

Optimisation of a 1,000 Year-Old Antimicrobial Recipe

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Summary

Leechdoms is a term given to medieval era Anglo-saxon remedies which the historian ML Cameron believed to be a meticulous collection of medical texts and knowledge from several cultures. His hypothesis was that these recipes, for years dismissed as useless and ineffective, had merit as medical treatments. In 2015, Harrison *et al.* published a paper in which they recreated a recipe from a 10th century book known as Bald's Leechbook. This recipe was that of an eyesalve designed to treat a stye in the eye. The authors showed that their preparation of Bald's eyesalve proved to be a powerful antimicrobial, effective against antibiotic resistant *S. aureus* strains. This thesis builds on this work in two ways. First to simplify and optimise the recipe without sacrificing efficacy. Second to refine and isolate active components from the eyesalve to narrow down the list of candidate active components. Attempts to simplify the recipe showed that the wine required in the recipe is key to activity, but is independent of the type of wine used for the preparation. It was demonstrated that the number of days it takes to prepare an active batch of eyesalve can be reduced from nine days to one. The eyesalve remained stable when incorporated into molten agar which was demonstrated to be useful in screening some species for sensitivity to the eyesalves antimicrobial effects. Four different species, *S. aureus*, *K. pneumoniae, A. baumanii* and *E. coli* were screened for growth on eyesalve treated plates and only *S. aureus* was found to grow, demonstrating that the salve may specifically target certain species of bacteria. Fractionation experiments generated fractions with antimicrobial activity, however the specific method used was not very sensitive and as such narrowed the potential active components to a relatively broad spectrum of compounds. In summary, the data presented here provides significant advancements into understanding how old recipes may have functioned against ancient bacterial infections and provides a unique novel avenue for the discovery of antibiotics.

Table of Contents

INTRODUCTION **4**
Anglo-Saxon Medicine 4
S. species and Associated Antimicrobial Resistance 5

MATERIALS & METHODS **10**
Making and testing Bald's eye salve 10
Bacterial strains and culture conditions 11
Culturing and treatment of synthetic wound infections 11
Pre-Treatment of *in vitro* Collagen Wounds 11
Fractional Isolation of Bald's Eyesalve 12
Statistical analysis 13

CHAPTER 1: EYESALVE RECIPE OPTIMISATION **14**
The Importance and Non-specificity of Wine to the Efficacy of Bald's Eyesalve 15
Figure 1.1 ..... *15*
Figure 1.2 ..... *16*
Resting Period is important but does not need to be 9 days 17
Figure 1.3 ..... *17*
Development of Methods of Screening for Eyesalve Sensitivity 18
Figure 1.4 ..... *18*
Figure 1.5 ..... *19*
Figure 1.6 ..... *20*

CHAPTER 2: ISOLATION OF ACTIVE COMPONENTS **21**
Isolating Active Fractions of the Eyesalve 22
Figure 2.1 ..... *22*
Figure 2.2 ..... *23*
Figure 2.3 ..... *24*
Anti-microbial Yield of Combined Fractions 25
Figure 2.4 ..... *25*

DISCUSSION **27**
The Importance of Wine 27
Resting the Eyesalve is Important 31
Bald's Eyesalve is *S. aureus* Specific 31
Isolation of Active Components from Bald's Eyesalve 33
Developing a Unique Pipeline for the Discovery of Novel Antimicrobials 35
Figure 3.1 ..... *37*
Future Directions 39

REFERENCES **41**

Introduction

Anglo-Saxon Medicine

From a scholarly and practical point of view, Anglo-Saxon medicine has, for many years, been regarded with great reticence. Many liken Anglo-Saxon leechdoms, a term coined by Thomas Oswald Cockayne (Cockayne, 1864) to describe the recipes and remedies from this period, to snake oil salesmen. In the sense that what they used was of very little practical efficacy and the practitioners that used leechdoms were merely carving themselves a niche in society. In the early 1990's, the historian M.L. Cameron published a seminal text, making the argument that some Anglo-Saxon medical texts, specifically Bald's Leechbook (Cockayne, 1864), were in fact methodical collections of medical knowledge from an array of cultures including Greek, North African and Byzantine (Cameron M.L., 1993). Academically a bold perspective, but Cameron's arguments were generally unchallenged as his research into the texts was meticulous and his points were well presented.

In 2005, a group of researchers from Wheaton College took on the challenge of recreating a recipe from Bald's *Leechbook*. In their paper, they translated and recreated a specific recipe pertaining to the treatment of a "*wen"* or an eye sty. They tested the prepared recipe against several different species of bacteria, including but not limited to, *E. coli, Streptococcus mutans* and *S. aureus* using the laboratory method known as the Kirby-Bauer method (Brennessel et al., 2005). This method involves placing filter paper discs saturated with the agent to be tested on bacterial "lawns" grown on a solid nutrient medium (Bauer et al., 1966). They found almost no anti-microbial activity. In fact, they observed less anti-microbial activity than the individual components of the recipe when tested in isolation.

A decade later Bald's Leechbook was revisited. Researchers recreated and tested the same recipe as Brenessel *et al. (*2005)*,* but this time using more modern approaches to the testing of the efficacy against *S. aureus*, because this is the primary causative pathogen for sties of the eye (Tamparo and Lewis, 2011) (Harrison et al., 2015). Testing the combined recipe in both planktonic cultures and an *in vitro* collagen wound model that allows for the formation of mature bacterial biofilms, they found the recipe to be a highly effective anti-staphylococcal (Harrison et al., 2015). Furthermore, Harrison et al. also showed that the eyesalve was effective at treating *S. aureus* infected wounds in mice (Harrison et al., 2015)(Dalton et al., 2011). These results not only add weight to Cameron's theories of a scholarly approach to medieval medicine, but they also open a potential new pipeline to discover new antimicrobial agents from ancient texts. Bald's Leechbook is just one of several key ancient medical texts that can be explored for potential novel antimicrobials. Other texts that both preceded and superseded Bald's Leechbook include *De Materia Medica* (Norton, 2006), *Historia Naturali* (Pliny the, 1982)*, Hildegrad's Medicine* (Strehlow and Hertzka, 1988) and the *Compendium of Medicine* by Gibertus Anglicus (Handerson, 1918).

Staphylococcal species and Associated Antimicrobial Resistance

Staphylococcus is a genus of Gram-positive bacteria comprised of approximately 40 currently known species. The majority of species are harmless and are a normal part of the skin and mucosal flora of humans and other organisms (Madigan et al., 2009)(Harris et al., 2002). The two most common species of *Staphylococcus* found as part of the natural skin flora of humans, are *S. epidermidis* and *S. aureus*. *S. aureus*, unlike *S. epidermidis*, is an opportunistic pathogen generally causing minor problems like pimples, but is capable of causing far more severe infections like pneumonia, meningitis or osteomyelitis (Madigan et al., 2009). *S. aureus* infections are broadly categorized into hospital acquired (HA) and community acquired (CA).

Historically, in the case of CA *S. aureus* infections, it is a leading cause of bacteraemia and, prior to the discovery of antibiotics, had a mortality rate of 65-70% (McNeal et al., 1942). Today, even with appropriate treatment, the thirty-day mortality rate for patients with Staphylococcemia is between 20-40% (Melzer and Welch, 2013).

As an opportunistic pathogen, *S. aureus* infects susceptible individuals such as the immunosuppressed or those with chronic diseases like diabetes (del Rio et al., 2009). Patients frequently admitted to hospital are susceptible to HA-*Staphylococcal* infections. The key concern with HA *S. aureus* infection compared to CA *S. aureus* infections, is that the multi-drug resistant *S. aureus* strains are much more prevalent in HA cases due to increased selection pressure placed on the strains in that environment (Dominguez, 2004). One of the most serious concerns in modern medicine with regards to the treatment of *S. aureus,* in particular drug resistant strains, is the cost of treatment. In the EU, MRSA infections in particular have resulted in over 1,000,000 additional days of extra hospitalization for patients and cost approximately €380 million to treat (Report, 2009). A US study of 1,355,347 admissions in 55 hospitals estimated a cost per infection of $12,197, but studies in France indicate a mean cost per case of $39,500 (Curtis, 2008). The differences in these figures can be attributed to various factors, generally associated with differences in healthcare systems, but they still serve to highlight the high cost each individual MRSA infection. An interesting systematic review of the economic costs of MRSA infections in Canada estimate the cost per infection to be $12,216. This report however is able to identify that 81% of the costs can be attributed to hospitalization rather than the cost of treatment of infections (Goetghebeur et al., 2007). Clearly *S. aureus* is a threat to both patient welfare and the healthcare economy.

S. aureus was one of first bacterial species to be targeted with Penicillin in the early 1940's (Raju, 2000). However, overuse and unregulated prescription of this effective drug led to *S. aureus* becoming one of the first microbes to evolve resistance to an antibiotic. By the late 1940's and early 1950's *S. aureus* had become resistant to penicillin (Kirby, 1944) (Bynoe et al., 1956). Methicillin was produced in 1959 as a way to counter the rise of resistance to penicillin (Landau et al., 1999). Although a beta-lactam antibiotic itself, methicillin is a penicillinase resistant compound, meaning it is capable of preventing cleavage by *Staphylococcal* resistance enzymes. This move came with risks. Methicillin has the same mechanism of action as other β-lactam penicillins but it has a much higher rate of normally rare complications such as interstitial nephritis. By 1961, only two years after its introduction, scientist's identified strains of *S. aureus* resistant to Methicillin. These strains were named Methicillin Resistant *S. aureus* or MRSA. In 1968, MRSA was identified in a human patient in the USA (Barber, 1961) (Crisóstomo et al., 2001). MRSA is resistant to all β-lactam penicillins including penicillin, oxacillin, amoxicillin, methicillin and many more. MRSA was one of the earliest multi-drug resistant pathogens in the world. Vancomycin is now one of the few drugs left to treat MRSA infections (Crisóstomo et al., 2001).

Interestingly vancomycin was discovered before Methicillin (Geraci and Hermans, 1983). Vancomycin and methicillin both target cell wall synthesis, making them effective against Gram-positive bacteria. As stated earlier, methicillin falls into the β-lactam class of antibiotics, meaning it targets penicillin binding proteins (PBP). PBP's catalyse crosslinking of peptidoglycans during cell wall synthesis and β-lactam antibiotics bind to these proteins and inhibit peptidoglycan crosslinking (Tipper and Strominger, 1965). Vancomycin achieves the same goal albeit by a slightly different method. Vancomycin is a hydrophilic molecule that forms strong hydrogen bonds with the D-alanyl-D-alanine moieties of N-acetylmuramic

acid/N-acetylglutamic acid (NAM/NAG), preventing crosslinking between said peptidoglycans (Geraci and Hermans, 1983). Despite its efficacy in treating *Staphylococcal* infections, vancomycin never became the first line drug for several reasons. From a pharmacokinetics point of view, vancomycin has very poor bioavailability and must be administered intravenously. This means one cannot prescribe a home course of antibiotics for a patient to take, which results in the patient being admitted and a rise in the treatment cost of that patient. The development of semisynthetic anti-*staphylococcal*'s like methicillin and it's successors (nafcillin, cloxacillin) proved safer alternatives due to early vancomycin trials resulting in severe ototoxicity and nephrotoxicity for many patients (Torok et al., 2009).

Due to the rise in multi-drug resistant strains of MRSA, vancomycin has joined the ranks of several drugs that are reserved for the treatment of multi-drug resistant infections (Torok et al., 2009). While the drug of choice will depend on the infection type, the fact remains that treatment options are in desperately short supply for MRSA infections. Many of the dominant antibiotics were either discovered in or derived from compounds discovered between 1940 and 1960, a period which has become referred to as the golden age of antibiotics. In the 1990's modern approaches to antibiotic discovery were employed such as high-throughput screening, an automated instrumental process for detecting the binding or activity of hundreds of thousands of compounds to an isolated receptor target or whole cells, thereby identifying worthwhile leads for development, and rational drug design (Coates and Hu, 2007). These failed to yield as many new and effective antibiotics as was hoped.

A novel pipeline of discovery is needed to help us identify new antimicrobial compounds. The data presented in this thesis helps support the case that there is potential in mining medieval texts for antimicrobial recipes and refining the effective recipes into novel antimicrobial

compounds. This thesis has two primary aims which are presented as two distinct chapters. Firstly, to optimize and refine the recipe further without sacrificing the anti-bacterial effect of the eyesalve. Secondly, to isolate an active component or components of the eyesalve with the goal of narrowing down an active compound(s).

Materials & Methods

Making and testing Bald's eye salve

The methodology of Harrison *et al.* was used to replicate Bald's eye salve in the lab. When translated into modern English the recipe reads as follows: *"Make an eyesalve against a wen: take equal amounts of cropleac [an Allium species] and garlic, pound well together, take equal amounts of wine and oxgall, mix with the alliums, put this in a brass vessel, let [the mixture] stand for nine nights in the brass vessel, wring through a cloth and clarify well, put in a horn and at night apply to the eye with a feather; the best medicine"* (Harrison et al., 2015). It was previously determined that due to ambiguity of the word "cropleac", leek and onion were both used to provide a second Allium species. Some of the experiments here pertain to both leek and onion versions, but later experiments focus on just onion based recipes. It was previously shown that copper was not an active ingredient in the recipe so it was excluded from our methodology (Harrison et al., 2015).

Equal amounts of each ingredient were specified; 25 ml of bovine bile (87 mg/ml in Cyclone water) was mixed with 25 ml of wine and 25 ml of peeled and finely chopped onion and garlic. The onion and garlic were crushed together using a sterile mortar and pestle for 2 minutes. Wine, bovine bile, onion and garlic were all combined in a sterile glass bottle and left covered in foil for 9 days at 4°C to avoid sunlight or fluctuations in temperature affecting the mixture. The onion and garlic were bought from a local green grocer, the wine from Pennard Organic and bovine bile from Sigma Aldrich. The recipe was filtered through a 5 μm filter so it was small enough to remove the large particles of vegetable matter therefore mimicking straining through cloth described in the ancient recipe. This was then briefly vortexed to homogenize the suspension because a white precipitate of bile forms in the mixture. All preparations were made as described unless otherwise specified.

Bacterial strains and culture conditions

A standard laboratory wild-type strain of *S. aureus* (Newman) was used for all experiments, except when screening *Klebsiella penumoniae*, *Escherichia coli* and *Acinetobacter baumanii* for sensitivity to the eyesalve. Synthetic wound fluid (SWF) (50% [wt/vol] fetal bovine serum, 50% peptone water) (Werthén et al., 2010) was used as the growth medium for all experiments. All cultures were grown aerobically at 37°C in an orbital shaker at 200 RPM.

Culturing and treatment of synthetic wound infections

Synthetic wounds comprised 200 µl polymerized collagen (2 mg/ml collagen, 0.01% acetic acid, 60% [vol/vol] SWF, 0.01 M sodium hydroxide) (Werthén et al., 2010). Cultures of *S. aureus* Newman were grown for 6 hours (mid-log phase) in SWF at 37°C on an orbital shaker**.** The cultures were then diluted to an OD_{600} of 0.05 – 0.1 in SWF, and 50 µl amounts added to wells of 48-well cell culture plates containing synthetic wound medium. Synthetic wounds were incubated aerobically at 37°C for 24 hours to allow the establishment of bacterial biofilms within the collagen matrix. After 24 hours, 100 µl eye salve onion was added. The wounds were then incubated for 24 hours for the eye salve to take effect. After a further 24 h, 300 µl of 0.5 mg/ml collagenase was added to each wound then homogenized through pipetting after 1 hour. Serial dilutions of each wound ($0 – 10^{-7}$) were made in PBS and 10µl was spotted onto LB agar plates. The colonies on each plate were then counted after 24 hours and the CFU were calculated.

Pre-Treatment of *in vitro* Collagen Wounds

Synthetic wounds were prepared as described above. Once the collagen matrices polymerized, aliquots (30 µl for 200 µl wounds and 60 µl for 400 µl wounds) of the onion version of the eyesalve were allowed to soak into the wounds completely for approximately 1 hour in a 37°C

humidified incubator. Cultures of *S. aureus* Newman were grown for 6 hour (mid-log phase) in SWF at 37°C on an orbital shaker**.** The cultures were then diluted to an OD_{600} of 0.05 – 0.1 in SWF, and 50 µl amounts added to wells of 48-well cell culture plates containing synthetic wound medium. Synthetic wounds were incubated aerobically at 37°C for 24 hours to allow the establishment of bacterial biofilms within the collagen matrix. After a further 24 hours, 300 µl of 0.5 mg/ml collagenase was added to each wound then homogenised after 1 hour. Serial dilutions of each wound ($0 – 10^{-7}$) were made in PBS and 10 µl was pipetted onto LB agar plates. The colonies on each plate were then counted after 24 hours and the CFU was calculated.

Fractional Isolation of Bald's Eyesalve

Two methods were used to isolate compound fractions from Bald's eyesalve; liquid phase separation and reverse phase solid phase extraction (rpSPE). Fractions eluted by liquid phase separation were generated by applying 5 ml of solvent (chloroform, ethyl acetate, acetone and hexane) to 2 ml of onion eyesalve and vortexed in glass tubes. The mixtures were subsequently centrifuged at 3000 RPM for 10 minutes. The resulting layers (where obtained) were separated and dried. Once dried the fractions were reconstituted in 200 µl of ethanol and made up to 2 ml with water. Reverse phase solid phase extraction was performed under the following conditions. Strata™ -X 33 µm polymeric reverse phase cartridges (200mg/6ml) (Phenomenex) were used. Cartridge columns were preconditioned with 5mls of methanol followed by 5 mls of water. 2 ml volume of sample was applied to the column and the subsequent elutions and washes applied are as follows:

- Fraction 1 is unretained sample
- 25% methanol wash = 5 ml 25% methanol solution
- 50% methanol wash = 5 ml 50% methanol solution
- 75% methanol wash = 5 ml 75% methanol solution
- 100% methanol wash = 5 ml 100% methanol solution

- 5 ml mobile phase B (10% water 10% acetonitrile 80% isopropanol with 0.1% ammonium acetate)
- 100% ethyl acetate

All of the above fractions were dried and reconstituted in 200 µl ethanol then made up to 2 ml with water.

Statistical analysis

Means were calculated for all data sets. The data for *S. aureus* grown in synthetic wound models were analysed to compare the means of each concentration of eye salve. ANOVA tests were performed but the data was too variable as was seen by graphical representation and did not meet the assumptions that variance was homogenous or that there was normality of error. Therefore non parametric Kruskal-Wallis tests were used to compare the medians and then Mann-Whitney U test to compare the medians in each neighbouring sample. A Bonferroni correction was then applied as two tests were carried out on each data set.

Chapter 1: Eyesalve Recipe Optimisation

The Importance and Non-specificity of Wine to the Efficacy of Bald's Eyesalve

The contribution of wine in the recipe to the efficacy of the eyesalve operated under the hypothesis that the wine may have acted as a solvent to extract compounds. To test this, several different batches of eyesalve were made. Two variants using onion as the sole allium species were prepared, one using the wine as described in Materials and Methods and the other using an equivalent percentage solution of ethanol (11% v/v). Two similar preparations using leek as the sole allium species were also constructed along with two using both leek and onion together as the allium species. Figure 1.1 illustrates the efficacy and the variation between the different recipes against *S. aureus* Newman in the *in vitro* collagen wound model.

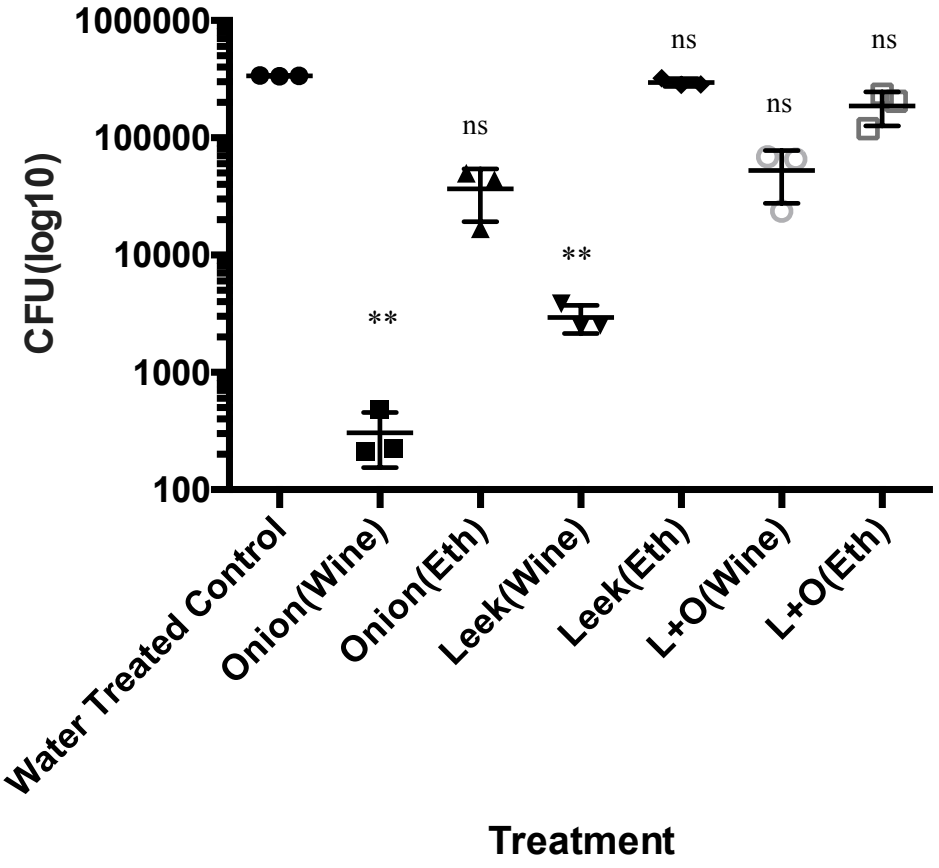


Figure 1.1 **The efficacy of wine and ethanol eyesalve preparations.** Bar graph of the colony forming units (CFU) on a $\log_{10}$ scale of *S. aureus* recovered from *in vitro* collagen wounds after 24-hour treatment with various eyesalve recipes. Onion(Wine/Eth): onion variant prepared with either wine or 11% ethanol solution. Leek(Wine/Eth): Leek variant prepared with wine or 11% ethanol solution. L+O(Wine/Eth): Leek and onion combined together with wine or 11% ethanol solution. N = 5. Error bars represent standard deviation. ** = $p < 0.01$. ns= not significant.

Only recipe variants prepared with wine demonstrated statistically significant anti-microbial activity ($p<0.01$). A significant reduction in anti-microbial activity was observed when the wine was replaced with an 11% v/v ethanol solution, with the leek variant prepared with ethanol showing almost no difference compared to the control. When using equal amount of leek and onion together in a preparation, there was very little anti-microbial activity observed.

Because wine is important to the anti-microbial activity of the recipe, a further experiment was undertaken to determine whether varying the type of wine matters. Five preparations of the onion variant of the eyesalve were made up on the same day. In each of the five preparations, a different wine was used. The wine used in Fig. 1.1 was used along with four alternative wines, two reds and two whites.

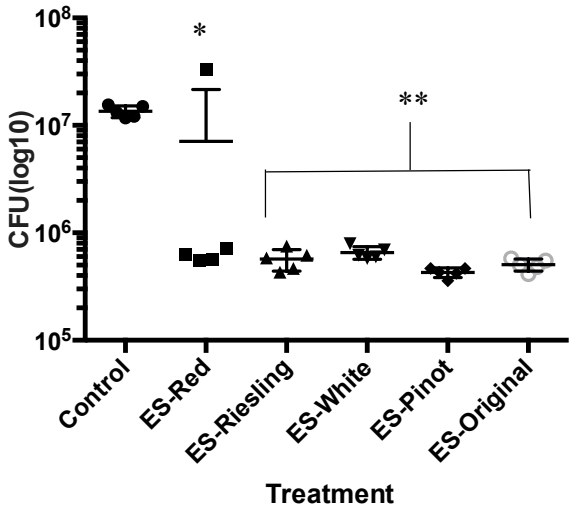


Figure 1.2 **Effects of wine type and variety on eyesalve efficacy.** The above figure is an X-Y dot plot of the number of Colony forming units (CFU) on a $\log_{10}$ of *S. aureus* cells recovered from an *in vitro* collagen wound model after 24 hour treatment with several recipe variants. Control: Water treated control. ES-Red: Onion eyesalve recipe prepared with a red wine of unknown varietal and origin. ES-Riesling: Onion eyesalve recipe prepared with a white Riesling from Australia. ES-White: Onion eyesalve recipe prepared with white wine of unknown varietals and origin. ES-Pinot: Onion eyesalve recipe prepared with a red Pinot Noir from Macedonia. ES-original: Original eyesalve recipe preparation using the same English white wine as described in Material & Methods. N =5. * = $p < 0.05$. ** = $p < 0.01$.

There was no observable difference in the efficacy of the eyesalve between the different wine types and the species of grape used (Fig. 1.2). A slightly lower degree of killing of *S. aureus* by the eyesalve is observed compared to other batches described in this thesis. This can be attributed to user variation in the preparation phase.

Resting Period is important but does not need to be 9 days

The recipe in Bald's *Leechbook* calls for the mixture to be left for 9 days before being filtered and used, and this was previously shown to be important for efficacy, because a freshly made batch had little anti-microbial activity compared to a 9 day old batch (Harrison et al., 2015).

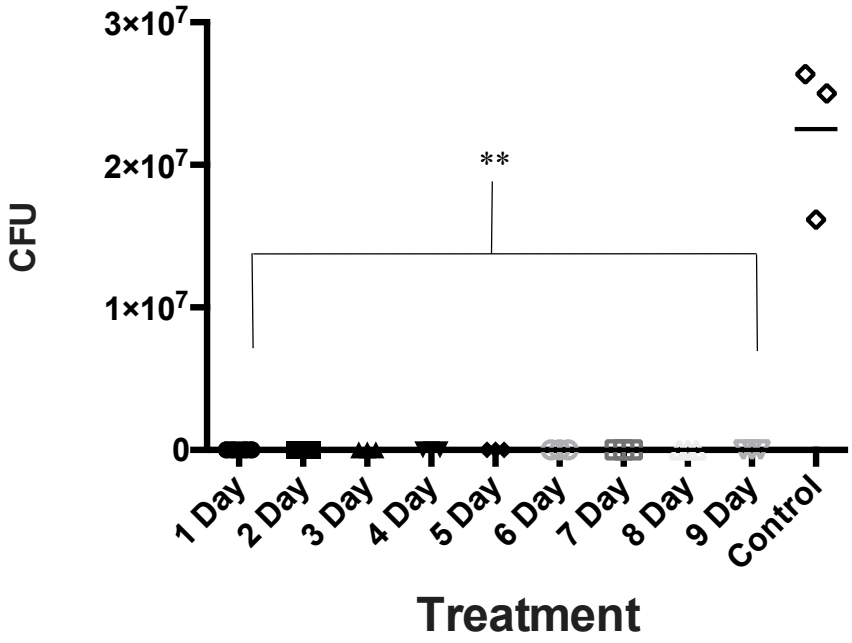


Figure 1.3 **The effect of preparation time on eyesalve efficacy.** Colony forming units (CFU) of *S. aureus* recovered from an *in vitro* collagen wound model after 24 hour exposure to different variations of the recipe. 9 identical preparations of the onion version of the eyesalve recipe were prepared. The titles of the treatments on the X axis describe the number of days the recipe was allowed rest before filtration. Control: water treated control. ** = $p<0.01$. N=3. Scale is linear as some values are zero and cannot be plotted on a log scale.

Nine identical batches of the onion version of the eyesalve were prepared. One batch was filtered after 24 hours and then different batches were filtered after each subsequent 24 hour period for 9 days. There was no observable difference between the anti-microbial efficacy between the batches. The large degree of killing compared to other batches can be attributed to user variation in batch preparation (Fig. 1.3). This shows the importance of a 'resting period' which was first described in the original recipe (Cockayne, 1864). However, the resting period required is much less than that described in the original recipe. Either the resting period is more efficient in our replications of the recipe due to the use of more modern techniques and storage methods, or the original specified resting period was for 9 days because it was out of superstition.

Development of Methods of Screening for Eyesalve Sensitivity

To determine whether the eyesalve has any effects on other species of bacteria, it was incorporated into Luria broth (LB) agar. Onion eyesalve was incorporated into molten LB agar as described above in the Materials & Methods section of this thesis.

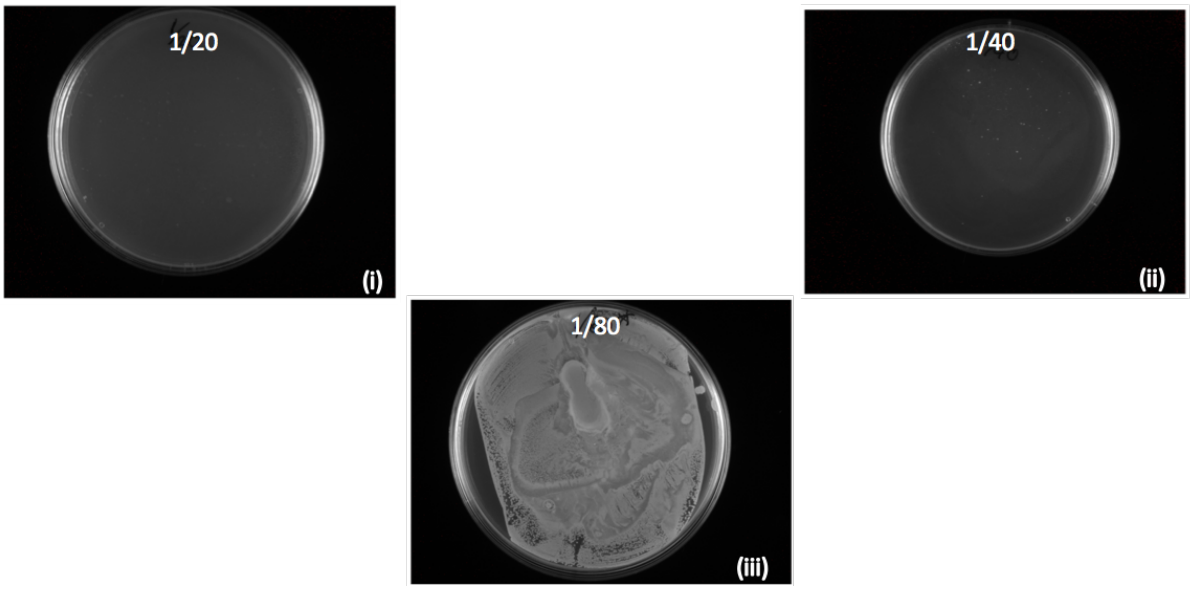


Figure 1.4 **Eyesalve agar plates can select for *S. aureus*.** The above images show three 20ml agar plates poured with the onion variant of the eyesalve incorporated in at various concentrations. Plates were inoculated once dry by spreading fresh *S. aureus* cultures on them. (i) Agar plate with eyesalve at a concentration of 1 in 20. (ii) Agar plate with eyesalve incorporated at a concentration of 1 in 40. (iii) Agar plate with eyesalve incorporated at a concentration of 1 in 80.

Figure 1.4 illustrates an experiment whereby a lawn of *S. aureus* was spread onto LB agar plates with the onion eyesalve. The image shows three plates containing various concentrations of eyesalve. In plates (i) and (ii) there was no observable growth of *S. aureus*. However a healthy lawn of *S. aureus* can be seen on plate (iii).

With the knowledge that the eyesalve is effective when incorporated in LB agar, eyesalve plates were used to screen several bacterial species for sensitivity at various concentrations. The species selected for screening were *K. pneumoniae*, *A. baumanii* and *E. coli* in addition to *S. aureus*.

Species	Concentration	Growth (+/-)
S. aureus	1/10	-
K. pneumoniae	1/10	+
A. baumanii	1/10	+
E. coli	1/10	+
S. aureus	1/20	-
K. pneumoniae	1/20	+
A. baumanii	1/20	+
E. coli	1/20	+
S. aureus	1/40	+
K. pneumoniae	1/40	+
A. baumanii	1/40	+
E. coli	1/40	+
S. aureus	1/80	+
K. pneumoniae	1/80	+
A. baumanii	1/80	+
E. coli	1/80	+

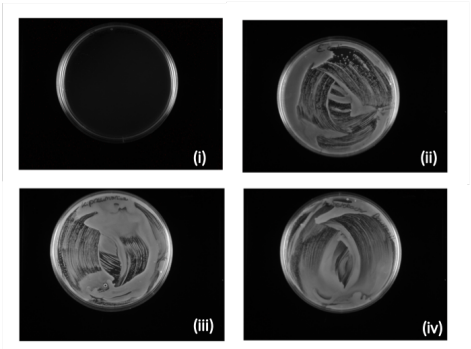


Figure 1.5 **Screening multiple species for eyesalve sensitivity.** The results of using pre-incorporated eyesalve LB agar plates to screen multiple species. The table on the left indicates if each of the fours species tested were capable of growing at one of the four concentrations of eyesalve. All species grew at concentrations 1/80 and 1/40. *S. aureus* was the only species unable to grow at a concentration of 1/20 or higher. The plate images on the right show the growth of each of the four species on plates with the highest concentration of eyesalve, 1/10. (i) *S. aureus* shows no growth (ii) *E. coli* exhibits normal growth (iii) *K. pneumoniae* exhibits normal growth (iv) *A. baumanii* exhibits normal growth.

Figure 1.5 shows that at concentrations below 1/20, none of the four species exhibited any noticeable sensitivity to the eyesalve. However, at concentrations of 1/20 and above only *S.*

aureus was unable to grow. Eyesalve was next incorporated into the *in vitro* collagen wound model. The purpose of this was to determine whether the eyesalve could prevent or reduce the colonisation of *in vitro* wounds by *S. aureus*. Prior to inoculation with *S. aureus* Newman, 50 µl of the onion eyesalve was allowed to soak completely into the wounds.

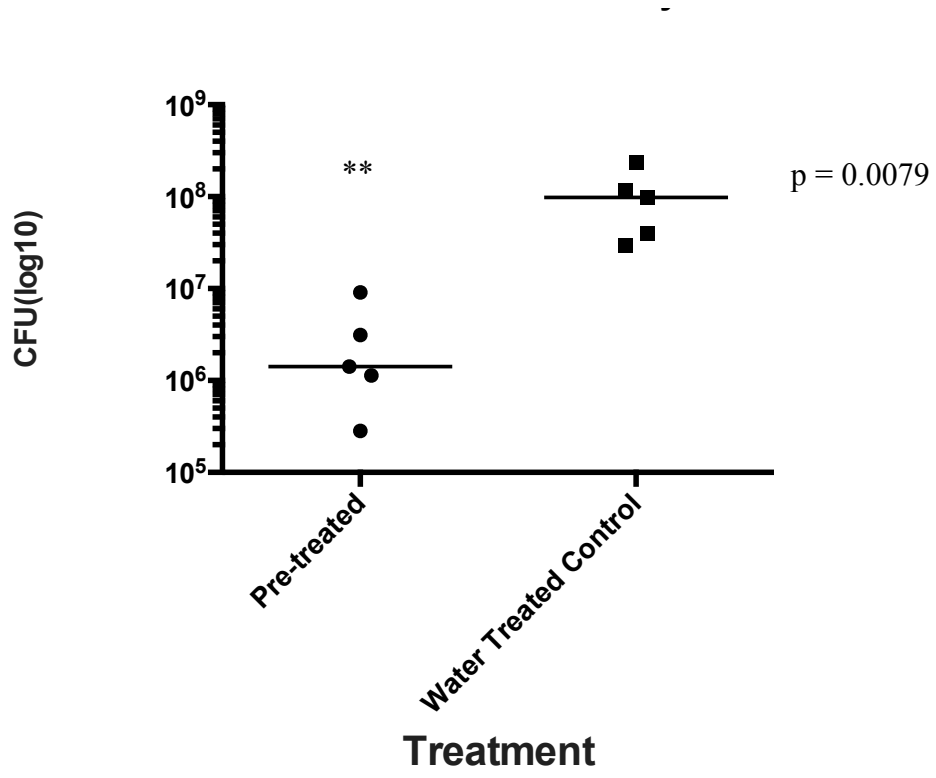


Figure 1.6 **Pre-treating *in vitro* wounds with eyesalve.** Colony forming units plotted on a log10 scale of *S. aureus* recovered from *in vitro* collagen wounds saturated with eyesalve prior to inoculation compared to *in vitro* collagen wounds saturated with water prior to inoculation. N= 5. Mann-Whitney-U test was used to compare the means.

2 log fold less bacteria were recovered from wounds pre-treated with eyesalve prior to inoculation compared to control wounds pre-treated with water (Fig. 1.6).

Chapter 2: Isolation of Active Components

Isolating Active Fractions of the Eyesalve

The first step towards isolating the active compound(s) of the eyesalve mixture was fractionation of the eyesalve. This was achieved by employing two different methods, liquid phase separation and solid phase extraction, as described in the Materials & Methods section of this thesis. Each solvent was selected in order to elute very polar to very non-polar molecular fractions from the eyesalve (Fig. 2.1).

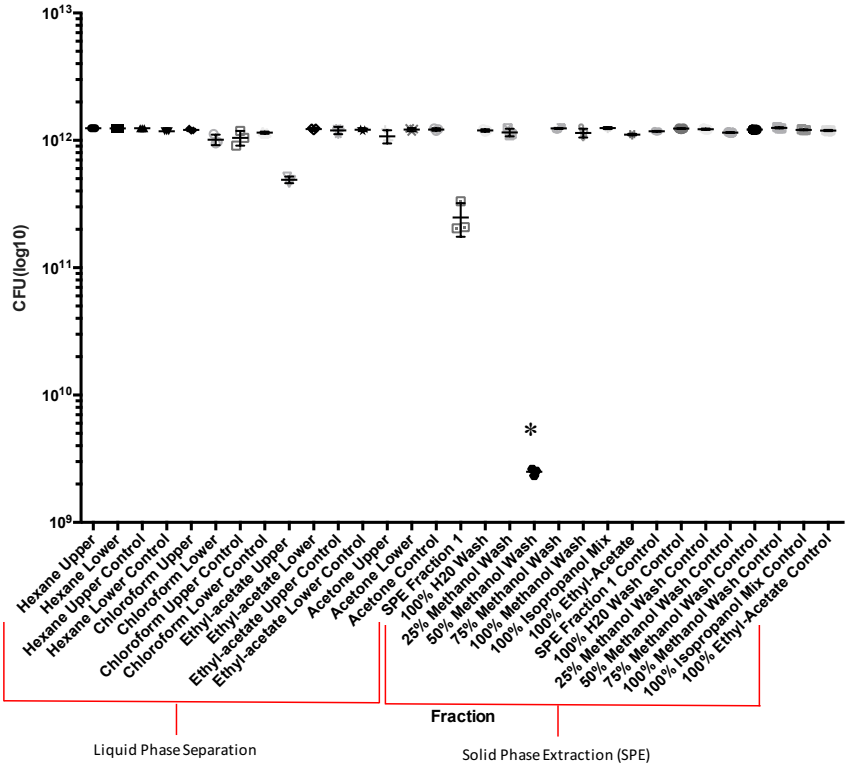


Figure 2.1 **Testing of eyesalve fractions in *in vitro* wounds.** Graph illustrating the efficacy of fractions of eyesalve against *S. aureus* Newman. The figure plots number of CFU of *S. aureus* Newman recovered from *in vitro* collagen wounds after 24 hour exposure to the fractions. Two methods were used to fractionate the eyesalve. Liquid phase separation which generates an upper and lower phase when a solvent is added to the eyesalve which can be separated. Solid phase extraction which passes the eyesalve through an affinity column and is washed with various solvents of different concentrations. Solid phase extraction elutes increasingly non-polar molecules in the fractions from left to right in the graph. Three fractions have some anti-microbial activity compared to the controls; Ethyl-acetate upper, SPE fraction 1 and 50% methanol wash. * = $p < 0.05$. N=3.

Three fractions of eyesalve exhibited some degree of antimicrobial activity; ethyl-acetate upper, SPE fraction 1 and 50% methanol wash. The fraction titled 50% methanol produced the most significant degree of anti-microbial killing (Fig. 2.1).

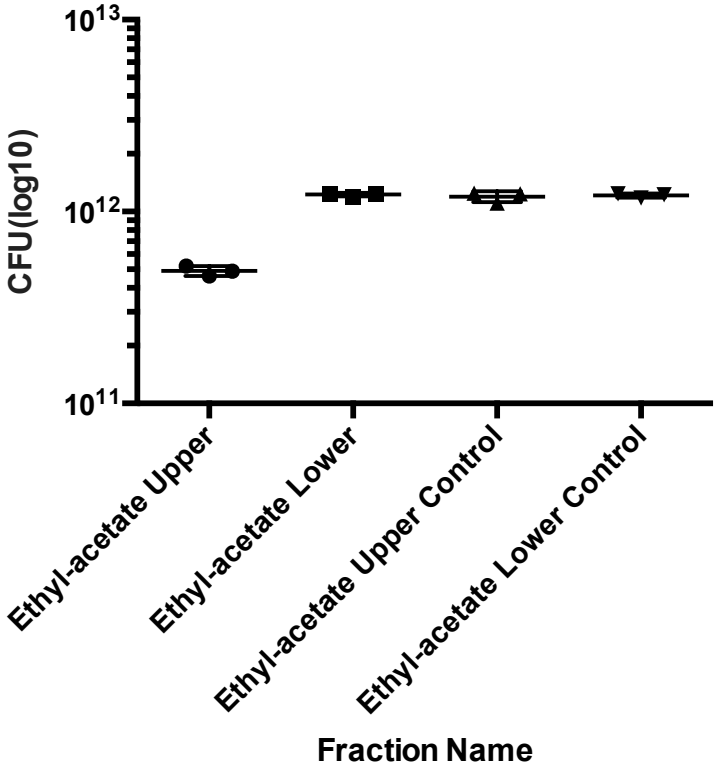


Figure 2.2 **Ethyl-acetate fraction efficacy at killing *S. aureus*** The above figure focuses in on one of the active fractions, Ethyl-acetate upper. The figure plots number of CFU of *S. aureus* Newman recovered from *in vitro* collagen wounds after 24 hour exposure to the fractions. A small degree of anti-microbial activity is observed in the fraction compared the controls which were prepared using the same method applied to water.

Figure 2.2 highlights the ethyl-acetate fractions generated by liquid phase separation. The upper phase generated from the eyesalve when ethyl-acetate was applied to the solution, shows a certain amount of anti-microbial activity. Highly hydrophobic molecules are the ones most likely to be contained within this fraction. Control fractions were prepared by applying the solvent to water. This shows that the solvent phases themselves are not lethal to the bacteria.

Figure 2.3 shows the fractions generated by solid phase extraction as described in the Materials & Methods section. The fractions entitled SPE fraction 1 and 50% methanol wash exhibited anti-microbial activity compared to controls, the latter of the two has more noticeable activity.

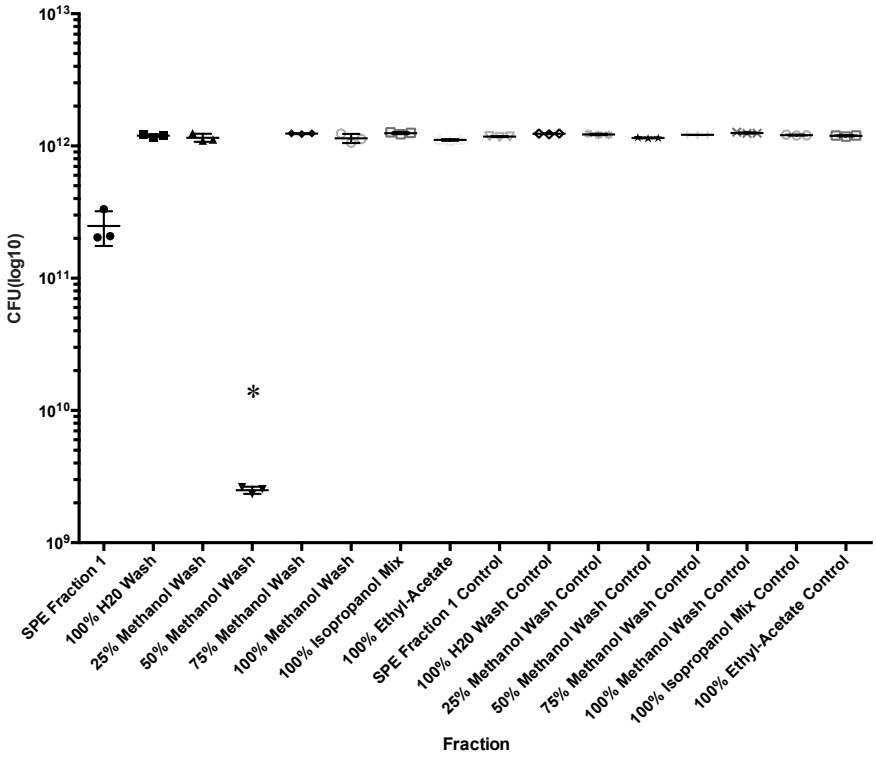


Figure 2.3 **Efficacy of SPE fractions at killing *S. aureus*.** The above figure focuses on the fractions generated using solid phase extraction. The figure plots number of CFU of *S. aureus* Newman recovered from *in vitro* collagen wounds after 24 hour exposure to the fractions. As can be seen in the above figure two fractions show a degree of efficacy in killing *S. aureus*; SPE fraction 1 and 50% methanol wash. 50% methanol wash is more effective than SPE fraction 1. 50% methanol wash was the only statistically significant fraction tested.. * = $p < 0.05$. N=3.

From SPE fraction 1 to 100% Ethyl-acetate wash, the resulting eluted fractions become increasingly non-polar in their contents (Fig. 2.3). Therefore, SPE fraction one should contain very hydrophilic compounds while 50% methanol was should be in the mid-range, eluting molecules that are amphillic in nature. The matching control fractions did not exhibit any killing effect demonstrating that the solvents used did not contribute to the killing effects observed. Overall, 50% methanol wash was the only fraction to yield a statistically significant

result. However, given the lack of specificity in separation technique used, the fractions that show observable anti-microbial activity but not statistically significant activity (Ethyl-acetate upper and SPE fraction 1) should still be considered as candidates for exploration and testing.

Anti-microbial Yield of Combined Fractions

After determining there are fractions that contain significant anti-microbial activity, the next stage was to combine the active fractions, as well as inactive fractions, to see if the anti-microbial activity of a single fraction can be enhanced or activated through synergistic action with other fractions (Fig. 2.4).

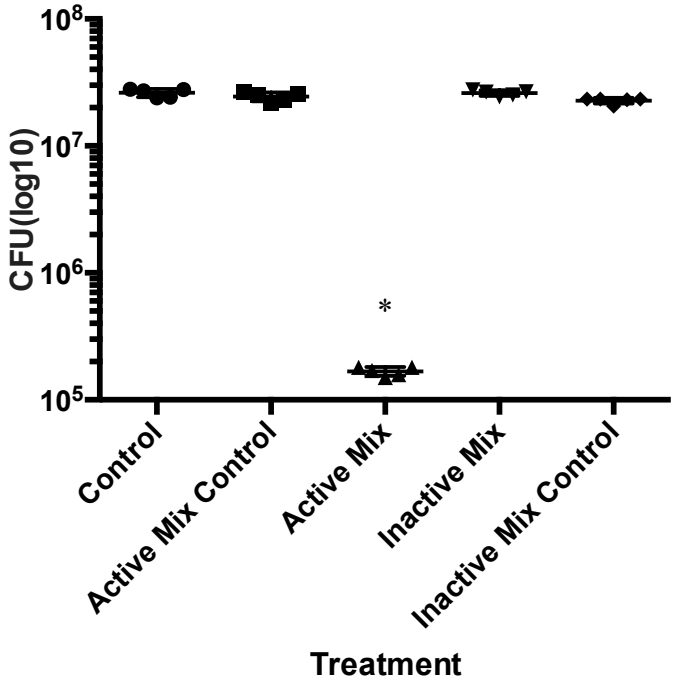


Figure 2.4 **Combined active fractions yield better activity than combined inactive fractions.** The above graph illustrates the effect of combining active and inactive solid phase extraction fractions together. The figure plots number of CFU of *S. aureus* Newman recovered from *in vitro* collagen wounds after 24 hour exposure to the fractions. Active mix consists of equal quantities of SPE fraction 1 and 50% methanol wash. Active mix control consists of the matching control fractions mixed in equal quantities. Inactive mix consists of all of the other fractions not included in the active mix combined in equal quantities. Inactive mix control consists of the matching control fractions mixed in equal quantities. A greater than 2 log fold reduction in observed in the active mix compare to the water treated control. The other combinations exhibit no noticeable anti-microbial activity. * = $p < 0.05$. N=5.

Active Mix was ethyl-acetate upper, spe fraction 1 and 50% methanol wash combined together in equal quantities. Active mix control: matching control fractions combined in equal quantities. Inactive mix: All other fractions from both fractionation methods were combined in equal quantities. Inactive mix control: matching control fractions combined in equal quantities. The active mix treatment was the only mixture with anti-microbial activity (Fig. 2.4). It cannot be ascertained whether this is a superior anti-bacterial effect to the individual fractions in this case as they were not tested in parallel in this experiment.

Discussion

I used *S. aureus* Newman as a laboratory standard for the testing of different variations of Bald's eyesalve and refined fractions of the recipe. I found that: (1) The wine is important to the recipe's antibacterial activity and the contribution of wine is independent of factors like ethanol concentration, wine type and wine origin (Figs. 1.1 and 1.2); (2) The resting period is important but does not need to be as long as described in the original recipe (Fig. 1.3); (3) The eyesalve can be incorporated into solid nutrient media and is selective specifically for *S. aureus* (Figs. 1.4 and 1.5); (4) Active and inactive components can be yielded through crude fractionation of the eyesalve with active components being found in hydrophilic, hydrophobic and amphillic fractions (Figs. 2.1, 2.2 and 2.3).

The Importance of Wine

Our initial hypothesis regarding the wine in the recipe was that it was chosen as an alternative to water. We can assume that the writers of Leechbooks would have had no idea about molecular polarity and solvent extraction, and it is likely that wine was used as a 'safe liquid' that was cleaner than most water sources available at the time (Hagen, 1992). However, the ethanol contained in wine could act as a solvent to extract certain compounds from the allium species in the recipe, thus generating anti-microbial activity. The results (Fig. 1.1) demonstrate that using an equivalent percentage (v/v) solution of ethanol does not yield the same efficacy in recipe preparations compared to wine, resulting in more questions about the recipe than it does answers. If the ethanol within the wine is not important to the efficacy of Bald's eyesalve, then somewhere in the plethora of organic compounds contained within *vitis vinifera* grapes there must be a key compound(s). This makes isolation and identification of active compounds and optimization of the recipe even more challenging.

An interesting additional result to the experiment described in figure 1.1, is the almost complete lack of anti-microbial activity in the eyesalve when both leek and onion are added together (where one-part allium species is called for in the recipes 0.5 parts of both leek and onion are used). This result is interesting as preparations using either leek or onion yield anti-microbial activity and it is a reasonable assumption that using them together would lead to an effective batch. As observed however (Fig. 1.1), this is not the case. It is difficult to surmise why this may be. Initially, with anti-bacterial activity observed in both leek and onion preparations, it was assumed that the same useful compound was shared between the allium species. The results would indicate that this is not the case. If the same effective compound was being elucidated from both allium species, then we would expect some anti-microbial activity to be observed. However, the observation suggests that different compounds may be elucidated from each allium species, which are interfering with one another in some fashion during the recipe preparation. The minutia of this observed effects can be difficult to understand however without knowing what compounds we are dealing with.

Given that wine is important to the efficacy of Bald's eyesalve, it was then important to determine whether the type of wine is important. Wine grapes around the world, regardless of whether they are red or white, all come from the same species of grape vine, *vitis vinifera* (Galet, 2000). Despite this, viticulture is a vast industry built upon the idea that not only does each grape variety have its own specific characteristics, but that every vine growing region in the world has distinct soil characteristics and climates that contribute to making batches of grapes distinct from others. Determining whether or not this array of factors combines in some unique fashion to generate a singular wine that happens to make the eyesalve recipe effective as an antimicrobial is important. If wine makes a difference, then it would also be significant from a humanities point of view. The wine, Pennard Organic, originally selected by Harrison

et al. (2015) was chosen for several reasons. It is an English wine, so we know it can grow in the generally inhospitable English climate and the grapes would have likely grown in medieval England. It is also a wine from a vineyard that has allegedly, been in the same locale since the Roman empire. These factors made the wine an ideal choice when it came to recreating the recipe as accurately as possible (Harrison et al. 2015). An experiment testing the wines contribution examined three confounding factors; (1) red or white; (2) variety of grape and (3) region of growth. Researchers from the University of Copenhagen published a paper in which the authors sequenced DNA from ancient grape seed samples to examine how similar they are to modern grape varieties. Of particular interest was their study of an ancient grape sample from York in England circa 1300 AD. Their data suggests that the sample from York is most similar to modern Riesling or Gerwürtraminer varietals (Wales et al., 2016). With this in mind, Riesling was selected as the single white varietal grape to study, as this data suggested it would have likely grown in England at the time of the recipes conception. It was suggested that the red variety of grape, Pinot Noir, would be relatively resistant to cooler weather (Mills et al., 2006) and if they had red grapes to make wine in medieval England, this was the most likely grape they would have used. As well as a single origin Riesling and a single origin Pinot Noir, very basic white and red wines with a mix of grape varieties from indeterminate regions were also used. The resulting tests of recipes prepared with those wines, along with a recipe prepared using the original wine, showed there was no observable or statistically significant difference in the efficacy of the recipes (Fig. 1.2). This result demonstrates that the activity of the recipe is largely independent of the three confounding factors mentioned above and the question now becomes what are the common features between these grapes.

Wine is a rich mixture of organic compounds and trying to determine the commonalities between grapes is an arduous task. However, figure 1.1 clearly demonstrates that one of the

most obvious common ingredients, ethanol, is not directly a contributing factor to the efficacy of the recipe, which is useful to know because each of the wines used all have different percentage volumes of ethanol. Similarly, red and white grapes don't share several types of compounds. Flavan-3-ol's are a group of compounds that, depending on the specific compound, are found either exclusively in red wines or are found in much higher concentrations in red wines. The compounds that can be included in this family are catechin, epicatechin gallate, epigallocatechin, epigallocatechin gallate, proanthocyanidins (tannins), theaflavins, thearubigins (González-Paramás et al., 2004). Considering the data presented in figure 1.2 it is unlikely that any of these compounds, neither their presence or absence or concentration, have an effect on the anti-microbial activity of the recipe as they would be in wildly different concentrations, even between the two red wines studied.

As was pointed out by Harrison *et al.* (2015) *Allium* species produce a range of antimicrobial compounds (Lanzotti et al., 2013). Ajoene, which is found in garlic (*A. sativum*), and allicin, found in garlic, ramsons (*A. ursinum*), and spring onions (*A. fistulosum*), may prevent biofilm formation by *S. aureus* and other bacterial species (Nidadavolu et al., 2012) and interfere with quorum sensing and virulence in *Pseudomonas aeruginosa* (Jakobsen et al., 2012). Other antimicrobial compounds produced by *Allium* species include flavonoids, such as quercetin, kaempferol, and their derivatives, found in leek (*A. ampeloprasum*/*A. porrum*) and onion (*A. cepa*) (Lanzotti et al., 2013), and the antimicrobial peptide Ace-AMP1, so far only characterized in onion (Soininen et al., 2014). It was pointed out in Harrison *et al.*'s paper that none of the individual plant components were as effective at killing *S. aureus* as the combined recipe. It may be that the combination of ingredients may be especially effective at extracting these antimicrobials but further experiments are needed to confirm this.

Resting the Eyesalve is Important

The original recipe contained within Bald's *Leechbook,* specifies that the eyesalve be left for 9 days in the dark. Harrison *et al.* demonstrated that a batch of eyesalve that was prepared as described and immediately filtered without leaving for nine days, yields little to no anti-microbial activity. That result indicates either synthesis is occurring within the recipe mixture during those nine days, yielding a novel anti-microbial compounds, or that something is being extracted slowly from the plant materials within the mixture. Most likely it is a combination of both these things. To test this hypothesis further, an experiment was designed to test the effects of the number of days the recipe was left "stewing". The results, outlined in figure 1.3, showed that the eyesalve has the same efficacy whether it is left for one day or nine days. This result is important for future testing of the eyesalve as it speeds up the workflow for preparation and testing. It also helps dispel the idea that the specificity of the recipe instructing the user to leave the salve for nine days was written after a trial and error method of preparation. If it had been observed that there was a gradual increase in the activity of the eyesalve over a number of days, then this would make a case for some sort of slow synthesis process. It is most likely that the extraction of active compounds or pro-compounds occurs within a 24 hour period. The experiments presented do not give much insight as to the processes involved in making the eyesalve active, the results do however accelerate the workflow for future users to explore this recipe further.

Bald's Eyesalve is *S. aureus* Specific

To date, the eyesalve has been treated for what it is, a mixture of plant and animal materials that yield an anti-microbial effect when prepared in a certain fashion. Advancing our knowledge of the eyesalve necessitates pushing our usage of the eyesalve further. Treating the eyesalve like any other laboratory antibiotic was a key step forward in the process. One of the most common usages of antibiotics in the laboratory is incorporation into agar plates.

Laboratory antibiotics are generally very stable when added to molten agar and don't lose their anti-microbial activity, which is useful as it allows for selected growth of resistant bacteria on agar. The onion variant of the eyesalve was prepared and added to molten agar at specific concentration and plates subsequently poured and set. The images in figure 1.4 show the effects the three different concentrations of eyesalve in agar plates had on *S. aureus* Newman when a culture was spread onto the plates. It is clear from the 1/20 and 1/40 concentrations that the eyesalve is still able to kill *S. aureus* when incorporated into molten agar. This can allow for simple and rapid screening of various bacterial species for their susceptibility to the eyesalve. It also demonstrates that the active components of the eyesalve are relatively stable at higher temperatures, otherwise they would denature and lose their activity when incorporated into the molten agar. This also demonstrates that the eyesalve is specific to *S. aureus*, which demonstrates that the eyesalve itself is not a "bleach" that kills all forms of microbial life that it comes in contact with. This suggests the eyesalve is targeting something specific about the biology of *S. aureus.* This specificity makes it an excellent agent for the targeted elimination of *S. aureus* potentially even in complex multi-species infections.

The idea of incorporating the eyesalve into growth media was expanded by soaking in vitro collagen wounds with the eyesalve prior to inoculation. The aim here was to determine whether the eyesalve could prevent or reduce S. aureus' ability to colonise the wound model effectively. It was observed that there was a 2 log fold reduction in the number of cells recovered from wounds pre-treated with the eyesalve (Fig. 1.5). This experiment demonstrates that the eyesalve can limit S. aureus' ability to colonise wounds effectively. This could be translated to a clinical setting although it will likely take several years before the eyesalve can be purified into active compounds and subjected to clinical testing. However, none of the individual ingredients contained within the eyesalve recipe are considered unsafe to use on humans. In its

current form, it could hypothetically be used in wound dressings to help prevent the risk of colonisation. Prophylactic prescription of broad-spectrum antibiotics in patients with deep wounds as well as those who are post-surgery to prevent a nosocomial infection can be a contributing factor in the emergence of antibiotic resistance (Enzler et al., 2011). Thus, if an alternative such as Bald's eyesalve could be used in place of prophylactic antibiotics, it would decrease the usage of those antibiotics and subsequently reduce the emergence of resistant strains (O'Neill, 2016).

Isolation of Active Components from Bald's Eyesalve

A crucial step in using Bald's eyesalve as a new antimicrobial is isolating the active compound(s). The eyesalve mixture by nature is incredibly complex with likely thousands of individual compounds making up the organic mixture. Isolating individual compounds would take a number of years and tedious fractionation through distillation. An easier and slightly more rapid first step is the generation of fractions of eyesalve that contain a broad spectrum of compounds. Two methods of fractionation of the eyesalve were described in Chapter 2 of this thesis; liquid phase separation and solid phase extraction. Both methods isolate compounds in broad spectrums but by slightly different means. In liquid phase separation, different solvents are applied to the eyesalve which generates an upper and lower phase which broadly discriminates on the types of compounds it extracts. For example, ethyl acetate is a strong polar solvent which generates an upper phase of hydrophobic elements and a lower phase containing hydrophilic components. This method is useful for generating largely dissimilar fractions but is of little benefit for the isolation of specific compounds. As presented in figure 2.1 and highlighted in figure 2.2 this method did yield a fraction with some anti-microbial activity, namely the upper phase generated when ethyl acetate was applied to the eyesalve. Ethyl acetate being a strongly polar molecule, generates a hydrophobic upper phase. An interesting

consequence of the control fraction of ethyl acetate to demonstrate that the solvent itself is not lethal to *S. aureus* allows us to eliminate ethyl acetate as an active component from the eyesalve mix. Ethyl acetate is the most common ester to be found in wine as it is the product of acetic acid in wine. As stated this method does not tell us specific compounds to examine but it points towards the direction of hydrophobic molecules.

The second method of fractionating the eyesalve is reverse phase solid phase extraction (rpSPE). The eyesalve is passed through a column that retains hydrophobic molecules through Van der Walls or dispersion forces. By washing the column with increasingly non-polar solvents one can slowly elute increasingly hydrophobic fractions of the eyesalve. While this method is one of the least selective approaches in solid phase extraction methods due to its inability (much like liquid phase separation) to distinguish between individual compounds, it is more discriminatory than liquid phase separation by giving a gentle gradation in the hydrophilic to hydrophobic compounds being eluted (Sigma-Aldrich, 1998). With that in mind it is a reasonable conclusion that the results from the rpSPE are of more value due to the greater degree of resolution in the method. The results in figure 2.1 and highlighted in figure 2.3 show two fractions with anti-microbial activity compared to controls; SPE fraction 1 and 50% methanol wash. SPE fraction 1 is the eyesalve that was not retained in the column and thus would consist of purely hydrophilic compounds in theory. 50% methanol wash would remove compounds that are amphillic in nature i.e. they are neither very hydrophilic nor very hydrophobic. A shortfall in the experimental design when testing the anti-microbial activity of the active fractions combined (Fig. 2.4) was not testing the individual fractions in parallel with the the combination. This means we can only compare different experiments, which does not control for confounding factors like day variation and other inter-experimental factors.

Developing a Unique Pipeline for the Discovery of Novel Antimicrobials

Exploring recipes from Bald's *Leechbook* and other *leechdoms* from earlier or later era's, could be expanded as a new pipeline of discovery for novel antibiotics. With such a rapid rise in the number of resistant strains of bacteria emerging in the world today, many researchers are turning to alternative methods of "mining" for antibiotics. One recent example is the discovery of teixobactin, a novel antibiotic effect against Gram-positive bacteria like *S. aureus* and *Mycobacterium tuberculosis* without detectable resistance (Ling et al., 2015). This antibiotic was discovered through a novel discovery pipeline which utilised a technology known as an isolation chip (iChip). iChip is a method devised as a way to overcome what is commonly referred to as "The Great Plate Count Anomaly", a term which describes the difficulty in culturing 99% of bacterial species in the laboratory despite high bacterial concentrations in environmental samples (Epstein, 2013). The basic theory behind this phenomenon is that the various nutrients present in the soil cannot be effectively utilised for culturing purposes in the lab. iChip overcomes this issue by allowing the bacteria to be cultured in their native soil. Once the bacterial species have been cultured, they can be tested for their ability to inhibit the growth of different bacterial species. The iChip group found that the soil bacteria currently named *Eleftheria terrae* (Ling et al., 2015) was producing a substance inhibiting *S. aureus* and they eventually isolated teixobactin as the active component.

iChip has been praised for its novelty, ease of use and high-throughput nature of culturing. Teixobactin was the first antibiotic to be successfully isolated, stereochemistry elucidated and mechanism of action understood from the iChip method. What this paper demonstrated was that there is scope for novel pipelines of discovery in antibiotic research. In a similar facet Bald's *Leechbook* and other well understood *leechdoms* could be explored as part of a new discovery pipeline in the search for new antibiotics.

While Bald's *Leechbook* has been the only Anglo-Saxon text so far to generate results that merit further exploration of medieval recipes, it poses the question whether or not more of these recipes also have potential anti-microbial effects not yet realised. As such, in the light of the results outlined in this thesis as well as previously published (Harrison et al., 2015), I propose a new discovery pipeline for the mining and testing of Anglo-Saxon and medieval medical recipes in the search for new antibiotics.

First and foremost a database must be established of all the Anglo-Saxon and medieval texts that describe various *leechdoms* or remedies for ailments that we know today would be caused by bacterial agents. This database would have to contain not only the translations proposed by the group submitting the text to the database, but also should contain the direct text as well as images of the recipe itself where possible. This is to ensure that the translations are continually updated though suggested revisions by experts that can view the database independently and without charge. By operating in this fashion, it would be ensured that the best and most accurate translations of the texts are at hand whenever the database is accessed. Research groups would then be free to access the translations they wish to explore and recreate for testing. An added facility would be the linking of papers that have already explored a given recipe, which would facilitate groups in ensuring they are not exploring a recipe that has not yielded any anti-microbial activity.

The methodology for testing the recipes would ideally be standardised such that the efficacy of the recipes would be equivocated. Using the collagen wound model devised by Werthén *et al.* (2010) and used by Harrison *et al.* (2015) as well as in this thesis, could be one such standard method as it is one step beyond testing in planktonic cultures. Having a standardised method

of testing of the recipes would also allow for a rigorous meta-analysis of the collective efficacies of the recipes as more and more recipes are tested. Such meta-data would be essential in making the case for the "ancientbiotics" field, as well as support ML Cameron's hypothesis on the efficacy of ancient *leechdoms*.

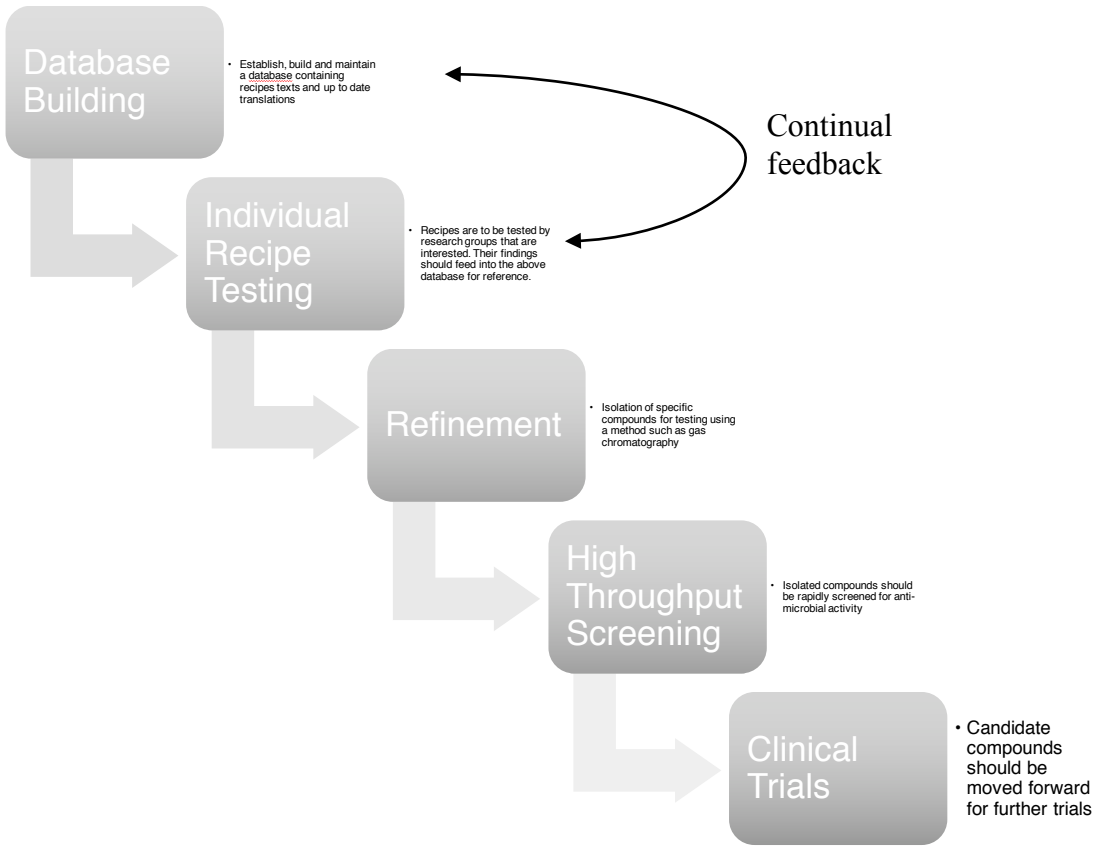


Figure 3.1 **Discovery pipeline for "ancientbiotics".** The proposed phases of a new discovery pipeline are outlined in the above figure. Each phase is has been equally proportioned to the other although some phases may take longer than others. Phase 2, individual recipes testing feeds back into Phase 1 due to the nature of keeping an up to date database.

Advancing and refining the recipes that prove to be effective is the next and potentially most challenging phase of this proposed discovery pipelines. As was outlined in this thesis, refining and understanding the active components of recipes such as Bald's eyesalve can be quite challenging. The degree of challenge will vary depending on the complexity of each individual recipe mixture. A prudent and logical step in the refinement of any of the recipes would be the

isolation of individual compounds from the mixture. This would require the application of a very sensitive separation technique like gas chromatography (Pavia et al., 2006). Gas chromatography works on the same principle as fractional distillation in the sense that it separates the components of the mixture by their specific heats of vaporisation, but where fractional distillation is useful on a large scale for a mixture like crude oil gas chromatography is ideal for smaller scale work. Complex organic mixtures like Bald's eyesalve and other recipes from leechdoms would likely yield hundreds if not thousands of individual fractions, each of which would have to be tested for their anti-microbial activity. Large numbers of compounds would require a high-throughput method of testing in order to prevent this phase of the pipeline from taking an enormous amount of time. One such method of high throughput screening was proposed by a team from Harvard (Moy et al., 2009). Their method of screening uses *Caenorhabditis elegans* nematodes infected with *Enterococcus faecalis* and is fully automated to assess worm survival in the presence of potential antimicrobial compounds. Their methodology allows for the rapid *in vivo* screening of 37,200 natural product extracts in a relatively short space of time. This method would be ideal for this pipeline due to the expected large numbers of compounds to be isolated from potential candidate recipes. The stereochemistry and mechanism of action of effective compounds should be elucidated also at this point.

The final and potentially longest phase of this proposed discovery pipeline is clinical trials. Assuming a specific compound or group of compounds is isolated from a recipe and found to be an effective anti-microbial in laboratory tests the compound(s) in question should be put forward for clinical trials. As history has shown us, clinical trial phases for new antibiotics can be incredibly long and arduous, with approximately 1 in 5 drugs making it to market (Hay et al., 2014). Not only this, but the length of time for a drug to go from Phase 1 clinical trials to

market can take up to 6 years according to the United States Food and Drug Administration (DiMasi et al., 2003).

Although the time that it takes to go through this discovery pipeline can be quite long but that should not deter one from exploring it as a route of research. “Anceintbiotics” may be an untapped source of knowledge for mining new and exciting antimicrobials. Only time, research and resources will unlock the true potential of this proposed research path.

Future Directions

While the work presented within this thesis has answered some of the questions surrounding Bald’s eyesalve as an antimicrobial, there is much still to be understood. The recipe itself has been refined in several ways so far. Despite this refinement there is still a degree of variation and imprecision in the efficacy of the recipe when different users prepare it. This is to be expected as the method of preparation leaves room for margins of error. For example, in the present recipe preparation, equal volumes of each of the ingredients are called for. Measuring the plant matter by volume can be inaccurate and variable, depending on the fineness of the chop. In would be more accurate, both in the lab and in a historical context, to measure all ingredients by weight rather than volume. This is likely what was done during the recipe’s conception as medieval practitioners would not have had precision volumetrics, only mass balances. A change like this would help enhance the replicability of the recipes effects for future users.

An important next step from this thesis would be to perform gas chromatography, as suggested in the above proposed discovery pipeline, to extract the specific compounds that make up an active batch of Bald’s eyesalve. While this thesis has presented fraction data, the data was not

generated by a sensitive separation technique such as gas chromatography or liquid-chromatography-mass-spectrometry. Identifying the active component or components of Bald's eyesalve is the most important next step in this line of research. Without a pure compound(s) to study, it is near impossible to investigate the mechanism of action of the eyesalve.

In summary, in this thesis I have explored the optimisation and refinement of a 1,000-year-old antimicrobial recipe. The data presented in this thesis demonstrate that aspects of the recipe such as the wine are of great importance to the activity of the eyesalve, however other components such as the length of time of the recipe is left before use are of less importance. Fractional isolation experiments have helped narrow the search for an active compound or group of compounds within the eyesalve which is a major step forward in turning this antimicrobial into something that has clinical benefits. More specific isolation of individual compounds is the most logical next phase for this project. This thesis helps confirm the replicability and efficacy of Bald's eyesalve as presented in Harrison *et al.*'s paper. Finally, this adds weight to the proposition that exploring similar Anglo-Saxon recipes, through a pipeline similar to that outlined in this thesis, may be a useful means of mining for new antibiotics.

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