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**UNIVERSITY OF SOUTHAMPTON**

**FACULTY OF MEDICINE, HEALTH AND LIFE SCIENCES**

School of Medicine

**Alternative treatments for female urinary tract infections:  
microbiological analysis of a herbal medicinal product, and  
qualitative study into patients' perspectives**

by

**Jeanne Trill**

Thesis for the degree of Doctor of Philosophy

July 2017



UNIVERSITY OF SOUTHAMPTON

# **ABSTRACT**

FACULTY OF MEDICINE, HEALTH AND LIFE SCIENCES

Doctor of Philosophy

## **ALTERNATIVE TREATMENTS FOR FEMALE URINARY TRACT INFECTIONS: MICROBIOLOGICAL ANALYSIS OF A HERBAL MEDICINAL PRODUCT, AND QUALITATIVE STUDY INTO PATIENTS' PERSPECTIVES**

**By Jeanne Trill**

Urinary tract infections (UTI) are one of the most common female conditions treated by general practitioners, and most patients are prescribed antibiotics. As bacterial resistance to antibiotics is on the increase there is a need to find alternative treatments to alleviate the uncomfortable symptoms of this condition. The herbal medicinal product (HMP) *Arctostaphylos uva-ursi* (uva-ursi) has traditionally been used to treat UTI and was, for the first time, being tested in a major clinical trial. This study aimed to support the trial by examining the quality of HMPs, assessing dosing and safety issues, and analysing the possible antibacterial effect and mechanism of action of uva-ursi. In addition, a qualitative study was conducted which explored patients' views on using an HMP for symptom relief of a UTI.

This study found that both uva-ursi and its metabolite hydroquinone (Hq), regarded as the main active constituent, demonstrated antibacterial activity against several named bacteria (including *E. coli*) in concentrations ranging from 32–512 µg/mL. The literature reported that urine required alkalisation for the release of Hq from uva-ursi metabolites, but that activity of Hq itself was unaffected by pH. However, this study determined that Hq increased activity at alkaline pH *in vitro* against *E. coli*. Moreover, the successful inhibition of growth of *E. coli* in the urine of healthy volunteers (at 50 colony forming units/mL) after taking uva-ursi was unaffected by an alkaline pH. In 2 out of the 4 volunteers antimicrobial activity increased the longer uva-ursi was taken.

Aggregation of *E. coli* was observed in the presence of the HMP compared to control samples *in vitro*, which may cause the bacteria to be flushed out of the urinary tract, suggesting there may be more than one mode of antimicrobial action.

The qualitative study identified that the participants were aware of antibiotic resistance, but that this knowledge did not deter them from seeking and expecting antibiotics if they wanted symptom relief from a UTI. Were an HMP shown to alleviate symptoms of UTI, based on this sample there would be few barriers to adopting it as an alternative treatment.



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# DECLARATION OF AUTHORSHIP

I, Jeanne Lindsay Dennett Trill, declare that the thesis entitled

**Alternative treatments for female urinary tract infections:  
microbiological analysis of a herbal medicinal product, and  
qualitative study into patients' perspectives**

and the work presented in the thesis are both my own, and have been generated by me as the result of my own original research. I confirm that:

- this work was done wholly or mainly while in candidature for a research degree at this University;
- where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
- where I have consulted the published work of others, this is always clearly attributed;
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*The Investigator's Brochure on ATAFUTI* - submitted to the Medicines and Healthcare Regulatory Agency

Signed: .....

Date: .....



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# Definitions and Abbreviations

## Abbreviation

Ar	Arbutin
$\beta$ GL	$\beta$ -Glucosidase
$\beta$ -GLn	$\beta$ -Glucuronidase
CFU	Colony forming unit
DAD	Diode Array Detector
EI	Electron impact
EMA	European Medicines Agency
EtOH	Ethanol
EU	European Union
G/g	gram
GC-MS	Gas Chromatography-Mass Spectrometry
HCL	Hydrochloric acid
H <sub>2</sub> O	Water
HMP	Herbal medicinal product
HPLC	High performance liquid chromatography
Hq	Hydroquinone
MeOH	Methanol
MHRA	Medicines and Healthcare Products Regulatory Agency
MIC	Minimum inhibitory concentration
MW	Molecular weight
<i>m/z</i>	Mass-to-charge-ratio (in mass spectrometry)
NaOH	Sodium hydroxide
nm	Nanometer
NIMH	National Institute of Medical Herbalists
NSAID	Non Steroidal Anti-inflammatory Drug
PBS	Phosphate buffered saline
tds	<i>ter die sumendus</i> (three times a day)
<i>t<sub>R</sub></i>	Retention time
$\mu$	Micro
UTI	Urinary tract infection
UV	Ultraviolet
v/v	Volume/volume ratio
WHO	World Health Organisation



# Chapter 1: Introduction & overview of thesis

## 1.1 Introduction

Prior to the advent of antibiotics in the 1930s infectious diseases were the most common cause of death in the world (Cohen, 2000). The increasing success in the prevention and treatment of bacterial infections with antimicrobial agents in the following sixty years lead to a steady decline in mortality due to infection related diseases (Griffiths and Brock, 2003). And yet since the 1990s there has been an emergence in the resistance of bacterial organisms to antibiotics, which has in part been influenced by unnecessary use of as well as inappropriate prescribing of these drugs (Cohen, 2000; Johnson, Ashiru-Oredope and Beech, 2015). Common infections and minor injuries that may otherwise have been treatable with antibiotics could now lead to fatalities, and it is estimated that by the middle of this century there may be an average of 10 million deaths a year globally as a result of antibiotic resistance. The current level is estimated to be 0.7 million a year (Cecchini, Langer and Slawomirski, L., 2015).

The World Health Organisation (WHO) has endorsed a global action plan to try to tackle the problem, which includes improving awareness of how antibiotic resistance develops as well as ensuring that there is continual investment in new medicines (WHO, 2015). With a potentially poor capital return on investing in new antimicrobial agents, and the lengthy lead-times that drug companies require from the inception of a new drug to testing them in clinical trials, pharmaceutical companies have been reluctant to finance the development of new antibiotics (Christoffersen, 2006).

Whilst there has been a reduction in investment by drug companies, research continues to be conducted by university academics as well as the pharmaceutical industry into antimicrobial properties of phyto-medicines and drugs derived from natural products. This is both in terms of *in vitro* assays and clinical studies (Butler, Robertson and Cooper, 2014; Wright, 2017). This has resulted in the discovery of a number of plant-based and traditional herbal medicines which have demonstrated antimicrobial action *in vitro* (Gibbons, 2005), and they require further research through clinical investigation.

One of the challenges with testing phytomedicines in clinical trials is that there is little incentive for entrepreneurs to invest in such a venture if the end product cannot be patented. However, with the consequential risk to human health, and the financial cost to the NHS in trying to treat infections which may have become resistant to antibiotics, academic institutions such as the University of Southampton have become involved in initiating and running both feasibility studies and clinical trials on the potential for plant based medicines to reduce antibiotic consumption either through antimicrobial effects or relief of symptoms.

With the aim of reducing antibiotic prescribing one such trial, set up to test the effectiveness of a traditional herbal medicine in alleviating the uncomfortable symptoms of urinary tract infections, is *Alternative Treatments for Adult Female Urinary Tract Infections* (ATAFUTI). This is a double-blind randomised placebo controlled clinical trial run by the University of Southampton and funded by the National Institute for Health Research (NIHR).

This PhD has been aligned closely to ATAFUTI. It has supported the trial through investigating the dose and safety of the proposed herbal treatment *Arctostaphylos uva-ursi* (uva-ursi), the quality control and subsequent selection of a suitable herbal medicinal extract to use, an examination of the potential antimicrobial activity and mode of action of the chosen extract and a qualitative study amongst patients and GPs related to the ATAFUTI trial. All the laboratory work was conducted at the UCL School of Pharmacy as a joint venture with the University of Southampton.

The main aims of the thesis are outlined below, together with a breakdown of the overall design and approach of the PhD.

## 1.2 Research Aims

The primary aims of this PhD were to:-

- Determine an appropriate dosing strategy for the extract, and investigate any issues regarding safety.
- Both source and conduct quality testing on three commercial extracts of *Arctostaphylos uva-ursi* with the aim of selecting a suitable HMP to be administered to patients in the ATAFUTI trial.
- Assess the potential antimicrobial activity of uva-ursi, and investigate the mode(s) of action.
- Explore patient attitudes towards the prescribing of a herbal medicinal product for relieving the symptoms of urinary tract infections.

## 1.3 Research Strategy

Before any experiments could be conducted for this PhD it was necessary to determine a dosing strategy for uva-ursi as well as address any safety issues, and then obtain three samples of an extract of *Arctostaphylos uva-ursi* produced to good manufacturing practice (GMP) standards. Following the EU Directive into the licensing of herbal medicines (Kroes, 2014) commercial products of uva-ursi disappeared off the shelves of UK outlets, so three European samples were sourced.

A flow diagram outlining the process of the PhD is shown in **Figure 1**.



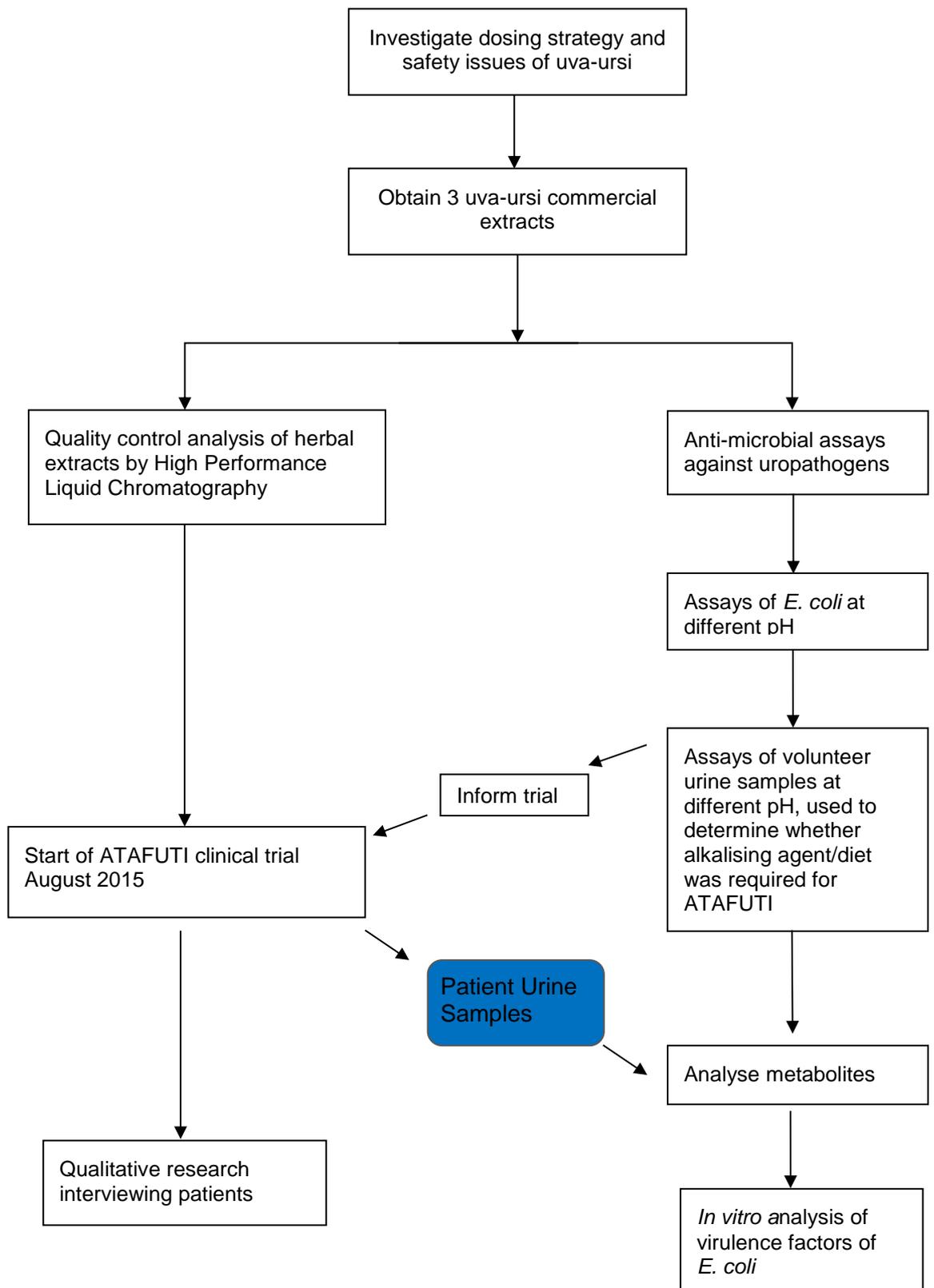


Figure 1: Flow diagram of the process of the PhD



## 1.4 Outline of Thesis

Following this introductory chapter the thesis comprises a further ten chapters. An outline of each is below:

**Table 1:** Outline of contents of each thesis chapter

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**Chapter 1 Introduction & overview of thesis**

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**Chapter 2 Urinary tract infections and management strategies**

An introductory narrative review of the literature into the problem of tackling urinary tract infections related to the increase in resistance to antibiotics. As lower urinary tract infections (cystitis) are the main focus of ATAFUTI the chapter concentrates mainly on cystitis and the associated bacterial organisms known to cause the condition.

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**Chapter 3 *Arctostaphylos uva-ursi* (L.) spreng**

A narrative literature review on the traditional herbal medicine *Arctostaphylos uva-ursi*. The review incorporates published papers on the herb, as well as herbal monographs. It discusses the pharmacological properties of uva-ursi including the potential antimicrobial activity. It also reviews the literature pertaining to advice that an alkaline environment may be necessary for uva-ursi to be effective in clinical use.

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**Chapter 4 Investigation into safety issues**

An investigation into the safety of uva-ursi related to discussions with members of the ATAFUTI Trial Management Group (TMG). This comprises a focused review of the literature which, in addition to assessing the safety of the herb itself, investigates citations relating to the safety of the metabolite hydroquinone.

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**Chapter 5 Determination of therapeutic dose of *Arctostaphylos uva-ursi***

An investigation into what might be the most appropriate therapeutic dose of uva-ursi to be prescribed to patients on the ATAFUTI clinical trial. This comprises a focused review of all published material on uva-ursi, including monographs, herbal texts and data from previous small clinical studies.

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**Chapter 6 Quality control of herbal medicinal product**

An analysis into the quality of the uva-ursi commercial extracts which were being considered for the clinical trial. This was conducted using high performance liquid chromatography (HPLC).

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**Chapter 7 Antimicrobial assays**

An investigation into the potential antimicrobial activity of the herbal extract both *in vitro* and *in vivo* at different pH.

The *in vitro* assays were conducted on pathogens involved in UTI, and the pH assays focused specifically on *E. coli*.

The *in vivo* assays were based on urine samples from healthy volunteers. This was to help address the question of whether the herb needs to be taken with an alkalisising agent or in conjunction with an alkalisising diet (as reported in some monographs and the literature). The results would be used to advise the ATAFUTI TMG.

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**Chapter 8 Determination of metabolites in urine samples**

An analysis of 60 urine samples from patients who have taken part in ATAFUTI. This was to assess whether the metabolite hydroquinone, (believed to be the active constituent) was present. The analyses would be compared to the un-blinded results of the trial to help determine a potential mode of action of uva-ursi should the trial be successful.

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**Chapter 9 Effects of uva-ursi on *potential E. coli* virulence factors**

Additional laboratory assays were conducted on uva-ursi to investigate potential effects against virulence factors of *E. coli*.

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**Chapter 10 Qualitative study into patients' perspectives of herbal treatment as an alternative to antibiotics**

A qualitative study amongst 20 patients who were approached about or were recruited for ATAFUTI. The study explores the patients' awareness of and views on antibiotic resistance, the delayed prescribing of antibiotics, the use of herbal medicine for the treatment of urinary tract infections, and the patients' experience of the clinical trial. This was with a view to gaining an understanding of how the respondents would feel about being prescribed a herbal medicine for a urinary tract infection.

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**Chapter 11 Discussion and conclusion**

An overall discussion and conclusion of the findings of the PhD.

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## Chapter 2: Urinary tract infections and management strategies

### 2.1 Introduction

Urinary tract infections (UTI) are one of the most common bacterial infections worldwide amongst women. They are becoming increasingly more difficult to treat due to growing antibiotic resistance, and in some instances can lead to sepsis (Peirano and Pitout, 2010; Barber *et al.*, 2013). It is estimated there may be 130-175 million cases a year worldwide, with 1 in 3 women experiencing an infection by the age of 24, and as many as 50% of women enduring at least one episode during their lifetime (Hacker and Kaper, 2000; Arinzon *et al.*, 2012; Barber *et al.*, 2013). UTI are now reportedly the most common infection acquired in hospital, where they are responsible for at least 35% of nosocomial cases (Al-Hasan, Eckel-Passow and Baddour, 2011; Barber *et al.*, 2013).

Multi-drug resistance (MDR) to the pathogens responsible for UTI is extensive and on the increase (Barber *et al.*, 2013). For example, in the USA resistance to the antibiotics ciprofloxacin and ampicillin, by *Escherichia coli* (*E. coli*), rose from 3% to 17% and from 38% to 43% respectively in the first decade of this century (Sanchez *et al.*, 2012). Antibiotic resistance is receiving greater publicity, but has not yet led to the development of new treatments that have been approved for use (Barber *et al.*, 2013). The British Medical Journal (BMJ) reported in 2014 that more than 1 in 10 cases of monotherapy prescribing of antibiotics for UTI resulted in failure of treatment between 1991 and 2012 (Currie *et al.*, 2014). This is contributing to an increasing financial burden to medical providers including the National Health Service (NHS). It affects direct costs related to visits to General Practitioners (GPs), as well as prescription costs and hospitalisation (Foxman, 2003; Turner *et al.*, 2010).

Antimicrobial misuse, such as overprescribing and patient non-adherence of the prescription (Bronzwaer *et al.*, 2002), means that the treatment itself may not only be contributing to the problem, but in the case of uncomplicated UTI antibiotics might not always be necessary. It has been demonstrated, in randomised placebo controlled trials, that the condition may be self-limiting (Christiaens *et al.*, 2002; Richards *et al.*,

2005; Linhares *et al.*, 2013). Almost 50% of women taking the placebo had a natural resolution of infection within a week, although the symptoms endured (such as frequency and dysuria, discussed further in Section 2.6) may prove distressing to some over this length of time (Christiaens *et al.*, 2002; Nicolle *et al.*, 2006). It also means that 50% may, therefore, require an intervention.

## **2.2 Classification of UTI**

### **2.2.1 Upper and Lower UTIs**

Female urinary tract infections can involve the urethra, bladder or kidneys, and are classified according to anatomical location. The most common UTI, accounting for 95% of all symptomatic infections is cystitis, which is inflammation of the bladder. This, together with urethritis (inflammation of the urethra) affects the lower urinary tract, and is the main area of investigation for this study owing to the connection to the clinical trial ATAFUTI. Where necessary, to provide greater depth of understanding of the condition and put it into context, this literature review does include other elements of UTI (for example upper urinary tract and recurrent infections).

Inflammation of the renal parenchyma, pyelonephritis (or kidney infection), refers to infections of the upper urinary tract and is much less common (believed to be just 5%), although data to support this may not be entirely accurate (Hooton, 2000).

### **2.2.2 Complicated versus Uncomplicated UTI**

For the purposes of identifying the most appropriate treatment strategy UTI are further classified as either 'complicated' or 'uncomplicated'. The majority are uncomplicated, occurring in women who are otherwise healthy and have a structurally normal urinary tract. Complicated infections, in either the upper or lower tract, are often associated with an underlying condition or associated treatment which has impaired the immune system or caused long-term inflammation, possibly damaging the uroepithelium (Grabe *et al.*, 2009). Complicated UTI may also be due to anatomical abnormalities (for

example, obstruction by renal stones), poor bladder emptying, a recent urinary tract intervention or catheterisation (Grabe *et al.*, 2009; Mittal *et al.*, 2009). They are associated with a broader range of pathogens than uncomplicated UTI (Anderson *et al.*, 2004; Grabe *et al.*, 2009). By definition UTIs in pregnancy are classified as complicated as they may be associated with both premature birth (reducing the gestational age to under 37 weeks) as well as neonatal mortality (Grabe *et al.*, 2009). There is also a greater risk in pregnancy (up to 40%) of a lower uncomplicated UTI leading to pyelonephritis (Glaser and Schaeffer, 2015).

### **2.2.3 Asymptomatic and Recurrent UTI**

Asymptomatic urinary infection (asymptomatic bacteriuria) may also occur and is quite common; it refers to a specified quantity of bacteria causing infection but without any detectable signs or symptoms (Grabe *et al.*, 2009; Moura *et al.*, 2009).

Some women (27% to 48%) may experience repeat infections. These are due to a relapse after the course of treatment has finished or a reinfection with a different pathogen. They are known as recurrent UTI, and can cause significant distress as they continually impact on the quality of life (Hooton, 2000; Foxman, 2003).

## **2.3 Aetiology**

Several bacterial species are known to be responsible for causing infections of the lower urinary tract, with Gram-negative organisms belonging to the Enterobacteriaceae family reported to be the most prevalent (Barber *et al.*, 2013; Linhares *et al.*, 2013). *Escherichia coli* (Enterobacteriaceae) is believed to account for at least 75% of cases, with some studies putting this estimate as high as 90% (Moura *et al.*, 2009). Staphylococci, including *Staphylococcus saprophyticus* and *aureus*, have been found present in up to 15% of infections (Jancel and Dudas, 2002). These and other bacteria which cause UTI are discussed more fully in Section 2.8.

In addition to infection, cystitis may also be triggered by bruising of the bladder following sexual intercourse, or a build-up of bacteria in the bladder following a delay in post-coital urination (Linhares *et al.*, 2013). It has been established there is an increase in bacterial counts after intercourse, but the precise cause is still not entirely certain (Ronald, 1996). The use of condoms, contraceptive diaphragms and spermicides are all linked with increasing the risk of developing a UTI. Spermicides in particular can cause problems because they can alter the natural vaginal flora, thus increasing susceptibility to colonisation with pathogens (Handley *et al.*, 2002).

The incidence of UTI often increases in postmenopausal women. This is thought to be due to the thinning of vaginal and vulvar tissues as a result of declining oestrogen production (Caretto *et al.*, 2017). The fall in oestrogen may also relate to a subsequent loss of an acidic environment which may be hostile to *E. coli*, and this may predispose postmenopausal women to infection (Bishop, 2004).

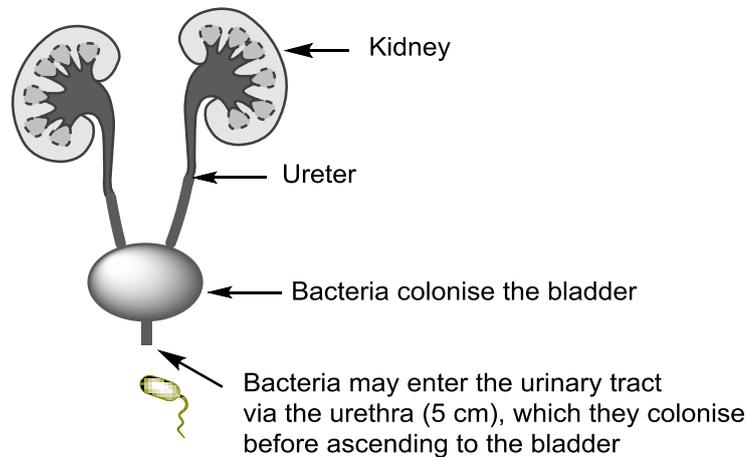
A weakened immune system, for example amongst diabetics, has been found to be a risk factor for a higher incidence of lower UTI (Grigoryan *et al.*, 2017). Diabetics may experience 2-4 times the number of incidence of infection compared to non-diabetics with a wider range of pathogens likely to be the cause (Foxman, 2003).

Women who have taken antibiotics for treatment of UTI are at risk of a recurrent infection two weeks later as the drugs themselves can disrupt the natural flora that would normally prevent an overgrowth in bacteria (Hooton, 2000). Furthermore, antibiotic resistance may develop, with the pathogens mutating during treatment (Moura *et al.*, 2009).

## **2.4 Pathophysiology**

Urine should flush out micro-organisms as it passes through the urinary tract. However, there is greater frequency of UTI amongst women than men, and anatomy is believed to play a major role (Barber *et al.*, 2013). Although bacteria may reach the urinary system via haematogenous or lymphatic spread, it is believed they usually invade from the exterior via the short female urethra; this is only 5 cm compared to 20 cm in a man. The close urethral proximity to the vaginal introitus and anus, where

bacteria are commonly present and may have originated from rectal flora, is considered a strong contributing factor (Hooton, 2000).



**Figure 2:** Bacterial colonisation of the urinary tract via the urethra

Invading pathogens subsequently adhere to the stratified epithelium (known as the uroepithelium) by means of fimbriae (proteinaceous hair like organelles). The bacteria bind to receptors, colonising the periurethral and distal urethral before finally entering the bladder (Klemm and Schembri, 2000; Finer and Landau, 2004). It is believed that bacterial motility is facilitated by flagella (Haiko and Westerlund-Wikström, 2013). Ascending uropathogens may eventually reach the kidneys, instigating pyelonephritis, but the mechanism of ascension is not proven (Moura *et al.*, 2009).

The normal pH of urine extends between 4.8 to 7.5, which is a wider range than serum (Nicolle, 2005). With infection it has been shown that the pH can alter depending on the pathogen responsible, and this can affect the choice of treatment; each antimicrobial drug has an optimum pH for efficacy (Nicolle, 2005).

An increased susceptibility to recurrent infection is believed to be genetic, with uropathogenic coliforms demonstrating a greater propensity to attach to both the uroepithelium and the vaginal epithelial cells (Schilling and Hultgren, 2002).

Chronic UTI may also be associated with the patient having greater frequency and colonisation of the vaginal introitus with *E. coli* than those women who do not regularly suffer from a urinary infection, thus leading to constant reinfection (Schilling and Hiltgren, 2002). This in turn may not only be associated with recurrent UTI but it has also been linked to symptoms of an overactive bladder (Balanchandran *et al.*, 2016).

## 2.5 Immune Response to UTI

The uroepithelium initially acts as a barrier to infection, thus forming part of the immune system - whereby it also activates both innate and adaptive immune responses.

Activation of the immune system is necessary for the eradication of the pathogen, but the process can also cause damage to local tissue (Bien, Sokolova and Bozko, 2012). Bladder epithelial cells express Toll-like receptor 4, and upon recognition of bacterial lipopolysaccharides they induce the innate response, primarily recruiting neutrophils (Anderson *et al.*, 2004; Umpiérrez *et al.*, 2013).

The epithelial cells also secrete cytokines, chemokines and immunoglobulin A, (IgA) (Hacker and Kaper, 2000). A pro-inflammatory cytokine IL-8 has been detected in the urine of infected patients, especially when *E. coli* is the causative agent, and an increase in IL-8 concentration has been shown to correlate with the numbers of neutrophils present. *In vitro* experiments have demonstrated that IL-8 can influence neutrophil chemotaxis and degranulation (Ulett *et al.*, 2013).

If the pathogen is not flushed out of the urinary tract urine can promote the growth of *E. coli*, providing nutrients in the form of amino acids, glucose and uric acid. It is, however, a poor source of iron, which is required for growth by several micro-organisms including *E. coli* (Moura *et al.*, 2009). Section 2.9 discusses the individual pathogens involved in UTI in more detail.

## 2.6 Symptoms

Symptoms of uncomplicated lower UTI may evolve suddenly over a few hours to a day. It has been reported that they last an average of 6 days, but can endure for several weeks (Ferry *et al.*, 2004; Moura *et al.*, 2009). Symptoms that last more than 7 days may be an indication of a potential complicated UTI (Grabe *et al.*, 2009), or in cases of clinically treated conditions could suggest resistance to the antimicrobial agent (Moura *et al.*, 2009). Those infected may present with dysuria, nocturia, suprapubic discomfort, low back pain and a frequent need to urinate. The frequency to urinate is often accompanied by a reduced output of urine which may be cloudy and odorous. Around 30%-40% may have haematuria, and a low grade fever may or may not be present (Grabe *et al.*, 2009; Moura *et al.*, 2009; Arinzon *et al.*, 2012).

The prevalence of symptoms based on a sample of over 300 adult female patients is shown in **Table 2** (Mclsaac *et al.*, 2007).

**Table 2:** Clinical presentation of patients presenting with cystitis

Symptom	% of Patients Presenting in Clinical Practice
Frequency of urination	92%
Urgency	83%
Dysuria	79%
Decreased urine output	75%
Abdominal pain	25%
Fever	4%

## **2.7 Diagnosis of Urinary Tract Infections**

### **2.7.1 Clinical Presentation**

Diagnosis of an otherwise healthy female of child bearing age is initially based on clinical assessment, with probability of bacteriuria estimated to be between 50-80% in those presenting with symptoms of acute UTI (Little *et al.*, 2009).

A combination of dysuria and frequency is regarded as an almost certain indication of a UTI, the likelihood being 90% (Little *et al.*, 2006; SIGN, 2012). Nevertheless, clinical assessments cannot be relied upon alone as predictors of infection, especially if there is only one symptom present (Little *et al.*, 2009). Signs are not a typical indication of UTI, excepting 10-20% of women who present with suprapubic tenderness (Colgan and Williams, 2011). Should a vaginal discharge or irritation be reported, a gynaecological infection is considered more likely than a UTI (Grabe *et al.*, 2009). Patient history should, therefore, be followed up either by dipstick or urinalysis, using a 5–10 mL clean-catch midstream urine specimen (MSU). An MSU is taken to reduce the risk of contamination by perineal organisms, although it has been demonstrated that an MSU does not necessarily reduce contamination rates as patients do not always take the sample correctly (Lifshitz and Kramer, 2000; SIGN, 2012).

### **2.7.2 Dipstick Analysis**

Dipstick analysis for the presence of nitrites (a by-product of bacteria), as well as leukocyte esterase and blood, is a simple test conducted in GP surgeries which is regarded as both convenient and cost effective (Little *et al.*, 2009). It has been reported to detect around 90% of all UTI, making it potentially useful if minimal symptoms are present (Nickel, 2005b). An in-depth study which analysed the detection of nitrites, leukocytes and blood in UTI found that having nitrites or both leukocytes and haematuria had a positive predictive value (PPV) of 81%, and negative predictive value (NPV) of 65%, which was moderately sensitive (77%) and specific (70%) (Little *et al.*, 2009). However, the specimen has to be tested rapidly, within 24 hours, to avoid unreliable results due to potential bacterial replication in the container (Shankel, 2012; LaRocco *et al.*, 2016).

Whilst the above tests may suggest the presence of an infection they have a serious limitation in that they are unable to identify the pathogen responsible. This information is necessary for selecting the appropriate antimicrobial treatment (Nickel, 2005b).

### 2.7.3 Bacterial Culture Analysis

Assays for urinalysis may detect leucocytes and the total number of colony forming units (CFU)/mL of bacteria in urine (Bishop, 2004). This quantitative analysis of bacteriuria (amount of bacteria in urine) is an important investigative tool, but in reality the amount of significant bacteriuria has been shown to vary across different types of UTI. Defining specific quantities can, therefore, prove to be too rigid for diagnosis (Grabe *et al.*, 2009).

The current guidelines produced by the European Association of Urology (EAU) for classification of a UTI state that a urine sample taken mid-stream with  $\geq 10^3$  CFU/mL is present in women with acute uncomplicated cystitis, CFU/mL  $\geq 10^4$  might indicate acute uncomplicated pyelonephritis, and  $\geq 10^5$  CFU/mL could indicate a complicated infection (Grabe *et al.*, 2009).

**Table 3:** Diagnosis of conditions according to the number of CFU/mL in urine as specified by the European Association of Urology

CFU/mL	Diagnosis (female UTI)
$\geq 10^3$	acute uncomplicated cystitis
$\geq 10^4$	possible pyelonephritis
$\geq 10^5$	complicated UTI

Nevertheless, the upper and lower limits for acute uncomplicated cystitis can range as widely as  $10^2$  to  $10^5$  CFU/mL (Bishop, 2004; Barber *et al.*, 2013). The American Society of Microbiology (ASM) suggests that samples should analyse as low as  $10^2$  CFU/mL for the detection of *E. coli*, *S. saprophyticus*, *Proteus spp*, and *Klebsiella spp* when diagnosing cystitis (Bent and Saint, 2002).

In the UK counts of  $\geq 10^4$  CFU/mL with a single organism are classed by Public Health England (PHE) as an infection, as well as those comprising  $\geq 10^5$  CFU/mL of mixed organisms but with one prevailing species. This is with the exception of *E. coli* and *S. saprophyticus* which are regarded as infections at a count of  $\geq 10^3$  CFU/mL (PHE, 2017a). Moreover, UK quality control guidelines for testing of organisms advise that counts of  $10^2$  CFU/mL may be the cause of symptoms in acute cases of UTI (PHE, 2017b). Where counts are high, at  $10^7 - 10^8$  CFU/mL for example, an infection is considered likely but it is also advised that the quality of the specimen be taken into account in case there is possible contamination (PHE, 2017b). The Scottish Intercollegiate Guidelines Network is slightly different again, advising that laboratory counts of  $\geq 10^5$  CFU/mL are generally used for confirmation of a suspected UTI. They advise that there is no specific bacterial count which can be an unequivocal diagnosis of a UTI (SIGN, 2012).

## 2.8 Management of urinary tract infections

Acute lower UTI rarely (5%) develops into symptomatic upper urinary tract infection and is not usually expected to cause adverse effects to renal function, even amongst recurrent infection (Moura *et al.*, 2009). As much as 50%-70% of lower UTI may resolve successfully without any treatment (Grabe *et al.*, 2009).

Nevertheless, the unpleasant and disruptive symptoms often lead the sufferer to initiate clinical help, especially since symptoms can occasionally endure for several months (Grabe *et al.*, 2009). A recent survey (2015) reported that 95% of women ( $n = 892$ ) who had ever had an uncomplicated UTI had consulted a healthcare professional about their most recent infection. Over half cited severe or persistent symptoms as the reason for seeking help (Butler *et al.*, 2015).

The treatment of UTI aims to resolve the symptoms and inhibit and eliminate the pathogen. The most critical initial step of management is the correct diagnosis, and choice of appropriate antibiotic. Clinical presentation may lead directly to the prescribing of empirical antimicrobial therapy rather than obtaining urine culture for testing or performing urinalysis (Car, 2006). This approach is considered appropriate based on the rationale that the test results would be predictable (Jancel and Dudas, 2002).

The general therapeutic approach to treating cystitis amongst women who have a positive dipstick result is to also prescribe empirical antibiotics. In the above survey 76% of those who consulted a practitioner had their urine tested, and 74% were prescribed an antibiotic (Butler *et al.*, 2015).

Treatment of patients presenting with symptoms but who do not show a positive dipstick urinalysis is more mixed, with some women receiving antibiotics and others not (Richards *et al.*, 2005). This may mean that some cases do not get treated which should receive antibiotics, as dipsticks are not always accurate (Richards *et al.*, 2005). An observational study which investigated nine GP practices found that only 40% of patients who were prescribed empirical antibiotics (which comprised 61% of the total sample of 111 women) had bacteria in their urine (O'Brien *et al.*, 2007). Assaying (quantitative analysis) of bacterial culture, is therefore, considered the 'gold standard' for diagnostic confirmation of an infection (O'Brien *et al.*, 2007).

The current recommendation in the UK with women who have  $\leq 2$  symptoms is to prescribe pain relief and consider a delayed prescription for antibiotics. With  $\geq 3$  symptoms the advice is to prescribe the antibiotic nitrofurantoin if the glomerular filtration rate is  $> 45$  mL/minute, and trimethoprim if it is under 45 (PHE, 2017c).

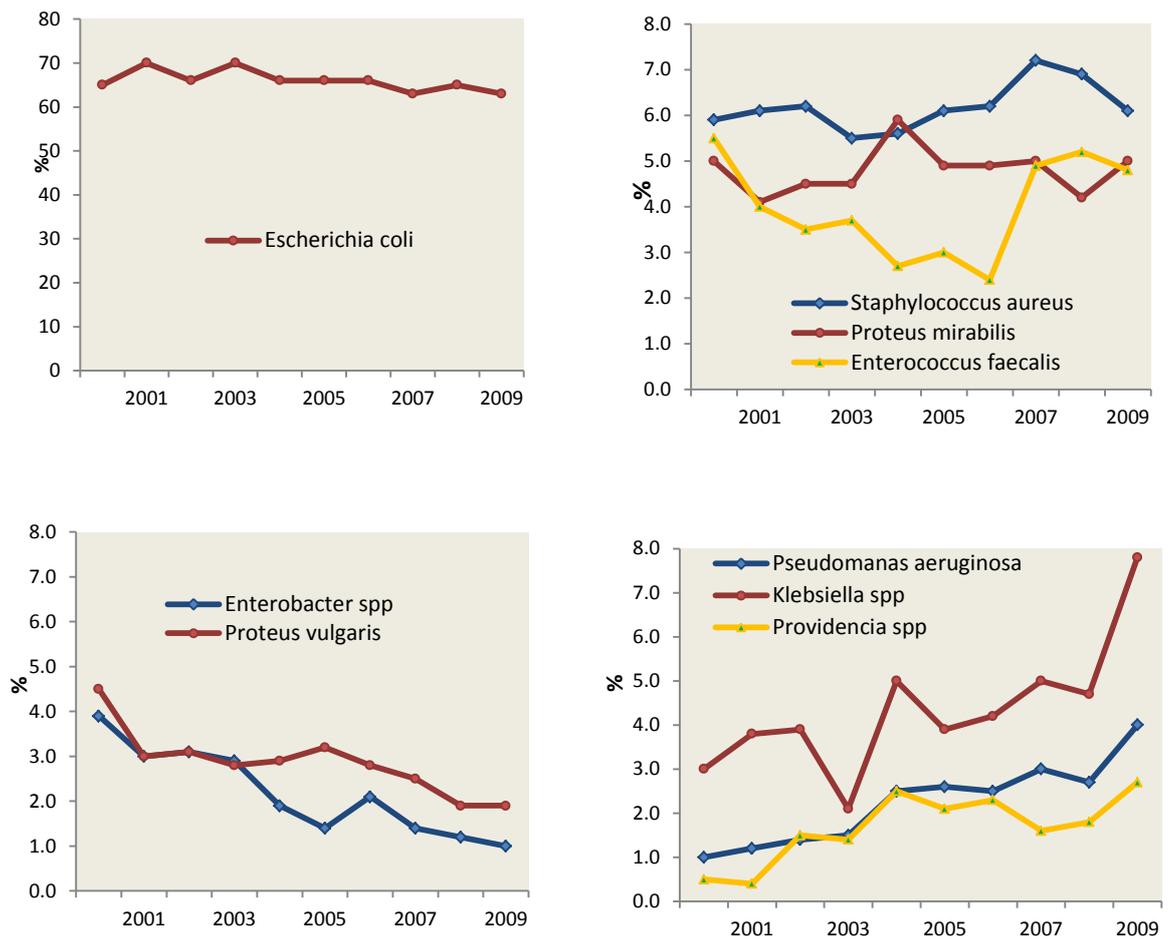
Antimicrobial drugs need to be in their active form as they filter through the kidneys into the urine, and be effective in urinary pH against the most common uropathogens. The trend in recent years has been to adopt short courses of antimicrobial treatment, defined as 3 days or less rather than 7-10 days. This is to encourage compliance, reduce adverse effects on the vaginal and gut flora, as well as keep cost to a minimum (Jancel and Dudas, 2002; Grabe *et al.*, 2009). This approach has been deemed effective when resistance has not developed towards the drug.

## 2.9 Pathogens

*Escherichia coli* is the predominant bacterial agent present in the majority of community acquired uncomplicated female UTIs (circa  $\geq 80\%$ ) possibly due to its natural presence in intestinal flora (Finer and Landau, 2004; Barber *et al.*, 2013). *E. coli* has consequently warranted a more in-depth investigation in this study.

Several pathogens constitute the remainder of UTIs. In a UK study published in 2003 with patients aged under 65 years of age (n=397) community acquired infections due to *E. coli* comprised 77%, *Proteus mirabilis* (4.3%), *Enterococcus faecalis* (3.8%), *Klebsiella pneumoniae* (3.5%), *Staphylococcus saprophyticus* (2.0%) and *Pseudomonas aeruginosa* (1.8%). A total of 5.8% of other species (unlisted) were responsible for the remainder (Farrell *et al.*, 2003).

A large community study conducted in Portugal, a country where antibiotics are not permitted to be bought over the counter or via the Internet without a prescription, and nor have been found to be purchased in this way (WHO, 2014), showed similar percentages. A total of 156K samples were analysed over 10 years and 12.1% of them tested positive for bacterial infection. Over the course of the survey the prevalence of *E. coli* (65-70%), *Staphylococcus aureus* (6%), *Proteus mirabilis* (4.7%) and *Enterococcus faecalis* (3.6%) remained fairly static, whilst infections caused by *Proteus vulgaris* (2.7%) and *Enterobacter sp.* (2%) declined. Whilst only a minority of infections were due to *Pseudomonas aeruginosa* (2.4%) *Providencia sp.* (2.5%) and *Klebsiella sp* (8%), these pathogens generated a steady growth in incidence over the 10 year period and all have shown a marked increase in resistance to antimicrobials (Linhares *et al.*, 2013). **Figure 3** below illustrates the results of the study according to the prevalence of individual uropathogenic bacteria (based on  $\geq 10^5$  CFU/mL) as a percent of total community acquired cases between 2001 and 2009.



**Figure 3:** Prevalence of uropathogens in community acquired UTI from 2001 to 2009 as a % of the total (based on data produced by Linhares et al., 2013)

### 2.9.1 *Escherichia coli*

The wide diversity of strains of *E. coli* (Gram-negative, Enterobacteriaceae) related to UTI are classed as uropathogenic *E. coli* (UPEC). They express a range of virulence factors (the ability to cause disease) which are lacking in the enteropathogenic strains (EPEC) (Moura *et al.*, 2009). Hacker & Kaper believe this variation is due to encoding by large blocks of genes known as pathogenicity-associated islands (Hacker and Kaper, 2000), but the pathways and triggers for gene expression are still not fully understood (Holden and Gally, 2004). Existence of virulence factors in UPEC which are different to those present in EPEC could put into question where the bacteria

actually originate, as well as the factors which cause the pathogen to develop virulence in the urinary tract. A very small recent study with just 4 women has reportedly discovered that *E. coli* can migrate back and forth between the gut and the bladder (Chen *et al.*, 2013), and this requires further investigation.

### 2.9.1.1 Adhesions

UPEC virulence factors include an array of adhesins, essential for establishing the pathogen in the urinary tract and avoiding possible removal by hydrodynamic forces (Klemm and Schembri, 2000; Wiles, Kulesus and Mulvey, 2008). For example, both P fimbriae (pyelonephritis-associated pili) and variants of the FimH Type 1 adhesin, which is expressed by more than 95% of UTI caused by *E. coli* equip the organism with the ability to attach to the urothelial surface (Tchesnokova *et al.*, 2011). Here they can subsequently invade the cells and proliferate, leading to enhanced virulence of the pathogen and persistent cases of bladder colonisation and inflammation (Klemm and Schembri, 2000; Finer and Landau, 2004; Moura *et al.*, 2009). P fimbriae bind to oligosaccharide receptors and FimH to mannose receptors ( $\alpha$ -D-mannosylated proteins) which are abundant in the bladder. This is the first step in *E. coli* induced cystitis, and can be a target for antibiotics (Bower, Eto and Mulvey, 2005; Ulett *et al.*, 2013). A third group of appendages, the O75X also known as the Dr family of adhesins, may participate in UTI but are mainly present amongst infected children and pregnant women (Nowicki, Selvarangan and Nowicki, 2001).

### 2.9.1.2 Hydrophobicity and Aggregation

The binding capability of *E. coli* to mucosal epithelial cells is believed to be enhanced by cell surface hydrophobicity, expressed by most strains of UPEC. However, the importance of this factor has not been conclusively identified. It is associated with aggregation of the bacterial cells, which could lead to adhesion of the microbe to the epithelium, may trigger the immune system or could damage the bacterial organism itself (Seong and Matzinger, 2004; Krasowska and Sigler, 2014). A close correlation has been observed between Type 1 fimbriae and cell surface hydrophobicity *in vitro*, but there is disagreement over whether P fimbriae are linked to elevated surface hydrophobicity. Moreover, UPEC strains have been isolated which do not express this

factor, indicating that it may not always be essential for virulence (Lachica, 1990; Sharma, Bhat and Shenoy, 2007), and non-fimbriated bacteria express a hydrophilic surface (Lindahl *et al.*, 1981). (Hydrophobicity and aggregation are discussed further in Chapter 9 related to an assay involving uva-ursi).

### **2.9.1.3 Secreted Toxins**

Many of the secreted toxins present in UPEC but lacking in the enteropathogenic strains may be of greater significance to *E. coli* virulence in addition to the iron sequestration system which is thought in some, but not all strains, to be mediated via the production of aerobactin (Bower, Eto and Mulvey, 2005; Katouli *et al.*, 2005; Ulett *et al.*, 2013). Iron is required by UPEC for growth and survival; it is scavenged from the host through lysis of epithelial cells via the toxin  $\alpha$ -hemolysin (Bower, Eto and Mulvey, 2005; Ulett *et al.*, 2013). Aerobactin is also considered to be an important mechanism for acquiring iron in its own right. In one study it was shown to be present in 73% of urinary isolates compared to only 38% which produced  $\alpha$ -hemolysin (Opal *et al.*, 1990).

The hydrophilic toxin cytotoxic necrotizing factor (CNF1) secreted by a third of uropathogenic *E. coli* strains is argued to be capable of promoting bacterial colonisation and virulence through enhancing adherence and entry to epithelial monolayers via a low pH endocytic pathway, as well as decreasing bacterial phagocytosis. Evidence of this is conflicting, and the mechanisms are still not fully understood (Hofman *et al.*, 2000; Bower, Eto and Mulvey, 2005). However, Mills *et al* showed that CNF1 may induce apoptosis in the bladder epithelium speculating that this could lead to increased bacterial access to underlying tissue and possible recurrent infection (Mills, Keysick and O'Brien, 2000).

### **2.9.1.4 Replication in the Urinary Tract**

UPEC replicate as they adhere to the surface of the uroepithelium, a process which is considered synonymous with causing an infection. However, it has been determined that the organisms can also invade the epithelial cells (Mulvey, Schilling and Hultgren, 2001). Once inside the epithelial tissue UPEC can quickly replicate to  $10^5$  bacteria per

cell. This leads to intracellular bacterial communities (IBC); these possess biofilm-like properties which protect UPEC from antibiotics and host defences (Kline *et al.*, 2009).

#### 2.9.1.5 Optimum pH

*E. coli* may survive and grow in a pH range from 2-10, but its optimum survival is in a concentration range between pH 6-7 (Small *et al.*, 1994).

#### 2.9.2 *Klebsiella pneumoniae*

*Klebsiella pneumoniae* (Gram-negative, Enterobacteriaceae) (*K. pneumoniae*) is considered the most opportunistic pathogen, affecting the immunocompromised. It is often responsible for hospital acquired infection but still accounts for at least 3% of cases of cystitis (Colgan and Williams, 2011). Although *K. pneumoniae* belong to the same bacterial family as *E. coli* the *Klebsiella* species (sp) does not possess flagella and the organisms are, therefore, non-motile. *K. pneumoniae* do, however, utilise Type 1 fimbriae adhesions and also express an additional Type 3 which may help them to adhere to bladder tissue as well as potentiating an ability to form biofilms (El Fertas-Aissani *et al.*, 2012).

#### 2.9.3 *Pseudomonas aeruginosa*

*Pseudomonas aeruginosa* (Gram-negative, Pseudomonadaceae) (*P. aeruginosa*) is an opportunistic pathogen capable of inhabiting and contaminating water. It can resist antiseptics and many antibiotics, and like *K. pneumoniae* it is a particular problem in hospitals amongst the immunocompromised, especially since it has the ability to form biofilms on the surface of urinary catheters (Mittal *et al.*, 2009).

The pili and outer membrane protein may mediate adherence. A polysaccharide alginate allows micro-colonies to form where organisms are protected from

opsonisation, phagocytosis and antibiotics. These, together with the proteases and cytotoxins it secretes (exotoxins A and S, haemolysins and elastase) are amongst the many virulent factors it possesses (Mittal *et al.*, 2009).

#### **2.9.4 *Proteus sp.***

*Proteus mirabilis* and *vulgaris* (Gram-negative, Enterobacteriaceae) grow in both aerobic and anaerobic conditions and can survive in a wide range of both temperature and pH. The species possess a diverse range of virulence mechanisms which enable them to infect the host. Primarily they are motile, which enable them to swim and colonise the urinary tract (Schaffer and Pearson, 2015). Their attachment to urinary epithelium is mediated via adherence pili. This pathogen also produces urease, which breaks down urea to ammonia and carbon dioxide. The process raises pH and can lead to the formation of crystals, which may assist in protecting the organism from immunoglobulins as well as antibiotics (Coker *et al.*, 2000).

#### **2.9.5 *Staphylococci***

The *Staphylococcus* (Gram-positive) genus comprises over 26 species, of which *Staphylococcus aureus* (*S. aureus*), *Staphylococcus epidermidis* (*S. epidermidis*), *methicillin-resistant Staphylococcus aureus* (MRSA), and *Staphylococcus saprophyticus* (*S. saprophyticus*) are the most common causes of UTI (Muder *et al.*, 2006; Namvar *et al.*, 2014). *S. aureus* and *S. saprophyticus* (in particular) are reportedly the biggest cause of UTI amongst young women in the USA and Europe after *E. coli* (Rupp, Soper and Archer, 1992).

Similar to other species which cause UTIs *S. saprophyticus* colonises the gastrointestinal tract, as well as the vagina. For reasons not yet established it has been found to cause over 40% of UTI incidences amongst women aged 16-24. It is consistently present across the urethra, cervix and rectum in around 40% of sexually active young women (Raz, Colodner and Kunin, 2005; Kline and Lewis, 2016). Not only may the bacteria be passed via sexual intercourse, one study has shown a

correlation between women with an *S. saprophyticus* infection and concurrent vaginal candidiasis, suggesting that colonisation by the organism may occur more easily when the vaginal flora has been disrupted (Raz, Colodner and Kunin, 2005).

*S. aureus* produces coagulase which stimulates the conversion of fibrinogen to fibrin; this is thought to form a protective barrier around the organism to prevent phagocytosis. It adheres to host cell receptors via matrix proteins, and produces lytic enzymes which aid the invasion of host cells (Vanassche, Peetermans and Verhamme, 2013).

### **2.9.6 *Enterococcus faecalis***

Traditionally forming part of the microflora in the human gut *Enterococcus faecalis* (Gram-positive, Enterococcaceae) (*E. faecalis*) has evolved to become a nosocomial pathogen of the urinary tract, relying on its ability to produce biofilms to enable colonisation (López-Salas *et al.*, 2013). Although still only responsible for less than 4% of the incidence of UTIs *E. faecalis* may account for over 80% of infections caused by the Enterococcaceae species and has shown increasing resistance to several antibiotics including penicillin and ciprofloxacin (Miskeen and Deodhar, 2002).

## **2.10 Antibiotic Use for Treatment of Urinary Tract Infections**

The class of antibiotic chosen for treatment should be dependent on the bacteria responsible for infection, as well as patient tolerability of the drug and resolution of a previous UTI. It may require a spectrum of activity (Holmes *et al.*, 2016).

Sulfanilamide (an enzyme inhibitor) was the first antimicrobial agent used for UTI, introduced in 1937 against *E. coli*, *Pseudomonas* and *Salmonella*, but as it caused severe side effects, such as cyanosis and gastrointestinal upset, it was replaced in 1953 by nitrofurantoin. In addition to being a much safer antimicrobial agent with greater tolerability, as a nitrofuran it is effective against *E. coli*, damaging the microbe's

DNA (Nickel, 2005b; Nicolle *et al.*, 2006). It also has activity against several other uropathogens, including *K. pneumoniae*, *E. faecalis* and staphylococci. However, it is not effective against *P. aeruginosa* or *Proteus sp.* Antimicrobial levels are not attained in the blood, it does not permeate urinary tract tissue and may, therefore, be unhelpful against complicated UTI (Moura *et al.*, 2009). Moreover, it has been linked to serious adverse effects, such as pulmonary fibrosis (Nickel, 2005b).

$\beta$ -lactams, which target bacteria by inhibiting cell wall synthesis, vary in their activity against different pathogens. Aminopenicillins such as amoxicillin have been routinely prescribed for UTI when *E. coli* and *Proteus mirabilis* are present, but resistance to amoxicillin is now widespread, reportedly as high as 50% of cases in North America (Gupta, Scholes and Stamm, 1999; Nickel, 2005b). This has led to trimethoprim, a dihydrofolate reductase inhibitor which blocks DNA synthesis though inhibiting folic acid (first introduced in 1962), being used alone or in combination with sulfamethoxazole (a sulphonamide). The latter is an antibacterial which inhibits the use of *para*-aminobenzoic acid (PABA) necessary in the same folic acid pathway (Nicolle *et al.*, 2006).

Quinolones followed in 1962 with the origination of nalidixic acid, and were found to easily penetrate urinary tract tissue (Nickel, 2005b, Bisacchi, 2015). This class of antibiotics, which inhibit DNA gyrase, have been further developed and are effective against complicated as well as uncomplicated UTI caused by Gram-negative species. Ciprofloxacin (a quinolone introduced in the 1980s) is one of the few classes of oral antibiotic which has demonstrated activity against *Pseudomonas* (Nickel, 2005b).

As mentioned above (Section 2.8) the recommendation by PHE is for empirical treatment with nitrofurantoin, kidney function permitting (PHE, 2017c). The official guidelines published by The European Association of Urology (EAU) advise that fosfomycin, trometamol, pivmecillinam (a penicillin), and nitrofurantoin are used as first-line therapies, with fluoroquinolones, cephodoxime proxetil, the sulphonamides and trimethoprim prescribed when local resistance is less than 20% (Linhares *et al.*, 2013).

## 2.11 Emerging Antibiotic Resistance to UTI

With infectious diseases (such as pneumonia and tuberculosis) one of the major causes of mortality in the world, and still the highest cause of death in low-income countries (WHO, 2011), effective treatment with antibiotics is essential. In fact for most people born since the Second World War (when penicillin first became commercially available), there is a common belief that infections can easily be cured with antibiotics (Hancock, 2007). This assumption, however, may be outdated. In recent years, not only have several multidrug resistant (MDR) bacterial strains emerged, such as *Staphylococcus aureus* and *Klebsiella pneumoniae*, there has been a dramatic slowdown in the discovery of new antibiotics (Alanis, 2005; Theuretzbacher, 2009; Gootz, 2010).

In order to survive, bacilli (both Gram-positive and Gram-negative) have developed the means of defending themselves against antimicrobials, becoming increasingly resistant to most antibacterial classes, especially amongst the immunocompromised who, as a result of being more prone to infections, may be prescribed a greater number of antibiotics (Pournaras, Iosifidis and Roilides, 2009).

As early as 1945 Sir Alexander Fleming predicted that misuse of penicillin might lead to resistance of mutant forms of *Staphylococcus aureus*. Within a year he was proved right (Alanis, 2005), and although a more potent derivative, methicillin was introduced in 1960, resistance developed within 6 months (Bassetti, Nicco and Mikulska, 2009).

With increasing consumption of antibiotics, this problem of selective pressure has accelerated. In fact a surveillance report carried out in the USA ascertained that US hospitalisations with resistant infections doubled in the first 5 five years of the 21<sup>st</sup> Century, particularly amongst the elderly (Zilberberg, Shorr and Kollef, 2008). A systematic review of 24 studies, which included 5 randomised trials, reported that primary care patients in the UK who took antibiotics for UTI could develop resistance to the antibiotic in the month following the initial prescription, and that resistance to the drug may also be detected as long as 12 months later (Costelloe *et al*, 2010). Similar scenarios have been reported in Europe, with growth in resistance directly correlated to use. Pneumococcal resistance to penicillin in Spain, for example, where antibiotics are bought over the counter, is significantly higher than in the Netherlands, where consumption is low (Austin, Kristinsson and Anderson, 1999; Bronzwaer *et al.*, 2002).

In 2002 a global surveillance program (The Study for Monitoring Antimicrobial Resistance Trends – SMART) was established for monitoring susceptibility of Gram-negative bacteria to antibiotics. In 2009 this study was extended to hospitalised patients with UTI due to increasing resistance to trimethoprim/sulfamethoxazole, amoxicillin and ciprofloxacin (Hoban *et al.*, 2011). Widespread empirical treatment for uncomplicated UTI in the community could arguably be contributing to the development of antibiotic resistance (Nicolle *et al.*, 2006). Deaths reported in the UK due to *E. coli* bacteraemia increased from 5.4K in the year 2013 to 5.9K four years later. UTI comprised the majority of primary focus cases at 46% of the total, and 11% of *E. coli* bacteraemia isolates were resistant to cephalosporin (PHE, 2016).

### 2.11.1 Mechanisms of Resistance

Bacteria have developed and utilise a variety of mechanisms to evade antimicrobial agents, which has served to escalate the problem of resistance - especially since multiple methods have evolved in individual pathogens (Hooper, 2005; D'Costa, Griffiths and Wright, 2007).

The first observed mechanism was the production by Gram-negative bacilli of the enzyme  $\beta$ -lactamase; this breaks open the  $\beta$ -lactam ring in the antibiotic, and in so doing can degrade most classes of  $\beta$ -lactams (Rossolini, 2005).

Many microbes also contain proteins that act as efflux pumps, effectively pumping out antibiotics to ensure their survival. *Pseudomonas aeruginosa* has at least ten such efflux systems removing drugs of several different types (Poole, 2005). Resistance to fluoroquinolones, which interact with DNA gyrase and topoisomerase IV to prevent replication, originated through mutations in the coding of the gyrase subunits and DNA topoisomerase (Hawkey and Jones, 2009).

Of major concern in recent times is the finding of a new type of carbapenem resistance gene – New Delhi metallo- $\beta$ -lactamase 1 (NDM-1), which renders nearly all antibiotics ineffective against *Escherichia coli* and *Klebsiella pneumoniae* (Kumarasamy *et al.*, 2010).

Once a resistance mechanism has evolved, it can be passed to other bacteria through vertical and horizontal gene transfer, or via plasmids; an incredibly efficient means of building up resistance on a significant scale (Hawkey and Jones, 2009). Its spread further afield is facilitated globally via human travel (Okeke and Edelman, 2001).

## 2.12 Alternative Treatment of Urinary Tract Infections

Symptoms of UTI have been treated for thousands of years, long before any knowledge or recognition of related causative bacteria existed. Written texts on papyrus from ancient Egypt proposed herbs including myrrh for both urine retention and excessive urination, and traditional Chinese medicine utilized herbs and acupuncture as long ago as 2637 BC (Nickel, 2005a).

Phyto-pharmaceuticals and probiotics, as well as a variety of nutrient interventions, are all now taken to treat UTI as alternatives to antibiotics (Yarnell, 2002; Head, 2008).

### 2.12.1 Herbal Treatment of Urinary Tract Infections

A number of herbal medicines are used across the world for treating UTIs, especially cystitis (Yarnell, 2002). The perceived efficacy is commonly based on traditional use, with only a small minority undergoing scientific analysis, usually *in vitro*. With the exception of *Vaccinium macrocarpon* (cranberry) clinical studies are lacking (Yarnell, 2002; Vasileiou *et al.*, 2013).

Herbs which have traditional use for treating UTIs are *Althea officinalis* (marshmallow), *Arctostaphylos uva-ursi* (bearberry) (the focus of this PhD), *Barosma betulina* (buchu) used in South Africa, *Equisetum arvense* (horsetail), *Juniperus communis* (juniper), *Solidago virgaurea* (golden rod), *Thymus vulgaris* (thyme), *Zea mays* (corn silk) and *Berberis vulgaris* (barberry) (Yarnell, 2002; Head, 2008). *B. vulgaris* is known to contain berberine, a compound which may inhibit of *E. coli* through blocking the binding of fimbriae (Sun, Abraham and Beachey, 1988). There is little known about the actions of the other herbs, except *Arctostaphylos uva-ursi*.

### 2.12.1.1 *Vaccinium macrocarpon* (Cranberry)

Possibly the most well-known botanical treatment is based on cranberry fruit *Vaccinium macrocarpon* (Ericaceae). Containing flavonoids (anthocyanins and proanthocyanidins), catechins, triterpenoids and other phenolic acids, cranberries have been the focus of several clinical studies as well as four systematic Cochrane reviews. The most recent Cochrane update was 2012 (Amalaradjou and Venkitanarayanan, 2011, Jepson, Williams and Craig 2012).

The first controlled clinical study in 1994 amongst 192 participants reported a reduction of bacteria in urine compared to placebo over a six month period upon consumption of 300 mL daily of cranberry juice. Bacteriuria and pyuria were present in 14% of the cranberry juice group compared to 28% of placebo versus baseline (Avorn *et al.*, 1994). Whilst this indicated that cranberries may reduce bacteria, it was conducted amongst healthy participants. Since then there have been a number of clinical trials evaluating the effect of cranberry intake on UTI, with mixed results, but those reporting a favourable outcome involved cranberry consumption for at least 6 months acting as a prophylactic rather than treatment for symptom relief (Vasileiou *et al.*, 2013).

The 2012 Cochrane review concluded that taking cranberry juice for UTI was less effective than had previously been determined. The addition of 14 new studies to the original 10 reviewed in 2008, which concluded that cranberry showed efficacy against the symptoms of UTI, resulted in the revised conclusion that there was not enough evidence to support the recommendation of taking cranberry juice for the prevention of UTIs. The authors also remarked that there needed to be greater standardisation of constituents for other cranberry preparations, such as powders, before their clinical efficacy could be evaluated (Jepson *et al.*, 2012). As yet there have not been any studies which have reported a conclusive dosage or specific recommendation for the duration of treatment (Vasileiou *et al.*, 2013)

With regard to the possible mechanism of action, it was initially hypothesised that the acidic profile of the fruit was responsible for exerting an antimicrobial effect. However, this was later discounted as cranberries did not lower pH sufficiently enough in the urinary tract to cause a bacteriostatic effect (Amalaradjou and Venkitanarayanan, 2011; Vasileiou *et al.*, 2013). Sobota *et al.* were the first to demonstrate that cranberry juice may reduce the ability of *E. coli* to adhere to the host (Sobota, 1984). It has since been shown *in vitro* that fructose can block the mannose sensitive fimbriae, and the

proanthocyanidins may block the mannose resistant adhesins of UPEC (Vasileiou *et al.*, 2013). However, not only is fructose a very common sugar present in many fruits, it is metabolised before reaching the urinary tract so is unlikely to exert efficacy *in vivo*. In contrast, A-linked proanthocyanidins are unique to *Vaccinium* (cranberries and blueberries), and may act as a prophylactic by preventing adhesion of uropathogenic *E. coli* (Nowack and Schmitt, 2008; Vasileiou *et al.*, 2013). This could warrant further investigation.

Finally, cranberries also contain arbutin, the compound considered the active constituent of *Arctostaphylos uva-ursi* (Bearberry), the main focus of investigation for this PhD (discussed in Chapter 3). In cranberries it is present in a much smaller quantity, and has not even been cited in a recent review of its efficacy (Vasileiou *et al.*, 2013).

## **2.12.2 Probiotics and Nutrient Interventions**

### **2.12.2.1 Probiotics**

Probiotics, such as *Lactobacillus*, are live organisms which are consumed to help restore or increase commensal microflora, to maintain balance in the microbial ecosystem (Head, 2008). Moreover, one study has reported that they can reduce and maintain an acidic pH  $\leq 4.5$ , which facilitates their growth and multiplication, as well as produce antimicrobial toxins such as bacteriocin and hydrogen peroxide (Amalaradjou and Venkitanarayanan, 2011).

Due to *E. coli*'s presence in gut flora, and the understanding that the organism easily migrates externally to the female urinary tract, *in vitro* studies have been conducted to investigate the effect of the *Lactobacillus* species on inhibiting both the growth and epithelial adhesion of uropathogens. The results have been mixed. In a study amongst 15 species, *Lactobacillus crispatus* showed the greatest activity against *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* and the least against *Staphylococcus aureus* and *Proteus mirabilis* (Osset *et al.*, 2001). *Lactobacillus crispatus* was then found to attach to vaginal epithelium, from which it was deduced that it may block adhesion (and subsequent multiplication) of uropathogens *in vivo*.

However, amongst 5 *Lactobacillus* species tested for activity against *E. coli* strains responsible for UTI *Lactobacillus* showed no activity (Kwok *et al.*, 2006).

#### **2.12.2.2 Nutrient Interventions**

D-mannose is a six carbon sugar related to glucose and fructose which is produced by the body, and is also found in many fruits. In the 1980s it was hypothesized that its consumption as a supplement might increase the amount of mannose present in urine to an amount sufficient enough to block the mannose binding receptors of *E. coli* and thus prevent the microbe adhering to the uroepithelium. This theory was subsequently tested on rats and mice with some success (Michaels *et al.*, 1983). Since then it has been sold as a supplement for the treatment and prevention of UTI.

Nevertheless, some strains of *E. coli* do not possess mannose-sensitive pili and pili saturated by mannose could also make it more difficult for *E. coli* to be recognised and destroyed by macrophages (Van Den Bosch *et al.*, 1980). Furthermore, not all UTI are caused by *E. coli*.

#### **2.12.3 Alkalisiation of Urine**

Taking alkalisating agents, such as sodium citrate and sodium bicarbonate, to increase urinary pH for symptomatic relief of cystitis has long been practiced in many countries in the world (O’Kane *et al.*, 2016). However, there is a lack of both research and credible evidence to support their efficacy. There have not, as yet, been any published double-blind placebo controlled trials or randomised controlled trials on the effect of alkalisating agents. Three observation studies have been conducted, and the results are outlined below.

One investigation which assessed the effect of alkalisiation, concluded that sodium citrate could provide symptom relief amongst women who did not have evidence of a bacterial infection (Spooner, 1984). The study was uncontrolled, did not use a placebo, and nor did it measure the pH of the urine. Furthermore, almost 80% of the sample (n = 205) was abacteriuric, which is double the 40% who reportedly present with a UTI in

clinical practice (Spooner, 1984; Little *et al.*, 2006). A second single arm study conducted on the same product six years later evaluated the effect on 64 patients. There was symptomatic improvement in 70% of patients but once again there was a higher than average inclusion of participants (70%) who were abacteriuric and the pH of the urine was not recorded (Munday and Savage, 1990). Amongst the 19 patients who had a proven infection the response was varied. Less than half reported an improvement in frequency and urgency, whilst just over half showed reduced urethral pain and dysuria (Munday and Savage, 1990).

A publication which studied the mechanism of action in terms of the relationship between urinary pH to symptoms of lower UTI concluded that there was no difference in symptoms with alkaline urine compared to the symptoms presented when the urine was acidic. The patients who took part in the research were not given an alkalisng agent, but were administered antibiotics (Brumfitt *et al.*, 1990).

## **2.13 Conclusion**

Reviewing the pathophysiology of lower UTI, together with the bacterial organisms and the relevant number of colonies which are known to cause cystitis, was important for this study in order to ensure that the antimicrobial activity of uva-ursi and its related constituents could be assayed against the pathogens involved in UTI (Chapter 7).

The review also facilitated the investigation of virulence factors pertinent to *E. coli*, the pathogen mainly responsible for UTI. As a result, the activity of uva-ursi was also tested to determine whether it may impact on aggregation and motility, and what implications this might have for efficacy (see Chapter 9).

Investigating the effect of alkalisng agents for symptom relief of UTI was particularly relevant to this thesis because there was a question of whether it was necessary to alkalisng urine for uva-ursi to be effective in the urinary tract (discussed in Chapter 3, and analysed in Chapter 7).

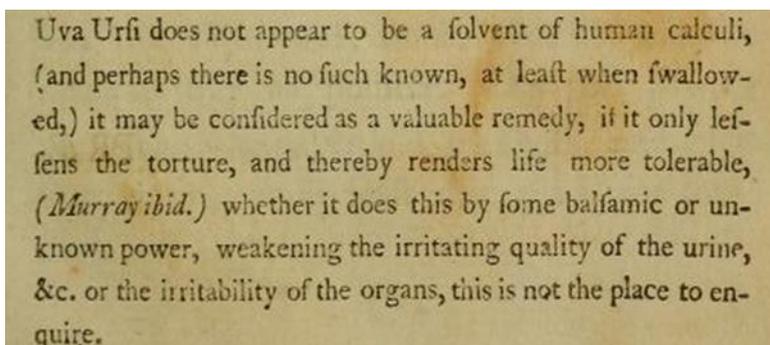
The research conducted on the effect of urinary pH facilitated co-authorship of the systematic Cochrane review published in 2016 “*Urinary alkalisiation for symptomatic uncomplicated urinary tract infection in women*” (O’Kane *et al.*, 2016).



## Chapter 3: *Arctostaphylos uva-ursi* (L.) spreng

### 3.1 Introduction

*Arctostaphylos uva-ursi* (L.) Spreng (Ericaceae) (uva-ursi) is commonly known as bearberry: the Latin epithet uva-ursi literally means 'bear's grape' since bears are known to eat the fruit (Bruneton, 1999). It is a small shrub which grows in the mountains of the northern hemisphere, and the therapeutic constituents for treating urinary tract infections are reportedly present in the leaves (Gallo *et al.*, 2012). Its use is claimed to date back to the 13<sup>th</sup> Century pharmacopoeia of the Welsh physicians of Myddfai (McKenna *et al.*, 2002), and over four hundred years later it appeared in the *Materia Medica* of the London Pharmacopoeia in 1788 for treating *diseases of urinary passages* (Healde, 1788).



Uva Urſi does not appear to be a ſolvent of human calculi, (and perhaps there is no ſuch known, at leaſt when ſwallow- ed,) it may be conſidered as a valuable remedy, if it only leſ- ſens the torture, and thereby renders life more tolerable, (*Murrayibid.*) whether it does this by ſome balſamic or un- known power, weakening the irritating quality of the urine, &c. or the irritability of the organs, this is not the place to en- quire.

**Figure 4:** Excerpt on uva-ursi from the London Pharmacopoeia 1788 (London & Healde, 1788)

### 3.2 Botany

Uva-ursi is a small, creeping to semi-erect evergreen shrub (10-15 cm high), with long (1.5 m) prostrate branches which may form mats as they trail the ground. The flat leathery leaves are up to 2 cm long, dark green on the surface, light green on the

underside, and oblong to obovate in shape. The flowers, which appear in early summer, are globular with 3 to 15 together. They have greeny-white or pink petals, and are followed by glossy red berries (Bown, 1995; Barker, 2001).



**Figure 5:** Photo of *Arctostaphylos uva-ursi* shrub  
Photo by Jeanne Trill

### 3.3 Phytochemistry

The chemistry of the leaves of *Arctostaphylos uva-ursi* has been well studied. It contains phenols, including the phenolic glycosides arbutin (hydroquinone- $\beta$ -D-monoglucopyranoside) (6-10%) and methylarbutin (0.5% to 1%), which are believed to be the constituents primarily necessary for activity (Bruneton, 1999; Schindler *et al.*, 2002). Other compounds present are tannins, especially the polyphenol gallic acid (15-20%), triterpenes (such as ursolic acid, uvaol,  $\alpha$ - and  $\beta$ -amyrin), flavonoids (mainly rutin, quercetin, hyperoside, kaempferol and myricetin), catechin and epigallocatechingallate (ESCOP, 2003), monotropin (an iridoid), and picein (a glucoside of 4-hydroxyacetophenone) (Bruneton, 1999; Heinrich *et al.*, 2004; Olennikov and Chekhirova, 2013).

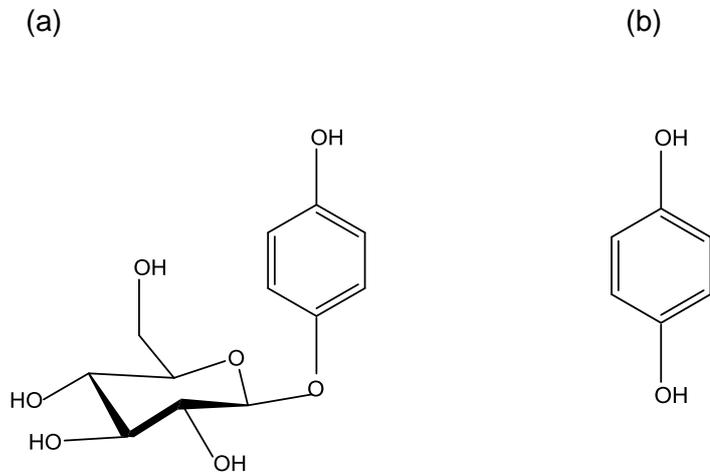
Arbutin is converted in the body to hydroquinone (see Section 3.4.1), and it is hydroquinone which is commonly and historically believed to exert an antiseptic effect in the urinary tract (Frohne, 1970; Gallo *et al.*, 2012). A fact usually not reported in herbal monographs is the presence of a small amount of free hydroquinone (<1.2% in the dried leaves), which is almost certainly due to hydrolysis of arbutin (Sticher, Soldati and Lehmann, 1979).

It is believed that the plant produces arbutin (and methylarbutin) as a form of defence against diseases such as fire blight, caused by the bacteria *Erwinia amylovora* (Hildebrand C, Powell Jr. and Schroth, 1969). The quantity of arbutin present has been shown to vary depending on the geographical location of the plant as well as its time of harvest. For example, within one plant population in Spain the level of arbutin ranged from 6% in the spring to 9.39% in the autumn (Parejo *et al.*, 2002). This could, therefore, have a bearing on the amount of commercial uva-ursi product which should be consumed.

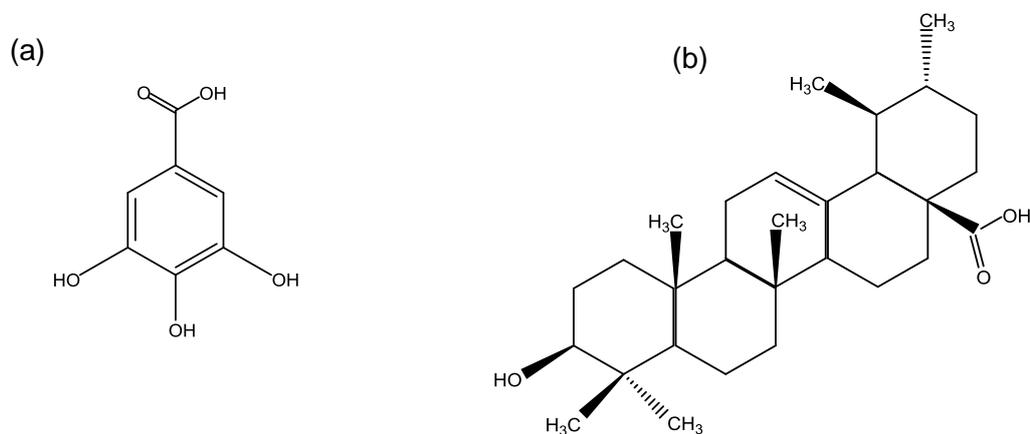
Arbutin is also present in smaller amounts than uva-ursi in the leaves and bark of other plants such as cranberry, cowberry, and bilberry, as well as in certain foods such as pears (especially the skins) wheat germ and whole wheat bread (Deisinger, Hill and English, 1996). Free hydroquinone has been found in coffee, broccoli, red wine, wheat and oat cereal in small amounts ( $\geq 0.1 \mu\text{g/g}$ ) (Deisinger, Hill and English, 1996).

### 3.3.1 Chemical Structures

The chemical structure of arbutin and its related aglycone hydroquinone is depicted below in **Figure 6**. Gallic acid and ursolic acid are in **Figure 7**.



**Figure 6:** Chemical structure of the glycoside arbutin (a), which is hydrolysed to release the aglycone hydroquinone (b)



**Figure 7:** Chemical structures of (a) gallic acid, and (b) ursolic acid

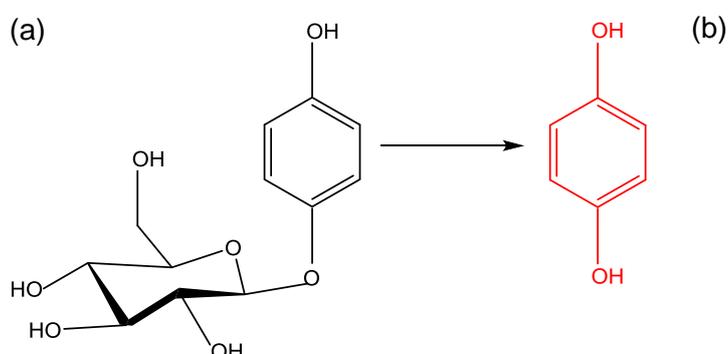
## 3.4 Pharmacological Properties

### 3.4.1 Pharmacokinetics

#### 3.4.1.1 Absorption of Arbutin

Studies on urinary metabolites after consuming uva-ursi have shown that hydroquinone conjugates and possibly a very small amount of free hydroquinone itself are eliminated in urine as quickly as two hours after taking the herb, but that no arbutin is present (Siegers *et al.*, 1997; Glockl, Blaschke and Veit, 2001; Quintus *et al.*, 2005).

The means by which polyphenols are fully metabolised is still not entirely determined, and there have been several mechanisms proposed about how the glycoside arbutin is absorbed in the body and converted to the aglycone hydroquinone (Manach *et al.*, 2004). MacDonald and colleagues hypothesized that it might be hydrolysed in stomach acid to release hydroquinone (McDonald *et al.*, 2001), but the incubation of 2mM of arbutin in synthetic gastric juices for two hours at pH 2 failed to produce the aglycone (Blaut *et al.*, 2006). It has also been proposed that glycosides may be absorbed intact across the enterocytes of the small intestine via the sodium ( $\text{Na}^+$ ) dependent glucose transporter (SGLT1) (Manach *et al.*, 2004), as phenyl glucosides are known to be absorbed in this manner (Lostao *et al.*, 1994). The glycoside (arbutin) would subsequently be cleaved by the enzyme  $\beta$ -glucosidase ( $\beta$ GL) into the sugar and its aglycone (Hq), a process known as deglycosylation (Dabek *et al.*, 2008).



**Figure 8:** Deglycosylation of arbutin (a) hydrolysed to release the aglycone hydroquinone (b)

### 3.4.1.2 Deglycosylation of Arbutin into Hydroquinone via $\beta$ -Gucosidase

It was originally thought that the deglycosylation of arbutin must take place in the colon, as human intestinal microflora have been shown to produce free hydroquinone from arbutin (Kang *et al.*, 2011; Khanal *et al.*, 2011). Moreover,  $\beta$ GL has been found present in over 20 types of colonic bacteria, particularly Gram-positive *Firmicutes* related to *Roseburia* and *Eubacterium rectale*, as well as the *Bifidobacterium* species and *Bacteroides thetaiotaomicron* (Dabek *et al.*, 2008). Eight species of intestinal bacteria in total, including *E. coli*, have been tested for their ability to convert arbutin to hydroquinone, but just one, *Bifidobacterium distasonis* DSM 20101, was able to transform it in its entirety. Two others (*Bifidobacterium adolescentis* and *Enterococcus casseliflavus*) could partially convert it over 72 hours.

Arbutin has also been efficiently converted to hydroquinone through incubation with human fecal slurries over a 24 hour period (Blaut *et al.*, 2006). Whilst individual hourly data for the conversion is unavailable, the study's time-graph illustrated that there is minimal release of hydroquinone during the first 3 hours of incubation (Blaut *et al.*, 2006). Since arbutin metabolites (hydroquinone glucuronide, hydroquinone sulphate and also free hydroquinone), have been detected in urine within 2-3 hours of taking uva-ursi, the process of deglycosylation in the colon may not act fast enough to produce the urinary metabolites detected (Kedzia *et al.*, 1975; Quintus *et al.*, 2005).

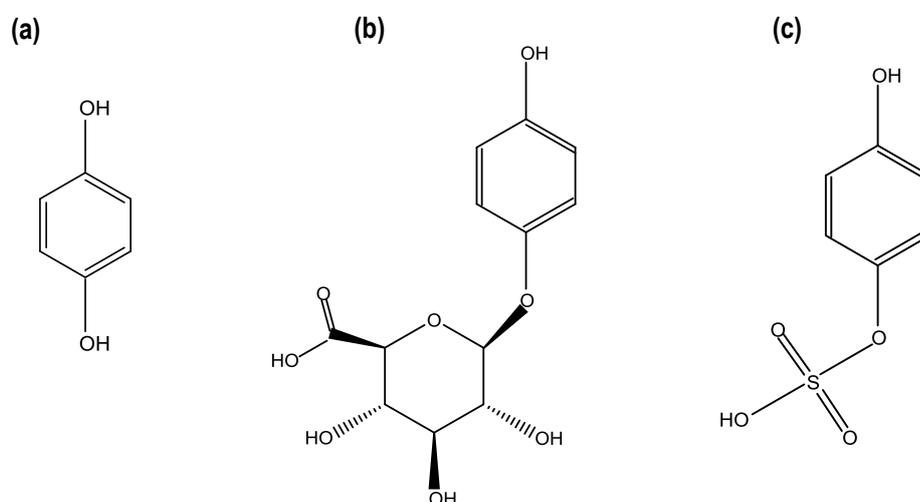
The enzyme  $\beta$ GL is also believed to be present in bacteria in the stomach, small intestine, liver, spleen and kidneys (Daniels *et al.*, 1981; Németh *et al.*, 2003; Nemeth and Piskula, 2007). It is, therefore, possible that the process of deglycosylation occurs in the liver or kidneys. However, the main metabolites are produced in the liver, making this the most likely site (see Section 3.4.1.3 and **Figure 10** for a summary of the proposed metabolic pathway).

### 3.4.1.3 Metabolites from Arbutin

Once hydroquinone has been released from arbutin it then has to be converted to non-toxic hydrophilic metabolites that can be excreted safely from the body. This prevents any unwanted cellular reactions which may be caused by hydroquinone (Attia, 2010). The main urinary metabolites are conjugates of hydroquinone with glucuronic acid and sulphuric acid, namely hydroquinone glucuronide and hydroquinone sulphate (Glockl,

Blaschke and Veit, 2001). These metabolites have been detected in urine as quickly as two hours after taking uva-ursi, and between 70% and 99% of the original dose of the herb has been shown to be passed within 24 hours (Kedzia *et al.*, 1975; Quintus *et al.*, 2005). Less than 1% of free hydroquinone has been shown to be eliminated, and in one study none at all was detected in urine samples of half the volunteers tested (Quintus *et al.*, 2005).

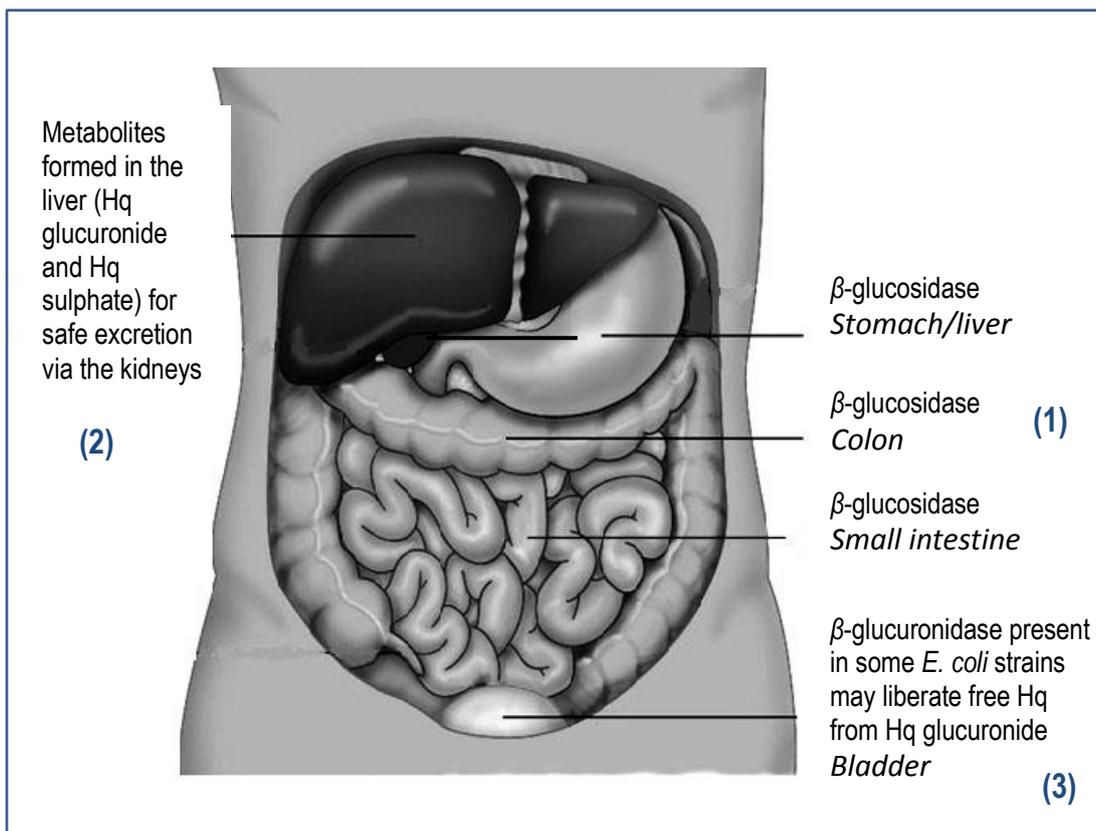
Glucuronidation, the addition of glucuronic acid (a carboxylic acid) to a substrate via a glycosidic bond, usually takes place in the liver, and is catalysed by the enzyme uridine 5'-diphospho-glucuronosyltransferase which is present in most major organs including the intestine and kidneys. The process enables a lipophilic compound to become more hydrophilic for safe excretion via the kidneys. Reaction with the enzyme sulfotransferase produces the sulphate, also for the purpose of safe elimination (Gamage *et al.*, 2005).



**Figure 9:** Chemical structures of metabolites. (a) hydroquinone; (b) hydroquinone glucuronide; (c) hydroquinone sulphate

The enzyme  $\beta$ -glucuronidase ( $\beta$ -GLn) catalyses the breakdown of complex sugars, such as glucuronides for reabsorption, and is for example involved in the metabolism of steroids and flavonoids, as well as the conversion of conjugated bilirubin for reabsorption (Schoenfield, Bollman and Hoffman II, 1962; O'Leary *et al.*, 2003). Similar to  $\beta$ GL the enzyme  $\beta$ -GLn is also present in micro-organisms. It is able to liberate free hydroquinone from the glucuronide (Wester *et al.*, 1998).  $\beta$ -GLn is

believed to be produced in the body mainly by intestinal bacteria such as *E. coli* and *S. aureus*, but it has also been found in other tissues such as the liver, spleen and kidneys as well as the urinary tract (Mroczyńska et al., 2013; Zenser, Lakshmi, & Davis, 1999).  $\beta$ -GLn is expressed by over 94% of *E. coli* strains (Jefferson, Burgess and Hirsh, 1986; Ralovich et al., 1991). The presence of  $\beta$ -GLn in *E. coli* may, therefore, play a role in the self-destruction of the micro-organism when treating a UTI with uva-ursi, as it may free hydroquinone from the glucuronide in the urinary tract.



**Figure 10:** Summary of the metabolism of arbutin to hydroquinone, and subsequent elimination of hydroquinone metabolites via the bladder

**(1)** Arbutin is hydrolysed by  $\beta$ -glucosidase to free the aglycone Hq in the stomach, small intestine, liver and colon, with the liver being the most likely site; **(2)** The aglycone Hq is then converted into Hq glucuronide and Hq sulphate in the liver for safe elimination out of the body via the urinary system; **(3)** Hq may be liberated from Hq glucuronide by  $\beta$ -glucuronidase expressed by *E. coli* if this organism is present in the bladder.

(Illustration adapted by Jeanne Trill from an outline diagram 'Anatomy of Human Abdomen' by van Brussel, 2010).

### 3.4.2 Pharmacodynamics

The principle traditional therapeutic use of uva-ursi is the treatment of UTIs. This has led to the instigation of several studies to investigate its potential antimicrobial activity against different microbes. The importance of this aspect has warranted a separate section in this thesis, and it is, therefore, discussed in detail in Section 3.5 below.

In addition to the possible antimicrobial action of uva-ursi, necessary to the treatment of UTI may be the fact that the herb has also been reported to have demonstrated both diuretic and anti-inflammatory activity (Beaux, Fleurentin and Mortier, 1999; Farah-Saeed and Ahmad, 2016).

A hypotonic saline solution (45%) containing 50 mg/kg of uva-ursi administered intraperitoneally induced significant diuresis in rats when compared to the control (Beaux, Fleurentin and Mortier, 1999).

The reported anti-inflammatory activity has also been observed in animal rather than human studies. The dried herb as well as a concentrated extract at doses of 500 mg/kg and 300 mg/kg body weight respectively showed significant anti-inflammatory effect compared to the control ( $P < 0.05$ ) on the volume of rats' paws (Farah-Saeed and Ahmad, 2016). This would be more than double the standard dose in adults, based on an adult weighing 58 kg. Moreover, uva-ursi was administered orally 30 minutes before an injection of carrageenan (used to stimulate inflammation) without acknowledgement of the time it takes for the herb to be metabolised.

Other animal studies on mice and rats have indicated anti-inflammatory activity of uva-ursi in conjunction with steroids. Co-administration of an uva-ursi methanolic extract (oral) at 100 mg/kg of body weight and prednisolone (subcutaneous) inhibited picryl chloride-induced swelling in mice more significantly than prednisolone or the uva-ursi extract alone. The extract alone also showed a therapeutic effect at the same dose 24 hours after its administration (Kubo *et al.*, 1990). A water extract of uva-ursi (2% in an ointment) did not on its own inhibit ear swelling or paw oedema in rats and mice, but did increase the anti-inflammatory effect of dexamethasone ointment ( $p = 0.005$ ) without increased side effects (Matsuda *et al.*, 1992).

#### **3.4.2.1 Antioxidant Activity**

It is not unusual for antioxidant activity to be demonstrated by polyphenolics (such as flavonoids) and extracts of uva-ursi (both ethanol, and ethanol/water) have demonstrated antioxidant activity when assayed with meat products such as pork. This can prevent lipid oxidation, and subsequent deterioration of the product (Samoilova *et al.*, 2014a; Azman *et al.*, 2016).

#### **3.4.2.2 Biofilms**

One paper has reported that low concentrations of uva-ursi may stimulate biofilm formation of the enteric strain of *E. coli* BW25113 (Samoilova *et al.*, 2014b). The publication was based on using a water extract of uva-ursi at a concentration that was shown by the authors to exhibit a 'slight bacteriostatic effect'. The publication found conflicting results for two plant extracts when compared to a previous study which had investigated biofilm formation, but not tested uva-ursi (Wojnicz *et al.*, 2012). However, the method which was used by Samoilova *et al.* (2014b) was based on a modification of a previously published method. The authors of the original method reported that the results of testing the formation of biofilms varied depending on the strain(s) assayed as well as the culture conditions. They had concluded that any results of such an assay should be treated with caution, as there were many variables involved (Naves *et al.*, 2008).

## 3.5 Antimicrobial Activity of Uva-ursi

### 3.5.1 In vitro Assays

Over half the antimicrobial assays on uva-ursi were conducted in the 1970s/80s, with only two carried out since then. Moreover, there is a lack of uniformity in the procedures previously employed to assess the anti-bacterial potential of uva-ursi, thus making it impossible to compare the results of all the studies conducted. These are summarised in **Table 4**, illustrating the range of assays conducted.

The EMA and WHO have reported that uva-ursi's anti-microbial activity may be dependent on the extraction solvent used. They have maintained that a high concentration of ethanol (95%) is less effective than a 30% extraction (WHO, 2002b; EMA, 2012a). Further examination of the studies they cite does not support this conclusion. An antibacterial test was over 60 years old: it reported negative results for cold and boiling water extracts as well as a 95% ethanol extraction (Gottshall, Lucas and Roberts, 1949). The constituents including arbutin are highly water soluble, and an undiluted decoction of uva-ursi (a water based extraction) has since been shown to inhibit 20 strains of *E. coli* (Turi *et al.*, 1997). A second report cited also failed to show antibacterial activity using a 95% ethanol extract. The authors of the original publication had concluded that the concentration may have been too weak, but the EMA and WHO did not refer to this in their reports (Dykes, Amarowicz and Pegg, 2003; EMA, 2012a). The study which used only 30% ethanol demonstrated inhibitory activity against six bacteria including *E. coli* (Leslie, 1978). However, this comprised a tincture with an unspecified amount of original plant material, making the results hard to interpret. Not only have tests been conducted to look at the possible anti-microbial properties of arbutin and hydroquinone, other constituents in uva-ursi could also possibly contribute to anti-bacterial effects. Ursolic acid, for example, has also demonstrated anti-microbial activity (Scalon Cunha *et al.*, 2007; Wolska *et al.*, 2010).

**Table 4:** Summary of *in vitro* antimicrobial studies on uva-ursi

Study	Extract Preparation/ Solvent	Results
<p><i>In vitro</i> efficacy of extracts of <i>Arctostaphylos uva-ursi</i> on clinical isolated <i>E. coli</i> and <i>Enterococcus faecalis</i> strains (Vučić <i>et al.</i>, 2013)</p>	<p>10 g dried leaf extracted in 200 ml of solvent, and extracted twice more</p> <p><b>H<sub>2</sub>O</b> <b>Ethanol</b> <b>Ethyl acetate</b></p> <p>Macro-dilution using DMSO</p>	<p><i>E. coli</i> – antimicrobial activity at 0.625-5.0 mg/mL with H<sub>2</sub>O; 5-10 mg/mL with ethanol; 10 mg/mL with ethyl acetate.</p> <p><i>E. faecalis</i> – antimicrobial activity at 1.25-2.5 mg/mL with all 3 solvents.</p> <p>Amoxicillin – antimicrobial activity ranged from 3.9-5000 µg/mL for <i>E. coli</i>, and 0.2-1.0 µg/mL for <i>E. faecalis</i></p>
<p>Antimicrobial activity of some Finnish Ericaceous plants (Holopainen <i>et al.</i>, 1988)</p>	<p><b>80% ethanol</b> 1:5 maceration. 10 ml evaporated to dryness, and 10 ml evaporated to remove ethanol only. Residue diluted with H<sub>2</sub>O (1:1) and extracted with ethylacetate. Solvent evaporated off.</p> <p>(Aerial parts mentioned in Abstract, leaves cited in main body of report)</p>	<p>Activity against all micro-organisms tested</p> <p><i>E. coli</i> <i>P. vulgaris</i> <i>S. faecalis</i> <i>E. aerogenes</i></p> <p><b>Ethylacetate slightly more effective extract than 80% ethanol</b></p> <p>At 5% concentration both extracts ≤ 20 mm Ø inhibition against <i>E. coli</i>; At 10% concentration 21-23 mm Ø (ethanol) and ≥ 24 mm Ø (ethylacetate). Both concentrations were ≥ 21 mm Ø for <i>P. vulgaris</i></p> <p>Activity of uva-ursi was 1/100 compared to streptomycin for ethylacetate, and 1/500 for ethanol</p>

Study	Extract Preparation/ Solvent	Results
Antimicrobial activity of selected plants employed in the Spanish Mediterranean area (Rios, Recio and Villar, 1987)	<p><b>Methanol</b></p> <p><b>Chloroform</b></p> <p>1:5 (w/v), yield 27.3% extract (1.36g)</p> <p>5 g/25 mL chloroform extract</p> <p>Dried down extracted in 25 mL MeOH</p>	<p><b>Methanol</b></p> <p><b>MIC g/L extract</b></p> <p><i>K. pneumoniae</i> – 1 (Gentamicin, 0.01)</p> <p><i>S. aureus</i> – 0.5 (Gentamicin, 0.01)</p> <p><i>M. phlei</i> &gt; 1 (Gentamicin, 0.01)</p> <p>MIC original dried plant material g/L</p> <p><i>K. pneumoniae</i> – 4.0</p> <p><i>S aureus</i> – 2.0</p> <p><i>M. phlei</i> &gt; 4</p> <p><b>Chloroform – no activity</b></p>
Antikikrobnalni pusobeni arbutinu a ekstraktu z listu medvedice lecive in vitro (Jahodar <i>et al.</i> , 1985)	Tested pure arbutin, and a decoction from leaves with $\beta$ -glucosidase	<p>Arbutin deemed responsible for most of antibacterial activity, found to be reliant on the presence of <math>\beta</math>-glucosidase.</p> <p>Minimum inhibitory concentration of arbutin was 0.4%-0.8%.</p>
Preliminary screening of Far-Eastern ethnomedicinal plants for antibacterial activity (Moskalenko, 1986)	<p><b>70% ethanol</b>, 1:5 maceration 10 days</p> <p>Added 400 <math>\mu</math>g/disc</p>	<p>Zone of inhibition</p> <p><i>E. coli</i> – 10-15 mm</p> <p><i>S. aureus</i> – 15-20 mm and 10-15 mm</p> <p><i>B. subtilis</i> – 15-20 mm</p> <p><i>Shigella sonnei</i> – 10-15 mm</p> <p><i>Shigella flexneri</i> – 10-15 mm</p>
Studies on dental caries prevention by traditional Chinese medicines (Part 1) Screening of crude drugs for antibacterial action against <i>Streptococcus mutans</i> . (Namba <i>et al.</i> , 1981)	<p><b>Water</b></p> <p><b>50/50 Methanol/water</b></p> <p><b>Methanol</b></p> <p>5g uva-ursi</p> <p>All extracted 1:20, Resulting powder prepared for test solution 1:1</p>	<p>Inhibitory action against <i>Strep. mutans</i></p> <p><b>Water – produced an inhibitory zone from 1.2 mg of extract</b></p> <p>Methanol, and methanol/water had indistinct inhibitory zone</p>

Study	Extract Preparation/ Solvent	Results
A pharmacometric evaluation of nine bio-strath herbal remedies (Leslie, 1978)	<b>30 % ethanol</b>  MIC (%)  <i>In vitro</i>  <i>Tested in vivo on rats and mice 2.5 ml/kg</i>	MIC – given as a % of formula  <i>E. coli</i> 1.25% <i>S. aureus</i> – 0.31% <i>B. subtilis</i> – 1.25% <i>Salm typhimurium</i> – 1.25% <i>Serr. marcescens</i> – 1.25% <i>P. aeruginosa</i> – 1.25%  <i>Caused zones of inhibition - unspecified</i>
The occurrence of antibacterial substances active against <i>Mycobacterium tuberculosis</i> in seed plants. (Gottshall, Lucas and Roberts, 1949)	<b>Cold Water</b> <b>Boiling water</b> <b>Ethanol</b>  Actual w/v extracted unstated	All 3 extractions tested against <i>M. tuberculosis</i> , <i>E. coli</i> and <i>S. aureus</i>  No effect against any bacteria.

### 3.5.2 Urine Samples and Alkalinity

It has been reported in herbal monographs as well as the European Medicine Agency's (EMA) Assessment Report on the herb that uva-ursi may require an alkaline environment for efficacy (Mills and Bone, 2005; EMA, 2012a; Bone and Mills, 2013). More specifically that free hydroquinone, considered the active constituent metabolised from arbutin, may only be detected in therapeutic concentrations in urine which has a pH over 8 (EMA, 2012a). In support of this view, the EMA cited papers by Frohne (1970) and Paper (1993) (EMA, 2012a), but in both these publications the claim is unsubstantiated (see below).

Frohne, a German scientist, undertook research on uva-ursi to clarify why, in his view, there were conflicting reports of the herb's efficacy as a urinary tract antiseptic, and to

investigate previous claims that uva-ursi required an alkaline environment to be effective. He conducted antimicrobial tests on the urine of healthy volunteers who had consumed one dose of either uva-ursi tea or an extract containing 800 mg of arbutin (Frohne, 1970). Frohne determined that there was no difference in antimicrobial activity of hydroquinone at alkaline pH *in vitro*, but reported that urine samples taken following ingestion of either uva-ursi or pure arbutin, had antimicrobial activity upon adjusting the pH to 8 using potassium hydroxide (KOH) compared to the control. He surmised that alkalinity was necessary to enable free hydroquinone to be deconjugated from the metabolite hydroquinone glucuronide, which he described as having 'apparent instability' in an alkaline environment (Frohne, 1970). Frohne concluded that for the herbal drug to be effective it required a large dose of the glycoside arbutin, in addition to alkaline urine (Frohne, 1970). However, it does not appear that the stability of the glucuronide itself was tested at pH 8, nor does the study quantify the amount of hydroquinone detectable after adjusting the urine to pH 8.

A Polish publication by Kedzia and colleagues followed up Frohne's work and tested 74 strains of bacteria isolated from patients with UTI (Kedzia *et al.*, 1975). Pure arbutin, as opposed to uva-ursi, was administered to one patient at doses of 1 g and 0.1 g. To alkalis the urine the volunteer was given 0.25 g of the medication Diuramidu. Urine was collected 3 hours after the administration of arbutin, or the arbutin/Diuramidu. The microorganisms were subsequently added to the urine and incubated at 37 °C for 18 hours. The results showed bactericidal activity against *E. coli*, *Proteus mirabilis* and *Pseudomonas aeruginosa* from 1 g of arbutin occurred between a pH range of 7.8 to 8.2. There was slight inhibitory activity against them from 0.1 g under the same conditions, but none at all at either dosage with acidic urine (pH 6) (Kedzia *et al.*, 1975). Whilst there was no antimicrobial activity from Diuramidu on its own, further investigation has shown that this product contains Acetazolamide – a drug sometimes used as a diuretic in heart failure (Kuzyp, 2007). This study, therefore, demonstrated antimicrobial activity from 1 g of arbutin at alkaline pH. This is higher than the total recommended daily dose of 840 mg (which is usually divided over 3-4 doses) and was based on only one volunteer. The study did not test uva-ursi per se.

The research by Paper and colleague's (1993) was unable to detect free hydroquinone at concentrations greater than 1 µg/mL in urine samples post ingestion of an uva-ursi extract taken with sodium bicarbonate (an alkalis agent). This was from two 250 mg tablets of uva-ursi containing a total of 100 mg of arbutin. The authors concluded that

sodium bicarbonate was deficient at alkalisng the urine sufficiently to produce therapeutic amounts of hydroquinone. Despite not having evidence to support Frohne's recommendation that alkaline urine was necessary for efficacy they stated that their findings agreed with Frohne (Paper, Koehler and Franz, 1993a). Nevertheless, a supplement publishing the results of a lecture on this study in a different journal (same authors) claimed that the formation of hydroquinone at pH 8 could not be confirmed (Paper, Koehler and Franz, 1993b).

The most recent research into the question of the efficacy of uva-ursi in an alkaline environment was conducted in 2003 by Siegers and colleagues. This contradicted the earlier findings, concluding that alkalinity was not necessary to free hydroquinone in urine after taking uva-ursi (Siegers *et al.*, 2003). The study focused specifically on the enzymes used to free hydroquinone from the metabolites hydroquinone glucuronide and hydroquinone sulphate, namely  $\beta$ -glucuronidase ( $\beta$ -GLn) and sulfatase. Following a preliminary study the authors demonstrated that incubating *E. coli* ATCC 6538 at  $10^6$  colony forming units/mL was more efficient at liberating hydroquinone than glusulase: an enzyme solution containing both  $\beta$ -GLn and arylsulfatase (to deconjugate hydroquinone from hydroquinone glucuronide and hydroquinone sulphate). Since glusulase deconjugates hydroquinone from both the metabolites it would be logical to conclude that there might be a greater amount of free hydroquinone present from this process than liberated via a strain of *E. coli* which expresses  $\beta$ -GLn but not sulfatase.

Further investigation into the method and conclusion revealed several weaknesses in the study. The *E. coli* strain used (ATCC 6538) is actually *S. aureus* not *E. coli* (American Type Culture Collection, 2014). It is not known whether the number is incorrect or it was a different organism. The enzyme solution glusulase may be most active at pH 5, whilst the optimal pH of  $\beta$ -GLn from *E. coli* is believed to be close to 7 (Zenser, Lakshmi and Davis, 1999; Feng, Elsohly and Duckworth, 2001). In fact, one study reported that *E. coli*  $\beta$ -GLn had an optimal pH of 6.3, and activity halved at both pH 4.3 and 8.5 (Leung *et al.*, 2001). Finally, the 4 participants were asked to follow a vegetarian diet to increase their urinary pH to  $\geq 6.5$  (Siegers *et al.*, 2003); vegetables as opposed to animal protein can increase the alkalinity of urine, but this aspect was not mentioned in their conclusion and the final pH achieved is unreported (Remer and Manz, 1995; Kanbara *et al.*, 2012). It should also be noted that one of the authors worked at the company who produced the uva-ursi product tested (Cystinol®) (Siegers *et al.*, 2003).

To date all the investigations into whether alkalinity of urine is necessary for the herb's efficacy have been on healthy volunteers. There have not been any studies on an individual currently suffering from a UTI.

### **3.6 Clinical studies**

There have not previously been any clinical trials to test the efficacy of uva-ursi, but it has been tested in combination with other phyto-medicines. In a double-blind randomised study with 57 women a product containing uva-ursi and dandelion root was shown to have a prophylactic effect against recurrent cystitis (Larsson, Jonasson and Fianu, 1993). To qualify for the study the patients had to have had 3 urinary tract infections in the previous 12 months, including at least one in the preceding 6 months. At baseline the patients both underwent a gynaecological examination to determine that they did not have a physical disorder which may cause recurrent cystitis, and also provided a urine sample to confirm whether they currently had a bacterial infection associated with cystitis. They were re-examined and re-tested at 6 months and 12 months. The study does not state how many of the previous infections had been confirmed by a bacterial culture or whether they may have had symptomatic abacteriuria (Larsson, Jonasson and Fianu, 1993).

The study concludes that the medication may act on bacteria which cause UTI, such as *E. coli*. Nevertheless, no assays were conducted either *in vitro* or *in vivo* to support this deduction. The introduction describes uva-ursi has having a possible bacteriostatic effect, but does not state against which organism (Larsson, Jonasson and Fianu, 1993).

A limitation of the study is the lack of data regarding the actual product in terms of quantifying the amount of uva-ursi and dandelion present in each tablet. This aspect is covered further in Chapter 5 (Section 5.6.2).

### **3.7 Safety Issues**

Potential toxicity is an important aspect of pharmacodynamics. Due to the planned use of the uva-ursi HMP in the ATAFUTI clinical trial this topic warranted a separate chapter (see Chapter 4).

## Chapter 4: Investigation into safety issues

### 4.1 Introduction

Concern has been raised over the fact that arbutin, considered the active constituent in uva-ursi, is metabolised in the gut to produce hydroquinone, because in large quantities hydroquinone may produce unpleasant side effects and could potentially be carcinogenic (NAT, 2006; EMA, 2012a).

#### 4.1.1 Uva-ursi and Arbutin – Side Effects

The European Medicines Agency (EMA) cite that there are no known cases of toxicity related to single or repeated doses of uva-ursi, nor are there any reported drug reactions (EMA, 2012a). Furthermore, the Agricultural and Food Agency in Canada, where bearberry is commonly grown and used, advise that the effective dose (see Chapter 5) and the toxic dose are so far removed from each other that the benefits of treatment outweigh the risks for self-medication (Agriculture and Agri-Food Canada, 2012). Nevertheless, a single case of bull's-eye maculopathy (damage to the retina) has been reported, reputedly due to uva-ursi ingestion (Wang and Del Priore, 2004). In this instance, it should be noted that a tea was consumed regularly over a three year period (Wang and Del Priore, 2004); this is considerably longer than the recommendations for uva-ursi or HMP's in general (EMA, 2012a).

A letter published on renal failure believed to be due to a herbal detoxification remedy branded as 'CKLS' (colon, kidney, liver, spleen) lists uva-ursi amongst 10 featured ingredients, but concludes that two other components (including cascara sagrada, a potential cause of nephrotoxicity) could have been responsible (Adesunloye, 2003). The same paper cites that uva-ursi has been associated with albuminuria, hematuria, and urine casts, but the statement is unsupported (Adesunloye, 2003).

Animal studies on the whole herb are virtually non-existent. The only example is an

aqueous extract of uva-ursi leaves administered to mice at a level of 50–100 mg extract/kg body weight, with no evidence of carcinogenic activity (WHO, 2002b). Nor are there any cited instances of toxicity in humans related to the ingestion of arbutin (EMA, 2012a). Studies on mice undertaken for 2 weeks have reported that there were no signs of toxicity at a dose of 8g/kg bodyweight (Li *et al.*, 1982).

Nevertheless, despite the lack of toxicological reports the advice regarding consumption of uva-ursi is to restrict it from 5 to 7 days (EMA, 2012a). The high tannin content could potentially lead to nausea and vomiting in people with sensitive stomachs although uva-ursi does not have a reputation for inducing symptoms of this nature (Blumenthal *et al.*, 1998). The main concern is likely to be due to the potential conversion of arbutin to the aglycone hydroquinone; hydroquinone is a metabolite of benzene, reported to be carcinogenic (Smith *et al.*, 2000).

#### **4.1.2 Hydroquinone**

Hydroquinone has for many years been a component of various commonly used chemical agents, such as photographic developing fluid, paints and motor fuels (DeCaprio, 1999; Sirajuddin *et al.*, 2007). It has also been incorporated as an ingredient of cosmetic creams as a skin lightening agent. Whilst its inclusion is now prohibited in skin creams in Europe and the United States, the glycoside arbutin is still permitted. Bacteria on the skin may in these circumstances convert arbutin to hydroquinone (Bang, Han and Kim, 2008). The risk of potential absorbance into the body via creams and inhalation of photographic developing chemicals has led to extensive animal studies being conducted on the toxicity of hydroquinone (Bang, Han and Kim, 2008).

##### **4.1.2.1 Hydroquinone Animal Studies**

The oral LD<sub>50</sub> of hydroquinone ranges from 300 to 1300 mg/kg body weight in rodents and dogs, but is only 42 to 86 mg/kg body weight in cats, because cats have a reduced ability to form glucuronide metabolites (Court and Greenblatt, 1997). Acute exposure of rats to high doses of hydroquinone (over 1300 mg/kg body weight) caused severe

effects on the central nervous system, including hyper-excitability, tremor, convulsions, coma and death (Inchem, 1994).

#### **4.1.2.2 Human Studies**

Human studies are minimal, as might be expected from what may be a potentially toxic substance. However, it has been reported that there were no pathological alterations in blood and urine on ingestion of 500 mg hydroquinone daily for 5 months (2 males), and 300 mg daily for 3 to 5 months (17 male/female volunteers) (Carlson and Brewer, 1953).

#### **4.1.3 Side Effects of Hydroquinone**

Exposure to hydroquinone is reported to cause tinnitus, nausea, dizziness, increased respiration, vomiting, pallor, muscular twitching, headache, dyspnoea, cyanosis, delirium, convulsions and even, at a dose of 1g (in an adult) collapse, and can be fatal at a dose of 5-12 g (Mills and Bone, 2005; Barnes, Anderson and Phillipson, 2007). Precautionary advice regarding these side effects and toxicity has been cited in pharmacological reports and herbal monographs. Amongst these are the European Medicines Agency's Assessment Report on uva-ursi (EMA, 2012a), the Merck Index (Merck Index, 2006), as well as US toxicological documents and manuals relating to hazardous materials, including the United States Centres for Disease Control (CDC) and Prevention database (CDC/NIOSH, 1994). A general exploration of the Google search engine focusing on keywords (hydroquinone, collapse, convulsions) in conjunction with just 3 of the cited side effects (tinnitus, syncope, and collapse), returns over two million hits for these phrases. Adding the specified affiliated dosages of 5-12 g to the search string still results in over 150,000 hits.

Whilst the cited fatal intake of 5-12 g, and even the potentially hazardous amount of 1 g, is much higher than the expected presence of hydroquinone following a therapeutic dose of uva-ursi (see Chapter 5), in light of the forthcoming clinical trial it was deemed prudent to investigate the original source(s) of these reports as well as the inclusion of the toxicity statements in herbal monographs on uva-ursi.

## 4.2 Aims

- To determine the basis of the citations relating to toxicity symptoms due to hydroquinone (for example, tinnitus, syncope, collapse).
- To investigate the origin of a 5-12 g dose of hydroquinone leading to a fatality.

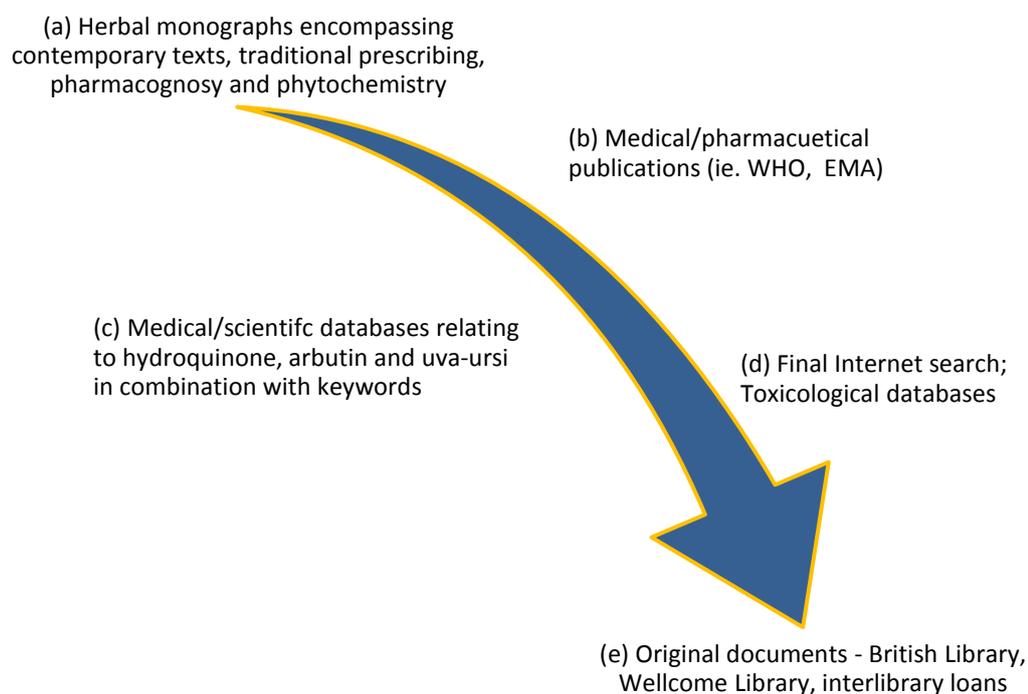
## 4.3 Method

A focused literature review searching the aforementioned symptoms and specified fatal dose was conducted between August 2012 and October 2014. For specialist knowledge both traditional and contemporary herbal text books and monographs were investigated, and their references tracked to the original source(s). International health agency publications which incorporate guidelines on herbal medicines (such as the WHO and EMA) were also examined.

To identify relevant physiological and chemical studies, a search was performed across 5 medical and scientific databases (The Cochrane Library, PubMed including Medline, Science Direct, SpringerLink, and Wiley Online Library). This was in addition to toxicological databases ToxNet, the Hazardous Substances Data Bank (HSDB), the National Toxicology Programme (NTP), the Environmental Protection Agency (EPA), and the US government Centres for Diseases Control and Prevention (CDC). The results incorporated journal articles, publications and toxicology reports on hydroquinone, arbutin and uva-ursi. At least one of these substances had to be featured with the following key-words and phrases singularly or in combination - 'toxicity', 'fatality', 'collapse', 'convulsions', 'tinnitus', 'syncope', 'dosage', 'side effects', '1 g', and '5-12 g'.

All study types (excluding animal assays) qualified for inclusion (for example, literature reviews, systematic reviews, meta-analyses, case control and observational studies). Searches included records featuring the relevant terms in the title, abstract and the text. Where abstracts were unavailable, and the title included hydroquinone and any of the keywords, the full text was acquired.

Original copies of relevant papers and journals were subsequently obtained from the British Library, Wellcome Library and via university inter-library loans in their original language (English, French, German, Polish and Czech). When required the results were translated into English. An overview of the search strategy is illustrated in **Figure 11**.

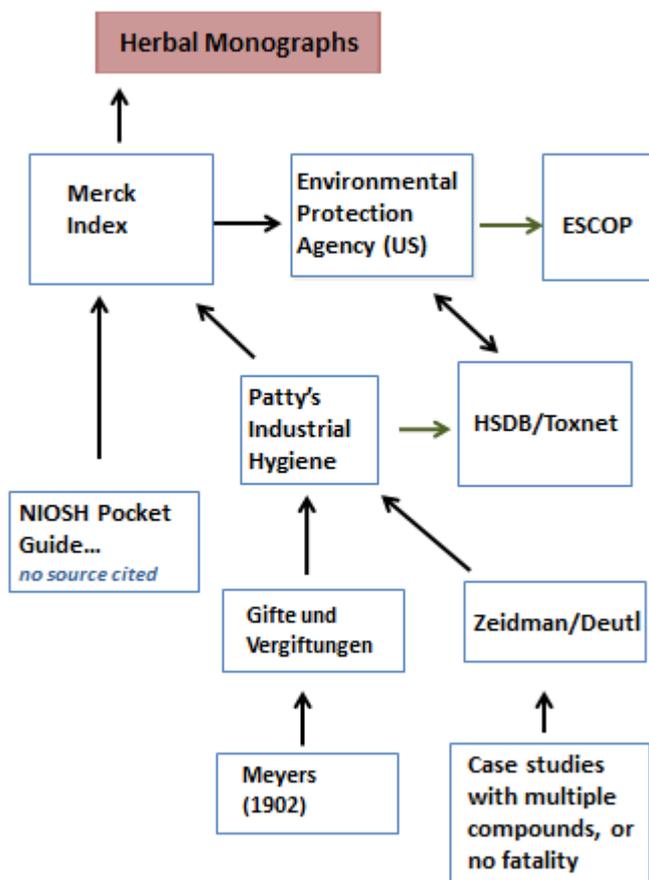


**Figure 11:** Flow diagram of sources/databases searched relating to toxicity of hydroquinone

## 4.4 Results

### 4.4.1 Herbal Texts/Monographs and Related Sources

Five out of a total of 14 reputable herbal texts (listed in Appendix A), including a natural medicines database, cited in their monographs the keyword symptoms/dosages related to the individual side-effects. These were the Essential Guide to Herbal Safety (Mills and Bone, 2005), Herbal Medicines (Barnes, Anderson and Phillipson, 2007), the ESCOP Monographs (ESCOP, 2003), the Clinicians Handbook of Natural Medicine (Pizzorno, Murray and Joiner-Bey, 2007), and Pharmacognosy, Phytochemistry, Medicinal Plants (Bruneton, 1999). Of these publications two (Barnes, Anderson and Phillipson, 2007; Pizzorno, Murray and Joiner-Bey, 2007) had sourced the Merck Index 11<sup>th</sup> edition and one (ESCOP, 2003) the EPA. The fourth did not publish a source (Bruneton, 1999), and the fifth did not provide a reference for the citation of 5-12 g being fatal (Mills and Bone, 2005). This latter text did, however, quote a reference for the symptoms including tinnitus, but this turned out to be an animal study (Woodard, Hagan and Radomski, 1949).



**Figure 12:** Origination of references leading to citations of 5-12 g of hydroquinone causing fatality

The EPA (cited by ESCOP) sourced the U.S. Department of Health and Human Services National Toxicology Information Programme, the Merck Index (11<sup>th</sup> Edition) as well as its own previously published documents (Arroll *et al.*, 2003). The Merck

handbook, credited as the basis of information regarding the symptoms and hazardous dose by at least 3 of the above publications, quoted both Patty's Industrial Hygiene & Toxicology and the National Institute for Occupational Safety and Health (NIOSH) Pocket Guide to Chemical Hazards as its sources (Merck Index, 2006).

The NIOSH Pocket Guide does not cite any origin at all for its data (NIOSH, 1997), whilst Patty's Industrial Hygiene & Toxicology refers to two sources. These comprise a 1929 German text entitled *Gifte und Vergiftungen (Poisons and Poisoning)* by Lewin for symptoms following the ingestion of 1 g hydroquinone, as well as a journal article by Zeidman & Deutl (1945) pertaining to a fatality post ingestion of 5-12 g (Clayton, 1981).

Of these *Gifte und Vergiftungen* contains a chapter on hydroquinone which reports on the effects that it has on rabbits at dosages of 0.75 g to 1.0 g, as well as the side effects of 0.8 g to 1.0 g on humans (including dizziness, tinnitus, increased respiration, delirium and collapse). However, it does not provide a source for this information. A separate chapter on uva-ursi in the same book recounts side effects of vomiting, cyanosis, irregular breathing and pulse and references a Dutch article from 1902 by Meyers (Dutch spelling Meijers).

The publication by Zeidman & Deutl refers to a case study of not 5-12 g hydroquinone, but to 15 g of a compound mixture of hydroquinone & monomethyl-p-aminophenol sulphate which resulted in two fatalities (Zeidman and Deutl, 1945). It also makes reference to 3 other publications, namely Halbron *et al* (1931), Busatto (1939) and Remond/Colombies (1927). The first two of these pertain to fatalities, but concern hydroquinone in combination with other compounds (4.5 mg benzcatechine plus 0.5 g methyl-p-aminophenol sulphate, and 2 g methyl-p-aminophenol sulphate respectively). The report by Remond/Colombies does actually refer to consumption of 12 g hydroquinone, but there was no fatality - the victim recovered after 24 hours (Remond and Colombies, 1927).

The 1902 (Meijers) article cited in *Gifte und Vergiftungen* pertains to a 48 year old female prescribed an uva-ursi decoction of 30:300 water (units unstated) who had suffered a cough for several weeks which triggered involuntary urination. She took a tablespoon on two occasions (afternoon/ evening of the same day). At night she experienced respiratory distress, nausea and vomiting. The next morning her pulse was irregular, and she had a rash resembling urticaria. Later that day her pulse and breathing normalised (Meijers, 1902).

#### 4.4.2 Organisations – Medicines and Health

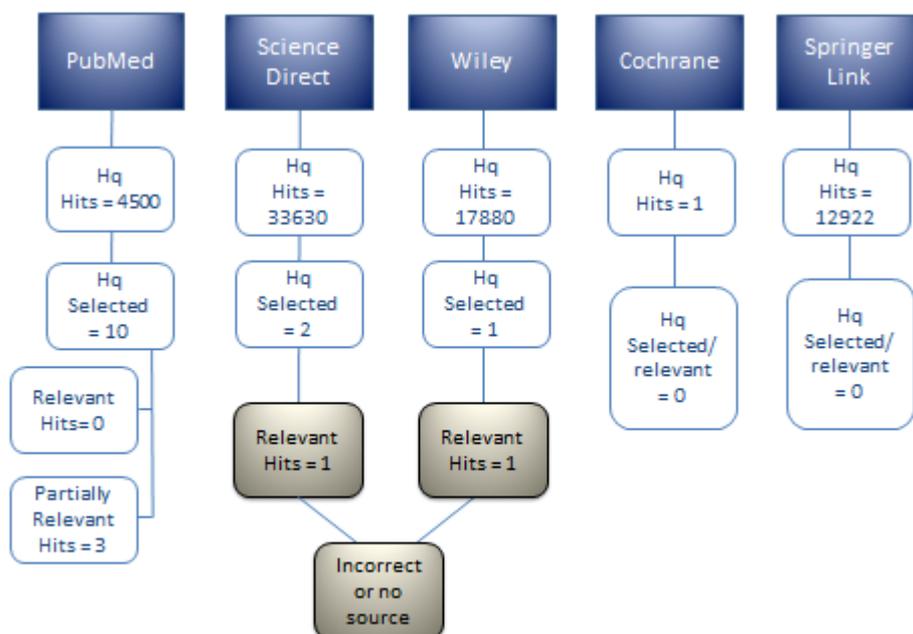
The Assessment Report on uva-ursi by The European Medicine's Agency describes the above symptoms and also cites a dose of 5 g causing fatality (EMA, 2012c). This organisation referenced Herbal Medicines 2008, an online database incorporated into Medicines Complete (Baxter, 2013), which in turn sourced the aforementioned edition of the Merck Index (11<sup>th</sup>) and the US Environmental Protection Agency.

The International Programme on Chemical Safety (INCHEM), published under joint sponsorship with the WHO and the United Nations Environment Programme, does not cite the keyword symptoms, with the exception of 'headache, dyspnoea, cyanosis and convulsions'. In section 1.7 entitled *Effects on Humans* it notes these symptoms occurred after ingesting hydroquinone alone or as a component of photographic developing agents. It also reports fatalities upon ingestion of developing agents containing hydroquinone, but no fatalities due to hydroquinone alone. No references are provided for this in a section 1.7 of the report, but sources relating to poisoning by hydroquinone as well as photographic developers containing hydroquinone are cited under section 8.1 *General Population Exposure* (Inchem, 1994). Fatalities are reported due to ingestion of developing fluids containing amounts of 3-12 g hydroquinone (80-200 mg/kg body weight), but the reference for this quantity is not provided.

The book of monographs by the WHO does not mention side effects in humans from oral intake (WHO, 2002a).

#### 4.4.3 Medical and Scientific Journals

Two papers on PubMed partially satisfied the inclusion criteria by virtue of the fact they contained a couple of the keyword side effects and mentioned 12 g (DeCaprio, 1999; de Arriba, Naser and Nolte, 2013). Finally, one paper on Science Direct mostly fulfilled the criteria in terms of keywords but its two references (International Agency for Research on Cancer 1977, and a second cited *Anonymous* which proved to be Sax's Dangerous Properties of Industrial Materials) did not correlate with the text (Devillers *et al.*, 1990). **Figure 13** illustrates the total number of results found on the databases.



**Figure 13:** Results of literature search on scientific databases

#### 4.4.4 Internet Search and Toxicological Databases

A final search on toxicological databases and Google returned a document which appeared in two separate sections. This was also present in its entirety on the CDC website, and contained relevant information pertaining to the commonly cited symptoms and dosages of 1 g, 5 g and 12 g (NIOSH, 1978). This report was originally produced by the US Department of Health Education and Welfare in association with NIOSH (1978) but its existence and referenced sources were omitted from the aforementioned NIOSH Safety Handbook. Further investigation into the sources established that these precise expressions and quantities were documented in 7 journal articles (British, French, German and American) across a range of reported cases from 1919 onwards. These articles together with the amount of hydroquinone ingested, the presenting signs and symptoms, and the outcome of the victim are summarised in **Table 5** together with a partially relevant original Polish paper on PubMed (Grudzinski, 1969).

**Table 5:** Toxicity case reports related to hydroquinone in chronological order

Original Source	Dose Ingested	Symptoms & Signs	Outcome
Mitchell and Webster, 1919	Estimated 20 grains (1.3 g) hydroquinone from 4 pints stomach washings. Total amount and actual substance ingested unknown (21 yr old female)	Unconscious, fast pulse, subnormal temperature, blood-stained foam at mouth. Convulsions. No diarrhoea. Clear lungs. Dark urine.	Recovered within 24 hours
Remond and Colombies, 1927	12 g hydroquinone (36 yr old male), attempted suicide	Tinnitus, swollen tongue, sense of suffocation, dyspnoea. Cyanotic skin, extreme fatigue. Fast, but regular pulse. Dark urine. Normal BP. No digestive problems.	Improved after 24 hours. Clear urine after 4 days. Blood normal after 13 days
Halbron, Bosquet and Tiffeneau, 1931	5g hydroquinone in photographic developing fluid, also present 4.5 g o-isomer hydroquinone (pyrocatechol), + 0.5 g methyl-p-aminophenol sulphate (female)	Unconscious in 15 minutes. Regained consciousness. Pain right hypochondrium. Dark urine + stool. Anuria day 9. Haemorrhagic kidneys.	Fatality Died day 12
Busatto, 1939	6 g hydroquinone + 2 g methyl-p-aminophenol sulfate ingested on 2 consecutive days (29 yr old female)	Hypotension, weak pulse, cyanosis, exhaustion, jaundice. Dark urine. No hydroquinone found in urine or stools.	Fatality Died day 6
Zeidman and Deutl, 1945	15 g = mixture of hydroquinone & monomethyl-p-aminophenol sulfate. Proportion unknown. (2 males)	Abdominal pain, vomiting, cyanosis, tachycardia.	Fatality Died 73 and 92 hours after ingestion.
Guyot, Bachelier-Notter and Dupret, 1966	Poisoning, autopsy = 1.35 g stomach, 17 mg/kg blood, 18 mg/kg liver, 17.5 mg/kg kidney, 9mg/ l urinary system. (27 yr old male)  <i>Quoted Sax</i> 5-12 g Photographic substances	Not reported	Fatality (suicide)

Original Source	Dose Ingested	Symptoms & Signs	Outcome
Grudzinski, 1969	3 to 5 g photographic developer 80% hydroquinone, 20% methyl-p-aminophenol sulphate (18 month child)	Pale skin, abdominal pain. Occasional spasms of limbs. Died day 2.	Fatality
Larcán <i>et al.</i> , 1974	Half glass photographic developer - approx. 0.8 g hydroquinone, + 4 other chemicals (0.15 g phenidone, 15 g sodium carbonate, 10 g sodium sulphite, 0.15 g potassium bromide (28 yr old female)	Convulsions. Posterior pharyngeal wall and palate suffered lesions. Acidosis, anaemia.	Recovered after 12 days

## 4.5 Discussion

Investigation into the original source of data published in herbal monographs regarding the potential side effects of hydroquinone (as cited above) and fatalities, at doses of 1g and 5-12 g respectively, has either lead to no citation of the evidence sourced or to a trail of misinformation.

There are several publications dating from 1919, which could potentially be the original source of concerns regarding the specific aforementioned doses of 1g and 5-12 g. The first of these, a 1919 British Medical Journal article, had investigated previous reports of slight hydroquinone poisoning from 0.5 to 1 gram, but claimed not to be able to find any detailed records (Mitchell and Webster, 1919). Their own study, which formed the basis of their paper, concerned a 21 year old female who was admitted unconscious to hospital suspected of hydroquinone poisoning. She suffered convulsions, but recovered three days later. Stomach washings produced around 20 grains (1296 mg) of hydroquinone from 4 pints, but the precise total amount of the compound originally ingested could not be determined (Mitchell and Webster, 1919).

The Remond/Colombies report of 1927 (published in French) concerned a 36 year old man who swallowed 12 g hydroquinone in an attempt to commit suicide (Remond and Colombies, 1927). This could perhaps be the basis for citations referring to 12 g of the compound causing fatality; it is the only mention of this precise amount in any of the sources. However, the victim did not die, but made a complete recovery in 24 hours (Remond and Colombies, 1927). Four years later this paper was followed by an article by Halbron *et al*, who recounted a fatality due to hydroquinone. This refers to 5 g hydroquinone, and could be the origin of subsequent citations relating to this quantity. Scrutiny of the article shows that the victim did not in fact ingest just 5 g of hydroquinone, but actually consumed photographic developing fluid containing 5 g hydroquinone, as well as 4.5 g of an *o*-isomer of hydroquinone (pyrocatechol) plus 0.5 g methyl-*p*-aminophenol sulfate (Halbron, Bosquet and Tiffeneau, 1931). The quantity ingested was, therefore, double the amount of hydroquinone along with 0.5 g of a compound which, reportedly if swallowed in quantities less than 150 g, can cause breathing difficulties and symptoms of cyanosis as well as being potentially fatally harmful (Ernst, 1999).

Ziedman and Deutl (1945) addressed the physical effects of previous experimental studies on mice and cats and the aforementioned cases highlighted by Remond and Colombies and Halbron *et al*, as well as reporting on the physiological effect of cases of two men who had each taken around 15 g of developing powder, which contained a mixture of hydroquinone and mono-methyl-paraminophenol sulphate, which they had confused with Epsom salts (magnesium sulfate). Ziedman and Deutl hypothesised on this basis that hydroquinone could cause fatality through affecting the central nervous system. This deduction may be justifiable but was based on dosages injected into mice which were as high as 17% of their body weight, and studies on cats. There was no mention that cats do not have the ability to form hydroquinone glucuronide for safe excretion. These were in addition to case studies on humans which referred to the consumption of compound products containing hydroquinone as well as one case of pure hydroquinone where the victim survived.

The Polish publication by Grudzinski, which was partially relevant, refers to the keyword of 5 g amount of hydroquinone but as a suggested upper limit of an estimated 3-5 g range of intake. As well as mentioning three of the keyword symptoms (pallor, vomiting and convulsions), it led to a fatality (Grudzinski, 1969). The responsible agent was a photographic developer containing hydroquinone rather than the pure substance, but the amounts and symptoms could have reinforced existing perceptions.

All the papers examined either related to the ingestion of hydroquinone as a component of photographic developing fluid, or in combination with another potentially lethal substance. In the only examples on hydroquinone where it is adduced as the sole constituent the victim either recovered (Remond and Colombies, 1927) or the precise total amount consumed is not known but surmised from an autopsy examining different organs (Guyot, Bachelier-Notter, and Dupret, 1966). This latter paper makes a direct reference to a cited danger of 5-12 g of hydroquinone, and uses Sax as its authority for this information. However, the Sax publication does not provide a source (Sax, 1963).

The 1902 publication (Meijers), the origin of resultant evaluations on hydroquinone, was based on a case study whereby the patient suffered symptoms related to two tablespoons of an aqueous decoction 30:300 (w/v). A decoction involves boiling followed by simmering the herb until the liquid is reduced by at least a quarter. It is usually used to extract constituents from woody plant materials rather than leaves. In this article there is no information about how the decoction was prepared and to what extent the water was evaporated, or what units of herb were used. For 30:300 it would likely be 30 g in 300 mL (approximately a cup) of water. The maximum daily dose for an infusion, a weaker preparation than a decoction, is 12 g divided over 3 to 4 cups. The patient in this instance had a preparation based on 2.5 times the advised total daily dose and consumed it in half a day.

The two papers which partially qualified for inclusion in the results as they contained a couple of keywords (DeCaprio, 1999; de Arriba, Naser and Nolte, 2013) were unrelated to the chain of publications connected to herbal monographs, health agencies and pharmaceutical manuals. One of these mentioned symptoms related to ingestion of photographic developing fluids, but did not reference any primary sources (de Arriba, Naser and Nolte, 2013). The second, published in 1999, sourced original papers from 1919, which are cited in this chapter, and it reported on ingestion of 12 g hydroquinone when the victim recovered (DeCaprio, 1999). This paper was not referenced by any of the herbal texts or monographs which referred to a fatality at a dose of 5–12 g. It was published after the pharmaceutical and toxicology texts (for example, the Merck Index 11<sup>th</sup> edition, and Sax's *Dangerous Properties of Industrial Materials*) which were used as major sources of information.

## 4.6 Conclusion

There were no reports of toxicity in the literature from taking uva-ursi, despite it being used for many centuries.

Whilst there are reasons to be cautious and aware of the potential toxicity from a high intake of hydroquinone, the warnings regarding specific amounts of 1 g and 5-12 g do not appear to have been checked against the original sources, and are misleading. The amounts have only been reported as part of a cocktail of photographic development chemicals. This highlights the importance of referring to original documents whenever possible when producing reports for publication, rather than citing a secondary reference.

Finally, the quantities of 1 g and 5-12 g far exceed the dose of hydroquinone which could be ingested from herbal preparations. The traditional dose for uva-ursi is investigated in the next chapter, and the bioavailability of hydroquinone in the urinary tract is discussed further in Chapter 7.

## Chapter 5: Determination of therapeutic dose of *Arctostaphylos uva-ursi*

### 5.1 Introduction

Establishing an effective and safe dose of any medication is a vital requirement. There are as yet no statutory worldwide documents or publications which can be referenced for official Western herbal posology, despite being able to trace back over thousands of years the written evidence of the use of botanical medicines (Robson and Baek, 2008). The British National Formulary (BNF), which publishes pharmacological data and prescriptive recommendations on orthodox drugs for use in primary care, contains only a small amount of information and advice on herbal products. Limited to just senna and St John's Wort (Joint Formulary Committee, 2015), this is possibly due to a lack of clinical evidence regarding the efficacy of phyto-pharmaceuticals.

Pharmacopoeias for individual countries and continents, on the other hand, provide reference data for the quality control of herbal medicinal products (as well as orthodox drugs), and whilst most, including the European Pharmacopoeia (Ph. Eur.), are legally binding documents, not all are. Nor do all territories even produce their own pharmacopoeial monographs (Mandal and Mandal, 2011; Ph. Eur., 2013), and whilst these publications provide details of the constituents required in the verification of a finished herbal product they do not publish guidance on dosage for a herbal medicinal product (HMP). The constituents may be defined according to an established and approved therapeutic marker, if one is known. If not, an analytical marker of a phytochemical constituent of the plant may be used for quality control. In the case of uva-ursi the European Pharmacopoeia states that it needs to contain no less than 7% anhydrous arbutin, calculated according to the anhydrous herbal drug (Ph. Eur., 2013).

Changes to the law introduced in 2011 in the European Union (EU) over the sale and prescribing of herbal products resulted in the introduction of the European Traditional Herbal Medicinal Products Directive (THMPD) 2004/24/EC (MHRA, 2011). This in turn led to the publishing of phyto-pharmaceutical Assessment Reports and Community Herbal Monographs for individual herbs including a profile for uva-ursi (EMA, 2012a, 2012b). These monographs have been produced by the Committee on Herbal

Medicinal Products (HMPC) and include proposed dosages. They and the German Commission E, a German advisory board formed in 1978 who published monographs on 380 medicinal herbs between 1984 and 1994 (Blumenthal *et al.*, 1998), are the only official guidelines on dosages for different preparations of herbs for the EU (EMA, 2012b). None are legally binding.

The Medicines and Healthcare Products Regulatory Agency (MHRA), who have to ratify the clinical trial dose for ATAFUTI before the trial can commence, holds the view that the EMA reports may be sufficient when an application is made to register a traditional herbal product for commercial use, but with the proviso and reservation that they may not wholly agree with every aspect of the monograph (MHRA, 2007). Consequently it is important that not only the above EU monographs are adhered to when proposing a dose for the planned clinical trial, but that all additional sources of data on posology and prescribing are also examined and taken into account before submitting a dosing document to the MHRA for approval. These would encompass the EMA assessment report, as well as traditional herbal text books and compendiums containing monographs with recommendations on dosage. In addition, existing clinical studies with volunteers which have administered uva-ursi require scrutinising for dosages prescribed and any reported adverse effects. An alternative approach might be to also investigate the dosage taught by academics to herbal medicine students.

For the ATAFUTI clinical trial the proposed dose of the uva-ursi HMP has to be consistent with effectiveness observed in clinical practice, it needs to be safe and, so it cannot be identified as an herbal product, it must be matched by an identical placebo. Arbutin is commonly considered to be the active ingredient (Frohne, 1970), so the total amount of this constituent should be taken into account when determining the posology. There have been concerns expressed that the amount of tannins present could cause digestive upsets, as well as the possible carcinogenic activity of free hydroquinone (Yarnell, 2002), especially if the kidneys are continually exposed to this constituent. However, a major review on hydroquinone safety concluded that there was minimal nephritic risk to humans (DeCaprio, 1999).

## 5.2 Aims

- To determine an effective (and safe) therapeutic dose of uva-ursi for use in the ATAFUTI clinical trial, establishing the maximum daily acceptable quantity of arbutin.
- To attain authorised ratification of the proposed dose from the MHRA.

## 5.3 Method

### 5.3.1 Literature

A focused literature review was conducted to investigate official and traditional recommendations of dosages in July 2012 (at the planning stages of ATAFUTI) and reviewed in January 2014 (prior to submitting recommendations to the MHRA). This encompassed searching the sources detailed in sections (a) and (b) in **Table 6**.

**Table 6:** Sources of data/information on dosing used in search strategy

(a)	Contemporary English language herbal monographs in compendiums, text books and assessment reports used in academia, and also published by official bodies and institutions. Searched by hand and the Internet.
(b)	An Internet search across published peer-reviewed papers and articles on the databases PubMed, Science Direct, Embase and the Cochrane library in order to investigate dosages employed in clinical studies or administered by herbal practitioners using uva-ursi. Keywords ' <i>Arctostaphylos uva-ursi</i> ', 'arbutin', 'bearberry' (common name for uva-ursi), 'clinical', 'trial', 'dose(age)', 'herbal medicine', 'administration', 'metabolites', 'urinary tract infection', 'urine' and 'posology' were searched. There were no restrictions imposed regarding uva-ursi in combination with other herbs, or the published language. All selected papers were read in full.
(c)	Dosages advised by professionals and academics through writing directly to the individuals concerned, and requesting information on prescribing uva-ursi as experienced or taught in their professional capacity.

### **5.3.2 Analysis**

The minimum/maximum amount of arbutin estimated to be present in each individual and daily dose of uva-ursi was calculated according to the type of herbal preparation, the percent of arbutin generated by the method of extraction used, and the number of prescribed doses. Where the dried herb was referred to (either on its own or as the basis of another preparation), 7% arbutin was used. This is in accordance with the European Pharmacopoeia (Ph. Eur. 2013). The resultant quantities from each literature source were then compared.

In evaluating the recommended dose for the clinical trial the investigation into the potential safety of the constituents, especially the conversion of arbutin to hydroquinone, was also taken into consideration (see Chapter 4).

Based on the findings the proposed dose was presented to the ATAFUTI Trial Management Group for their approval, before being incorporated into the trial application submitted to the MHRA.

## **5.4 Results**

### **5.4.1 Monographs (Compendiums, Text Books, Assessment Reports)**

Sixteen monographs on uva-ursi were identified. Removing publications where the authors and dosages were duplicated reduced this total to twelve. A further five monographs had sourced at least one of the other seven as their reference material, and these were discounted in the final analysis.

**Table 7:** Monographs on uva-ursi

	Literature	Source of Original Monograph/Used in Analysis
(a)	Herbal Medicines (Barnes)	Sourced (e) and (f)
(b)	A Clinical Guide to Blending Liquid Herbs (Bone, K)	(c) <i>The Essential...</i>
(c)	The Essential Guide to Herbal Safety (Mills & Bone)	
(d)	Principles and Practices of Phytotherapy (Mills & Bone)	
(e)	British Herbal Compendium Volume 1	(e) .... <i>Compendium</i>
(f)	British Herbal Pharmacopoeia	
(g)	Complete German Commission E Monographs (The)	(g)
(h)	European Medicine Assessment	(h)
(i)	European Scientific Cooperative on Phytotherapy (ESCOP)	Frohne 1977* and (n)
(j)	Holistic Herbal (Hoffman, D)	(k) <i>Medical Herbalism</i> Sourced (e) and (g)
(k)	Medical Herbalism (Hoffman, D)	
(l)	Physicians' Desk Reference (PDR) (Gruenwald <i>et al</i> )	(l)
(m)	Weiss's Herbal Medicine	(m)
(n)	Wichtl (ed) Herbal Drugs and Phyto-pharmaceuticals	Sourced (g)
(o)	World Health Organisation	Sourced (g) and (j)
(p)	Medicinal Plants of the World (Van Wyk, B, Wink, M)	(p)

\* Frohne 1977 unpublished review at *Kooperation Phytopharmaka, Germany*

#### 5.4.2 Internet Search - Scientific Databases

Five papers reporting on clinical studies which met the criteria were attained via the database search. Of these, four related to small clinical studies involving volunteers (with less than 20 participants), and the fifth numbered 57 participants in a double-blind controlled trial who were administered a commercial product 'UVA-E'.

A major study conducted on uva-ursi by Frohne in 1970 assayed the amount of arbutin derived from the leaves via water extractions, both hot and cold, before administering uva-ursi to an undisclosed number of volunteers (Frohne, 1970). These results have been included to facilitate a comparison and analysis with the other clinical and volunteer studies.

No published papers pertaining to dosages of uva-ursi prescribed by herbal practitioners were found, but two journal articles on treatment of urinary tract infections by botanical medicines incorporated this plant. Both referred to the dosages supported by the German Commission E, either directly or through referencing one of the above volunteer studies (Yarnell, 2002; Abascal and Yarnell, 2008). The search also produced the aforementioned toxicity report on hydroquinone by de Arriba *et al* (see Chapter 4); this paper cited dosages published by the WHO, ESCOP and the German Commission E (de Arriba, Naser and Nolte, 2013).

### 5.4.3 Professional Opinion

Three practising and academic Medical Herbalists, two of whom are both authors of herbal texts, provided information on dosages taught.

## 5.5 Findings

### 5.5.1 Preparations and Traditional Dose

The literature revealed a high level of consistency across the seven monographs examined with regard to the maximum daily quantity of herb used – 12g. The maximum amount of arbutin present was 840 mg unless a concentrated dried standardised extract was produced when (total) estimates ranged from 640 mg to 760 mg. Recommended dosages for different preparations corroborated each other when converted to dried herb equivalent (Blumenthal *et al.*, 1998; WHO, 2002a; Mills and Bone, 2005; Gruenwald, Brendler and Jaenicke, 2008). **Table 8** summarises the recommended minimum to maximum daily dose according to the variety of specified preparations used, and provides the expected amount of arbutin present according to the traditional dose.

**Table 8:** The recommended dose of uva-ursi and estimated arbutin content for different herbal preparations for treating UTI (based on traditional dose)

Preparation	Uva-ursi Content Per Day	Arbutin Content Individual Dose	Frequency Of Dose/Day	Estimated Dose Per Day Arbutin*
Dried Herb	3–12 g	7% minimum	Divided over 4 doses	210 mg–840 mg
Dried Herb capsules	10-12 g	7% minimum	6 capsules 4 times a day	700 mg-840 mg
Extract Solvent: EtOH 60% 5.5:1	2.2 g (from 12 g)	23.5 – 29.3%	Divided over 3 doses	520 mg–640 mg
Extract Solvent: H <sub>2</sub> O 4.5:1	2.7 g (from 12 g)	20 – 28%	Divided over 3 doses	540 mg–760 mg
Infusion	3-12 g	7% minimum	Divided over 3–4 doses	210 mg–840 mg
Fluid Extract (1:1)	3–12 mL	7% minimum	Divided over 3 doses	210 mg–840 mg
Tincture (1:5)	6–12 mL (not direct comparison to fluid extract i.e. not 5 times the dose)	7% minimum	Divided over 2-3 doses	210 mg–840 mg

\* the recommended daily minimum amount of arbutin is 400 mg

(Bradley, 1992; Blumenthal *et al.*, 1998; van Wyk and Wink, 2004; Mills and Bone, 2005; Gruenwald, Brendler and Jaenicke, 2008)

The EMA guideline of dosage for all preparations is shown in **Table 9**. It includes the dried herbal powder which generates a maximum of 840 mg arbutin; this quantity equates with the traditional literature. The monograph also contains two dried extracts

based on two separate solvent extraction processes, namely 60% ethanol and 100% water (EMA, 2012a).

**Table 9:** EMA guideline on dose for uva-ursi by herbal preparation

Preparation/ Original Solvent	Arbutin Content/ Individual Dose	Frequency of Dose/ Day	Estimated Total Dose/Day Arbutin
1.5–4 g infusion or macerate infused in 150 mL boiling water for 10-15 mins, or cold for 30 mins  Maximum 8 g dried herb *	<i>not stated – will vary</i>	2 – 4	<i>not stated – will vary</i>
Powdered herb	100–210 mg hydroquinone derivatives calculated as anhydrous arbutin	2 – 4	200–840 mg
Dry extract (3.5-5.5:1) ethanol 60% (v/v)	23.5%-29.3% hydroquinone derivatives calculated as anhydrous arbutin	2 - 4	200–840 mg hydroquinone derivatives
Dry extract (2.5-4.5:1) water	20-28% hydroquinone derivatives calculated as anhydrous arbutin	2 – 4	200–840 mg

(EMA, 2012b)

### 5.5.2 Clinical Dose

The only double-blind controlled trial to be published utilised a commercial brand 'UVA-E' produced to treat cystitis (Larsson, Jonasson, & Fianu, 1993). This also contained *Taraxacum officinalis* (dandelion root), and was tested successfully as a prophylactic against UTI with 57 women. Three tablets were administered three times a day, but the actual amount of uva-ursi present in the product was unspecified (Larsson *et al.*, 1993). It was prescribed for 4 weeks, which is longer than the 7 days advised (EMA, 2012b); no side effects were recorded.

Out of the four small studies conducted with healthy volunteers on pharmacokinetics two used a small amount of the proposed daily dose - only 945 mg, reported to contain 22% arbutin (Glockl, Blaschke and Veit, 2001; Schindler *et al.*, 2002). The third employed a commercial product 'Cystinol', with a total quantity of 420 mg arbutin (Siegers *et al.*, 2003), whilst the fourth administered an aqueous extract over four doses which totalled 600 mg of arbutin (Quintus *et al.*, 2005).

The dosages from the volunteer studies and the trial, together with the amounts of arbutin generated from the *in vitro* study, are featured in **Table 10**.

**Table 10:** Dosages used in previous clinical studies on uva-ursi, including the total arbutin content (chronological order)

Study/ Sample Size	Uva-ursi Content	Arbutin Content	Frequency of Dose/Day	Estimated Total Arbutin Dose/Day
<i>In vivo</i> study followed by <i>in vitro</i> antimicrobial analysis. Sample size unspecified (Frohne, 1970)	2g/150 mL water	168mg-189mg	N/A	168mg-189mg
	10g/150 mL water 10 minute hot water; 30 minute cold water extraction	447mg-864mg	N/A	447mg-864mg
57 women (Prophylactic effect of UVA-E) (Larsson, Jonasson and Fianu, 1993)	Dose not reported	N/A	3 tablets tds	N/A
Sixteen volunteers (50/50 male/female) (Schindler <i>et al.</i> , 2002)	945 mg  Either dry extract from leaf (472.5 mg x 2 tablets) or aqueous solution from 945 mg	210 mg (22%)	One dose	210 mg
One volunteer (Glockl, Blaschke and Veit, 2001)	945 mg extract	210 mg	One dose	210 mg
Four volunteers (Siegers <i>et al.</i> , 2003)	Extract 3.5 5.5:1 dry uva-ursi = 238-297.5 uva-ursi  Extracted ethanol 60% v/v  Cystinol (trade name)	70 mg	2 tablets tds (6 tablets)	420 mg
Three volunteers (Quintus <i>et al.</i> , 2005)	Unspecified amount aqueous leaf extract based on German Commission E	150 mg	Four doses	600 mg

### **5.5.3 Professional Opinion on Dosing**

The dosages used by academics who responded to the enquiry varied as follows:

Kerry Bone, principal of the Australian College of Phytotherapy, a practising Medical Herbalist, industrial chemist, and author of several herbal publications, bases his dose of 12 g over 3 – 4 doses on the British Herbal Pharmacopoeia, the German Pharmacopoeia and the German Commission E.

Julie Whitehouse, Medicinal Herbalist and a former course leader of the BSc in Herbal Medicine at the University of Westminster, prescribes 25-30 mL a week of tincture, which is about half the maximum traditional dose.

Michael McIntyre, Chair of the European Herbal and Traditional Medicine Practitioners Association (EHTPA) and also a practising Medical Herbalist and author of several herbal books, prefers to administer a tea. At 20 gm infused in 500 mL of water (across 2-3 doses daily) he prepares a higher dose than the traditional dose in **Table 8**.

## **5.6 Discussion**

### **5.6.1 Traditional Dose**

Uva-ursi has been used as a traditional herbal medicine for UTI for hundreds of years, but as yet there is no legal agreed dosage for it. Nevertheless, seven published monographs derived from text books and assessment reports from the UK, Europe and Australia on Western phytotherapy, including the German Commission E, showed a high correlation across the advised posology. These publications had also been referenced by at least a further five monographs, including the World Health Organisation, who also cited the German Commission E. The total amount of dried herb to be administered daily was 12 g, divided over 3 or 4 doses - thus providing a maximum of 840 mg arbutin. The variety of herbal preparations included in the recommendations (powder, concentrated extract, dried herb for tea, tinctures) reinforced the consistency of the total quantity of arbutin expected to be present regardless of the form in which the herb may be administered.

The dosage guidance in the EMA assessment report for the powdered herb and extract corresponds to 100–210 mg anhydrous arbutin 2 to 4 times daily, thus also providing a maximum total of 840 mg arbutin (EMA, 2012a). This is consistent with a 12 g dose of dried uva-ursi. Nevertheless, the EMA proposed posology for an infusion was limited to a maximum daily dose of 8 g (dried herb). The latter is restricted due to an expected variation in the quantity of arbutin following spectrophotometric analysis by an herbal tea company; they discovered 10% arbutin present rather than the expected 8% (EMA, 2012a). This difference could provide almost 1 g of arbutin in a day. It is a variation which can be controlled with the manufacturing of a standardised extract.

### **5.6.2 Scientific Papers and Reviews on Botanical Treatment of UTI**

Aside from two reviews on botanical medicines for UTI there were no published research papers on scientific databases which provided information or advice regarding an effective and safe dosage of uva-ursi for UTI. Nor did the literature search return any published papers which had specifically explored dosing practices of herbal medicine in general. Of the two reviews, one directly cited the German Commission E monograph (Yarnell, 2002), whilst the second referred to a dose of 3 g used in an analysis by Quintus *et al* (Abascal and Yarnell, 2008). Although this latter study qualified the amount of arbutin ingested according to recommendations by the German Commission E, the actual quantity of raw herb administered in their investigation was unspecified (Quintus *et al.*, 2005); this second review, therefore, appears to have made an assumption that 3 g was ingested.

The remaining 3 volunteer studies (which investigated pharmacokinetics rather than therapeutics) together with the only double-blind trial to be conducted on uva-ursi did not qualify their dosing strategy or rationale for the amount prescribed. The studies on pharmacokinetics (also previously discussed in Chapter 3) reported that free hydroquinone was detected in the urine of only half the participants who took uva-ursi, and was at a level less than 1% the original dose (Schindler *et al.*, 2002, Quintus *et al.*, 2005). The amount present from an infected person (with a UTI) is not known. The double-blind trial, which returned promising results for its product as a UTI prophylactic, simply stated that it contained an aqueous/alcohol extract of the leaves with a

standardised amount of arbutin and methyl-arbutin (Larsson, Jonasson and Fianu, 1993) – thus prohibiting any comparison to the traditional dose. Providing the actual quantity of uva-ursi could have benefitted future trials especially as there were no reported side-effects, despite taking the herb four times longer than is usually recommended.

### 5.6.3 Primary Sources of Data

The German Commission E, which was established as a result of a new German Drug Act in 1978, produced the oldest of the monographs retrieved and is possibly the most influential. Its monographs have been adopted by several others, in addition to the dose being used in the investigative study by Quintus (Quintus *et al.*, 2005). However, it does not provide any reference for its data, and the publication has previously come in for criticism due to the brevity of the information, the possible lack of impartiality of the authors, and the lack of evidence for the suggested pharmacological actions (Ernst, 1999).

The in-depth study on uva-ursi by Frohne investigated the amount of arbutin extracted by hot and cold water, and reported a maximum of 683 mg arbutin from 10 g uva-ursi from 15 minutes (hot) extraction, and 864 mg post 30 minutes (cold) extraction (Frohne, 1970). In light of the solubility of arbutin in water, and the proven anti-microbial properties of a decoction<sup>1</sup> of uva-ursi, these preparations should achieve a dose close to that recommended in the monographs.

Frohne cites the amount of 10 g as being the recommended dose by Hagar's Pharmaceutical Practice (1938) and Kreitmair's Pharmazie (1953), and may have assayed 10 g on this basis. However, whilst the average volume of arbutin produced from the cold water extraction (864 mg) virtually equated both the quantity in the German Commission E and now suggested by the EMA (840 mg) as the daily dose, the original recommendation by both Hagar and Kreitmair was to drink 10 g not once but several times a day, thus ingesting a much greater amount. Frohne wrote an unpublished paper in 1977 which supports the total of 840 mg used by the German Commission E and ESCOP (ESCOP, 2003), but the paper is not accessible. It is

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<sup>1</sup> Preparation derived from plant material which has been boiled

deposited at the BfArM in Berlin, and can only be viewed by an attorney or scientific organization in relation to the German Medical Act (American Botanical Council, 2013). The 840 mg could possibly relate to the amount of free hydroquinone released in the body and its proposed therapeutic effect, but without the paper there is no means of substantiating this theory or providing a firm conclusion as to why the amount of consuming 10 g *several times a day* has not been adhered to. It may even be unconnected to the amount of hydroquinone present and could relate to the concern regarding the amount of tannins (known to cause digestive upsets).

An investigation into a statistically robust sample of doses taught by the academic community was not practical. However, renowned academic and scientific herbalists were invited to advise what dosage regimes they taught and used in their herbal practice. Out of the three who were approached one adhered to the guidance of the German Commission E, the second used around half this dose, whilst the third preferred to prepare infusions using a larger dose, thus illustrating how herbalist's preparations and posology may vary depending on their experience in practice as well as potential differences in the products sourced.

#### **5.6.4 Recommended Dose for Clinical Trial**

To keep the number of capsules to a minimum for ease of administration, and to correlate with the dosing regime of ibuprofen (which was another arm of the ATAFUTI trial), it was necessary to prescribe an extract of uva-ursi rather than 12 g of the pure powdered herb. Standardised extracts are permitted under the EU Directive, providing it can be demonstrated that the proposed herbal product does not exceed the traditional dose (MHRA, 2012).

A medicinal herbal capsule size '0' holds 400 mg to 500 mg of powder, depending on the density of plant material. Therefore, in order to provide a dose of arbutin equivalent to 12g of uva ursi (therapeutic dosing range of 400 mg to 840 mg arbutin) it was necessary to use a 5:1 extract (eg. 2.4 g per day) to contain not less than 20% arbutin. The resultant six capsules would be divided across three doses. Splitting the total quantity across three doses would support both the traditional dosing regime, and the

discussion in Section 3.4.1 reporting that the metabolites appear in the urine as quickly as 2-3 hours of taking uva-ursi.

The recommended duration of use in the trial is a minimum of 3 days and up to a maximum of 5 days; this will be in line with the published guidelines of a maximum of 7 days for uva-ursi (Blumenthal *et al.*, 1998).

## **5.7 Conclusion**

Despite the absence of an official consensus on posology, not just for this herb but for any botanical medicine, there was remarkable consistency of dosing recommendations for uva-ursi across the range of publications and sourced data. These dosages enabled an estimated quantity of arbutin to be calculated, providing a range from the minimum to the maximum expected amount present. The lowest content, derived from 3 g of pure dried leaf converted to 210 mg arbutin, and the highest comprised 840 mg arbutin from 12 g.

There can quite clearly be variations in the amount of arbutin present, but a standardised extract produced in line with the EMA recommendations of a maximum dose of 840 mg arbutin may have therapeutic efficacy based on traditional use, and be within a safe amount of hydroquinone, but it is yet to be verified within a clinical trial. The pharmacokinetic studies on volunteers did not provide therapeutic data.



## **Chapter 6: Quality control of herbal medicinal product**

### **6.1 Introduction**

#### **6.1.1 Background**

In order to proceed with the ATAFUTI clinical trial it was necessary to source and identify a suitable powdered herbal extract which met approved 'good manufacturing practice' (GMP) guidelines. The GMP regulations were originally set out by the World Health Organisation (WHO) for pharmaceutical products, and the European Union has since formulated similar GMP requirements (WHO, 2005).

A product could then be produced in the UK which could conform to the 'investigational medicinal product' (IMP) standards required for testing in a clinical trial. This chapter discusses the specifications which need to be accommodated for quality control in choosing a product, and how the HMP extract was subsequently selected through testing by high performance liquid chromatography (HPLC).

#### **6.1.2 Official Guidelines**

The European Medicines Association (EMA) and the European Pharmacopoeia (Ph. Eur.) both publish recommendations concerning the quality control of HMPs, with the EMA advising that the Ph. Eur. analytical procedures are adhered to whenever appropriate (EMA, 2011; Ph. Eur., 2013). These specifications cover macroscopic and microscopic examination of the herbal product, the phytochemical characterisation of known constituents of therapeutic value, or which are deemed valuable markers, as well as the method of preparation of the herbal drug and the detection of impurities. Chromatography tests such as HPLC and thin layer chromatography (TLC) densitometry, or a combination into a single procedure such as HPLC/UV-diode array

or Liquid-Chromatography-Mass Spectrometry (LC-MS), may be accepted for identification (EMA, 2011).

There are no recommended assays to cover stability testing of each individual HMP due to the complexity of the products. The guidelines provide a range of assays and procedures for consideration, but recommend that the manufacturer propose their own tests on stability for detecting changes during the shelf-life (EMA, 2011). Whilst these may include accelerated stability tests (EMA, 2014) without any prior testing of the product or herb in question there could initially be unforeseen problems which develop over a period of time, such as degradation of the main compound. Any degradation is not considered acceptable should it occur within 3 months of the original manufacturing date (EMA, 2014).

### **6.1.3 Standardised Extracts**

The quantity of desired constituents in phytomedicines can vary due to the environment in which the plants are grown, such as the soil and the climate, as well as the season and growing stage when they were harvested. These factors can, therefore, influence the quality of the final product (Applequist and Miller, 2013). The drying and storage of plant material, as well as methods of extraction can also affect the quality of the herb (Heinrich, 2015).

To help avoid such variation, standardised extracts are prepared specifically to contain a precise and reportedly safe quantity of a defined constituent which is usually known or believed to have therapeutic activity. The standardisation is often achieved through adjusting the preparation with the addition of excipients (EMA, 2010). Based on the dosing strategy (Chapter 5), in order to contain up to 840 mg of arbutin, the product proposed for use in the ATAFUTI clinical trial needed to be a standardised extract to contain a minimum of 20% arbutin.

Regarding *uva-ursi* the Ph. Eur. outlines tests for the dried herb which include HPLC and thin layer chromatography (TLC) analyses, using reference standards of arbutin and hydroquinone for quantification. The individual monograph itself does not yet

contain any specific guidelines for a concentrated extract such as a product containing 20% arbutin (Ph. Eur., 2013).

#### **6.1.4 HPLC**

HPLC is a technique commonly used in analytical chemistry for separating compounds of different polarities, and is now often used in the analysis of natural products. It works on the principle that there needs to be a strong attraction between the solvent(s) used in the mobile phase and the analytes of interest. Using pressure, these two components are both passed through, and will subsequently be retained on, a column (usually) of the opposite polarity. The time the analyte(s) retains on the column, known as the retention time ( $t_R$ ) is recorded via a detector. The detectors commonly use UV light which is absorbed by the compounds at varying wavelengths. Chromatograms are then produced, according to the UV spectrum recorded, which show a series of peaks at different  $t_R$ . Each of the peaks represents a compound contained in the plant (Heinrich *et al.*, 2004).

#### **6.1.5 Voucher Specimens**

Voucher specimens of plants usually comprise pressed and dried herbarium specimens. These include a mixture of plant parts for identification (for example, the flower, fruit, roots), with recorded data of when and where they were collected (Eisenman, Tucker and Struwe, 2012). The plant material is identified and verified by a qualified botanist using a published source for corroboration, and then deposited in a certified collection. The specimens serve as physical evidence to confirm the identification of a species, and are important in natural product research for reproducibility (Eisenman, Tucker and Struwe, 2012).

The use of voucher specimens is essential for the correct identification of medicinal plants, in order to help verify the chemical content (which can vary between species), and to determine the presence of adulterants and contaminants (Applequist and Miller, 2013). Anatomical features are used to help identify unprocessed plant material, such

as anatomical and morphological characteristics of the leaf, flower, fruit and roots. Processed plant parts for commercial use cannot be authenticated visually, so there is a risk that powdered, or even simply cut material, could be adulterated with a less valuable species, or even just contaminated with weeds during harvesting (Applequist and Miller, 2013).

#### 6.1.6 Sample GMP Products of *uva-ursi*

Three commercial GMP samples for consideration for the clinical trial were obtained for quantification of arbutin and hydroquinone, as well as for the qualitative comparison to voucher specimens. These GMP products were sourced from Germany and Serbia. The details of the products are shown in **Table 11**, which also lists the original extraction solvents used. The most efficient method of extraction is important when analysing the desired chemical components. For example, a mixture of ethanol and water (in particular) is efficient at extracting phenolic compounds, whilst ethanol alone extracts both polar and nonpolar compounds due to its alcohol and methyl groups.

**Table 11:** Country of origin and the original extraction solvents used for the GMP herbal products assayed for potential use in the clinical trial

GMP Sample	Country of Origin	Extraction Solvent
HMP A	Germany	100% H <sub>2</sub> O
HMP B	Serbia	60/40 EtOH/H <sub>2</sub> O
HMP C	Germany	60/40 EtOH/H <sub>2</sub> O

## 6.2 Aims

In order to select a product for the trial it was necessary to:-

- Determine which of the three commercial HMP products consistently contained 20% arbutin during repeated analyses.
- Certify that the product is *Arctostaphylos uva-ursi*, through comparison with an authenticated plant specimen.

## 6.3 Methods

### 6.3.1 Chemicals and Reagents

Analytical HPLC-grade water and methanol were supplied by Fisher Scientific (UK). Hydroquinone (purity 99%+) and arbutin (purity 98%+) reference standards were obtained from Sigma-Aldrich and Alpha Aesar respectively, and were stored at -20°C. Gallic acid, used for identification rather than quantification, was obtained from Sigma-Aldrich.

The three HMP extracts were stored in their original containers in a refrigerator under laboratory conditions.

### 6.3.2 Extraction

Samples for HPLC analysis were extracted according to two methods:-

*Method A:* in order to comply with the Ph. Eur. assay for *uva-ursi folium* the powdered extract (200 mg) was added to 20 mL distilled H<sub>2</sub>O and heated under a reflux condenser on a water-bath for 30 minutes, cooled, and filtered through a plug of absorbent cotton. The cotton was added to the residue and re-extracted in 20 mL H<sub>2</sub>O

under identical conditions. The filtrates were combined and diluted to 50 mL, then filtered through filter paper and the initial 10 mL was discarded. The supernatant was analysed by Reversed-phase (RP) HPLC in a mobile phase of 90:10 H<sub>2</sub>O:MeOH (Ph. Eur., 2013).

*Method B:* was adapted from a previously tested extraction procedure using 100 mg powdered extract sonicated in 25 mL distilled H<sub>2</sub>O for 30 minutes (Parejo *et al.*, 2001). Four different solvent ratios of water and methanol were assayed to determine the most efficient extraction: 100% H<sub>2</sub>O, 95:5 H<sub>2</sub>O:MeOH, 100% MeOH, 50/50 H<sub>2</sub>O:MeOH. The supernatants were analysed by HPLC in a mobile phase of 95:5 H<sub>2</sub>O:MeOH.

All samples were filtered through 0.45 µm PTFE membrane filters (Millex), and centrifuged in eppendorffs at 13000 rpm for 15 minutes before pipetting into 1 mL vials for HPLC.

### 6.3.3 HPLC

Chromatography samples were analysed using an Agilent 1200 series comprising an Agilent 1200 degasser (G1322A), quaternary pump (G1311A), standard auto-sampler (G1329A), thermostatted column compartment (G1316A) coupled to an Agilent 1200 diode array and multiple wavelength detector SL (DAD) (G1315D). Samples of 20 µL were injected via the autosampler onto an Agilent Zorbax Eclipse RP (5 µm) XDB-C18 column (4.6 x 150 mm) maintained at 30°C.

UV spectra were recorded in the range of 220 to 360 nm at a sampling rate up to 80 Hz, with analysis via Agilent 1200 ChemStation software. The gradient flow rate was set at 1 mL/minute with mobile phase for method **A** 90:10 H<sub>2</sub>O:MeOH and for method **B** 95:5 H<sub>2</sub>O:MeOH for 0-4 minutes, followed by a linear gradient to 70:30 from 5-30 minutes for both methods, and 100% MeOH from 31-35 minutes. An equilibration period ran for 5 minutes before the next injection. Blanks containing the mobile phase were run at the start and end of each analysis to check for any contaminants in the system or on the column.

### 6.3.4 Identification and Quantification of Arbutin and Hydroquinone

Arbutin and hydroquinone were identified through comparing their retention times on the UV spectra with the standard reference compounds, and the purity of the peaks were verified via the DAD analysis. For quantification, seven concentrations (serially diluted) of the arbutin reference standard (Ar) were analysed at: 1 mg/mL, 0.75 mg/mL, 0.5 mg/mL, 0.375 mg/mL, 0.25 mg/mL, 0.187 mg/mL and 0.125 mg/mL. The hydroquinone reference was prepared by diluting 2.5 mg of the standard in 10 mL of the mobile phase, and to 5 mL of this solution 2.5 mL of arbutin was added and re-diluted to 10 mL with the mobile phase (Ph. Eur., 2013). This was analysed at concentrations of 0.125 mg/mL, 0.094 mg/mL, 0.063 mg/mL, 0.047 mg/mL and 0.032 mg/mL.

UV detection for arbutin and hydroquinone was set at 280 nm, band-width 4, with reference signal 'off'. For confirmation of the compounds, additional signals were recorded at 220 nm, 254 nm and 290 nm.

Calibration curves ( $y = ax + b$ ) were produced by plotting the peak areas ( $y$ ) versus the concentrations ( $x$ ) of the calibration standards. Quantification of the HMPs were then confirmed against the calibration curve and the formula in the Ph. Eur (Figure 14) (Ph. Eur., 2013). Microsoft Excel © was used for data analysis.

$\frac{F_1 \times m_2 \times p}{F_2 \times m_1}$	$F_1$ = HPLC peak area due to arbutin in the HMP $F_2$ = HPLC peak due to arbutin reference compound $m_1$ = mass of the drug to be analysed $m_2$ = mass of <i>arbutin R</i> in the reference solution $p$ = percentage content of arbutin in reference compound
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Figure 14: Quantification calculation for arbutin and hydroquinone

The commercial samples were analysed in duplicate, and the sample with the most consistent results (sample **A**) received further investigation. This was analysed a third time, and the procedure was repeated in triplicate (inter-day) 12 months later to assess

any changes in the quantity of arbutin and hydroquinone. The sample and reference compounds were prepared afresh for the repeat runs.

### **6.3.5 Method Validation**

#### **6.3.5.1 Specificity**

Specificity is the ability of the method to unequivocally assess the study analytes in the presence of other components in the sample. In this study specificity was assessed through adopting the European Pharmacopoeia method for preparation of the standards, evaluating the peak separations and comparing the chromatograms of the blank, the standards and the sample solution (ICH, 1996).

#### **6.3.5.2 Sensitivity**

The limit of detection (LOD) and limit of quantification (LOQ) were calculated from the calibration curves using the standard deviation of the response ( $S_y$ ) and the slope ( $S$ ) according to the following equations:  $LOD = 3 \times S_y/S$ , and  $LOQ = 10 \times S_y/S$  (ICH, 1996).

#### **6.3.5.3 Linearity**

The correlation coefficient ( $R^2$ ) for the calibration curves (linear regression of the peak area versus concentration levels for arbutin and hydroquinone) were used to evaluate the linearity, thus demonstrating that the detector and data acquisition may show a linear response over the concentration ranges of the reference compounds (LoBrutto and Patel, 2007).

#### **6.3.5.4 Accuracy**

Accuracy was verified by the recovery test: through adding known quantities of the reference solutions of arbutin and hydroquinone to a known quantity of the GMP herbal extract. These were added at three different concentrations for arbutin (0.38 mg/mL, 0.5 mg/mL and 1 mg/mL) and two concentrations for hydroquinone (0.031 mg/mL and 0.063 mg/mL). Each solution was injected (20 µL) in triplicate, intra-day. The recovery was calculated by taking the mean result of the three spiked injections.

#### **6.3.5.5 Precision**

To ensure repeatability the precision was evaluated through inter-day variability tests, analysing the reference samples and a GMP product on six different days.

#### **6.3.6 Qualitative Analysis**

Several voucher specimens from different eras and locations were obtained from Kew (supply agreement No. MSA2-2013). The voucher codes for the samples are included in Appendix B. A photo of a sample used in the analysis is shown in **Figure 15**.

The voucher specimens were extracted using the same methodology (w/v) as the commercial products, using 95:5 H<sub>2</sub>O/MeOH.



**Figure 15:** Herbarium sample from Kew used in HPLC analysis. Collected in August 1995 from France (photos by Jeanne Trill)

## 6.4 Results

### 6.4.1 Sample Preparation - Extraction Solvent

HMP (**A**) dissolved quickly and without residue in 100% H<sub>2</sub>O, 95:5% H<sub>2</sub>O:MeOH and 50:50 H<sub>2</sub>O:MeOH. When dissolved in 100% MeOH some sediment remained and required a further extraction.

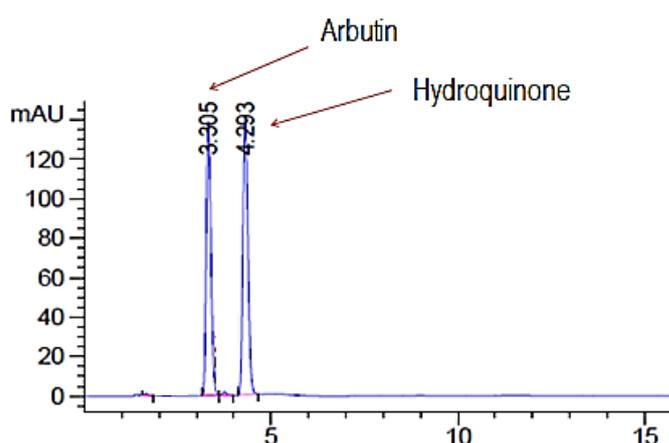
HMP (**B**) dissolved readily in 100% MeOH and 50:50 H<sub>2</sub>O:MeOH. It also dissolved in 100% H<sub>2</sub>O and 95:5% H<sub>2</sub>O:MeOH but the supernatant in both these latter two solvent ratios was more cloudy than for HMP (**A**).

HMP (C) dissolved completely in 100% MeOH and 50:50 H<sub>2</sub>O:MeOH, but required two further extractions to remove all solids when extracted with the more aqueous solutions of 100% H<sub>2</sub>O, and 95:5% H<sub>2</sub>O:MeOH.

#### 6.4.2 Optimisation of HPLC Method

The extraction of 90:10 H<sub>2</sub>O:MeOH in an equivalent mobile phase eluted with retention times ( $t_R$ ) of 2.4 and 3.4 minutes for arbutin and hydroquinone respectively, their identities confirmed by comparison with the reference compounds. This was 1.5 minutes faster than the proposed Ph. Eur. of 4 minutes for arbutin. Altering the mobile phase to 95:5 H<sub>2</sub>O:MeOH improved retention on the column, and delayed the  $t_R$  by one minute. The peaks for this method were characterised by 3.3  $t_R$  for arbutin and 4.3  $t_R$  for hydroquinone (reference samples): this was the method which was then adopted for analysing the commercial products. Gallic acid was also identifiable, and showed an earlier peak of 1.4  $t_R$ .

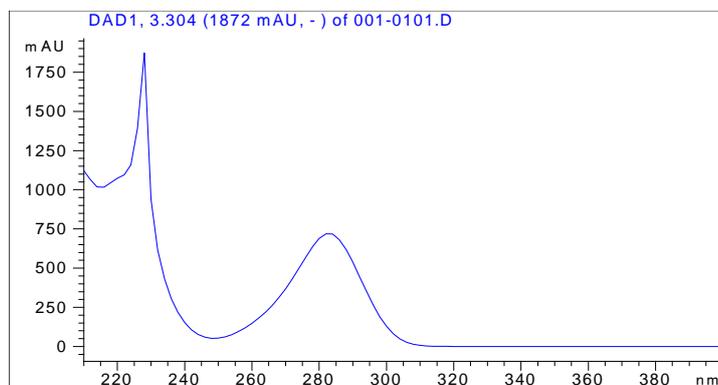
Sharp and symmetrical peaks were generated for the two reference compounds to be quantified at 280 nm (see **Figure 16**).



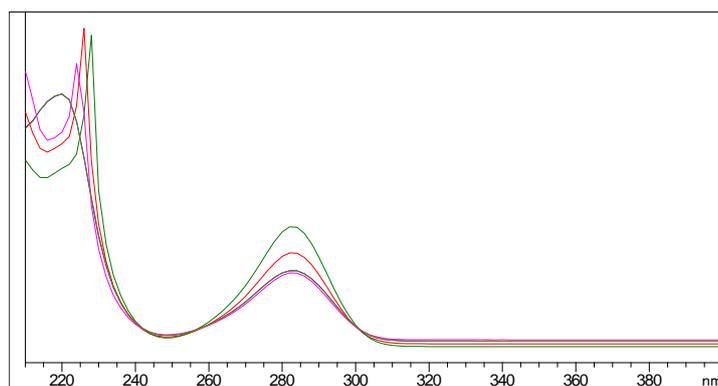
**Figure 16:** HPLC chromatogram of reference samples for arbutin and hydroquinone. Arbutin shows a ( $t_R$ ) 3.3, and hydroquinone a ( $t_R$ ) 4.3

At 3.3 minutes ( $t_R$ ) the UV-DAD spectra of the signals depicted a large sharp peak at 220 and a smaller peak at 280 for arbutin. At 4.3 minutes ( $t_R$ ) the spectra showed a broader large peak at 220 with a smaller peak at 290 for hydroquinone. These, together with spectra showing the purity of signals, are shown in **Figure 17**.

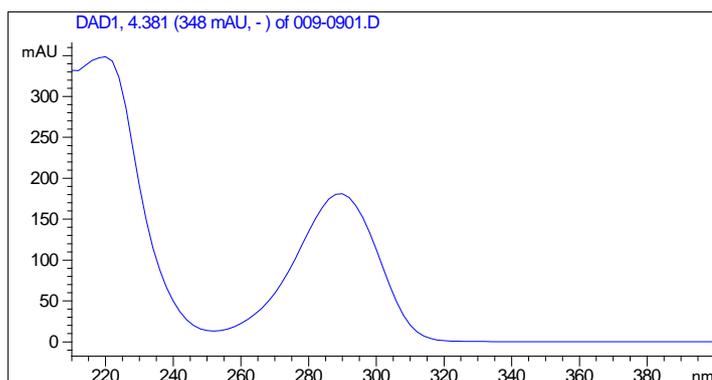
(a)



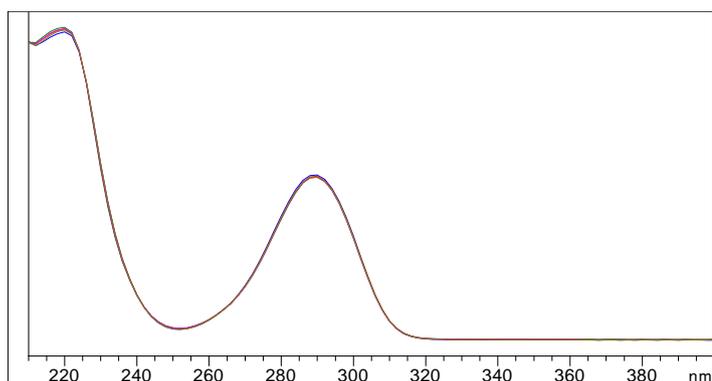
(b)



(c)

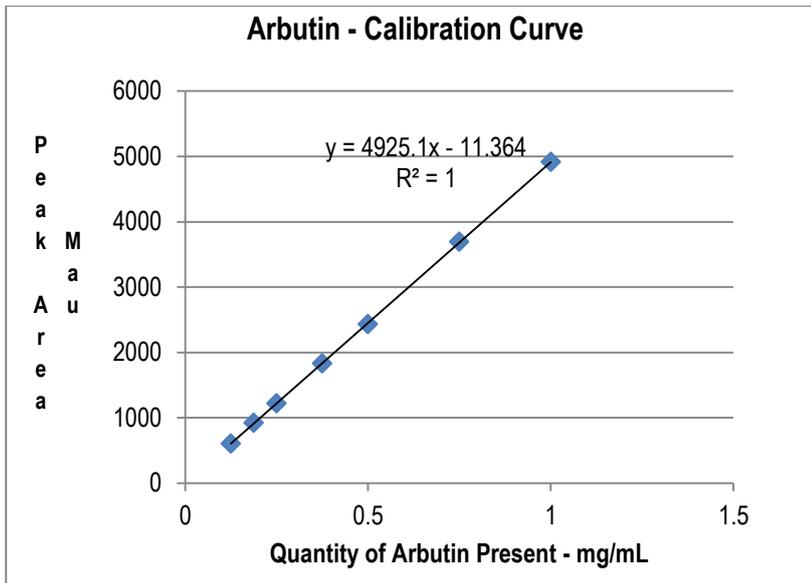


(d)

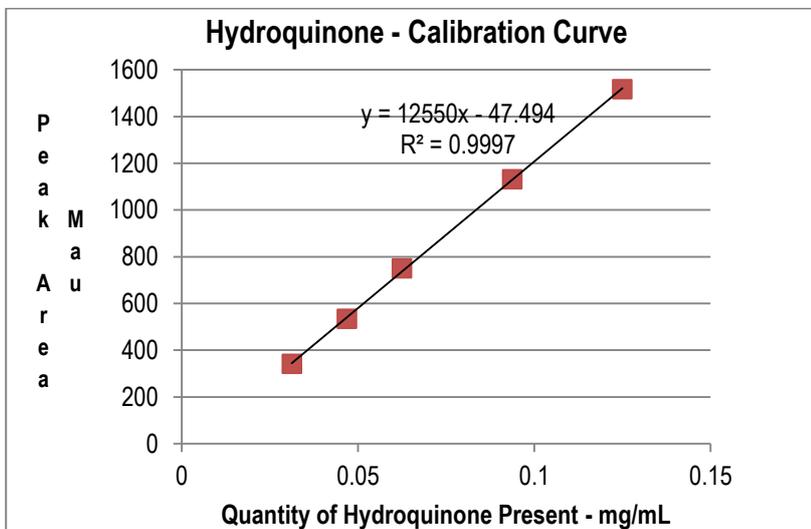


**Figure 17:** HPLC UV spectra showing purity of signals for arbutin and hydroquinone (a) arbutin profile at 3.3 minutes; (b) purity of signal for arbutin (all reference signals); (c) hydroquinone profile at 4.3 minutes; (d) purity of signal for hydroquinone (all reference signals)

The linear calibration curves for both arbutin and hydroquinone showed a good correlation between the peak areas measured and the concentrations of the standards. The calculated coefficient of determination for measuring the linearity based on the seven concentrations of arbutin was  $R^2 = 1$  (Figure 18). For the five concentrations of hydroquinone it was  $R^2 = 0.997$  (Figure 19).



**Figure 18:** Calibration curve for arbutin



**Figure 19:** Calibration curve for hydroquinone

The limits of detection (LOD) for arbutin and hydroquinone based on 20  $\mu$ L injections are depicted in **Table 12** below.

**Table 12:** HPLC Limit of Detection and Limit of Quantification of arbutin and hydroquinone reference compounds

	LOD µg/mL	LOQ µg/mL
Arbutin	0.61	2.0
Hydroquinone	0.52	1.7

The average recoveries (%) for arbutin and hydroquinone spiked in the HMP extract are shown in **Table 13**.

**Table 13:** Results of accuracy based on percentage recovery of known concentrations of arbutin and hydroquinone

	Amount Spiked mg/mL	Mean Recovery (%)	Precision (RSD %) Intraday
Arbutin	1	102.3	0.14
	0.5	103.5	1.11
	0.38	105.3	0.51
Hydroquinone	0.063	99.3	0.49
	0.031	105.8	0.50

### 6.4.3 Quantification of Arbutin and Hydroquinone (via DAD)

The total quantity of arbutin present in HMP (A) and HMP (B) comprised 20% in each product. The results of quantification of HMP (C) were inconclusive using the same solvent extraction ratio of 95:5 H<sub>2</sub>O:MeOH.

**Table 14:** % of arbutin contained in each commercial GMP product

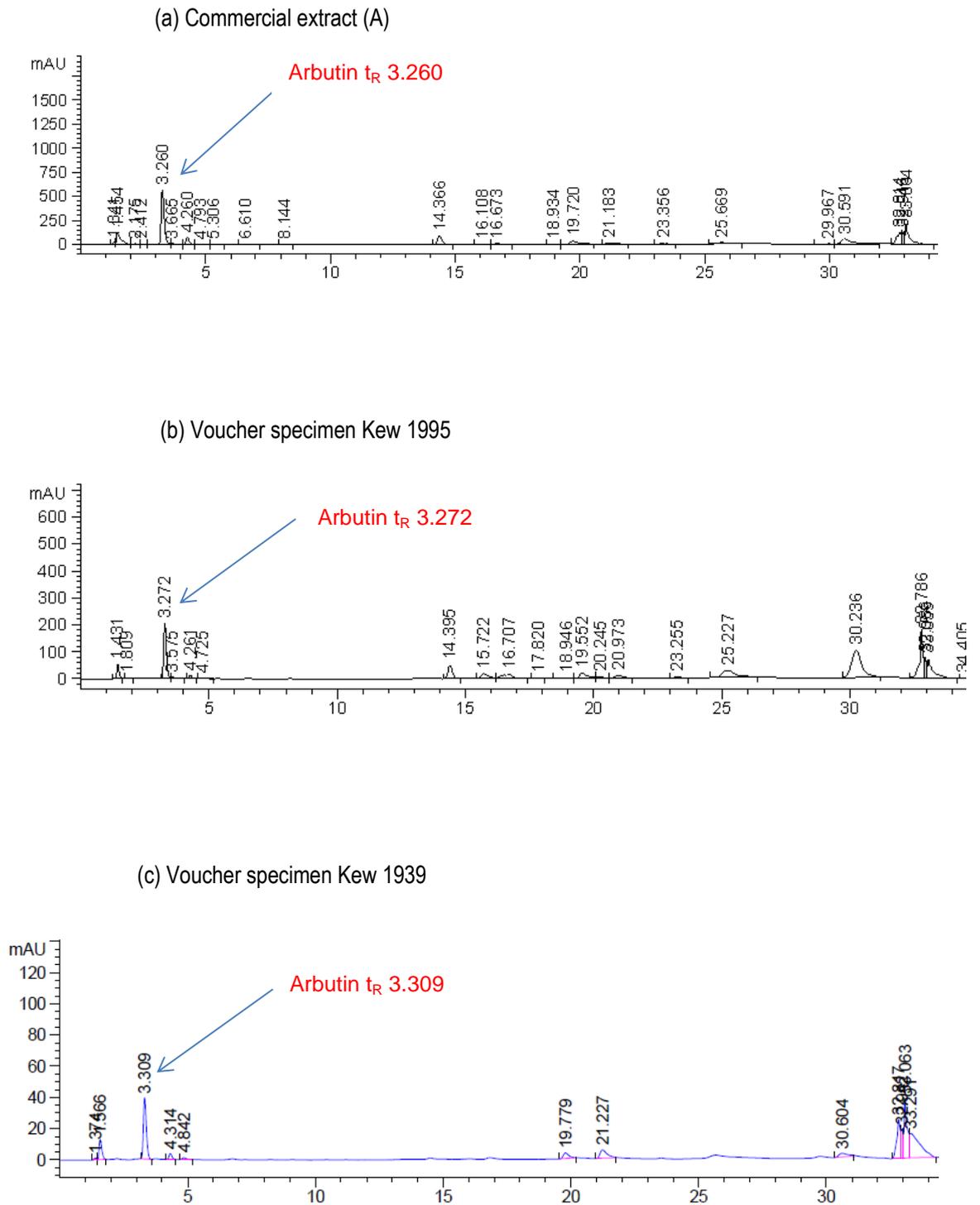
GMP Product	% Arbutin
HMP A	20.05
HMP B	19.9
HMP C	Required 50% MeOH in order to dissolve

The mean amount of arbutin in HMP (A) tested in triplicate in May 2013 was 20.046%. Assayed in triplicate one year later it measured 20.998%. This is not a statistically significant difference (with a 95% confidence interval).

HMP (A) contained 1.86% hydroquinone. A year later it totalled 1.2%.

### 6.4.4 Qualitative Analysis – Voucher Specimens

The commercial extract (A) displayed an almost identical HPLC chromatogram to a voucher specimen from France (1995). There were seven small additional peaks (peak areas under 100 mAU) for the GMP product compared to the voucher specimen. Older voucher specimens (prior to 1995) contained far fewer peaks, but all contained arbutin and hydroquinone. Chromatograms of the commercial extract and two of the Kew voucher specimens are shown in **Figure 20**.



**Figure 20:** HPLC chromatograms of commercial uva-ursi sample compared to voucher specimens from Kew  
 (a) commercial uva-ursi extract (A); (b) voucher specimen collected in France in 1995;  
 (c) voucher specimen collected in Poland in 1939

## 6.5 Discussion

### 6.5.1 HPLC Method Development

The method development of the sample extraction and the chromatographic conditions resulted in good resolution and separation of peak areas for both the standards to be quantified: arbutin and hydroquinone. This is critical for quantitative analysis. The method also facilitated the identification of a third compound relevant to the integrity of the plant material: gallic acid. As arbutin is more polar than hydroquinone due to the glucose moiety, it eluted one minute earlier in the polar mobile phase of 95:5 H<sub>2</sub>O:MeOH at 3.4 minutes compared to 4.4 minutes. Gallic acid is even more polar than arbutin and hydroquinone, and it eluted earlier at 1.4 minutes.

When compared with other reported analyses for quantification of arbutin and hydroquinone by HPLC, this method showed both similar and/or higher sensitivity and precision. The LOQ for arbutin and hydroquinone were 2 µg/mL and 1.7 µg/mL respectively. The study by Parejo and colleagues, on which the method of extraction was based, did not report either the LOD or the LOQ achieved by their HPLC analysis (Parejo *et al.*, 2001). Rychlinska and Nowak, on the other hand, who published a quantification study in 2012 on the presence of arbutin in 16 plants, reported slightly higher LOQ for arbutin and hydroquinone at 3.07 µg/mL and 1.5 µg/mL. This was also based on an injection of 20 µL (Rychlinska and Nowak, 2012).

### 6.5.2 Extraction Solvent and Quantification of Compounds of Interest

The highest concentration of arbutin was demonstrated in the commercial samples dissolved in 100% H<sub>2</sub>O. Since arbutin is highly polar, this could be expected. This finding resulted in water being used as the solvent for the extractions in the follow-up analyses one year later (using 95:5 H<sub>2</sub>O:MeOH solvents for the mobile phase and reference samples). Product (**A**) provided precisely the 20% arbutin content required for use in the clinical trial, and was consistent across the repeat runs. Product (**B**) was similar to (**A**), but was slightly below 20%. Product (**C**) was rejected as it did not provide equally reproducible amounts of arbutin during repeated analyses. Moreover,

the powder for Product (C) did not dissolve easily in water and required a higher concentration of MeOH. This may be due to the excipient used, but it does imply that it would be less bioavailable than a product which dissolves easily in 100% water.

The repeat runs of product (A) conducted 12 months later had a slightly higher percentage of arbutin. When taking into account the results of the voucher specimens this could be due to degradation of other components in the plant material (see below in Section 6.5.3). The amount of hydroquinone displayed did not show the same consistency of results as arbutin, and varied very slightly. This compound is prone to oxidation, and this may be the reason why it proved less stable (Andrade *et al.*, 2006).

### 6.5.3 Confirmation of Plant Material via Voucher Specimens

With neither macroscopic nor microscopic identification possible due to the powdered nature of the product, the plant was verified via comparison with the voucher specimens. There was a strong correlation between the specimen from Kew collected in France in 1995 and the GMP product (A). The HMP produced seven more peaks, but it is to be expected that the latter would have additional compounds as it would also include the excipients.

It is possible that some compounds may have degraded from the voucher specimen as it was over 20 years old. It is known that leaves on a single plant can show a variation in constituents if the leaves are different ages (Applequist and Miller, 2013), and the older the specimens from Kew the fewer the number of peaks displayed on the chromatograms (Figure 20). Nevertheless, all, without exception, showed peaks for arbutin and hydroquinone. This suggests that these constituents may be more stable than the others, and that they will possibly not degrade as much within a commercial product.

It is the case that finished GMP products have to record when and where they have been grown and collected, but do not have to be tested immediately upon the collection of plant material. It, therefore, cannot be known how quickly the constituents might degrade from when they are initially harvested (European Commission, 2008).

## 6.6 Conclusion

It is expected that the three commercial products should all contain 20% arbutin as they were reported to be of GMP standard. However, they did vary in quality. The HPLC method which was developed using 5% MeOH instead of the 10% proposed by the Ph. Eur. proved to be the most reliable, and the accuracy was more reproducible. This enabled a suitable product (HMP A) to be chosen for the trial.

## Chapter 7: Antimicrobial assays

### 7.1 Introduction

Almost all the previous antimicrobial assays on uva-ursi were conducted over 25 years ago (see Section 3.5.1). The wide variation in the range of extractions and methods which were used has made it impractical to compare the results (see Table 4).

Furthermore, although some studies have examined antimicrobial activity of uva-ursi against *E. coli*, none have focused specifically on the pathogens responsible for causing UTIs, which comprise both Gram-positive and Gram-negative organisms.

With resistance to antibiotics partly due to continual mutations of bacteria it is logical to conclude that the results of preceding tests could also now be invalid and that new assays are warranted. Moreover, the lack of uniformity of the earlier studies coupled with improved, standardised methods for testing antimicrobial susceptibility justifies the need for contemporary assays.

Not only is it necessary to re-examine the potential antimicrobial effects of uva-ursi, the opportunity may be used to investigate and compare the activity of each of the 3 commercial herbal medicinal products (HMPs) being considered for the forthcoming clinical trial, as well as analysing the effect of the individual constituents arbutin and hydroquinone.

Finally, there is the question of whether an alkaline environment is necessary for antimicrobial activity of uva-ursi, either to facilitate the expression of the enzyme(s) involved in the release of free hydroquinone or to enhance antimicrobial activity of the herb. As mentioned in Chapter 3 it has been proposed that an alkaline environment is required for efficacy, but there have previously been conflicting results concerning the effect of an alkaline pH (see Section 3.5.2).

All the antimicrobial assays conducted in this study are covered in this chapter, with the first half (Section 7.2) relating to *in vitro* assays and the second (Section 7.3) concentrating on the antimicrobial activity of *in vivo* samples.

The enzymes involved in the metabolism of arbutin to hydroquinone are also included in the assays. As discussed in Chapter 3 the initial step in arbutin's metabolism involves  $\beta$ -glucosidase ( $\beta$ -GL), which converts arbutin to hydroquinone. This is used in the *in vitro* assays when analysing whether arbutin itself has any activity, or whether the presence of the enzyme is required.

The second enzyme  $\beta$ -glucuronidase ( $\beta$ -GLn) is used in the *in vivo* assays as it releases hydroquinone from the metabolite hydroquinone glucuronide in urine. It is present in most strains of *E. coli*, so the assays were conducted with and without  $\beta$ -GLn to determine whether it was necessary to add it as well for anti-microbial activity.

## 7.2 Minimum Inhibitory Concentration

### 7.2.1 Background

A broth microdilution assay using a 96 well micro-titre tray provides reliable reproducible screening of several compounds at the same time, and can demonstrate acceptable growth of the majority of pathogens (Reller *et al.*, 2009). The assays involve subjecting the standardised bacterial suspension of  $1-5 \times 10^5$  colony forming units (CFU)/mL to a series of concentrations of the antimicrobial product, which is diluted in Müller-Hinton broth (Reller *et al.*, 2009). The serial two-fold dilutions commonly range from 512 µg/mL down to 1 µg/mL. The lowest concentration, at which growth of the pathogen is completely inhibited, is determined by the absence of visible growth. This is known as the 'minimal inhibitory concentration' (MIC) (Andrews, 2001).

Müller-Hinton broth (MHB) is the favoured medium for susceptibility testing of commonly isolated, fast growing aerobic organisms. This method provides a quantitative result. A large amount of data to which the results can be compared have been accumulated on assays performed with this medium by the Clinical and Laboratory Standards Institute (CLSI, 2012).

The microdilution method was chosen for this study due to the large number of bacteria which required testing. The disadvantage is that colonies of bacteria are treated by a static drug concentration, and this would probably vary *in vivo* (Amsterdam, 2005).

### 7.2.2 Aims

- To determine whether the three commercial uva-ursi herbal medicinal products (HMP), and their related constituents (arbutin and hydroquinone), demonstrate antimicrobial activity (*in vitro*) against common uropathogens.
- To examine the potential antimicrobial susceptibility of *E. coli* to the same products and constituents at acidic and alkaline pH, both with and without the presence of  $\beta$ -glucosidase ( $\beta$ GL).

### 7.2.3 Method

#### 7.2.3.1 Compounds and Materials

The three powdered commercial uva-ursi HMPs previously examined in this study by High Performance Liquid Chromatography (HPLC) to quantify their arbutin content were assayed alongside pure compounds of arbutin ( $\geq 98\%$ ) and hydroquinone ( $\geq 99\%$ ) (Sigma-Aldrich).

Norfloxacin (Roche), a broad spectrum antibiotic prescribed for both Gram-positive and Gram-negative bacteria, and licensed for complicated and uncomplicated UTI, was used as the positive control (Sharma, Saneja, & Jain, 2008). It has an expected MIC of 0.06  $\mu\text{g}/\text{mL}$  against *E. coli* 10418 (Andrews, 2001). Norfloxacin was selected instead of trimethoprim and nitrofurantoin, which are the antibiotics used in clinical practice for UTI in the UK, as it has a greater range of activity against the bacteria to be tested (see 7.2.3.2). For example, *Pseudomonas aeruginosa* and *Proteus spp* are resistant to both trimethoprim and nitrofurantoin (Andrews, 2001, Steinke 2001), and both antibiotics have higher MICs against *E. coli* 10418 at 4  $\mu\text{g}/\text{mL}$  and 0.12  $\mu\text{g}/\text{mL}$  respectively (Andrews, 2001).

Müller-Hinton broth (MHB) (Oxoid CM0405) and nutrient agar (Oxoid CM0003) comprised the growth media. Dimethyl sulfoxide (DMSO) (Applichem, cell culture grade), a polar solvent which dissolves both polar and non-polar compounds, was applied to facilitate the dissolution of the HMP and its constituents (Balakin, Savchuk and Tetko, 2006). Analytical grade 70% Ethanol (EtOH) (Fisher Scientific) was used for sterilisation.

### 7.2.3.2 Bacterial Strains

Strains of reference microorganisms were supplied from the UCL School of Pharmacy stock (stored on agar slopes). These are routinely checked for purity before assays are conducted through preparing streak plates to isolate the colonies. The strains comprised *Enterococcus faecalis* 13379 (*E. faecalis*), *E. coli* NCTC 10418 which is used for primary testing of urine and is a fully susceptible strain used for controlled studies (PHE, 2014), *Klebsiella pneumoniae* 17 (*K. pneumoniae*), *Pseudomonas aeruginosa* 10663 (*P. aeruginosa*), *Methicillin-resistant Staphylococcus aureus* (MRSA), *Proteus species* 10830 (*Proteus. sp*), *Staphylococcus aureus* 12981 (*S. aureus*), and *Staphylococcus epidermidis* 11047 (*S. epidermidis*).

The Gram-positive organism *S. saprophyticus*, more common amongst 16-24 year old sexually active women, was not tested as the ATAFUTI trial was targeting a much broader demographic of 18-70 year old females.

### 7.2.3.3 Microdilution Assays - Aseptic Preparation

The 96 well microtiter plates (Nune<sup>®</sup>) were pre-sterilised. Nutrient agar, MHB, phosphate-buffered saline solution (PBS), and pipette heads were autoclaved (in a Classic Prestige Medical 2100) at 121°C for 18 minutes to destroy living cells and spores.

Platinum loops for bacterial sample collection, and glass spreaders for applying inoculum to nutrient agar plates, were sterilized through immersing into EtOH, allowing EtOH to evaporate and then flaming.

#### **7.2.3.4 Inoculum Preparation**

The organisms were sub-cultured and incubated overnight for 18-24 hours on nutrient agar in petri dishes at 37°C in a Genlab incubator. A bacterial colony was then collected from the sub-culture with a sterilized loop, and scraped onto the side of a 7 mL bijoux containing 2 mL of sterile phosphate buffered saline (PBS). One mL of this solution was transferred to a cuvette and the absorbance measured with a spectrophotometer (Therma Electron Helios Alpha Beta UV-visible) pre-calibrated to zero using a cuvette containing 1 mL of PBS at a wavelength ( $\lambda$ ) of 590 nm. The required optical density (OD) of the inoculum was 0.1, corresponding to  $1 \times 10^8$  CFU/mL (equivalent to a 0.5 McFarland turbidity standard). The remaining 1 mL of inoculum in the bijoux was subsequently diluted 1:100 in MHB to yield  $10^6$  CFU/mL. The concentration of the final inoculum upon adding 0.1 mL to the 96 well plates was  $5 \times 10^5$  CFU/mL.

Growth control agar plates (without antibacterial agents) were initially prepared to confirm the CFU/mL and to double-check for possible contamination according to the CLSI recommendations (CLSI, 2012).

#### **7.2.3.5 Preparation of Plates**

Uva-ursi extracts, arbutin, hydroquinone and norfloxacin were weighed, dissolved in DMSO, and further diluted in MHB to achieve starting concentrations of 512  $\mu\text{g/mL}$  for the extract and constituents, and 64  $\mu\text{g/mL}$  for norfloxacin. The microtiter plates were incubated under optimal conditions of 37°C for 18- 24 hours (Genlab 75 L incubator). All assays were run in duplicate (interday) for confirmation of the results.

#### 7.2.3.6 pH Assays

The pH was altered by adding either 0.5 mL of 1M hydrochloric acid (HCl) (for acidification) or 1M sodium hydroxide NaOH (for alkalization) to 250 mL of MHB (Hindler, Hochstein and Howell, 2010), and was verified via a pH meter (Thermo Scientific Orion 3 Star Desktop) and an electrode (Fisher Scientific FB68800) calibrated at pH 4, 7 and 10. The pH of MHB may increase slightly (approximately 0.1 to 0.2 pH units) with autoclaving; this was taken into account when preparing the medium and checking the pH again post autoclaving.

MIC assays on *E. coli* (only) were initially undertaken at pH 6.7, 7.4, 8.0 and 8.4. Subsequent pH assays were all conducted at pH 7.2 and pH 8.2. Control plates were run with MHB without the herb or hydroquinone to ensure that the addition of HCl and NaOH in the medium did not affect the results.

#### 7.2.3.7 $\beta$ -Glucosidase

$\beta$ -Glucosidase a lyophilized powder from almonds (Sigma-Aldrich G4511), was reconstituted at a concentration of 1 mg/mL in pH 7 phosphate buffer. It was assayed twice using different quantities of enzyme to determine the most active number of units, both 1 unit (100  $\mu$ cl) and 5 units of enzyme (500  $\mu$ cl) were dissolved with MHB (post autoclaving). Enzyme assays were routinely tested in MHB prepared at pH 7.2 and pH 8.2, with controls comprising just the enzyme (without uva-ursi) and norfloxacin.

#### 7.2.3.8 HPLC Detection of Hydroquinone and Arbutin

After sterile filtering the samples (via a 0.22  $\mu$ m Millex filter) and then centrifuging them, an aliquot (0.5 mL) was taken for determining the presence of hydroquinone via HPLC. The same method was used to run the samples as was developed in Chapter 6 (Section 6.3.3).

A control reference sample of pure hydroquinone was also filtered and prepared with the same method to ensure that hydroquinone would still be present after being passed through the 0.22  $\mu$ m filter.

## 7.2.4 Results

### 7.2.4.1 MIC at pH 7.2

Activity of the compounds is affirmed when there is no visible bacterial growth in the wells at a concentration of  $\leq 512 \mu\text{g/mL}$ . The results (of the two duplicate plates) showed that the three uva-ursi HMPs all demonstrated equal antimicrobial activity (to each other) against all the organisms tested. The MICs for *S. epidermidis* and *MRSA* were both  $256 \mu\text{g/mL}$ , and for *S. aureus* and *E. coli* they were  $512 \mu\text{g/mL}$ . None of the other organisms were susceptible to the herbal medicinal product.

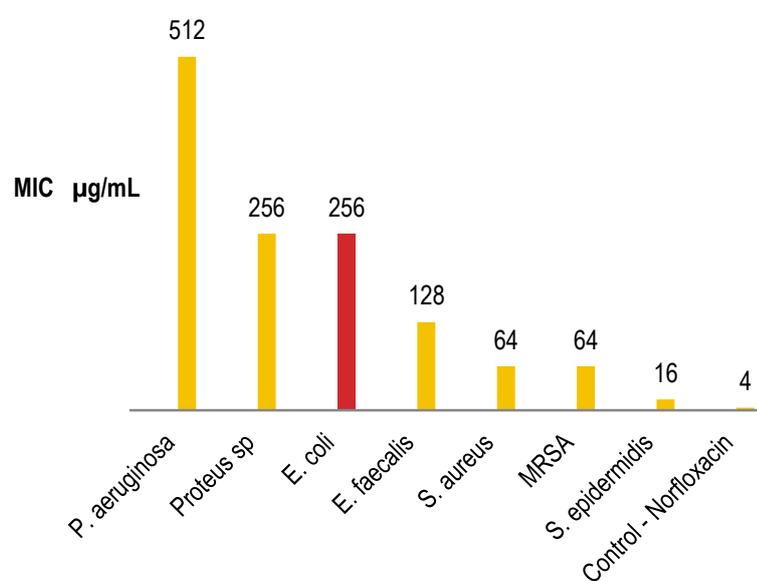
Hydroquinone demonstrated greater antimicrobial activity than the HMPs: all the pathogens with the exception of *K. pneumoniae* were susceptible to this compound. *S. epidermidis* showed the greatest susceptibility at  $16 \mu\text{g/mL}$ , whilst *E. coli*, responsible for most UTI, was inhibited at  $256 \mu\text{g/mL}$ .

None of the micro-organisms were susceptible to the parent glycoside of pure arbutin.

The results of all the compounds assayed are summarised below in **Table 15**, and the activity of hydroquinone versus all the bacteria tested is illustrated in **Figure 21**.

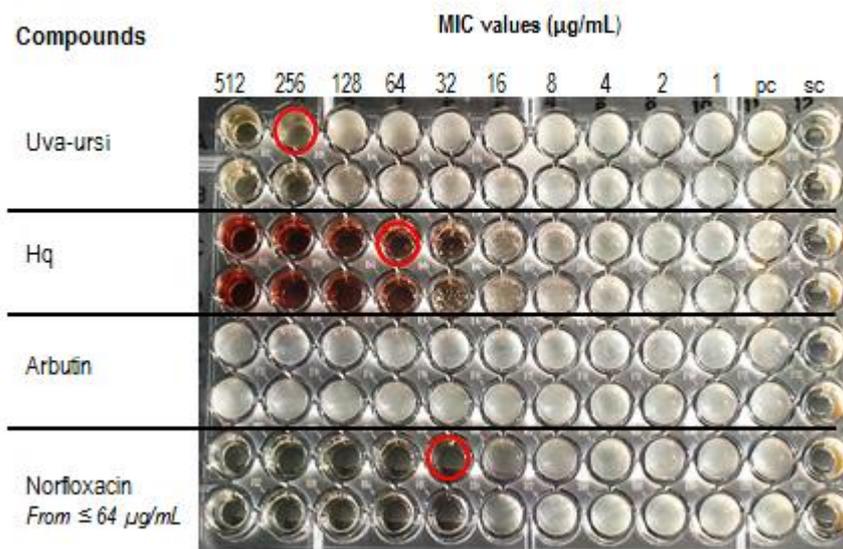
**Table 15:** MICs of hydroquinone, uva-ursi, arbutin and norfloxacin (positive control)

Bacteria (strain)	Antimicrobial Susceptibility MIC ( $\mu\text{g/mL}$ )			
	Hq	Uva-ursi HMP	Arbutin	Norfloxacin
<i>S. epidermidis</i> 11047	16	256	> 512	0.125
MRSA 13373	64	256	> 512	1.0
<i>S. aureus</i> 12981	64	512	> 512	1.0
<i>E. faecalis</i> 13379	128	> 512	> 512	2.0
<i>E. coli</i> NCTC 10418	256	512	> 512	0.125
<i>Proteus</i> sp 10830	256	> 512	> 512	0.125
<i>P. aeruginosa</i> 10663	512	> 512	> 512	0.125
<i>K. pneumoniae</i> 17	> 512	> 512	> 512	> 512



**Figure 21:** MICs of hydroquinone versus the uropathogens at pH 7.2

A photo of the MIC plate with MRSA is shown in **Figure 22**. It illustrates both the inhibition of growth by the uva-ursi HMP at 256  $\mu\text{g/mL}$ , and that hydroquinone was more effective at the lower MIC of 64  $\mu\text{g/mL}$ . The solution containing hydroquinone, originally a white powder, turned red as it oxidised (Foxman and Brown, 2003).



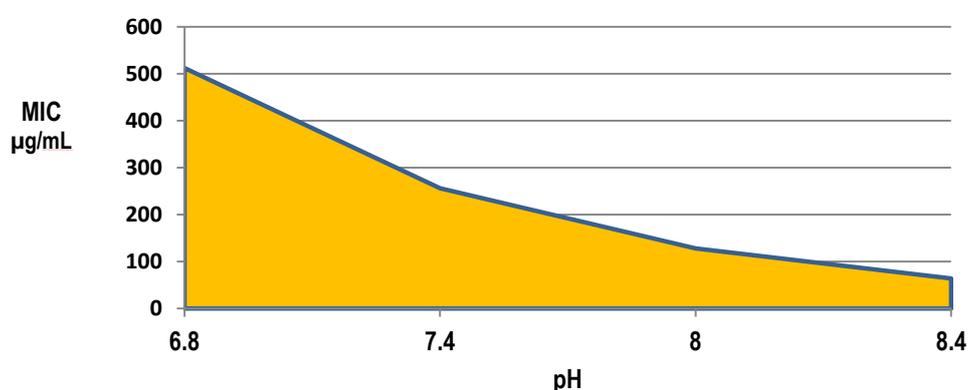
**Figure 22:** MIC of uva ursi, hydroquinone, and arbutin versus MRSA.

Uva-ursi MIC = 256  $\mu\text{g/mL}$ ; Hq = 64  $\mu\text{g/mL}$  (slight inhibition at 32  $\mu\text{g/mL}$ );  
 Arbutin no inhibition  $\leq$  512  $\mu\text{g/mL}$ ; Norfloxacin (negative control) = 1  $\mu\text{g/mL}$ ;  
 pc = positive control; sc = sterile control

#### 7.2.4.2 pH Sensitivity - *E. coli*

Increasing the pH of the broth from acid to alkaline improved the activity of both hydroquinone and uva-ursi. At pH 6.7 hydroquinone showed activity at 512  $\mu\text{g/mL}$  and this activity increased at pH 8.4 with a reduction in MIC of -87.5% to 64  $\mu\text{g/mL}$  (illustrated in **Figure 23**). The activity of the uva-ursi HMP was unchanged with increasing alkalinity and only demonstrated activity at 512  $\mu\text{g/mL}$  at pH values of 7.2 and 8.4.

Arbutin did not show activity at any pH.

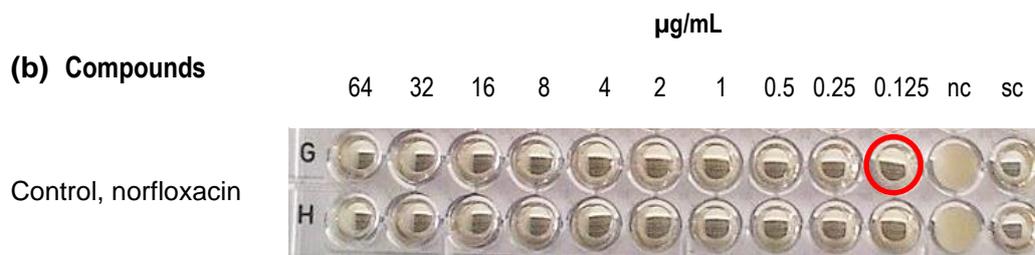
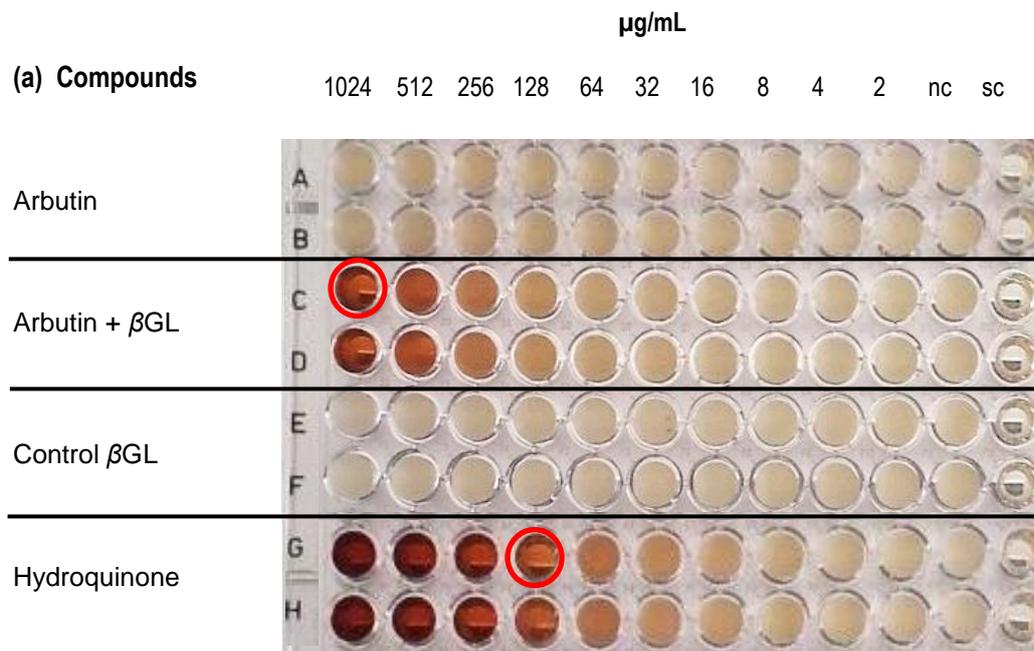


**Figure 23** Increased antimicrobial activity of hydroquinone against *E. coli* at increasing pH

#### 7.2.4.3 Assays Incorporating $\beta$ -glucosidase

The addition of either 1 or 5 units of  $\beta$ -glucosidase ( $\beta\text{GL}$ ) did not induce any activity of arbutin against *E. coli* at pH 7.2 or pH 8.2 at the highest standard concentration evaluated (512  $\mu\text{g/mL}$ ), nor did it enhance the antimicrobial effect of the HMP.

Increasing the concentration to 1024  $\mu\text{g/mL}$  incorporating 1 unit of  $\beta\text{GL}$ , *E. coli* showed susceptibility to arbutin at pH 8.2 (illustrated in **Figure 24**), but not at pH 7.2.

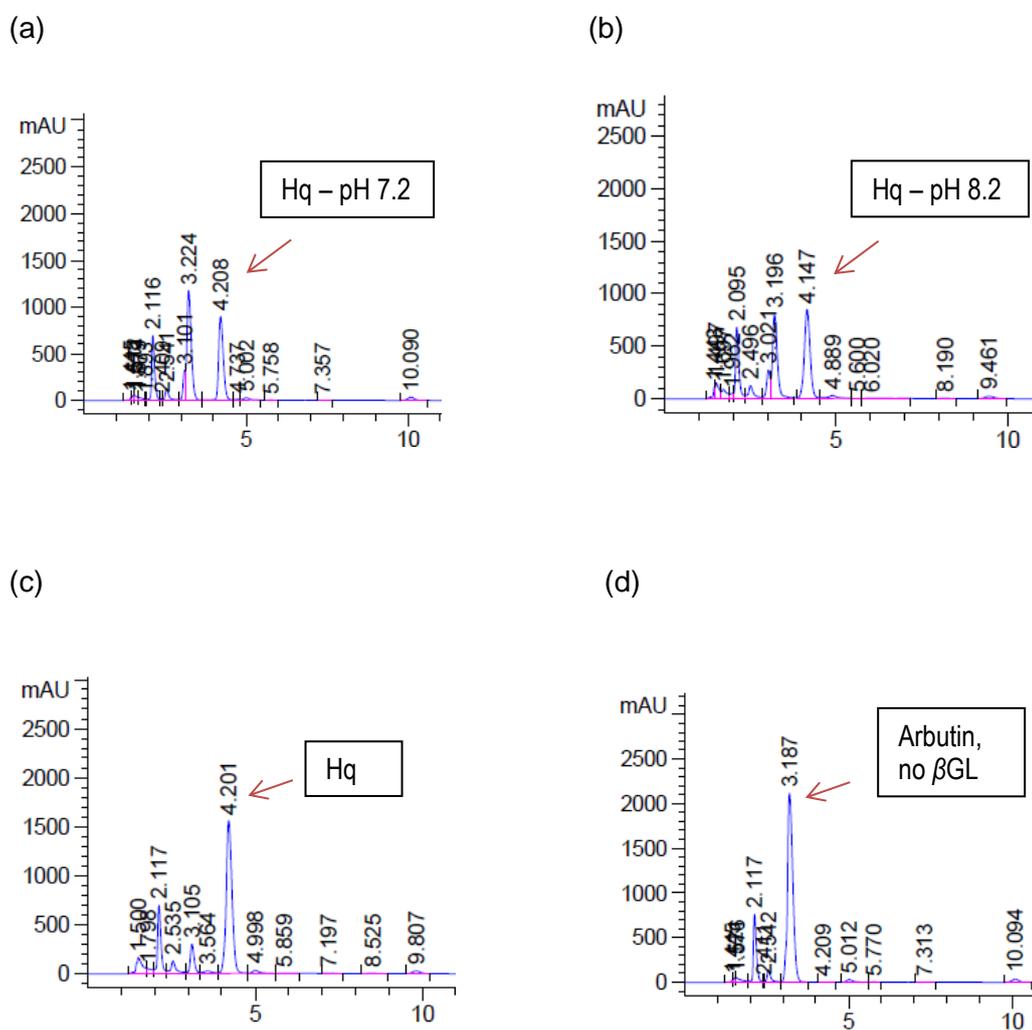


**Figure 24:** MIC of arbutin against *E. coli* in the presence of  $\beta$ GL at pH 8.2 in 96 well microassay.

Plate (a) arbutin growth (no  $\beta$ GL) (rows A/B); activity of arbutin at 1024  $\mu$ g/mL with the enzyme added (rows C/D);  $\beta$ GL controls show growth (rows E/F); activity from hydroquinone at 128  $\mu$ g/mL (rows G/H) (previously 64  $\mu$ g/mL at the slightly higher pH of 8.4); Plate (b) antimicrobial activity of norfloxacin (positive control) at all dilutions no growth (rows G/H). nc = negative control (no antibiotic), sc = sterile control (no bacteria)

#### 7.2.4.4 Results of HPLC

The presence of hydroquinone was detected by HPLC after incubating the arbutin samples with  $\beta$ GL and comparing them to the control samples with reference standards of hydroquinone and arbutin. Hydroquinone was detected in the arbutin/*E. coli* samples post the addition of  $\beta$ GL at both pH levels (pH 7.2 and pH 8.2) (see Figure 25).



**Figure 25:** Detection of the presence of hydroquinone in *E. coli*/arbutin samples after incubation with  $\beta$ GL at pH 7.2 and pH 8.2 by HPLC

(a) pH 7.2 and (b) pH 8.2, compared with controls of (c) hydroquinone (Hq) and (d) arbutin without  $\beta$ GL

## 7.2.5 Discussion

### 7.2.5.1 Antimicrobial Activity Standard pH (7.2)

The HMPs and hydroquinone both showed greater activity against Gram-positive strains of bacteria (*S. epidermidis* 11047, *MRSA* 13373, *S. aureus* 12981, *E. faecalis* 13379) compared to the Gram-negatives (*E. coli* 10418, *Proteus sp* 10830, *P. aeruginosa* 10663, *K. pneumoniae* 17). Gram-negative bacteria generally tend to be more resistant to antibiotics and natural antimicrobial agents compared to Gram-positives (Nikaido, 1998; Silhavy, Kahne and Walker, 2010). This is believed to be due to the outer membrane, not present in Gram-positive organisms: it acts as a selective barrier protecting the peptidoglycan cell wall and the contents of the cell within, without affecting the organism's ability to diffuse nutrients, disseminate waste and efflux (Beveridge, 1999; Delcour, 2009).

The MIC of the three HMPs was high compared to the MIC of the antibiotic norfloxacin, the positive control, the lowest results observed were  $\geq 256 \mu\text{g/mL}$  compared to  $\leq 1.0 \mu\text{g/mL}$ , respectively. Whilst it would appear from these assays that the herb as a whole may not have potential as an antimicrobial agent, these results are still encouraging. It is more common practice for individual compounds to be extracted from plants and then tested for their antimicrobial activity rather than testing the plant material itself. It is the efficacy of the inherent constituents either extracted or released through metabolic processes which have the most significance (Gibbons, 2004; Mena and Llorach, 2017). The fact that there was no difference in the results between the three commercial GMP products tested demonstrated consistency in potency, and facilitated making a decision regarding the commercial extract to use in the ATAFUTI trial.

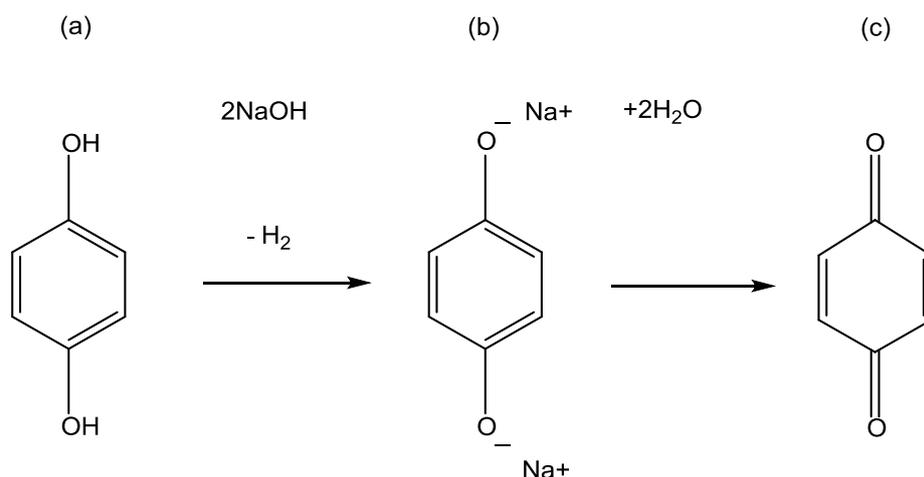
Pure hydroquinone was more effective than the HMP against the Gram-positive organisms. It was at least 4 times more active against *E. faecalis* (the least susceptible), and 16 times more effective against *S. epidermidis* (the most susceptible reaction). There was little difference between the results of the HMP and hydroquinone versus Gram-negative organisms. The antimicrobial activity was either equal or hydroquinone showed increased activity at one two-fold dilution lower than the HMP. This is not a marked difference as the 'true' MIC may lie anywhere between the two points. It would imply that neither hydroquinone nor the HMP was easily able to

penetrate the outer membrane of the Gram-negative bacteria and/or were subjected to drug efflux.

#### 7.2.5.2 Activity against *E. coli* at alkaline pH

In this study *E. coli* was shown to have greater susceptibility to hydroquinone at pH 8.2 than pH 7.2. This contradicted the result by Frohne, who found no difference in activity by altering the pH to alkaline in an *in vitro* agar hole punch assay (Frohne, 1970). He demonstrated that 60 µg/mL of hydroquinone could completely inhibit the growth of *E. coli* and *S. aureus* and that 180 µg/mL of methylhydroquinone inhibited the growth of *E. coli* - but neither were affected by alkalisation (Frohne, 1970). Whilst the agar hole punch is a standard assay, it has been observed that results can be negatively affected by the thickness, permeability and homogeneity of the agar (Bonev, Hooper and Parisot, 2008).

In an alkaline environment hydroquinone may act as both a reducing agent and a weak acid. The hydrogen ions from the OH groups will react with the sodium ( $\text{Na}^+$ ) from the NaOH and result in the loss of the hydrogen ions and a two electron reduction from the oxygen: this will cause the oxygen atoms to become ionised and have a negative charge. The  $\text{Na}^+$  will associate with the  $\text{O}^-$  (see **Figure 26**). Moreover, when exposed to air hydroquinone will oxidise and turn red, and in an alkaline solution, such as NaOH, hydroquinone oxidation can occur rapidly to form 1-4, benzoquinone, which turns the liquid a darker red colour (Kung and McBride, 1988). This reaction appeared to occur as the resultant liquid was redder in colour than when hydroquinone was present in the MHB at pH 7.2.



**Figure 26:** Possible chemical reactions between hydroquinone and sodium hydroxide. (a) = hydroquinone, (b) = anion and cation formed from alkaline solution, (c) 1-4, benzoquinone

In an acidic solution oxidation may be much slower (Kung and McBride, 1988). This might explain why at pH 6.7 hydroquinone had a higher MIC (512  $\mu\text{g}/\text{mL}$ ) than at pH 8.4 (64  $\mu\text{g}/\text{mL}$ ).

The growth of *E. coli* is unaffected between pH 5 to 9 (Small *et al.*, 1994). However, micro-organisms including *E. coli* may show increased or decreased susceptibility to certain classes of agents if the pH is altered. For example, a study which investigated changes to antimicrobial resistance with uropathogens found that *E. coli* became susceptible at pH 8 to the aminoglycosides gentamicin and tobramycin after being resistant at pH 5. This organism also became more susceptible to quinolones at pH 8 than in an acidic environment.  $\beta$ -lactams, on the other hand, demonstrated activity against *E. coli* at pH 5 but none at pH 8 (Yang *et al.*, 2014). It is believed that ionic interactions with porins and lipid membranes at alkaline pH may increase the uptake of quinolones into the bacterial cells, and that electrical activity could also be responsible for increased bacterial uptake of aminoglycosides (Piddock, 1991; Yang *et al.*, 2014).

### 7.2.5.3 The addition of $\beta$ -glucosidase

To continue the discussion on the relevance of pH to the antimicrobial activity of uva-ursi it is also necessary to consider the optimum pH of the enzymes involved in its metabolism. A study on broad specificity  $\beta$ -glucosidases from the liver have demonstrated that maximum expression of the enzyme may occur between pH 5 and 6 (Daniels *et al.*, 1981), which is in line with the protocol for the enzyme used in this study. It has been reported that there are bacterial  $\beta$ -glucosidases in the colon which are more active at pH 8, but as already discussed it is unlikely that deglycosylation of arbutin occurs in the colon (see 3.4.1.2) (Kim *et al.*, 1994).

The susceptibility of *E. coli* to pure arbutin after the addition of  $\beta$ GL was the first time arbutin showed antimicrobial activity. At 1024  $\mu\text{g/mL}$  at pH 8.2 (and no activity at pH 7.2) the MIC was quite high. This was weaker than the MIC of the HMP which was active at 512  $\mu\text{g/mL}$  at pH 7.2 without adding the enzyme. Adding  $\beta$ GL to pure arbutin may have liberated free hydroquinone: the arbutin samples became a dark red colour so would have hydrolysed to release hydroquinone, and this was confirmed by HPLC. However, the total quantity of arbutin in the HMP (20%) may have been too low to generate activity  $\leq 512 \mu\text{g/mL}$ . For *E. coli* to be more susceptible to the HMP than it was to pure arbutin with the addition of the enzyme suggests that either there may be another active compound in the herb, which has antimicrobial activity, or that there is a small amount of free hydroquinone present.

### 7.2.5.4 Strengths and Limitations

Not using clinical strains of *E. coli* found present in UTI may be a limitation of the research. However, it was a controlled study and the strain which was used was approved for urine infections and is known not to be resistant. Using clinical strains may have produced a variety of responses.

The activity of the HMP was not tested against *S. saprophyticus* as this mainly causes UTI amongst sexually active 16-24 year old women and the ATAFUTI trial encompassed the broader demographic of 18-70 year old females.

### 7.2.6 Conclusion

Previous research into the antimicrobial effect of uva-ursi and its constituents has concluded that urine containing metabolites of arbutin require an alkaline environment for efficacy, maintaining that free unconjugated hydroquinone is more likely to be present at a pH over 7. No publication on uva-ursi or arbutin has reported the efficacy of hydroquinone itself at an alkaline pH, and these results show that hydroquinone improved activity against *E. coli* at increasing pH, but was unchanged in an acidic environment. Arbutin required the presence of  $\beta$ GL for activity, and was unaffected by pH at  $\leq 512$   $\mu\text{g/mL}$ , possibly due to the smaller amount of hydroquinone released. Bacterial organisms were susceptible to the herb itself at a lower MIC than arbutin. Further investigation would have to be conducted on the other constituents to determine what if any antimicrobial properties they may express and how they compare with hydroquinone.

The antimicrobial action of hydroquinone may be related to the ions present, as it easily oxidises in an alkaline solution. Additional research into the activity of the anions produced would have to be conducted to determine whether this was the case. Moreover, there is still the question of whether this result could be replicated *in vivo*, which is investigated in the second part of this chapter (Section 7.3).

## 7.3 Volunteer Urine Samples Containing Metabolites of *uva-ursi*

### 7.3.1 Background

There have been a variety of investigations into the theory that an alkaline environment is necessary for efficacy of *uva-ursi in vivo*, with emphasis placed on the optimum pH required to induce the enzyme activations involved in its metabolism, particularly  $\beta$ -glucuronidase ( $\beta$ -GLn). This liberates free hydroquinone from the main metabolite hydroquinone glucuronide: the compound is believed to account for 55% of the total metabolites from arbutin (Glockl, Blaschke and Veit, 2001). This peaks within the first 2 – 3 hours after taking *uva-ursi* (Paper, Koehler and Franz, 1993a; Quintus *et al.*, 2005). The papers and findings previously discussed in Section 3.5.2 show conflicting and inconclusive results. The *in vitro* antimicrobial assays in Section 7.2.4.2 (see above) demonstrated that hydroquinone increased activity against *E. coli* when the pH of the broth was made alkaline. This was contrary to the aforementioned publication by Frohne, which reported that the effect of hydroquinone was unchanged in an alkaline environment *in vitro*. Only the urine samples he tested, which included *uva-ursi* metabolites, showed increased antimicrobial activity at pH 8 compared to pH 7 (Frohne, 1970).

In light of the forthcoming clinical trial on *uva-ursi* and the need to clarify whether an alkaline environment is necessary for efficacy of the herbal treatment, it was decided to investigate the antimicrobial activity of urine samples post ingestion of *uva-ursi* against *E. coli* amongst healthy volunteers. This would facilitate a decision on whether either an alkalisating diet or agent should be recommended for patients in the trial to take alongside the herb.

Quantification of the hydroquinone derivatives eliminated from the body after 24 hours have varied from 65% to 99% (Schindler *et al.*, 2002; Quintus *et al.*, 2005). With the mechanism of action of *uva-ursi* still not entirely determined, and the antimicrobial activity having previously been examined after taking a single dose of the herb, the opportunity can also be used to analyse the HMP's potential effectiveness over a longer period of time with additional doses.

Determination of antimicrobial activity in urine samples after ingesting the herb can be accomplished by streaking urine containing metabolites of the *uva-ursi* HMP with

*E. coli* onto agar plates to see how many colony forming units (CFU) (viable counts) are able to grow.

### **7.3.2 Aims**

- To investigate the potential antimicrobial activity of uva-ursi in urine samples versus *E. coli* after ingestion of the HMP, and assess whether this improved in an alkaline versus acidic environment.
- To determine whether taking the HMP over two days, compared to one, affected the antimicrobial activity of urine containing uva-ursi metabolites.

### **7.3.3 Methods**

#### **7.3.3.1 Recruitment of Volunteers and Ethics**

It was decided to recruit a minimum of three healthy volunteers (to equal previous studies on uva-ursi metabolites) and a maximum of eight over 18 years old who might be interested in the project, but exclude students from UCL where the study was being conducted in order to maintain anonymity. The participants were given a Participant Information Sheet and Consent Form to read and sign, and told that they would be free to withdraw at any time without having to provide a reason. They were also informed that all urine samples would be anonymised, and that the consent forms would be stored in a locked filing drawer. Ethics for the study was granted by UCL Research Ethics Committee on 24/2/15, Project ID: 6376/001. A copy of the Ethics Approval is included in Appendix C, and the Volunteer Information Sheet and Consent Forms are included in Appendix D. The study ran between March and April 2015.

### 7.3.3.2 Uva-ursi HMP and Dose

An over-the-counter tablet (450 mg) of the same GMP product being tested in the ATAFUTI clinical trial was used. Details of the product were also approved by the UCL Ethics Committee (above). Each dose comprised 2 tablets (900 mg of herb containing approximately 180 mg arbutin) providing a total of 3600 mg of HMP and 720 mg of arbutin each day.

### 7.3.3.3 Dosing Regime and Collection of Urine Samples

Prior to consuming the uva-ursi HMP on Day 1 a control urine sample was taken. A minimum of three doses of the HMP were then consumed before any further urine specimens were obtained. Urine was then collected (midstream) two hours after the third dose of uva-ursi and again one hour later in case the amount of metabolites varied. A fourth and final dose of the HMP was taken immediately afterwards, and a final urine sample was collected three hours later. The dosing and urine collection regime was repeated the next day (see **Figure 27**). All urine samples were collected in sterile 30 mL Sterilin Collection tubes, labelled chronologically (a) to (g) together with the time and date. They were then immediately frozen.

Time	Urine Sample Collection	Dosing regime
9 am	→ Collect sample (a) (Day 1)	→ Take 2 uva-ursi
12 pm	→	Take 2 uva-ursi
3 pm	→	Take 2 uva-ursi
5 pm	→ Collect sample (b); Day 2 (e)	
6 pm	→ Collect sample (c); Day 2 (f)	→ Take 2 uva-ursi
9 pm	→ Collect sample (d); Day 2 (g)	

**Figure 27:** Outline of procedure for taking HMP medication and collecting urine samples on both days. Samples are labelled (a) to (g)

#### 7.3.3.4 Preparation of Urine Samples and Inoculum

All urine samples were sterile filtered through a 0.22 µm filter (Millex GP) in a laminator flow cabinet to remove cells and bacteria, the pH was measured and recorded at baseline. The samples were then stored at -20° (for short-term storage) and assayed either soon after or within a few days. At storage temperatures of -20°C the pH of urine has been shown to be relatively stable over a two week period (Cook *et al.*, 2007).

The pH readings of the seven urine samples from the first volunteer, which were all acidic, were averaged and the mean reading (pH 6.2) was used to help determine at what pH two separate mixtures of broth should be prepared for creating acidic and alkaline environments for the *E. coli* inoculum. Double-strength broth was then prepared at pH 7.2 and pH 8.6 (for alkalisation method see Section 7.2.3.6), and these two pH levels were used with all the samples. The larger variation in pH in this experiment compared to the *in vitro* assays (run at pH 7.2 and 8.2) was to allow for the fact the pH of the broth would reduce once the urine specimens were introduced (in equal volumes). Double-strength broth was necessary for the same reason: to support the growth of the organism diluted in urine (Blake *et al.*, 2003).

The same strain of *E. coli* NCTC 10418 was used as for the antimicrobial assays. This strain has been shown to express β-glucuronidase activity, either registered as NCTC (Turner and Burton, 2016) and also tested as ATCC 10536, the American collection of the same strain (Baudart *et al.*, 2009). It was sub-cultured (as per Section 7.2.3.4), and colonies measured using the spectrophotometer calibrated to 0.1 OD to confirm 10<sup>8</sup> CFU/mL in PBS. The inoculum was then diluted in the double strength MHB to give 10<sup>2</sup> CFU/mL. To check whether there might be activity at a stronger concentration of bacteria the first experiment was also conducted with 10<sup>4</sup> CFU/mL. Urine samples and inoculum were then added in equal measure (0.5 mL v/v) to a bijoux container (7 mL), to give final concentrations of 0.5 x 10<sup>2</sup> CFU/mL. Controls were prepared with the same method using the first urine sample of Day 1 without the HMP (sample a), and a separate water sample (run in duplicate).

The bijoux containing the inoculum and urine samples were prepared in duplicate to allow the addition of β-glucuronidase (β-GLn) to be tested. The overall volume of broth and urine was adjusted to allow for the addition of the enzyme (an adjustment of 9 µL in total). The bijoux containers (with lids closed) were then incubated at 37°C for 18 hours.

The acidity/alkalinity of a random selection of urine samples (10%) was measured once they were removed from the incubator to check whether the time and temperature had affected the pH.

#### 7.3.3.5 $\beta$ -Glucuronidase

Lyophilized  $\beta$ -GLn from *E. coli* (Sigma-Aldrich G7646) was reconstituted in sterile distilled water at 1 mg/mL and stored at -20° C. The enzyme was initially tested by adding either 50 units (9  $\mu$ L) or 10 units (2  $\mu$ L) to 1 mL of urine (post ingestion of the HMP) to determine which quantity may effectively produce free hydroquinone from the specimens. After sterile filtering (via a 0.22  $\mu$ m Millex filter) and centrifuging the samples the presence of hydroquinone with 9  $\mu$ L was confirmed via HPLC using the method developed in Section 6.3.3, so this was the amount used in the assays.

Antimicrobial activity of the enzyme itself was assayed using 96 well microtiter plates (as per method in Section 7.2.3) to ensure that the bacteria were not susceptible to the enzyme or any compounds it may have been prepared or stored with.

To activate the enzyme 9  $\mu$ L of  $\beta$ -GLn was added to the *E. coli* in MHB and incubated for 30 minutes at 37°C.

#### 7.3.3.6 Plating the Inoculum/ Urine

Enumeration of viable counts of bacterial growth of *E. coli* + urine ( $0.5 \times 10^2$ ) with and without  $\beta$ -GLn was measured by spreading 20  $\mu$ L of the culture evenly onto sterile nutrient agar plates (in duplicate). Where necessary, to further quantitate any growth the cultures were diluted by adding 20  $\mu$ L to 180  $\mu$ L PBS, and then serially diluting a further six times in microtiter plates. All samples from volunteers 1 and 2 were plated at both pH levels. For volunteers 3 and 4 only the very first (a) and last samples (g) were plated at both pH. The plates were then inverted and incubated under aerobic conditions at 37°C for 18 hours. Results were measured by counting the colonies across two duplicated plated samples, and using the mean number from both plates. The CFU/mL of the average of the two plates was then calculated according to the original dilution ( $0.5 \times 10^2$ ).

## 7.3.4 Results

### 7.3.4.1 Volunteer Sample and Safety of HMP

Four healthy adult volunteers were recruited (three female and one male) with a mean age of 49 years. None had taken any antibiotics in the preceding 2 months. The HMP was well tolerated and all volunteers completed the study without any adverse events.

### 7.3.4.2 pH of Urine Samples

At baseline, 26 of the 28 volunteer urine samples tested were acidic (**Table 16**). Samples ranged from pH 4.9 to 7.1, with a mean pH of 6.3. After adding the samples in equal quantities to both the pH 7.2 and pH 8.6 broth containing the *E. coli*, the resultant mean pH levels of the broth were 6.7 (acidic), and 7.5 (alkaline).

**Table 16:** Urinary pH of samples before adding broth containing inoculum

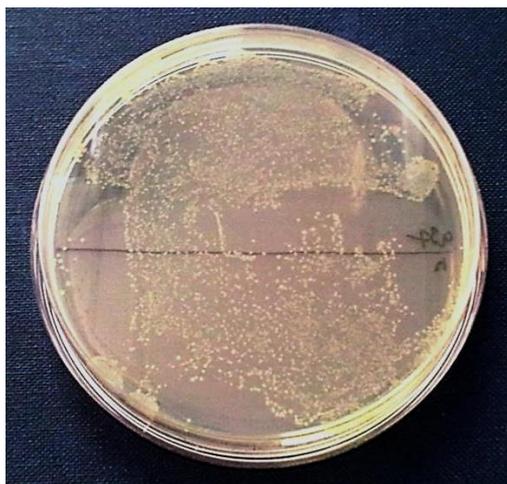
Urine Sample		Urinary pH at Baseline By Volunteer			
	Time	No. 1	No. 2	No. 3	No. 4
Day 1	09.00 (a)	5.84	6.45	6.43	6.65
	17.00 (b)	6.68	7.32	6.71	5.98
	18.00 (c)	6.94	6.82	6.60	6.23
	21.00 (d)	5.73	6.12	6.11	5.95
Day 2	17.00 (e)	5.91	5.52	6.90	4.94
	18.00 (f)	6.41	5.76	6.71	5.95
	21.00 (g)	6.20	5.71	5.84	7.10
<b>Mean pH</b>		<b>6.24</b>	<b>6.24</b>	<b>6.47</b>	<b>6.11</b>

Half of the 10% of the samples which were checked at random post incubation had fallen by  $\leq 0.2$  pH. None increased their pH.

#### 7.3.4.3 Urine Samples Containing HMP Metabolites – Inhibitory Effect by Day

At baseline there were  $0.5 \times 10^2$  CFU/mL of *E. coli* present in each of the broth/urine samples. This means that plated out on agar, prior to incubation, there should be visible growth of 50 colonies.

The results showed that post incubation either too many colonies to count (TNTC) had grown or a bacterial film had developed in all the control samples (of the volunteer urine prior to taking the HMP, and the water samples). This was at both pH 6.7 and pH 7.5. This compared with either reduced growth or no growth in the samples from all four volunteers post ingestion of the HMP. **Figure 28** below shows the reduced growth of *E. coli* after taking the HMP compared with the control urine sample (without the herb) for volunteer 4 at the same concentration of bacteria.



Sample (a) control (no HMP) pH 6.7



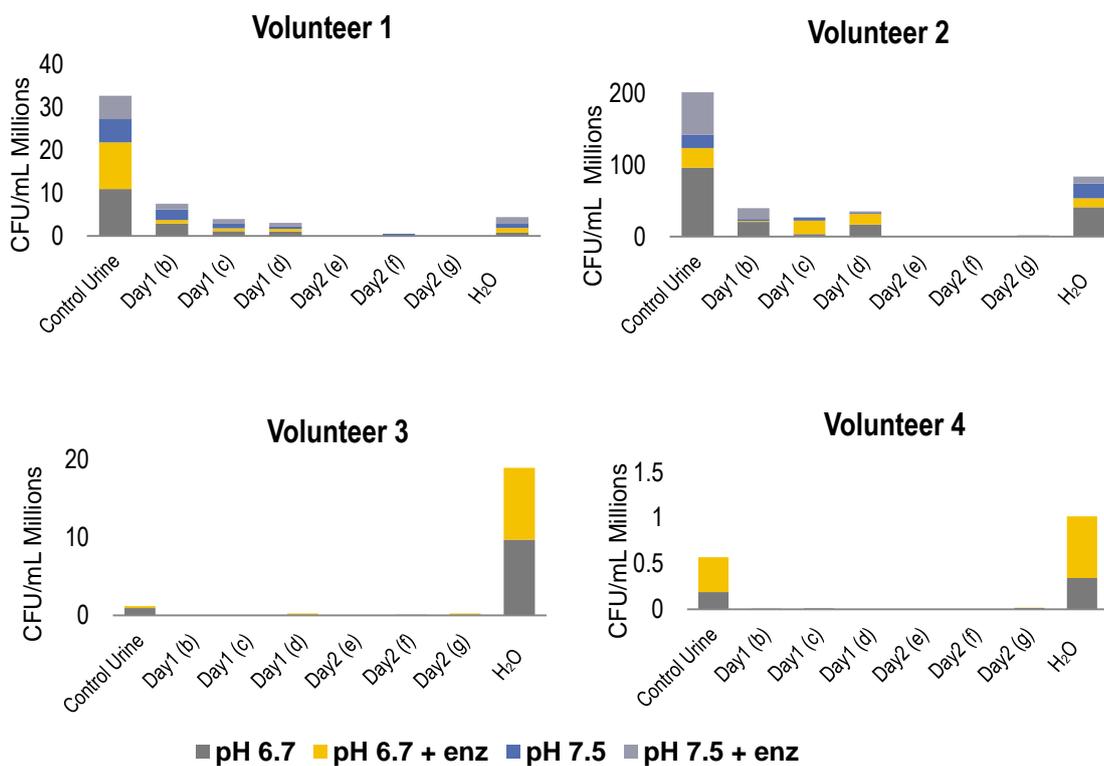
Sample (g) with uva-ursi HMP pH 6.7

**Figure 28:** Growth of CFU *E. coli*  $0.5 \times 10^2$  on agar from volunteer 4 urine samples at pH 6.7 without the addition of  $\beta$ -glucuronidase

The results for volunteers 1 and 2 showed complete inhibition of activity against *E. coli* from the first urine sample on Day 2 (sample e). None of their urine specimens on

Day 1 completely inhibited growth of the organism, but all except one (sample b of volunteer 1) showed less growth than the control samples. Samples from volunteers 3 and 4 demonstrated activity against *E. coli* compared to the controls from the first sample of urine taken on Day 1 (sample b), with almost no variation across the subsequent specimens. All showed less growth than the controls containing urine without the herb, and water (see Figure 29).

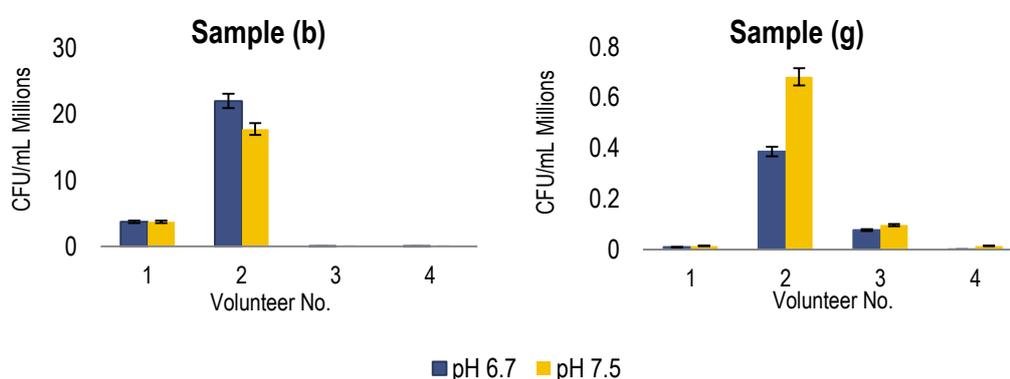
There was no inhibition of growth of *E. coli* at  $10^4$  in any of the samples tested, regardless of pH or addition of the enzyme.



**Figure 29:** CFU/mL for all 4 volunteers by urine sampled across Days 1 & 2 compared to controls of both H<sub>2</sub>O and urine without metabolites of the HMP

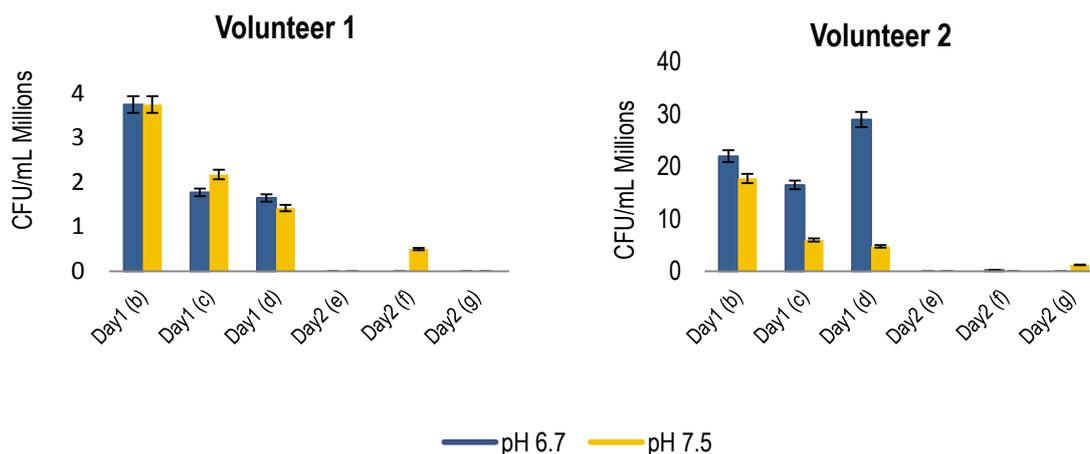
#### 7.3.4.4 Results by pH

The inhibitory effects of the urine samples post ingestion of uva-ursi assayed at alkaline pH (7.5) compared to acidic pH (6.7) fluctuated and did not show any consistent patterns. When all the results from the first urine samples taken post ingestion of the HMP on Day 1 (sample b) were added to all the final urine samples taken on Day 2 (sample g) there was an equal number of CFU/mL for each pH. (These were the two samples which were plated at both pH levels for all four volunteers). The individual results for urine samples (b) and (g) for all four volunteers are illustrated below in **Figure 30**.



**Figure 30:** CFU/mL of *E. coli* in urine for the first (b) and last samples (g) taken for all four volunteers by pH after consuming the HMP. The results include the CFU/mL of the samples containing the enzyme  $\beta$ -GLn, and are the mean of two experiments.

All the urine samples from the first two volunteers were tested at both pH values, and they did not show consistent results when the growth was analysed. The samples from volunteer 1 had slightly more growth (+9.4%) when total colonies were compared at pH 7.5 to the growth at pH 6.7, whilst the CFU/mL for volunteer 2 demonstrated greater fluctuations: three of the four samples had more growth at pH 6.7 compared to pH 7.5 (especially sample d). The growth of CFU/mL for both these volunteers at both pH levels is presented in **Figure 31**.



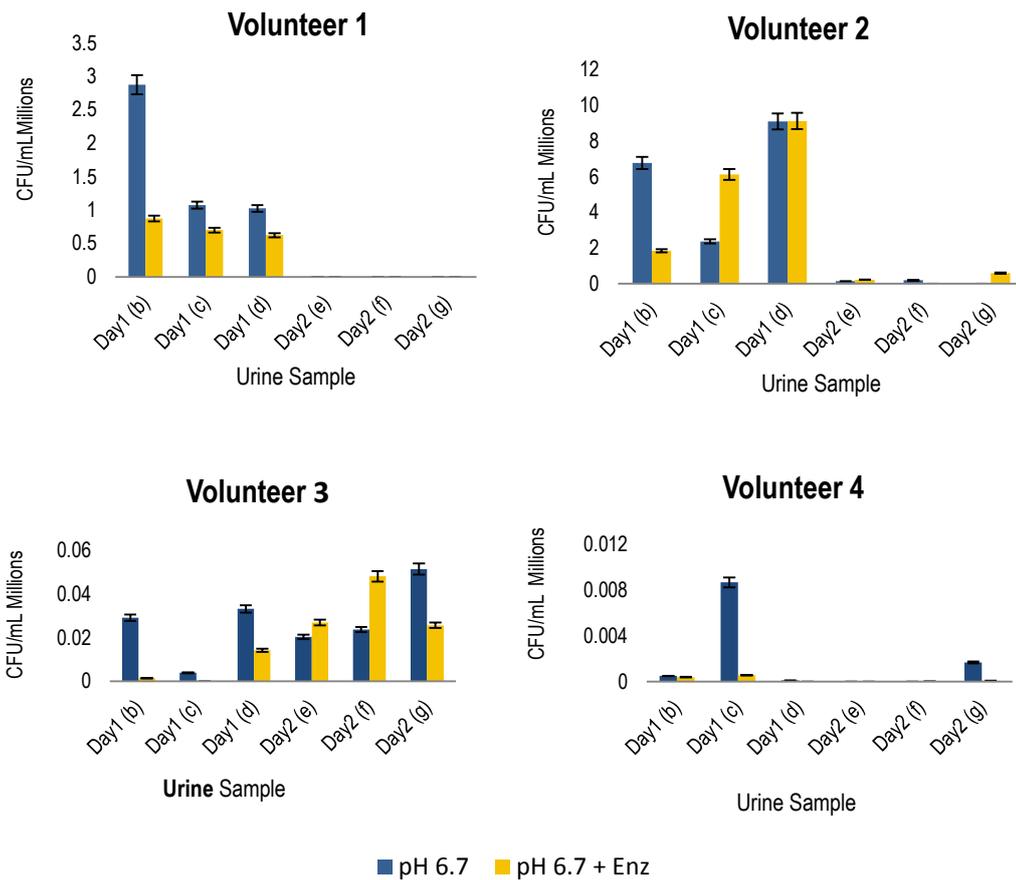
**Figure 31:** CFU/mL of *E. coli* in the urine samples post ingestion of the uva-ursi HMP for volunteers 1 and 2 by pH (including the samples with  $\beta$ -GLn)

#### 7.3.4.5 $\beta$ -Glucuronidase

The addition of the enzyme  $\beta$ -glucuronidase to the urine samples diluted with the broth containing *E. coli* did not consistently affect the visible number of colony forming units. The samples from two of the volunteers (1 and 4) showed less growth at pH 6.7 with the enzyme added for all of their samples compared to those at pH 6.7 without the enzyme, but growth from the samples from the other two volunteers varied ( **Figure 32**).

Adding  $\beta$ -GLn at pH 7.5 did not consistently affect the results.

As mentioned in the methods Section 7.3.3.5 the enzyme itself was checked by HPLC to confirm that its activity was positive. The results showed that hydroquinone appeared in the chromatogram at the same  $t_R$  as the reference compound once the enzyme was added to the samples containing uva-ursi metabolites. Hydroquinone was not present in the control urine samples, with or without the enzyme. Of note, was that a major peak area was present in the samples containing the metabolites before the enzyme was added, which decreased in size once hydroquinone was detected. This may have been the glucuronide. The peak areas from the chromatograms are in Appendix E.



**Figure 32:** CFU/mL for all samples at pH 6.7 with and without the addition of the enzyme  $\beta$ -GLn. The samples for volunteers 1 and 4 showed less growth after the enzyme was added, but the results from the samples from volunteers 2 and 3 were inconsistent.

### 7.3.5 Discussion

Urine samples from the four healthy volunteers who had taken the HMP inhibited the growth of *E. coli* which had been added *in vitro* at  $0.5 \times 10^2$  CFU/mL when compared to the control urine and water samples. There was no difference in the results at pH 6.7 compared to pH 7.5, regardless of whether the enzyme  $\beta$ -GLn was added.

### 7.3.5.1 Antimicrobial Results by Day

This is the first study which has tested the susceptibility of *E. coli* in urine samples following the intake of uva-ursi over a two day period. The urine from two of the four volunteers showed complete inhibition of *E. coli* compared to the control samples (urine or water without the HMP) following the very first dose of taking the herb on Day 1. The results from the other two volunteers did not show complete inhibition of growth until the first sample was tested on Day 2 (the fourth specimen to be assayed).

The last dose of the HMP in this study was administered 33 hours after the first dose, with the final urine sample taken three hours later. Previous reports have either assayed the antimicrobial activity from a urine sample following a single dose of uva-ursi or arbutin (Frohne, 1970; Kedzia *et al.*, 1975), or, have investigated the quantity of metabolites excreted up to 36 hours later (Siegiers *et al.*, 1997; Schindler *et al.*, 2002). The minimum and maximum amount of metabolites reported was 65% and 75% of the total dose (Frohne, 1970; Siegiers *et al.*, 1997; Schindler *et al.*, 2002). The only study (n = 3) which has analysed metabolites following the administration of more than one dose (a total of 4 doses in 16 hours) calculated that within 24 hours one volunteer excreted 99% of the total possible arbutin metabolites during this period, a second totalled 96% and the third only 79% (Quintus *et al.*, 2005).

The detection methods employed for quantification across all the previous studies have varied. The two most recent used HPLC, but they also reported the lowest and highest quantities of metabolites detected (65% and 99%) (Schindler *et al.*, 2002; Quintus *et al.*, 2005).

Based on the above, whilst it has been demonstrated that metabolites produced from arbutin after taking uva-ursi may start to be eliminated in urine as quickly as two hours after taking the herb (Kedzia *et al.*, 1975), it is still possible that not all metabolites may be excreted entirely within 24 hours from either single or multiple doses of the herb taken in one day. The process of elimination would depend on a person's individual rate of metabolism according to their genetic makeup. There is still limited kinetics research about how efficient the enzymes are at hydrolysing polyphenols to free their aglycones: most studies investigating bioavailability have focused on single rather than multiple doses (D'Archivio *et al.*, 2010). Moreover, there is still no official published half-life of uva-ursi.

### 7.3.5.2 Acidic versus Alkaline Urine

The volunteers' urine samples showed a mean acidic pH level of 6.3 at baseline. This is in-line with the results of a large nutritional study, which investigated the urinary pH of over 22K adults and reported that the pH of 82% of the participants fell between 5.0 and 6.5 (Welch, Mulligan, Bingham, & Khaw, 2008). Adding the urine samples to the two different preparations of broth containing *E. coli* resulted in the samples being tested at an average acidic pH of 6.7 and an alkaline pH of 7.5.

The *in vitro* assays in this study tested the urine from pH 6.7 to pH 8.4 and found a steady increase in the activity of hydroquinone as the pH level increased. It would not have been realistic for the *in vivo* investigation to test the urine at a level as high as 8.4, or even at pH 8 - the level which showed activity in Frohne's assays (Frohne, 1970). The procedure would have to be easily replicated in a clinical trial.

Attempting to increase the pH by following an alkalising diet or taking an alkalising agent can be a slow and imprecise process, and it is possible that the urine may neither reach pH 8 nor maintain this level once it was achieved. In a small study in which participants (n = 26) adopted an alkalising diet, it took three days for the urine to reach a steady pH of 6.7 from a baseline of 6.1 (Kanbara, Hakoda and Seyama, 2010). In another study where volunteers (n = 5) were administered a 4 g dose of sodium citrate (a standard method for alkalising urine) urinary pH increased from 6.2 to 7.3 over three hours compared to the control (without alkalisation), but it then started to drop (Gargan, Hamilton-Miller and Brumfitt, 1993). In this latter publication it was also reported that urinary pH increases over the course of the day, a phenomenon termed diurnal change. In this study that did not occur: half the samples taken at the end of the day had the lowest pH.

Furthermore, in order to test the urine samples at a pH  $\geq$  8, the broth containing the inoculum would have had to have been prepared at a minimum of pH 10 for diluting with the urine. Growth of *E. coli* may be negatively affected by a pH over 9.2 over the course of 24 hours, and can be killed at pH 9.4 over the same time period. The strongly alkaline environment may affect the expression of the genes and enzymes necessary to support its growth (Parhad and Rao, 1974; Padan *et al.*, 2005). Finally, the enzyme  $\beta$ -GLn used in the study (which was derived from *E. coli*) has an optimum pH of 6.8 - 7.0 (Zenser, Lakshmi and Davis, 1999). It is reported to be half as active at

pH 8.5 compared to its ideal range of pH 5.0 - 7.5. The fact that all the urine samples from two of the volunteers (1 and 4) showed reduced growth of *E. coli* when the enzyme was also added at pH 6.7 may be significant. Human endogenous  $\beta$ -GLn, is most likely to be present in the urinary tract of individuals with a local carcinoma. It has also been detected in healthy adults, but may not be at a high enough concentration to induce metabolism (Ho, 1985; Zenser, Lakshmi and Davis, 1999): it has been found to be most active at the acidic pH 5.5 (Jefferson, Burgess and Hirsh, 1986; Zenser, Lakshmi and Davis, 1999). Detecting the expression of  $\beta$ -GLn is not a precise science, and may vary according to the method used (Rice, Allen and Edberg, 1990; Kopen, Prockop and Phinney, 1999).

The pH of the urine at baseline fluctuated across each of the volunteers' samples and there was no correlation between the original pH and the subsequent growth of *E. coli*. The sample which showed the greatest difference in growth between the alkaline and acidic broth (-83% CFU/mL at pH 7.5 compared to pH 6.7) (see **Figure 31**), was not the most alkaline pH at baseline (sample d from volunteer 2). It had an original pH of 6.12, whilst sample (b), which had the highest pH of all those tested at baseline (pH 7.32), demonstrated -19% less growth in the alkaline broth compared to the acidic. Sample (g) from volunteer 4 showed higher growth in the alkaline broth (+717%) compared to the acidic medium despite having an alkaline pH at baseline (7.1), but sample (b) from the same volunteer was inhibited in the alkaline broth but not the acidic: this had a low pH at baseline (pH 5.98).

One study has demonstrated that urine samples may increase their pH by as much as one unit if left to stand for 24 hours (Cook *et al.*, 2007). The report measured the samples with a dipstick, but this method has been shown to be inaccurate when compared to using a pH meter especially at a higher pH. There can be a 1 in 4 risk of producing clinically significant differences when compared to a pH meter with resultant pH differences >0.5 (Kwong *et al.*, 2013; Omar *et al.*, 2016). The 10% of urine samples which were checked at random in this study did not show an increase in pH after incubation, but half reduced by  $\leq 0.2$  pH units. This was not sufficient to alter the alkaline samples to acidic. None of the previous publications on uva-ursi report whether they re-checked the urinary pH at the end of their investigations, including Frohne, who analysed his samples after 48 rather than 24 hours (Frohne, 1970).

### 7.3.5.3 Presence of Free Hydroquinone

Existing antimicrobial studies on uva-ursi testing urine at alkaline pH have focused on hydroquinone after taking either the herb itself or pure arbutin (Frohne, 1970; Kedzia *et al.*, 1975; Siegers *et al.*, 2003). Frohne and Kedzia both used a single high dose of arbutin in their investigations (840 mg and 1 g respectively) whereas it is recommended to split the total amount of uva-ursi, between 3-4 doses over the course of a day, partly because it is metabolised quickly and partly because this will reduce the amount of tannins administered at any one time which can upset the gut (Chapter 5). In so doing it will reduce the amount of arbutin in any one dose to a maximum of 210- 280 mg.

In separate investigations into the herb's metabolites, free (unconjugated) hydroquinone has previously been detected in some but not all urine samples (3.4.1.3). Out of a total of 31 healthy volunteers across three separate studies combined, only 15 participants eliminated free hydroquinone (Siegers *et al.*, 1997; Schindler *et al.*, 2002; Quintus *et al.*, 2005). This comprised a maximum of 0.6% of the original amount of arbutin, and was excreted in the first 3-6 hours after taking uva-ursi, derived from a maximum amount of 210 mg per dose (Schindler *et al.*, 2002; Quintus *et al.*, 2005). It has been estimated that approximately 0.66 µg/mL of hydroquinone may be present in urine after taking a dose of 420 mg of arbutin. However, this is based on averages of male and female participants (n = 11), where the females eliminated more than double the amount of free hydroquinone in the first six hours of the study compared to the men, and some people did not eliminate any free hydroquinone at all. The minimum and maximum quantities for each participant were not reported (Siegers *et al.*, 1997, de Arriba, Naser and Nolte, 2013).

If 0.66 µg/mL were to comprise a realistic quantity of free hydroquinone present in urine, based on the antimicrobial MIC obtained in this *in vitro* study it would not be sufficient to be effective against any of the three Gram-positive organisms (*S. epidermidis*, MRSA, *S. aureus*) or the five Gram-negative organisms (*E. faecalis*, *E. coli*, *Proteus Sp.*, *P. aeruginosa*, and *K. pneumoniae*) which were assayed at a concentration of  $5 \times 10^5$  CFU/mL.

If an individual with a UTI is infected with *E. coli* then the quantity of free hydroquinone should increase in the presence of β-Gln (expressed by the organism) and may be higher than the estimate above, which was calculated without the enzyme, but the *in*

*in vivo* studies with the four volunteers, which aimed to determine the difference in activity at different pH, inhibited growth of *E. coli* at  $0.5 \times 10^2$  CFU/mL and did not inhibit it at  $0.5 \times 10^4$  CFU/mL. The clinically accepted level for diagnosis of cystitis is  $\geq 10^3$  colonies bacteria/mL (Grabe *et al.*, 2009).

#### 7.3.5.4 Strengths and Limitations

A possible limitation of the volunteer study may be that the last dose of uva-ursi on Day 1 was taken at 6 pm rather than before bed to facilitate the collection of urine three hours later, and the next dose of the HMP was not taken until 9 am the next day. However, this regime did not appear to negatively affect the results. The complete inhibition of growth of bacteria was achieved amongst all volunteers by the first urine sample taken on Day 2 and it was not affected by pH.

#### 7.3.6 Conclusion

The *in vitro* experiments in the first part of this chapter demonstrated that hydroquinone may have greater antimicrobial activity with increasing alkalisation. This finding was not replicated with the urine samples from the four volunteers after taking the HMP. Growth of *E. coli* was inhibited in the urine specimens, but it was unaffected by adjusting the pH to alkaline. The duration of the study (over more than one day) had a more significant effect on the results.

There are many variables involved *in vivo*. The enzyme necessary for producing free unconjugated hydroquinone from the glucuronide  $\beta$ -GLn has an optimum pH of 6.8 - 7.0, which is lower than the pH required for optimum antimicrobial activity of hydroquinone (over pH 8). The pH of the urine samples varied for all four volunteers over the course of the day, and even if an alkalisating agent could increase an individual's urinary pH to alkaline long enough to enhance the activity of hydroquinone, there is no certainty that a sufficient amount would be produced by  $\beta$ -GLn to inhibit bacterial growth.

Previous recommendations regarding alkalisiation of urine for efficacy of uva-ursi cannot, therefore, be substantiated. Uva-ursi had a traditional use as a urinary antiseptic long before there was any investigation into how it worked and whether it required an alkaline environment. The possible presence of hydroquinone may enhance the antimicrobial activity of uva-ursi depending on the environmental conditions present, or there may also be another active compound or mechanism of action involved. Nevertheless, no study has ever investigated the metabolites produced from an individual infected with a UTI, and this will be looked at further in Chapter 8 in conjunction with patients taking part in the clinical trial (ATAFUTI). Chapter 9 investigates other potential mechanisms of action for uva-ursi.



## Chapter 8: Determination of metabolites in urine samples

### 8.1 Introduction

Chapter 7 reported that the urine samples of four healthy volunteers showed inhibitory activity against *E. coli* regardless of the urinary pH. It is possible that this activity might be due to hydroquinone should a sufficient quantity be present. Existing literature has described detecting the presence of hydroquinone in the urine samples of healthy volunteers by high performance liquid chromatography (HPLC), capillary zone electrophoresis and photometric methods (Paper, Koehler and Franz, 1993b; Wittig, Wittemer and Veit, 2001; Schindler *et al.*, 2002; Quintus *et al.*, 2005).

Whilst spiking samples with a pure reference compound is considered a reliable method of detecting compounds by HPLC, there have not, as yet, been any studies which have categorically confirmed the presence of hydroquinone in urine samples through analysis of molecular weight using mass spectrometry (MS) coupled to gas chromatography (GC). Neither have there been any studies which have investigated the metabolites of patients who have a urinary tract infection. It is important to examine the urine of an infected person to determine whether hydroquinone may be present, or may still need to be liberated from the parent hydroquinone-glucuronide with the addition of the enzyme  $\beta$ -glucuronidase ( $\beta$ -GLn).

With the prospective ATAFUTI clinical trial the opportunity existed to use HPLC to examine the urine samples of patients who may have a UTI, and who may have been assigned one of the two uva-ursi treatment arms. As a double-blind randomised placebo controlled trial it would not be possible to know which participants would have been given uva-ursi. At the end of the trial, when the results are un-blinded, the HPLC analysis of the patient samples can then be compared with the medication provided to each of the participants.

In order to be certain of analysing a reasonable number of active samples (in this case containing hydroquinone) the trial criteria stipulated that a minimum of 20 specimens would need to be examined. Owing to the large number of urine samples required, a

reliable and reproducible HPLC method would have to be developed for detecting hydroquinone whereby the machine and HPLC column would also be protected from salts and proteins. Through first analysing a volunteer urine sample using gas chromatography linked to mass spectrometry (GC-MS), the HPLC method could also be double-checked for determining the unequivocal presence of hydroquinone.

## 8.2 Aims

- To develop an HPLC method for detecting the presence of hydroquinone in the urine samples of patients on the ATAFUTI clinical trial.
- To confirm whether the peak observed in the HPLC chromatogram is hydroquinone through analysis of the molecular weight using GC-MS.
- To analyse a minimum of 20, and a maximum of 60, patient urine samples from the ATAFUTI trial for the presence of hydroquinone.

## 8.3 Methods

### 8.3.1 Chemicals and Reagents

Sep-Pak Vac 35 cc (10g) C18 cartridges (Waters) were used as the stationary phase to extract hydroquinone from urine. Analytical HPLC-grade methanol and deionised water were supplied by Fisher Scientific (UK). Hydroquinone (purity 99%+) and arbutin (purity 98%+) reference standards were obtained from Sigma-Aldrich and Alpha Aesar respectively, and were stored at -20° C.

Powdered  $\beta$ -glucuronidase ( $\beta$ -GLn) was from the same origin (*E. coli*) as the product used in the volunteer urine samples in Chapter 7 (Sigma-Aldrich), and was reconstituted in distilled water.

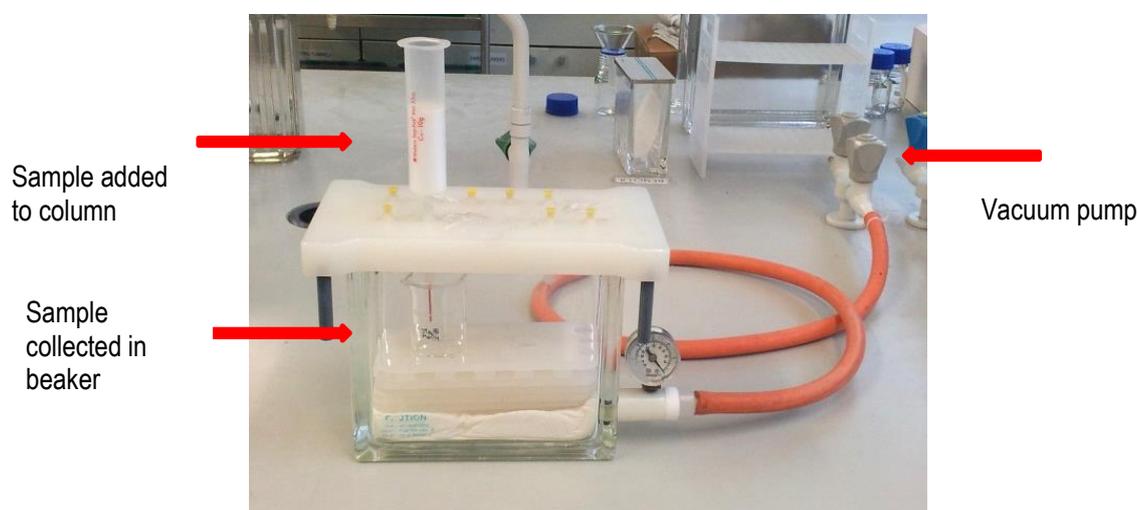
## 8.3.2 Extraction and Preparation of Samples for GC-MS Analysis

### 8.3.2.1 Extraction

In order to obtain a reasonably clean sample of hydroquinone from urine it was first necessary to use a liquid/liquid extraction procedure. This was done by passing the urine through the Sep-Pak C18 cartridge. This column has a non-polar stationary phase which retains non-polar compounds more efficiently than polar. This would enable hydroquinone (polar) to be washed off with polar solvents ( $\text{H}_2\text{O}$  and MeOH).

One of the volunteer urine samples used in Chapter 7, which was taken two hours after ingestion of the herbal extract, was filtered through a  $0.22\ \mu\text{m}$  Millex filter unit to remove existing bacteria. A total of  $9\ \mu\text{L}$  of  $\beta$ -glucuronidase per  $0.5\ \text{mL}$  urine was added and incubated for one hour at  $37^\circ\text{C}$  to deconjugate hydroquinone from the parent glucuronide.

The Sep-Pak column was conditioned with  $130\ \text{mL}$  MeOH, followed by  $130\ \text{mL}$   $\text{H}_2\text{O}$ , and then  $10\ \text{mL}$  urine was added to the column. This was washed with  $90\ \text{mL}$   $\text{H}_2\text{O}$ , and subsequently eluted with  $90\ \text{mL}$  MeOH. Both the  $\text{H}_2\text{O}$  and MeOH fractions were collected in separate beakers. An aliquot of each was removed ( $1\ \text{mL}$ ), added to an Eppendorf and centrifuged at  $13000\ \text{rpm}$  for HPLC analysis.



**Figure 33:** SepPak extraction of hydroquinone from urine.

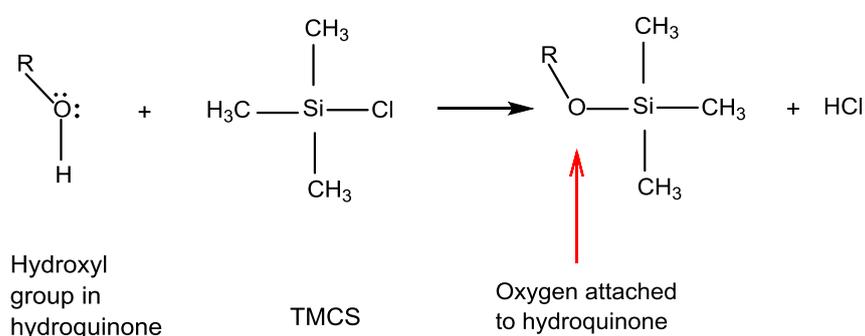
The urine sample was added to the column and collected in the beaker, after washing the column with either  $\text{H}_2\text{O}$ /MeOH. A vacuum was applied to draw the sample through the column.

A 400  $\mu\text{L}$  aliquot was then transferred into a 1 mL HPLC vial. A separate aliquot for GC-MS analysis (400  $\mu\text{L}$ ) was transferred to a pre-weighed vial, dried down and the weight recorded.

### 8.3.2.2 Derivatisation

Derivatisation is necessary when functional groups such as hydroxyls (-OH), which are non-volatile, can be problematic for detecting with GC-MS. The aim of the process is to produce a compound which is less polar and more volatile. Small molecules such as hydroquinone, for example, may have low volatility due to attractions between the hydroxyl groups (-OH), or the -OH groups could adsorb to the packing material on the surface of the column (Sobolevsky *et al.*, 2003; Poole, 2013).

*N,O*-bis(trimethylsilyl) trifluoroacetamide with 1% trimethylchlorosilane (BSTFA-TMCS) derivatisation is a silylation process whereby TMCS acts as a catalyst and increases the reactivity of the BSTFA. The active hydrogens (or protons) from an -OH group are replaced with an alkylsilyl group  $\text{Si}(\text{CH}_3)_3$ : this is usually trimethylsilyl (TMS). The resultant compound is less polar, thermally more stable, and more volatile and, therefore, suitable for vaporisation with gas chromatography (Poole, 2013). The expected chemical reaction is outlined below in **Figure 34**.



**Figure 34:** Silylation mechanism - reaction of an -OH molecule from hydroquinone with TMCS

To facilitate dissolving the samples in the derivatising reagent which comprised BSTFA-TMCS, both the hydroquinone reference compound and the dried urine sample potentially containing hydroquinone were separately re-dissolved in MeOH.

The reference standard of hydroquinone was prepared by dissolving 1 mg hydroquinone in 2 mL MeOH (99.99% for GC-MS). The MeOH was evaporated off (overnight in a fume cupboard), and 1 mL of BSTFA-TMCS was added to the dried residue. This was heated gently in an ultra-bath sonicator at 65 °C for 30 minutes until all the hydroquinone was dissolved and 300 µL were transferred to a vial for GC-MS.

The dried urine sample (from 400 µL) was prepared in a similar manner, through adding 1 mL MeOH and then evaporating this off. The resultant dried residue weighed 0.02 mg. This was dissolved in 100 µL BSTFA-TMCS.

### **8.3.3 Preparation and Analysis of Patient Urine samples**

Patients on the ATAFUTI trial were asked to take a urine sample two hours after taking their trial medication on the fourth day of participating in the trial. They then posted the sample to the laboratory as soon as possible in the secure packaging provided. The packaging was in accordance with the Royal Mail PI 650 guidelines (IATA, 2011).

On receipt in the laboratory all samples were initially filtered through a 0.22 µm Millex filter to remove cells and any existing bacteria, and 1 mL of urine was then added to each of two 7 mL bijoux containers. The first was used as a control sample. To the second, 20 µL of the enzyme  $\beta$ -GLn was added and incubated for 30 minutes at 37° C. This was for deconjugating free hydroquinone from hydroquinone glucuronide.

In order to develop a workable method for analysing the patient urine specimens by HPLC, the urine samples from two of the volunteers from the antimicrobial study were tested (Section 7.3). (The analysis of metabolites from these volunteer samples was included in the aforementioned UCL ethics approval - Project ID: 6376/001). This involved adopting an existing published method for precipitating proteins and salts in order to protect the HPLC apparatus and column from contamination (McGuffey *et al.*, 2014). Prior to centrifuging the filtered urine samples for HPLC, 200 µL of urine was

transferred to an Eppendorf, and diluted with 200  $\mu\text{L}$  of distilled water. To this 850  $\mu\text{L}$  of acetone was added. The samples were placed for 10 minutes in a fridge to precipitate out the proteins, and then centrifuged before transferring an aliquot (400  $\mu\text{L}$ ) into HPLC vials.

Three vials for each patient were prepared, one control, and two containing  $\beta\text{-GLn}$  samples. The vials were left with lids off (but covered) for 24 hours to evaporate the acetone. One of the vials containing the enzyme  $\beta\text{-GLn}$  was then spiked with an equal amount (1:1 v/v) of a 0.125 mg/mL hydroquinone reference sample (diluted in 95:5  $\text{H}_2\text{O}/\text{MeOH}$ ). This was for confirming the presence of hydroquinone according to the retention time ( $t_R$ ).

This final HPLC method was tested (results below) and then included in the publication of a laboratory manual (Appendix K), for submission in the ethics application for the ATAFUTI trial. Ethics approval was provided by the South Central – Hampshire Research Ethics Committee (REC No. 14/SC/1143).

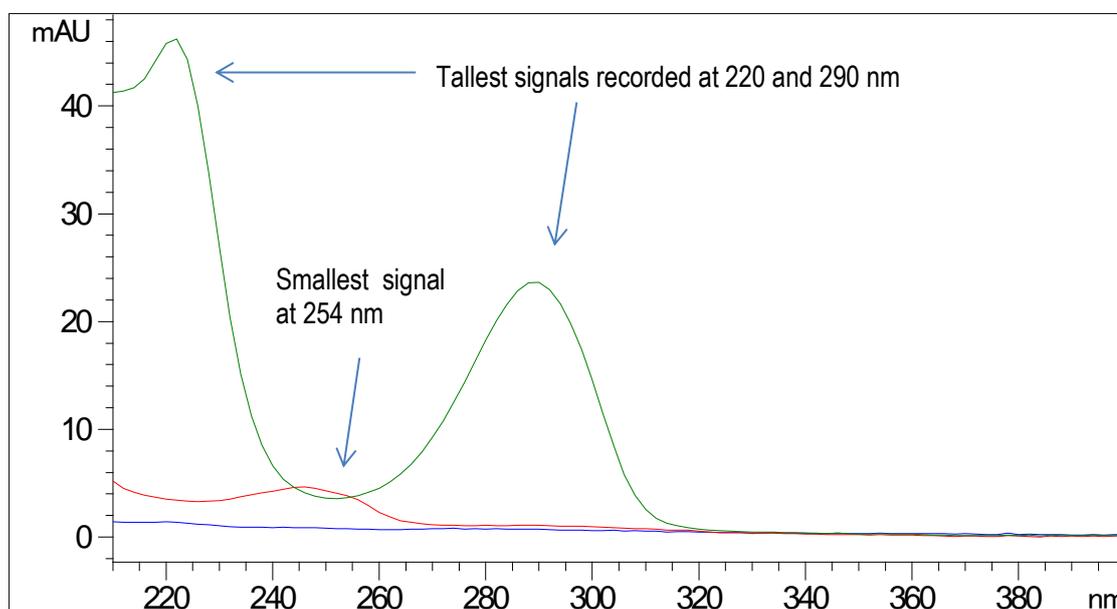
#### **8.3.4 HPLC Analysis**

The identical Agilent 1200 equipment, Diode Array Detector and UV spectra ranges were employed to run and detect the samples as used in Section 6.3.3. The column and method were altered to accommodate a longer time and slower flow rate. These comprised: a reversed-phase C18 Supelco Ascentis column (25 cm x 4.6 mm, 5  $\mu\text{m}$ ) for the stationary phase. The gradient flow rate was 0.9 mL/minute with mobile phase 95:5  $\text{H}_2\text{O}:\text{MeOH}$  for 0-4 minutes, followed by a linear gradient of 70:30 from 5-11 minutes, and 100% MeOH from 12-16 minutes. An equilibration period comprising the starting ratio of the mobile phase ran for 10 minutes before the next injection. Blanks containing the mobile phase were run at the start and end of each analysis to check for any contaminants in the system.

A control sample of the hydroquinone reference compound diluted in the mobile phase, with acetone added, was run to check that the acetone did not affect the hydroquinone peak. Acetone has a UV absorbance at 260 nm.

A control of the enzyme  $\beta$ -GLn (in control urine without the herbal extract) was also run to compare with a sample containing the herb (in urine) with  $\beta$ -GLn added. A reference sample of arbutin was run to check whether this compound may be present in the urine.

In addition to comparing the retention time of the urine samples with that of the spiked samples containing hydroquinone, recording the data at all the main UV wavelengths produced by hydroquinone could enable a more precise analysis of the results. Data were, therefore, recorded at the highest expected peaks of 220 nm, 280 nm and 290 nm, as well as the lowest peak of 254 nm. An example spectra produced by the reference compound during the method development is depicted in **Figure 35**.



**Figure 35:** Shape of UV signal for hydroquinone reference compound recorded at 220 nm

### 8.3.5 GC-MS Analysis

Electron impact (EI) ionization has historically been the method of choice for detecting molecules with a low molecular weight. It would, therefore, be the preferred method for

hydroquinone which has a molecular weight of 110. The constant ionization energy, usually at 70 eV, produces sufficient fragments visible in the spectra to be able to identify the chemical structure (Scheubert, Hufsky and Böcker, 2013).

A Thermo Trace 1300 GC system coupled to an ISQ Single Quadrupole MS and connected to a Thermo Scientific TriPlus RSH autosampler was used for analyses. The analytes were separated on a Trace TR 5MS universal column (30 m x 0.25 mm ID, 0.25  $\mu\text{m}$  film thickness), 5% Phenyl Polysilphenylene-siloxane from Thermo Scientific. Helium was used as the mobile phase at a flow rate of 1.5 mL/minute. The oven temperature was set at 60 $^{\circ}$  C for 1 minute, ramped to 250 $^{\circ}$  C at a rate of 30 $^{\circ}$  C/minute to 7.25 minutes, and then held for 6 minutes. The injector and mass transfer line temperatures were set at 230 $^{\circ}$  C. The injection was splitless, and the volume injected was 1  $\mu\text{L}$ . The MS detector operated in electron impact (EI) ionisation mode, with the ion source temperature set at 230 $^{\circ}$  C. The scan range was 50-800  $m/z$  with a scan time of 0.2 seconds.

The hydroquinone sample already derivatised with BSTFA-TMCS (10  $\mu\text{L}$ ) was diluted in dichloromethane (DCM) (90  $\mu\text{L}$ ).

Hydroquinone was identified through matching the retention times and mass spectral data with the pure reference compound of the same. A control sample (without hydroquinone) of BSTFA + TMCS (1%) diluted in DCM (1% solution) was also injected (1  $\mu\text{L}$ ) to be able to identify and compare its fragmentation.

## **8.4 Results**

### **8.4.1 HPLC**

#### **8.4.1.1 Optimisation of HPLC Method**

The retention time ( $t_R$ ) for hydroquinone for the volunteer sample was recorded at 10.02 minutes. The calculated coefficient of determination for measuring the linearity (based on 4 concentrations of hydroquinone, from 5  $\mu\text{g/mL}$  - 15  $\mu\text{g/mL}$ ) was  $R^2 = 0.999$ .

The limits of detection (LOD) and quantification (LOQ) of hydroquinone based on a 20 µL injection are shown in **Table 17**. These were calculated from the linearity using the standard deviation of the response (Sy) and the slope (S) according to the following equations:  $LOD = 3 \times Sy/S$ , and  $LOQ = 10 \times Sy/S$  (ICH, 1996).

**Table 17:** LOD and LOQ for hydroquinone

	LOD µg/mL	LOQ µg/mL
Hydroquinone	0.5	1.6

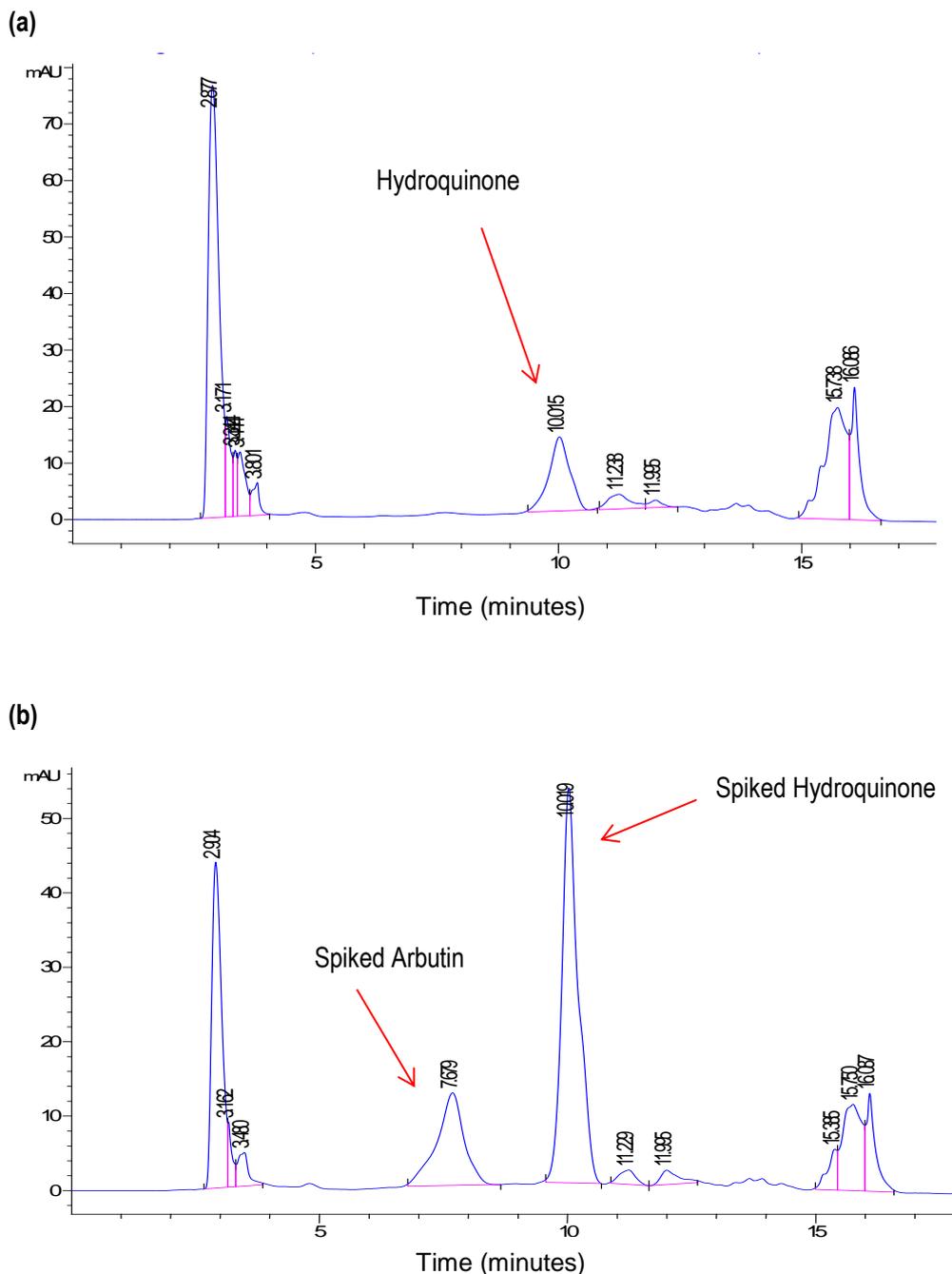
The accuracy of the method was validated by the recovery of hydroquinone from the spiked (volunteer) samples of urine with three different concentrations measured at both 220 nm and at 290 nm. These are recorded in **Table 18**.

**Table 18:** Recovery (%) of hydroquinone spiked in volunteer urine sample at three different concentrations (intra-day)

Quantity of Hydroquinone Spiked mg/mL	Recovery (%) 220 nm	RSD % Intraday	Recovery (%) 290 nm	RSD % Intraday
0.125	94.00	2.25	105.4	3.69
0.094	92.31	1.97	106.3	1.56
0.031	91.00	1.40	105.2	2.00

### 8.4.1.2 Presence of Hydroquinone Detected in H<sub>2</sub>O and MeOH Extractions

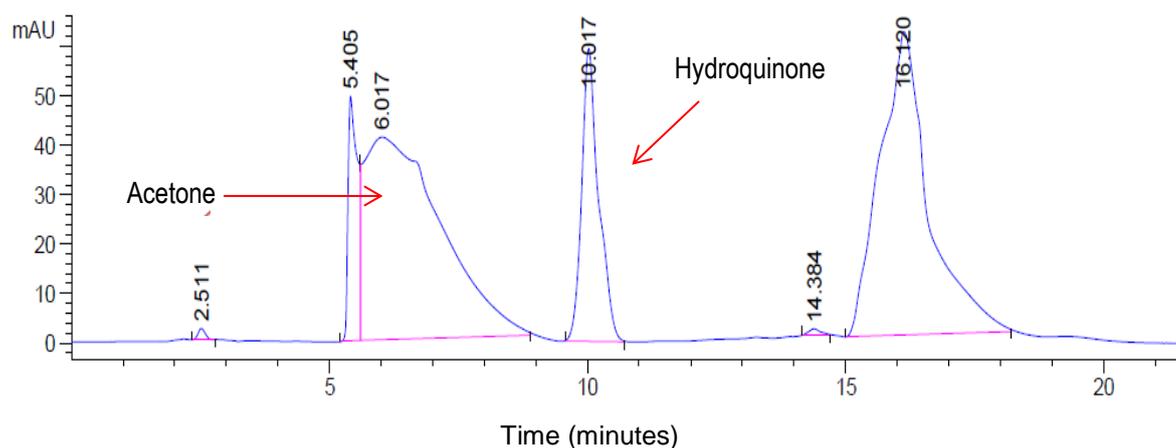
Hydroquinone was detected in both the water and MeOH fractions when the retention times were compared to the hydroquinone reference compound. The MeOH sample produced a cleaner chromatogram than the water extract, and this together with the sample spiked with the hydroquinone reference standard is illustrated in **Figure 36**.



**Figure 36:** HPLC chromatograms showing (a) hydroquinone extracted from urine sample at 10.02 minutes, and (b) the same spiked with pure hydroquinone reference compound

No arbutin was detected as being present in the extracted urine sample (confirmed by spiking the compound).

The sample with just hydroquinone and acetone showed a clear separate peak for hydroquinone. The peak was sharpest both at 220 nm (especially) and at 290 nm. These signals had a larger peak area than at 280 nm, and would be relevant for detecting hydroquinone in the patient urine samples.



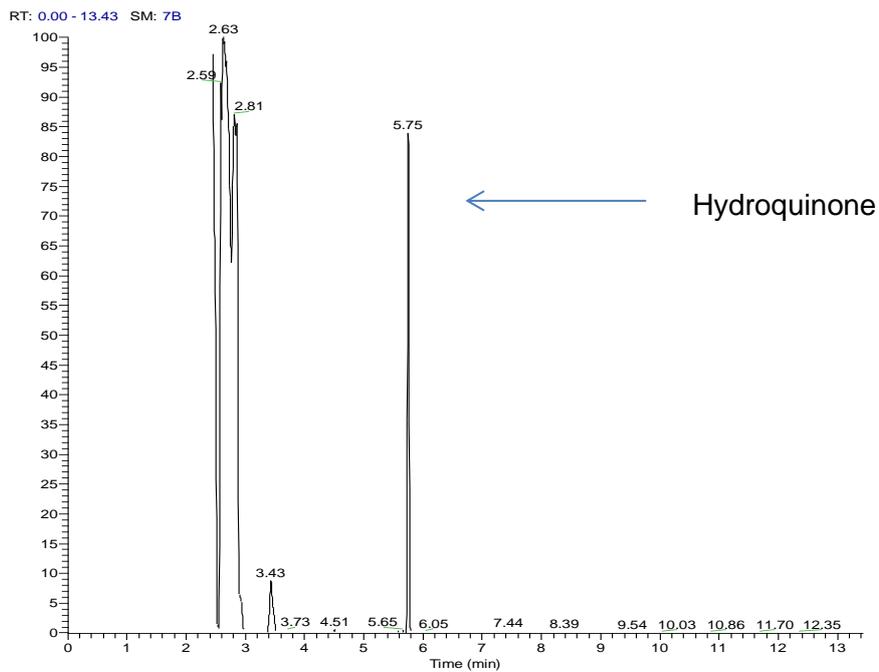
**Figure 37:** Sharp hydroquinone peak visible at 10.017 minutes. Acetone peak visible at 6.017 minutes.

Following the above results the method was then adopted for analysing the patient urine samples.

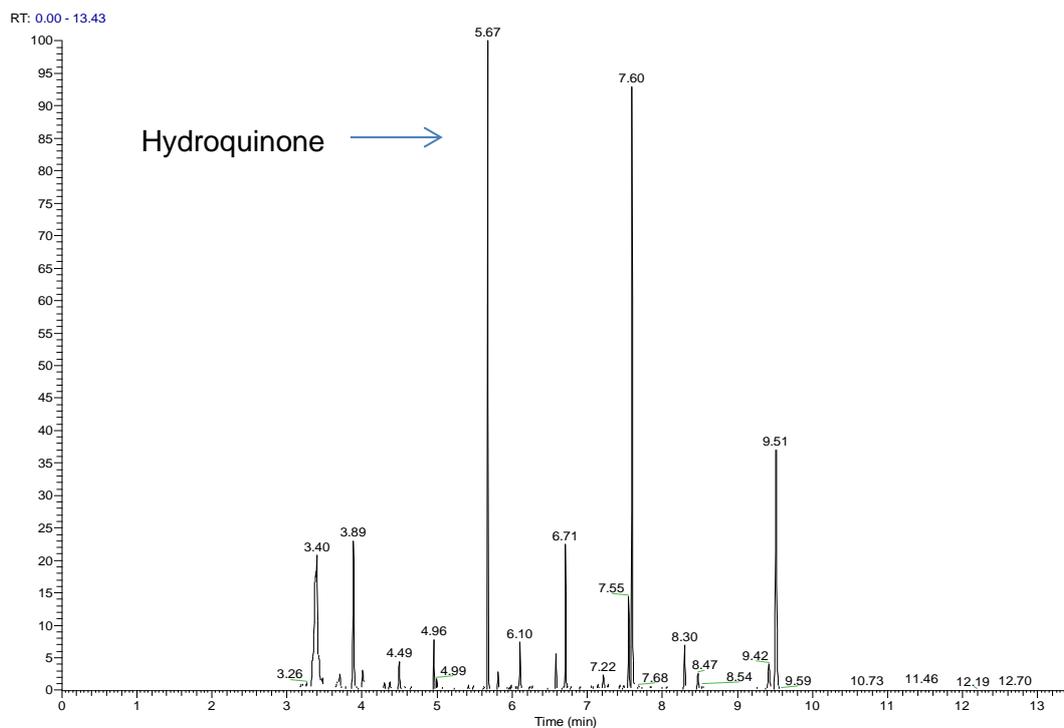
#### 8.4.2 GC-MS

The chromatogram of the control sample of BSTFA-TMCS (without either hydroquinone or the dried urine sample) showed a tall peak at 2.57 minutes. The control sample of hydroquinone eluted at 5.67 minutes. The same retention time ( $t_R$  5.67) was also visible in the chromatogram of the derivatised extract from the urine sample. These are both shown in **Figure 38**.

(a)

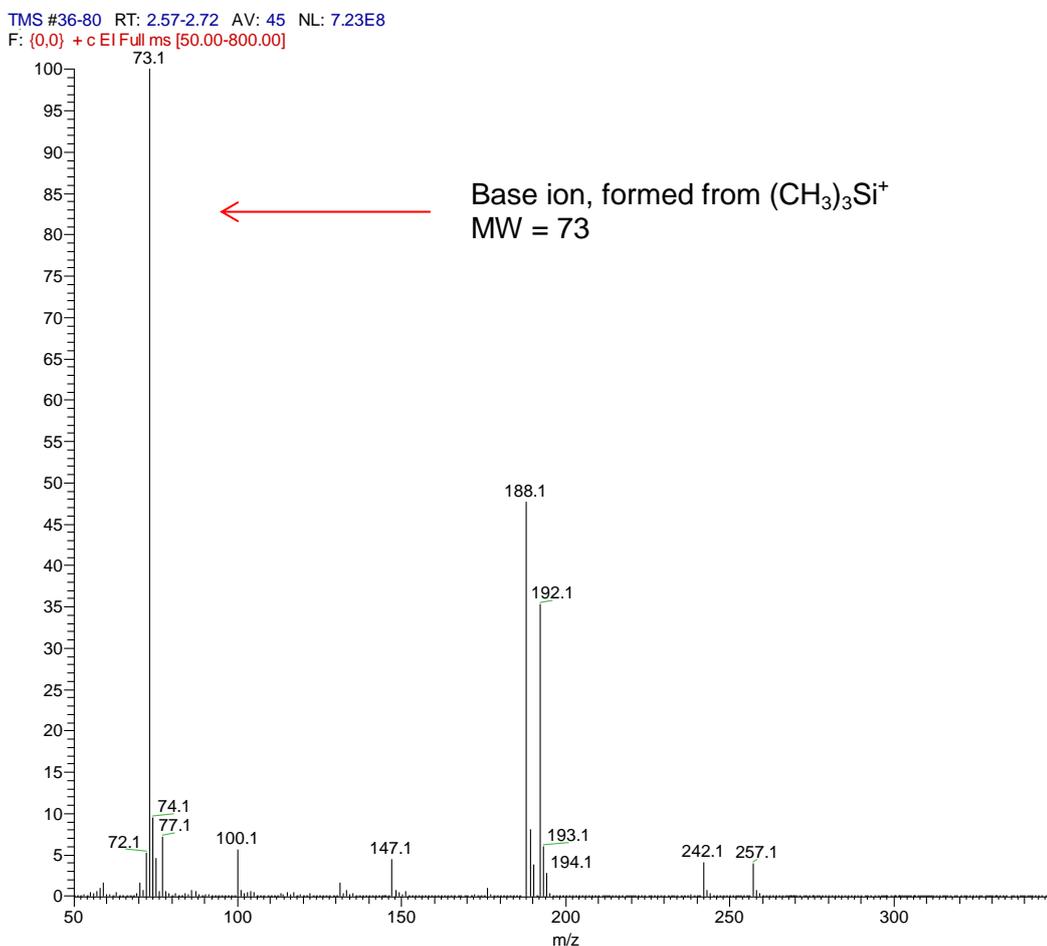


(b)



**Figure 38:** Chromatograms of (a) hydroquinone reference standard derivatised with BSTFA-TMCS (1%) eluting at 5.67 minutes, and (b) dried urine sample also containing hydroquinone peak at 5.67 minutes

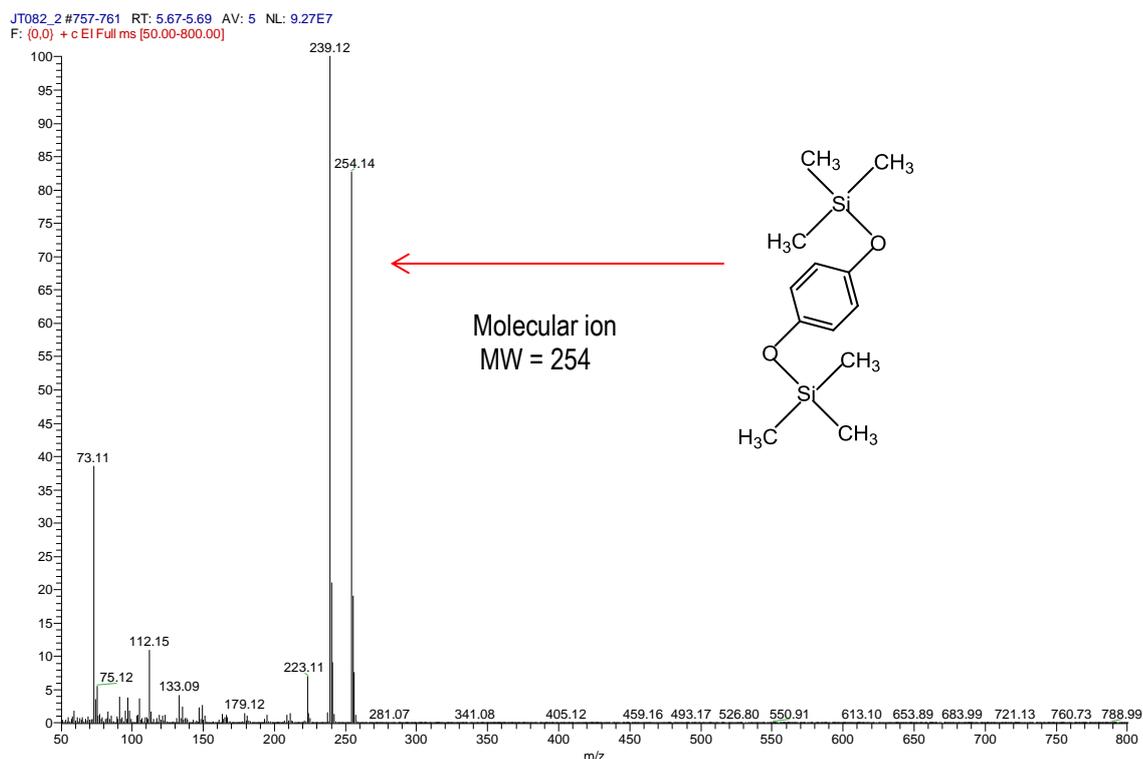
Through derivatisating hydroquinone with BSTFA-TMCS the molecular weight of the overall compound is increased, and ion fragments of all these constituents were detected. An ion which should commonly be found, formed from three methyl groups bonded to a silicon atom in the TMS, comprised the base ion at a mass to charge ratio ( $m/z$ ) of 73 in the control sample of just the BSTFA-TMCS at 2.57 minutes.



**Figure 39:** Ion fragments present in control sample containing BSTFA-TMCS at 2.57 minutes with base ion at 73  $m/z$

The ion fragments produced by both the hydroquinone reference sample and the dried urine sample potentially containing hydroquinone comprised 73  $m/z$ , 239  $m/z$  and 254  $m/z$ . These are relevant to both the methylsilyl groups in TMS and the hydroquinone molecule. The proposed chemical structure of the derivatised molecule based on the

molecular ion at 254  $m/z$  is shown in **Figure 40**.



**Figure 40:** Chemical structure of derivatised hydroquinone molecule with 2 trimethylsilyl ethers from TMS at 254  $m/z$  at 5.67 minutes. Base ion was present at 239  $m/z$ .

### 8.4.3 Patient Urine Samples

A total of 60 samples from the clinical trial were received for analysis. Hydroquinone was detected in 24 of these by HPLC, and ten of these required the addition of the enzyme  $\beta$ -GLn before hydroquinone was observed in the chromatogram. Exactly half the samples (30) did not show any hydroquinone, and six samples showed a retention time which corresponded with hydroquinone but not the correct UV shape of the spectrum (as portrayed in **Figure 35**). The peak area of the signal recorded at 254 nm was higher than the peak areas of the other signals (220 and 280) when it should have been lower. The results are summarised below in **Table 19**.

**Table 19:** Number of patient urine samples in which hydroquinone was detected both before and after adding  $\beta$ -GLn

Detection of hydroquinone	Number of samples
Hydroquinone present	24
<i>After adding enzyme <math>\beta</math>-GLn (n = 24)</i>	10
No hydroquinone	30
Possible hydroquinone, but 254 nm signal too high	6
<b>Total samples processed</b>	<b>60</b>

## 8.5 Discussion

The aim of the study was to develop a method using HPLC and GC-MS to confirm the presence of hydroquinone in urine after taking uva-ursi. This was achieved, and used to detect hydroquinone in the urine samples of patients on the ATAFUTI trial.

### 8.5.1 HPLC

The chromatogram of the spiked volunteer urine sample depicted in **Figure 36** shows that hydroquinone could be determined by HPLC following the sample extraction via the Sep-Pak column, and that the chromatogram was relatively free of other peaks.

Arbutin was not detected, and would most likely have been hydrolysed during the digestive process.

The accuracy of the method was confirmed through good recovery (between 91% and 106%) of the spiked volunteer sample with known concentrations of hydroquinone. The results were unaffected by adding acetone to the vials, and the method was, therefore, considered suitable for testing the patient urine samples for the presence of hydroquinone.

### 8.5.2 Confirmation of Hydroquinone by GC-MS

Owing to the molecular ion present at 254  $m/z$ , which could be formed from two methylsilyl groups replacing the hydrogen atoms in hydroquinone, the presence of hydroquinone in the volunteer urine sample was confirmed (see **Figure 40**).

Other molecular ions detected were characteristic of the fragmentation pattern due to the silylation with TMS. For example, the base ion at 239  $m/z$  would comprise the same chemical structure as that depicted in **Figure 40** but with the loss of one of the methyl groups ( $\text{CH}_3$ ) which has an  $m/z$  of 15. These results corresponded with the literature reporting the ionization of a molecule of the same mass (110) and similar structure (catechol) which had been derivatised with BSTFA-TMCS (Kiprop, Pourtier and Kimutai, 2013).

### 8.5.3 Patient Urine Samples

The antimicrobial studies in Chapter 7 demonstrated that, after being taken by healthy volunteers, the uva-ursi herbal extract may have activity against *E. coli* in urine. Whilst the results were positive, and did not require an alkaline pH for activity, they were not consistent when the enzyme  $\beta$ -GLn (which is expressed by *E. coli*) was added. It was not clear whether a sufficient amount of hydroquinone could be deconjugated to produce antimicrobial activity without separately adding this enzyme: there was less growth of the bacteria with two of the volunteer samples at pH 6.7 once the enzyme

had been added, but its inclusion did not affect the samples of the other two volunteers at this pH.

Previous literature has reported that hydroquinone has been detected in some, but not all urine samples after taking uva-ursi. In fact it has appeared in just under half those sampled (Siegers *et al.*, 1997; Schindler *et al.*, 2002; Quintus *et al.*, 2005). Urine samples from a person infected with a UTI have never before been tested. It is expected that hydroquinone may be released from the parent glucuronide should *E. coli* be the cause of an infection (Siegers *et al.*, 2003).

In this study a total of 60 urine samples from patients on ATAFUTI were received and processed for analysis by HPLC. Of these, 24 showed the presence of hydroquinone with the correct retention time and corresponding shape of the UV signal, but just under half (10/24) also required the addition of the enzyme  $\beta$ -GLn before hydroquinone was observed in the chromatogram.

A further 30 samples did not show any hydroquinone present. This could be expected as it was a placebo controlled trial, although it is also possible that the patient(s) did not have a proven infection. The remaining six samples analysed displayed the correct retention time, but not the correct shape of UV signal (see **Figure 35**).

The fact that some of the samples indicated the presence of hydroquinone after adding the enzyme  $\beta$ -GLn, but did not show the metabolite in the original sample without the enzyme, suggests that either the patients may have had a UTI which was not caused by *E. coli* or, if it was, then not enough  $\beta$ -GLn was produced by the bacterial organism to deconjugate hydroquinone from the parent glucuronide. If the presence of hydroquinone is required for antimicrobial activity, then these (ten) samples may not have been effective against the bacteria. Nevertheless, the precise mechanism of antimicrobial action is still to be determined.

The six patient samples which were not possible to analyse sufficiently to confirm the presence of hydroquinone, despite there being the appropriate observed retention time for this compound, could have possibly been affected by another medication. Some patients on the trial were also given advice to take ibuprofen alongside the trial medication as this formed another arm of the ATAFUTI trial as an alternative treatment. This was prescribed as an anti-inflammatory rather than an antimicrobial as several studies have investigated the antimicrobial activity of ibuprofen against *E. coli* and

shown the MIC to be high at 2.5 mg/mL (Hussein and Al-Janabi, 2010). However, the retention time for ibuprofen was checked via HPLC (both with and without uva-ursi) and this medication did not interfere with the results.

The presence of hydroquinone in the urine samples may give an indication of patient compliance with taking the medication on the trial. Nevertheless, it is also possible that there may have been another source of the hydroquinone which was detected: it can be produced through consumption of certain foods, for example pears, which contain arbutin but in smaller amounts than uva-ursi, and also through smoking (see Section 3.3) (de Arriba, Naser and Nolte, 2013). However, urine samples in this study could not be analysed at baseline. The specimens had to be provided after the patients had started taking the intervention in order that they potentially contained metabolites of the HMP.

A limitation of the study was that it was not possible to quantify the amount of hydroquinone present in the urine samples. It would not have been practical to monitor the liquid intake of the patients whilst they were on the trial.

## **8.6 Conclusion**

The presence of hydroquinone could be detected in urine after ingesting the herbal extract through the method developed by HPLC, and confirmed through analysis of the molecular weight using GC-MS.

The results of the patient urine samples suggested the presence of hydroquinone in less than half of those tested. The analysis needs to be compared further with the unblinded data from the patients in the clinical trial, once the information is available. This will help determine whether hydroquinone was present in the urine of patients who were assigned uva-ursi. The results can then also be compared to their clinical outcome.

Future work might entail repeating the method to quantify the amount of hydroquinone detected to determine whether it might be sufficient to produce antimicrobial activity.

## **Chapter 9: Effects of uva-ursi on potential *E. coli* virulence factors**

### **9.1 Introduction**

In addition to demonstrating antimicrobial efficacy it is perhaps relevant to consider that uva-ursi may have other therapeutic mechanisms of action relating to its pharmacodynamic properties as indicated in Section 3.4.2 (such as anti-inflammatory activity). These might potentially work synergistically within the body and the immune system.

With *E. coli*'s ability to enhance its potential to cause disease via virulence factors such as motility and aggregation (a process believed to be enhanced by hydrophobicity) (see Section 2.9.1) it was considered important to investigate whether uva-ursi's therapeutic properties could affect either of these actions. The background to both the mechanisms and the assays required to determine the potential effect by uva-ursi are covered separately within this chapter.

### **9.2 Hydrophobicity and Aggregation**

#### **9.2.1 Background**

Several aspects of the cell surface of a micro-organism contribute to its ability to interact with its host environment, including hydrophobic forces more commonly referred to as cell surface hydrophobicity. These forces of attraction between the cell and other hydrophobic surfaces, which have weak affiliation with aqueous environments, are believed to contribute to the organism's potential to aggregate, adhere to the host mucosal epithelium and potentially also to form biofilms (Popovici *et al.*, 2014). In fact bacterial aggregation is believed to be linked to and enhanced by an increase in the cell surface hydrophobicity of *E. coli*. This in turn may lead to bacterial

adhesion to the host epithelium via either Type 1 or P fimbriae and the subsequent colonisation of pathogens in the urinary tract (see Section 2.9.1) (Doyle, 2000).

In a small study it has been shown that almost all strains (73%) of *E. coli* present in lower urinary tract infections demonstrate hydrophobic properties and are, therefore, aggregative (Puzova *et al.*, 1994), but how important this mechanism is to the establishment of a UTI in humans is still not certain as most reports related to Type 1 fimbriae adhesins have been conducted on mice (Tchesnokova *et al.*, 2011). Furthermore, hydrophobic molecules may aggregate to each other to form larger aggregates, which may then be more easily detected by the immune system (Seong and Matzinger, 2004). A glycoprotein, uromodulin (also known as Tamm-Horsfall) is produced in the kidney, and is part of the innate immune response. It has been found to bind to Type 1 fimbriae in uropathogenic *E. coli* during a UTI. Uromodulin causes *E. coli* to aggregate whilst preventing the organism from interacting with the uroepithelium, resulting in the bacteria being flushed out of the urinary tract (Abraham and Miao, 2015). Studies on mice demonstrated that knocking out the gene for producing uromodulin resulted in a longer duration of bacterial infection compared to those where the gene was present (Bates *et al.*, 2004, Mo *et al.*, 2004).

Five *in vitro* studies have investigated the effect of traditional plant based medicines on the hydrophobicity and aggregation of bacteria known to cause UTI, including *E. coli* and *S. aureus*. In addition to testing uva-ursi the effect of other herbal plants on aggregation was also examined. These traditional medicines included *Vaccinium vitis-idaea* (cowberry), *Matricaria recutita* (chamomile), *Calendula officinalis* (calendula), *Mentha piperita* (Peppermint), and *Hypericum perforatum* (St John's Wort). They were tested on bacteria derived from a variety of human, animal and food sources (Turi *et al.*, 1997; Annuk *et al.*, 1999; Türi *et al.*, 1999; Dykes, Amarowicz and Pegg, 2003). An outline of the results is summarised in **Table 20**. The findings are not directly comparable to each other as different methodologies were used, and the original paper featuring *M. piperita* (cited in Türi *et al.*, 1999) is unavailable, but they provide a good indication of the difference in activity between the plants tested.

The reports concluded that uva-ursi and *V. vitis-idaea*, plants whose main constituents are arbutin and tannins, modulated the cell surface of bacteria and in so doing increased the hydrophobicity and aggregation of the organism. *H. perforatum* had a weaker aggregative effect than uva-ursi on non-aggregative strains. *M. recutita*,

*C. officinalis* and *M. piperita*, on the other hand, attenuated aggregation in *E. coli* in both aggregative and non-aggregative strains (Turi *et al.*, 1997; Türi *et al.*, 1999; Dykes, Amarowicz and Pegg, 2003).

**Table 20:** Results of aggregation assays including uva-ursi vs *E.coli*, *H. pylori*, and *S. aureus*. + indicates level of aggregation scored visually from + to +++ with 3 being the most aggregative

	Plant/Solvent Extraction					
	Uva-ursi EtOH	Uva-ursi H <sub>2</sub> O	<i>V. vitis- idaea</i> H <sub>2</sub> O	<i>H. perforatum</i> H <sub>2</sub> O	<i>M. recutita</i> H <sub>2</sub> O	<i>C. officinalis</i> H <sub>2</sub> O
<i>E. coli</i> Aggregative strain	n/a	+++	++	+++	-	-
<i>E. coli</i> Non-aggregative strain	-	++	++	++	-	-
<i>H. pylori</i>	+	n/a	++	-	-	-
<i>E. coli</i>	++	n/a	n/a	n/a	n/a	n/a
<i>S. aureus</i>	-	n/a	n/a	n/a	n/a	n/a

(Adapted from: Annuk *et al.*, 1999; Dykes *et al.*, 2003; Türi *et al.*, 1999; Turi *et al.*, 1997)

Whilst uva-ursi is a traditional treatment for UTI, the three plant extracts which had no effect on enhancing aggregation (*C. officinalis*, *M. recutita*, *M. piperita*) are not traditionally deemed ‘specifics’<sup>2</sup> for this purpose (Mills and Bone, 2005). Moreover, none contain arbutin, and in the case of *M. recutita* and *M. piperita* they contain minimal amounts of tannins. *H. perforatum*, which had a weaker reaction than uva-ursi contains tannins but not arbutin (Barnes, Anderson and Phillipson, 2001; Germ *et al.*, 2010).

Three of the above studies, which were author related, deduced that stimulating the

<sup>2</sup> Herbal treatment of a specific symptom or condition

bacteria to aggregate could be part of uva-ursi's mechanism of action in alleviating symptoms of UTI. After first testing the plant for antimicrobial activity and determining that uva-ursi demonstrated what they described as a 'moderate' antimicrobial effect Turi and co-workers concluded that inducing aggregation of bacterial cells could interfere with the development of a UTI through facilitating the excretion of microbial particles in a cluster via the urine (Turi *et al.*, 1997). Nevertheless, the fourth and the most recent study by Dykes and colleagues, proposed the opposite theory: that an increase in hydrophobicity may be associated with increased pathogenicity (Dykes, Amarowicz and Pegg, 2003).

There was a marked difference which affected hydrophobicity from two extraction methods. A cold maceration<sup>3</sup> left for 4 days enhanced aggregation of both hydrophobic strains and non-hydrophobic strains of *E. coli* more efficiently than either a decoction<sup>4</sup> or heated maceration. The latter needed to contain 256 times more extracted constituents than a cold maceration to produce aggregation of a hydrophobic strain, and 64 times more for a non-hydrophobic strain. The authors concluded that the constituents affecting aggregation were, therefore, negatively affected by heat (Türi *et al.*, 1999).

With the aim of establishing what may be the active phytochemical constituents Turi and co-workers investigated the total amounts of arbutin and tannins extracted from both macerating and decocting uva-ursi and *V. vitas-idaea*. They determined the total weight of compounds from each method extracted to be identical. Of this, the percentage of tannins and arbutin in uva-ursi was slightly greater for the decoction. This comprised 49.9% vs 46.4% (tannins) and 23.6% vs 19.8% (arbutin) (Türi *et al.*, 1999). When compared to *V. vitas-idaea* the amount of arbutin produced via maceration was almost identical to uva-ursi (19.8% uva-ursi vs 20.3% *V. vitas-idaea*), but tannins were virtually double for uva-ursi (46.4% vs 23.7%). The authors found that three times more extracted constituents from *V. vitas-idaea* were required to produce the equivalent aggregation of *E. coli* (Türi *et al.*, 1999). This implies that the tannins may be the active ingredient.

An additional study further analysed the effect of individual constituents, including tannic acid. This was on *Helicobacter pylori* instead of *E. coli* yet it showed that

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<sup>3</sup> Plant material steeped in distilled water at 4°C

<sup>4</sup> Plant material heated for 30 minutes at 100°C

aggregation was enhanced by tannins, thus reinforcing the above supposition that tannins may be the most relevant constituent (Annuk *et al.*, 1999).

As aggregation of *E. coli* in the urinary tract could possibly be influenced by uva-ursi it was decided to repeat the previous tests with the clinical trial (ATAFUTI) herbal medicinal product (HMP) using the same strain of *E. coli* tested in the anti-microbial assays (see Chapter 7).

### 9.2.2 Salt Aggregation Test (SAT)

The SAT was originally devised by Lindahl and co-workers to investigate the aggregative and hydrophobic properties of bacteria (Lindahl *et al.*, 1981). The technique is based on the principle that proteins can be precipitated by salts. Therefore, once bacteria with hydrophobic properties are immersed in a solution of inorganic salt such as ammonium sulphate  $(\text{NH}_4)_2\text{SO}_4$ , a non-aggregating strain will aggregate together. The lower the concentration needed to aggregate the organisms, the more hydrophobic the cell surface of the bacteria (Lindahl *et al.*, 1981), whilst hydrophilic bacteria require more time and a higher salt concentration before aggregation will occur (Doyle, 2000). Using a weaker salt than  $(\text{NH}_4)_2\text{SO}_4$  such as sodium chloride present in PBS will cause aggregation of an auto-aggregating strain (Lindahl *et al.*, 1981).

Lindahl's original assay demonstrated that pH only slightly affected the endpoint, a strain aggregating at 0.04 M  $(\text{NH}_4)_2\text{SO}_4$  at pH 5.2 aggregated at 0.05 M at pH 7, but, would aggregate better in lower salt concentrations at 37°C than at 4°C (1.2 M versus 1.4 M respectively) (Lindahl *et al.*, 1981).

Both time and bacterial cell concentration also affected the reaction. An increase in incubation time reduced the molarity required (from 0.05 M to 0.03 M from 2-10 minutes respectively), and the endpoint could be observed more readily at  $5 \times 10^9$  than at  $5 \times 10^{10}$  microbial cells/mL (Lindahl *et al.*, 1981).

### 9.2.3 Method

A 1:10 aqueous dilution (cold maceration with distilled water) of the powdered trial HMP was prepared, left for 30 minutes, and filtered through absorbent cotton.

*E. coli* (NCTC 10418) was sub-cultured on agar plates and grown at 37° C for 18-24 hours. Inoculum suspensions (in PBS) were adjusted to  $3 \times 10^9$  microbial cells/mL, and 0.8 mL of the inoculum was added to 0.8 mL of the aqueous herbal extract. This was left to stand at room temperature for 5 minutes. Ammonium sulphate solutions of 0.1, 0.5, 1.0, 1.5, and 3.0 M were prepared in PBS (pH 6.8). Equal amounts (0.15 mL) of  $(\text{NH}_4)_2\text{SO}_4$  and bacterial/herbal solutions were pipetted into wells of U-shaped microtitre plates, to give final concentrations of 0.05, 0.25, 0.5, 0.75 and 1.5 M ammonium sulphate and  $0.75 \times 10^9$  microbial suspensions.

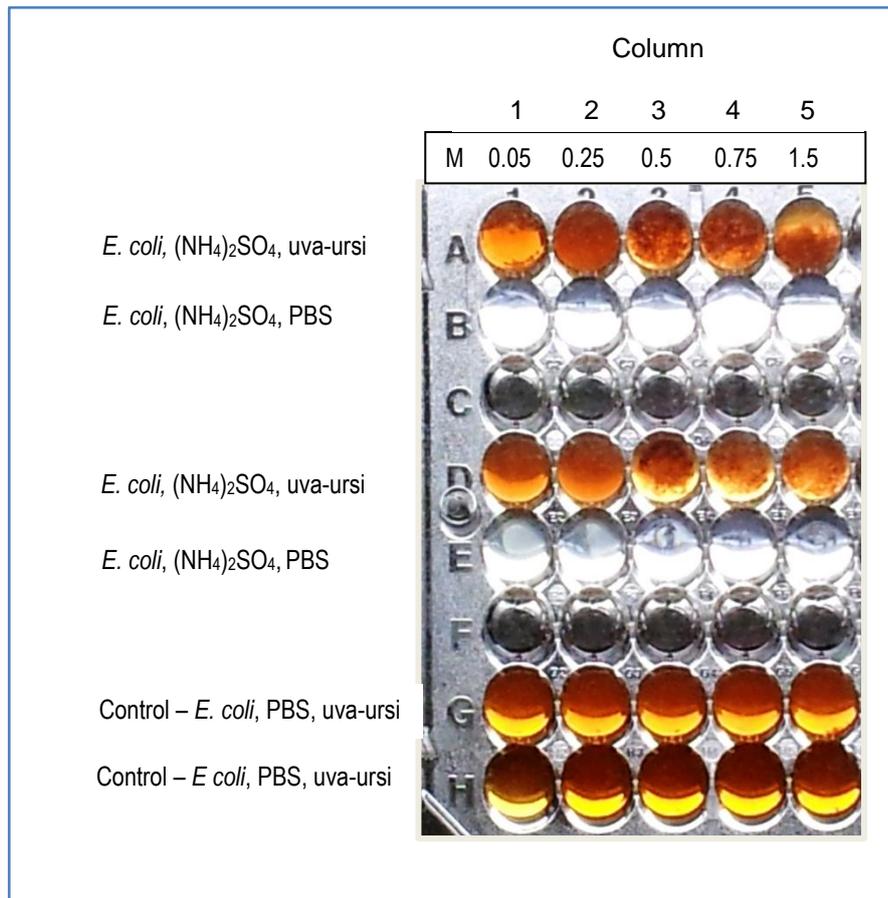
Controls were prepared using 0.8 mL PBS instead of the herbal extract, PBS with the extract but with no  $(\text{NH}_4)_2\text{SO}_4$ , and *E. coli* with the extract plus PBS instead of  $(\text{NH}_4)_2\text{SO}_4$ . All assays were repeated five times (inter-day).

Plates were shaken gently for 5 minutes. Tests were designated positive if aggregation was clearly visible to the naked eye and negative if no aggregation was observable. For confirmation, results were also examined under a microscope.

### 9.2.4 Results

Aggregation of *E. coli* was visible after 5 minutes in all five of the repeated (inter-day) assays which included the trial herbal extract. This was at concentrations of 0.5 M, 0.75 M and 1.5 M ammonium sulphate. Aggregation was not visible at the weaker concentrations of 0.05 M and 0.25 M. It was not present in any of the controls; *E. coli* was non-aggregative in PBS and at all dilutions of  $(\text{NH}_4)_2\text{SO}_4$  (see **Figure 41**).

After leaving the plates for 24 hours aggregation was visibly pronounced in all dilutions of  $(\text{NH}_4)_2\text{SO}_4$  containing the HMP, including those where aggregation had not previously occurred.



**Figure 41:** Plate showing results of aggregation assay  
 Aggregation of *E. coli* is visible in columns 3 - 5 of rows A and D which contain the uva-ursi HMP and (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>. M = molar dilution of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> in each column.

### 9.2.5 Discussion

Ammonium sulphate is a hydrous compound, and a solution concentrated with (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> will reduce water availability. In this study aggregation of *E. coli* (a non-aggregative strain) was not detectable in the (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> solution alone but visible in the 3 highest concentrations (0.5 M, 0.75 M and 1.5 M) which contained a 1:10 cold maceration of the uva-ursi HMP. This reinforced the findings of the aforementioned tests on a variety of bacteria (including several strains of *E. coli*) once the same

strength dilution of uva-ursi was added (Turi *et al.*, 1997; Annuk *et al.*, 1999; Türi *et al.*, 1999).

Nevertheless, the previous investigations by Turi and co-workers showed clumping together of both aggregative and most non-aggregative strains in just PBS once a 1:10 decoction of uva-ursi had been added (Turi *et al.*, 1997). The current assay only showed clumping in the strongest concentrations of  $(\text{NH}_4)_2\text{SO}_4$  despite using a cold maceration which should have been more efficient at stimulating aggregation. This may simply be down to the strain of *E. coli*, but could also be due to the shorter maceration time – just 30 minutes compared to four days. The results of these assays were not comparable to the study by Dykes and colleagues who employed a different methodology; the bacterial attachment to hydrocarbon (BATH) assay instead of SAT (Dykes, Amarowicz and Pegg, 2003). It was decided to adopt the methods by Turi and co-workers, as they had focussed their studies more specifically on uva-ursi and its constituents (Turi *et al.*, 1997; Türi *et al.*, 1999).

It is thought that the potential for bacteria to aggregate is related directly to their surface structures, with stronger hydrophobicity also leading to a greater capacity for adhesion (Lindahl *et al.*, 1981). Moreover, the capacity of an organism to exhibit hydrophobicity may also relate to its surface charge or surface-associated proteins, which can differ between strains and species (Krasowska and Sigler, 2014; Kumar *et al.*, 2015).

It has been hypothesised that bacteria have developed different virulence factors such as hydrophobicity to aid their adhesion to tissues for cell survival and colonisation (Doyle, 2000). This in turn would possibly lead to the development of biofilms; the formation and structure of which may help to secure the organisms' survival against the flow of urine in the urinary tract, the host immune system, as well as treatment by antibiotics (Wojnicz *et al.*, 2012).

Since the original studies were conducted, theories regarding the adhesion of bacteria and aggregation have evolved to demonstrate that the momentum and force of flow in the urinary tract could have a bearing on an organisms' ability to attach to the uroepithelium (Thomas *et al.*, 2004; Björnham and Axner, 2010). Although hydrostatic forces in the urinary tract have been generally accepted as an effective means of both preventing bacterial attachment and flushing out microbes, studies have shown that

increasing the force of the fluid flow may have the opposite effect on Type 1 fimbriated bacteria bound to monomannose surfaces, thus increasing the binding capacity (Thomas *et al.*, 2004; Björnham and Axner, 2010). This phenomenon is believed to be dependent on the expression of the structural bonds of FimH; these can vary and exist in two differing conformations (Tchesnokova *et al.*, 2011). One theory is that 'slip-bonds' (non-covalent bonds between receptor and ligand) may weaken under a tensile force whilst so-called 'catch-bonds', which are considered responsible for FimH adhesion via Type 1 pili on *E. coli*, become more adherent under faster flow rates (Björnham and Axner, 2010). A lower force may decrease the initial rate of binding, but an increased force may enhance the length of time the *E. coli* may remain bound (Thomas *et al.*, 2004; Tchesnokova *et al.*, 2011). These fimbriae may then mediate the ability to bind to single mono-mannose coated surfaces (Thomas *et al.*, 2004).

Nevertheless, the above studies regarding the mechanisms of adhesion have been assayed *in vitro* examining specific aspects of the immune system. They have not tested all the elements of the innate immune response together, which work in unison as a multi-layered defence against uropathogens. This includes the aforementioned uromodulin, which is believed to cause bacteria to be flushed out of the urinary tract as a result of inducing bacterial aggregation (Abraham and Mio, 2015). The plants which stimulated the greatest amount of aggregation in the studies conducted by Turi and colleagues have a specific traditional use for treating UTI, whilst the ones which showed weaker or no aggregation do not (Mills and Bone, 2005).

From the earlier works it seems probable that tannins could be the main constituent responsible for inducing aggregation (Turi *et al.*, 1997; Annuk *et al.*, 1999; Türi *et al.*, 1999). The amounts of these compounds were determined for uva-ursi and *V. vitis-idaea*, which both stimulated aggregation, but were not examined in *H. perforatum* which also had an effect (Turi *et al.*, 1997). This latter plant also contains tannins, but in order to draw firm conclusions the total amount needs to be compared to uva-ursi.

Tannins are known to precipitate proteins, but not all the original studies were conducted on *E. coli*. The conclusive assay in this field involving both uva-ursi and tannic acid was carried out on *Helicobacter pylori* (Annuk *et al.*, 1999).

Limited studies on humans have detected the presence of tannins in the urinary tract over 24 and 48 hour periods, so it may be possible that these compounds are

bioavailable (Shahrzad and Bitsch, 1998; Mennen *et al.*, 2008).

## 9.2.6 Conclusion

The results of this study show that uva-ursi does promote aggregation under experimental conditions. It is plausible this might be part of the mechanism of action if it causes *E. coli* to aggregate together and be flushed out of the urinary tract. It is also possible that the mechanism could make a UTI worse were it to cause bacteria to adhere to the epithelium. However, as uva-ursi has a well recorded traditional use (EMA 2012a; Barnes, Anderson and Phillipson 2007) for treating UTI going back hundreds of years the latter supposition might be considered unlikely.

Without further *in vivo* testing it is not yet possible to determine how the HMP may work in the body in conjunction with the immune system. Moreover, whilst several studies have been conducted on the environmental and metabolic aspects of cell surface hydrophobicity and aggregation, understanding of this field is still limited.

Further work needs to be conducted on different strains of *E. coli* and gallic acid, the main tannin present in uva-ursi, as well as a metabolic study to determine whether gallic acid, a polyphenol, is bioavailable within the urinary tract. This would need to be conducted over a 2-3 hour period to compare it with the excretion of hydroquinone, and also to a 24 hour excretion level – in line with previous studies on tannins.

## 9.3 Motility

### 9.3.1 Background

Bacterial motility is considered a recognisable although not essential virulence factor of some uropathogens in the establishment of an infection in the urethra and bladder (including *E. coli*, *Proteus mirabilis* and *Pseudomonas aeruginosa*) (Lane *et al.*, 2005; Wright, Seed and Hultgren, 2005; Kaya and Koser, 2012). The phenomenon enables

an organism to swim and propel itself toward nutrient rich surfaces such as the uroepithelium before eventually attaching and colonising the urinary tract (Lane *et al.*, 2005).

The mechanism is mediated by flagella of which *E. coli* typically possess between four and eight located at random on the surface of the organism. The flagella are approximately 10 µm long and 20 nm in diameter. They comprise a filament attached to a basal body (via a hook) and this traverses the bacterial membranes (Maki *et al.*, 2000; Kaya and Koser, 2012). The filaments are able to rotate and spin from several hundred to over a thousand revolutions a second, enabling the micro-organism to perform tumbling and swimming cycles in its dissemination to new sites within the urinary tract (Manson, 2010; Kaya and Koser, 2012).

It has been demonstrated that motile strains with flagella may cover a greater surface area of the epithelium compared to non-motile strains of the same species, which contributes to their greater propensity to establish an infection compared to non-motile bacteria (Klausen *et al.*, 2003; Hidalgo, Chan and Tufenkji, 2011). The mechanism allows them to potentially ascend from the lower to the upper urinary tract and kidneys, where they may then also cause a secondary infection (Hidalgo, Chan and Tufenkji, 2011).

Plant extracts (including uva-ursi) and natural compounds have previously been tested for their ability to inhibit bacterial motility (Samoilova *et al.*, 2014b). Constituents which have shown efficacy include alkaloids from *Piper nigrum* (black pepper) and *Rauwolfia serpentina* (Indian Snakeroot), as well as proanthocyanidins (condensed tannins) from *Vaccinium macrocarpon* (cranberry) (Hidalgo, Chan and Tufenkji, 2011; Dusane *et al.*, 2014). *In vitro* analyses have indicated that these compounds may impede motility due to inducing a reduction in the expression of the flagellin (or fliC) genes (Hidalgo, Chan and Tufenkji, 2011; Dusane *et al.*, 2014).

In the uva-ursi study it was reported that the herb reduced motility to 37% of the control sample after 22 hours incubation with *E. coli* (strain BW25113). This was at a concentration that demonstrated a slight bacteriostatic effect against this organism (Samoilova *et al.*, 2014b). It was, therefore, decided to investigate whether the ATAFUTI trial HMP of uva-ursi could potentially disrupt motility, and as a result contribute to an antimicrobial effect.

### 9.3.2 Method

The method used was adapted from the study on natural product alkaloids in combination with a motility stab assay (Murinda *et al.*, 2002; Dusane *et al.*, 2014). Both a motile strain (NCTC 10418) and a non-motile strain (OP50) of *E. coli* together with a strain of *S. aureus* 12981, an organism which does not possess flagella and is therefore non-motile (Harris, Foster and Richards, 2002), were sub-cultured for 18-24 hours on (soft) nutrient agar at 37°C. Soft agar (Sulfide Indole Motility Medium, 0.3%, Sigma Aldrich) was autoclaved and left to cool to 55°C before being supplemented with uva-ursi extract (diluted in sterile distilled water) to give final concentrations of 5 µg/mL and 50 µg/mL. These were more than 10 times weaker than the MIC of uva-ursi (512 µg/mL), and replicated the above study on alkaloids (Dusane *et al.*, 2014). A stock solution of 5 mg HMP was initially diluted in 10 mL of sterile distilled water, and 1 mL of this was re-diluted in soft agar to provide the final concentrations. The soft agar was added to a bijoux container (7 mL) and allowed to set overnight at room temperature.

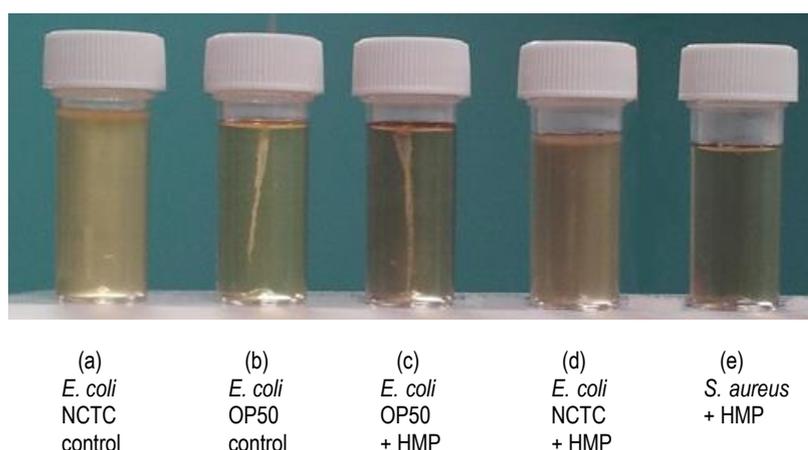
The assays were subsequently repeated at 64 µg/mL and 128 µg/mL of HMP, both with and without the addition of Triphenyl-Tetrazolium Chloride (TTC) at a concentration of 5 mL/litre of a 1% sterile solution. Running the assays with and without TTC would ensure that the results would be visible, and confirm that this compound had not interfered with the ability of *E. coli* to migrate through the agar. TTC is colourless when oxidised but as it diffuses into a respiratory organism it accepts electrons (via the enzyme dehydrogenase) to produce red TTC crystals, thus leaving a red trail as the bacteria swims (Luna *et al.*, 2005).

Dilutions of *E. coli* were prepared in MHB (pH 7.2) to give a final concentration of 10<sup>6</sup> CFU/mL. This is a concentration which would allow the observation of a diffuse cloudy growth if motility were present. The organisms were transferred via an aseptic metal needle to the centre of the soft agar and inserted in a straight line vertically down towards the bottom of the bijoux container. They were then incubated overnight at 37°C. Results were determined by observation after 24 hours, and again at 48 hours.

Experiments were performed five times for validity and reproducibility.

### 9.3.3 Results

A positive result to this assay, indicating the presence of motility, would be a diffuse area of growth spreading from the line of inoculation. A negative result, where no motility is present, would show growth along the line of inoculation only. After 24 hours motility of *E.coli* NCTC 10418 was visibly present in the initial assays containing the lowest concentrations of the HMP (5 µg/mL and 50 µg/mL) in all five duplicated experiments. The positive control of *E.coli* NCTC 10418 also showed motility, thus confirming this to be a motile strain (see **Figure 42**).



**Figure 42:** Results of motility assay utilising 50 µg of uva-ursi HMP.

Motility present in (a) *E. coli* NCTC 10418 control sample without the HMP, and (d) *E. coli* NCTC 10418 containing uva-ursi. Growth visible along the inoculation stab lines in both samples of the non-motile strain of *E. coli* OP50 (b and c)

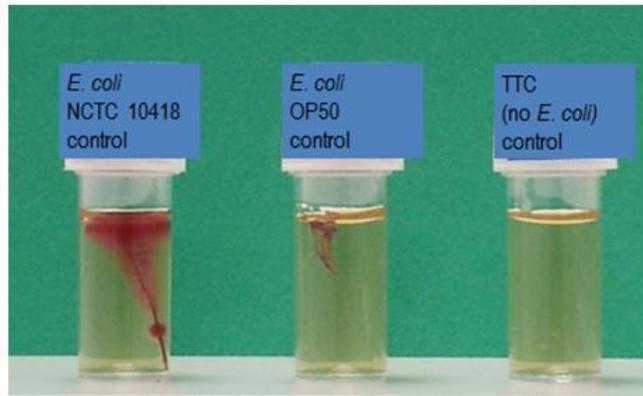
The repeated assays which contained stronger concentrations of the HMP (64 µg/mL and 128 µg/mL), also clearly showed motility after 24 hours. This was both with and without TTC. The control samples with TTC without the herbal extract and those which contained 128 µg/mL of uva-ursi are shown in **Figure 43**.

No motility was visible in any of the samples of *E.coli* OP50 which included all the dilutions of the HMP as well as the control. *E.coli* OP50 was, therefore, confirmed as a

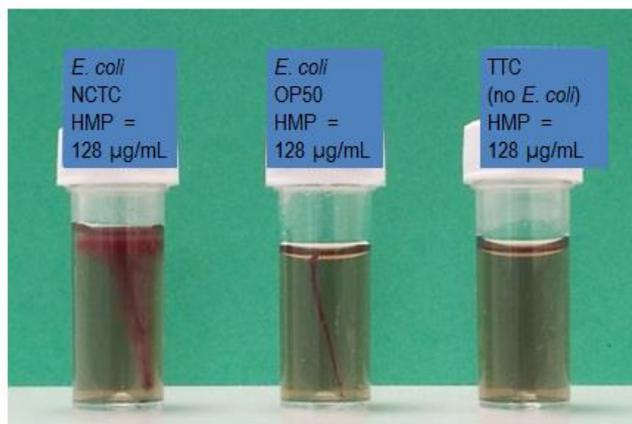
non-motile strain. Motility was not visible either in any of the samples containing *S. aureus* which is a non-motile organism (Harris, Foster and Richards, 2002).

There was no discernible difference in the appearance of any results after 48 hours compared to 24 hours.

(a)



(b)



**Figure 43:** Results of motility assays with TTC

**(a)** control samples without uva-ursi (*left to right*) *E. coli* NCTC 10418 shows motility; *E. coli* OP50 no motility visible; and TTC only (negative control no *E. coli*); and **(b)** utilising 128 µg/mL uva-ursi (*left to right*) *E. coli* NCTC with uva-ursi shows motility; *E. coli* OP50 growth along 'stab' line no motility visible; and TTC with uva-ursi (negative control no *E. coli*)

### 9.3.4 Discussion

Although not crucial for the colonisation of the bladder, the expression of flagellum mediated motility is considered to play an important role in the early stages of establishing a UTI, as organisms swim towards nutrient rich surfaces (Lane *et al.*, 2005; Kaya and Koser, 2012). The highest strength of uva-ursi tested in these assays to investigate its possible effect on hindering motility of *E.coli* was 128 µg/mL. This is four times weaker than the MIC (512 µg/mL) demonstrated in Chapter 7, and is therefore neither sub-inhibitory nor sub-lethal, but it did not appear to prevent or impair the ability of the organism to swim.

Strains of motile bacteria will not only grow around the immediate area of 'needle' inoculation, displaying a virtually straight line of growth, but they will also diffuse and swim into the surrounding soft agar producing a cloudy, turbid appearance; thus demonstrating their ability to migrate through the medium. This was confirmed in the results, illustrated in **Figure 43** by the control sample of the motile strain of *E. coli* (NCTC 10418). The non-motile organisms, *E. coli* OP50 and *S. aureus*, only grew along the line of the needle stab (Murinda *et al.*, 2002).

A natural progression of this study would be to inoculate petri dishes rather than bijoux and measure the distance travelled by the organism at different time-points instead of just after 24 hours. This could then be compared to the time uva-ursi may be present in the body, prior to elimination. However, there can be difficulties in incubating petri dishes with soft agar. These cannot be inverted upside down, and any condensation developing in the lids may then easily interfere with the results.

Were the assays to be run again, an additional control would be to include a sample with the antibiotic ciprofloxacin, to see if dead *E. coli* cells can still diffuse through the soft agar. However, the non-motile strain used in the above assay, together with *S. aureus* which do not possess flagella, should be reasonable comparisons.

Various natural compounds extracted from plants (such as alkaloids and salicylic acid) (Chow *et al.*, 2011; Dusane *et al.*, 2014), together with plants which have traditionally been used to treat UTI (*Vaccinium macrocarpon* and *Equisetum arvense*), have been tested for their potential effect on motility. This is in terms of both observational studies

as well as detecting inhibition of the expression of the relevant flagellin gene (Hidalgo, Chan and Tufenkji, 2011; Wojnicz *et al.*, 2012).

Whilst uva-ursi had previously been tested for anti-microbial activity *in vitro*, as well as its effect on aggregation and inflammation, there has only been one published report on whether it may reduce or inhibit motility (Samoilova *et al.*, 2014b). This aforementioned study reported that the motility of a strain of *E. coli* could be negatively affected by the presence of uva-ursi (Samoilova *et al.*, 2014b). The concentration of extract tested was reported to be slightly bacteriostatic. It could, therefore, be disputed that the concentration used to test motility was too close to the MIC for the results to be viable. It may have already been inhibiting cell growth and thus affecting motility in the process. The authors used the same method for all 18 plant extracts tested and the two which had the greatest effect on reducing cell survival (uva-ursi and *V. vitis-idaea*) also showed the strongest inhibition of motility compared to the other 16 tested (Samoilova *et al.*, 2014b).

### **9.3.5 Conclusion**

Based on these *in vitro* assays, repeated five times in order to generate acceptable data due to the results being qualitative rather than quantitative, the HMP extract of uva-ursi was not able to inhibit the motility of a motile strain of *E. coli*. However, without testing individual constituents present in the urinary tract after metabolism of the herb (such as hydroquinone and gallic acid), as well as against different strains of *E. coli* (in case the results are strain specific) the *in vivo* effect of uva-ursi on the motility of *E. coli* in the bladder is as yet undetermined. This could be further investigated via microscopy in a future study analysing urine metabolites of the herb.

# Chapter 10: Qualitative study into patients' perspectives of herbal treatment as an alternative to antibiotics

## 10.1 Introduction

### 10.1.1 Background

In order to test the efficacy of the herbal medicinal product (HMP) uva-ursi as an alternative treatment for symptom relief of UTI, both on its own and alongside a non-steroidal anti-inflammatory drug (NSAID) ibuprofen, the ATAFUTI trial adopted a strategy of delayed prescribing of antibiotics. This approach has previously been undertaken with several health conditions (respiratory tract infections, otitis media, sore throat and cough lasting seven days) to try to reduce antibiotic prescribing (Arroll *et al.*, 2003). The strategy has also already been trialled with uncomplicated UTI (Little *et al.*, 2010), as well as amongst patients prescribed ibuprofen for this condition (Bleidorn *et al.*, 2010; Gágyor *et al.*, 2015). The latter treatment (ibuprofen) has been reported to have provided similar levels of symptom relief to antibiotics (Bleidorn *et al.*, 2010).

Previous qualitative research into attitudes towards delayed prescribing of antibiotics for UTI determined that some women were happy to adopt this route if an alternative treatment resulted in an avoidance of antibiotics; this was mainly due to expected side-effects (Leydon *et al.*, 2010). Had patients already tried alternative treatments then there was a high expectation of receiving antibiotics once symptoms were discussed with the GP (Leydon *et al.*, 2010). Furthermore, a minority were reluctant to receive a delayed prescription; they claimed that their experience of how their condition was likely to progress was not being given due consideration by their GP. In the study they were reassured that a prescription was available for them at the GP surgery should they require it (Leydon *et al.*, 2010).

A quantitative study in the UK determined that over half the women who had ever experienced a UTI had first tried to manage the symptoms of their most recent infection

with an alternative to antibiotics. This comprised 27% who tried cranberry juice, 21% who had taken analgesics and 17% had tried using a cystitis sachet (Butler *et al*, 2015).

An investigation commissioned by the Medicines and Healthcare Products Regulatory Agency (MHRA) amongst 2300 adults in 2008 reported that 35% had used herbal medicine, and 26% had purchased an HMP in the last two years (MHRA, 2008). There is limited data regarding the prescribing of herbal medicine by GPs in the UK. In the 1990s it was indicated that less than 5% of clinicians either wrote prescriptions for herbal medicine, or referred their patients to a Medical Herbalist (White, Resch and Ernst, 1997; Perry and Dowrick, 2000). The only study which has been conducted in the UK since, a study in Scotland amongst 323 GP practices which ended in March 2004, concluded that 32% had prescribed a herbal medicine in the preceding 12 months (Ross, Simpson and McLay, 2006). It is not possible to know whether the higher percentage of prescribing is due to a general increase over the two time periods studied, or whether Scotland is representative of the UK. It compares with over 60% in Germany who have an involvement with complementary therapies per se (such as osteopathy and acupuncture), and a minimum of 70% of German primary care physicians who actually prescribe herbal medicine (White, Resch and Ernst, 1997). Moreover, in Germany 15% of cases of mild UTI are treated by GPs with non-antibiotic drugs such as phyto-medicines and antispasmodics (Hummers-Pradier *et al.*, 1999).

Not only might there be a low referral of HMPs by GPs in the UK, but there is evidence that the majority of patients do not inform their practitioner they are taking a botanical product. This is either because patients are not asked (just over a quarter of GPs enquire about herbal medicine when taking patient history), patients do not feel it is important to tell their GP, or they may be apprehensive about the doctor's response (Vickers, Jolly and Greenfield, 2006; Lisk, 2012).

A quantitative survey amongst 452 participants reported that in practice 97% of responders who asked for an antibiotic prescription in the previous 12 months (any condition) were granted their request. Almost a quarter of these did not involve any patient/practitioner discussion regarding their illness (McNulty *et al.*, 2013). It has been reported that GPs may feel pressure to prescribe antibiotics from patients who expect to receive a prescription, or some GPs have a perception (which might be unfounded) that patients expect medication (Little *et al.*, 2004; Flower *et al.*, 2015). Moreover,

research indicates that prescribing decisions can be habitual, and that the adoption of new medicinal treatments may depend entirely on early successful experience of a new drug (Wood, Simpson and Butler, 2007).

There has been a small qualitative study amongst GPs on their experience of treating *recurrent* UTI and their attitudes towards possible herbal alternatives, which concluded that practitioners were reluctant to recommend a herbal alternative without sufficient evidence of efficacy and safety (Flower *et al.*, 2015). There are no qualitative studies at all amongst either patients or practitioners into the use of herbal medicine for alleviating uncomplicated UTI symptoms. Such a study could provide insight into both the patients' experience of UTIs, and the approach to treatment. The opportunity could also be taken to follow-up previous findings into delayed prescribing of antibiotics, and explore current awareness and perceptions regarding antibiotic resistance.

### **10.1.2 Qualitative Perspective**

The origins of qualitative research are derived from several doctrines and disciplines, including sociology, psychology and linguistics (Forman *et al.*, 2008). The methods are viewed as holistic as the aim of the researcher is to discover, understand and interpret the opinions and perceptions of the sample that cannot be explained by a few simple variables (Patton, 2002). It is a flexible methodology concerned with the questions of interpretation, such as 'why' and 'how', rather than in generalisations or evaluating numbers of responses. In so doing, and using participants' language to illustrate the findings, a qualitative investigation can provide profound insight into human behaviour, and may help avoid the potential ambiguities of a quantitative survey (Ormston *et al.*, 2013).

The philosophical theories of qualitative research are based on how the social world can be studied, with some rooted in an ontological approach concerned with the nature of reality, and others based on an epistemological perspective with the aim of focusing on how knowledge is best acquired (Ormston *et al.*, 2013). There is no one single and preferred approach for conducting qualitative studies.

In the first decade of this century at least 12% of randomized controlled trials were run with a qualitative research element. Research into studies conducted between January 2008 and September 2010 reported that the majority of qualitative projects (71%) focused on the intervention being trialled, with only 15% exploring attitudes into the trial process and just 1% investigating attitudes towards the outcomes (O’Cathain *et al.*, 2014). With a lack of research into opinions on treatment with herbal medicines there existed an opportunity to further investigate this area.

For this study qualitative in-depth interviews nested within the main ATAFUTI trial were considered the most appropriate approach for gaining an understanding of these issues (see 10.3.1). Due to the pragmatic nature of the research question thematic analysis was considered the most appropriate means by which to analyse the data (see 10.4.3). It is helpful when there is a large amount of diverse data, which needs to be reduced and managed in order to gain an understanding of the thoughts and experiences of the participants and relate them back to the research question (Braun and Clarke, 2006). Other methods, such as thematic discourse analysis and grounded theory are more appropriate when it is necessary to develop a theory (Bradley, Curry and Devers, 2007).

## **10.2 Aims**

The aims of this study were to:-

- Explore and evaluate patients’ views and opinions towards delayed prescribing of antibiotics for urinary tract infections in favour of an alternative symptom treatment such as HMPs.
- Identify any barriers towards the prescribing of herbal medicine for UTI.
- Investigate how patients may learn about the findings of the trial.

## 10.3 Qualitative Research Approach and Methods

### 10.3.1 In-depth Interviews

Semi-structured individual in-depth interviews can encompass a wide and diverse range of research themes, and may offer a deeper understanding of social phenomena from a patient's perspective than either focus groups or quantitative research (Dicicco-Bloom and Crabtree, 2006). The basis of in-depth interviewing is to allow the interviewer to explore personal and social issues more fully than within a focus group, where the more public setting could inhibit respondents' personal expression regarding sensitive topics (Dicicco-Bloom and Crabtree, 2006). Focus groups may be helpful for prompting discussion and raising issues amongst participants, as well as for gaining insight into collective views (Gill *et al.*, 2008), but as personal views were required on questions which some might make some participants uneasy group discussions were considered inappropriate for this study.

It was, therefore, decided to conduct in-depth semi-structured interviews amongst patients connected to ATAFUTI as part of a nested study within the main clinical trial. Patients who took part in the ATAFUTI trial, as well as those attending the surgery with a UTI but either not qualifying or wishing to be considered for the clinical study, were eligible to be interviewed. This would allow respondents who had taken part on the trial to be followed-up on their experience of participating, as well as exploring any views they may have about taking alternative treatments such as herbal medicine.

Using in-depth semi-structured interviews for exploring the views and experiences of the patients connected to ATAFUTI facilitated the development of an iterative process of discovery. The semi-structured nature of the interview allowed for some guidance for broaching topics crucial to the research, whilst also providing the flexibility for participants to introduce pertinent issues. It was necessary to remain impartial and observant that the topics may fluctuate and vary from those expected when the project was initially conceived (Britten, 1995).

All interviews were conducted over the telephone between one and three months after the participants had been on the trial, and were between 30 - 60 minutes in length.

Topic areas were covered according to a pre-planned interview guide. The questions for the guide were developed from the outlined proposal in the qualitative section of the ATAFUTI trial protocol (see Appendix F) as well as existing literature. The guide was approved by the qualitative research supervisor (Dr Caroline Eyles), the main PhD supervisor (Professor Michael Moore), as well as the NHS ethics committee.

Ethics approval for the whole qualitative study was applied for within the application for the main ATAFUTI clinical trial. It was granted by NHS Ethics on 15/5/15 (REC 14/SC/1143). It was registered with the European clinical trials database in 2013 - EudraCT reference number 2013-003327-11. The University of Southampton was responsible for the sponsorship and insurance. The NHS research passports were provided by the local district NHS trusts.

#### **10.3.1.1 Data Saturation**

The total number of participants planned to take part in a qualitative research study is driven by the research questions, the method of data collection, possible comparison with similar studies, the researcher's experience as well as a need to advise ethics committees (Pope, Ziebland and Mays, 2000; Tran *et al.*, 2016). In practice, data saturation is achieved when new information does not provide additional insights or themes, and will produce little or no change to the codes being analysed (see section 10.4.2). There should be a high level of consistency across the data collection and findings for data saturation to be achieved (Tran *et al.*, 2016).

In this study the ethics committee approved the interviewing of 20-30 patients.

#### **10.3.2 Sample - Recruitment Procedure**

Participants who took part in the ATAFUTI trial, and those who had been approached but did not qualify for the study, were opportunistically recruited in GP surgeries by way of a letter of invitation (outlining the project) with a reply slip (Appendix G); this was to return via a pre-paid envelope if they were interested in taking part in an interview. They were also given a Participant Information Sheet (PIS) (Appendix H) to read which explained the reason for the qualitative study, together with contact details should they

have any questions. They were sent a Consent Form (Appendix I) to sign and return in a stamped-addressed envelope, together with another copy of the PIS in case this had been mislaid. The PIS advised the potential participant that the interview would be audio recorded, and that all data would be anonymised for confidentiality. At the start of the interviews the respondents were reminded that they were being recorded and asked if they were still happy to proceed.

### **10.3.3 Data Collection and Management**

#### **10.3.3.1 Audio Recordings and Field Notes**

All interviews were audio-recorded on two Olympus-WS digital voice-recorders. The audio files were uploaded onto a password locked University of Southampton computer as well as the University of Southampton M Drive. They were not accessible to anyone else other than the PhD supervisors involved in the project. The original files were then deleted from the voice-recorders.

The recordings were listened to immediately after the interviews were conducted and prior to transcribing. This was to check for quality, become familiar with the data (see 10.4.1) and determine whether all topic areas from the discussion guide had been covered.

Field notes were made regarding topic areas that needed further investigation in follow-up interviews, and these also acted as an aide memoir when assessing whether any areas needed further expansion.

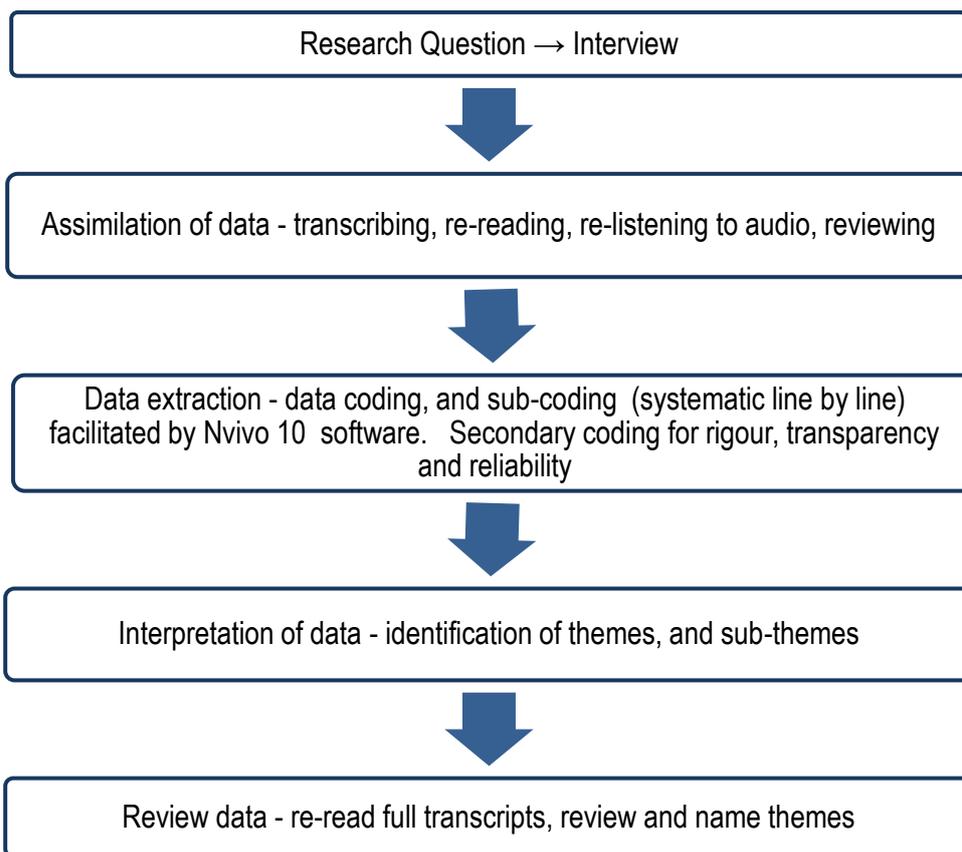
#### **10.3.3.2 Transcriptions**

Recordings were sent to a professional transcriber. Transcriptions were anonymised, with patient names converted into codes, and within this process respondents were ascribed a number. Proper names were also removed to maintain anonymity and confidentiality.

Transcriptions were verbatim, including word for word dialogue, all pauses, false starts, repetitions and laughter in order to facilitate a richness and depth of data. As part of an audit trail of data analyses which could be accessed by the project's supervisors the transcripts were also uploaded onto the University of Southampton M Drive.

Once transcribed the manuscripts were imported from Word into NVivo (version 10) data management software.

The full methodological process including data analysis is illustrated in **Figure 44**.



**Figure 44:** Overall development of interview process and thematic analysis

Methodology adapted from Braun & Clark (Braun and Clarke, 2006)

## **10.4 Data Analysis**

### **10.4.1 Familiarisation with Data**

All the audio recordings were listened to at least twice for full immersion into the data, whilst the transcripts were checked concurrently for any errors. Where necessary the field notes were also expanded upon to assist further questioning. This process facilitated familiarisation with the data, and allowed topic areas which required further exploration to be identified (Noble and Smith, 2014).

### **10.4.2 Coding**

Creating a coding framework - a list of codes into which data can be classified - enables the large amount of interview data collected to be managed more efficiently (Noble and Smith, 2014).

In this study, after transcripts were uploaded into NVivo 10 they were analysed systematically line by line before generating and labelling individual codes and sub-codes. The latter were necessary so that the original contextual meaning did not get over-looked.

Coding commenced after the first three interviews had been conducted (for both patients and clinicians), in order to facilitate an iterative process of data collection and analysis. Where necessary some data was replicated under different codes, which potentiated it being used in more than one theme. This was helpful in the analytical process as it could indicate that it was possibly a priority code (Silver and Lewins, 2014). The procedure helped expedite a cycle of induction and deduction whereby results could be compared within and between interviews, and tentative themes identified and followed-up in subsequent interviews (Pope, Ziebland and Mays, 2000; Noble and Smith, 2014).

### 10.4.3 Themes

The process of thematic analysis involves the identification of emerging themes derived from both the data, as well as the researcher's prior understanding of the topic/phenomenon being explored (Ryan and Bernard, 2003).

A theme may focus on an element of the data which relates strongly to the original research question (Braun and Clarke, 2006). The role of the researcher is to determine patterns and themes which originate during the interviews, and analyse and report them to the reader. With in-depth interviews the analytical process starts during the period of data collection in order to refine the questions and enable new topic areas pertinent to the study to emerge and be followed up (Pope, Ziebland and Mays, 2000). As a project progresses this method can expedite an understanding of views and opinions as it continually adapts and augments an iterative-inductive approach (Gantley *et al.*, 1999).

In this study, through highlighting and then comparing and contrasting the coded data in NVivo, a table was developed to encompass themes and related sub-themes. These were identified from repeated patterns amongst the text within similar codes. An example of a theme and related sub-themes identified from the codes is illustrated in **Table 21**.

**Table 21:** Example of codes leading to sub-themes and main overall theme

<b>CODE</b>	<b>SUB THEME</b>	<b>THEME</b>
Previous antibiotic treatment of UTI		
Awareness of antibiotic resistance When first became aware of antibiotic resistance		
Unsuccessful alternative treatment	Expectation that antibiotic will work	
Expected diagnosis of UTI		Anticipation of antibiotic prescription
Feel they will always need a prescription		
Immediate GP appointment		
Already tried alternative treatment		
UTI affecting daily activities	Belief they have infection	
Range of UTI symptoms experienced		

#### **10.4.4 Reviewing Data**

Finally the initial themes and sub-themes were reviewed and refined before and during the writing-up of the findings as it became evident that some of the coding data was too diverse to justify their inclusion. Furthermore, during this process some themes had to be broken down further into new themes, or ultimately renamed in order that they provided an accurate and cohesive portrayal of the essence of the data and related directly back to the aims of the study (Braun and Clarke, 2006).

#### **10.4.5 Quality and Rigour**

The taxonomy and diversity of frameworks used in qualitative research methods in relation to quality and validity have historically been the source of much debate, particularly when compared to quantitative research (Ravenek and Rudman, 2013). For example, from a quantitative perspective 'validity' relates to the accuracy of individual measurements, whilst in qualitative research it may refer to 'truth' or the extent to which the findings illustrate the social phenomena or the data collected (Long and Johnson, 2000; Silverman, 2005). Seale argues that quality can be maintained by the researcher drawing upon valuable lessons from a variety of concepts and terminology rather than adopting the criteria proposed by one specific philosophical approach (Seale, 1999). This is a view supported by Creswell who favours adopting a pragmatic approach rather than adhering to a definitive philosophy (Creswell, 2007). The credibility of the results of qualitative research can always be questioned; the analysis and findings might be perceived to be the subjective assessment and views of the researcher (Forman *et al.*, 2008; Noble and Smith, 2015). Therefore, in order to produce credible and trustworthy research to validate the findings within this study a number of criteria were adhered to – namely Reflexivity, Reliability and Transparency (see below). Without these techniques it may prevent the reader from being able to critically assess the interpretation of the findings (Mays and Pope, 1995; Noble and Smith, 2014).

##### **10.4.5.1 Reflexivity**

Reflexivity comprises an awareness of how both the approach to the study and the researcher themselves may have an influence on how the data is collected and subsequently analysed (Forman *et al.*, 2008). In this study open ended questions were developed for the interview guide in order to try to avoid influencing the opinions of the participants. Whilst it was essential to initially put the respondent at ease and build a rapport, it was also necessary to avoid commenting on the replies of the participants in order to maintain neutrality. Repeating words and phrases from the responses was helpful to signal understanding or a need for further clarity (Dicicco-Bloom and Crabtree, 2006). Maintaining field notes to examine in between interviews as well as a personal reflexive diary also helped a reflexive process, through providing the opportunity to review the questions as well as the approach to the study.

Counting the codes and assessing NVivo software calculations which assessed coverage by individual participants was necessary to determine how many participants contributed to the themes and sub-themes rather than relying on the subjectivity of the researcher's interpretation of the data (Green and Thorogood, 2014).

The overall process also benefitted from discussions with the PhD supervisors over the course of the study. This was in order that alternative interpretations of the data could be explored and conclusions arrived at through consensus of opinion (Forman *et al.*, 2008).

#### **10.4.5.2 Reliability**

Within qualitative research reliability refers to the consistency of the findings were a similar study to be conducted by an independent group of researchers (Green and Thorogood, 2014). In order that the analysis is reliable in terms of identifying common traits within the data the researcher needs to be methodical, and develop a systematic and uniformed approach (Silverman, 2005). Verbatim transcriptions and employing computer software to facilitate scrutiny of the interview transcripts allowed for a second researcher within this study (the qualitative supervisor) to double code the findings; this process also helped to maintain transparency (see Section 10.4.5.3). The first three transcripts were double coded by the qualitative researcher as part of the iterative analytical process, prior to proceeding with the study. As the project progressed all the remaining transcripts and coding were made available on NVivo for the qualitative supervisor to access on-line and double code. These codes were subsequently discussed and refined where necessary.

A verbal summary of the interview findings was read back to the respondents at the end of each interview. This was to compare the researcher's understanding with that of the participants' account to explore whether the findings were a reasonable portrayal of their experience, and to help ensure validity and reliability of the data (Braun and Clarke, 2006).

### 10.4.5.3 Transparency

The researcher needs to be open about the steps taken during the research process, from the data collection through to how the analysis was conducted and the themes identified (Mays and Pope, 2000).

Making available all the interview recordings, the transcripts, the updated codes and the theme table on a regular basis on the University M drive helped to create an audit trail. It also made it possible for both supervisors to look over the data and its progression into identifiable themes before discussing them. This facilitated a consensual analysis.

## 10.5 Results

### 10.5.1 Sample

#### 10.5.1.1 Patients

Sampling continued until data saturation was achieved at 20 respondents, 15 of whom had taken part in ATAFUTI and 5 who had not been in the trial (because they did not meet the criteria) but suffered from UTI. None of the participants who had taken part in the trial knew whether they had received the herbal medicine or a placebo, and they expressed similar views regardless of whether their condition had resolved or not whilst on the trial.

The interviews were conducted between November 2015 and May 2016.

The final sample illustrated below in **Table 22** comprised an equal mix of working and retired women. Half were aged 20-49 and the rest over 50 years. Their exact age has not been included to help protect their anonymity.

**Table 22:** Demographics of female patients

Patient ID	Age Group	Occupation	ATAFUTI Trial Participant	Experience of UTI
P1	60+	Retired	√	One
P2	60+	Retired	No	Several
P3	20-29	Nanny	√	Two a year
P4	60+	Retired	√	One every 3 years
P5	40-49	Company secretary	√	First UTI
P6	40-49	Teaching Assistant	√	Three
P7	40-49	GP	√	Several
P8	30-39	Health care	√	First UTI
P9	30-39	Retail Manager	√	Several
P10	20-29	Ambulance service	√	Two a year
P11	60+	Retired	√	Two a year
P12	40-49	Office administrator	No	Several
P13	50-59	Health service	No	Two a year for 20 years
P14	60+	Retired	√	Four
P15	50-59	Teaching Assistant	√	Several
P16	60+	Retired	No	Several
P17	40-49	Haematologist	√	Two
P18	50-59	Housing Developer	√	Two
P19	30-39	Teacher	√	First UTI
P20	50-59	Support Worker	No	6 - 7 a year

Marital status was omitted as this was not deemed a relevant factor in the results of previous studies (Little et al., 2010).

### 10.5.2 Themes – Patient Interviews

The coding and analysis lead to the identification of four overarching themes and their corresponding sub-themes as illustrated in **Table 23**. The full theme table relating to all the original codes is included in Appendix J.

**Table 23:** Patient themes and sub-themes

Theme	Sub-themes
1 Anticipation of antibiotic prescription	<ul style="list-style-type: none"> <li data-bbox="651 450 1075 483">[ Expectation that the antibiotic will work</li> <li data-bbox="651 506 932 539">[ Coping with an infection</li> </ul>
2 Factors that influence decision to participate in trial	<ul style="list-style-type: none"> <li data-bbox="695 640 1190 674">Reassurance of delayed prescription for antibiotics</li> <li data-bbox="651 696 935 730">[ Clear explanation of trial</li> <li data-bbox="651 752 1007 786">[ Would there be any side effects</li> <li data-bbox="695 819 983 853">Reluctance to take antibiotics</li> </ul>
3 Desire that herbal treatment will work	<ul style="list-style-type: none"> <li data-bbox="651 954 1078 987">[ Willingness to try alternative treatments</li> <li data-bbox="651 1010 1206 1043">[ Accessibility and trustworthiness of herbal treatment</li> </ul>
4 Barriers to adoption of herbal medicine	<ul style="list-style-type: none"> <li data-bbox="651 1111 1155 1144">[ Anticipated problems of taking herbal medicine</li> <li data-bbox="651 1167 1023 1200">[ Learning about the trial outcomes</li> </ul>

The four main themes and their associated sub-themes helped to gain an understanding of patients’ views regarding antibiotic resistance and treatment with herbal medicine. They are detailed below and illustrated with verbatim quotations from the respondents. The participant’s identification number is provided in parenthesis following the quotes.

**Theme 1: Anticipation of antibiotic prescribing**

A fundamental theme which emerged across the sample was the expectation that an antibiotic would be prescribed should the patient make an appointment with their GP.

This theme consisted of two sub-themes: *Expectation that the antibiotic will work*; and *Coping with an infection*.

*“Well I always say to them, just give me what you gave me last time and they generally do”. (P20)*

### ***Sub-theme 1.1: Expectation that the antibiotic will work***

Most of the participants described suffering from and having been successfully treated for a previous UTI with antibiotics. These respondents, therefore, had confidence that this form of treatment would clear the infection. From experience several also had reason to believe that the antibiotic would take effect quite quickly, sometimes within 24 hours, and had faith in taking them.

*“I had a short course, three days' worth, so pretty strong ones but it was almost instantly – it was – within a day, a day and a half, I didn't have the urge to go, it didn't hurt. It was pretty quick”.  
(P19)*

Whilst the majority of the participants had heard of the possibility that antibiotics may one day not work for them most had not contemplated what implications this might have for them in the future. Nearly all had difficulty describing exactly what antibiotic resistance was. Several imagined that the body would just get used to the drugs and the medication would simply, therefore, no longer be effective.

*“I do know that in layman's terms, I think the more antibiotics you have in your system and throughout your life, eventually there are going to be some sort of things they are not going to help you. But that may be a simplified version”. (P1)*

*“The more you take them, the less likely they are to work; your body's going to become – whatever it is – become immune to them”. (P9)*

Almost a third of the sample had already suffered previous bacterial infections where they had to get a new course of antibiotics because the first course had not worked. Whilst a couple of them were concerned about this most of these participants had not contemplated the possible implications for the future. One lady had just assumed that she had not been given enough tablets.

*“... Well I can't say I've – I've not really thought about it that deeply, to be quite honest with you”.*  
(P20)

Despite some personal experience of antibiotics not working for them in the past, as well as the high awareness amongst the whole sample of antibiotic resistance, once they suffered a UTI they were inclined to ask for a prescription. Several participants were driven by their need for symptom relief, rather than concern that using antibiotics may one day mean that this class of medication may no longer be effective.

*“Well it depends when you've got cystitis and it's really uncomfortable, to be honest, I don't really care, I just want to get better”.* (P12)

Around a quarter of the total sample were aware that infections may cease to be brought under control by antibiotics, and were fearful for the future of themselves and their families. Just one participant thought that there may come a time when existing antibiotics did not work at all. A couple of other participants pondered on this possibility but assumed that new antibiotics would be discovered.

### ***Sub-theme 1.2: Coping with an infection***

Due to the fact most participants had previously experienced a UTI they were certain that they could self-diagnose a new infection based on their symptoms, and described being keen to get checked and treated as soon as they could.

*“... by that night I knew I had a bit more than a slight irritation”.* (P1)

Over the course of previous urinary infections they had endured a range of symptoms with the most common being dysuria, and frequency. Over half had also suffered from lower abdominal pain.

Although the patients tended not to take time off work during a UTI several were very concerned that the condition negatively affected their ability to do their job, or even simply manage to function on a day to day basis. One lady admitted that she was unable to think about anything else during a bout of infection, and felt it took over her life.

*“In some ways it’s one of the most distressing things that you can have. And I think people would – do anything not to have it”. (P2)*

Trying to manage the symptoms (especially dysuria and frequency) usually meant making an early appointment with the GP, but a few women had in the past first also tried out an alternative treatment for one to three days. If the alternative had not alleviated their discomfort then they were very likely to want to take antibiotics.

*“It says 3 sachets, really, on the first day, which I did, but by that night I knew I had a bit more than a slight irritation. I was in real pain so I decided not to take anymore and just go to the GP”. (P1)*

*“At the moment it gets so bad I just take what I can take to relieve the symptoms”. (P9)*

## **Theme 2: Factors that influenced the decision to participate in the trial**

The majority of the respondents were happy to take part in ATAFUTI because they considered it worthwhile to be testing an alternative treatment for antibiotics, and regarded it important to help. There were certain aspects of the trial which contributed to their decision which are outlined in the sub-themes below.

### ***Sub-theme 2.1: Reassurance of delayed prescription for antibiotics***

The delayed prescription for antibiotics to take away was reassuring to most respondents who took part in the trial. Without this prescription several would have been unlikely to participate, especially if they had already been experiencing symptoms for at least two or three days by the time they saw their GP.

*“Once the doctor said, look, I’m going to give you antibiotics anyway, so if it gets worse, you just start taking them; that kind of reassured me that – actually – it’s a win win situation here because I can help in some way with the trial, but I can still help myself get better. I knew that, you know, I could try and let’s see but if it didn’t work, it didn’t work.” (P19).*

Just a minority admitted that they would not have gone ahead with the trial without a delayed prescription in their hand. This was due to a concern that they believed they ran the risk of contracting a more serious infection.

*“No, I wouldn’t have taken part, because of my other health issue, you know, I can become very unwell if an infection gets hold”. (P4)*

Several did not feel that they would want to wait more than a couple of days before taking the antibiotic prescription if their symptoms had not shown signs of alleviating.

*“Well I was quite happy to do it – but I have to admit – I mean my immediate thought was – I don’t mind doing it but I really want to get this sorted now and is this just going to delay things”. (P6)*

There was a cost implication for one lady who went on the trial, who confessed she had taken part purely because the treatment was free.

*“.....and then she said I could do that and it was free, obviously, and saved me paying out for a prescription. So it was brilliant”. (P10)*

### **Sub-theme 2.2: Clear explanation of trial**

Understanding what was expected of them on the trial, a clear description of the products being tested, and being able to contact their GP surgery for support should they have any questions once they had enrolled were the main criteria which had inspired confidence in the participants.

The explanations of the trial by the GP or NP were found helpful and informative. This made it easier for the respondents to follow and understand the literature handed out. The patients described it as ‘thorough’, and all except one were undeterred by either the amount of paperwork they were given to read, or the diary they had to complete. Overall, they observed that there was more work for the practitioner to have to do than they did themselves.

*"I think it was just about right. It seemed a lot at the time when she was handing it over; she gave me a carrier bag. But when I got home and sat down and had a look at it all, it was quite informative". (P1)*

In fact the layout of the diary was considered quite simple, and several respondents described it as being self-explanatory and 'straight-forward' to fill in.

*"...it was explained to me, I understood it. I understood, you know, each step along the way what would happen and I left the surgery happily knowing, you know, what to do. And then it was repeated in the literature and the diary was straightforward, so I think I left the surgery feeling confident in what I was going to do, what might or might not happen". (P4)*

One lady expressed the view it would have been beneficial to include a simple step-by-step guide within the information pack to make the overall process easier to follow. She was concerned that she may forget to do something important. Nobody else had any reservations after everything had been explained to them at the surgery, and they also knew that they could ring up to ask any questions.

*"I had letters from one person, letters from another, letters from the surgery – and it would have been easier, I think, to have more – more guidance because, you know, some people doing it would find that quite a lot and go, actually, I'm not going to do this. There were times when I thought – what am I doing, what am I doing – but no, I kind of persevered as long as I could". (P19)*

The only negative issue raised about the trial documentation related to the safety of the herbal product. One patient recalled reading that it may alter the colour of urine. She had then taken the trouble to search for uva-ursi on the internet and discovered additional information relating to the time limit the herb could be taken for treatment. This concerned her enough to want to know the reasons behind why the duration of its prescribing may be restricted. She felt this information should have been included in the PIS, and thought it would also be helpful in a product leaflet.

### **Sub-theme 2.3: Would there be any side-effects?**

Related to the above concern expressed about the potential change in the colour of urine, a couple of participants were worried that there might be side-effects from taking the herbal medicine. They mentioned that they had specifically asked about this when signing up for the trial. They felt a little daunted by the fact they did not really know anything at all about what they were going to be taking.

*"I wanted to know what the side-effects were. That's it, that's all I asked her". (P10)*

### **Sub-theme 2.4: Reluctance to take antibiotics**

Whilst increasing resistance to antibiotics did not worry most of the patients enough to deter them from asking for a prescription, the fact that they could possibly suffer side-effects or experience an allergic reaction to antibiotics was of greater concern.

*"I woke up during the night to go to the toilet and my urine was dark brown and I was bright red all over and I didn't know what was happening. I've heard people say that they've had an allergic reaction to antibiotics, but I didn't realise that's what it was. So I got in a bit of a panic, ringing 111: I'm bright red and my urine's got blood in it". (P11)*

Digestive problems (including nausea) were the most common complaint, and this aspect was, therefore, a major factor which contributed to their reluctance towards taking them.

*"...they upset my tummy so I get diarrhoea and they just make me feel generally unwell, slightly nauseous and just, you know, I prefer not to take them, however, you know, needs must, if you get one of these infections". (P4)*

### **Theme 3: Desire that herbal treatment will work**

A few, but not all the participants had previously been keen to self-medicate either to put off having to make a doctor's appointment or to avoid having to get antibiotics. Were an effective remedy to be available, all the respondents except one shared the

view that they would prefer to use it for symptom relief. There was not an aversion to antibiotics, but most people did express greater reticence to take this form of medication.

Just over half the participants (twelve) had used a herbal medicine previously, and shared a perception that herbs had traditionally been used to treat a variety of conditions. Only one person expressed surprise that an HMP was being tested in a clinical trial, but all agreed it made sense to test a herbal medicine.

*“Well actually I was surprised, but surprised in a good way because I just feel it’s something not to be dismissed”. (P1)*

*“Mother Nature is a good teacher – if you know where to look and you know how to use them”. (P12)*

### **Sub-theme 3.1: Willingness to try alternative treatments**

A variety of alternative treatments for UTI had been tried by the respondents ranging from cranberry juice to over-the-counter alkalisng products, as well as drinking more fluid in an attempt to flush out the bacteria. Most of those who had taken alternative remedies found that some could reduce symptoms but not alleviate them completely. This then resulted in a visit to the GP. Cranberry was the most common over-the-counter treatment sampled (by over half the respondents), but with minimal success.

*“It didn’t clear it but it helped if I took it along with any other tablets. So I’d say it helped but didn’t clear it”. (P3)*

A couple of women believed that cranberry was not effective once there were physical signs of an infection. One had read that the berries were useful for preventing the bacteria from adhering to the wall of the uroepithelium, but that they were unlikely to be of benefit once the infection had taken hold. She felt that it was misleading to tell people to take cranberry to alleviate an infection.

*“.....apparently once you’ve got the infection the cranberries don’t do anything, because it’s like shutting the gate after the horse has got out”. (P11)*

Two other respondents disliked drinking commercial cranberry juice because it was deemed to contain too much sugar.

A perceived benefit of using herbal medicine as an alternative was that it was more 'natural' rather than 'chemical' when compared to an antibiotic, and would therefore be safer and less likely to produce side effects. This view was partly based on the fact that herbs per se are consumed on a regular basis in cooking or as teas, whilst one participant believed that they were harmless because they came from plants; they did not think of them as medicines.

*"Well you sort of tend to think that these are more natural, rightly or wrongly, but that's – that's what you tend to think. I mean, you know, when you say – herbs – you use herbs all the time, don't you?" (P2)*

The opinion that a herbal medicine might be less harmful to take than an antibiotic may also potentially be reinforced if the HMP is commonly available over-the-counter from a chemist or health food shop and not require a prescription.

*"If you can buy it yourself – then I feel it's a safer product to – to have". (P10)*

One lady imagined that because it was a plant it meant she could investigate and understand more fully what it was she was putting into her body, compared to an antibiotic.

*"Obviously it would be natural; you haven't got any strange name that you don't know what you are putting into you. It's a plant you could search it easier, I suppose, and understand what's in it more, that would be good. Some of the pills they give you have really long weird names, haven't they?" (P3)*

Another respondent was heartened by the fact that a herbal medicine was being tested through primary care. She felt that perhaps the possible benefits of complementary medicines were being given greater consideration, and hoped they would be looked at alongside orthodox treatments under one umbrella.

*“...I think it's good now there's all this – there's all different kinds of ways of treating things now and I think it's nice – it's all going in the right direction where all these different types of medications and herbal things and – complementary medicines are all working altogether now, as a team, rather than people saying, oh no, don't do this, do this”. (P5)*

### **Sub-theme 3.2: Accessibility and trustworthiness of herbal treatment**

The convenience of potentially being able to obtain a herbal medicine over-the-counter compared to needing a GP appointment for an antibiotic prescription appealed to several participants. This was tied into the early recognition of their symptoms. They wanted immediate relief before the infection had a chance to get hold, and also shared the view it would save the doctor's time.

*“Yeah I think with a urine infection, 99% of the time you know that you've got one, and you kind of feel a bit of a waste going, you know - wasting the doctor's time in a way. I think it would be good to be able to get it from a shop or a pharmacy”. (P3)*

They, nevertheless, agreed that they would have more confidence in the medication were it accessible through their GP, but also appreciated the fact they might be able to buy it themselves prescription-free.

*“I think it's – it's – if it came from a known source like yourselves, it wouldn't be a problem but it's, you know, when you hear of people taking things that aren't – actually what they say on the tin, that's when I'd be more wary”. (P13)*

Health food shops were considered convenient, but a couple of women had more faith in purchasing an HMP via a professional body such as a pharmacist. They believed that sham medications might be sold in both health food shops and over-the-internet.

*“There's a huge industry ... sort of these herbal medicines are a huge industry, but I think taking something which is – has a clinical trial, proven to work, and you can either be prescribed it or it can be recommended, you know, by a pharmacist that this is actually something that does work – I wouldn't have any problem with”. (P10)*

#### **Theme 4: Barriers to adoption of herbal medicine**

The majority of the sample did not believe they would have any reservations about taking herbal medicine for a UTI, providing it had been demonstrated that the herb in question could alleviate their symptoms. Based on this sample there were, nevertheless, a couple of problems that might need to be addressed or reassurances made to potential patients were the results of ATAFUTI to be positive.

##### ***Sub-theme 4.1: Anticipated problems of taking herbal medicine***

The large size of the tablets on the trial (herbal/placebo) had alarmed a couple of women, especially as there were three tablets in a dose, but this was not enough to have put them off taking part. It was clear, however, that they would have preferred to have been prescribed a greater number of smaller pills.

*“They were like the size of some sort of horse tablet. That’s the one thing, when I opened the packet and I thought Oh no I’ve got to take three of these, three times a day, they were a bit daunting. I would rather have taken more but smaller”. (P18)*

Further to the discussion about the potential ease of accessibility of the HMP two patients showed concern about where a herbal product might have been produced. They had reservations about whether an HMP would be precisely what it claimed, were it made available without a prescription. They wanted to be reassured that the herb came from a creditable source, having some awareness that it might not be a genuine product.

*“...there’s so many – sort of horror stories of people buying herbal medicines from various countries around the world– I’ve really steered clear of them”. (P13)*

*“..I have read a lot about these preparations, but I don’t believe in buying them over-the-counter. You don’t know how they’re manufactured, you don’t know where they’ve come from, you don’t know how strong they are”. (P18)*

The participants were keen to take something to relieve their symptoms and were happy to use a herbal medicine providing they knew that it was likely to be effective. A couple of women had been sceptical that the treatment would actually work when they

started the trial, whilst another lady, who had not taken part in the clinical trial, claimed she would only take a herbal medicine in the future providing she was also given a back-up prescription for antibiotics.

Finally, one participant, who had not taken part in ATAFUTI, suffered a health condition which worried her enough to put her off trying anything herbal in case she experienced a bad reaction. This was regardless of whether it had been proven safe in a clinical trial.

*"I think I'd rather stay clear of herbs altogether; yes, I think I would prefer to keep off". (P20)*

#### **Sub-theme 4.2: Learning about the trial outcomes**

Should the results of the trial be favourable the participants hoped that they would read about it in the press, or that their GP would inform them. They imagined that the GP would simply give them the option of using the HMP instead of an antibiotic, and/or give them a leaflet on the herb per se.

*"Well I'd like to think that because I go to the doctor's frequently for this type of problem, that they would give me that information – but otherwise, through the media, I guess". (P15)*

They were not entirely sure whether they would actually learn about the trial results had they not themselves taken part.

*".....newspapers often take up some trials, don't they?" (P7)*

Although several believed that leaflets providing information about the herb may be made available in their local surgery not all of the women took the time to read the ones on display. They needed it to be drawn to their attention. A couple said they would be more likely to read a leaflet handed out at their local pharmacist than to pick up literature in the GP practice.

*"I think I have done in the past, but not recently, no, not unless it's something specific that I want to know about, I'd probably ignore it. I'd probably ignore – yes – the information boards and stuff, I don't really take any notice". (P11)*

One lady suggested that a poster providing information on the HMP could be displayed on the inside of the toilet door at her local surgery. This approach was subsequently suggested to several other respondents, who all favoured the idea. Running a promotion video on the TV screens in the surgeries was also considered more beneficial than providing leaflets to read.

*“And the screen, of course, you can’t avoid, can you? You can avoid everything else but you can’t avoid a screen”. (P2)*

### **10.5.3 Discussion**

#### **10.5.3.1 Main Findings**

Twenty patients were recruited into the study. Most had taken part in ATAFUTI, but the sample also included five who had not participated in the main trial. The majority felt confident and reassured in taking part in ATAFUTI due to being handed a delayed prescription for antibiotics were their symptoms to persist or worsen whilst they participated. Most respondents had heard about antibiotic resistance but this information had not affected their decision to seek and expect to be prescribed antimicrobial treatment for a UTI. Their desire to obtain symptom relief was more pressing than the potential consequences that prescribing might have on contributing to antibiotic resistance in the future.

Of greater concern to a few respondents regarding taking antibiotics was the possibility of experiencing either an allergic reaction or suffering side effects. This had inspired most to take part in ATAFUTI, and had also previously motivated around half to self-medicate with alternative treatments including herbal medicine.

There were no major barriers identified to adopting herbal medicine as an alternative treatment to antibiotics for symptom relief of UTI. A few of the respondents were concerned about the origin of the herbal product were it not available via their GP or pharmacist, and there were some reservations about the size of the tablets. This latter concern was not enough to cause them to withdraw from the trial.

### 10.5.3.2 Scope and Limitations of the Study

Including participants who suffered from UTI but did not qualify for ATAFUTI enabled a broader spectrum of views to be explored, and resulted in respondents being included who would not have been influenced by experiencing the trial. Conducting the interviews a month after their attendance on ATAFUTI, but not longer than three months, meant that the respondents were still able to recall details of the trial but would not be overloaded with tasks too close together.

The drawback of the recruitment method was that there might be bias amongst those who took part in the trial and experienced a positive outcome compared to those who did not, or vice versa. In reality, when data saturation was reached in this sample, there was little difference of opinion concerning the trial set-up between those who experienced symptom relief and those who needed to take their antibiotic prescription.

This sample is drawn from those who have consulted their GP or health practitioner. A different attitude may be expressed by those who may spend more than 2 or 3 days waiting for their symptoms to resolve or who are simply against taking antibiotics *per se*.

Conducting interviews over the telephone instead of face-to-face may have been a weakness of the study as it would not have been possible to observe the non-verbal reactions of the respondents as they were questioned. Nevertheless, telephone interviews meant that the respondents could be interviewed in the familiar surroundings of their own home rather than the more formal setting of the GP surgery. This could be beneficial when asking them to disclose sensitive information to a researcher they have never met (Novick, 2008).

### 10.5.3.3 Comparison with Previous Literature

A new finding derived from this PhD study was that the participants' desire to obtain relief from the uncomfortable symptoms of UTI was more important to them than the risk that antibiotics might not work for them in the future. Most appeared either unaware or unconcerned that by asking for a prescription they might be contributing to the build-up of antibiotic resistance. They were driven by an over-riding need to alleviate their symptoms; most within 2 to 3 days of the first signs of an infection

regardless of whether they had tried to self-medicate first. The most common symptoms experienced were dysuria and frequency.

This study showed that patients who frequented their GP with a UTI made the appointment with the expectation that they would be prescribed antibiotics, regardless of whether they had already tried self-care. This view confirms previous findings (Little *et al.*, 2004).

All the participants had heard of antibiotic resistance, even if they had not come across the precise term before, but they did not have any knowledge of how resistance developed. When prompted for further definition, they either believed that the body got used to the medication and did not respond to it any more, or imagined it simply no longer worked. There was no understanding demonstrated that bacteria were able to adapt to defend themselves against antibiotics (Hawkey and Jones, 2009). This finding reinforced the results of a 2014 systematic review (incorporating both quantitative and qualitative data) regarding the public's misconceptions and lack of knowledge about antibiotic resistance (McCullough *et al.*, 2016), as well as a 2013 qualitative study (conducted in New Zealand) where the participants used the words 'immunity' and 'resistance' interchangeably when describing the body as becoming accustomed to the medication (Norris *et al.*, 2013).

Most felt confident in taking part in ATAFUTI due to being handed a delayed prescription for antibiotics were their symptoms to persist whilst on the trial. The literature review identified three previous qualitative research publications regarding how people feel about being given a delayed prescription for a UTI. These showed mixed responses, with just over half willing to try the strategy, and the others wanting to take antibiotics. Those that did not want to delay felt that they knew their own body well enough to decide whether they needed treatment, or their symptoms were interfering with their ability to work (Little *et al.*, 2009; Leydon *et al.*, 2010; Duane *et al.*, 2016).

There has been one qualitative study amongst GPs exploring the use of herbal medicine for urinary tract infections, but none amongst patients (Flower *et al.*, 2015).

#### **10.5.3.4 Implications for Antibiotic Resistance**

As mentioned above, although the patients in this sample had some perception or awareness that antibiotics might not work for them in the future most appeared either unaware or unconcerned that by asking for a prescription they might be contributing to the build-up of resistance. It is, therefore, necessary that an alternative symptom relief for cystitis is found otherwise this condition will continue to contribute to the growth of antibiotic resistance. The findings suggest that by giving patients the option of having a delayed prescription will probably result in them going to obtain the medication if the symptoms do not subside within 2 to 3 days.

#### **10.5.3.5 Implications for Prescribing or Recommending Herbal Medicine for Cystitis**

With side effects and safety issues dominating the responses to questions on antibiotics most of the participants were open to using a herbal medicine in the future for symptom relief of UTI. Even though more than half the women in this study had endeavoured to but failed to treat a UTI with an alternative treatment in the past (such as taking cranberry), this had not put them off wanting to try something else.

Providing an HMP has been proven to alleviate symptoms in a clinical trial, based on this sample there were very few barriers, if any, to adopting this line of treatment instead of antibiotics. This was despite the fact that just under half had never used a herbal medicine in the past. Only one respondent claimed that she would prefer to take antibiotics for symptom relief in case she experienced an adverse reaction to the HMP.

If the herbal treatment proves successful they would expect their GP surgery to bring the findings of the trial to their attention. Once informed they would be likely to accept a prescription for the HMP, or seek it out themselves at a pharmacy or health food-shop. Just buying the herb at a health food-shop was not considered a satisfactory option by most women, although it was viewed as convenient. They wanted to know they were getting the correct extract, and not one which might have been produced anywhere in the world with the risk it might not be authentic. Should the clinical trial prove successful it may be advisable to produce a branded product which receives publicity and is readily available.

### **10.5.3.6 Design of a Future Trial**

The majority did not express any reservations about taking part in ATAFUTI once offered and, therefore, reassured by the (delayed) antibiotic prescription. Nearly all the participants had found the process and paperwork straightforward and expressed a willingness to participate in a future trial. This suggests that the overall design of the trial could be replicated for a future study without needing any major alterations.

## **10.6 Conclusion**

Should the results of ATAFUTI show that relief of UTI symptoms can be achieved through taking the trial dosage of uva-ursi, then based on this sample there is a suggestion that there should be a favourable response to being prescribed this HMP amongst those patients who are unhappy about taking antibiotics. Fears of antibiotics not working for future infections due to bacterial resistance to this class of medication was not a major concern when it came to enduring UTI symptoms, but experiencing possible side-effects of the medication were.

# Chapter 11: Discussion and conclusion

## 11.1 Introduction

The reasons behind the PhD stemmed from a need to help tackle the increasing problem of antimicrobial resistance across a range of antibiotics used for treating lower urinary tract infections. There has been a call for the discovery of new antibiotics by the World Health Organisation (WHO), who for the first time published a list in February 2017 of micro-organisms for which new antimicrobials are urgently required. These included *E. coli* a major cause of urinary tract infections (WHO, 2017).

The aims of the PhD were in part to support the double-blind placebo controlled clinical trial *Alternative Treatments for Adult Female Urinary Tract Infections* (ATAFUTI). The trial itself was testing an alternative treatment, an extract of the herb *Arctostaphylos uva-ursi*, to determine whether the herbal extract could reduce symptoms of urinary tract infections (UTI) and as a result whether antibiotic prescribing may potentially be reduced.

With few trials run on herbal medicines it posed certain questions regarding how to determine an appropriate dose, what the potential antimicrobial activity and mechanism of the herb might be and what barriers might exist to adopting a herbal medicine into clinical practice.

In order to fulfil the objectives of the research the PhD utilised a range of mixed methods. These comprised focused reviews, laboratory investigations and a qualitative research study amongst patients who had either taken part or been approached about ATAFUTI.

The study is unique in its investigation of a herbal treatment for urinary tract infections in conjunction with a clinical trial.

## 11.2 Findings

### 11.2.1 Dosage and Safety Issues

Of priority was to determine the safety of uva-ursi. Researchers have an ethical obligation to not put the health of participants at risk and any medication to be tested in a clinical trial has to be evaluated for safety (Kimmelman and Federico, 2017). Since uva-ursi is classed as a traditional herbal medicine under the EU directive, and has also been granted marketing authorisations in several European countries, it is acknowledged that the herb had already been used for a minimum of 30 years (including 15 years within Europe) and has an acceptable level of safety. Bibliographic efficacy and safety data have been used to produce an overall assessment report by the European Medicine's Agency (EMA, 2012a).

Nevertheless, monographs on the herb together with the EMA assessment report have referred to a high dose of one of the constituents metabolised from uva-ursi as being potentially lethal. This concern related specifically to hydroquinone at a level of 5 g, derived from the equivalent of 30 – 100 g of dried plant material (EMA, 2012a; Bone and Mills, 2013). A single dose of the herb is 3 g, and, therefore only a tenth of the lowest end of the range considered to be of risk. Moreover, the investigation into the quality of the GMP extract by HPLC (Chapter 6) determined that there was a maximum of 1.86% hydroquinone present in the leaf, which decreased to only 1.2% after a year. This would mean that at least 269 g - 500 g would have to be consumed to obtain even the putative toxic dose of 5 g. Moreover, any free hydroquinone that was present would likely be conjugated in the liver to glucuronide and sulphate metabolites for safe excretion.

However, the issue had to be addressed and researched further to determine the source of the information. The investigation led to papers going back over a hundred years that related to the ingestion of photographic developing fluid. These developing solutions contained not only hydroquinone but a further 5 g of an isomer of hydroquinone, as well as additional chemical components which were toxic. This enabled concerns relating to the potential toxicity of hydroquinone to be put into context. It also demonstrated the importance of sourcing and reading original material rather than citing secondary references.

The investigation into the dosing strategy showed there was consistency amongst the published sources as to the maximum quantity of 840 mg arbutin believed to be required for treatment. This is based on using 12 g of the dried leaf, which contains approximately 7% arbutin. Arbutin is considered the main ingredient, which is then metabolised to hydroquinone in the body. Whilst the total quantity of arbutin may have originally been derived from an *in vitro* assay and subsequently used in clinical studies on pharmacokinetics, involving between 1 and 16 volunteers, it was also based on doses administered in traditional use.

Uva-ursi has not previously been tested in a clinical trial, and a dose response study has not, as yet, been conducted. The published studies on pharmacokinetics using healthy volunteers ascertained that less than 1% of free hydroquinone was available in around half the urine samples analysed after taking uva-ursi, whilst the other 50% did not contain any. However, it is not known how much free hydroquinone is present in urine after it may have been released from the parent glucuronide during a UTI. This release is due to the enzyme  $\beta$ -glucuronidase, expressed by *E. coli*. The mechanism has been demonstrated and quantified *in vitro* (Siegiers *et al.*, 2003), but never quantified *in vivo* during an infection. The amount of free hydroquinone present in the course of an infection was, therefore, not available at the time of calculating the dose.

### **11.2.2 Antimicrobial Activity of uva-ursi**

The *in vitro* experiments in this PhD demonstrated that *E. coli* and several other organisms responsible for causing UTI were susceptible to uva-ursi and hydroquinone. The study has brought up to date and provided uniformity to antimicrobial assays with uva-ursi and hydroquinone.

Whilst existing literature reported that urine had to be alkaline for uva-ursi to be effective (believed necessary to hydrolyse hydroquinone glucuronide to hydroquinone), but that alkalinisation itself did not improve the efficacy of hydroquinone (Frohne, 1970), this study determined that this was not the case. *E. coli*, for example, was more susceptible to hydroquinone at an alkaline pH. Furthermore, the experiments which tested the urine of 4 healthy volunteers who had consumed uva-ursi showed that increasing the pH from acidic to alkaline did not consistently affect the results of these

antimicrobial assays. This suggests that either there may be another mechanism of action, or that hydroquinone is not liberated in sufficient quantities to be effective. Moreover, the pH of the enzyme responsible for liberating hydroquinone from the glucuronide ( $\beta$ -glucuronidase) has an optimum pH activity (of between 6 and 7) which is lower than that of the pH required for optimum efficacy of hydroquinone (pH >8). This may be of significance with regard to how efficient the mechanism of action is within the urinary tract if hydroquinone is central to antimicrobial activity.

Using high-performance liquid chromatography to analyse the urine of patients who participated in ATAFUTI, who were diagnosed with a UTI, showed that it was necessary to add the enzyme  $\beta$ -glucuronidase (to liberate hydroquinone from the glucuronide) in almost half the samples before hydroquinone was detected. It suggests that either the infections were not caused by *E. coli* or that the enzyme may not always be efficient at deconjugating hydroquinone from the glucuronide.

The results of the volunteer study showed that there was greater inhibition of the growth of *E. coli* in the samples of 2 out of the 4 volunteers after taking uva-ursi over 48 hours compared to 24 hours. There are no previous reports on this finding.

The investigations to determine whether uva-ursi may negatively affect virulence factors of *E. coli* reinforced earlier *in vitro* findings that it potentiated aggregation of this organism. In so doing it may cause the bacteria to clump together and be flushed out of the urinary tract, or it could possibly enhance virulence. The latter theory is less likely as uva-ursi has a known traditional use for treating UTI rather than exacerbating it, but this would require further investigation *in vivo*.

The *in vitro* assays which assessed uva-ursi's effect on motility showed no impact on this mechanism. It is not known, however, whether individual constituents or metabolites from uva-ursi may have a different effect *in vivo*.

### **11.2.3 Qualitative Study**

The qualitative study identified the issue that despite the fact that patients were aware of the problem of antibiotic resistance, and knew that they may be negatively affected

by how well this class of medication may work for them in the future, they may not be deterred from seeking antibiotics if they wanted symptom relief of a UTI. The most common symptoms they complained of were dysuria and frequency, and they had usually experienced some symptoms for at least 2 to 3 days before consulting their health-care practitioner for treatment. Once they got to this stage most expected to be given an antibiotic prescription.

The current guidance by Public Health England is to consider a back-up or delayed prescription for treatment in mild cases of urinary tract infection ( $\leq 2$  symptoms) (PHE, 2017). Further education may be needed regarding how antibiotic resistance might impact on more serious health conditions in the future. Based on this study, the participants agreed to delay taking antibiotics, but they had the possibility of receiving a potential alternative relief for their symptoms in the trial. Without providing an alternative treatment they may just collect the antibiotics. Perhaps it should be noted that there is no system in place for GPs to know how many prescriptions are taken up.

With a successful clinical trial, based on these respondents there should not be any major barriers to using an alternative (herbal) treatment for symptom relief of UTI. Advice on what commercial extract to use would be valuable to avoid the pitfalls of obtaining an adulterated product.

### **11.3 Implications for the Clinical Trial *ATAFUTI***

The investigations into the toxicity and dosing, and proposed dosing strategy, were addressed before the Investigator's Brochure on the herbal extract was published. This was in order that this information could be submitted to the MHRA for consideration in using the extract in *ATAFUTI*.

Investigations into the safety of uva-ursi dispelled concerns regarding the potential side effects of hydroquinone as reported in the literature. Whilst, according to the prescribed dose for the trial it would be highly unlikely if not impossible to generate 5 g of free hydroquinone through metabolising uva-ursi, tracing the source of these citations showed that there was no evidence that this amount had previously proved lethal.

Based on the dosing study the recommended dosage for the HMP for the trial was 840 mg per day of arbutin derived from 3 g of plant material (leaf). The product selected for the trial, the quality of which was confirmed by HPLC in this study, was a 2.5 - 4.5:1 concentrated extract containing 20% arbutin. The recommended dose for ATAFUTI was to take up to 840 mg arbutin divided across 3 doses. It was necessary to divide the dose partly because uva-ursi is known to be metabolised quickly (within 2-3 hours) and partly so that the tannins would not cause gastrointestinal upset. There is no reason that it should not be taken for the duration required by the trial of 3 - 5 days.

As herbal monographs have reported that urine needs to be alkaline for efficacy of uva-ursi, prior to running the volunteer study it was necessary to investigate how alkalinisation itself may affect the results in case it became a confounding factor. The literature study into alkalinisation of urine, the data extraction of which contributed to a systematic Cochrane review, showed that there have not, as yet, been any randomised controlled trials to provide credible evidence to support the recommendation and practice of taking alkalinising agents (such as sodium citrate) for the relief of the symptoms of cystitis (O'Kane *et al*, 2016). Not only is there a lack of firm evidence to endorse this treatment, further investigation revealed that it can take several days to alkalinise urine via the diet, and taking alkalinising agents cannot be relied upon for altering and maintaining an alkaline urine pH (Gargan, Hamilton-Miller and Brumfitt, 1993).

The Investigator's Brochure containing the above information on dosing and safety was subsequently submitted to and approved by the MHRA. The review on alkalinisation, combined with the volunteer study into the antimicrobial activity of uva-ursi at alkaline pH, enabled a decision to be made that patients taking part in ATAFUTI would not be required to follow an alkaline diet or take an alkalinising agent.

Based on the volunteer study there should be an inhibitory effect against a low grade bacterial urinary infection caused by *E. coli*. The *in vitro* experiments indicate that there might also be an effect against *S. epidermidis*, *MRSA*, *S. aureus* and *E. faecalis*.

## 11.4 Contributions to Science

Work related to this PhD has contributed to the following:

- *The Investigator's Brochure on ATAFUTI* - submitted to the Medicines and Healthcare Regulatory Agency
- Co-author of Cochrane Review - Urinary alkalisiation for uncomplicated urinary tract infections (O'Kane *et al.*, 2016)
- World Congress on Integrative Medicine and Health 2017 (Berlin) Workshop (Oral Presentation) *From Margins to Mainstream* Scientific analysis
- Westminster Ethnopharmacy Seminars and Talks 2017 (London) Oral Presentation
- Register of Chinese Herbal Medicine Seminar 2016 (London) *Back to the future: the antibiotic sparing role of herbal medicines in the treatment of acute infections* Oral Presentation: The ATAFUTI trial: uva-ursi for acute UTIs
- Society for Primary Academic Care Conference 2015 (Birmingham) Elevator Pitch *Are safety issues concerning *Arctostaphylos uva-ursi* based on distorted evidence relating to ingestion of photographic developing fluid?*
- Society for Primary Academic Care Conference 2014 (Bristol) Oral Presentation *Alternative Treatments of Adult Female Urinary Tract Infection (UTI): Quality Control and Antimicrobial Activity of a Herbal Medicinal Product*

## **11.5 Future work**

### **11.5.1 Metabolites in Patient Urine Samples**

Once the trial is un-blinded it will be possible to compare the results of the analysis on metabolites in this study (Chapter 8) with the medication which was given to the patients. It will first be necessary to confirm the presence of a urine infection as well as investigating how the patients responded to treatment. This will help determine the accuracy of the results analysed by HPLC, and assess whether hydroquinone was likely to be present in the urine according to the medication allocated to the patient. This would facilitate an understanding of whether hydroquinone may have efficacy in the body.

A future study would also be to quantify how much hydroquinone may be present in the urine of an infected person. This would help determine whether the concentration of this compound is sufficient to have therapeutic effect *in vivo*, based on the results of the *in vitro* study, or whether there could be another metabolite and/or mechanism of action.

### **11.5.2 Gallic Acid**

It may be beneficial to further investigate what role the compound gallic acid may play, if any, in antimicrobial activity of uva-ursi if the trial proves successful. It is a tannin, and comprises 50% of uva-ursi and there is some evidence that it is implicated in colony aggregation which may play a role in the pathogenicity of *E. coli*.

### **11.5.3 Qualitative Study**

Interviews with GPs and Nurse Prescribers regarding their opinions of alternative treatments to antibiotics, including herbal medicines, could be helpful. This may contribute to an understanding of how health practitioners view the prescribing of herbal medications as an alternative treatment to antibiotics should the trial be successful. Such a study could also highlight any issues clinicians feel need to be addressed in the actual running of a future clinical trial on testing interventions, especially alternative treatments.

It may be also worthwhile to conduct a qualitative study amongst patients suffering from UTI who have been given a delayed prescription for antibiotics and then collected the antibiotics to clarify how long they waited before taking the medication.



# Appendices



## Appendix A Herbal Texts Searched in Literature Review

	Title of Publication
1)	A Clinical Guide to Blending Liquid Herbs (Bone, 2003)
2)	British Herbal Compendium Volume 1 (Bradley, 1992)
3)	Clinicians Handbook of Natural Medicine (Pizzorno, Murray and Joiner-Bey, 2007)
4)	European Scientific Cooperative on Phytotherapy (ESCOP) (ESCOP, 2003)
5)	Herbal Medicines (Barnes, Anderson and Phillipson, 2007)
6)	Medical Herbalism. The Science and Practice of Herbal Medicine (Hoffman, 2003)
7)	Medicinal Plants of the World: An Illustrated Scientific Guide to Important Medicinal Plants and Their Uses (van Wyk and Wink, 2004)
8)	Physicians' Desk Reference for Herbal Medicines (Gruenwald, Brendler and Jaenicke, 2008)
9)	Pharmacognosy, Phytochemistry, Medicinal Plants (Bruneton, 1999)
10)	Principles and Practices of Phytotherapy (Mills and Bone, 2000)
11)	Principles and Practices of Phytotherapy (2 <sup>nd</sup> edn) (Bone and Mills, 2013)
12)	The Essential Guide to Herbal Safety (Mills and Bone, 2005)
13)	Weiss's Herbal Medicine (Weiss, R., 2000)
14)	World Health Organisation Monographs on Selected Medicinal Plants, Vol. 2. Folium Uvae ursi

## Appendix B Kew Voucher Specimens - *Arctostaphylos uva-ursi*

HPLC Analysis Number	Location	Collected	Reference
K1	Austria, August 1932	J Bornmüller	H2013/649
K2	France, August 1995	Brummitt 19132	H2013/649
K3	Canada, August 1959	Garton 6978	H2013/649
K4	Spain, August 1980	F & H 38	H2013/649
K5	Scotland, June 2001	Crawley 346	H2013/649
K6	Poland, July 1939	Kobendza 454	H2013/649
K7	Scotland, May 1900	Marshall	H2013/649
K8	Italy, June 1964	Brummitt 4725	H2013/649
K9	Yorkshire, May 1939	Sledge 37	H2013/649

## Appendix C UCL Ethics Committee Study Approval Letter

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UCL RESEARCH ETHICS COMMITTEE  
ACADEMIC SERVICES



Professor Simon Gibbons  
School of Pharmacy  
UCL

24 February 2015

Dear Professor Gibbons

**Notification of Ethical Approval**

**Project ID: 6376/001: Alternative treatments of adult urinary tract infection: anti-microbial activity of a herbal medicinal product**

I am pleased to confirm in my capacity as Chair of the UCL Research Ethics Committee that I have approved your study for the duration of the project i.e. until **February 2016** on condition that as a measure of risk minimization:

- (a) the medicines are segregated from other stock and stored at temperatures specified by the manufacturer (ideally in a monitored setting).
- (b) as the investigational tablets will be taken home by participants they should be appropriately labelled.
- (c) accountability records are maintained to capture receipt, dispensing and returns.

as detailed in the attached letter from Mitesh Kunwardia, the Regulatory Manager (Pharmaceuticals).

Approval is also subject to the following conditions:

1. You must seek Chair's approval for proposed amendments to the research for which this approval has been given. Ethical approval is specific to this project and must not be treated as applicable to research of a similar nature. Each research project is reviewed separately and if there are significant changes to the research protocol you should seek confirmation of continued ethical approval by completing the 'Amendment Approval Request Form':
2. It is your responsibility to report to the Committee any unanticipated problems or adverse events involving risks to participants or others. Both non-serious and serious adverse events must be reported.

**Reporting Non-Serious Adverse Events**

For non-serious adverse events you will need to inform Helen Dougal, Ethics Committee Administrator ([ethics@ucl.ac.uk](mailto:ethics@ucl.ac.uk)), within ten days of an adverse incident occurring and provide a full written report that should include any amendments to the participant information sheet and study protocol. The Chair or Vice-Chair of the Ethics Committee will confirm that the incident is non-serious and report to the Committee at the next meeting. The final view of the Committee will be communicated to you.

**Reporting Serious Adverse Events**

The Ethics Committee should be notified of all serious adverse events via the Ethics Committee Administrator immediately the incident occurs. Where the adverse incident is unexpected and serious, the Chair or Vice-Chair will decide whether the study should be terminated pending the opinion of an independent expert. The adverse event will be considered at the next Committee meeting and a decision will be made on the need to change the information leaflet and/or study protocol.

On completion of the research you must submit a brief report (a maximum of two sides of A4) of your findings/concluding comments to the Committee, which includes in particular issues relating to the ethical implications of the research.

With best wishes for the research.

Yours sincerely



**Professor John Foreman**  
Chair of the UCL Research Ethics Committee

Enc.

Cc: Jeanne Trill, Applicant

Academic Service, 2 Taviton Street,  
University College London Gower Street London WC1E 6BT  
Tel: +44 (0)20 3108 4312  
Email: [ethics@ucl.ac.uk](mailto:ethics@ucl.ac.uk)  
<http://ethics.grad.ucl.ac.uk/>



### VOLUNTEER STUDY INFORMATION SHEET

**Project Title:**

**Alternative Treatments of Adult Urinary Tract Infection: Antimicrobial Activity of an Herbal Medicinal Product**

This study has been approved by the UCL Research Ethics Committee (Project ID Number): 6376/001

Dear

We would like to invite you to take part in a research study, which is being conducted by a PhD researcher in conjunction with the School of Pharmacy (at UCL). Before you decide, we would like you to understand why the research is being done and what it will involve for you. Please take time to read this carefully. Your participation would be entirely voluntary, and you should not at any stage feel obliged to take part.

Thank you for taking the time to read this and to consider whether you wish to take part.

**Why is the study being conducted?**

Due to growing resistance to antibiotics alternative treatments are being sought for certain conditions, such as urinary tract infections. This study is being conducted to investigate the possible antibacterial effect of a traditional herbal medicine for urinary tract infections.

**What is being tested?**

The herb *Arctostaphylos uva-ursi* (known as Bearberry) – the leaves of which have been used for hundreds of years for relieving the symptoms of urinary tract infections. A commercially available tablet is being used in the study.

**What is the purpose of the study?**

The purpose of the study is to find out whether, after having taken the herb *uva-ursi*, the person's urine demonstrates any antibacterial properties (particularly against *E coli*). The study will also be used to confirm which constituents from the herb may be present in the urine.

**Why have I been invited?**

There are up to 8 healthy volunteers (over the age of 18) being recruited for the study. There are no specific reasons why you have been approached other than this. We require a minimum of 3 people, but are asking more in case volunteers change their mind and so that nobody feels any obligation to take part.

**For how long will my participation be needed?**

Two days

**What do I have to do?**

You would have to take 2 herbal tablets four times a day over a 2 day period, and produce urine specimens at certain time periods (as outlined on the attached procedure). There will be 7 urine samples in total required (one prior to taking the herb, and then 3 urine specimens on Day 1 and 3 on Day 2 after taking the herbal tablets).

The PhD researcher will liaise with you regarding a time to participate which is convenient for you.

**Where will the study be conducted?**

The samples will be analysed at the School of Pharmacy, UCL.

**Do I have to take part?**

No. It is up to you to decide whether or not to take part. You are free to withdraw at any time and without giving a reason. You just have to contact the researcher (details below) and let them know. Your decision will be respected.

**What are the possible disadvantages and risks of taking part?**

The dosage you will be given to take is within the dose approved by the European Medicines Association – but for a shorter duration. It is uncommon to have any side effects with this dose. However for your general information, in higher dosages taken for a long time it may cause an upset tummy.

If you should experience any side effects at all then please contact the PhD researcher to inform them and do not take any further tablets.

**What are the possible benefits of taking part?**

It is not known whether you will have any personal benefit from taking part in this study. However, your participation may help to give important information about how to treat people with urinary tract infections in the future.

**Are there any benefits or losses to the researcher if I decline?**

There are no financial benefits or losses to the researcher if you decline. The researcher can approach other possible volunteers if you decide not to take part.

**Will my taking part in this study be kept confidential?**

Your name will not be required on the samples and your participation will be anonymous.

**How long will my samples be used and stored for?**

Your samples will only be kept for a few days (less than 7) whilst they are being analysed.

**What will happen to the results of the research study?**

The samples will be analysed by a PhD researcher, and the results entered into a thesis. They may at a later stage be published in a medical journal, but will remain anonymous at all times. As a participating volunteer you would be welcome to have a copy of the thesis and/or any published material which is produced as a result of this study.

**Who is organising and funding the research?**

This study is being run by the School of Pharmacy, University College London (UCL).

**Who has reviewed the study?**

All research is looked at by independent group of people, called a Research Ethics Committee, to protect your interests. This study has been reviewed by the UCL Research Ethics Committee.

**Further Information and contact details**

Please contact the PhD researcher (Jeanne Trill) if you have any further questions: via email or phone: [Jeanne.Trill@btinternet.com](mailto:Jeanne.Trill@btinternet.com), or 07770-623956.

The PhD researcher is being supervised by Professor Simon Gibbons (Head of the Department of Pharmaceutical and Bio Chemistry at the School of Pharmacy, UCL). His contact details, should you wish to ask him any questions about the study, are: [simon.gibbons@ucl.ac.uk](mailto:simon.gibbons@ucl.ac.uk)

Any data in this study will be handled in accordance with the Data Protection Act 1988.



**Informed Consent Form for the study: Alternative Treatments of Adult Urinary Tract Infection: Antimicrobial Activity of an Herbal Medicinal Product**

This study has been approved by the UCL Research Ethics Committee (Project ID Number): 6376/001

**Please tick appropriate box:**

Yes, I would like to take part in this study

No, I do not want to take part in this study

**If Yes, please complete the following by initialling each box:**

1. I have read the Information Sheet (Version 1 dated 10/12/14) about the study on **Antimicrobial Activity of an Herbal Medicinal Product**

2. I understand that I do not have to take part in this study if I do not want to.

3. I understand that I can withdraw at any time without giving a reason.

4. I have had the opportunity to ask any questions I wish to ask.

5. I understand that any data will be handled in accordance with the Data Protection Act 1988.

6. I have access to the name and contact details of the researcher in case I have any questions in the future.

I consent to giving urine samples to be tested by the PhD researcher

**My Name:** \_\_\_\_\_

**Signature:** \_\_\_\_\_ **Date:** \_\_\_\_\_

**Researcher's Statement**

I .....confirm that I have carefully explained the purpose of the study to the participant and outlined any reasonably foreseeable risks or benefits

Signed:

Date:

**Procedure for Taking Herbs and Producing Urine Samples**

**Alternative Treatments of Adult Urinary Tract Infection: Antimicrobial Activity of an Herbal Medicinal Product**

**Day 1**

(a)	Morning time (this would ideally be around 9 am or before). Please take a mid-stream urine specimen (this is from the middle of the flow rather than the beginning).
(b)	9 am - please consume 2 herbal <i>uva-ursi</i> tablets as provided, with a little water (On Day 1 this would be straight <u>after</u> taking the first urine specimen).
(c)	Three hours later (eg. 12 pm) please consume 2 herbal <i>uva-ursi</i> tablets
(d)	Three hours later (eg. 3 pm) please consume 2 herbal <i>uva-ursi</i> tablets
(e)	Two hours later (eg. 5 pm) please take a urine specimen
(f)	One hour later (eg. 6 pm) please take a urine specimen
(g)	Please then consume your final 2 herbal <i>uva-ursi</i> tablets for the day (eg. 6 pm)
(h)	Three hours after your last dose (eg. 9 pm) please take a urine specimen

**Day 2**

Please repeat Day 1 from (b) to (h). There is no need to take the urine specimen labelled (a) first thing in the morning on Day 2.

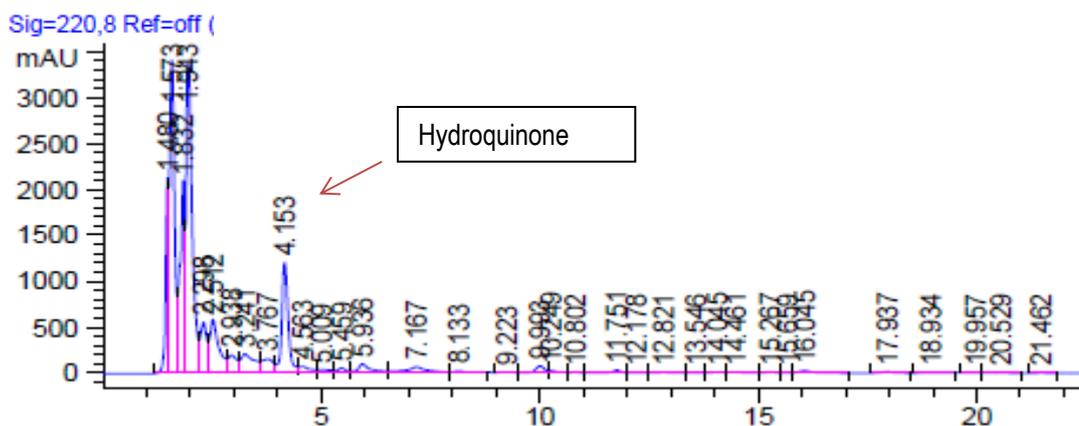
## Appendix E HPLC Analysis of Volunteer Urine Samples

Chromatograms of volunteer sample (d) at 220 nm and 280 nm.

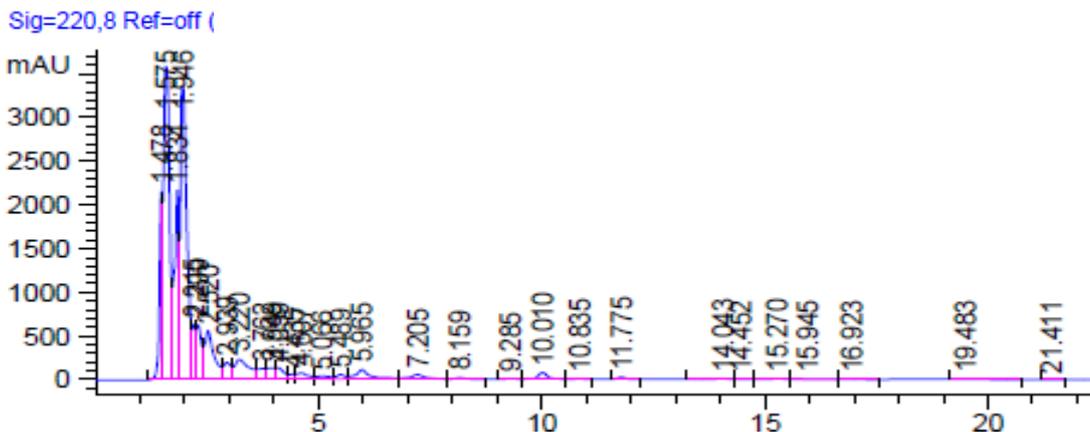
Hydroquinone is present post the addition of the enzyme  $\beta$ -glucuronidase

### Signal at 220 nm

$\beta$ -glucuronidase present. Peak at 4.153 minutes = hydroquinone

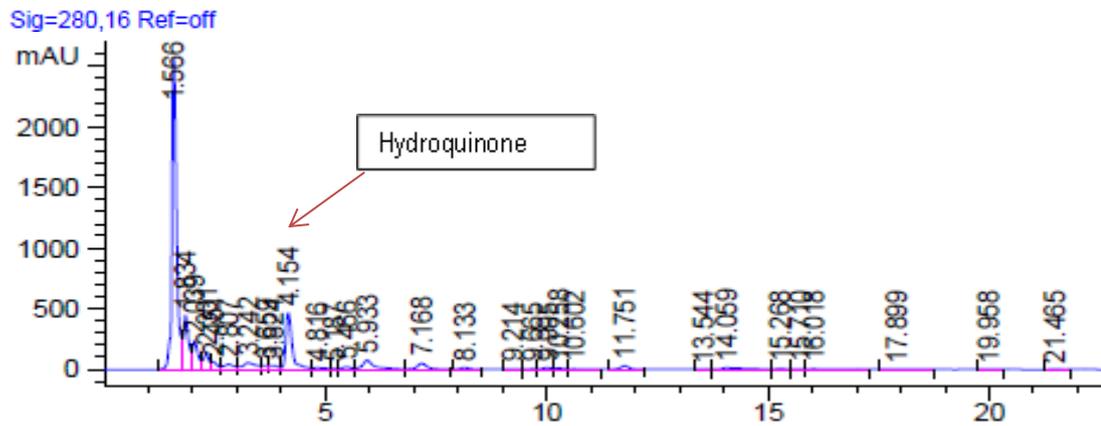


No  $\beta$ -glucuronidase present, and no hydroquinone peak

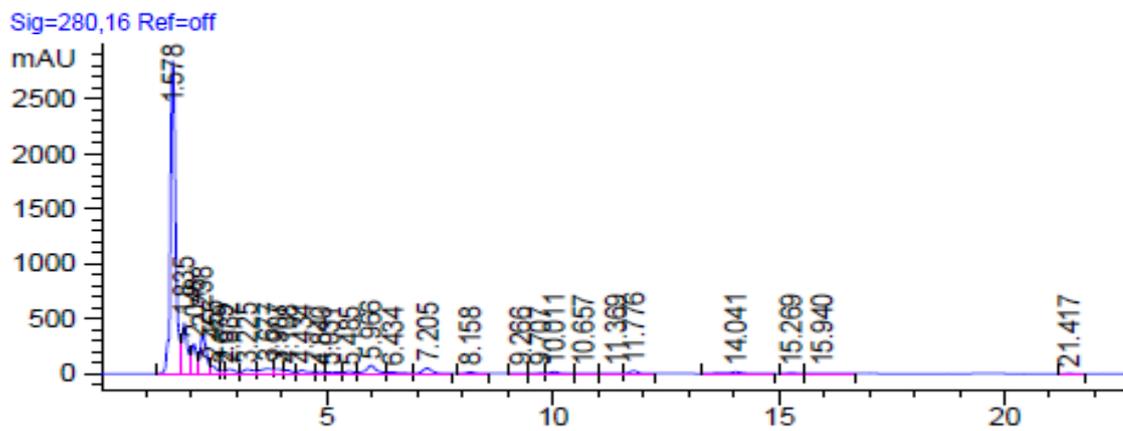


### Signal at 280 nm

$\beta$ -glucuronidase present. Peak at 4.154 minutes = hydroquinone



No  $\beta$ -glucuronidase present, and no hydroquinone peak



**Attitudes towards Alternative Treatments to Antibiotic Prescribing for Urinary Tract Infections**

**Patient In-depth Qualitative Interviews: Outline Interview Guide**

**Aims and Objectives**

The aim of this study is to explore patients' attitudes towards delayed prescribing of antibiotics for urinary tract infections ((UTI) in favour of symptom treatment with ibuprofen and/or herbal medicine

Below is a list of topics to be discussed in this study. The qualitative work will remain flexible with respect to participants' agendas but will cover the broad areas below. It is common in qualitative work to iteratively develop topics and questions as new ideas emerge from early data collection. Therefore, new topics may be added as the interviews progress and data collection continues, but the key subject areas will remain the same.

**Introduction**

---

*Aim: To introduce the research and set the context for the proceeding interview.*

- Introduce self
- Introduce the study: who it is for, what it is about
- Talk through key points:
  - purpose of the interview
  - length of the interview
  - voluntary nature of participation and right to withdraw
  - reasons for recording interview
- Confidentiality, and how findings will be reported
- Any questions they have

## 1. Background and personal circumstances

---

*Aim: to introduce participant, and highlight any key background issues that might influence their views.*

- Age, household circumstances
- Main daytime activity
  - whether working or not: details
- Other interests/activities

## 2. Urinary Tract Infections

---

*Aim: to gain understanding of the participant's experience of UTI*

- Occurrence/frequency of infection(s)
- Symptoms experienced
- How UTI might affect their day to day activities
- How and when they would normally treat the UTI
  - previous GP treatments
  - reasons for attending GP on this occasion
  - previous experience of antibiotics
  - alternative treatments tried if any

## 3. Antibiotics

---

*Aim: to elicit their attitudes towards antibiotics*

- Frequency of being administered antibiotics, and for what condition(s)
- Perceived benefits and drawbacks of taking antibiotics
- Awareness of antibiotic resistance
  - when first became aware
  - perceptions of what it means
  - likelihood to affect them

#### **4. Experience of the Clinical Trial** (if took part)

---

*Aim: to explore their views towards delayed prescribing of antibiotics for UTI*

- Feelings about being asked to delay taking antibiotics
  - perceived implications for their symptoms/duration of condition
- Whether they felt comfortable about the alternative treatments
- Personal experience of the trial/treatment
- The amount of information they were given about the trial
  - too much/too little
  - accessibility

#### **5. Herbal Medicine and Ibuprofen**

---

*Aim: to learn about their experiences of herbal medicine and attitudes towards its use for UTI*

- Awareness of herbal medicine, and perceptions of conditions it's used for
- Their experience of using herbal medicine, if any
- Perceived benefits and drawbacks of taking a herbal medicine
- How they felt about being offered herbal medicine and/or ibuprofen as an alternative to antibiotics
  - expectations of treatment for both herbal medicine and/or ibuprofen
- Likelihood to inform GP about alternative treatments

#### **6. Information Provision**

---

*Aim: to elicit views on how participants expect to find out about the results of the trial (i.e. should there be positive findings regarding alternative treatments).*

- How they find out about treatments
- Sources of information used
- Views on who should be providing information

**Final steps**

Thank the participant. Check whether they have any remaining questions about the research.

Reassure them about confidentiality and anonymity

Ask if they would like to be informed of the outcomes of the research

## Appendix G Qualitative Research Letter of Invitation

Dear Patient

**Ethics number: 14/SC/1143**

**ATAFUTI: A Trial Investigating Alternative Treatments of Adult Female Urinary Tract Infection.**

We are writing to invite you to take part in a research study being led by investigators from the Universities of Southampton, Bristol and Oxford. *<insert GP Practice>* is taking part in this study and we would be grateful if you could read the enclosed information and think about whether you would like to be interviewed as part of this study.

You are being invited to participate in this trial as you have recently been diagnosed with a suspected urinary tract infection. Your experience of urinary tract infections and your views on current and alternative treatments would make a valuable contribution to this research.

Enclosed is a Participant Information Sheet (PIS) explaining the study and telling you more about the interview which will be conducted by Jeanne Trill, a PhD researcher.

If you are interested in taking part, please complete the reply slip attached to this letter and return it in the FREEPOST envelope provided. There are contact details included in the Participant Information Sheet should you need any further information.

After receiving your reply slip Jeanne Trill will contact you directly about your participation in the interview as explained in the PIS.

Thank you very much for taking the time to read this letter and the enclosed information sheet.

**Study:**  **ATAFUTU**

Your name: .....

Your address:

1<sup>st</sup> Line .....

2<sup>nd</sup> Line .....

Town/City .....

County .....

Post Code .....

I am interested in taking part in the above study and would like to be contacted.

My preferred contact details are:

mobile .....

home .....

work.....

email .....

Please post this slip in the FREEPOST envelope provided.

Thank you

FREEPOST RTHY-TBHY-ZJJR, ATAFUTI-Q TRIAL, University of Southampton, Southampton  
Clinical Trials Unit, MP 131, Southampton General Hospital, Southampton, SO17 1YN

## Appendix H Participant Information Sheet - Patient



NUFFIELD DEPARTMENT OF  
PRIMARY CARE  
HEALTH SCIENCES



UNIVERSITY OF  
**Southampton**



### Participant Information Sheet

#### Patient Interview

#### ATAFUTI

**A Trial Investigating Alternative Treatments for Adult Female Urinary Tract Infection.**

Version number v3 17/03/15

Ethics number: 14/SC/1143

We would like to invite you to participate in an interview as part of a research study looking into alternative treatments for urinary tract infections. The interviews are being undertaken as part of a PhD Thesis at the University of Southampton. Before you decide whether or not to take part, we would like you to understand why the research is being carried out and what it will involve. Please take time to read the following information carefully.

Please ask us if there is anything that is not clear or if you would like more information.

## Part 1

### **What is the purpose of the study?**

Urinary Tract Infections are very common, and alternative treatments to antibiotics are being sought to relieve the symptoms. We want to learn about your experience of urinary tract infections, and are interested in your views about being offered an alternative treatment such as a herbal medicine. If you recently agreed to take part in the trial to test these treatments we would also like to find out how you felt about being asked to delay taking antibiotics to relieve your symptoms.

### **Why have I been invited?**

You have been chosen because you have recently been diagnosed by your GP with a urinary tract infection.

### **Do I have to take part?**

No. It is up to you to decide whether or not to take part. A decision not to take part, will not affect the standard of care you receive.

### **What will happen to me if I agree to take part?**

After carefully reading this information sheet and asking any questions you may have about the study you will be asked to provide the researcher with your contact details by completing the reply slip at the bottom of the accompanying letter and returning this using the prepaid addressed envelope enclosed. The researcher will contact you at a later date, but within three months of your participation in the trial (by email/letter/telephone) to see if you are still willing to take part. You will then be sent a Consent Form for the in-depth interview, which you will be asked to sign and return to the PhD Researcher. The consent form will confirm that you have read and understood the information in this document.

If you agree to take part in the informal interview, you will be contacted by the PhD Researcher by phone/email to arrange a convenient time. The informal interview will last 30-60 minutes and be conducted in your home unless you would prefer to come to the GP practice instead or be interviewed over the telephone. The interview will be conducted by a female PhD Researcher. You will be asked questions about your Urinary Tract Infection symptoms and any treatment you have received. You will also be asked about your views on antibiotics, as well as on an alternative treatment such as herbal medicine.

The interview will be audio recorded so that the researchers can write it up and study it at a later day. Your name is not used in this process and any quotes from the interview will remain anonymous.

### **What are the other possible disadvantages and risks of taking part?**

You will be giving up some of your time to attend the interview. Slightly embarrassing issues (i.e. urinary tract infections) will be talked about.

### **What are the possible benefits of taking part?**

Your participation may help to give important information about how best to treat people with Urinary Tract Infection in the future.

### **This completes Part 1 of the Information Sheet.**

If the information in Part 1 has interested you and you are considering participation, please read the additional information in Part 2 before making any decision.

## Part 2

### **What will happen if I don't want to carry on with the study?**

You can withdraw from the study at any time you don't have to give a reason. The standard of your care will not be affected. Information collected up to the time you withdraw may still be used.

### **What if there is a problem?**

If you have a concern about any aspect of this study, you should speak to the researcher Tel: 07935 280202 who will do their best to answer your questions or contact the trial co-ordinator – contact details below.

If you remain unhappy and wish to complain formally, you can do this through the normal National Health Service complaints Procedure. Details can be obtained through the following NHS web site.

<http://www.nhs.uk/choiceintheNHS/Rightsandpledges/complaints/Pages/AboutNHScomplaints.aspx>

### **Will my taking part in this study be kept confidential?**

All information which is collected about you during the course of the research will be kept securely, anonymously and strictly confidential. Your name will not be used in the collection, storage or publication of any research material.

### **What will happen to the results of the research study?**

The findings from this study will be written up as part of a PhD thesis after the study data has been analysed (approximately during 2016). No identifiable information will be included which could compromise the confidentiality of the study participants. The findings may also be written up and published in a medical journal. Any participant who wishes to obtain a copy of the publication should contact the Southampton Clinical Trials Unit, MP131, Southampton General Hospital, Tremona Road, Southampton, Hants, SO16 6YD.

If results are conclusive they may be used to influence future NHS guidelines for treatment of Urinary Tract Infection.

### **Who is organising and funding the research?**

This study has been organised by the Universities of Southampton, Oxford and Bristol and the trial is being run by the Southampton Clinical Trials Unit. The study is funded by a grant from the National Institute for Health Research, National School for Primary Care Research. The study Sponsor is the University of Southampton.

### **Who has reviewed the study?**

All research in the NHS is looked at by independent group of people, called a Research Ethics Committee, to protect your interests. This study has been reviewed by NRES Committee South Central – Hampshire A.

**Further Information and contact details**

If you have any questions, please contact:

PhD Researcher, Jeanne Trill

Tel: Tel: 07935 280202

Email: [Jeanne.Trill@btinternet.com](mailto:Jeanne.Trill@btinternet.com)

Or Trial Co-ordinator, Catherine Simpson at the Southampton Clinical Trials Unit,

Tel: 023 8120 5171

Email: [CTU@soton.ac.uk](mailto:CTU@soton.ac.uk)

If you have any further questions about your illness, please discuss them with your GP.

For further information about Urinary Tract Infection you may also find it helpful to look at the following NHS internet link:

<http://www.nhs.uk/conditions/Urinary-tract-infection-adults/Pages/Introduction.aspx>

**Thank you for taking the time to read this information sheet.**

## Appendix I Consent Form – Patient Interview



UNIVERSITY OF  
**Southampton**

**Ethics number:**

14/SC/1143

**Centre ID:**

**Participant's Initials:**

(If no middle initial insert '-')

**Participant's Month & Year of Birth:**

**Participant Trial ID(if applicable):**

### **ATAFUTI**

A Trial Investigating Alternative Treatments of Adult Female Urinary Tract Infection.

### **CONSENT FORM – PATIENT INTERVIEW**

Name of Researcher: Professor Michael Moore

The informed consent consists of two parts – the information sheet and this consent form.

Please Initial Box

1. I confirm that I have read and understand the participant information sheet (version 3 dated 17/03/15) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.
2. I understand that my participation in the interview is voluntary and that I am free to withdraw at any time without giving any reason, and without my medical care or legal rights being affected.

- |   |   |  |
|---|---|--|
| 3 | I consent to the collection and use of information about me in accordance with the participant information sheet.   | <input style="width: 40px; height: 30px; border: 1px solid black;" type="checkbox"/> |
| 4 | I give permission for my interview to be audio recorded. I understand that my name will not be used in this process and that any quotes used will remain anonymous. | <input style="width: 40px; height: 30px; border: 1px solid black;" type="checkbox"/> |
| 5 | I understand that any data collected, at the interview, up to the time of my withdrawal may continue to be used in the above study.                                 | <input style="width: 40px; height: 30px; border: 1px solid black;" type="checkbox"/> |
| 6 | I agree to take part in the interview for the above study.  | <input style="width: 40px; height: 30px; border: 1px solid black;" type="checkbox"/> |

*For the purposes of the Data Protection Act 1998 the data controller is the University of Southampton and any inquiries relating to your personal information may be addressed to the Southampton Clinical Trials Unit, MP131, Southampton General Hospital, Southampton, SO16 6YD.*

Name of Patient	Signature	Date

Name of Person taking consent	Signature	Date

When completed:  
 1 (original) signed consent form to be kept in Medical Notes  
 1 copy for the participant  
 1 copy for the researcher site file



## Appendix J Patient Theme Table Developed from Codes

CODE	SUB THEME	THEME
Previous antibiotic treatment of UTI	Expectation that antibiotic will work	T1: Anticipation of antibiotic prescription
Awareness of antibiotic resistance		
When first became aware of antibiotic resistance		
Unsuccessful alternative treatment		
Expected diagnosis of UTI		
Feel they will always need a prescription		
Immediate GP appointment	Belief they have infection	
Already tried alternative treatment		
UTI affecting daily activities		
Range of UTI symptoms experienced		

CODE	SUB THEME	THEME
Reassured by antibiotic prescription	Reassurance of delayed prescription for antibiotics	T2: Factors that influence decision to participate in trial
Will only delay prescription for 2 – 3 days		
Treatment is free		
Attitude to going on trial - concern		
Sufficient information	Clear explanation of trial	
Easy to complete diary		
Attitude to going on trial - concern		
Concern there would be side effects	Would there be any side effects?	
Reassured herb was coming from GP practice		
Post-trial follow-up GP appointment		

CODE	SUB THEME	THEME
Previous alternative treatment	Reluctance to take antibiotics	T2: (Continued) Factors that influence decision to participate in trial
Desire to find alternative treatment		
Side effects of antibiotics		
Attitude towards being prescribed herbal treatment for clinical trial		
Awareness of mechanism of action	Option of taking ibuprofen	
Intolerance		

CODE	SUB THEME	THEME
Awareness of antibiotic resistance	Willingness to try alternative treatments	<b>T3: Desire that herbal treatment will work</b>
Side effects from taking antibiotics		
Opinion of herbal medicine being used for UTI		
Reassured herb was coming from GP practice		
Positive attitude towards being prescribed herbal medicine		
Perceived benefits of herbal medicine		
Attitude towards being prescribed herbal medicine - unsurprised		
How they would be able to obtain the HMP	Accessibility and trustworthiness of herbal treatment	
Confidence in receiving a prescription		
Convenience of self-purchase of medication		
Use herbal medicine		

CODE	SUB THEME	THEME
Publicity of results	Learning about the trial outcomes	<b>T4 Barriers to adoption of herbal medicine</b>
Health concerns about herbal medicine	Anticipated problems of taking herbal medicine	
Tablets too big		
Would there be any side effects		
Sceptic that treatment will work		
Feel they would always require a prescription		



# Lab Manual



Alternative Treatments of Adult Female Urinary Tract Infection: a double blind, placebo controlled, factorial randomised trial of uva-ursi and open pragmatic trial of ibuprofen

Role	Name	Signature	Date
Authors	J. Trill		
	C. Simpson		
Approval	Prof Gibbons		





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## 1. INTRODUCTION

### 1.1 TRIAL TITLE

Alternative Treatments of Adult Female Urinary Tract Infection: a double blind, placebo controlled, factorial randomised trial of Uva ursi and open pragmatic trial of ibuprofen

### 1.2 TRIAL OBJECTIVES

#### Primary Objective:

- To evaluate whether Uva ursi compared to placebo or the advice to take ibuprofen compared to no advice provide relief from urinary symptoms in adult women with suspected UTI.
- 

#### Secondary Objectives:

- To evaluate whether Uva ursi compared to placebo or the advice to take ibuprofen compared to no advice result in reduced antibiotic use in adult women with suspected UTI.
- To determine the patient / practitioner barriers to implementation of a delayed antibiotic prescription approach and use of herbal medication.

### 1.3 TRIAL OUTCOME MEASURES

#### Primary outcome:

- Symptom severity day 2-4 using validated diary data

#### Secondary outcomes:

- Use of antibiotics
- Duration of moderately bad symptoms
- Re-consultation in one month with UTI from notes review
- Re-consultation with UTI from notes review in the next 3 months

#### Exploratory analysis:

- Differential effects on primary outcome depending on culture results

### 1.4 TRIAL IDENTIFIERS

This Trial has the unique identifying numbers:

This Trial has the unique identifying numbers:

- EudraCT reference number: 2013-003327-11
- Ethics reference number: 14/SC/1143

## 1.5 PURPOSE OF LABORATORY MANUAL

The purpose of this manual is to describe the collection, transportation, storage and processing of urine samples for the analysis of metabolites of Uva ursi as described in the ATAFUTI protocol. All relevant staff contacts are given in Appendix 1. These details may be amended without the reissue of the entire laboratory manual. Any forms required to record sample processing/reagent preparation etc. are attached to this document as appendices. These may be photocopied, or printed off, as required.

## 1.6 SAMPLE LABELS

All labels must contain the following details:

**Trial Name** (ATAFUTI)

**Participant Trial ID:**

**Date:** Date of collection

**Time:** Time of collection

## 2 COLLECTION, SHIPMENT, PROCESSING, RECORDING AND STORAGE OF URINE SAMPLES

### 2.1 SAMPLE COLLECTION AND SHIPMENT

Urine samples will be collected by participants 2 hours after taking the first dose of Trial medication on Day 4 using the collection kit provided (see Appendix 2 for details). The date and time the urine sample is collected and the Participant Trial ID number will be recorded clearly on the monovette label. The sample will be placed in the pre-paid addressed packaging provided and posted the same day. The samples will be sent by next day delivery to the UCL School of Pharmacy. If for any reason samples cannot be posted the same day then they should be stored in the sealed, leak proof packaging in a refrigerator at around 4°C but for no longer than 24 hours.

### 2.2 SAMPLE RECEIPT

The packages containing the urine samples will be delivered to the reception at the School of Pharmacy, UCL. Reception staff will immediately notify the PhD Researcher by text/email/phone that a sample has arrived and place the sample in the refrigerator until it has been collected.

The PhD Researcher (MSc Pharmacognosy), designated by the Chief Investigator (CI) will be the sole person responsible for receipt, storage, processing and analysis of the samples.

The PhD Researcher will document receipt of the sample on the Participant Sample Log (Appendix 3). All urine samples will be assessed on arrival by the PhD Researcher to check their physical integrity. If urine samples have been compromised in transit the SCTU must be notified promptly.

On receipt, each sample should be examined to ensure that the label does not display information which may identify the trial subject. If information other than the clinical trial ID is recorded on the label which may compromise the trial subject's right to privacy, it should be masked or deleted. Care should be taken not to obliterate other information which may be needed to identify the sample during analysis.

On receipt samples should be filtered through a 0.22 µm sterile filter to remove existing bacteria and human cells and then be stored in a freezer at - 20°C until they are analysed.

Freezers will be checked on a daily basis to ensure that there has not been a malfunction, and

the PhD Researcher will be notified immediately if a problem occurs. Adequate provision will be made to ensure that laboratories have sufficient spare capacity for the storage of chilled and frozen samples, should a refrigerator or freezer malfunction. Adequate and appropriate storage conditions will be maintained that will protect sample integrity and prevent cross-contamination. The design of the facility will provide an adequate degree of separation of different activities to assure the proper conduct of the work.

## 2.3 REAGENTS, EQUIPMENT AND PRECAUTIONS

The following equipment is required for processing/storing the samples, or storing the reagents:-

- Millex GP sterile syringes and 0.22 µm sterile filter units
- Benchtop centrifuge
- Pipettes and sterile filter tips
- 70% ethanol (Fisher Scientific) for sterilising surfaces
- - 20°C freezer
- 1.5 ml autosampler HPLC vials and corresponding lids
- HPLC grade water (Fisher Scientific)
- HPLC grade Methanol (Fischer Scientific)
- HPLC grade Acetone (Fisher Scientific)
- β-glucuronidase (Sigma-Aldrich G7646)
- Hydroquinone (purity 99%+ Sigma-Aldrich) reference standard
- Arbutin (purity 98%+ Alpha Aesar) reference standard

A Risk Assessment has been carried out and a copy of the completed form is held at UCL and in the Trial Master file at SCTU.

## 2.4 SAMPLE RECORDS

The Participant Sample Log (Appendix 3) should be completed at time of processing by the PhD Researcher. The completed log will be kept in the ATAFUTI file locked in the lab-technicians office in Lab 201b.

## 2.5 PROCESSING URINE SAMPLES

All procedures must be performed using aseptic techniques in a laminar flow safety cabinet. A laboratory coat, mask and disposable gloves must be worn.

A Risk Assessment has been carried out and a copy of the completed form is held at UCL and in the Trial Master file at SCTU.

### Method

The following method has been validated 6 times with volunteer urine samples (interday), and replicated in triplicate (interday) with second volunteer urine samples. (Volunteer samples approved by UCL Ethics Committee).

1. Filter sample through 0.22 µm sterile filter
2. Split urine sample in two. Add 9µl of β-glucuronidase to one half of the sample and incubate at 37°C for 30 minutes to liberate hydroquinone from the glucuronide.
3. To 0.200 mL of urine add 0.200 mL distilled water (using sterilised pipettes) to dilute the sample in order to protect the HPLC machine and column. Add 0.850 mL acetone. Place sample in fridge at 4°C for not less than 10 minutes and then centrifuge for 30 minutes at 13000 rpm to precipitate proteins and phospholipids.
4. Remove supernatant and transfer to 1.5 mL HPLC vials for HPLC analysis.
5. Evaporate off the acetone overnight, covering the vials loosely with aluminium foil.

6. HPLC method using an Agilent 1200 series HPLC System comprising an Agilent 1200 degasser (G1322A), quaternary pump (G1311A), standard auto-sampler (G1329A), thermostatted column compartment (G1316A) coupled to an Agilent 1200 diode array and multiple wavelength detector SL (DAD) (G1315D).
  - a. Inject 20  $\mu$ L samples via the autosampler. Analyse samples on a Supelco C18 Ascentis (5  $\mu$ m) column (250 x 4.6 mm) maintained at 30°C. UV spectra recorded in the range of 220 to 360 nm at a sampling rate up to 80 Hz, with analysis via Agilent 1200 ChemStation software. The gradient flow rate set at 0.9 mL/minute with mobile phase 95:5 H<sub>2</sub>O:MeOH for 4 minutes, followed by a linear gradient to 70:30 from 5 to 11 minutes.
  - b. Calibration curves - prepared with reference standards of hydroquinone and arbutin. UV detection set at 280 nm, band-width 4, and reference signal 'off'. For quantification, 7 concentrations of arbutin reference standard were analysed at 1 mg/mL, 0.75 mg/mL, 0.5 mg/mL, 0.375 mg/mL, 0.25 mg/mL, 0.187 mg/mL and 0.125 mg/mL. Hydroquinone reference prepared by diluting 2.5 mg of the standard in 10 mL of the mobile phase, and to 5 mL of this solution 2.5 mL of arbutin added and re-diluted to 10 mL with the mobile phase. This is analysed at concentrations of 0.125 mg/mL, 0.09375 mg/mL, 0.0625 mg/mL, 0.046875 mg/mL and 0.03125 mg/mL.
  - c. Retention times (R<sub>f</sub>) for hydroquinone and arbutin recorded and compared with urine samples. Urine sample also spiked with equal amount of known quantity of hydroquinone (0.125 mg/mL) to confirm (R<sub>f</sub>) for presence of hydroquinone.

## 2.6 DATA RECORDING

All data will be recorded directly, promptly, accurately and legibly in a lab book used only for the ATAFUTI trial. The date on which the analysis was performed and the name of the person conducting the analysis will be documented.

## 2.7 DISPOSAL OF URINE SAMPLES

Urine samples to be analysed will be disposed of within 7 days. The samples will be placed in a clinical waste autoclave bag in a clinical waste box in Lab 201c. The waste is removed daily to be autoclaved and disposed of with the microbial waste.

### 3 REVISION HISTORY

Version No.	Date Effective	Revision History
1.0	Aug 2015	First issue

## 4 APPENDICES

### APPENDIX 1 CONTACT DETAILS

University College London	
PhD Researcher: Jeanne Trill (MSc)	
Address	Lab 201 UCL School of Pharmacy 29-39 Brunswick Square, London WC1N 1AX
Tel:	07770-623956
Fax:	
Email:	Jeanne.Trill@btinternet.com
Responsibility:	Receipt, storage, analysis and disposal of samples. First point of contact.

University College London	
Professor Simon Gibbons FRSC FLS	
Address	Faculty of Life Sciences School of Pharmacy UCL School of Pharmacy 29-39 Brunswick Square, London WC1N 1AX
Tel:	0207 7535913
Fax:	0207 7535964
Email:	<a href="mailto:simon.gibbons@ucl.ac.uk">simon.gibbons@ucl.ac.uk</a>
Responsibility:	Head of the Department of Pharmaceutical and Biological Chemistry

<b>Clinical Trial Unit Coordinator, Southampton:</b>	
<b>Catherine Simpson</b>	
Address	University of Southampton Clinical Trials Unit MP131 Southampton General Hospital Tremona Road Southampton, SO16 6YD
Tel:	023 8120 5171
Fax:	0844 7740621
Email:	<a href="mailto:catherine.simpson@soton.ac.uk">catherine.simpson@soton.ac.uk</a>
Responsibility:	First point of contact, study logistics.

<b>Clinical Trial Unit Manager, Southampton:</b>	
<b>Fran Webley</b>	
Address	University of Southampton Clinical Trials Unit MP131 Southampton General Hospital Tremona Road Southampton, SO16 6YD
Tel:	023 8120 3866
Fax:	0844 7740621
Email:	<a href="mailto:f.webley@soton.ac.uk">f.webley@soton.ac.uk</a>
Responsibility:	Trial Manager for the study

<b>Chief Investigator</b>	
<b><u>Prof. Michael Moore</u></b>	
Address	Primary Care and Population Sciences University of Southampton Aldermoor Health Centre Aldermoor Close Southampton SO16 5ST
Tel:	023 8024 1056
Email:	mvm198@soton.ac.uk
Responsibility:	Chief Investigator for the study



## APPENDIX 2 URINE COLLECTION KIT



Southampton Clinical Trials Unit



**ATAFUTU**

A Trial Investigating Alternative Treatments of Adult Urinary Tract Infection.

Day 4 Urine Collection Instruction Sheet.



Southampton

Thank you for agreeing to provide a urine sample for further laboratory testing to see if there are active ingredients of the trial medication in your urine. Please follow the instructions below.

1. The urine sample should be collected 2 hours after taking your first dose of the trial medication on Day 4.
2. Collect your urine sample using the collection kit given to you by the GP/Nurse and following the instructions on the bag containing the kit. These instructions are repeated for convenience on the reverse of this sheet.
3. Once the sample tube has been filled, according to the instructions, please package the sample for posting following the steps below:
  - a) Write your Participant Trial ID number (this can be found on the front of your diary and on your Trial Treatment Card) in the "name" section on the sample tube label along with the date and time the urine specimen was collected.
  - b) Please do **not** record your name or complete any other details on this label.
  - c) Place the sample tube inside the screw top container provided in the cardboard box.
  - d) Place the screw top container with the filled sample tube back into the cardboard box.
  - e) Seal the box with the enclosed sticky label to ensure that the box is secure.
4. Please post the sealed box back to the Freepost address as soon as possible after collection to ensure that it arrives at the laboratory the next day. If there is any delay between collection of the sample and posting, please keep in the refrigerator.

**THANK YOU!**  
You have made a valuable contribution to this medical research.



UKCRC  
Registered  
Clinical  
Trials Unit



National Institute for  
Health Research

FUTU Urine Collection Instruction Sheet v1.31-MAR-2015  
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Southampton Clinical Trials Unit



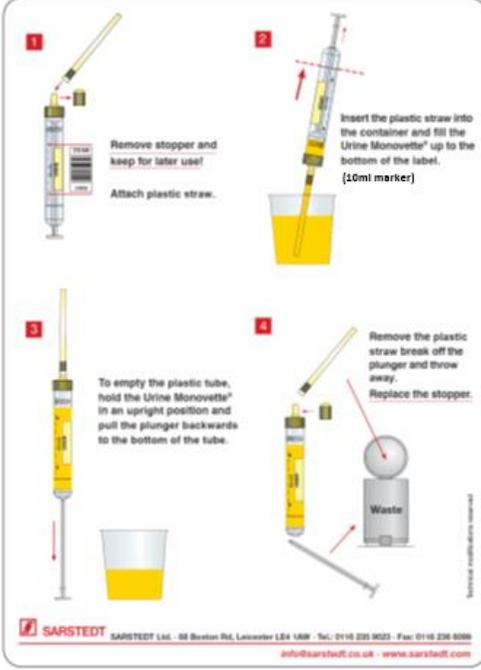
**ATAFUTU**

A Trial Investigating Alternative Treatments of Adult Urinary Tract Infection.



Southampton

### Urine Monovette® User Guide



**1** Remove stopper and keep for later use! Attach plastic straw.

**2** Insert the plastic straw into the container and fill the Urine Monovette® up to the bottom of the label. (10ml marker)

**3** To empty the plastic tube, hold the Urine Monovette® in an upright position and pull the plunger backwards to the bottom of the tube.

**4** Remove the plastic straw break off the plunger and throw away. Replace the stopper.

**SARSTEDT** SARSTEDT Ltd. - 68 Beckett Rd, Leicester LE4 1AW - Tel: 0116 239 9021 - Fax: 0116 239 8899  
info@sarstedt.co.uk - www.sarstedt.com



UKCRC  
Registered  
Clinical  
Trials Unit



National Institute for  
Health Research

FUTU Urine Collection Instruction Sheet v1.31-MAR-2015  
Page 2

### APPENDIX 3 PARTICIPANT URINE SAMPLE LOG



Southampton Clinical Trials Unit

UNIVERSITY OF  
Southampton



**PARTICIPANT URINE SAMPLE LOG**

Short Title	ATAFUTU
REC number:	14/SC/1143
EudraCT number:	2013-003327-11
Sponsor:	University of Southampton

Date Received	Received By	Participant Trial ID Number	Packaging (tick box if acceptable or comment)			Volume ml	Stored in freezer (-20°C) Date	Comments	Processed for Analysis (Sign and Date)	Safely Disposed of (Initial and Date)
			Pack intact	No Leaks	Label					



ATAFUTU Participant Urine Sample Log v1.02/AUG/2015



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