was tested in four ways to show its full activity against the *S. mansoni* parasite; inhibitory activity against the larval schistosomula (Figure 18), adult male, adult female and egg production (Figure 19 and Figure 20). Each of these factors are important to have a comprehensively active anthelminthic agent, the current drug praziquantel is lacking efficacy against the schistosomula and female unpaired worms. MNQ showed exceptional activity against the schistosomula with and IC50 of <1.88 mg/L (10 µM) but was less effective against the adult worms showing a slight preference for the female worm with an IC50 3.69 mg/L (19.61 µM) and IC50 4.73 mg/L (25.11 µM) against male worms. The efficacy of MNQ matches that of PZQ against adult worm and exceeds against the larval stage of the parasite, making MNQ a potential treatment for *S. mansoni* in the future.

2.4.7 – Cytotoxicity

For a potential drug to be a viable treatment option it must be safe to administer to humans, ideally with minimal side effects. Conflicting cytotoxicity results were obtained, firstly MNQ was found cause minimal lysis of red blood cells (RBC) indicating that MNQ is relatively non-toxic. Considering that naphthalene which is at the core of MNQ is known to damage human red blood cells which can lead to haemolytic anaemia, it is a sign that MNQ is less toxic as it was unable to lyse RBC easily. Exposure to large amounts of naphthalene can cause a range of negative effects such as confusion, nausea, vomiting, diarrhoea, blood in the urine, and jaundice (Sanctucci and Shah, 2000). The presence of naphthalene in the body is understood to be metabolised by cytochrome P450 and

its structure is altered in three ways resulting in either mercapturic acids, 1,2naphthoquinone, 1,4-naphthoquinone (Waidyanatha et al., 2002) or 1-naphthol. The cytotoxicity of these metabolites against mononuclear leucocytes were assayed; where 1,2-NQ, 1,4-NQ and 1naphthol were all found to be significant more cytotoxic than naphthalene (Table 22). Previous studies have shown that naphthoquinones are toxic to rat hepatocytes which is related to the disturbance of intracellular glutathione (Ollinger and Brunmark, 1991). Although the RBC lysis results were initially promising due to the high concentration required to lyse RBC, the fact that naphthalene metabolites are all highly toxic means that MNQ also toxic.

Table 22: The effect of naphthalene and its metabolites on mononuclear leucocytes (Wilson et al., 1996).

Metabolite	**Percentage cell death of mononuclear leucocytes**	**±**
naphthalene	19	10.00
1-naphthol	49.8	13.90
1,2-naphthoquinone	51.4	6.60
1,4-naphthoquinone	49.1	3.40

It was found that MNQ was an extremely potent inhibitor of HepG2 cells indicating cytotoxicity. Other studies have found that MNQ is a potent inhibitor of cancerous cell lines and has been investigated as a potential anticancer agent (Mori et al., 2011; Wang and Lin, 2012; Liew et al., 2014). HepG2 is a cancerous cell line with increased rate of metabolism, and other changes which are typical of cancerous cells this could have made HepG2 more susceptible to MNQ. Further in vivo studies were carried out using wax moth larvae which are thought to be a good indicator of compound toxicity in humans (Desbois and Coote, 2011). MNQ was found to be non-toxic >800 mg/L and an infection model experiment was carried out to determine the ability of MNQ to prevent an MRSA infection in the larvae. MNQ was unable to significantly prevent death by bacterial infection over 72 hours. These results indicated that in vivo MNQ would likely be very toxic and unable to successfully treat a bacterial infection.

2.5 – Conclusion

In conclusion MNQ was found to be a consistently potent antimicrobial all gram-positive bacterial tested but was ineffective against most of the gram-negatives bacteria. Synergistic assays also suggested the formation of ROS could play a crucial role in the way MNQ kills bacteria, due to its synergism with ciprofloxacin and antagonism with HNQ. MNQ was shown to be a potent inhibitor of the parasite *S. mansoni* with similar level of activity to praziquantel and exceeded the current drug of choice because MNQ was also a potent inhibitor of the larval schistosomula which praziquantel is ineffective against. There were mixed results with the cytotoxicity data and poor performance in the infection model.

MNQ has been shown to have antibacterial and anthelmintic activity equal to current drugs and seems to have a novel mode of action. Unfortunately, there is also a high probability that MNQ is toxic and cannot be used as a drug, although the cytotoxicity assays carried out are not exhaustive. The most valuable discovery in the fight against resistance would be the novel mechanism of action of MNQ. Therefore, further work will be carried out to understand why MNQ is such a potent inhibitor of antibiotics resistant *S. aureus*.

Chapter 3 Mode of action studies: Proteomics and Metabolomics

3.1 Introduction – Mode of action

3.1.1 - Chapter aims

As a potent antimicrobial compound has been identified from *I. glandulifera*, the mechanism by which it acts upon bacteria needs to be understood. Novel metabolomics and proteomics approaches will be used to further understand the mode of action (MoA) of MNQ against MRSA. In this chapter, comparisons will be drawn between MNQ and known antibiotics to cluster its activity with already understood MoA. In-depth metabolic pathway analysis will be carried out to uncover fine detail of the effect MNQ has on MRSA.

3.1.2 – 'Omics' and the central dogma of molecular biology

Over the past two decades since the beginning of the twenty-first century there has been rapid growth and advances made in the areas of analytical and informatic technologies driving the progress in 'omic' technologies, which involves the study of huge data sets. The term 'omics' is a suffix used to suggest the complete study of a particular area; for example, the genome refers to the complete genetic material of an organism whereas genomics is the study of genomes. There is a broad range of 'omics'; genomics, transcriptomics, proteomics, metabolomics and lipidomics. These are, respectively, the complete study of genes, transcription, proteins, metabolites, and lipids within a living system. When used collectively, these omics platforms are termed as systems biology (Hasin, Seldin & Lusis, 2017). At the time of writing, there have been 3941 eukaryotic species genomes sequenced and made publicly available via the National Centre for Biotechnology Information (NCBI) genome database. This revolutionised the field of biology, where instead of

studying one gene, protein or metabolite at a time it is now possible to study the effects of any physiological or pathological changes on the expression of all genes, proteins or metabolites.

To give metabolomics context it is worth considering its benefits and drawbacks in comparison to the other 'omics' and where it sits in the central dogma (CD) of molecular biology. This dogma states that the flow of genetic information begins with DNA passing through RNA into proteins and was first proposed by Francis Crick in 1958 (Crick, 1958). Simply put the theory suggests that each level had its own alphabet and that information was transferred residue-by-residue. For DNA, this alphabet consists of four base pairs adenine with thymine and cytosine with guanine, RNA shares three of the same letters as DNA but substituting thymine for uracil and proteins with their 20 naturally occurring amino acids. The CD states that there are three types of transfer; general, special and unknown transfers. General being the well-established transfer between DNA->DNA, DNA->RNA and RNA->Protein. Special transfers only occur in certain circumstances which are the transfer between RNA->DNA, RNA->RNA and DNA->Protein (Figure 24) (Crick, 1970). Crick realised that reverse transcription was essentially the interconversion of nucleic acids which relies on the universal rules of base complementarity therefore the conversion is relatively easy. The conversion of nucleic acids to amino acids is more complex than breaking down amino acids into nucleic acids but this conversion has been biologically beneficial and therefore has been selected for by evolutionary processes. The reverse, which in principle is theoretically possible, is hampered by the design of the translation system. Reverse translation would require an elaborate sequence of reactions that are not known to exist. Two fundamental steps being; i) recognition of nucleotide triplets (tRNA anticodons) by amino acid residues within a polypeptide chain, ii) joining of these triplets into an RNA molecule. Furthermore, given the degeneracy of genetic code, reverse translation would result in major loss of information (Koonin, 2012).

The CD where DNA drives all living systems appears comprehensive but fails to consider the impact of metabolism. This is surprising, as so many proteins are enzymes whose specific role is to

transform chemicals into metabolites some of which are the building blocks of living systems. The catalytic abilities of these metabolically important proteins are encoded in their amino acid sequence, therefore derived from the RNA sequence and ultimately from the DNA which rules living systems according to the CD. However, the residue-by-residue transfer of information does not apply in the metabolic context because chemical structures of metabolites or their fluxes cannot be predicted from the genetic code (de Lorenzo, 2014). Therefore, DNA is not driving the living system but reacting to its current metabolic environment.

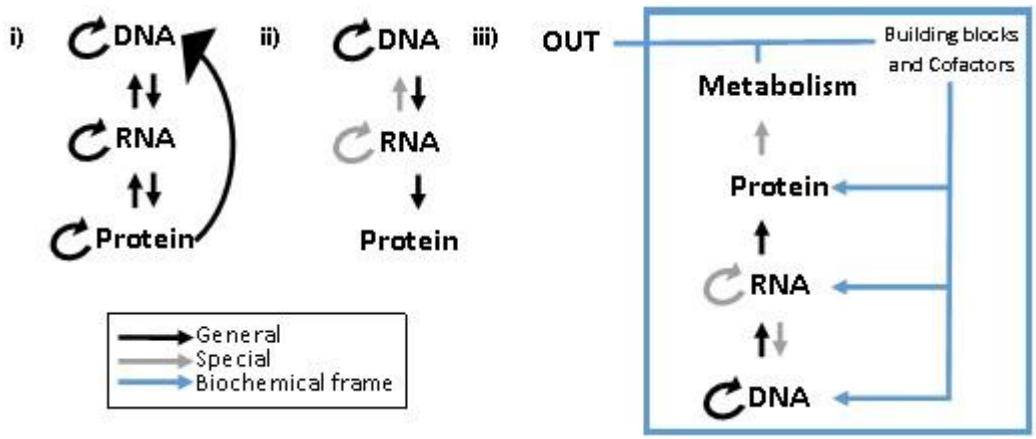


Figure 24: Growing expansion of the central dogma. i) All possible interaction between DNA RNA and protein ii) exclusions made by the development of the central dogma iii) CD considering the role of metabolism (adapted from de Lorenzo, 2014)

Metabolism is discounted from the CD because it does not have a direct impact on the sequences of DNA, RNA and proteins. But metabolism does have a massive effect on the regulation of genes therefore effecting RNA and proteins. There are many regulatory mechanisms which are genetically controlled in response to metabolites, usually mediated through regulatory proteins (Struhl, 1999), but interacting directly with mRNA (Nahvi et al., 2002). Therefore, it could be said that metabolism both drives and responds to genetics in terms of regulation if not in terms of sequence (Figure 24).

3.1.3 – Genomics

Genomics is the study of the entire DNA sequence of organisms and can focus on its function, structure or evolution. DNA sequencing technologies took off in the 1970s with two major publications describing different approaches to improve the speed at which sequencing can be carried out (Sanger and Coulson, 1975; Maxam and Gilbert, 1977). In particular, the development of the Sanger chain-termination method yielded the most used DNA sequencing technique to date (Sanger, Nicklen and Coulson, 1977). This method was used to sequence the human genome, which deepened our understanding of human evolution, causation of disease and the interplay between environment and heredity (Venter et al., 2001). Further, comparative genomics is a versatile tool able to highlight large scale similarities and differences between entire organisms (Rubin et al., 2000) and the small differ between genomes of the same species (Fournier et al., 2006; Feng et al., 2007; Rasko et al., 2008). Genomics can also be used for understanding the molecular mechanism of bacterial pathogenicity (Schoolnik, 2002; Zhang et al., 2012).

The emergence of "next-generation" (NGS) technologies has dramatically increased sequence throughput albeit at the expense of read length. NGS has also allowed targeted resequencing, discovery of transcription factor binding sites, and noncoding RNA expression profiling (Morozova and Marra, 2008). It is routinely being used on human genomes to understand genetic mutations associated with disabilities and diseases (Link et al., 2011; Sanders et al., 2012; Kan et al., 2013) and can be utilised in a similar way, by inducing genetic mutations which result in resistance to investigate the MoA of a drug against a particular bacteria:

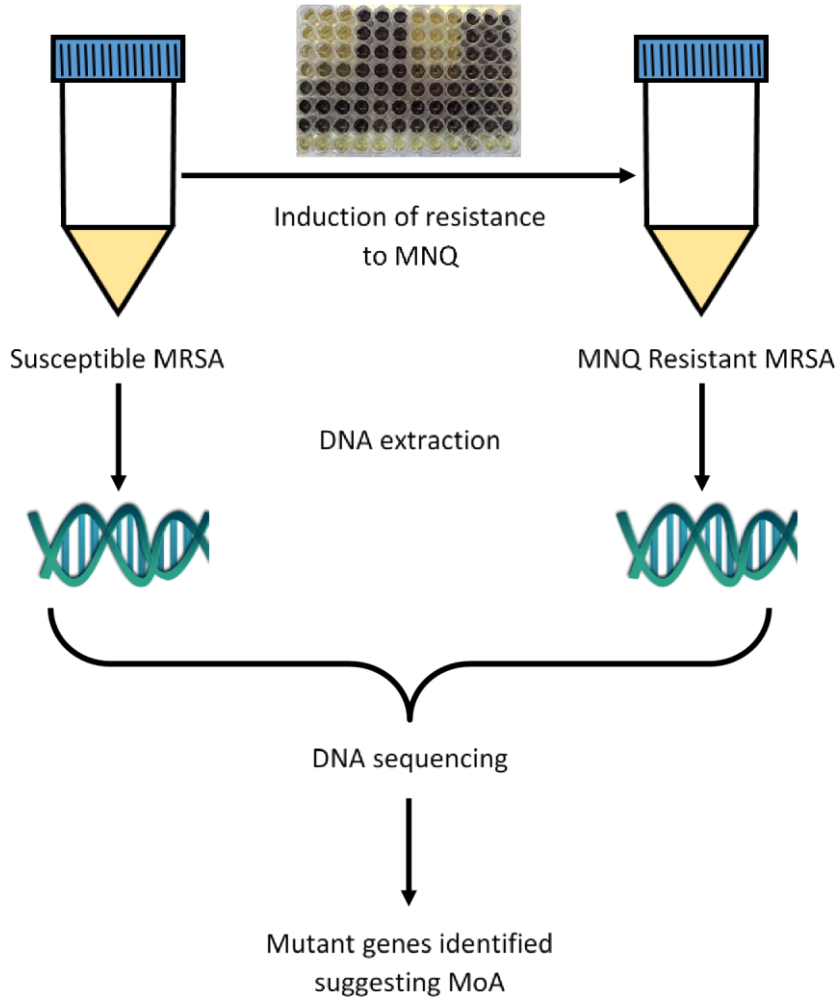


Figure 25: Genomic approach to MoA studies – Genes of interest can be identified by inducing resistance to the drug and comparing to the DNA sequence of a resistant and non-resistant bacteria. The genes which have changed convey a protective characteristic against the drug and can be used to infer the MoA.

3.1.4 – Proteomics

Proteomics is the large-scale analysis of proteins within organisms to gain important insights into many biological processes. Investigation of living systems at the protein level opened new horizons in many areas of life science. DNA microarrays and transcriptomics can provide data about gene function however proteins are the real mediators of physiological function. Changes in biological events such as disease, drug effects, or physiological activity will be reflected in the abundancy or processing of proteins.

Proteomics usually involves several processes; the extraction of the proteins followed by purification, fractionation, digestion into peptides which are separated and analysed by liquid chromatography mass spectrometry (LC-MS) to identify the proteins. Polyacrylamide gel electrophoresis (PAGE) is a technique commonly used to separate protein extracts. This provides a visual representation of all proteins present within samples and their relative quantities allowing the selection interesting proteins for further analysis (Ünlü, Morgan and Minden, 1997). Gel based proteomics can be challenging; 2D-PAGE typically visualising 30-50% of proteins tending to neglect membrane-associated proteins, or proteins whose isoelectric point, hydrophobicity, and/or molecular weight fall beyond the parameters of the separation method employed (Baggerman et al., 2005). LC-MS fundamentally changed the way in which proteins are identified and measured, due to its ability to measure thousands of proteins in parallel allowing the investigation of increasingly complex biological matrices. MS is now the de facto standard for quantitative measurements in proteomics (Bantscheff et al., 2012). With the advances in LC-MS and the inherent issues with gel-based proteomics gel-free methodologies were improved by using multidimensional capillary LC-MS/MS.

Even though MS has become the de facto method for proteomic analysis there are a range of different technologies available; metabolic labelling, chemical labelling or label free. The most popular metabolic labelling method is stable isotopic labelling with amino acids in cell culture (SILAC). Unlabelled and labelled protein populations can be mixed directly after harvesting and mass spectrometric identification is straight forward due to the isotopically amino acids in the treatments. This method requires limited sample manipulation, and with virtually 100% incorporation of the label and quantification is relatively easy (Ong et al., 2002). An alternative approach involves chemical labelling of peptides and proteins. For example, isobaric tags for absolute and relative quantification (iTRAQ) is a popular method which targets primary amines for the attachment of tags, which have identical masses but can be distinguished and accurately quantified according to MS/MS spectra (Wiese et al., 2007). Other techniques do not require labels (Bantscheff et al., 2012).

Shotgun proteome digestion approaches yield a complex mixture of peptide which have been digested using specific enzymes, so fragmentation can be predicted, and original protein structure elucidated from MS/MS data. This technique requires separation and nanoscale ion paring reversed phase LC-ESI-MS/MS is commonly used but there have recently been new technologies in this area, most notably the use of microfluidic chip-based technology has been emerging. These chips can aid in sample clean up, digestion separation, and throughput due to their ability to handle small quantities (Lee, Soper and Murray, 2009).

3.1.5 – Metabolomics

Metabolites are small molecules that are chemically transformed during metabolism which can be used to interpret the cellular state of an organism. Unlike genes and proteins discussed previously, whose functions are subject to epigenetic regulation and post translational modification, metabolites serve as direct signatures of biochemical activity. The study of the metabolome, defined as the collection of all small metabolites produced by cells, has become widely adopted in clinical diagnosis of cancer (Spratlin, Serkova and Eckhardt, 2009), diabetes (Griffin and Nicholls, 2006), cardiovascular diseases (Lewis, Asnani and Gerszten, 2008), asthma (Carraro et al., 2007), neurological diseases (Dunckley, Coon and Stephan, 2005), and many other diseases (Madsen, Lundstedt and Trygg, 2010). Metabolomics is not only an invaluable diagnostic tool it can also provide insight into the mechanism by which drugs act on a biological system, for example the effect of an antibiotic on bacteria. Metabolomics is an imperfect method due to the fact that databases used to identify metabolites are incomplete (Kind, Scholz and Fiehn, 2009), however metabolomics has made remarkable progress and has become the apogee of the omics trilogy (Patti, Yanes and Siuzdak, 2012).

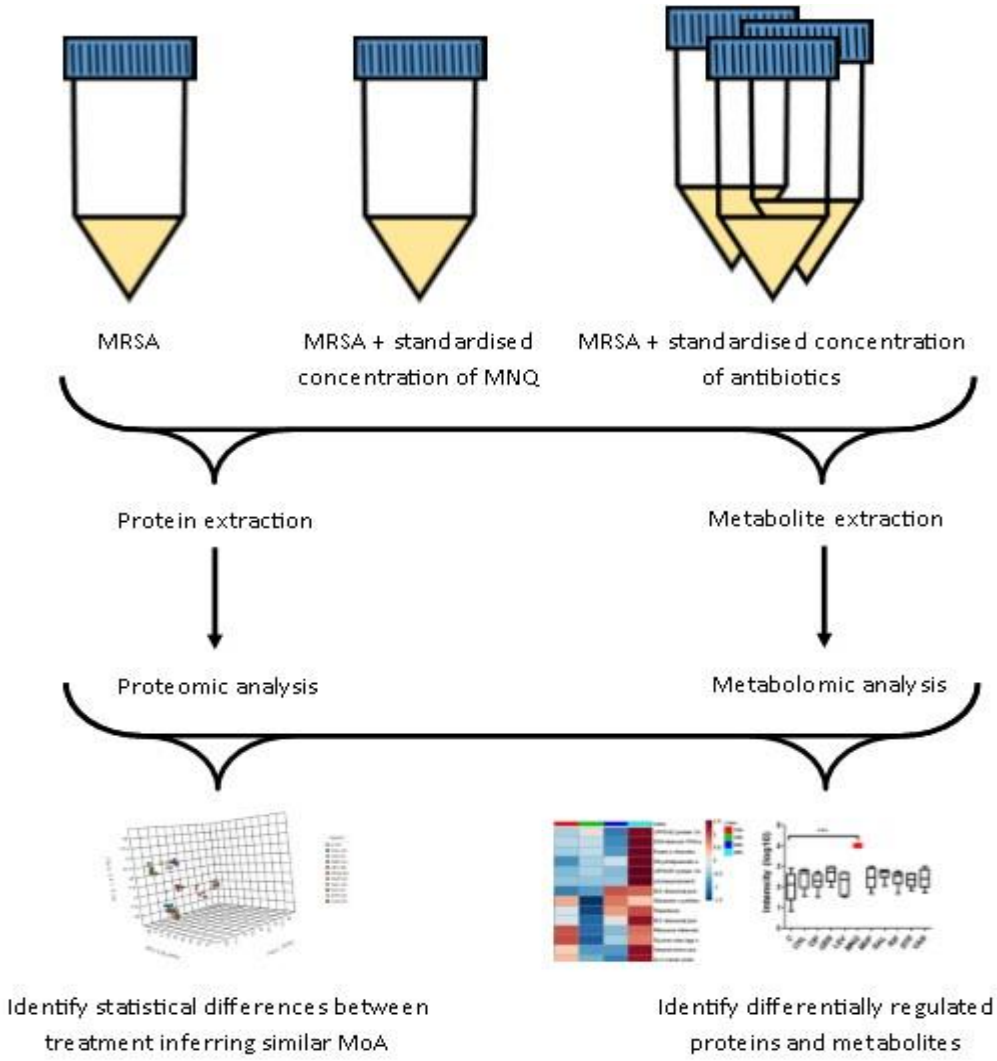


Figure 26: Proteomic and metabolomic approaches: the proteomic and metabolomic profile of a bacterium require treatment with the compound of interest along with known antibiotics for comparison. This is followed by the extraction of proteins and metabolites and then analysis using MS. The proteomic and metabolomic profiles are compared with known MoA, potentially leading to target identification. Further information can be obtained by looking at specific regulation of certain proteins/metabolites to ascertain MoA.

3.1.6 – 'Omics' for Antimicrobial Mechanism of Action Studies

Each of the three 'omics' discussed can be used to investigate the Mechanism of action (MoA) of antimicrobial compounds. Unlike genomics, proteomics and metabolomics can provide real-time information of the MoA of MNQ. By their nature proteins and metabolites are more reactive to changes in environment, such as the presence of a drug, therefore resistance will not need to be induced. The mere presence of the drug will have significant effects on protein expression and metabolite regulation. (Santos et al., 2016)

3.1.7 – Antibiotic modes of action

3.1.7.1 – DNA targeting antibiotics

4-quinolones such as nalidixic acid, ciprofloxacin and levofloxacin (Figure 27) are agents which target two essential bacterial enzymes, topoisomerase II (DNA gyrase) and IV. Nalidixic acid is a first generation naphthyridone quinolone predominantly used for urinary tract infections (Drlica & Zhao, 1997). Whereas ciprofloxacin and levofloxacin are second generation fluoroquinolone which have enhanced activity, broader spectrum, and able to treat a wide range of bacterial infections (Wolfson & Hooper, 1989).

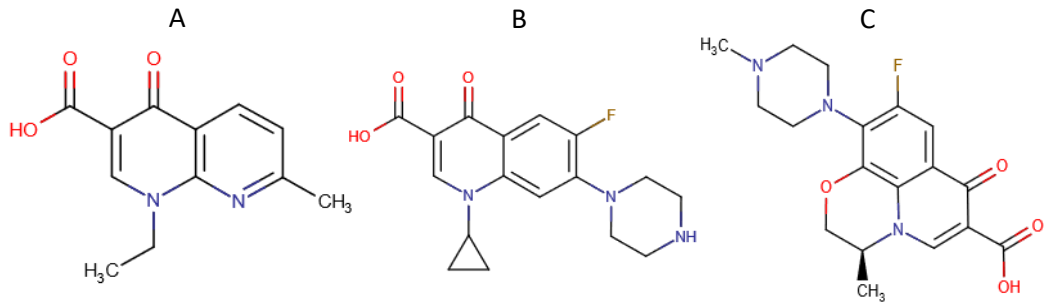


Figure 27: Chemical structure of nalidixic acid (A), ciprofloxacin (B), and levofloxacin (C).

These enzymes alter the topological state of DNA which determines the function of DNA in cells. Generally, type I topoisomerases cleave one strand of double helical DNA to relax supercoiled DNA, whereas type II use energy from the hydrolysis of ATP to cleave both strands and pass an intact DNA strand through this "gate" to introduce supercoils. DNA gyrase and topoisomerase IV are both type II involved in the supercoiling of DNA. In prokaryotes supercoiling of DNA results in torsion strain, and the DNA can be considered in an energetically active state which is crucial for DNA replication, transcription and genetic recombination (Gellert, 1981). DNA gyrase binds directly to DNA as a tetramer with two gyrA and two gyrB subunits. The B proteins have ATPase activity to obtain energy for the A subunits to cleave both DNA strand and increasing or decreasing the linking number by two, either increasing torsion leading to positive super coiling or decreasing torsion leading to negative supercoiling (Reece and Maxwell, 1991). Gyrase can also relax negative

supercoiling in the absence of ATP therefore the balance between the levels of ATP versus ADP is a key aspect of the supercoiling relaxation relationship, making gyrase and supercoiling sensitive to intracellular energetics, and many aspects of the extracellular environment (Drlica and Zhao, 1997). DNA gyrase interacts with DNA in multiple ways and there are many ways quinolones can interrupt this activity, summarised in Table 23.

Table 23: Summary of reactions DNA gyrase is capable which subunits are involved, whether ATP is required, and which reactions quinolones can interrupt (Reece and Maxwell, 1991)

The Reactions of DNA Gyrase		Subunits required	ATP required	Inhibited by quinolones
Supercoiling		A, B	Yes	Yes
Relaxation	Negative supercoils	A, B	No	Yes
	Positive supercoils	A, B	Yes	Yes
Catenation		A, B	Yes	Yes
Decatenation		A, B	Yes	Yes
Unknotting		A, B	Yes	Yes
DNA cleavage		A, B	No	No
ATPase		B	Yes	No

For example, quinolones can prevent 6 of the 8 functions of DNA gyrase by forming a quinolone-enzyme-DNA complex at the point where the double strand break occurs. There are two quinolone binding pockets between GyrA and GyrB (Heddle and Maxwell, 2002), when filled with a quinolone the complex is trapped in this open gate position, if gyrase is trapped in this form ahead of the fork blocking any further movement along the replication fork along the DNA inhibiting DNA synthesis (Kreuzer and Cozzarelli, 1979). The formation and dissociation of this complex leads to cell death through double strand breakage of the DNA which is lethal to cells (Krasin and Hutchinson, 1977).

3.1.7.2 – RNA/protein targeting antibiotics

Aminoglycosides, such as gentamycin and streptomycin (Figure 28) target the bacterial ribosome disrupting protein synthesis. Streptomycin was the first aminoglycoside discovered, isolated from Streptomyces griseus. Gentamycin is a 4, 6-disubstituted 2-deoxystreptamine class of aminoglycoside, there's also a 4,5-distrubyted 2-deoxystreptamines class although streptomycin is not classified in the same way (Ristuccia and Cunha, 1982). Aminoglycosides have a polycationic structure which have an affinity for negatively charged residues such as those in the outer membrane of gram-negative bacteria. This affinity allows the drug to electrostatically bind to the membrane of bacteria and access the periplasmic space through porin channels (Hancock et al., 1991). The antibiotic is then transported across the cytoplasmic membrane utilising the electron transport chain in an energy and oxygen dependant process. This is the rate limiting step and means that these antibiotics are less effecting in anaerobic conditions. Once in the cytosol the antibiotics bind 30S subunit, not preventing the formation of the of the initiation complex but perturbing the elongation of the nascent chain by impairing the proofreading processes controlling translation accuracy. These proteins may be inserted into the membrane leading to altered permeability and further stimulate uptake of aminoglycosides (Melancon, Tapprich and Brakier-Gingras, 1992).

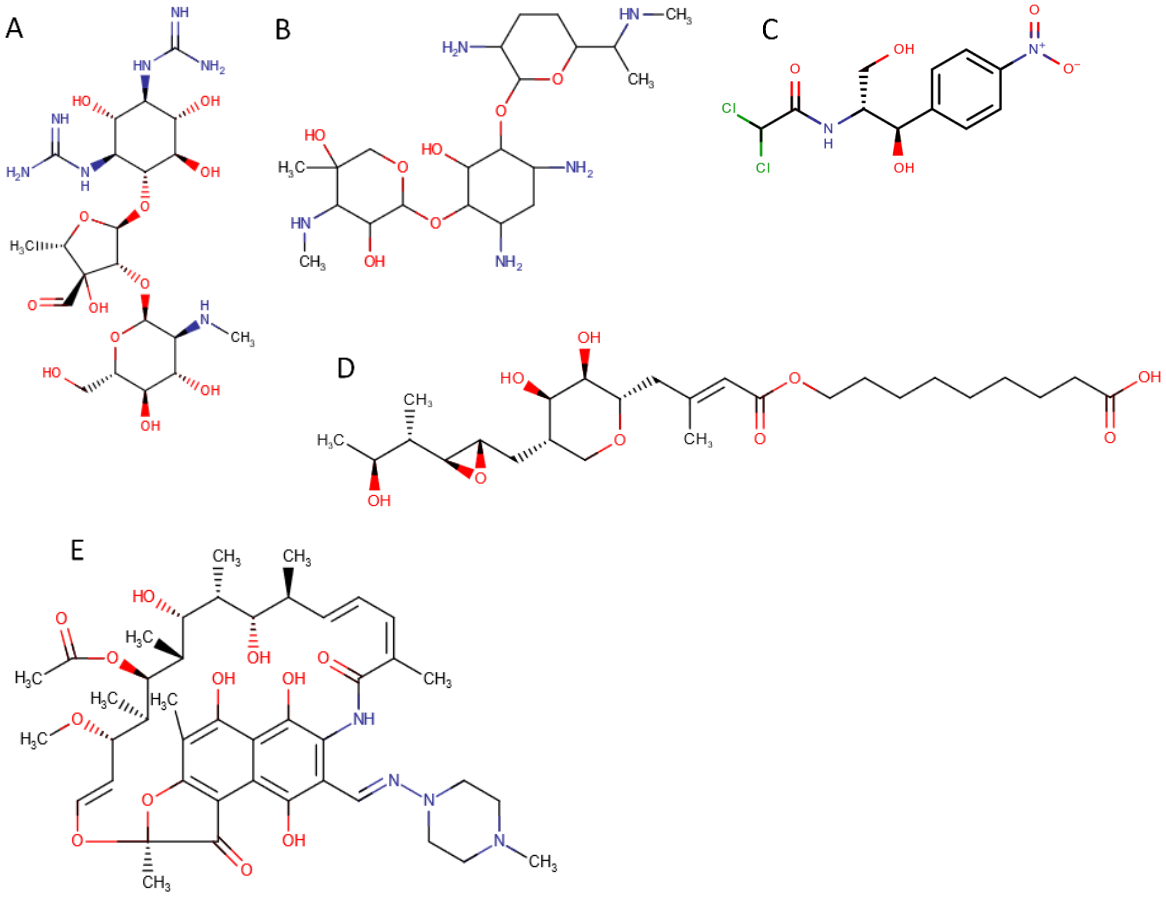


Figure 28: Aminoglycoside antibiotics streptomycin (A) and gentamycin (B). Chloramphenicol (C), Mupirocin (D) and Rifampicin (E)

Chloramphenicol (Figure 28c) was first isolated from Streptomyces venezuelae in 1947 and was later synthesised in 1949, making it the first antibiotics to be synthesised rather than extracted from microorganism. Unlike most antibiotics' chloramphenicol does not belong to a family of other similar drugs and is classed as "other". Chloramphenicol, like aminoglycosides, inhibit protein synthesis but in a different way, it is bacteriostatic, binding the L16 subunit of the 50S ribosomal protein. This protein binds directly to the 23S ribosomal RNA located at the peptidyl transferase centre which plays an essential role in subunit assembly (Murray et al., 1995). It directly interferes with substrate binding, whereas macrolides, a family of antibiotics which work in a similar way prevent the growth of the peptide chain (Hahn, Wisseman Jr and Hopps, 1955).

Mupirocin does not belong to a class of antibiotics with a unique mechanism of action. It was discovered in 1971 when it was isolated from Pseudomonas florescens (Fuller et al., 1971). Mupirocin binds to isoleucine-tRNA ligase, which catalyses the attachment of isoleucine to tRNA, although this is a unique MoA this in effect inhibits protein synthesis (Hurdle, O'neill and Chopra, 2004). Although this mainly effects protein synthesis this also negatively impacts DNA activity and cell wall formation (Hughes and Mellows, 1978).

Rifampicin is a polyketide with a naphthalene at the core (Figure 28e) belonging to a class of chemicals called ansamycins, it is a semisynthetic antibiotic derived from rifamycin antibiotics which are naturally produced by Nocardia mediterranei. Rifampicin was discovered in 1965 (Sensi, 1983). Rifampicin's bactericidal activity is due to its strong binding affinity to and the inhibition of bacterial DNA-dependant RNA polymerase, which catalyses the transcription of DNA into RNA using the four ribonucleoside triphosphate as substrates (Hartmann, 1967).

3.1.7.3 – Cell wall targeting antibiotics

Vancomycin (Figure 29) is a branched tricyclic glycosylated nonribosomal peptide first isolated from Amycolatopsis orientalis in 1953 (Griffith, 1981). Due to the large hydrophilic structure of vancomycin it prevents proper cell wall synthesis in gram positive bacteria it forms hydrogens bond interactions with D-Ala-D-Ala moiety of N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG) peptide subunits of peptidoglycan. Without these bonds the peptidoglycan layer cannot remain intact and fall away leaving the bacteria without a protective barrier eventually causing the death of the bacteria (Barna and Williams, 1984).

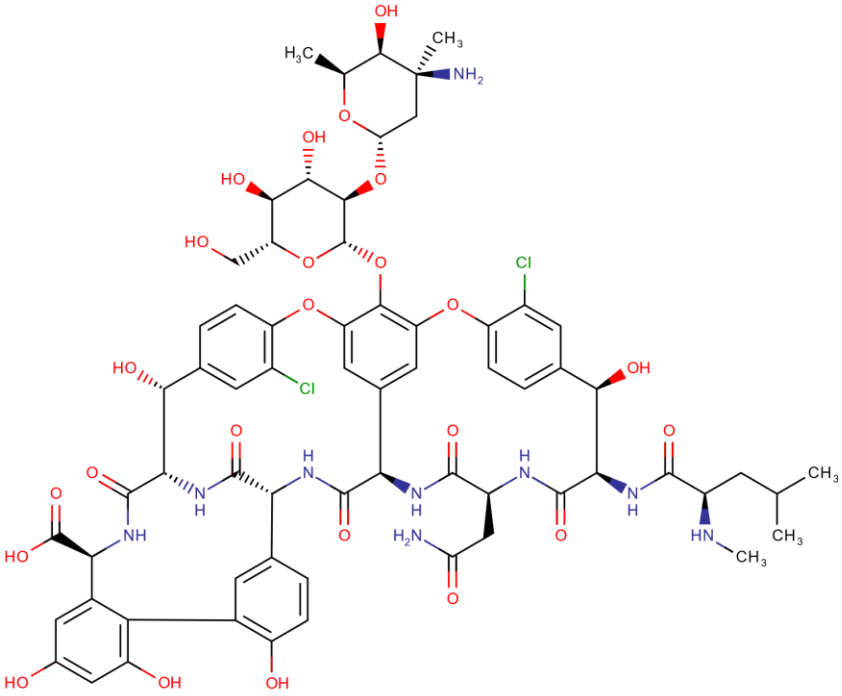


Figure 29: Chemical structure of Vancomycin

3.2 – Methods – Mode of action

3.2.1 – Bacterial strains and growth conditions

All experiments carried out in a biosafety containment level 2 using ST8:USA300 MRSA grown in Brain heart infusion (BHI) media incubated over night at 37 °C while shaking at 250 rpm (Vitko and Richardson, 2013). Optical density (OD) at 600 nm was used to determine bacteria concentrations as described in 1.2.5 – Antimicrobial susceptibility testing – 24-hour growth curve. This strain was chosen due to the clinical relevance of MRSA and MNQ has been shown to be particularly effective against *S. aureus*. The required inhibitory concentrations of all antibiotics used in this section was determined using this strain at these growth conditions.

3.2.2 – Standardisation of antibiotic treatment

All antimicrobial agents; chloramphenicol (CHL), ciprofloxacin (CIP), gentamycin (GEN), levofloxacin (LEV), mupirocin (MUP), nalidixic acid (NAL), rifampicin (RIF), streptomycin (STR),

vancomycin (VAN), and 2-methoxy-1,4-naphthoquinone (MNQ) were obtained from Sigma-Aldrich, Gillingham, UK. For both proteomics and metabolomics antibiotic treatments need to be standardised so each antibiotic is having an equal effect on the bacteria over 6 hours. In order to obtain enough material to accurately detect and quantify proteins and metabolites a higher concentration of bacteria was required. MIC susceptibility testing is standardised to 5 × 105 cfu/mL but for metabolomics it was found that 1x108 cfu/mL was required. Therefore, susceptibility tests were carried out against the higher concentration and over 6 hours. To investigate the MoA of these antibiotics the bacteria cannot be killed outright, this would not reveal how the drugs work therefore the amount of antibiotic required to reduce growth by half over 6 hours was used in the proteomic and metabolomic experiments (Table 24). This would allow the bacteria to react to the challenge of the antibiotics without dying. This also standardises the effect of each antibiotic so they should all have the same proteomic and metabolic impact on the bacteria.

Table 24: Concentration of antibiotic required to inhibit growth of USA300 MRSA by 50% over 6 hours at a bacterial concentration of 1x10^{8} cfu/mL and their mode of action.

Antibiotic	**Concentration (mg/L)**	**Mode of action**
CHL	250	RNA - Binds 50S subunit (L16) or rRNA inhibiting protein synthesis.
CIP	15.625	DNA – 4-quinolone - Inhibition of the enzyme topoisomerase II (DNA gyrase) & IV preventing DNA replication and transcription (Drlica and Zhao, 1997).
GEN	250	RNA – aminoglycoside - Binds 30S subunit (S12) of rRNA misreading mRNA and incorrect amino acid insertion.
LEV	10.625	DNA - 4-quinolone - Inhibition of the enzyme topoisomerase II (DNA gyrase) & IV preventing DNA replication and transcription (Drlica and Zhao, 1997).
MNQ	250	Unknown
MUP	5.313	RNA - Binds isoleucyl-tRNA synthetase inhibiting bacterial protein and RNA synthesis.
NAL	250	DNA - 4-quinolone - Inhibition of the enzyme topoisomerase II (DNA gyrase) & IV preventing DNA replication and transcription (Drlica and Zhao, 1997).
RIF	0.122	RNA - Inhibit DNA-dependent RNA polymerase supressing RNA synthesis.
STR	250	RNA - Binds 30S subunit (S12) of rRNA misreading mRNA and incorrect amino acid insertion.

VAN	10.625	Cell Wall - Prevents incorporation of N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG) peptide subunits from being incorporated into the peptidoglycan matrix. In addition, altering bacterial-cell membrane permeability and RNA synthesis.

3.2.3 – Proteomics – Bacteria treatment and standardisation

A 1 L culture of MRSA was grown overnight (section 3.2.1) and bacterial concentration standardised to 1x108 cfu/mL using a spectrophotometer OD600 (Hidex Sense Plate Reader LabLogic, Sheffield UK) by adding sterile growth media. This culture was aliquoted into twelve 50 mL sterile centrifuge tubes. Three antibiotic treatments were chosen; MNQ, Vancomycin (current treatment for MRSA), Levofloxacin (Table 24), and a no treatment control with three biological replicates. Following addition of antibiotics, the 50 mL cultures were mixed and incubated at 37°C shaking at 250 rpm. Two time points were sampled for proteomics, 0 hours (which is roughly 1 min after adding the antibiotic) and 6 hours after the addition. Upon sampling 14 mL was transferred into a new centrifuge tube, frozen in liquid nitrogen and stored in a -80°C freezer overnight. Samples were thawed and centrifuged (4°C, 4500 rpm) with the resulting pellet washed in 10 mL of cold saline solution (0.85% NaCl) and centrifuged again (4°C, 4500 rpm). All samples were adjusted to an OD600 of 1 in 200 µL of water (18.2 Ω) prior to protein extraction.

3.2.4 – Proteomics – Protein extraction and quantification

To the 200 µL samples was added 200 µL of 20% trichloroacetic acid in acetone and subjected to 50 cycles of sonication (1 minute), rapid freezing liquid nitrogen, thawing and vortex (1 minute). A 1 hour incubation at -20°C to precipitate proteins followed. Sample were centrifuged (4°C, 13,000 rpm), washed twice in acetone followed by further centrifugation, supernatant was removed, and samples left to dry at -20°C for 15-20 minutes. Protein concentration of all samples were calculated using the Braford method for protein quantification (Kruger, 2002). The lowest concentration of protein yielded in any sample was 514.844 µg, therefore all samples were made to

a concentration of 500 µg in 100 µL of 6 M urea buffer (2.0 g of urea, 1.25mLof 0.4 M Tris solution and 5mLwith Milli-Q H20).

3.2.5 – Proteomics – Protein Trypsin Digestion

To the 100 µL extracted and standardised protein solution in urea buffer (Supelco), was added 5 µL of the reducing agent dithiothreitol (DTT) (Sigma-Aldrich) (0.031 g of DTT in 750 µL of MilliQ-H2O, and 250 µL 0.4 M Tris solution(Millipore)), followed by vortex and 60 minutes incubation at room temperature. 20 µL of alkylating agent (0.037 g iodoacetamide (Sigma-Aldrich) in 750 µL of MilliQ-H2O, and 250 µL 0.4 M Tris solution (Millipore)) was added followed by vortex and 60 minutes incubation at room temperature. A further 20 µL of the reducing agent was again added then vortexed and incubated for 60 minutes at room temperature. Samples were diluted using 775 µL MilliQ-H2O and vortexed. Trypsin (Promega, Gold Mass Spectrometry Grade) was added at 1:50 ratio to the amount of protein, mixed and the digestion was carried out at 37°C overnight.

3.2.6 – Proteomics –Data analysis

Proteomics data was analysed using Spectrum Mill (Agilent, Santa Clara) and Swissprot (Swiss Institute of Bioinformatics, Lausanne). 261 proteins were identified using the proteome data available from Swissprot (STAPHUSA300TCH1516). Univariate and multivariate analyses were performed with MetaboAnalyst 3.0 (Xia Lab, McGill University). Differences in the proteomic profiles of samples were analysed with unsupervised principal component analysis (PCA) and supervised partial least-squares discriminant analysis (sPLS-DA). The significance of the cross-validated P-values, based on volcano plot comparisons between control and treatment at individual time points ($P<0.1$).

3.2.7 – Metabolomics – Bacterial treatment and standardisation

A 2 L culture of MRSA was grown overnight (section 3.2.1) and bacterial concentration standardised to 1x108 cfu/mL using a spectrophotometer OD600 using sterile growth media. This

was roughly a ½ dilution which provided around 4 litres of bacterial culture and 2.75 L were required in total. This culture was aliquoted into 55, 50 mL sterile centrifuge tubes. All antibiotic treatments were used at concentrations stated in Table 24, and a no treatment control using 5 biological replicates. Antibiotics were added to 50 mL cultures and mixed. Throughout the experiment the bacteria were incubated at 37°C shaking at 250 rpm. As the antibiotic was added (0 hour), after 2, 4, and 6 hours; 10 mL was transferred into a new 15 mL centrifuge tube and frozen in liquid nitrogen and stored in a -80°C freezer overnight. Samples were thawed and centrifuged (4°C, 4500 rpm) with the resulting pellet washed in 10 mL of cold saline solution (0.85% NaCl) followed by further centrifugation (4°C, 4500 rpm). All samples were adjusted to an OD600 of 1 in 200 µL of water prior to metabolite extraction.

3.2.8 – Metabolomics – Metabolite extraction and quantification

Aliquots of 200 µL of chloroform/methanol/water (1:3:1) solution were added to 200 µL samples. All samples were subjected to 5 freeze thaw cycles with periodic vortexing to extract all metabolites. Samples were then centrifuged at 4500 rpm to remove any particulate matter and 200 µL of supernatant was transferred to a new microcentrifuge tube. A second extraction was carried out by adding a further 100 µL of chloroform/methanol/water (1:3:1) to the pellet with further vortexing. After centrifugation, 150 µL of the supernatant was combined with supernatant from the first extraction. From these 350 µL metabolite extracts 50 µL was transferred to glass vials containing 0.2 mL flat bottomed micro insert for flow injection electrospray high-resolution mass spectrometry (FIE-HRMS) analysis. Master mixture samples were made up of all 5 biological replicates at all time points creating a total of 264 samples. These samples were randomised with methanol blanks equally distributed throughout the run.

3.2.9 – Metabolomics – Metabolite fingerprinting by flow injection electrospray high-resolution mass spectrometry

A high-throughput, non-targeting metabolite fingerprinting method which does not incorporate chromatography was used to analyse the metabolomes of MRSA treated with MNQ and other antibiotics (Beckmann et al., 2008). Flow injection electrospray high-resolution mass spectrometry (FIE-HRMS) was performed using an Exactive HCD mass analyser equipped with an Accela UHPLC system (Thermo Scientific) generating metabolite fingerprints in both positive and negative ionisation modes in a single run. Of the 50 µL samples, 20 µL was injected into a flow of 100 µL/min methanol/water (70:30, v/v). Ion intensities were acquired between m/z 50 and 1000 for 3.5 minutes at a resolution setting of 100,000 (at m/z 200) resulting in 3 (± 1) ppm mass accuracy. ESI source parameters were set according to Thermo Scientific recommendations. Raw files were exported to CDF-files, mass aligned and centroided in MATLAB (V8.2.0, The MathWorks) maintaining highest mass accuracy. Mass spectra around the apex of the infusion peak were combined in a single intensity matrix (runs x m/z) for each ion mode. Data from intensity matrix was log-transformed before further statistical analysis.

3.2.10 – Metabolomics –Data Analysis

Univariate and multivariate analyses were performed with MetaboAnalyst 3.0. Differences in the metabolomics profiles of samples were analysed with unsupervised principal component analysis (PCA) and supervised partial least-squares discriminant analysis (sPLS-DA). The significance of the cross-validated P-values, based on one-way analysis of variance (ANOVA), was set to $P < 0.05$. Multiple comparison and post hoc analysis used Tukey's Honestly Significant Difference (Tukey's HSD). Both ANOVA and Tukey's HSD allowed the identification of significant metabolite changes between groups (control or antibiotics). Metabolites that did not show significant differences between treatment and control were not further analysed. For each mass-ion (m/z) the annotation was made using a 3 ppm tolerance on their accurate mass. Metabolomic annotation was made using

MZedDB (Draper et al., 2009) (http://maltese.dbs.aber.ac.uk:8888/hrmet/index.html), the *E. coli* Metabolome Database (ECMDB, http://ecmdb.ca/) and LipidMaps (http://www.lipidmaps.org/) databases, considering the following possible adducts: [M + H]+ , [2M + H]+ , [M + 2H]2+ , [M + 3H]+ , [M + Na]+ , [M + Na + 2H]+ , [2M + Na]+ , [M + 2Na−H]+ , [M + 2Na + H]+ , [M + Na + H]2+ , [M + 2Na]+ , [M + 3Na]3+ , [M + K]+ , [M + K + H]2+ , [M + 2K + H]+ , [M−FA + H]+ , [M−H2O + H]+ , [2M + 3H2O + 2 H]2+ , [M + NH4]+ , [M + NH4 + H]+ , [M + NH4 + H]2+ , [2 M + NH4]+ , [M + ACN + H]+ , [M + ACN + Na]+ , [2M + ACN + H]+ , [M + ACN + 2H]+ , [M + 2ACN]2+ , [M + 2ACN + H]+ , [M + 2ACN + 2H]+ , [M + 3ACN + 2H]+ , [M + ACN + Na]+ , [M + CH3OH + H]+ ; [M−H]−, [2M−H]−, [3M−H]−, [M−2H]−, [M−3H]−, [M−H2O−H]−, [M + Cl]−, [M + FA−H]−, [2M + FA−H]−, [M + Hac−H]−, [M + Na−2H]−, [2M + Na−2H]−, [M + K−2H]−, [M + TFA−H]−. This was carried out using R statistical software package (R-project, version 3.6.1). A script was written to feed a csv file into the selected databases to output the most likely metabolite identification for each m/z value which was found to be significant (Appendix 5 – R-statistical package script). The targeted metabolites were mapped on to Kyoto Encyclopaedia of Genes and Genomes (KEGG) for pathway analysis (http://www.genome.jp/KEGG/pathway.html).

3.2.11 – Transmission Electron Microscopy

Overnight cultures of MRSA USA300 were adjusted to 1x108 cfu/mL and treated with standardised concentration of MNQ for 6 hours. 1 mL samples were taken at 0, 2, 4 and 6 hours, then centrifuged at 10,000 rpm for 5 minutes. The supernatant was removed, 1 mL of fixative (2.5% glutaraldehyde in 0.1M sodium cacodylate at pH 7.2) was added to the bacterial pellet, resuspended and stored at 2-8 °C. After 30 minutes fixation, the samples were centrifuged, and the supernatant discarded. The pellets were re-suspended in another 1 mL of fresh fixative and a further 30 minutes, samples were centrifuged, supernatant discarded and re-suspended in 1 mL 0.1 M sodium cacodylate wash buffer pH at 7.2. The samples were centrifuged, and the supernatant discarded. They were resuspended in 1 mL of a secondary fixative consisting of 1% osmium tetroxide (Agar

Scientific Ltd) made up in 0.1M sodium cacodylate buffer pH at 7.2 and after 30 minutes fixation, the samples were centrifuged, the supernatant carefully discarded and it was replaced with a quick rinse in 1 mL of wash buffer. After 5 minutes rinse, the samples were centrifuged, and the supernatant discarded. The pellets were re-suspended in another 1mL of wash buffer. The samples were centrifuged, and the supernatant discarded. The samples were re-suspended in 100 µl agarose solution at 25^{o}C and placed in a refrigerator to gel at 4^{o}C. After gelling overnight, the agarose containing the bacteria was cut from the Eppendorf tubes and transferred into 1 mL wash buffer in a capped 5 mL glass vials at 4^{o}C. After 30 minutes the gelled agarose pellets were placed in fresh wash buffer. Samples were then progressed through an alcohol series of 30%, 50%, 70%, 95% and three changes of 100% for at least an hour. The samples were transferred to a 1:2 mixture of ethanol to LR White hard grade resin (London Resin Company) then a 2:1 mixture of ethanol to resin and finally 100% resin overnight at 4^{o}C. The next morning, the resin was removed and replaced with fresh resin and later that day the samples were then placed in size 4 gelatine moulds (Agar Scientific), filled up with fresh resin and polymerised overnight in an oven at 60^{o}C. 2 µm thick sections containing the bacteria were cut and dried down on drops of 10% ethanol on glass microscope slides. They were stained with AMB stain (Merck, Darmstadt) and photographed using a Leica DM6000B microscope (Leica Biosystems, Wetzlar). Ultrathin 60–80 nm sections were then cut on a Reichert-Jung Ultracut E Ultramicrotome with a Diatome Ultra 45 diamond knife and collected on Gilder GS2X0.5 3.05 mm diameter nickel slot grids (Gilder Grids, Grantham, UK) float-coated with Butvar B98 polymer (Agar Scientific) films. All sections were double stained with uranyl acetate (Agar Scientific) and Reynold's lead citrate (TAAB Laboratories Equipment Ltd, Aldermaston, UK) and observed using a JEOL JEM1010 transmission electron microscope (TEM) (JEOL Ltd, Tokyo, Japan) at 80 kV. The resulting images were photographed using Carestream 4489 electron microscope film (Agar Scientific, UK) developed in Kodak D-19 developer for 4 minutes at 20 °C, fixed, washed and dried according to the manufacturer's instructions.

To visualise the presence of hydrogen peroxide (H2O2) produced by MRSA bacterial samples untreated and treated with MNQ and HNQ were placed in cerium chloride prior to fixation. Cerium chloride reacts with H2O2 resulting in the presence of cerium perhydroxide which shows up as black needle-like crystals under TEM (Bestwick et al., 1997).

3.2.12 – Antimicrobial Assay with antioxidants

This assay was repeated as discussed in 1.2.6 – Antimicrobial susceptibility testing – Minimum inhibitory concentration, MNQ was combined with catalase and ascorbate (100 mg/L).

3.3 – Results – Mode of action

3.3.1 – Proteomics

Label free proteomic methodologies were used to further understand how MNQ effects the growth of MRSA after immediate exposure and 6 hours. In addition, two antibiotics were also used for comparative purposes; vancomycin and levofloxacin.

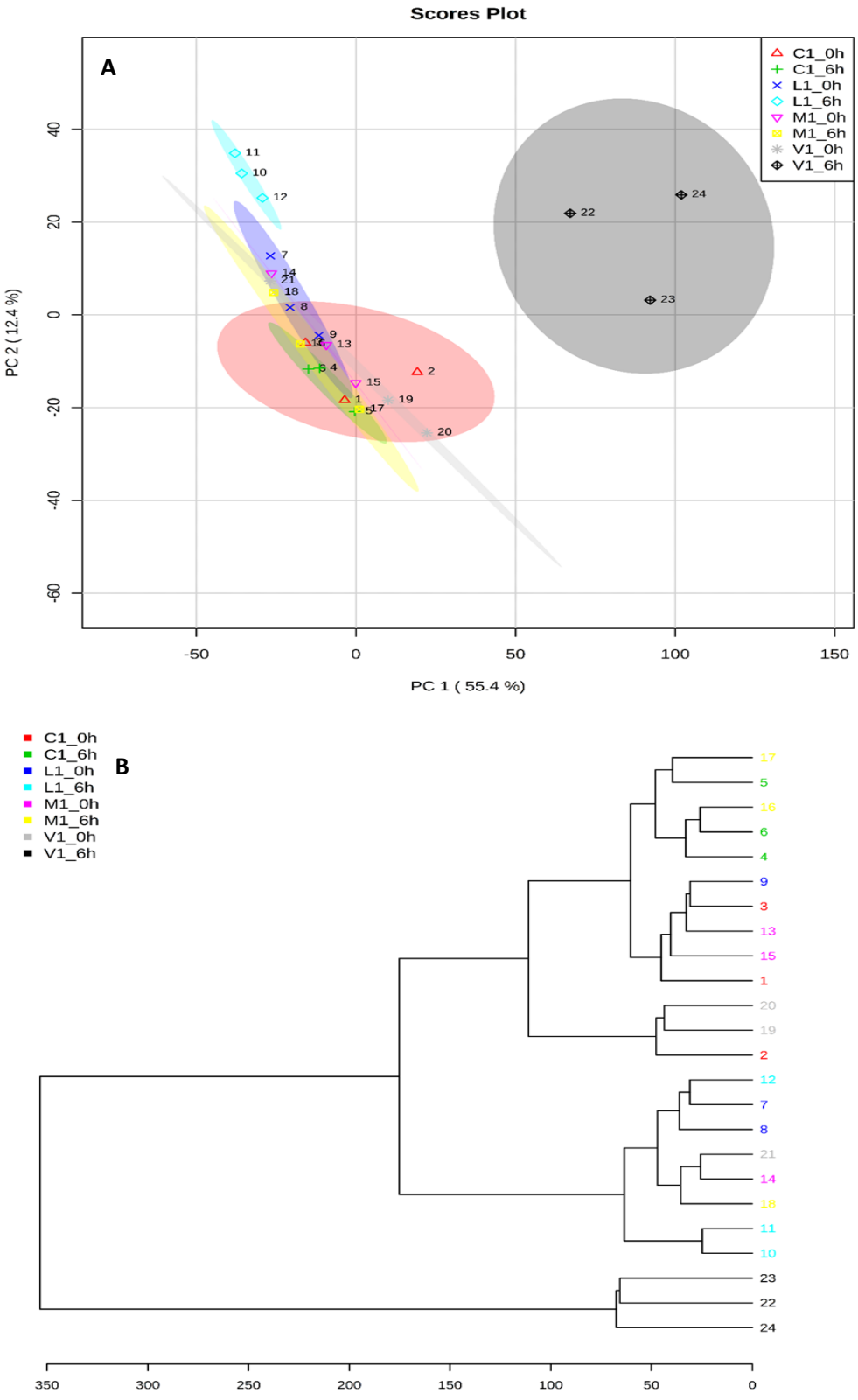


Figure 30: A – Principle component analysis showing the separation of all proteomic antibiotic treatments and time points based on the different quantities of proteins identified. B - Dendrogram of all proteomic antibiotic treatments and time points

When analysing all proteome data at once Vancomycin has had the most significant effect on the proteome of MRSA over 6 hours, showing clear separation in a principle component analysis and the dendrogram (Figure 30).

Table 25: Statistical analysis of all treatments and the total number of significantly affected proteins (n=3).

Treatment	**MNQ**		**LEV**		**VAN**	
Time Point (hours)	0	6	0	6	0	6
Volcano Plot (p<0.1)	3	4	16	52	3	128
Fold change (threshold 2)	94	51	134	111	71	228
ANOVA (p<0.05)	4	4	17	17	91	91

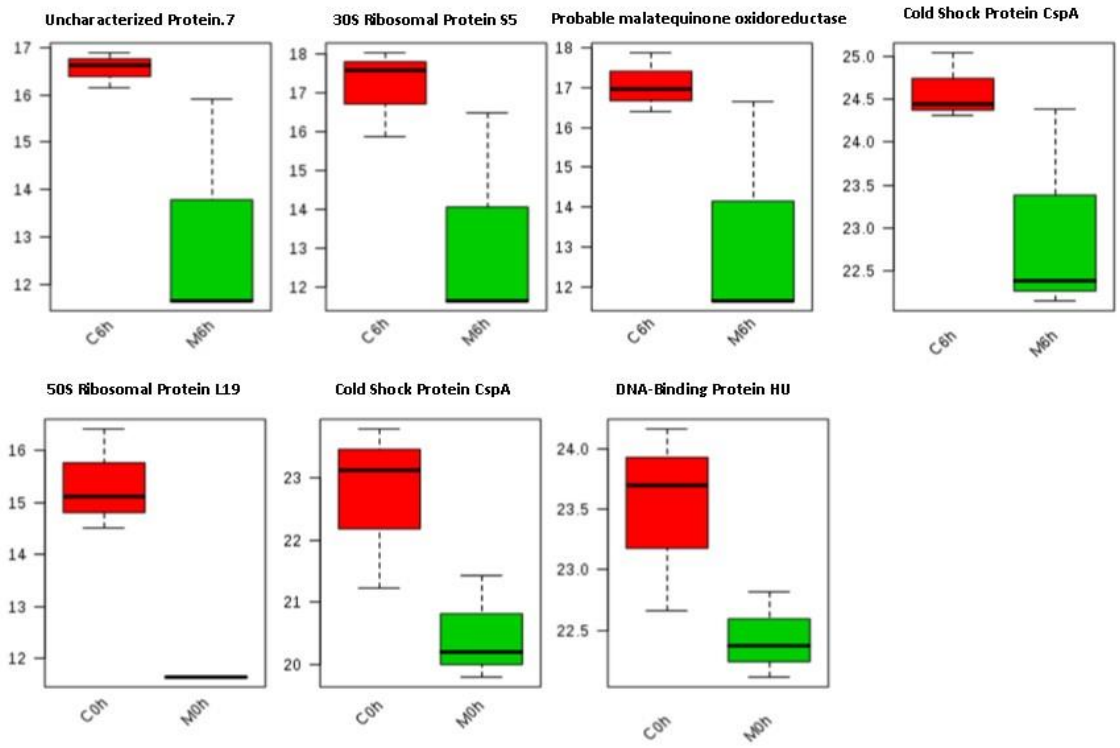


Figure 31: Box plots showing significantly (p<0.1) changed proteins when MRSA in exposed to MNQ at 0 and 6 hours.

Of the three antibiotics tested vancomycin had the largest number of significantly different proteins in all statistical tests, followed by Levofloxacin and MNQ had by far the fewest protein differences (Table 25). This indicates that vancomycin had the largest impact on the proteome of MRSA which is unsurprising given that it is the drug of choice for treating MRSA. MNQ however, had so few significantly perturbed proteins that it is difficult to suggest any MoA based on the proteomic data. The small number of proteins affected could indicate that the MoA of MNQ does not involve protein synthesis. The proteins identified using the volcano plot are shown in a box plot format in Figure 31.

3.3.2 – Metabolomics

3.3.2.1 – Metabolomics – Overview of the data

High throughput streamlined metabolomic methodologies were employed to compare the response of MRSA to MNQ and a wide range of antibiotics at four time points; 0, 2, 4, and 6 hours.

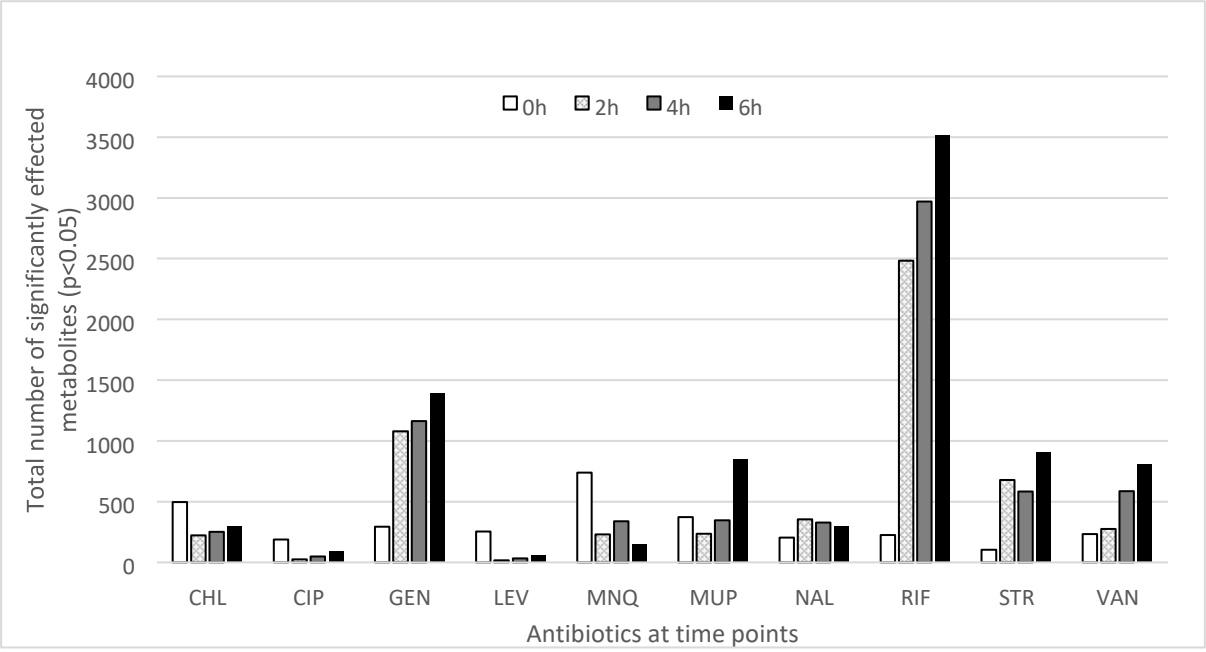


Figure 32: Bar chart representing the total number of negatively ionised detected which were found to be significantly different from the control across all time points.

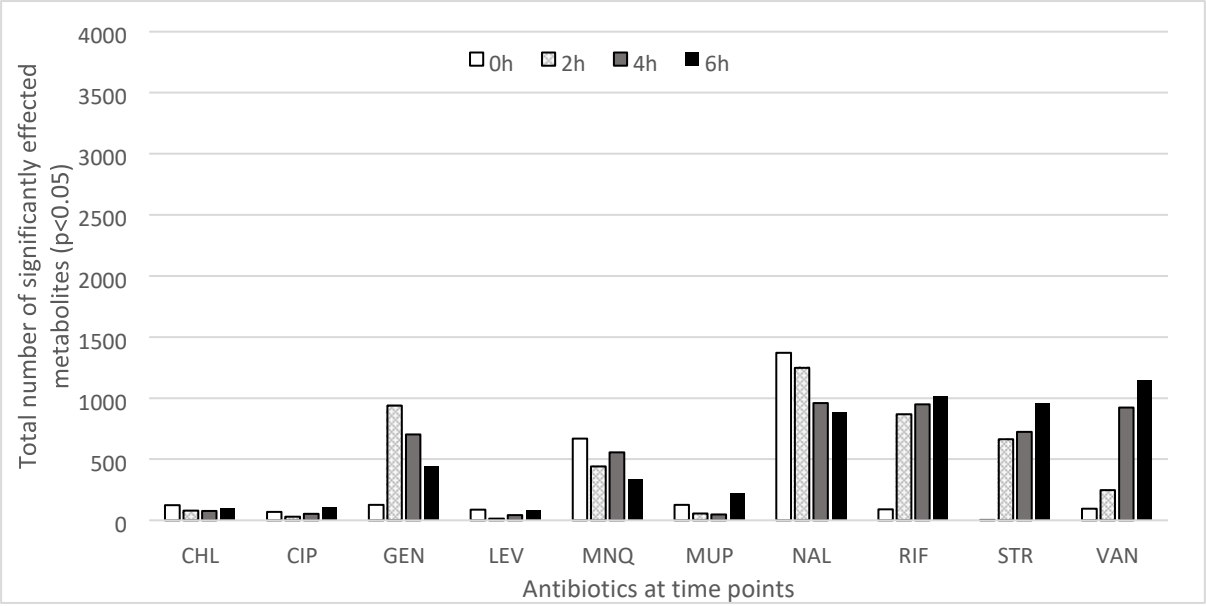


Figure 33: Bar chart representing the total number of positively ionised detected which were found to be significantly different from the control across all time points.

Figure 32 and Figure 33result from an ANOVA (P<0.05) carried out between the antibiotic and the control. Each bar represents the total number of metabolites which have been significantly perturbed from normal growing conditions. This allows the identification of the most crucial time point for each antibiotic suggestion how quickly each antibiotic is acting upon the bacteria. I would be expected that as time progresses the number of significantly altered metabolites would increase. This is true for gentamycin (-), rifampicin (+/-), vancomycin (+/-) and streptomycin (+/-), however in most cases the antibiotics are not having this effect. MNQ has the same time profile in both positive and negative ionisation, this highest number of perturbed metabolites at 0h, followed by 4h, then 2h and finally 6h. This could indicate that MNQ works very quickly.

These figures could also suggest how much impact each antibiotic has upon the metabolome of MRSA over time. Both rifampicin and gentamycin which are both protein targeting antibiotics seem to be having the largest impact on the metabolome. MNQ is having the 6th largest impact on metabolome of MRSA form the 10 antibiotics tested.

3.3.2.2 – Metabolomics – Statistical analysis

Figure 35, Figure 36, Figure 37, Figure 38, Figure 39, Figure 40, Figure 41 and Figure 42 show principal component analyses (PCA) of the metabolite profiles obtained with each antibiotic treatment at each time point. MNQ datapoints were significantly separated from the control at all time points along with many of the other antibiotics. However, each antibiotic seems to show a specific clustering pattern showing that each has had different effects on the metabolome of MRSA. As MNQ has not clustered closely to any other antibiotic at any time or in either charged form. This suggests that MNQ has a novel MoA.

The antibiotics used fall into three general targeting categories; DNA (ciprofloxacin, levofloxacin and nalidixic acid), RNA/Protein (chloramphenicol, gentamycin, mupirocin, rifampicin and streptomycin) and Cell wall (vancomycin). These were combined into groups and analysed again along with MNQ and the control (Figure 43). By combining all the antibiotics into their broad groups, it was hoped that MNQ would fall into one of the three categories. MNQ distinctly clusters away from these three groups as it did when all antibiotics were separate both in positive and negative mode. This clearly indicates that MNQ does not target DNA, RNA, proteins or the cell wall in the same way as any of the antibiotics tested.

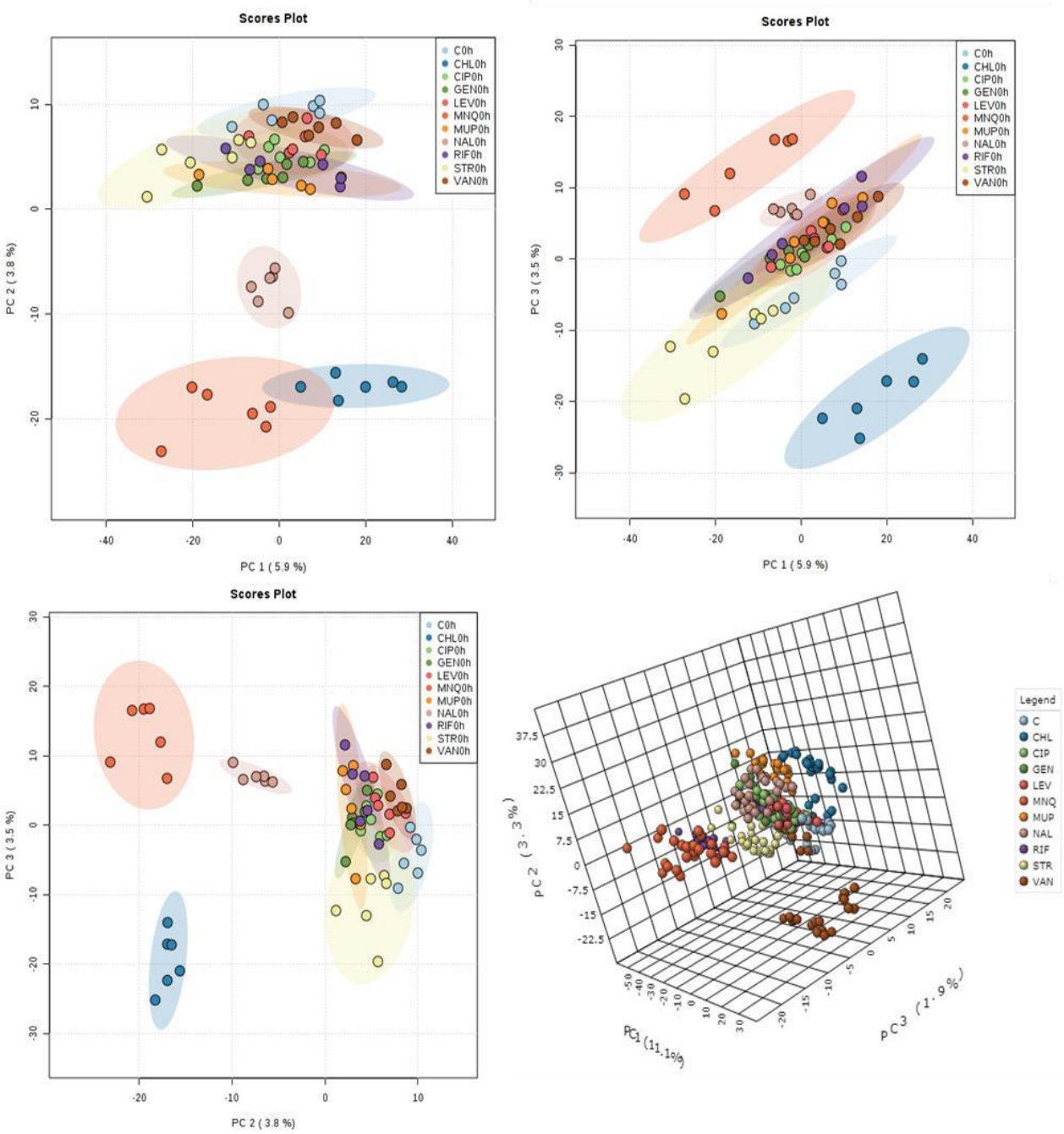


Figure 34: PCA combination of the three principal components of all antibiotics at 0 hours. A - PC1 vs 2 (neg). B – PC1 vs 3 (neg). C – PC2 vs 3 (neg). D - Three dimensional PCA with PC1, 2 and 3 (neg).

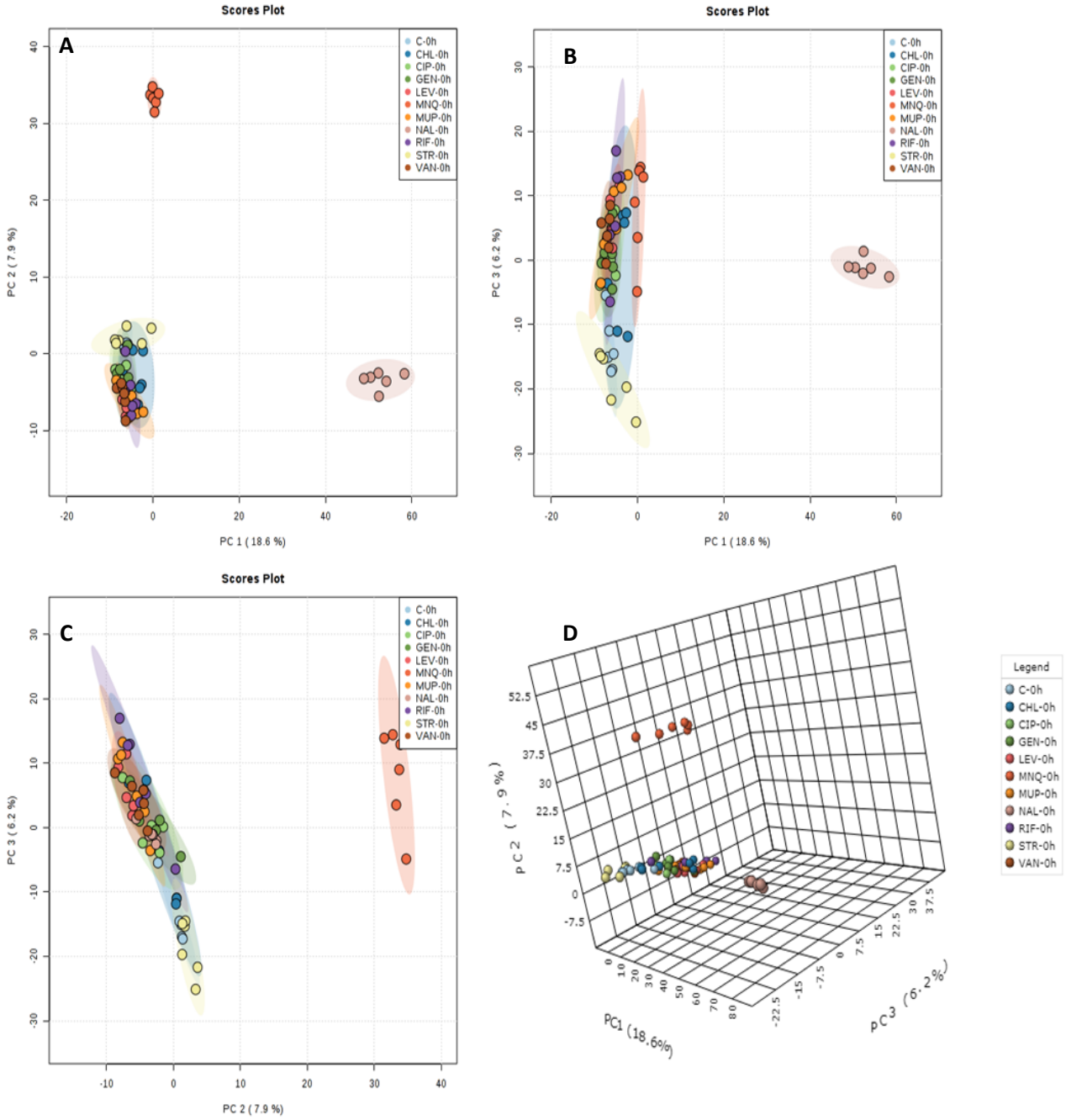


Figure 35: PCA combination of the three principal components of all antibiotics at 0 hours. A - PC1 vs 2 (pos). B – PC1 vs 3 (pos). C – PC2 vs 3 (pos). D - Three dimensional PCA with PC1, 2 and 3 (pos).

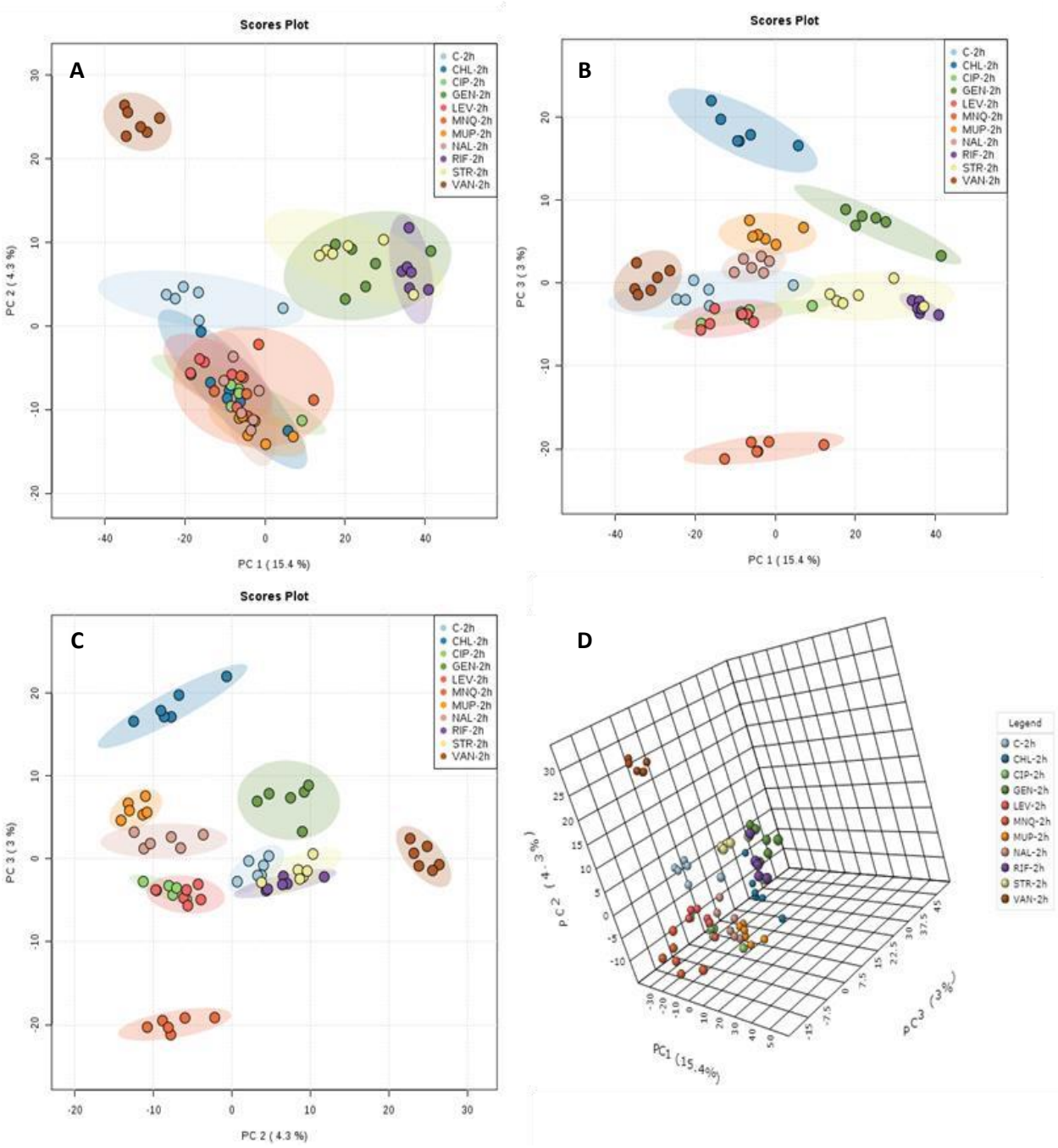


Figure 36: PCA combination of the three principal components of all antibiotics at 2 hours. A - PC1 vs 2 (neg). B – PC1 vs 3 (neg). C – PC2 vs 3 (neg). D - Three dimensional PCA with PC1, 2 and 3 (neg).

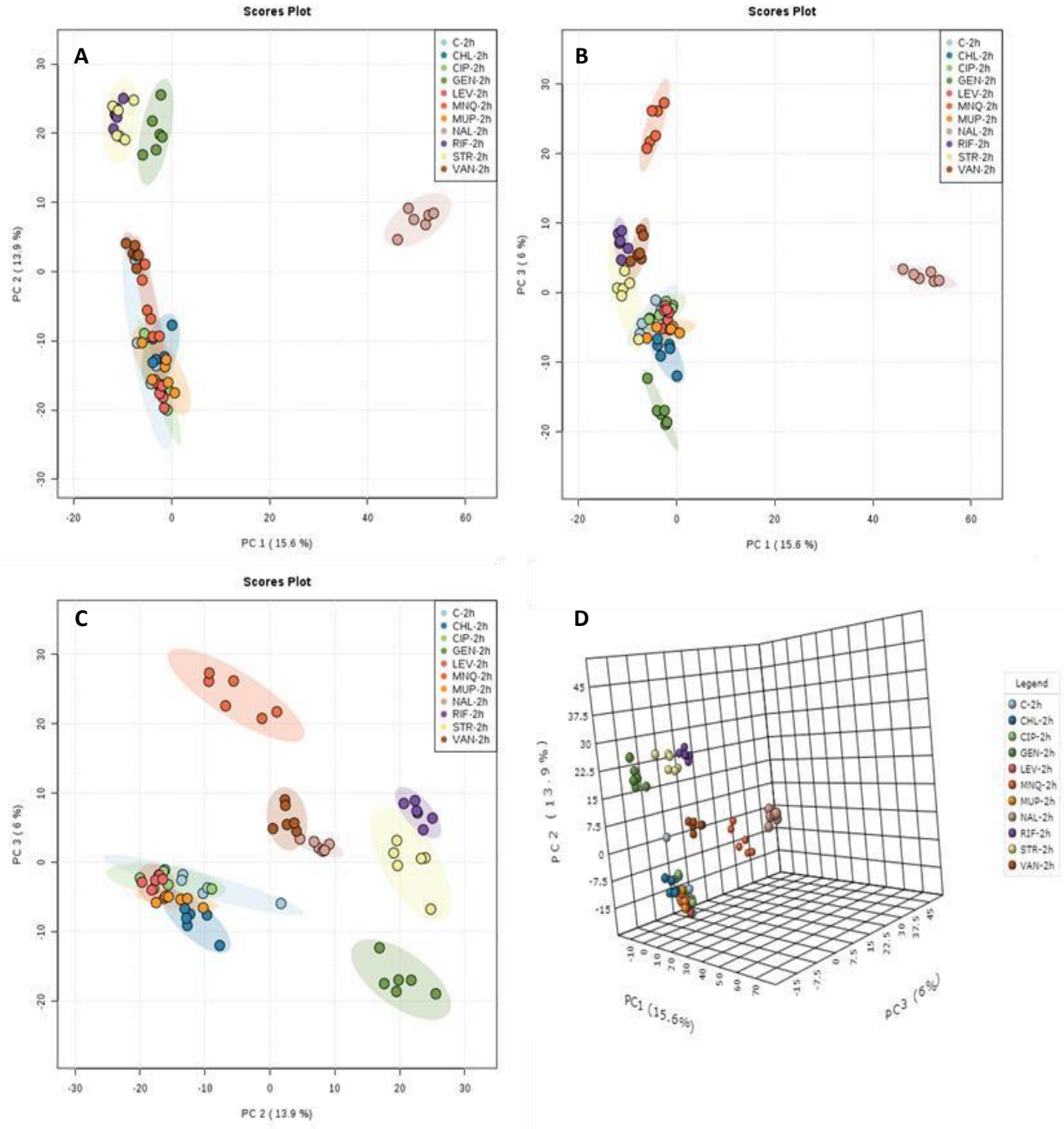


Figure 37: PCA combination of the three principal components of all antibiotics at 2 hours. A - PC1 vs 2 (pos). B – PC1 vs 3 (pos). C – PC2 vs 3 (pos). D - Three dimensional PCA with PC1, 2 and 3 (pos).

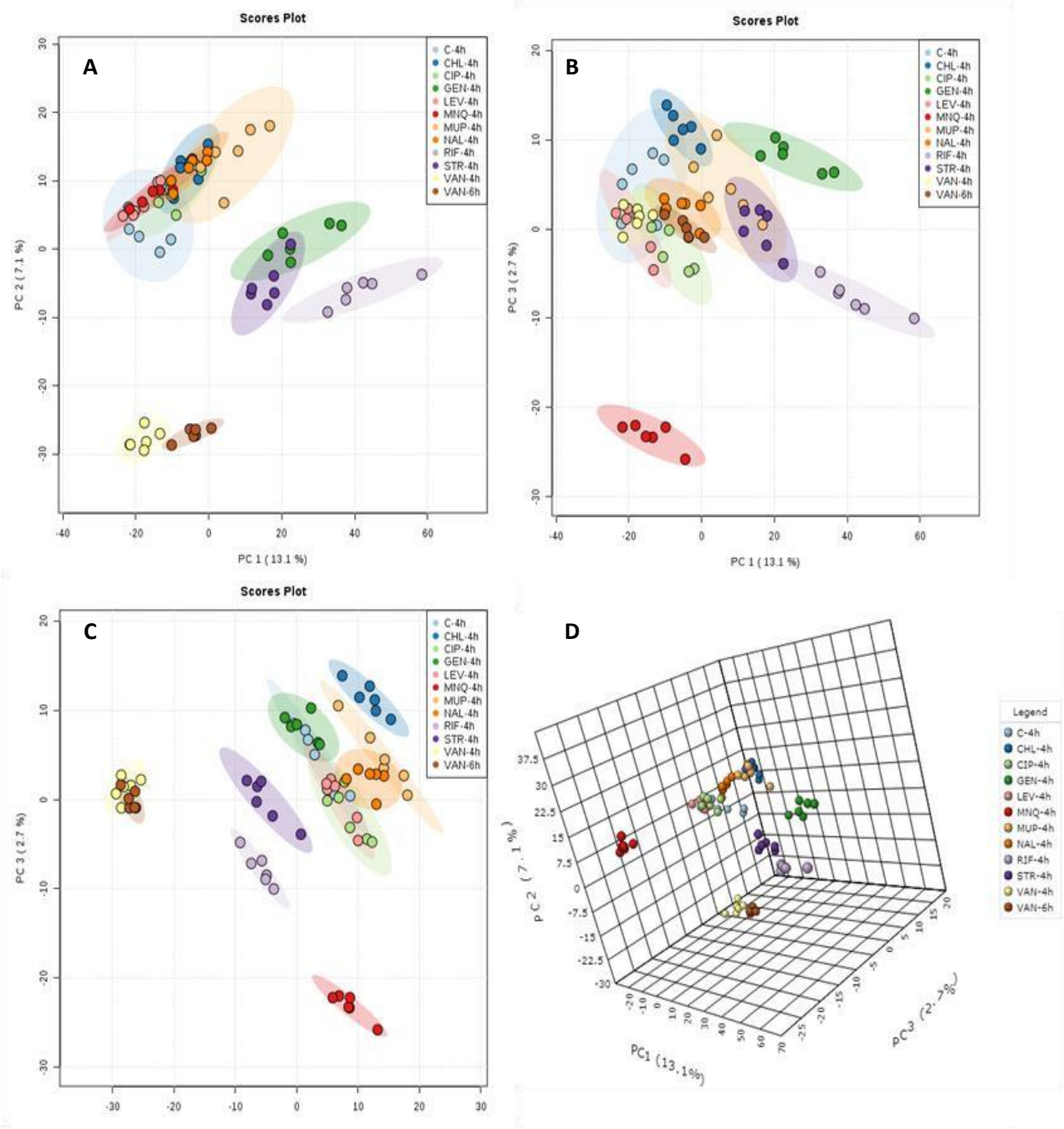


Figure 38: PCA combination of the three principal components of all antibiotics at 4 hours. A - PC1 vs 2 (neg). B – PC1 vs 3 (neg). C – PC2 vs 3 (neg). D - Three dimensional PCA with PC1, 2 and 3 (neg).

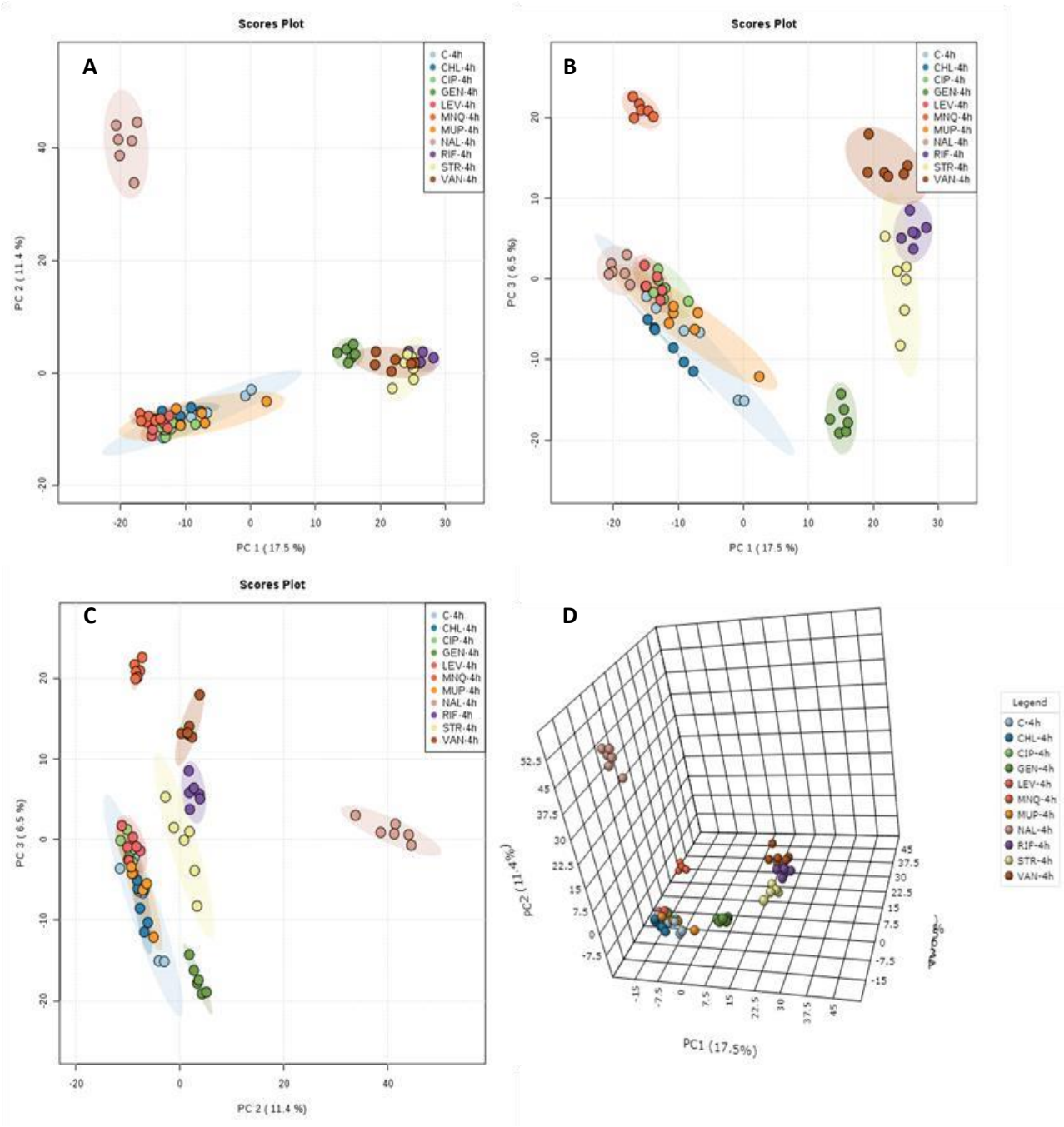


Figure 39: PCA combination of the three principal components of all antibiotics at 4 hours. A - PC1 vs 2 (pos). B – PC1 vs 3 (pos). C – PC2 vs 3 (pos). D - Three dimensional PCA with PC1, 2 and 3 (pos).

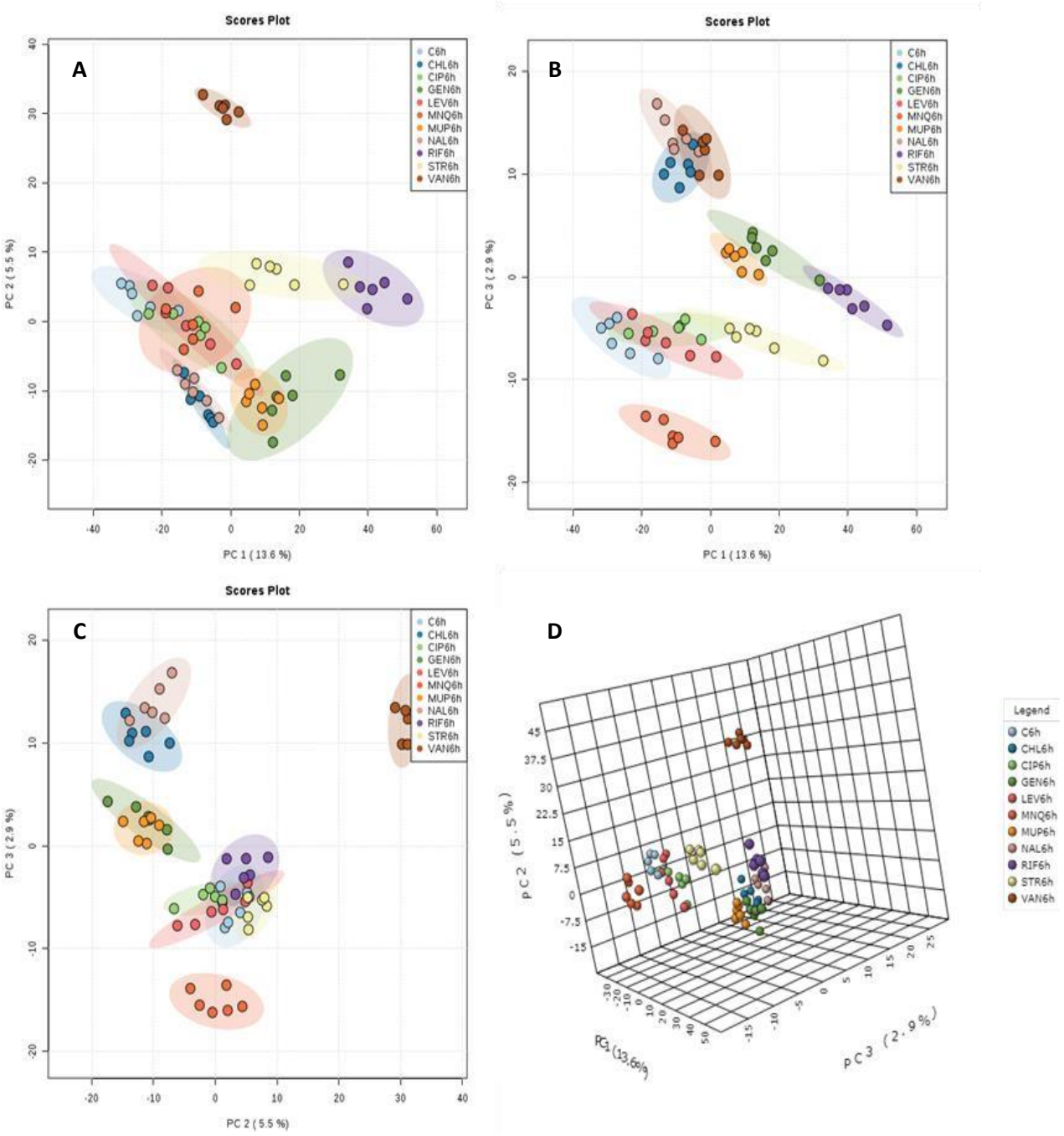


Figure 40: PCA combination of the three principal components of all antibiotics at 6 hours. A - PC1 vs 2 (neg). B – PC1 vs 3 (neg). C – PC2 vs 3 (neg). D - Three dimensional PCA with PC1, 2 and 3 (neg).

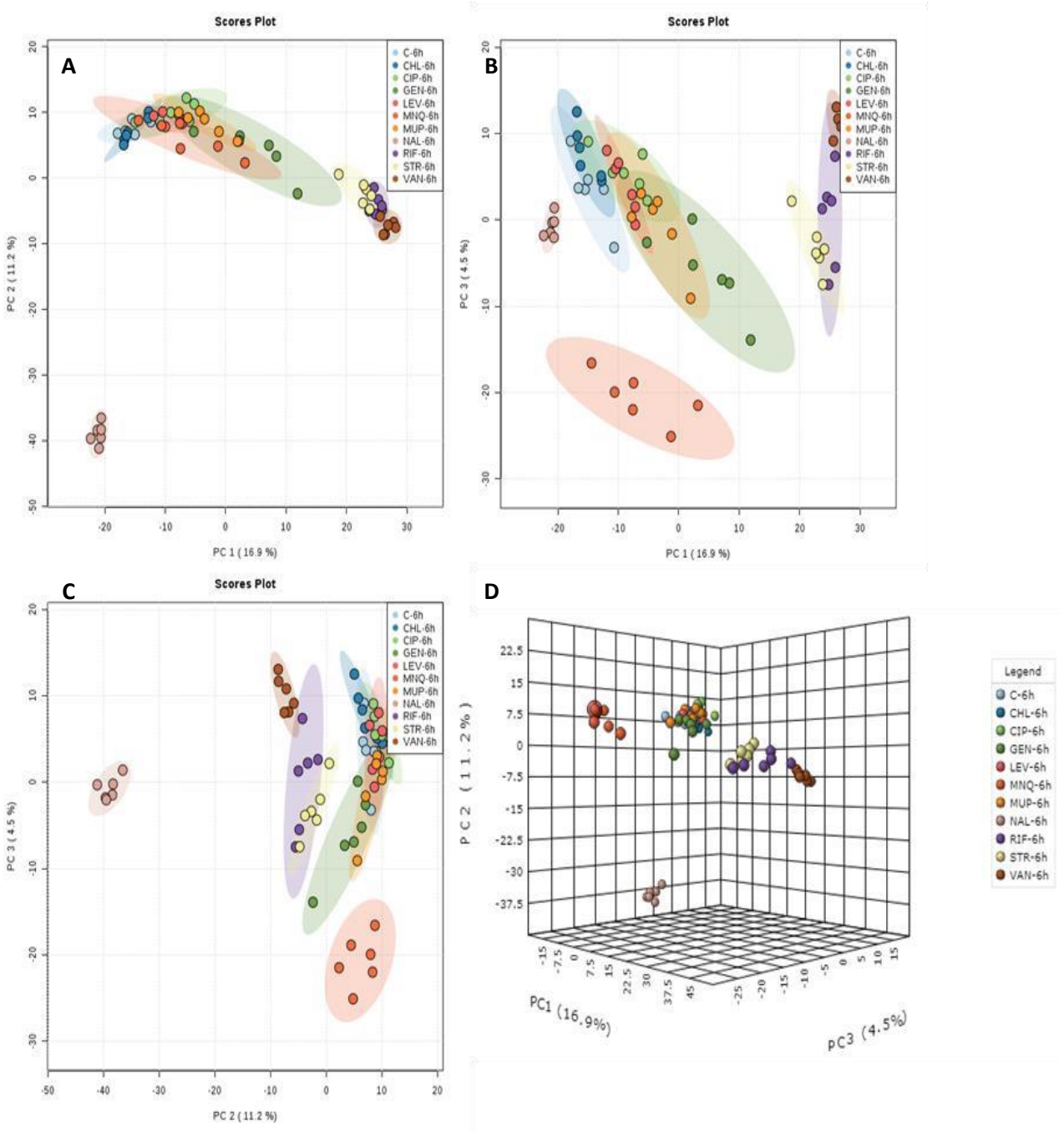


Figure 41: PCA combination of the three principal components of all antibiotics at 6 hours. A - PC1 vs 2 (pos). B – PC1 vs 3 (pos). C – PC2 vs 3 (pos). D - Three dimensional PCA with PC1, 2 and 3 (pos).

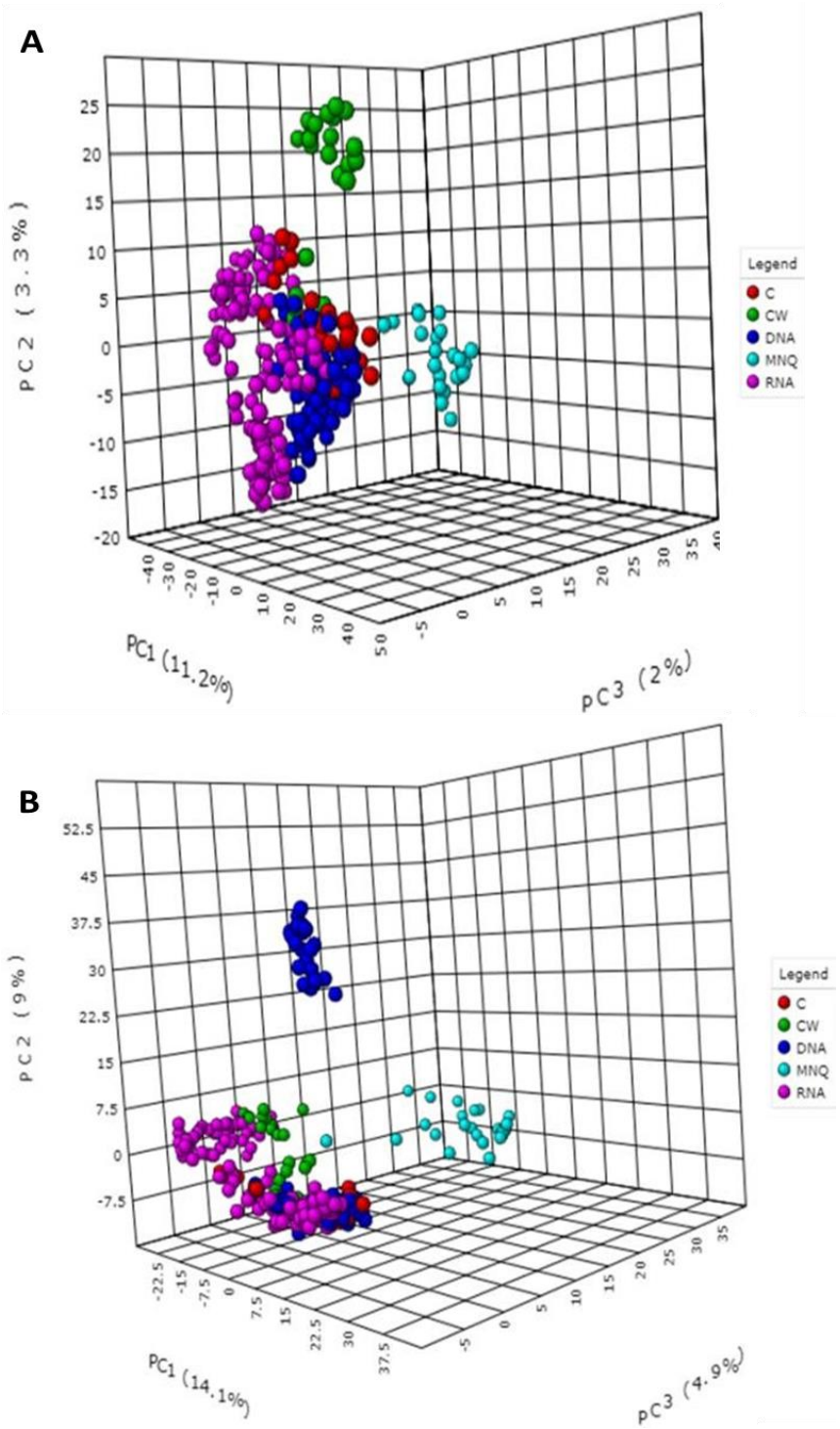


Figure 42: PCA combining all antibiotics into their general MoA; DNA (ciprofloxacin, levofloxacin and nalidixic acid) RNA/Protein (chloramphenicol, gentamycin, mupirocin, rifampicin and streptomycin) and Cell wall (vancomycin) and 2-methoxy-1,4-naphthoquinone (MNQ). A - Negative ionisation. B - Positive ionisation.

Table 26: Total number of metabolites significantly affected by each antibiotic within KEGG metabolic pathways at the most significant time point for each antibiotic (number of metabolites expressed as a percentage of the total number of metabolites disturbed by the antibiotic).

	CHL	CIP	GEN	LEV	MNQ	MUP	NAL	RIF	STR	VAN	RNA	DNA	CW
Carbohydrate metabolism	74 (20.5)	39 (27.7)	88 (17.0)	54 (25.6)	194 (24.7)	82 (21.8)	138 (19.6)	195 (17.2)	104 (14.6)	142 (16.0)	18.2	25.6	16.9
Energy metabolism	23 (6.4)	11 (7.2)	34 (6.6)	23 (10.9)	42 (5.3)	24 (6.4)	38 (5.4)	81 (7.2)	47 (6.6)	54 (6.1)	6.6	9.1	6
Lipid metabolism	23 (6.4)	10 (6.6)	23 (4.4)	11 (5.2)	38 (4.8)	14 (3.7)	50 (7.1)	63 (5.6)	40 (5.6)	44 (5.0)	5.1	5.9	5
Nucleotide metabolism	21 (5.8)	9 (5.9)	27 (5.2)	12 (5.7)	34 (4.3)	20 (5.3)	34 (4.8)	49 (4.3)	27 (3.8)	32 (3.6)	4.9	5.8	3.6
Amino acid metabolism	102 (28.3)	38 (25.0)	186 (35.8)	57 (27.0)	223 (28.3)	126 (33.5)	228 (32.4)	371 (32.8)	242 (34.0)	306 (34.5)	32.9	26	34.5
Metabolism of other amino acids	37 (10.2)	15 (9.9)	53 (10.2)	21 (10.0)	76 (9.7)	38 (10.1)	67 (9.5)	127 (11.2)	92 (12.9)	117 (13.2)	10.9	9.9	13.2
Glycan biosynthesis and metabolism	2 (0.6)	2 (1.3)	2 (0.4)	2 (0.9)	3 (0.4)	0 (0.0)	5 (0.7)	8 (0.7)	8 (1.1)	7 (0.8)	0.6	1.1	0.8
Metabolism of cofactors and vitamins	46 (12.7)	21 (13.8)	64 (12.3)	22 (10.4)	88 (11.2)	42 (11.2)	73 (10.4)	127 (11.2)	80 (11.3)	98 (11.0)	11.7	12.1	11
Metabolism of terpenoids and polyketides	3 (0.8)	0 (0.0)	10 (1.9)	0 (0.0)	8 (1.0)	2 (0.5)	14 (2.0)	21 (1.9)	12 (1.7)	11 (1.2)	1.4	0	1.2
Biosynthesis of other secondary metabolites	12 (3.3)	4 (2.6)	14 (2.7)	6 (2.8)	19 (2.4)	12 (3.2)	24 (3.4)	42 (3.7)	30 (4.2)	35 (3.9)	3.4	2.7	3.9
Xenobiotics biodegradation and metabolism	18 (5.0)	3 (2.0)	18 (3.5)	3 (1.4)	62 (7.9)	16 (4.3)	33 (4.7)	47 (4.2)	29 (4.1)	41 (4.6)	4.2	1.7	4.6
Total	361	152	519	211	787	376	704	1131	711	887	100	100	100

Table 26 shows the number of metabolites whose regulation has been perturbed significantly by each antibiotic and matched a KEGG metabolic pathway of *S. aureus* subsp. aureus USA300_TCH1516.

In terms of structural similarity and proportional effect on metabolic pathways of MRSA MNQ is most like that of DNA targeting antibiotics; with high numbers of disturbed carbohydrate metabolites and relatively low proportion of amino acids affected. Therefore, MNQ and the DNA gyrase targeting antibiotics were docked to the using mcule 1-click docking (https://mcule.com/apps/1-click-docking/). This online platform calculates how well MNQ fits into the active site of *S. aureus* DNA gyrase subunit B (UniProt: GYRB_STAAU) compared to the other antibiotics (Table 27 and Figure 43). Based on the structural similarities MNQ has similar docking cores to all the DNA targeting antibiotics, possibly revealing the MoA of MNQ.

Table 27: Top 4 docking scores of MNQ and quinolone antibiotics when bound to DNA gyrase subunit B

Docking position	MNQ	NAL	CIP	LEV
1	-6	-6.4	-7.3	-7
2	-6	-5.9	-7.1	-6.1
3	-5.7	-5.4	-7	-5.9
4	-5.5	-5.3	-6.3	-5.7

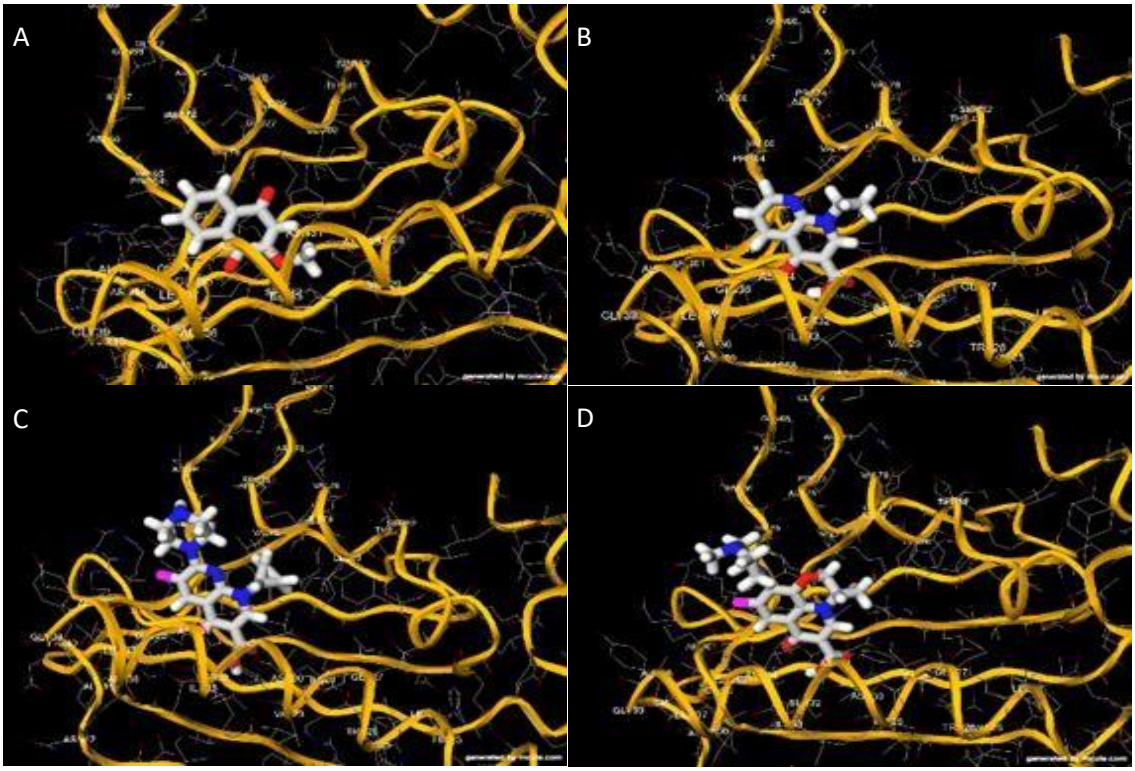


Figure 43: mcule 1-click docking. #1 docking orientation of 2-methoxy-1,4-naphthoquinone (A), nalidixic acid (B), ciprofloxacin (C), and levofloxacin (D)

3.3.2.4 – Metabolomics – Specific Pathway mapping – TCA Cycle

Using the significantly perturbed metabolite identities which are linked to KEGG pathway it is possible to see in detail how certain pathways are being affected by MNQ. Several highly significant metabolites were identified which were involved in the TCA cycle. Not only were they significantly different from the control but also the other antibiotics in most cases, indicating this a specific to the MoA of MNQ.

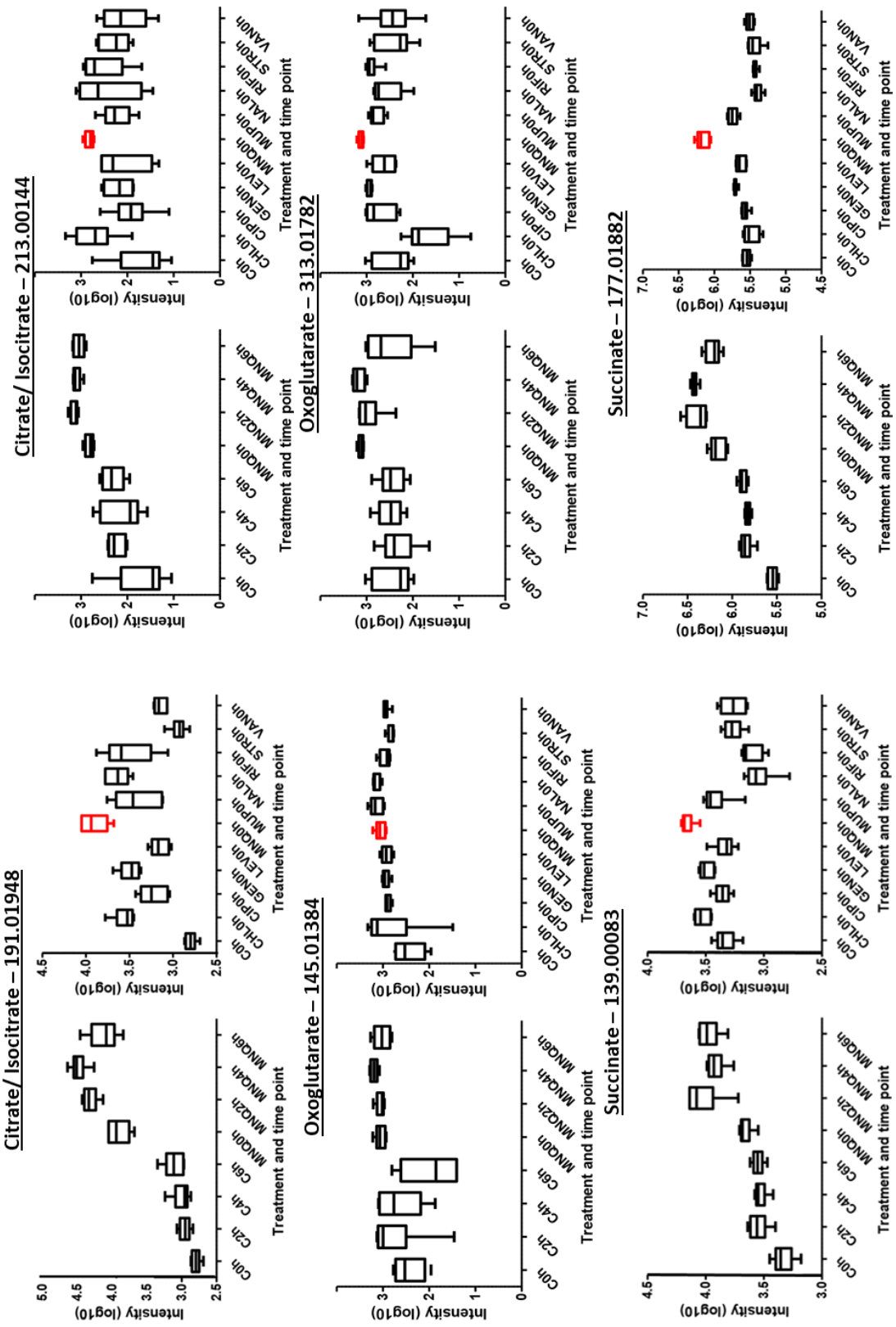


Figure 44: Left: Box plots indicating the regulation of specific metabolites involved in the TCA cycle across all time points between MNQ and no treatment control. Right: Box plots indicating the regulation of specific metabolites involved in the TCA cycle across all antibiotics at 0h time point

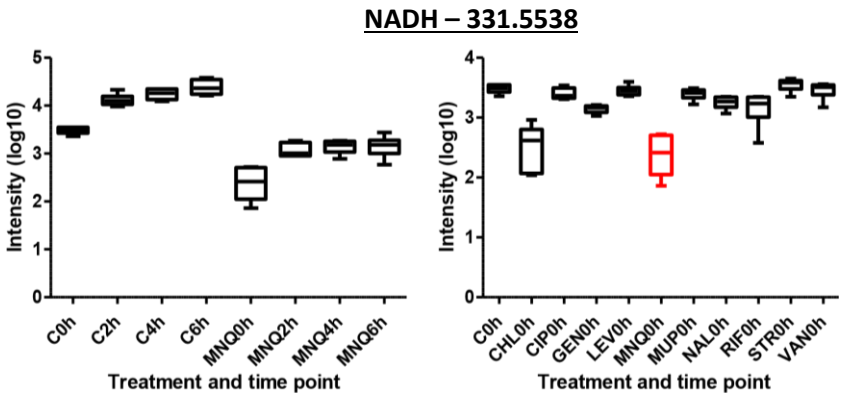


Figure 45: Left: Box plots indicatingthe regulation of nicotinamide adenine dinucleotide (NADH) across all time points between MNQ and no treatment control. Right: Box plots indicating the regulation of NADH across all antibiotics at 0h time point.

Figure 44 and Figure 45 show the unique effect which MNQ has upon the TCA cycle of MRSA. MNQ causes MRSA to upregulate all TCA cycle metabolites when compared to the control and most other antibiotics. NADH, a metabolite which is closely related to the TCA cycle, was also included as it shows a significantly decreased regulation. Multiple m/z have matched with adducts of the same metabolite which increases the confidence in these results.

3.3.2.5 – Metabolomics – Specific Pathway mapping – Sugar metabolism

As well as the TCA cycle sugar metabolism stood out as another metabolic pathway which was specifically and significantly affected by MNQ.

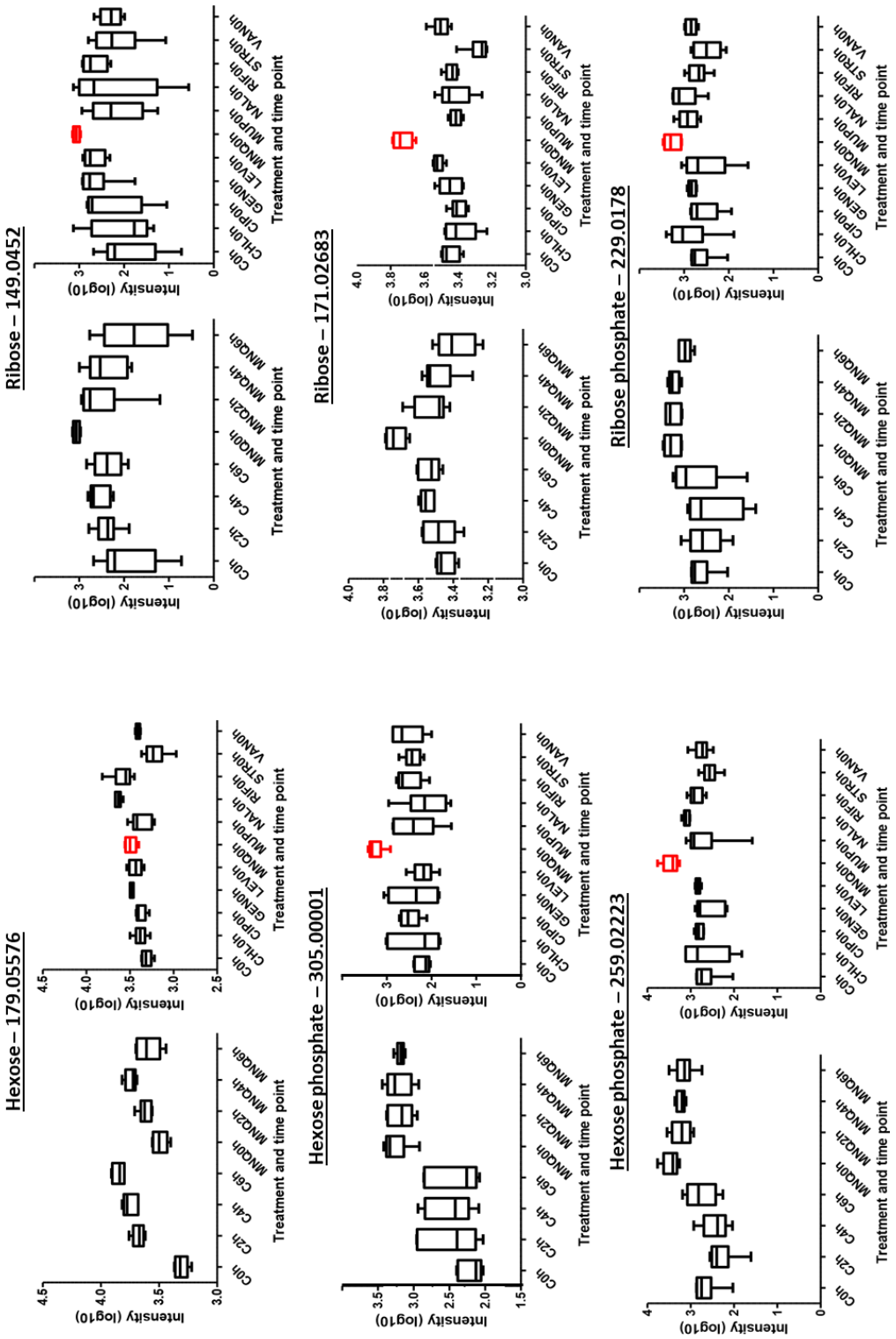


Figure 46: Left: Box plots indicating the regulation of specific metabolites involved in the sugar metabolism across all time points between MNQ and no treatment control. Right: Box plots indicating the regulation of specific metabolites involved in the sugar metabolism across all antibiotics at 0h time point

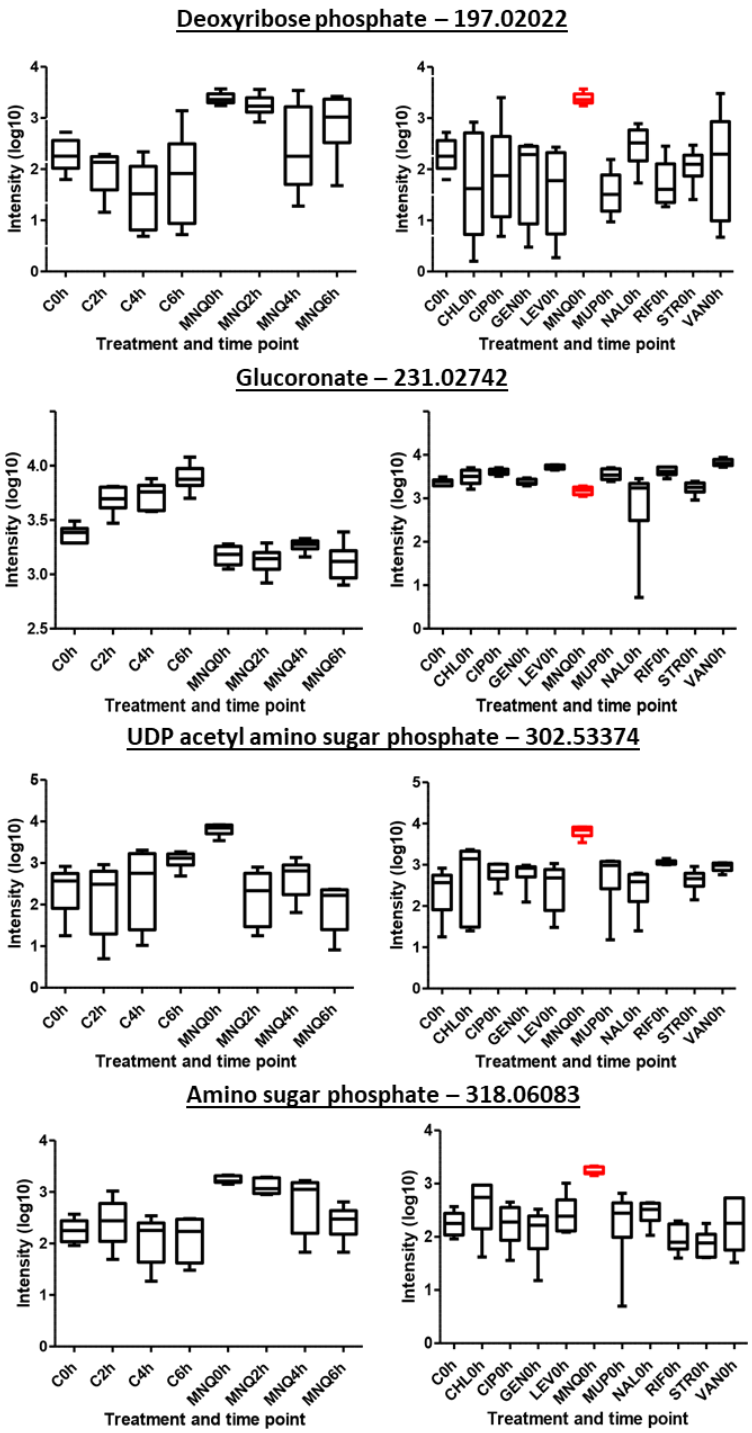


Figure 47: Left: Box plots indicating the regulation of specific metabolites involved in further sugar metabolism across all time points between MNQ and no treatment control. Right: Box plots indicating the regulation of specific metabolites involved in further sugar metabolism across all antibiotics at 0h time point.

The ability to metabolise sugars is crucial for the survival of organisms (Figure 46 and Figure 47), MNQ was found to have a significant effect on many metabolites which were found to be either

sugars or linked to sugar metabolism. These were also found to be unique to MNQ treatment and their differential regulation was not seen in any of the other antibiotics tested.

3.3.2.6 – Metabolomics – Specific pathway mapping – Lipid metabolism

In addition to the TCA cycle and sugar metabolism, MNQ also had a significant effect on lipid metabolism. MNQ was shown to influence most lipid types, especially the fatty acids (Table 28).

Table 28: The regulation of all lipid families identified as significantly different from the control

Lipid family	**LipidMaps classification**	**Up**	**Down**	**Total**
Fatty acid conjugates	FA01	84	21	105
Octadecanoids	FA02	1	0	1
Eicosanoids	FA03	3	0	3
Fatty aldehydes	FA06	2	0	2
Fatty esters	FA07	2	2	4
Fatty amides	FA08	5	0	5
Monoradylglycerols	GL01	2	0	2
Diradylglycerols	GL02	19	3	22
Triradylglycerols	GL03	2	0	2
Glycerophosphocholines	GP01	2	2	4
Glycerophosphoethanolamines	GP02	1	2	3
Glycerophosphoserines	GP03	2	0	2
Glycerophosphoglycerols	GP04	0	2	2
Glycerophosphates	GP10	2	1	3
Glyceropyrophosphates	GP11	0	1	1
Macrolides and lactone polyketides	PK04	1	0	1
Aflatoxins and related substances	PK10	1	0	1
Flavonoids	PK12	2	0	2
Aromatic polyketides	PK13	5	1	6
Acylaminosugars	SL01	2	0	2
Sphingoid bases	SP01	1	0	1
Phosphosphingolipids	SP03	1	0	1
Neutral glycosphingolipids	SP05	2	0	2
Sterols	ST01	2	0	2
Steroids	ST02	4	0	4
Secosteroids	ST03	11	5	16
Bile acids and derivatives	ST04	1	0	1

3.3.3 – Transmission Electron Microscopy

To visually inspect the impact MNQ is having upon MRSA transmission electron microscopy (TEM) was carried out. Figure 48 shows TEM of MRSA treated at a standardised concentration of MNQ at 2, 4, 6 and 24 hours. Although MNQ has been shown to impact the metabolome of MRSA, these changes are not visible when using TEM alone. However, at 24 hours (D) cell debris can be seen, cells are lighter grey and are not actively dividing, this is a clear sign of cell death. Although the metabolomics suggested that MNQ works quickly, this is not supported by the TEM images.

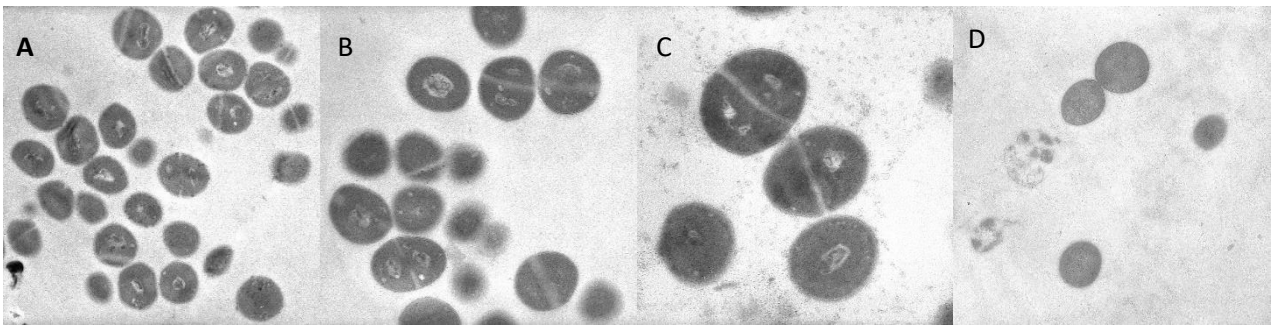


Figure 48: Transmission electron microscopy of MRSA; treated with a standardised concentration of MNQ at 2 (A), 4 (B), 6 (C), and 24 hours (D).

Figure 49¬show MRSA with cerium chloride untreated and treated with MNQ and HNQ. Cerium chloride reacts with H2O2 to form cerium perhydroxide which shows up as black needle-like crystals under TEM, this indicates that there could be generation of reactive oxygen species (ROS). These formations can only be seen in the presence of MNQ, it is absent when untreated and treated with HNQ.

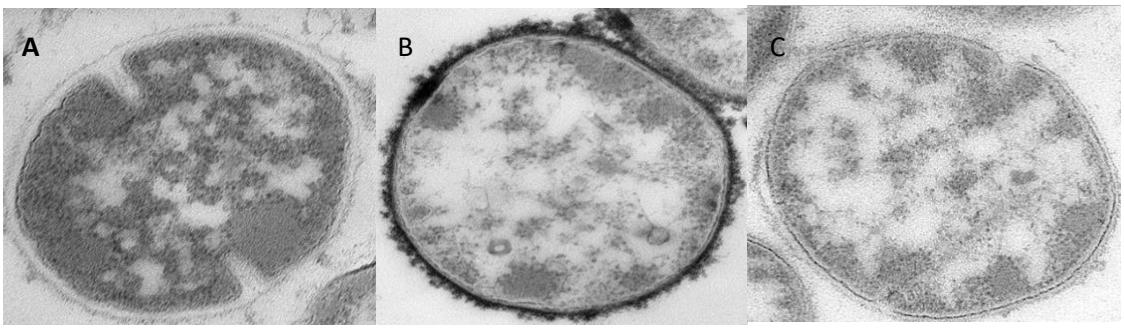


Figure 49: Transmission electron microscopy of MRSA with cerium chloride; untreated (A) and treated with a standardised concentration of MNQ (B), and HNQ (C).

3.3.4 – Antimicrobial Assay with Antioxidants

Catalase and Ascorbate are both antioxidants which are able react with H2O2 to reduce their negative effects. This assay was carried out to see if the antioxidant activity of catalase and ascorbate were able to reduce the efficacy of MNQ as an antimicrobial compound. The presence of these antioxidants reduced the activity of MNQ two-fold (Table 29). For example, at 12.5 mg/L of MNQ the OD600 increased by 12.31% over 24 hours, when catalase and ascorbate is combined with MNQ the OD600 increased by 23.11% and 56.54%, respectively. A negative OD600 result was obtained for MNQ treatment at 100 mg/L, this is not uncommon as MNQ is green in colour which absorbs light at a lower wavelength than the straw yellow media. At very high concentration the green pigment is mixed thoroughly over 24 hours and will reduce the maxima of the absorbance, therefore slightly decreasing the OD600 value obtained at 24 hours compared to the initial time point. This value can be considered as 0% growth and lower concentrations of MNQ do not have this issue.

Table 29: Minimum inhibitory concentration (MIC) of 2-methoxy-1,4-naphthoquinone (MNQ) and in the presence of catalase and ascorbate (n=3).

MNQ Concentration (mg/L)	MNQ	MNQ with Catalase (100 mg/L)	MNQ with Ascorbate (100 mg/L)
100	**-1.30**	**0.03**	**0.30**
50	**8.52**	**2.07**	**0.58**
25	**13.03**	**3.59**	**13.03**
12.5	**12.31**	23.11	56.54
6.25	50.61	95.29	100.46
3.125	103.95	88.89	109.35
1.5625	103.03	95.34	101.35

3.4 – Discussion

3.4.1 – Proteomics

The large-scale analysis of proteins has been used to further understand antibiotics mode of action (Bandow et al., 2003) as well as understanding the molecular roles of many active compounds including natural products (Lao et al., 2014). Proteomics approaches were employed to understand the effects on MNQ on MRSA, however, the regulation of very few proteins were affected by MNQ after 6 hours of exposure (Table 25). The low number of proteins changed by MNQ could indicate that MoA of MNQ does not impact on protein synthesis and regulation.

"Uncharacterised protein 7" was significantly down regulated under MNQ treatment at 6 hours. It is a helical and transmembrane protein and belongs to a family of cell wall-active antibiotic response proteins. These transmembrane proteins are involved in the LiaRS two-component system (TCS) which regulates cell envelope stress response especially following the exposure to antibiotics such as vancomycin which interfere with the lipid II and undecaprenol cycle (Jordan et al., 2006, 2007). Both vancomycin and levofloxacin also showed a downregulation of this protein therefore this is likely a generic stress response and not useful to decern the MoA of MNQ.

The expression of MRSA ribosome was significantly reduced by the presence of MNQ. The ribosome is made up of two subunits small (S1-31) and large (L1-44) and one protein of each subunit has been affected; S5 at 6 hours and L19 at 0 hours. L19 is known to be located at the interface between the small and large subunit and may play a role in the structure and function of aminoacyltRNA binding site (Brosius and Arfsten, 1978). Levofloxacin also significantly decreased the expression of both S5 and L19 whereas vancomycin significantly increased S5 with no effect of L19. Interestingly, the DNA targeting antibiotic, levofloxacin, shows a downregulation of ribosomal proteins. This would be expected as ribosomes are produced from DNA in the same way as all other proteins within bacteria (Crick, 1958). The upregulation of ribosomal in response to vancomycin is

likely a response to the cell wall breaking down. Peptidoglycan fragments are recovered and recycled as a part of cell wall biosynthesis; this also serves as a detection mechanism for cell-wall-targeting antibiotics leading to an upregulation of protective genes (Johnson, Fisher & Mobashery, 2013), which requires significant ribosomal activity.

Cold shock protein CspA was affected both at 0 and 6 hours. and was also significantly affected in vancomycin and levofloxacin. As the bacteria were flash frozen in liquid nitrogen and stored at -80˚C to halt protein activity, it was thought this process would have been fast enough to avoid any protein alterations due to this stress. The significant perturbation of cold shock proteins suggests otherwise.

DNA-binding protein HU is a part of the histone-like DNA-binding protein family which are a set of basic proteins that wrap around DNA stabilising it from denaturation under extreme conditions (Tanaka et al., 1984). MNQ decreased the expression of DNA-binding protein HU but vancomycin had the opposite effect; significantly increasing the amount of this protein. This indicates that MNQ does not inhibit bacterial growth in the same way as vancomycin.

Probable malate:quinone-oxidoreductase belongs to the family of proteins called malate:quinone-oxidoreductases (MQO). These membrane-associated enzymes are an alternative to the NAD-dependant malate dehydrogenase as part of the tricarboxylic acid (TCA) cycle. They play a critical role in bacterial survival during oxidative stress. Nitric oxide stress is an important immune response utilised by the host to clear a bacterial infection therefore these proteins also play a key role in virulence (Spahich et al., 2016.). Both MNQ and levofloxacin negatively affected this protein whereas vancomycin increased the amount of this protein. This suggests that MNQ seems to disrupt the TCA cycle and similar metabolic pathways likely acting through the generation ROS.

Given the low number of proteins and relatively low significance ($P<0.1$) of the disruption it is difficult to draw definite conclusions regarding the MoA of MNQ. This experiment would require repetition with different concentrations of MNQ over different periods of time to see a truly

significant proteomic impact. The fact that both vancomycin and levofloxacin were shown to effect large number of proteins is an indication that this experiment worked well. In addition, the few proteins which were affected by MNQ did match with the hypothesis that MNQ bactericidal action is brought about via the generation of ROS.

3.4.2 – Metabolomics

3.4.2.1 –Metabolomics – Overview of the data

When the metabolomics data are considered holistically, which is often done in literature (Maifiah et al., 2017; Schelli, Zhong and Zhu, 2017; Zampieri et al., 2017; Baptista et al., 2018), this provides broad differences and similarities between treatment groups. Figure 33 to Figure 43 show a combination of approaches which provide general information about all antibiotics used and how they compare to each other. Figure 32 and Figure 33 show the total number of metabolites affected by each antibiotic over 6 hours. The number of metabolites affected by each antibiotic in total and at each time point varies greatly. The antibiotics gentamycin, rifampicin, streptomycin and vancomycin affect an increasing number of metabolites over time, however MNQ along with chloramphenicol, ciprofloxacin, levofloxacin, and nalidixic acid have the largest effect on the metabolome of MRSA at the initial time point. This suggest that these antibiotics not only work in different ways but at different speeds. As eluded to in the introduction (Figure 24) metabolites are the front line in terms of response to a challenge. Metabolites are the first to respond to a change in environment, for example the presence of an antibiotic. Whereas proteins and especially DNA takes time to react to changes. The 0-hour time was originally to be used as a control time-point used for comparisons with later sampling points. However, the 0-hour point it is technically around 1 minute for each sample as it takes time to add, mix, aliquot and freeze samples, therefore significant metabolic effects can take place within this time. This first time point can reveal the true metabolic effect of a drug as it shows the primary metabolic effect, whereas later time points would show secondary and tertiary reactions as a result of the drugs action. A publication by Zampieri et

al., 2018 used methodologies like ours to predict MoA of uncharacterised antimicrobial compounds. The research measured the total number of metabolites associated with 7 antibiotic categories; folic acid biosynthesis, mycolic acid biosynthesis, cell wall, RNA synthesis, protein synthesis, quinolones, and DNA cleavage. The first four of which had the largest number of affected metabolites effected at the first time point measured and even protein synthesis could also be argued to have a very significant initial response.

3.4.2.2 –Metabolomics - Statistical analysis

PCA is an exploratory statistical procedure often used in metabolomics to draw meaningful conclusions between treatment groups (Xia et al., 2015). Figure 34 to Figure 42 show the PCA of all antibiotic treatments at each time point. At 0h MNQ has a distinct clustering pattern based on the top three principal components (PC) in both positive and negative ionisation. In addition to MNQ, chloramphenicol and nalidixic acid both cluster away from the control whereas the other treatments do not significantly separate from the control. This result correlates with the total number of disturbed metabolites because these three antibiotics have the largest effect at 0h. This indicates these antibiotics are having a rapid metabolic effect on the bacteria, these are likely the fastest acting antibiotics.

As time progresses other antibiotics begin to separate out from the control. MNQ is consistently separated from the control at every time point. At 2, 4, and 6-hours vancomycin has the most distinct clustering pattern in negative ionisation mode whereas nalidixic acid formed the most distinct cluster for positively ionised metabolites across all time points. Of all the antibiotics used there are three general targets, DNA, RNA and CW. The hypothesis of this experiment is that antibiotics which have similar MoA would show similar metabolic fingerprints, therefore forming distinct clusters within a PCA. All time points and treatment groups were combined into these groups and a PCA was carried out resulting in Figure 42. There are 3 clear clusters for each MoA and MNQ does not cluster with any of these suggesting that MNQ does not target DNA, RNA or the cell

wall in the same way these antibiotics do. This is also true for nalidixic acid, which was considered to have a DNA targeting MoA, however nalidixic acid points on the PCA are separate from all other DNA MoA treatments. Of the three DNA MoA antibiotics; nalidixic acid, ciprofloxacin, and levofloxacin, the latter two both second generation fluoroquinolone, whereas nalidixic acid is a first generation naphthyridone quinolone. In addition, a much higher dose of nalidixic acid was required compared to the more effective fluoroquinolone. As the antibiotic's concentrations were standardised to show the same level of growth inhibition over 6 hours, this higher concentration is unlikely to be the cause of this differential clustering. The fact that these small differences were clearly shown the PCAs adds confidence to the fact that we are inferring that MNQ works differently to all antibiotics tested in this metabolomic data set.

By looking at the data collectively we have established MNQ does not affect the metabolome of MRSA in the way other antibiotics do, leading us to conclude MNQ has a novel MoA. Confidence in this result is backed up by the fact that all other antibiotics cluster together when grouped by their MoA, apart from nalidixic acid which seems to be working differently.

3.4.2.3 –Metabolomics – Pathway mapping

To provide further detail to this metabolomic dataset the high-resolution m/z values which were identified as significant by the statistical analysis were matched to known metabolite identities using R-statistical package. Using R-statistical software, the m/z values were annotated with the most likely metabolite ID based on how likely certain adducts are to form and the ppm error. These metabolites can then be mapped onto MRSA metabolic pathways to elucidate the true metabolic effect of MNQ. The total number of m/z values matched to metabolites which are involved in MRSA metabolic pathway are shown in Table 26 separated into distinct metabolic pathways.

Amino acid metabolism had the highest number of perturbed metabolites, with at least a quarter of all metabolites affected being involved in amino acid metabolism. Carbohydrate metabolism was the second with the total number of affected metabolites ranging from 14.6 to

27.7%. The two exceptions to this general trend are ciprofloxacin and levofloxacin; which have a proportionally larger impact on carbohydrate metabolism. These two antibiotics are 4-quinolones which interfere with DNA replication and causing DNA breakage so understandably these antibiotics had a lesser effect on amino acid when compared to other antibiotics which target RNA polymerase which is directly involved in amino acid utilisation and protein synthesis. However, as we have seen previously nalidixic acid does not share the same metabolic fingerprint as the other DNA targeting antibiotics. Of all the metabolites perturbed by nalidixic acid 32.4% are involved in amino acid metabolism and 19.6% are involved in carbohydrate metabolism, this is patter is like that of RNA targeting antibiotics. The structural difference between the quinolone nalidixic acid and the fluorinated quinolones ciprofloxacin and levofloxacin could possibly explain the metabolic differences. Ciprofloxacin and nalidixic acid are known to affect *E. coli* in different ways; ciprofloxacin is able to kill non-growing cells whereas nalidixic acid cannot (Howard, Pinney and Smith, 1993), a resistance mutation within gyrA completely renders nalidixic acid ineffective whereas ciprofloxacin remains active (Lewin, Howard and Smith, 1991), and ciprofloxacin becomes less active in the presence of chloramphenicol and rifampicin (Chen et al., 1996). Although these differences cannot directly explain the metabolomic differences seen they do suggest that nalidixic acid has a very different activity profile to ciprofloxacin.

The metabolomic profile of DNA targeting antibiotics was discussed in detail as the metabolic profile of MNQs most closely matches that of ciprofloxacin and levofloxacin. MNQ has a relatively large effect on carbohydrate metabolism and a lesser effect on amino acids. MNQ also shares a similar chemical structure with 4-quinolone antibiotics (Figure 27) as they both possess a naphthalene core. This similarity in structure and profile was probed using mcule 1-click docking (mcule, Palo Alto) to see how well MNQ fits into the active site of *S. aureus* DNA gyrase subunit B (UniProt: GYRB_STAAU) shown in Table 27 and Figure 43. When docked ciprofloxacin showed the highest binding affinity with a docking score of -7.3, followed by levofloxacin with -7. As these are second generation quinolone antibiotics it is unsurprising that they have higher binding affinity than

that of nalidixic acid which is -6.4. MNQ has similar binding affinity of -6. This is a strong binding affinity considering this is compared to drugs which are known to target this protein.

This binding affinity combined with the similar metabolomic interference profiles could hint that MNQ interferes with DNA synthesis in some way. However, to understand exactly how MNQ inhibits the growth of MRSA individual pathway analysis needs to be carried out.

3.4.2.4 –Metabolomics - Specific Pathway Mapping – TCA cycle

MNQ had the third highest proportional impact on carbohydrate metabolism of the antibiotics with 24.7% and the second most individual metabolites with 194 significantly influenced metabolites within this pathway, this was only surpassed by rifampicin with 195. Therefore, carbohydrate metabolism was severely affected by MNQ. The TCA cycle (citrate cycle or Krebs cycle) is the primary metabolic pathway for all aerobic processes, it is essential for the complete catabolism of non-preferred carbon sources and the subsequent generation of reducing potential and biosynthetic intermediates (Vuong et al., 2005). It also plays an important role in glycolysis/gluconeogenesis, transamination, deamination and lipogenesis. It's an important aerobic pathway for the final step of oxidation of carbohydrates and fatty acids. The cycle begins with acetyl-CoA derived from glycolysis; the acetyl group is transferred to oxaloacetate to form citrate. A series of reactions causes the oxidation of two carbons from citrate supplying NADH for the use in oxidative phosphorylation (Akram, 2014).

Figure 44 and Figure 45 shows the regulation of all significantly perturbed TCA cycle metabolites over time in comparison to other treatments; MNQ showed a significant upregulation of citrate, isocitrate, oxoglutarate, malate, and succinate. We are assured in the identification of these TCA cycle metabolites as the same metabolite has been identified more than once due to adduct formation. For different m/z values to result in the same metabolite ID increases confidence in the identification process. This effect on the TCA cycle is specific to MNQ, no other antibiotics tested have the same activity profile a clear indication of the unique action of MNQ. Figure 50

depicts the TCA cycle with the 5 metabolites significantly affected by MNQ highlighted. These are the central metabolites within this pathway, and they are all upregulated in response to MNQ. NADH is a product of the TCA cycle therefore it would be expected also increase, however the opposite is true, there is a considerable down regulation of this metabolite.

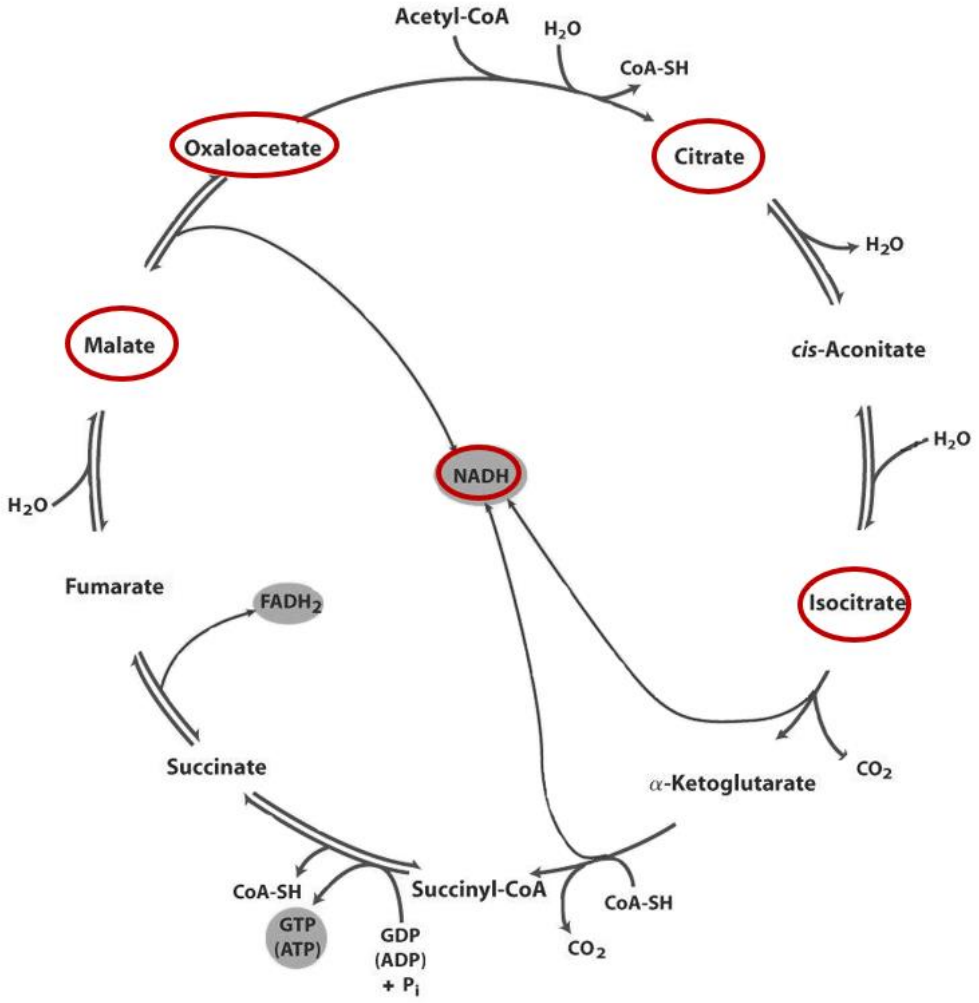


Figure 50: TCA cycle - metabolites which have been significantly affected by MNQ have been circled in red.

The literature states that clinical MRSA strains which are exposed to β-lactam antibiotics have been found to alter their metabolism to optimise energy production through the TCA cycle. An upregulation of TCA related activity was found using transcriptomics and metabolomics which suggests the bacteria is increasing its capability to produce more energy and increasing biosynthetic capability. These benefits allowed the bacteria to respond to the inhibition of cell wall synthesis by

β-lactams (Keaton et al., 2013). MNQ was shown to be the only antibiotic to upregulate all these metabolites so this is not generic stress response to an antibiotic, this is a specific response to MNQ. The upregulation of the TCA cycle metabolites could be an attempt to compensate for reducing compounds such as NADH being depleted. Fenton-mediated hydroxyl radicals which have been shown to be produced by many antibiotics irrespective of the molecular target. These radicals result in the depletion of NADH (Kohanski et al., 2007), which is demonstrated in the metabolic fingerprint of MNQ and the formation of H2O2 was observed using TEM (3.3.3 – Transmission Electron Microscopy)

3.4.2.5 –Metabolomics - Specific Pathway Mapping – Sugar metabolism

MNQ also severely affects sugar metabolism (Figure 46 and Figure 47). Sugar metabolism is linked to energy metabolism and feed directly into the TCA cycle. There's a significant upregulation of hexose and ribose sugars and their phosphates, as well as deoxy ribose and amino sugar phosphates and a down regulation of glucoronate. These changes in sugar metabolism are specific to MNQ treatment. Redox balance is known to be closely involved in sugar metabolism (van Dijken and Scheffers, 1986), this supports the hypothesis that the toxic effects of MNQ are due to the production of ROS.

3.4.2.6 –Metabolomics - Specific Pathway Mapping – Lipid metabolism

The metabolomics results to this point indicate the antibacterial activity of MNQ is due to toxic effect of redox cycling. The production of superoxide radicals required the use of reducing agents such as NADH by the bacteria to detoxify the ROS, which were found to be depleted by MNQ. This redox cycling is known to be caused by a range of compounds such as catechols, quinones, iron chelates, and aromatic nitro compounds causing membrane damage by peroxidative reactions of polyunsaturated fatty acids (Kappus and Sies, 1981). Metabolomic data of MRSA in the presence of MNQ has shown that there is redox cycling occurring and significant perturbation of lipid metabolism (Table 28).

3.4.3 – Transmission Electron Microscopy

Metabolomic data revealed that MNQ significantly upregulates TCA cycle metabolites whilst downregulating NADH. This would suggest that the redox state of MRSA has been disrupted. This oxidative stress caused by MNQ was supported with transmission electron microscopy using cerium chloride, which produces black crystals in the presence of hydrogen peroxide (H2O2) (Figure 49) (Bestwick et al., 1997). H2O2 is produced by bacteria due to oxidative stress, this can be seen in presence of MNQ. The same experiment was carried out with HNQ yet there was no H2O2 produced by the bacteria. This also means that the methoxy group present is crucial to the activity of MNQ.

3.4.4 – Antimicrobial Assay with Antioxidants

Metabolomics and TEM revealed that MNQ affects the growth of MRSA by forming hydroxyl radicals and changing with the redox state. This has resulted in the production of H2O2, which can be seen in the TEM, however, we do not know for certain if this production is lethal to the bacteria. This production could be a relatively harmless by-product of MNQ treatment, and the death of bacteria occurs by another mechanism. Therefore, MNQ was combined with the antioxidants catalase and ascorbate for a bacterial susceptibility assay. Antioxidants reduce the toxic effects of H2O2. If the addition of these compounds reduces the potency of MNQ, this proves the bactericidal effect of MNQ is due to the production of ROS. Table 29 shows that MNQ inhibits the growth on MNQ to just 12.3% at 12.5 mg/L, in the presence of catalase this increases to 23.1% and 56.54% in the presence of ascorbate. This indicates that the production of H2O2 does contribute to the inhibitory effect of MNQ.

Chapter 4 – General Conclusion

Of all the invasive weeds explored to discover new antimicrobial agents, one compound was found to be far more active than all others. This compound was identified as 2-methoxy-1,4-naphthoquinone (MNQ) using mass spectrometry and nuclear magnetic resonance spectroscopy. A broad range of biological assays were carried out, finding that MNQ had a broad range of activity especially against gram-positive bacteria and the parasite *Schistosoma mansoni*. As the focus of this project was originally targeted at MRSA, many strains with a range of resistance mechanisms were assayed, all of which were susceptible to MNQ. 2-hydroxy-1,4-naphthoquinone, which is a structural derivative of MNQ lacking a methoxy group was found to be 10x less effective against MRSA indicating the importance of the methoxy group.

Metabolomic methodologies were developed to discover the mechanism by which MNQ inhibits the growth of MRSA. MNQ was found to work differently to other antibiotics and there were three major effects on the metabolome of MRSA: i) TCA cycle metabolites were significantly upregulated while NADH was down regulated. ii) Sugar metabolism was also significantly affected which is closely related to both the TCA cycle and the regulation of NADH. iii) Lipids across all families were perturbed by MNQ, which is known to be a side effect of oxidative stress. These all indicate that MNQ is interfering with the redox state of MRSA. These results were backed up by TEM showing production of hydrogen peroxide and reduction of MNQ potency by the presence of antioxidants. These effects are exactly what would be expected by a quinone or other compounds which cause superoxide radicals. Although the metabolomic results indicate that MNQ inhibits MRSA by the production of superoxide radicals, this does not specifically address the improved potency of the methoxy groups.

This shows the accuracy and value of metabolomics which has been the least utilised “omic” technique (Figure 51). The applications of metabolomics are endless, and this research has shown how it can be successfully applied to mode of action studies. With further development, high-

throughput metabolomics could be used to streamline drug discovery and development leading to the approval of more antibiotics and other drugs.

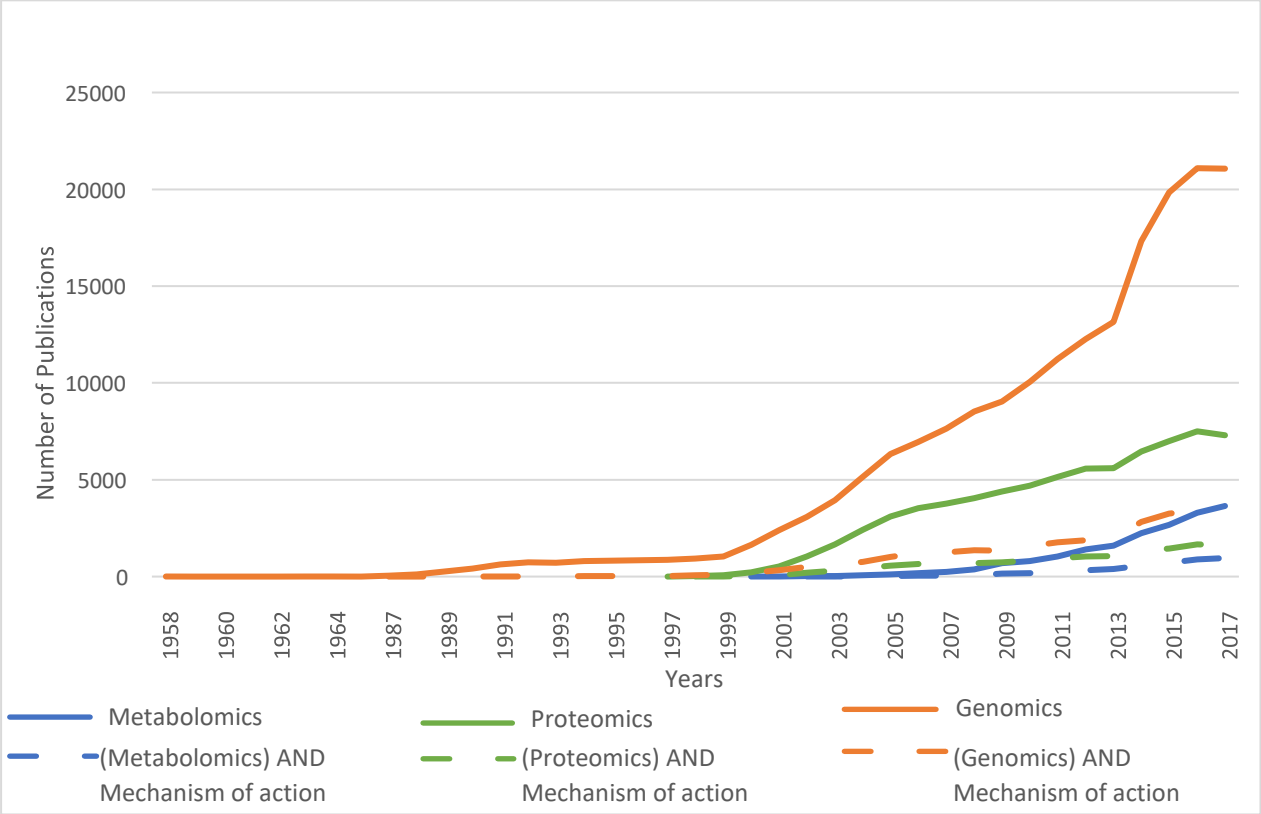


Figure 51: Total number of "omics" publications over time using the Google scholar search function.

As for the development of MNQ into an approved antimicrobial drug the issue with its cytotoxicity needs to be addressed. The cytotoxicity results obtained are worrying, however rather promisingly MNQ does share many structural similarities to current drugs. Synthetic alterations to this structure could yield a potent antimicrobial without the cytotoxicity. The significance of the methoxy group in terms of the specific effect of MNQ would also need to be investigated further.

Appendix 1 -HPLC-PDA-ESI-MS data for *I. glandulifera*

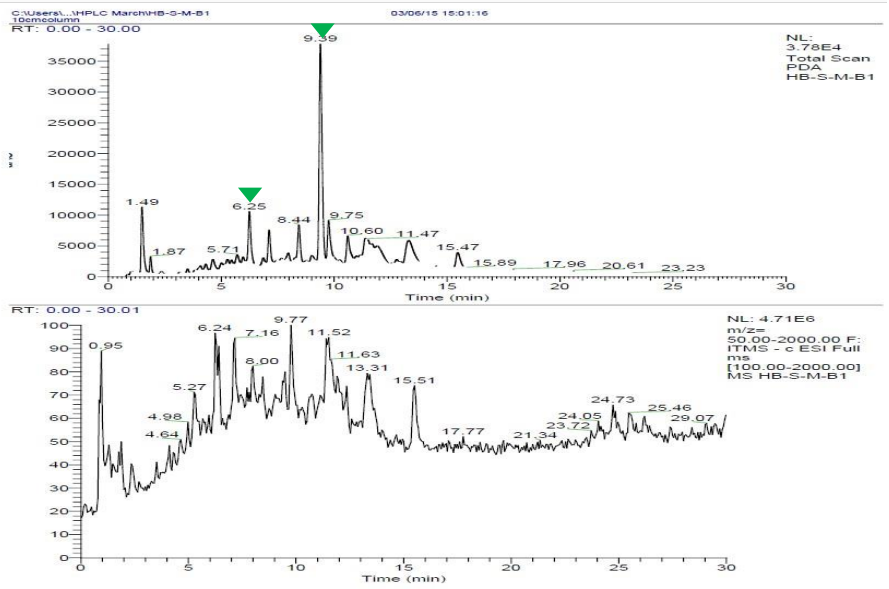


Figure 52: HPLC-PDA-ESI-MS chromatograms of IG1. The top graph represents the total UV absorption of the eluting analytes. The bottom graph represents the mass chromatogram of all the negatively charged ions detected after eluting from the column. There are two peaks of interest (green arrow) which show significant UV absorption and consistent molecular ions within the mass spectrum.

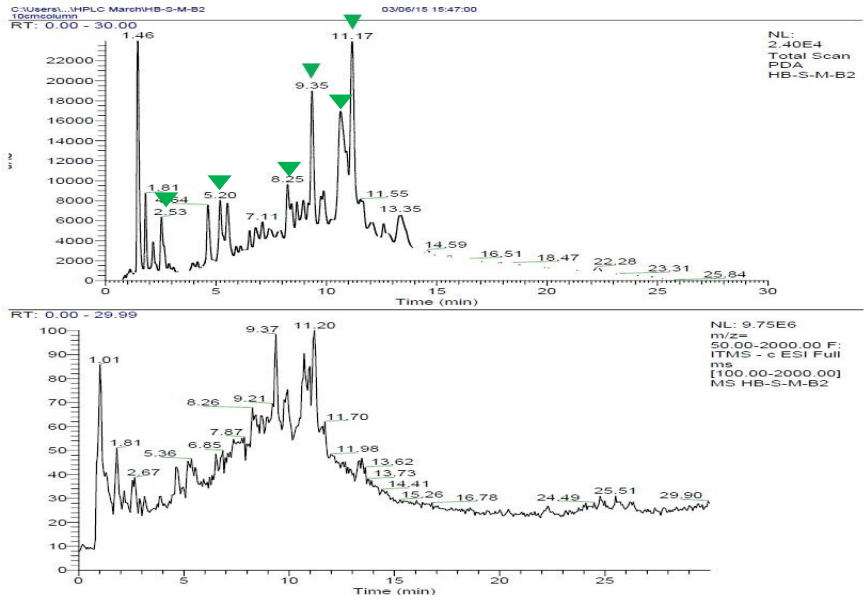


Figure 53: HPLC-PDA-ESI-MS chromatograms of IG2. The top graph represents the total UV absorption of the eluting analytes. The bottom graph represents the mass chromatogram of all the negatively charged ions detected after eluting from the column. There are 6 peaks of interest (green arrow) which show significant UV absorption and consistent molecular ions within the mass spectrum.

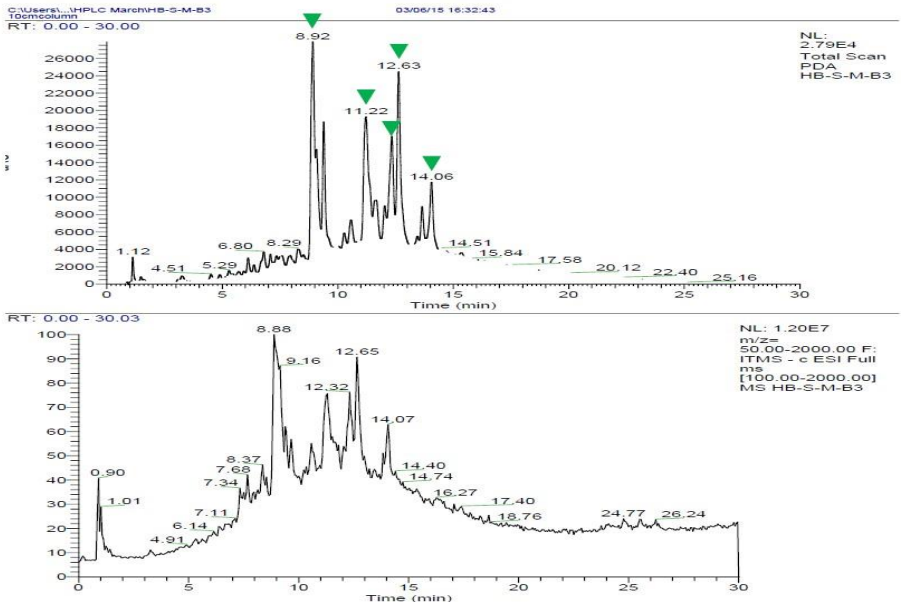


Figure 54: HPLC-PDA-ESI-MS chromatograms of IG3. The top graph represents the total UV absorption of the eluting analytes. The bottom graph represents the mass chromatogram of all the negatively charged ions detected after eluting from the column. There are 5 peaks of interest (green arrow) which show significant UV absorption and consistent molecular ions within the mass spectrum.

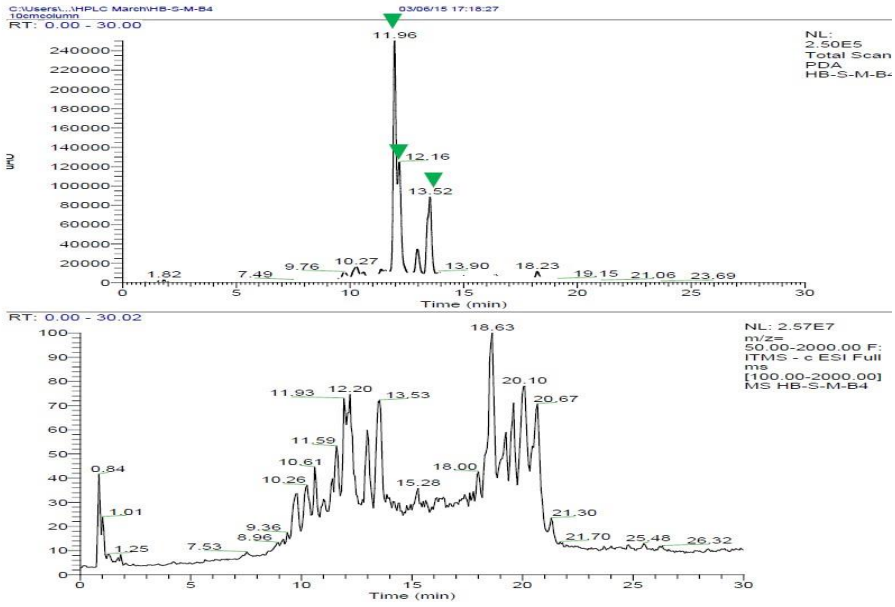


Figure 55: HPLC-PDA-ESI-MS chromatograms of IG4. The top graph represents the total UV absorption of the eluting analytes. The bottom graph represents the mass chromatogram of all the negatively charged ions detected after eluting from the column. There are 3 peaks of interest (green arrow) which show significant UV absorption and consistent molecular ions within the mass spectrum.

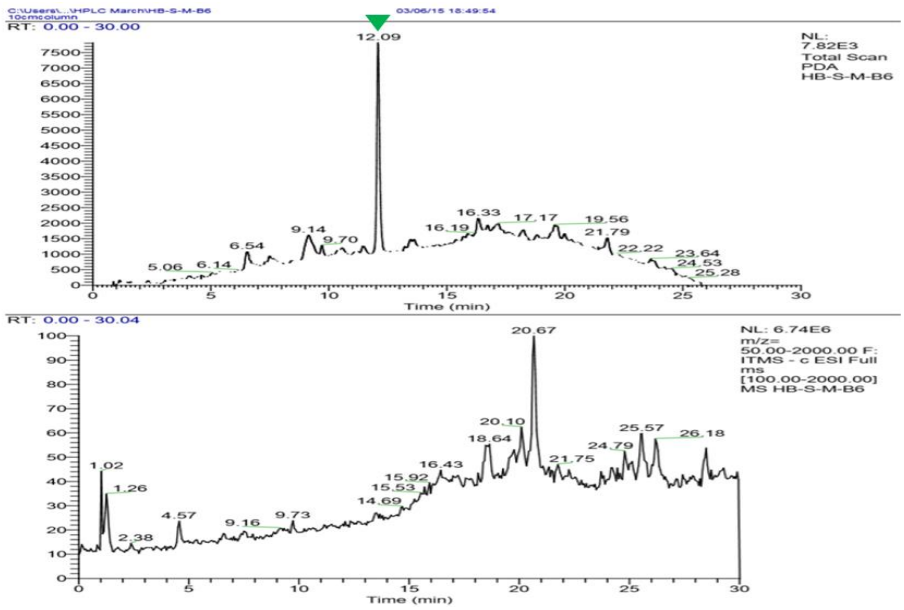


Figure 56: HPLC-PDA-ESI-MS chromatograms of IG5. The top graph represents the total UV absorption of the eluting analytes. The bottom graph represents the mass chromatogram of all the negatively charged ions detected after eluting from the column. There is 1 peak of interest (green arrow) which shows significant UV absorption and consistent molecular ions within the mass spectrum.

Appendix 2 - HPLC-PDA-ESI-MS data for *F. japonica*

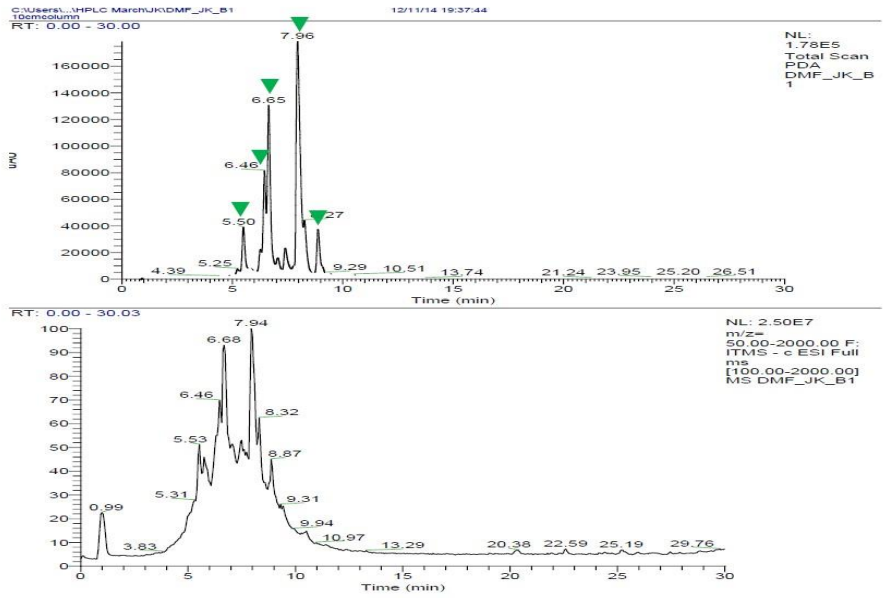


Figure 57: HPLC-PDA-ESI-MS chromatograms of FJ1. The top graph represents the total UV absorption of the eluting analytes. The bottom graph represents the mass chromatogram of all the negatively charged ions detected after eluting from the column. There are 5 peaks of interest (green arrow) which show significant UV absorption and consistent molecular ions within the mass spectrum.

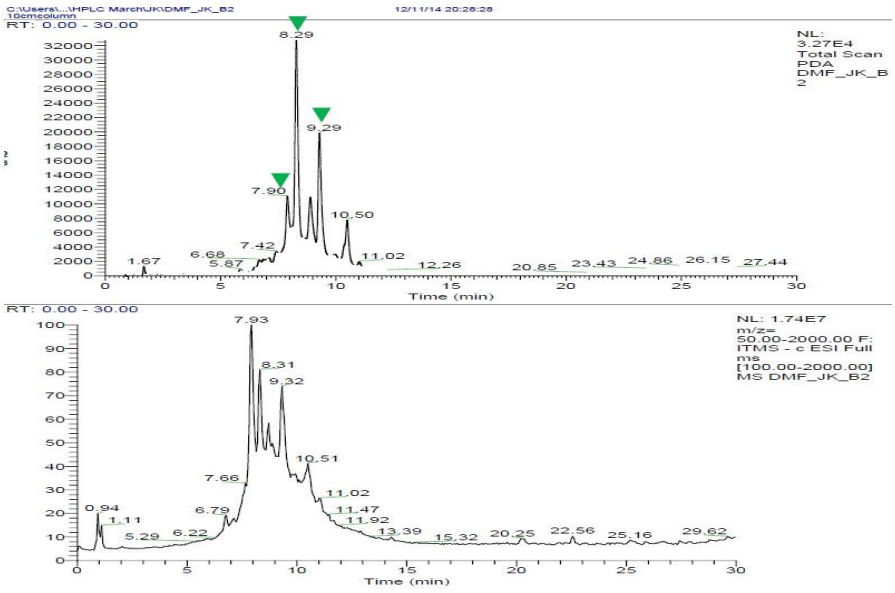


Figure 58: HPLC-PDA-ESI-MS chromatograms of FJ2. The top graph represents the total UV absorption of the eluting analytes. The bottom graph represents the mass chromatogram of all the negatively charged ions detected after eluting from the column. There are 3 peaks of interest (green arrow) which show significant UV absorption and consistent molecular ions within the mass spectrum.

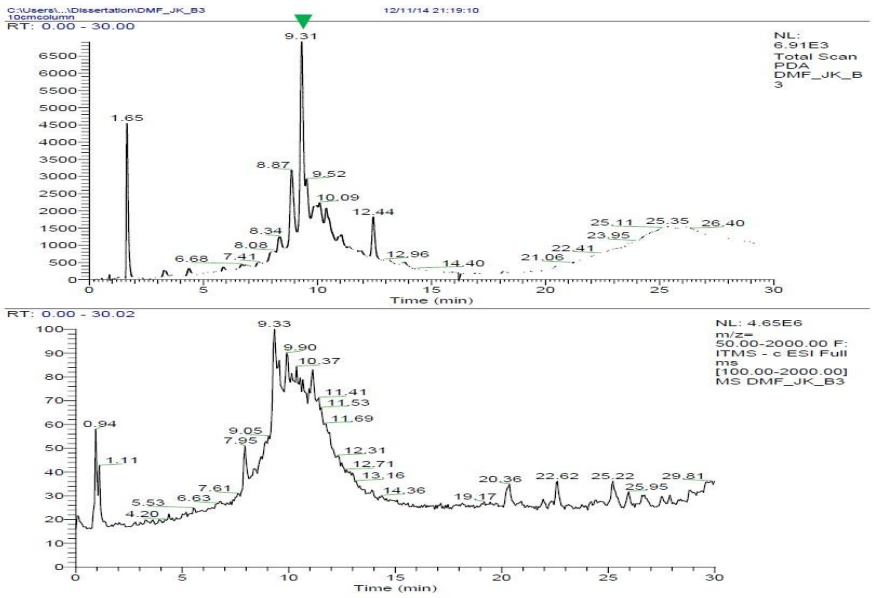


Figure 59: HPLC-PDA-ESI-MS chromatograms of FJ3. The top graph represents the total UV absorption of the eluting analytes. The bottom graph represents the mass chromatogram of all the negatively charged ions detected after eluting from the column. There is 1 peak of interest (green arrow) which shows significant UV absorption and consistent molecular ions within the mass spectrum.

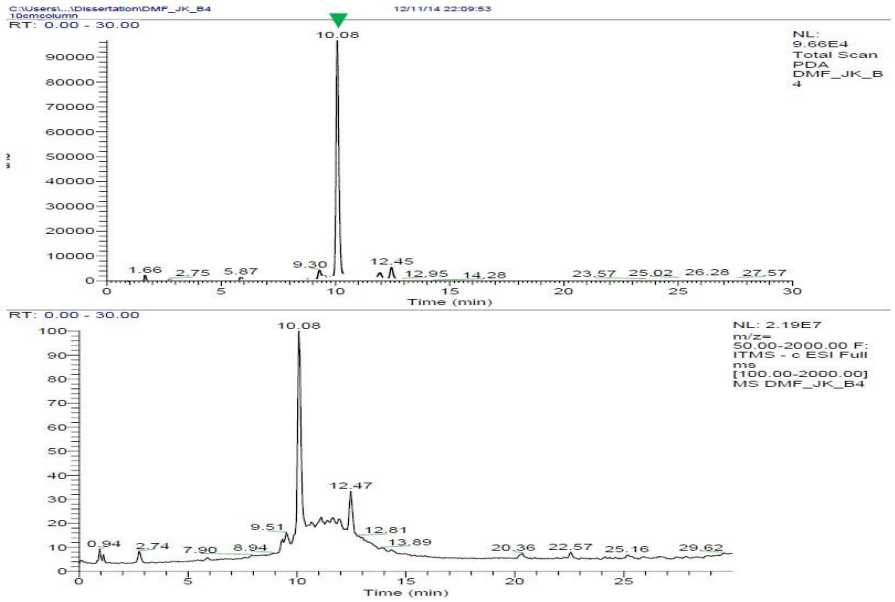


Figure 60: HPLC-PDA-ESI-MS chromatograms of FJ4. The top graph represents the total UV absorption of the eluting analytes. The bottom graph represents the mass chromatogram of all the negatively charged ions detected after eluting from the column. There is 1 peak of interest (green arrow) which shows significant UV absorption and consistent molecular ions within the mass spectrum.

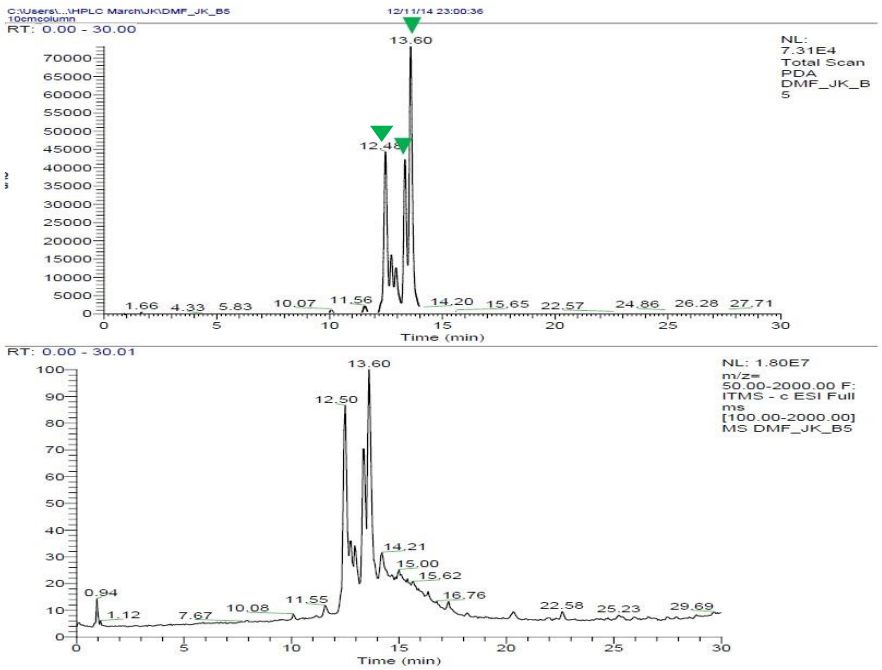


Figure 61: HPLC-PDA-ESI-MS chromatograms of FJ5. The top graph represents the total UV absorption of the eluting analytes. The bottom graph represents the mass chromatogram of all the negatively charged ions detected after eluting from the column. There are 3 peaks of interest (green arrow) which show significant UV absorption and consistent molecular ions within the mass spectrum.

Appendix 3 - HPLC-PDA-ESI-MS data for *R. ponticum*

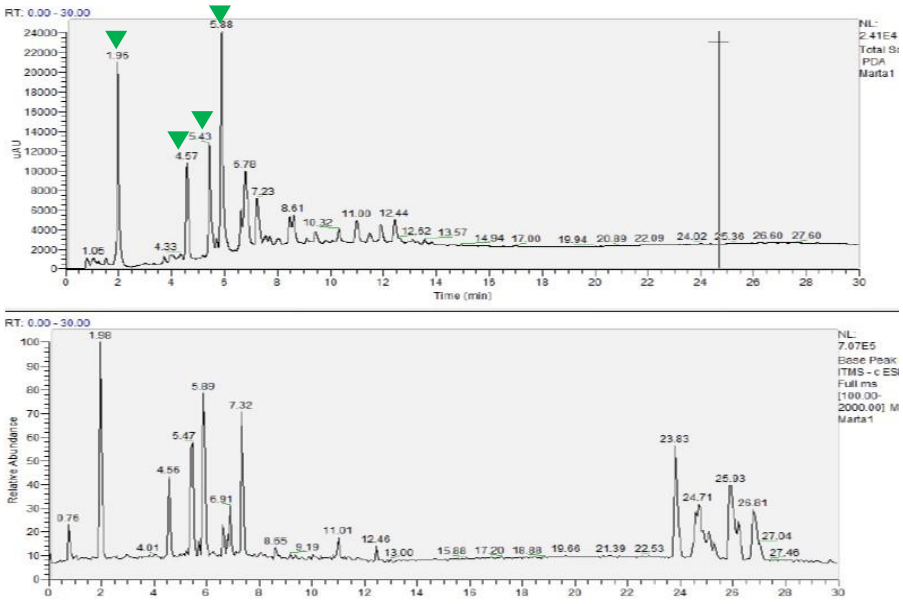


Figure 62HPLC-PDA-ESI-MS chromatograms of RP1. The top graph represents the total UV absorption of the eluting analytes. The bottom graph represents the mass chromatogram of all the negatively charged ions detected after eluting from the column. There are 4 peaks of interest (green arrow) which show significant UV absorption and consistent molecular ions within the mass spectrum.

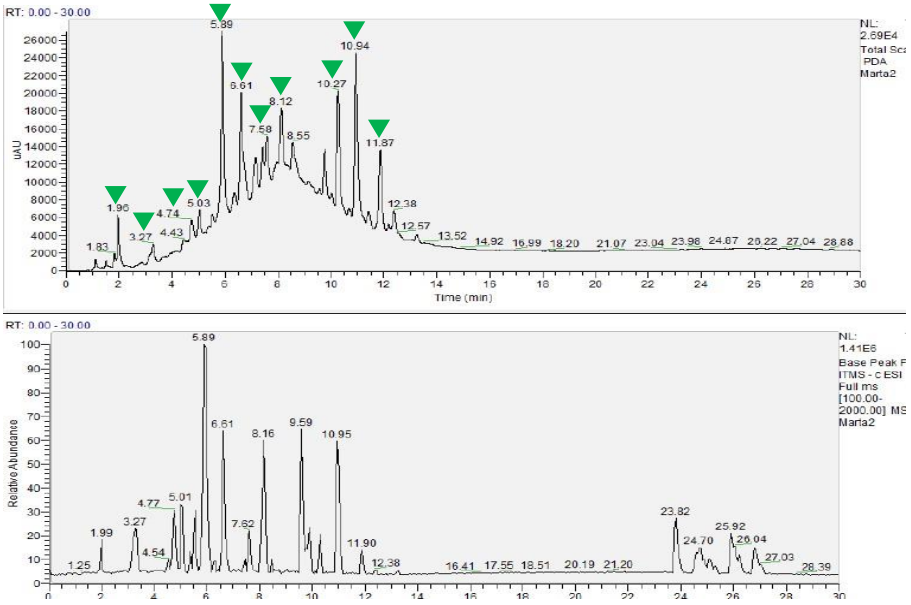


Figure 63: HPLC-PDA-ESI-MS chromatograms of RP2. The top graph represents the total UV absorption of the eluting analytes. The bottom graph represents the mass chromatogram of all the negatively charged ions detected after eluting from the column. There are 11 peaks of interest (green arrow) which show significant UV absorption and consistent molecular ions within the mass spectrum.

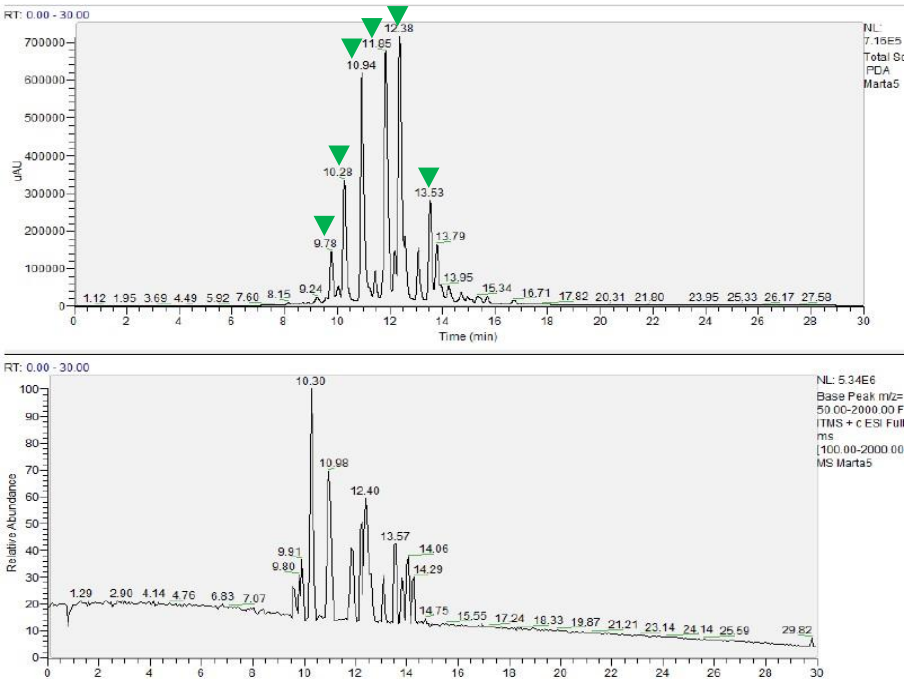


Figure 64: HPLC-PDA-ESI-MS chromatograms of RP3. The top graph represents the total UV absorption of the eluting analytes. The bottom graph represents the mass chromatogram of all the negatively charged ions detected after eluting from the column. There are 6 peaks of interest (green arrow) which show significant UV absorption and consistent molecular ions within the mass spectrum.

Appendix 4 – All bacterial strains

Table 30: List of all aerobic bacterial strains used for susceptibility testing and their mechanism of resistance to certain antibiotics

Strain no.	Organism	Resistances	Mechanism of resistance
572	*Acinetobacter baumannii*	-	-
NCTC 10661	*Burkholderia cepacia*	-	-
NCTC 12201	*Enterococcus faecalis*	Vancomycin	vanA
ATCC 51299	*Enterococcus faecalis*	Vancomycin	vanB
ATCC 29212	*Enterococcus faecalis*	-	-
ATCC 25922	*Escherichia coli*	-	-
ATCC 35218	*Escherichia coli*	Ampicillin	β-lactamase
NCTC 13353	*Escherichia coli*	3rd gen cephalosporins	CTX-M
353	*Escherichia coli*	Nitro / Trim	-
Controls	*Klebsiella pneumoniae*	Carbapenems	NDM
NCTC 13442	*Klebsiella pneumoniae*	Carbapenems	OXA-48
21856	*Klebsiella pneumoniae*	-	-
ATCC 700603	*Klebsiella pneumoniae*	4th gen cephalosporins	SHV-18
NCTC 10975	*Proteus mirabilis*	-	-
ATCC 27853	*Pseudomonas aeruginosa*	-	-
8204	*Salmonella enteritidis*	-	-
11051	*Staphylococcus aureus*	Tetracycline	-
ATCC 29213	*Staphylococcus aureus*	-	-
ATCC 33591	*Staphylococcus aureus*	Methicillin	mecA (III)
ATCC 700698	*Staphylococcus aureus*	Vancomycin	hVISA
ATCC BAA-977	*Staphylococcus aureus*	ERY/CLIND	MLSB
H-EMRSA-15	*Staphylococcus aureus*	Methicillin	mecA (IV)
NCTC 12493	*Staphylococcus aureus*	Flucloxacillin	mecA
ST8-USA300	*Staphylococcus aureus*	Methicillin	mecA (IV)
25760	*Staphylococcus epidermidis*	-	-
25495	*Staphylococcus epidermidis*	-	-
NCTC 11047	*Staphylococcus epidermidis*	-	-
19473	*Staphylococcus saprophyticus*	-	-
Wild strain	*Staphylococcus saprophyticus*	-	-
20456	*Streptococcus bovis*		
21816	*Streptococcus bovis*	-	-
21818	*Streptococcus bovis*	-	-
22358	*Streptococcus Group A*	-	-
22362	*Streptococcus Group G*	-	-
ATCC 49619	*Streptococcus pneumoniae*	-	-
18778	*Streptococcus pneumoniae*	ERY, TET	-
21394	*Streptococcus pneumoniae*	Penicillin	PBP
21395	*Streptococcus pneumoniae*	ERY, CLIND	MLSB

13121	*Streptococcus pneumoniae*	-	-
13122	*Streptococcus pneumoniae*	-	-

Table 31: List of all anaerobic bacterial strains used for susceptibility testing and their mechanism of resistance to certain antibiotics

Strain No.	**Organism name**	**Ribotype/Resistance mechanism**	**Meropenem susceptibility**
1579	*Bacteroides fragilis*	-	Sensitive
1580	*Bacteroides fragilis*	-	Sensitive
1581	*Bacteroides fragilis*	-	Sensitive
1582	*Bacteroides fragilis*	-	Sensitive
1583	*Bacteroides fragilis*	-	Sensitive
1584	*Bacteroides fragilis*	-	Sensitive
1585	*Bacteroides fragilis*	-	Sensitive
1591	*Bacteroides fragilis*	-	Resistant
1592	*Bacteroides fragilis*	-	Sensitive
1593	*Bacteroides fragilis*	-	Sensitive
1594	*Bacteroides fragilis*	-	Sensitive
1595	*Bacteroides fragilis*	-	Resistant
1596	*Bacteroides fragilis*	-	Sensitive
1597	*Bacteroides fragilis*	-	Sensitive
1598	*Bacteroides fragilis*	-	Sensitive
1599	*Bacteroides fragilis*	-	Sensitive
1600	*Bacteroides fragilis*	-	Sensitive
1601	*Bacteroides fragilis*	-	Sensitive
1602	*Bacteroides fragilis*	-	Sensitive
13350	*Bacteroides fragilis*	Met R	Sensitive
ATCC 25285	*Bacteroides fragilis*	-	Sensitive
R43812	*Clostridium difficile*	Ribotype 001	Sensitive
R43874	*Clostridium difficile*	Ribotype 027	Sensitive
R43875	*Clostridium difficile*	Ribotype 001	Sensitive
R43883	*Clostridium difficile*	Ribotype 014	Sensitive
R43935	*Clostridium difficile*	Ribotype 001	Sensitive
R43942	*Clostridium difficile*	Ribotype 078	Sensitive
R43943	*Clostridium difficile*	Ribotype 001	Sensitive
R43968	*Clostridium difficile*	Ribotype 001	Sensitive
R43997	*Clostridium difficile*	Ribotype 078	Sensitive
R43998	*Clostridium difficile*	Ribotype 078	Sensitive
R44000	*Clostridium difficile*	Ribotype 014	Sensitive
R44002	*Clostridium difficile*	Ribotype 014	Sensitive
R44003	*Clostridium difficile*	Ribotype 014	Sensitive
R44004	*Clostridium difficile*	Ribotype 078	Sensitive
R44007	*Clostridium difficile*	Ribotype 078	Sensitive
R44015	*Clostridium difficile*	Ribotype 014	Sensitive

Appendix 5 – R-statistical package script

```
# PIPsearches of ecmdb metabolite database Jasen Finch 12/10/2017
# Database downloaded in JSON format and converted to csv
##### Functions #####

ecmdbQueryPIP <- function(add, mz, ppm = 5, adducts = adducts, DB = DB){

  rule <- adducts$Rule[adducts$Name == add]
  mass <- ppmRange(mz, ppm)
  mass <- map(mass, calcM, adduct = add)
  DB <- DB %>% filter(moldb_mono_mass > mass$lower & moldb_mono_mass < mass$upper) %>%
    rename(Nch = moldb_formal_charge,
           Nacc = moldb_acceptor_count,
           Ndon = moldb_donor_count
           ) %>%
    mutate(Ionisation = eval(parse(text = rule))) %>%
    filter(Ionisation == T) %>%
    mutate(Adduct = add,`Measured m/z` = mz)

  return(DB)
}


ecmdbPIPsearch <- function(mz, ppm = 5, mode = 'n', adducts = adducts, DB = DB){
  if (mode == "p") {
    adductList <- adducts$Name[adducts$Nelec < 0]
  }
  if (mode == "n") {
    adductList <- adducts$Name[adducts$Nelec > 0]
  }
  if (mode == "ne") {
    adductList <- c("M")
  }

  res <- map(adductList,ecmdbQueryPIP,mz = mz, ppm = ppm, adducts = adducts, DB = DB) %>%
    bind_rows() %>%
    select(id:moldb_mono_mass,Adduct:`Measured m/z`) %>%
    rename(`Accurate Mass` = moldb_mono_mass)

  if (nrow(res) > 0) {
    res <- res %>%
      rowwise() %>%
      mutate(`Theoretical m/z` = calcMZ(`Accurate Mass`,Adduct), PPMerror = ppmError(`Measured
m/z`,`Theoretical m/z`))
  } else {
    res <- res %>%
      bind_cols(tibble(`Theoretical m/z` = numeric(), PPMerror = numeric()))
  }
```

```
  return(res)
}

########## Script starts here #############

## library load
packages <- c('magrittr',
              'purrr',
              'dplyr',
              'mzAnnotation',
              'parallel'
)
lapply(packages,library,character.only = T)

## Database and adduct preperation
# Set DBpath to the location of ecmdb.csv
DBpath <- 'C:/Users/dmf/Documents/ecmdb.csv'

DB <- DBpath %>%
  read_csv() %>%

select(id,name,description,kegg_id,moldb_formula,moldb_inchikey,moldb_mono_mass,moldb_acceptor_
count,moldb_donor_count,moldb_formal_charge)

adducts <- Adducts %>%
  filter(!grepl('Nnhh',Rule,fixed = T),!grepl('Noh',Rule,fixed = T),!grepl('Ncooh',Rule,fixed = T))

## PIPsearch

# Change mode and ppm threshold for searches accordingly
Mode <- 'n'
PPM <- 3

# set mzPath to the location of mz.csv
# mz.csv should contain a single column of masses with the heading "mz"
mzPath <- 'C:/Users/dmf/Documents/mz.csv'
MZ <- read_csv(mzPath)

clus <- makeCluster(detectCores())
clusterExport(clus,varlist = c(ls('package:magrittr'),
                               ls('package:purrr'),
                               'ecmdbQueryPIP',
                               ls('package:mzAnnotation'),
                               ls('package:dplyr')
                               ))
PIPhits <- parLapply(clus,MZ$mz,ecmdbPIPsearch,ppm = PPM,mode = Mode,adducts = adducts,DB = DB)
%>%
  bind_rows()
stopCluster(clus)
```

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